University of the Algarve
Faculty of Science and Technology

Development of the nutraceutical and pharmaceutical applications of plants selected from Portugal and Iran with presumptive health potentials

Vahid Farzaneh

Thesis submitted to MeditBio, Faculty of Sciences and Technology of University of the Algarve for Doctor Degree in Food Sciences and Biotechnology

Supervised by:
Professor Dr Isabel. S. Carvalho

Faro
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PhD Thesis

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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Vahid Farzaneh

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Abstract

All over the years scientists have studied an extensive range of medicinal and aromatic plants species which exist in nature and contain an unlimited category of bioactive compounds that could potentially be used within an extensive range of products including nutraceutical; pharmaceutical and even cosmetic. This seems to be more substantial since scientists have detected an extensive range of negative adverse effects among many of the synthetic compounds commonly used today.

In this research, we have selected plants which, are particularly well known among people as being healthy and could have therapeutic for chronic and acute diseases. Therefore, after the precise researches and discussions with native residents of the selected regions-including the northwest of Iran and south Portugal, with focus on the Algarve, about 14 different plant species were selected and identified for this research. Among them, different individual parts of plants including seeds (Portugues and Iranian *Pimpinella anisum* L., with *Coriandrum sativum* L., and *Levisticum officinale* W. D. J. Koch., from Iran); flowers (*Echium amoenum* Fisch & C.A. Mey., *Echinacea angustifolia* DC., *Matricaria chamomilla* L., and *Althaea officinalis* L., from *Iran*); leaves (*Thymus vulgaris* L., *Rosmarinus officinalis* L., *Salvia officinalis* L., and *Alisma plantago-aquatica* L. subsp. orientale (Sam.)Sam., from Iran and *Olea europaea* L. var. sylvestris., from Portugal) have been studied. Moreover the aerial parts of *Lavandula pedunculata* L. subsp. pedunculata., from Portugal were selected and studied in this research.

An extensive range of *in vitro* chemical and microbial experiments as well as optimization of different variables in the extraction process and *encapsulation efficiency* of the bioactive compounds have been performed on the different species of plants. Initially, the research intended to study the encapsulation of two of the selected plants' infusions including *Olea europaea*., and *Alisma plantago-aquatica*., with the preparation of calcium-alginate beads in combine of potato starch as a filling substance to study and optimise the release kinetics and *encapsulation efficiency* of the bioactive compounds in deionised water and *simulated gastric fluid* (*SGF*) (as solvents) respectively for each plant. The results confirmed the positive role of the potato starch, in a particular concentration, in the monitoring of release kinetics of *phenols* from the *CAS* (*Calcium Alginate Starch*) beads.

On the other hand, the performance of the applied models of *Response Surface Methodology* (*RSM*) and *Artificial Neural Networks* (*ANN*) on the optimisation of the

release kinetics and *encapsulation efficiency* of the bioactive compounds from the encapsulated infusion in *simulated gastric fluid* (*SGF*) as the solvent was compared, and the *ANN* design was reported as the more accurate tool compared to *RSM* in the prediction of the optimum range of the selected independent parameters including potato starch as one of the variables.

Portugues *P. anisum*., seeds was selected to optimise the extraction process of the bioactive compounds regarding the two selected independent variables, including the time and temperature of extraction using water as a solvent. Maximum desirability was obtained for $DPPH^{\cdot -}$ and TMA parameters. *Gas Chromatography-Mass Spectrometry* (*GC-MS*) analysis was performed on *P. anisum*., seeds' infusion to determine the major compounds profile. The obtained results demonstrated that, *fatty acids* with indicating 47.68% of the whole detected compounds are the most detected predominant compounds found in the tested extracts followed by *triterpenoids*; among *fatty acids, linoleic acid* in combine of *oleic* and *palmitic acids* were detected as major compounds.

In the current research, the authors also detected bioactive compound content alongside different *anti-oxidant* parameters of 10 different plants' infusions picked from Iran. In this regard, total *phenolic* content (TPC*)*; total *flavonoid* content (TFC*)*; total *chlorophyll* content (TCC*)* values along with total *anti-oxidant* activity (TAA*)*; reducing power (RP*)*; ferric reducing *anti-oxidant* power (FRAP*)*; *ABTS* free radical inhibition and the *DPPH free radical scavenging activity* of different plants species were determined. The extraction of the bioactive compounds was performed using different pathways; firstly: hot aqueous infusions were obtained at 90 ºC within 5 minutes' extraction time and cold aqueous extracts were obtained at room temperature within 2 hours blending. Most of the achieved values for the hot infusions were significantly higher compared to cold aqueous extracts. In some cases, a few exceptions were found in *P. anisum*., and *L. officinale*., species (seeds) justified by their tough and wooden textures (abnormality in extraction). The *Pearson correlation coefficient* established that the highest *anti-oxidant* parameters are related to *phenols*; *chlorophylls* and eventual further bioactive compound existence such as *triterpenoids*; *fatty acids*; *sterols* or others rather than *flavonoids* and further bioactive compounds. On the other hand, synergistic activity hypothesis among at least two or more compounds were proposed. Mostly, the examined leaves, particularly *T. vulgaris*., presented higher bioactivity than the other studied seeds and

flowers species in this research. *Gas Chromatography-Mass Spectrometry* (*GC-MS*) analysis was used in the identification of the bioactive compounds in the leaves and two seeds. The achieved results demonstrated an extensive range of compounds with eventual bioactivity including: *fatty acids*; *fatty alcohols*; *sterols*; *triterpenoids*; *monoacylglycerols*; aromatics; *alkanes*; *waxes*; *di-acids*; α-hydroxy *fatty acids* and sugars. It has been observed that in the leaves *triterpenoids* combined with *sterols* are the most abundant compounds demonstrating 63.42; 47.64 and 33.51% of the total characterised compounds respectively in *R. officinalis*., *S. officinalis*., and *T. vulgaris*., while in the seeds, *fatty acids* were discovered as the most predominant compounds, expressing 80.22, 49.04 and 47.68% of the specified compounds in Iranian *C. sativum*., and *P. anisum* from Iran and from Portugal., respectively. *Betulinic*, *maslinic*, *ursolic* and *oleanolic acids* were the most predominant *triterpenoids* in the leaves and *oleic*, *palmitic* and *linoleic acids* were identified as the most abundant *fatty acid* content in the seeds. The obtained results proposed that in the plants, the synergistic effects among different bioactive compounds might be expected.

According to the characterised and identified bioactive compounds of the plants' infusions in this study and their established potential health benefits, it could be expected that these plants might demonstrate further potential health benefits for human body such as *anti-carcinogenic*, *anti-diabetic*, *anti-Alzheimer's*, *anti-Parkinson's*, or others. Therefore, this research expanded the research and studied the potential health benefits including *anti-bacterial* and *in vitro anti-diabetic*, as well as *anti-Alzheimer's* disease, along with *anti-radical* and further *anti-oxidant* properties of the plants. Finally the relations between the achieved *in vitro* results with the characterised compounds of the *Gas Chromatography-Mass Spectrometry* (*GC-MS*) were interpreted.

Selected leaves' water infusions also were detected as stronger inhibitors in supressing of the enzymes attributed in revealing of *diabetes* and *Alzheimer's diseases*. The *anti-bacterial* potential of some of the plants against five different bacterial species showed that the selected Iranian plants, except *E. angustifolia*., (flower) inhibited *E. faecalis* in *MIC* value $\leq$0.156 mg. mL^{-1}, while other bacterial species showed activities in *MIC* values $\leq$10 mg. mL^{-1}. Among flowers *M. chamomilla*., and among leaves *S. officinalis*., alongside *R. officinalis*., revealed slightly higher *anti-bacterial* activities than others.

Alongside the abovementioned studies this research also studied the modelling of the extraction of monomeric *anthocyanins* using *microwave assisted extraction* (*MAE*) from *L. pedunculata.*, picked in Algarve-Portugal. Three independent variables were selected for two responses including TAA and TMA values, among variables microwave power showed 465 W as the optimized value for the extraction of monomeric *anthocyanins*.

In the final of this study, the inhibitory activities of Portugues *P. anisum*., seeds' infusion against *α-Amylase* and *Acetylcholinesterase* (*AChE*) were determined, confirming the eventual *anti-oxidant*; *anti-diabetic* and *anti-Alzheimer's* activities of this plant. *Free fatty acids* as well as *terpenoids* and *sterols* respectively, have demonstrated higher quantities in Portugues *P. anisum* L., seeds. With respect to the obtained results of inhibitory activities of this infusion against tested enzymes and free radicals, it has been approved that compounds with free radical suppressing activities might not able to participate in competitive enzymes suppressive reactions with substrates. Therefore the needs to *in vivo* tests for obtaining most reliable results are revealed.

This research aims to introduce novel and natural sources of bioactive compounds that might be used in promoting good health, in general, and in particular in the treatment of chronic disorders such as *Alzheimer's*, *diabetes*, *cancers* as well as *Parkinson's disease* along with microbial infections. This research revealed that the tested plants from Portugal and Iran could be potential sources of compounds with an extensive range of application for health benefits as well as in nutraceutical, pharmaceutical and cosmetic products.

Resumo

Ao longo dos anos tem sido estudadas uma enorme diversidade de plantas, aromáticas e medicinais, que existem na natureza e que contêm inúmeras categorias de compostos bioativos que podem potencialmente ser utilizadas numa grande variedade de produtos incluindo os nutracêuticos, farmacêuticos e até cosméticos. Este facto assume maior relevância atendendo a que alguns estudos já realizados detetaram uma variedade de efeitos adversos em muitos dos compostos sintéticos utilizados atualmente.

Neste trabalho, em particular, foram selecionadas plantas que têm, de acordo com conhecimento popular, propriedades importantes para a saúde no geral, podendo ainda potencialmente apresentar propriedades terapêuticas em doenças crónicas ou agudas. Assim, após a realização de estudos e conversas com as populações locais, do noroeste do Irão e do sul de Portugal, com foco na região algarvia, foram selecionadas e identificadas 14 diferentes espécies de plantas. De entre elas foram estudadas individualmente diferentes partes das plantas, incluindo sementes (*Pimpinella anisum* L., de Portugal e do Irão, e *Coriandrum sativum* L., e *Levisticum officinale* W.D.J. Koch., do Irão); flores (*Echium amoenum* Fisch & C.A. Mey., *Echinacea angustifolia* DC., *Matricaria chamomilla* L., e *Althaea officinalis* L., do Irão) e folhas (*Thymus vulgaris.* L., *Rosmarinus officinalis* L., *Salvia officinalis* L., e *Alisma plantago-aquatica* L. subsp. orientale (Sam.) Sam., do Irão e *Olea europaea* L. var. sylvestris., do Portugal). Além disso, foi estudada uma mistura homogénea da parte aérea de *Lavandula pedunculata* L. subsp. pedunculata., de Portugal.

Foi realizada uma gama extensa de testes, *in vitro*, químicos e microbiológicos assim como a otimização de diferentes variáveis do processo de extração e eficiência de encapsulação de compostos bioativos das diferentes espécies de plantas selecionadas. Inicialmente, pretendia-se estudar a encapsulação de infusões aquosas das duas plantas selecionadas *O. europaea*, e *A. plantago-aquatica*, com a preparação de cápsulas de alginato de cálcio combinadas com amido de batata, enquanto substância de preenchimento, com o objetivo de otimizar os modelos para a cinética de libertação e eficiência de encapsulação dos compostos bioativos utilizando como solvente água desionizada e *Fluído Gástrico Simulado* (*SGF*), respectivamente para cada planta. Os resultados confirmaram o papel positivo do amido de batata, numa determinada concentração, na monitorização da cinética de libertação de fenóis das cápsulas de alginato de cálcio e amido (*CAS*).

Por outro lado, foram comparados o desempenho dos modelos aplicados de metodologia de superfície de resposta (*RSM*) e de redes neurais artificiais (*ANN*) na otimização da cinética de libertação de eficiência de encapsulação dos compostos bioativos das infusões encapsuladas no *SGF*. O desenho por *ANN* foi reportado como uma ferramenta mais exata em comparação com o desenho por *RSM* na previsão da gama ótima dos parâmetros independentes selecionados incluindo o amido da batata como uma das variáveis.

As sementes de *P. anisum*, de Portugal foram selecionadas para otimizar o processo de extração dos compostos bioativos para duas variáveis independentes selecionadas, o tempo e a temperatura de extração utilizando água como solvente. O valor máximo foi obtido para os parâmetros $DPPH^{\cdot-}$ e TMA. Com o objetivo de determinar o perfil dos principais compostos presentes, foi ainda efetuada uma análise de cromatografia gasosa acoplada a um espectrómetro de massa (*GC-MS*) às infusões aquosas obtidas para as sementes de *P. anisum*. Os resultados mostram o teor em ácidos gordos como os compostos predominantes, sendo cerca de 47,68 % dos compostos totais detetados, seguido de triterpenóides. De entre os ácidos gordos, o ácido linoleico em combinação com os ácidos oleico e palmítico foram os principais compostos detetados.

No decorrer deste trabalho, foram ainda analisados o teor em compostos bioativos juntamente com diferentes parâmetros antioxidantes para as infusões de 10 diferentes plantas do Irão. Foram determinados os conteúdos de fenólicos totais (TPC), de flavonoides totais (TFC) e de clorofila (TCC) bem como as seguintes atividades: antioxidante total (TAA), poder redutor (RP), poder redutor férrico total (FRAP) e captura de radicais *ABTS* e *DPPH* das diferentes plantas. A extração dos compostos bioativos foi realizada utilizando diferentes métodos. Primeiramente, foram realizadas infusões aquosas a duas diferentes temperaturas, uma extração a quente a 90 °C por um período de 5 minutos e uma extração a frio à temperatura ambiente por um período de 2 horas. Na maioria dos casos, os resultados das infusões a quente foram significativamente superiores aos resultados da extração a frio, sendo a exceção as sementes, *P. anisum* e *L. officinale*, provavelmente devido à sua textura lenhosa. O coeficiente da correlação de Pearson estabeleceu que os parâmetros de antioxidantes mais elevados estão relacionados com os compostos fenólicos, clorofilas e eventualmente com a existência de outros compostos bioativos como os triterpenos, ácidos gordos, esteróis entre outros e não com os flavonoides e

outros compostos bioativos. Por outro lado, foi proposta a hipótese de existir atividade sinergética entre pelo menos dois destes compostos. As folhas examinadas, em particular as folhas pertencentes ao *T. vulgaris*, apresentaram na sua maioria melhores atividades bioativas quando comparados com os resultados obtidos para as sementes e para as flores estudadas. Foi efetuada uma análise de cromatografia gasosa acoplada a um espectrómetro de massa (*GC-MS*) para identificar os compostos bioativos em folhas e em duas sementes. Tendo sido encontrada uma grande diversidade de compostos com eventual atividade bioativa incluindo: ácidos gordos e álcoois gordos, esteróis, triterpenos, monoacilgliceróis, aromáticos, alcanos, ceras, diácidos, e açúcares. Foi observado para as folhas de *R.* o*fficinalis, S. officinalis*, e *T. vulgaris*, que os tripterpenos combinados com os esteróis são os compostos mais abundantes sendo respetivamente 63,42; 47,64 e 33,51% dos compostos bioativos totais caracterizados, por sua vez, as sementes têm maioritariamente ácidos gordos, na sua composição, contabilizando 80,22; 49.04 e 47.68% do total de compostos identificados, para *C. sativum*, do Irão e o *P. anisum*, do Irão e de Portugal, respetivamente. Os ácidos *betulinico*, *maslinico*, *ursólico* e *oleanólico* foram os triterpenos predominantes nas folhas enquanto que os ácidos *oleico*, *palmítico* e *linoleico* foram identificados como os mais abundantes nas sementes. Os resultados obtidos permitiram propor que existem provavelmente efeitos sinergéticos entre os diferentes compostos bioativos presentes nas plantas.

De acordo com os compostos bioativos, caracterizados e identificados nas infusões, das plantas usadas neste estudo e os seus potenciais efeitos benéficos para a saúde, podem ser ainda previstos outros efeitos benéficos para a saúde, nomeadamente atividade anticarcinogénica, antidiabética assim como efeito benéfico na prevenção de doenças de *Alzheimer* e de *Parkinson*. Assim, o estudo foi alargado para o potencial efeito benéfico, na saúde humana, ao nível antibacteriano e antidiabético, bem como no combate à doença de *Alzheimer*, conjuntamente com a atividade anti-radical e propriedades antioxidantes. Finalmente, os resultados obtidos, *in vitro*, foram interpretados e relacionados com os resultados dos compostos bioativos obtidos por *GC-MS*. Infusões aquosas, de folhas das plantas selecionadas, mostraram ainda ter forte inibição da actividade de enzimas específicos, que se sabem estar relacionada com o diabetes e Alzheimer.

O potencial antibacteriano de algumas plantas foi testado recorrendo a 5 espécies bacterianas. As plantas Iranianas, à exceção da *E. angustifolia*, (flor),

inibiram a *E. faecalis* (*MIC* <0.156 mg. mg^{-1}) enquanto outras espécies bacterianas mostraram atividade em valores *MIC* ≤10 mg. mL^{-1}. Entre as flores, a *M. chamomilla*, demonstrou atividade antimicrobiana ligeiramente maior enquanto nas folhas, a *S. officinalis*, juntamente com a *R. officinalis* foram as que tiveram os melhores resultados.

Adicionalmente, foi realizada a modelação da extração de antocianinas monoméricas utilizando um método de extração assistido por microondas (*MAE*) da *L. pedunculata*, colhida no Algarve-Portugal. Foram selecionadas três variáveis independentes para duas variáveis de resposta incluindo valores de TAA e TMA, entre as variáveis a potência de microondas apresentou um valor otimizado de 465 W para a extração de antocianinas monoméricas.

No final deste estudo, foi ainda determinada a atividade antidiabética e anti-*Alzheimer* das infusões obtidas a partir das sementes de *P. anisum*, Portuguesas, utilizando as enzimas *α-amilase* e *acetilcolinesterase* (*AChE*) respetivamente, confirmando assim a existência de atividade desta infusão. Os ácidos gordos livres, bem como os triterpenos e esteróis, foram os compostos presentes em maior quantidade nas sementes desta planta. Os resultados de inibição de atividade das enzimas e radicais livres testados para esta infusão aquosa demonstraram que os compostos com atividade sequestrante de radicais livres podem não participar de modo competitivo nas reações de supressão de enzimas com substratos, apesar destes dados são necessários testes, *in vivo*, para obter resultados mais conclusivos.

Este trabalho teve como objetivo principal identificar novas fontes naturais de compostos bioativos que possam ser utilizadas na promoção da saúde humana e em particular no tratamento de doenças crónicas, como o *Alzheimer*, diabetes, cancro e *Parkinson* e ainda infeções bacterianas. Este estudo mostrou que as plantas Portuguesas e Iranianas testadas podem ser fontes potenciais de compostos com uma grande diversidade de aplicação para a saúde, assim com aplicação em diferentes produtos nutracêuticos, farmacêuticos e cosméticos.

List of Publications

Publications in international peer-reviewed Journals

1. Vahid Farzaneh, Isabel S. Carvalho. 2014. A review of the health benefits potentials of herbal plant infusions and their mechanism of actions. Industrial Crops and Products, Volume 65, 247-258 (Published).
2. Vahid Farzaneh, Isabel S. Carvalho. 2015. Release kinetics and *encapsulation efficiency* of bioactive compounds through encapsulated *Olea europaea* L. var. sylvestris., Leaves' infusion. South African Journal of Botany (Under review).
3. Vahid Farzaneh, Jorge Gominho, Helena Pereira, Isabel S. Carvalho. Modelling of the extraction and screening of bioactive compounds of anise infusion using *GC-MS* analysis. Applied Biochemistry and Biotechnology (Under review).
4. Vahid Farzaneh, Isabel S. Carvalho. Modelling of the release kinetic and *encapsulation efficiency* of *phenolic* compounds in *simulated gastric fluid* (*SGF*) using *RSM* and *ANN*. Journal of Food process Engineering (Got revision).
5. Vahid Farzaneh, Jorge Gominho, Helena Pereira, Isabel S. Carvalho. Screening of the bioactivity potential of some reputed plants using *GC-MS* analysis. Journal of Food Science and Technology (Under review).
6. Vahid Farzaneh, Jorge Gominho, Helena Pereira, Isabel S. Carvalho. Medicinal chemistry, phytochemical profiles and the potential health benefits of a selected group of plants. Journal of the Science of Food and Agriculture (Under review).
7. Vahid Farzaneh, Isabel S. Carvalho. Modelling of microwave assisted extraction (MAE) of *anthocyanin*s (TMA). Journal of Applied Research on Medicinal and Aromatic Plants (Under review).
8. Vahid Farzaneh, Jorge Gominho, Helena Pereira, Isabel S. Carvalho. Screening of the *anti-oxidant* and enzyme inhibition potentials of Portugues *Pimpinella anisum* L., seeds by *GC-MS*. Medicinal Chemistry Research (Revised version submitted).

Publication in Proceedings of Scientific Meetings

1. Vahid Farzaneh, Isabel S. Carvalho. 2015. Optimization of the release kinetics of bioactive compounds of encapsulated *Olea europaea* L. var. sylvestris., infusions (Abstract). Journal of Food Processing and Technology (Published).

Oral Communication in Scientific Meeting

1. Vahid Farzaneh, Isabel S. Carvalho. Optimization of the release kinetics of bioactive compounds of encapsulated *Olea europaea* L. var. sylvestris., infusions. 5th Euro-Global Summit and Expo on Food & Beverages, 16-18 Jun 2015, Alicante, Spain.

Poster presentation

1. Vahid Farzaneh, Jorge Gominho, Helena Pereira, Isabel S. Carvalho. Screening of *hypoglycemia*, *anti-amnesic* and *anti-bacterial* potentials of some plant species by *GC-MS* analysis. 2º Simposio Nacional, Promocao de uma Alimentacao Saudavel e Segura, Qualidade Nutricional & Processamento Alimentar. SPASS 2015, 26 de novembro de 2015, Lisbon, Portugal.

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List of Abbreviations

AAD (*Absolute average deviation*)

Ab (Absorbance)

ABTS (*$ABTS^{\cdot+}$* free radical inhibition activity)

ACAT (*Acetyl-CoA C-acetyltransferase*)

AChE (*Acetylcholinesterase*)

AD (*Alzheimer's* disease)

AMU (*atomic mass unit*)

ANN (*Artificial Neural Network*)

ANOVA (*Analysis of Variance*)

ATCI (*Acetylthiocholine iodide*)

BA (*Betulinic acid*)

Bl% (bluish)

BO (*Borneol*)

CAS (*Calcium Alginate Starch hydrogels*)

CCD (*Central composite design*)

Chl (*Chlorophyll*)

CLA (*Conjugated linoleic acid*)

CNS (*Central nervous system*)

Colon (*HT-29*) cancer cell lines

Cyclooxygenase-2 (*COX-2*)

DAG (*Diacylglycerol*)

^{o}C (Degree Celsius)

D (Dimension)

DF (Factor dilution)

DMSO (*Dimethyl sulphoxide*)

DNA (*Deoxyribonucleic acids*)

DPA (*Disc Propagation Assay*)

DPPH (*$DPPH^{\cdot-}$ free radical scavenging activity*)

DNS (*Dinitrosalicylic acid*)

DTNB (*5,5'-dithiobis-(2-nitrobenzoic acid*))

Dw (Dry weight)

EE (*Encapsulation efficiency*)

EI (*Electron ionization*)

FCT (*Faculty of Science and Technology*)

FRAP (Ferric Reducing *Anti-oxidant* Power)

GAE (*Gallic acid equivalent*)

GC-MS (*Gas Chromatography-Mass Spectrometry*)

G (Gram)

Gr^- (*Gram-negative*)

Gr^+ (*Gram-positive*)

HDL (*High density lipoproteins*)

H (Hour)

IC50 (*Inhibition concentration*)

ITM (*Iranian Traditional Medicine*)

IL-1β (*Interleukin-1β*)

LDL (*Low density lipoproteins*)

LNCaP (*Prostate cancer cell lines*)

MAE (*Microwave Assisted Extraction*)

MAG (*Monoacylglycerols*)

MAX (Maximum)

M bar (Millibar)

MDA (*Malondialdehyde*)

Mg (milli gram)

MIC (*Minimum Inhibition concentration*)

Min (minute)

MLP (*Multi-layer perceptron*)

MTT (*3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyl tetrazolium Bromide*))

MSD (*Mass selective detector*)

MSE (*Mean square error*)

Mw (Molecular weight)

MXR (*Mitoxantrone*)

ND (Not detected)

NO (Nitric oxide)

$No^{\cdot}$ (*Nitric oxide radical*)

NOS (Nitric oxide synthase)

$OH^{\cdot}$ (*Hydroxyl radical*)

PCA (*Plate count agar*)

Peroxynitrite (*$ONOO^-$*)

pNPG (*p-nitrophenyl α-D-glucopyranoside*)

PSE (*Phytosterols*)

PUFA (*Polyunsaturated fatty acids*)

QE (*Quercetin equivalent*)

RCS (*Reactive chlorine species*)

RD% (Reddish)

R^2 (*Correlation coefficient*)

RMSE (*Root mean square error*)

RM (Room temperature)

RNS (*Reactive nitrogen species*)

ROS (*Reactive oxygen species*)

RP (Reducing Power)

RSM (*Response Surface Methodology*)

SD (*Standard deviation*)

SGF (*Simulated gastric fluid*)

Single oxygen (*$1O^-2$*)

STZ (*Streptozotocin*)

Superoxide anion (*O_2^-*)

TAG (*Triacylglycerol*)

TanH (*Hyperbolic tangent*)

TBA (*Thiobarbituric acid*)

TCC (Total *Chlorophyll* Contents)

TE (*Trolox equivalent*)

TFC (Total *Flavonoids* Contents)

TMA (Total monomeric *anthocyanin*)

TMS (*Trimethylsilyl*)

TPC (Total *Phenolic* Compounds)

TPTZ (*2,4,6-Tripyridyl-s-Triazine*)

Tumour necrosis factor-α (*TNS-α*)

µm (Micrometer)

Ye% (Yellowish)

CHAPTER 1.

Motivation, Aims and Thesis Outline

1.1.Thesis Motivation

Today, the consumption of natural products with medicinal potential is improving. Natural phytochemicals accessible in various sources such as leaves, branches as well as the roots and flowers of plants are noticed extensively due to their bioactivity; well-being advantages and functional ingredients. The various known advantages of herbaceous extracts, such as being *anti-diabetic, anti-Alzheimer's* disease, *anti-carcinogenic, anti-Parkinson's*; *anti-radical* as well as *anti-microbial* potentials have been estimated at different functions. Unlike chemical medicines that act with several negative physical and mental adverse effects, herbal medicines have presented the least adverse effects. Most of the reputed plants with diagnosed medicinal properties belong to the plant species *Compositae, Umbelliferae, Labiatae* (*Lamiaceae*), *Myrtacae* and *Lauracae*.

Previous findings have shown that the medicinal properties of the explored herbs are often caused by the existence of bioactive and mainly *phenolic* compounds [1]. The examined extracts have been often discussed by *in vitro* assays for the determination of potential *anti-oxidant, anti-radical, anti-microbial, anti-diabetic* and *anti-Alzheimer's* properties.

The foundation of the warp and woof process of extraction using various solvents has been screened in various studies [2, 3]. The target compounds' extraction is composed of multiple phases and depends on the nature of the sample matrix and the category and behaviour of target compounds within the matrix. The use of organic solvents, such as ethanol, methanol and acetone, as well as boiling water, might extract undesirable compounds. To avoid this phenomenon in the extraction procedure of this project, the authors have used water with two different temperatures (RT and 90 ºC) and have optimised the extraction procedure using *microwave-assisted extraction* (*MAE*) to prevent the aforementioned disadvantages. The main differences in the mechanism of extraction by convection (normal extraction) and *MAE* were mentioned. In the heat transfer in the convectional process, convection, conduction and radiation phenomena from external to internal surfaces play substantial roles, while in *MAE*, the energy is transferred directly to the materials through the conversion of electromagnetic energy into thermal energy [4]. Therefore, if the solvent (water in this study) is polarised and contains ions, it could therefore absorb microwave energy to heat up the environment, therefore the *MAE* method has been selected and performed in this thesis.

The study on the identification of various plants with potential health benefits and the characterisation of their bioactive compounds presenting different potential health benefits was the major aim in the expansion of this project. Several established facts have been considered when defining this thesis, from which the following are kept on mind as the predominant facts:

1. The parts of plants (leaves, seeds, flowers, roots), their tissue (wooden and and/or soft and crisp) and the structures of the prepared beads of the encapsulated extracts could reveal substantial impacts in the release of the bioactive compounds from the matrix into solvents. Hot water acts as the main influential factor in the extraction of available bioactive compounds of plant tissue. Although extraction at higher temperatures might decompose the bioactive compounds' profiles and this loss might limit the use of thermo-related extraction approaches of the bioactive compounds [5], the optimisation of the extraction conditions such as the time of extraction and extraction method could increase the extraction performance.
2. Solvent temperature express an effective impact on the physico-chemical properties (colour, viscosity) and bioactivity potentials of the extracts [6]. The bioactivity includes different parameters such as *anti-diabetic*, *anti-Alzheimer's* and *anti-Parkinson's diseases*, *anti-carcinogenic*, *anti-microbial* and other properties, some of which have been studied in this survey.
3. An extensive range of phytochemicals, mainly including *fatty acids*; *terpenoids*; *sterols* and others might be detected in extracts [7, 8]. On the other hand, several studies have revealed *phenols* as the major phytochemicals with bioactivity and potential health benefits in plants [9], and furthermore, the existence of the higher correlations between *phenolic* compounds and different detected bioactivity parameters of plants might be established [10].
4. The encapsulation of the infusions with bioactivity using the calcium alginate method might be efficient in controlling the release kinetics of compounds [11, 12]. The optimised concentration of filler substances such as casein and starch in sodium alginate beads could control the release kinetic of bioactive compounds in both water and *simulated gastric fluids*. *Artificial Neural Network* (*ANN*) and *Response Surface Methodology* (*RSM*) could be applied in the modelling of the extraction method and release kinetics of bioactive compounds. *ANN* and *RSM* tools might demonstrate a higher performance in the modelling of the release

kinetics of encapsulated compounds [13, 14]; therefore the authors track both of these and compare the obtained results.

1.2. Research Aims

In the current research, the major objectives were to discover the plants with the most potential health benefits including *anti-oxidant*, *anti-radical* reducing power, *anti-bacterial*, *anti-diabetic*, and *anti-Alzheimer's* as well as finding which parts of the examined plant species contain considerable quantities of bioactive compounds and which plant species show higher bioactivity. Moreover, the profile of bioactive compounds was characterised using *GC-MS* analysis to reveal what categories of the compounds are mostly associated with presenting potential health benefits.

To accomplish the main objectives, different specific objectives were considered throughout the study:

1. The development and validation of simple and/or environmentally authoritative extraction methods of bioactive compounds with the optimised conditions from plants used traditionally in Portugal and Iran with the least unwelcome extracted compounds.
2. The development and validation of an appropriate encapsulation method for the infusions combined with the optimal concentration of potato starch and a study of the physico-chemical properties of the prepared beads and release kinetics of the encapsulated bioactive compounds in different environments as solvents such as the simulated human gastro-intestinal environment (*simulated gastric fluid* (*SGF*)).
3. The development and validation of *in vitro* based experiments for the determination of reducing powers, *anti-radical*, *anti-diabetic* and *anti-Alzheimer's* disease potentials of various plant extracts as well as the *anti-bacterial* activities and a comparison of the obtained data.
4. A characterisation of the bioactive compounds profile of the plants' extracts using *GC-MS* analysis.
5. The modelling and optimisation of the extraction conditions of the bioactive compounds of plants and/or released kinetics of the encapsulated extracts using *Response Surface Methodology* (*RSM*) and *Artificial Neural Network* (*ANN*) tools.

1.3. Outline of the thesis

With respect to the main objectives of this study, the current thesis presentation is arranged into 10 different chapters.

In the **Current Chapter** (**Chapter 1**), the motivation, research objectives and the thesis outline have been organised.

In **Chapter 2**, the different potential health benefits of medicinal plants, including *anti-oxidant, anti-diabetic* and *anti-carcinogenic*, alongside the potential *anti-microbial* properties and their mechanisms of action have been reviewed, discussed and interpreted. Moreover, the *correlation coefficient* between the bioactive compounds and detected health potential parameters of the previous studies were revealed.

In **Chapter 3**, the encapsulated extracts of Portugues *Olea europaea* L. var. sylvestris., leaves using the calcium alginate method combined with different concentrations of potato starch (*Calcium Alginate Starch* beads (*CAS*)) along with the different extraction times of the beads were optimised for the release kinetics and *encapsulation efficiency* of the bioactive compounds with *anti-oxidant* properties. The impacts of the different concentrations of the potato starch on the visual and physical properties of the prepared beads were also screened.

In **Chapter 4,** Portugues *P. anisum* L., seeds were selected to model the extraction of bioactive compounds. Two different independent variables were optimised for eight selected responses (*anti-oxidant* parameters) by the *central composite design* (*CCD*) of the *Response Surface Methodology* (*RSM*). The bioactive compounds' profile was characterised using *GC-MS* analysis.

The *simulated gastric fluid* (*SGF*) solution has been used in the screening of the *encapsulation efficiency* and release kinetics of the encapsulated bioactive compounds of *Alisma plantago-aquatica* L., leaves' extract in **Chapter 5**. Three independent variables have been optimised using the *Response Surface Methodology* (*RSM*) and *Artificial Neural Network* (*ANN*) models for the optimisation of the *encapsulation efficiency* of TPC as a response. The reliabilities of the *RSM* and *ANN* models have been compared and interpreted.

The 10 selected Iranian plants, including three leaves; three seeds and four flowers were studied in **Chapter 6** for different bioactive compound contents and *anti-oxidant* parameters. Three leaves and two seeds were also characterised using *GC-MS* analysis to identify the bioactive compounds' profiles and their proportions.

In **Chapter 7**, the inhibition activities of 10 selected plant extracts against three different enzymes that might be attributed in revealing *diabetes* and *Alzheimer's* diseases were studied. The *anti-bacterial* potential of hot and cold aqueous infusions of those selected plants against five different bacterial species associated in revealing foodborne pathogens and wound infections were also determined. The relation between detected enzyme inhibition activities and characterised compounds with *GC-MS* analysis of the extracts were interpreted.

The extraction of the total monomeric *anthocyanins* (TMA) content from *L. pedunculata* L. subsp. pedunculata., has been modelled in **Chapter 8,** using the *Box-Behnken design* (*BBD*) of *Response Surface Methodology* (*RSM*). Three independent variables, including microwave irradiation power; irradiation time and solvent to sample proportion; have been optimised for the maximum values of the responses including TMA and TAA parameters. The reliability of the design with the optimal conditions has been reported.

In **Chapter 9**, the *anti-oxidant* and some enzymes (attributed in the revealing of chronic disorders) inhibitive activities of Portugues *P. anisum* L., seeds have been screened, the compound profiles of this plant have been characterised; the outcomes of the experiments were interpreted with the characterised compounds with *GC-MS* analysis.

Finally, **Chapter 10** reveals the overall conclusions of the results obtained and the main achievements of this study.

1.4. Work Plan of the Thesis

To accomplish the objective proposed in this thesis, this research has been expanded into four different elements including sampling, extraction of bioactive compounds, experiments and outcomes exhibition, regarding the progression presented obviously in Figure 1.1.

The major parts of this study have been performed in:

- The Food Science Lab (FSL), *Faculty of Science and Technology* (*FCT*), University of Algarve

GC-MS analysis has been carried out in:

- Center for forest studies, Instituto Superior de Agronomia, University of Lisbon.

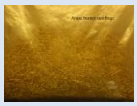
Phase 1: Sampling

Iranian plants including three leaves, three seeds and four flowers along with three Portuguese plants species including one seed, one leaf and one aerial part).


Garden thyme


Rosemary


Kitchen sage


Anise burnet saxifrage


Garden lovage


Coriander


Blacksamson Echinacea


Common borage


Water plantain


German chamomile


Common marshamallow


Erva-doce


Zambujeiro oliveira


Lavenders

Phase 2: Sample preparation and extraction

Sample preparation: Dry sample; grinding; homogenization; extraction and analysis.

- Hot and cold aqueous extraction along with *microwave assisted extracts* (*MAE*).
- Soxhlet extraction for *GC-MS* analysis.
- Encapsulation of the extracts using calcium-alginate method for release kinetics study.


Garden thyme


Anise burnet saxifrage


Kitchen sage


Coriander


Rosemary


Erva-doce

Phase 3: Sample analysis

Bioactivity tests

Bioactive compounds content

- Total *phenols* (TPC)
- Total *flavonoids* (TFC)
- *Total monomeric anthocyanins* (TMA)

- *Anti-oxidant* activity (TAA)
- *Anti-radical* (*DPPH*; *ABTS*),
- Reducing power (FRAP; RP)
- *Anti-diabetic* (*α-Glucosidase* and *α-Amylase* inhibition activities)
- *Anti-alzheimer* (*Acetylcholinesterase* inhibition)
- *Anti-bacterial*

***GC-MS* analysis**

- Simple *polyphenols* and bioactive compounds.

Phase 4: Outputs

Development of experimental outcomes, *GC-MS* analysis and modelling

Paper I: A review of the health benefit potentials of herbal plant infusions and their mechanism of actions.
Paper II: Release kinetics and *encapsulation efficiency* of bioactive compounds through encapsulated *Olea europaea* L. var. sylvestris., leaves' infusion.
Paper III: Modelling of the extraction and screening of bioactive compounds of anise infusion using *GC-MS* analysis
Paper IV: Modelling of the release kinetic and *encapsulation efficiency* of *phenolic* compounds in *simulated gastric fluid* (*SGF*) using *RSM* and *ANN*.
Paper V: Screening of the bioactivity potential of some reputed plants using a *GC-MS* analysis.
Paper VI: Medicinal chemistry, phytochemical profiles and the potential health benefits of a selected group of plants.
Paper VII: Modelling of *microwave assisted extraction* (*MAE*) of anthocyanins (TMA).
Paper VIII: Screening of the *anti-oxidant* and enzyme inhibition potentials of Portuguese *Pimpinella anisum* L., seeds by *GC-MS*.

Figure 1.1 Work plan performed through the development of this research.

The main experimental outcomes achieved during the three initial phases are presented as scientific articles revealed through Chapters 2 to 9 of this thesis.

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CHAPTER 2.

Health benefit potentials of herbal plant infusions and their mechanism of actions

This chapter reviews different studies performed on the potential health effects of medicinal plants. The main aim of this chapter is discussing the mechanism of actions of the potential health benefits of the studied plants

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A review of the health benefit potentials of herbal plant infusions and their mechanism of actions

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Abstract

Nowadays, there has been a universal propensity to application of natural phytochemicals accessible in diverse intrinsic rich sources such as fruits, leaves, branches as well as roots of different plants because of existence of substituents with bioactive potentials, well-being advantages and functional ingredients. The preservative consequence of many herbs and spices are proposing the existence of compositions with varied remedial specifications in their structures. Plants are classified by geographical status and inharmonious territory that created further than several thousands herbages with various advantages. The various superiority of herbaceous infusions, such as *anti-diabetic*, *anti-carcinogenic*, *anti-microbial* and *anti-oxidant* are appeared in diverse functions. *Phenolic* as well as secondary metabolite components have been reported as the major components having health superiority, and follow this, superb relationship between those advantages and various measured *anti-oxidant* values, such as reducing power, scavenging and inhibition capability of free radicals, metal gelation activity and etc. are observed. Hence, the current review intends to debate the effectual fragments of medicinal plants with vulnerary potentials and explains their mechanism of functions.

Keywords: Herbal plant infusions; *anti-oxidant*; *anti-diabetic*; *anti-carcinogenic*; TPC; TFC.

2.1. Introduction

Plants with medicinal potentials and their secondary metabolites have been identified and applicated in dishes from the earliest history of human settlement; herbal medicine in ancient systems as well as advanced medicine has created one of the most effectual science bases for security in different areas of the mankind. For many of years, herbal plants have been used for distinct goals. Herbal plants are generally defined as one year gramineous herbs with not any strict contexture. Dependent on the type of plants, one part including, (flowers, leaves, seeds, branch or roots) or whole sectors (aerial parts or roots) could be accomplished in treatment of acute and chronic diseases, food products and nutritional improvement. After entering herbs into digestive systems, body initiates consuming them and stave off the waste as well as free radicals bounded to the fibres of the herbs and in general purify useful contents into body cells. Due to unending advantages and considerable benefits, plants with medicinal potentials are anticipated to get applied widely in human nutrition to improve not only the healthiness of human's body cells but also ensure the mental or psychological health. Unlike chemical medicines that act with several negative physical and sometimes mental adverse effects, herbal treatments have shown minimal side effects or maybe no losses. There is a global tension in the application of recognized potent herbal plants as a food ingredient, medicines and cosmetic products. Regarding the fact; plants are widely applicable for treatment of diseases.

Anti-oxidative properties of the extracts usually are expressed, not only by some recognized compounds such as *carotenoids*, *flavonoids* or *polyphenols* available, but also by secondary metabolic agents through several mechanisms. The two main mechanism of actions of *flavonoids* are that they expose UV protection and express metal chelation effects (Aleksic & Knezevic 2014). *Polyphenols* are involved in a large variety of various compounds depending on the types of the target plants, such as *rosemarinic acid* (in rosemary). *Anti-inflammatory* properties of some herbal plants are proposed as one reason of their *anti-cancer* potentials as well. The relevant compounds decrease the inflammatory leg which betides as a proof of dermal ageing (Aleksic & Knezevic, 2014). However, the application of herbs against diseases is not widely popular as much as it might have been expected. The institute of national cancer researches has diagnosed more than about 30,000 herbs with *anti-carcinogenic* potentials (Kaliora *et al*., 2014). The reputed plants with

diagnosed medicinal properties appertain to the clan as follow: *Compositae, Umbelliferae, Labiatae (Lamiaceae), Myrtacae,* and *Lauracae*. Carcinogenics have generally been individualized in the complex and multistage functions involved in causing breaking out in the ruined cells (Franco *et al*., 2008). Former findings have shown that the medical properties of the explored herbal herbages are often created by the availability of *phenolic* compounds. The examined infusions are often discussed the extent of in *vitro* assay for characterization of *anti-oxidant* activities, including the reduction of Fe^{3+} and Fe^{2+}, the capability to scavenge $DPPH^{\cdot-}$and inhibit $ABTS^{\cdot+}$radicals and conservation against Fe^{2+} inducing lipid peroxidation or metal chelating potentials. Therefore, this research characterizes and discusses the *anti-oxidative*; *anti-diabetic* and *anti-microbial* activities of the selected plants and specifies the most bioactive components of different herbal plant species commonly used in Portugal and Iran and explains their mechanism functions against disorders attacking human cells.

2.2. Principle of the extraction by solvent

The foundation, warp and woof of the process of extraction by solvent is studied in various inquiries (Carvalho *et al*., 2011; Kaliora *et al*., 2014). Concisely, the performance of the extraction substantially depends on the nature of the sample matrix, the target type to be extracted and manner of the target compounds within the matrix. Presented flow of extraction of inharmonic samples by a model, pointed the sample particles are permeable and inscribed by an organic stratum. The target compounds extraction have been composed by multiple steps: Initially, to separate the target from the extraction locus, the compound is first absorbed from its primal site into the sample matrix, in second stage, target is dispersed from organic tissue to enclose to the matrix-fluid juncture. Then, target travels into the extraction disposition, where is distributed within the lenticels, thereby reaching the leg of the extraction that is impressed by relocation. The ultimate step of the extraction procedure is the locality and circumstances of the target within the sample structure. Thereupon, the extraction flow includes several stages as follows: (1) absorption to the similitude of the matrix; (2) dissolving in a solvent hole or absorption on the surface; (3) dissolving in a matrix micro hole and chemically bonded to the matrix; (4) dissolving in solvent (Pawliszyn, 2003). The specification of matrix has a central impress in the extraction procedure. Camel in 2001

introduced extraction method of targets, and solubilisation as most important stages in herbal plant infusions, in the other word determination the type of solvent is a critical leg in this subject (Camel, 2001). Application organic solvent, such as ethanol, methanol and acetone as well as boiling water, might extract some officious compounds (toxic compounds in case of existence); therefore, for the solvent extraction procedure, application of water at low temperature is more appreciated than organic solvent and boiling water, due to the feasibility to eliminate or diminish toxic components in the final obtained infusions. Thus, the applications of the obtained extracts in the final products have more advantages as well as the minimal negative adverse effects. Therefor it is tried in this study to extraction process get performed by water as a solvent.

2.3. *Anti-oxidant* activity

The most reputed groups of combinations with *anti-oxidant* potentials in herbs are presented as follows: known *vitamins*, such as *vitamins* E and C, *polyphenols*, *flavonoids* as well as pigments amongst *carotenoids* and *anthocyanins*. *Anti-oxidants*, even in low concentrations, significantly retard or prevent oxidation reactions of susceptible ingredients like lipids. In addition, *anti-oxidants'* actuality are associated with decreased *DNA* damages and lipid peroxidation, which adjust the immune performance and reduce virulent metamorphosis of the cells (Torbeyns, 2012-2013). Several studies have introduced, *phenolic* compounds as the principal bioactive phytochemicals with *anti-oxidant* activities and health advantages (Javanmardi *et al*., 2003). Furthermore existence highly strong *correlation coefficient* between *phenolic* compounds and various determined *anti-oxidative* activity parameters of several sectors of plants has been proved (Qingming *et al*., 2010). The TPC quantity in 23 varieties of *Ocimum basilicum* L. accession infusions from Iran were determined and expressed that total *phenolic* content varied in multiple accessions, also this study showed, the central sections of plants' structure are demonstrating the higher quantity of TPC than other parts; furthermore, total *anti-oxidant* activity (TAA) *(Y)* and total *phenolic* contents (TPC) of Iranian basils offered a good linear correlation coefficient together (R^2=0.71*)*, (Javanmardi *et al*., 2003).

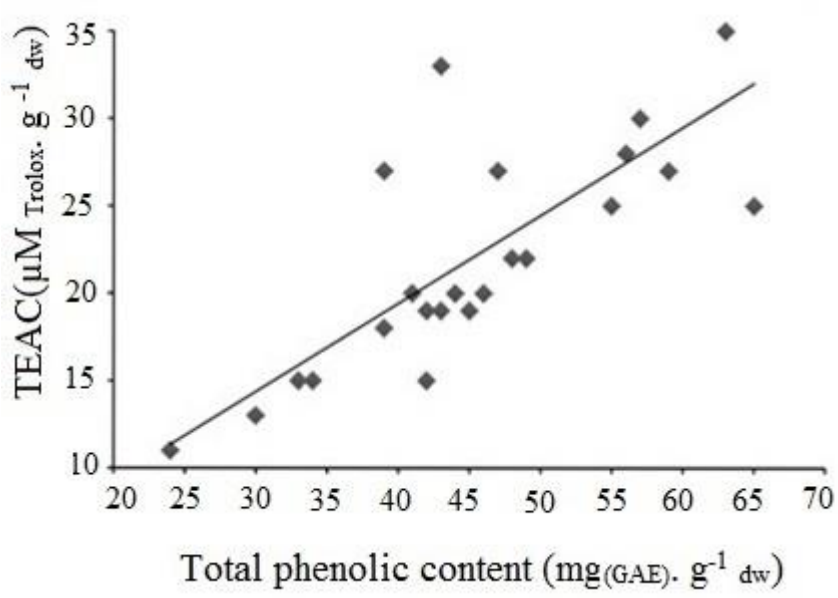


Figure 2.1 Linear correlation of *Trolox equivalent anti-oxidant* capacity (TEAC) (*Y*) versus the Barnes *phenolic* content (*X*) of 23 Iranian *Ocimum basilicum* L., using regression program (%) (Javanmardi *et al*., 2003).

Some researchers also proposed that Iranian *Ocimum accessions* L., which often are incorporated in Iranian diet daily, are strong *free radical scavengers* and could be announced as wealthy sources of innate *anti-oxidants* for pharmaceutical aspects as important as nutraceutical and commercial goals. Regarding, existence an extensive range of various indigenous *anti-oxidant* compounds involving *phenols* in Iranian *Ocimum accession* L., specification and evaluation of those compounds with contrast the contents of each species necessitates more researches (Javanmardi *et al*., 2003). Due to availability of various quantities of bioactive compounds, different plants offer different bioactivity. Each plant usually contains different *phenolic* compounds, which offer various strengths of *anti-oxidant* activity. By using *HPLC* analysis, *rosmarinic acid* has been determined as a predominant *phenolic* acid in Iranian basil accessions. *Phenols* and flavonoids are known as compounds with biological activities in herbs and have repeatedly been identified as natural *anti-oxidants* in wide range of fruits and vegetables species with bioactive and health avails potentials. For instance, organic acids, such as *vanillic*, *ferulic*, *caffeic* are available in extensive forms in some plant species. Also in the other research flavonoids and total *phenolic* contents of herbal plant extracts are identified as compounds with high radical scavenging capabilities (Torbeyns, 2012-2013). Studies on *phenolic* composition and *anti-oxidant* capacity of six *Artemisia* species showed a

highly linear relevancy (R^2=0.85) between the TPC and $DPPH^{\cdot-}$ scavenging activity values of *Artemisia* (Carvalho *et al*., 2011). After determination the $DPPH^{\cdot-}$ scavenging activity value of *Artemisia* leaf extract, it was approved; *Artemisia* species are portaging *free radical scavengers*. Inquiry on the *phenolic* content of herbs is extensive and focused on the proper subset plants of *Lamiaceae* clan due to existence of extensive confine of bioactive potentials they are demonstrating (Fecka & Turek, 2008). Some scientists have indicated, on some selected Greek herbal infusions examined, *R. officinalis* L., has shown the lowest contents of TPC value while the highest magnitude was observed in *St John's Wort*. According to the results, as have been expected *St John's Wort* exhibited the greatest bioactivity and *rosemary* the minim, whilst two other plants in the study, *i.e. Cretan dittany* and *Marjoram,* indicated the equal quantity. Moreover a positive substantial *correlation coefficient* among obtained value for $DPPH^{\cdot-}$ scavenging power with *flavonoids* and *polyphenols* contents was found (p<0.01) (Kaliora *et al*., 2014). To provide more, the similar outcomes were observed between p-OH *benzoic acid*, *protocatechuic acid*, *gallic acid*, *epicatechin*, *cathechin*, *kaempherol* and *querecetin* and *anti-free radical* activities (Kaliora *et al*., 2014). Some researchers have evaluated the *correlation coefficient* between *anti-oxidant* activity (obtained by different assays) and TPC value, and submitted existence an intense relevance in all carried out assays with an exception. Diverse *anti-oxidant* compounds also vary in displaying biological potential, and might act cooperatively, antagonistically or synergistically together or with other compounds available in green extracts. With notice to the observed results of different researches, it could be concluded that *polyphenols* are probably the most abundant and dominant *anti-oxidant* doer in herbal plant extracts. However it was also established that there are higher quantity of diverse compounds in plants that offer *anti-oxidant* potentials (Gonçalves *et al*., 2013). Regarding the observed findings, it is apprehensible that climate and geography perform a dominant role in the quantity and type of bioactive compounds in plants. The differences might be due to different light source intensity, available water supplies and physico-chemical properties of the cultivated soils; due to this fact, the differences in the *anti-oxidant* and medicinal potentials of plants have been observed, in addition of their species could be justified by the aforementioned environmental factors while the content of bioactive compounds depending on which part of plants are used have shown different values. Among the different Iranian

and British *Thymus* which were the purpose of one study, the higher *anti-oxidant* activity was observed in Iranian's, while the lower value was found in British plants. This phenomenon demonstrates the figure of environmental parameters, including physico-chemical properties of the cultivated soils, climate and geography conditions, available water supplies, intensity and period of sunlight, etc., on the quantity and type of bioactive factors. Those findings also approved that the great part of *anti-oxidant* activities in the herbs is created due to the accessibility of *phenolic* compounds. This finding is in line with the observation of prior researches that showed a similar *correlation coefficient* value between TPC and *anti-oxidant* activities in various target plants (Alizadeh *et al*., 2010). According to the former findings, it could be resulted that one *anti-oxidant* singly is unlikely able to protect the cells against wide range of acute and chronic disorders generated by a widespread diversity of known free radicals. Only a blend comprising of towering operators, with synergic authority, might create impressive and consistent protection against excess free radicals and boost the body's internal defence system.

2.4. $DPPH^{\cdot-}$ scavenging and $ABTS^{\cdot+}$ inhibition powers

The foundation of $DPPH^{\cdot-}$ scavenging and $ABTS^{\cdot+}$ inhibition activities is based on colour alteration of the solution containing extract due to the existence of *anti-free radical* factors (Singh *et al*., 2002). Reactions based on free radicals belong in both the live cells and food systems. Creating of reactive oxygen and nitrogen molecules are considered as an integral sector of the basic physiology of action of free radicals; these arisen molecules might react with bioactive molecules and create cellular injury, and in extremity, death. Therefore, they cause or enhance the rate of catching some chronic disorders such as *cancers* and those that outbreak the *cardiovascular* systems. Synthetic or chemical components with bioactivity properties have been used to extend the stabilities of sensitive food products containing *poly unsaturated fatty acids* (*PUFA*). Therefore, the application of bio active compounds to daily diet could improve internal cellular defense systems and facilitate debarment, experimental and clinical, of hurtful oxidative reflex, as important as causing a consequential factor in physiological arrangement. The application of synthetic or chemical preservatives, such as *butylated hydroxyl-anisole, butylated hydroxyl-toluene* and *propyl gallate* is performed in generative enormous scale to control *lipid oxidation* in final food products not only within production process but also

during warehousing period; however, application of those synthetic *anti-oxidant*s in food products has been questioned due to the potential health hazard and toxicity in alive cells. Detection bioactive compounds from available native sources has received grand regard and many researches have performed regarding seeking new sources of compounds that could be accounted as potent *anti-oxidants* with the lowest adverse effects instead of synthetic and chemical ones. Furthermore, they could be formulized in several themes that might diminish oxidative reactions from occurrence. A group of researchers have shown positive linear relations between the content of total *phenols* (TPC) and radical scavenging power (R^2*=0.85)* in both *Th. Vulgaris* L., from Britain and Iran (Alizadeh *et al*., 2010). In a study performed on some Mediterranean herbal plants the observed data indicated the highest free radicals scavenging capacity for the aqueous infusions extracted by hot water as a solvent. Interestingly, cold infusions of *P. lentiscus* L., showed a greater $DPPH^{\cdot}$ scavenging activity than hot ones, Therefore it appears that, bio-activity in plants is not caused only by *phenols* and could come from bioactive oils, bio-active *vitamins*, and *carotenoids* (Javanmardi *et al*., 2003). This might be resulted that hot aqueous might not have any positive impressions in the extraction of all bioactive compounds perhaps with hydrophobic terminals such as fugacious oils. Therefore studies declared *Th. Vulgaris* L., as a plant with high radical scavenging potential and possible source of bio-active compounds, and hot water extraction as an efficient way; moreover, *thymus* is advised as an herbal plant that might be used in traditional remedies as well as nutraceutical and pharmaceutical industries. In another study, scientists determined the $ABTS^{\cdot+}$ prohibition activity and have observed different properties of beetroot infusions, this plant is shown the most drastic *free radical scavenging activity* intra all the selected extracts, with about 92% prohibition. These scientists also characterized the $ABTS^{\cdot+}$ prohibition activity of mixed juices and found out that combined juices had a more ordinary inhibitory effect at specific percentages compared to individual ones (Wootton-Beard *et al*., 2011). On the rely of the results obtained by diverse researches, it could be indicated that the free radicals' prohibition potential of plant extracts might depend on the existence of *phenolic* components, *flavonoids* and bioactive oils as effective as natural *vitamins* with *anti-oxidant* activities, such as *vitamins E* and *D*, etc. *Anti-oxidant* synergy systems contain an admixture of highly effectual bio-active compounds; together represent grand-polyphase protection against an extensive

confine of free radicals including: *single oxygen*, *superoxide*, *hydroxyl radicals*, *fatty acid peroxides* and *oxidized proteins*. Table 2.1 shows some of the most vigorous noble rich sources of *anti-oxidant* compounds.

According to the prior researches, it has been proved that the *phenolic* content is directly responsible for decline of oxidative stress. Recently, as is cited, a composite of various infusions are able to present synergistic behaviours, additionally, a wide-spreading numeral of intrinsic herbaceous extractive and chemicals are declared to have lucrative potentials on *central neural system'* functions. Those potential effectivenesses for treatment super-sensible disorders are claimed on the obtained results of treatment on animal action models; however, reaching to a confident result, needs more treatments of the model on several animal systems followed by clinical experiments to approve development of the medicinal plants in formulization of nutraceutical, pharmaceutical and cosmetic products. Furthermore, with regard to discovering new potent sources of natural *anti-oxidant*s, perfect studies on the new bio-active origins are required for producing new products with highly *anti-oxidant* properties and health benefits.

2.5. Reducing activity

The most reputed and prominent *oxy free radical species* classified are *hydroxyl radical ($HO^{\cdot}$), hydrogen peroxide (H_2O_2), single oxygen (O_2^1) organic free radicals ($RO^{\cdot}$, $ROO^{\cdot}$)* and *superoxide radical anion ($O_2^{\cdot -}$),* that incorporate in active or passive form in lipid peroxidation reactions. Electron donor reducing compounds, such as *ascorbic acid* (*AA*), *phenolic* and *flavonoids*, *carotenoids*, *vitamins* with bioactivity etc. might act as $O_2^{\cdot -}$ and $HO^{\cdot}$ *scavengers*, as well as O_2^1 quenchers and, therefore, could be got engaged in the oxidative defence functions of plants. *Flavonoids*, available extensively in most herbal plant infusions, are especially common compounds whose *anti-oxidant* potentials are indicated and evaluated as nonenzymetic lipid peroxidation in diverse orders. Scientists expressed the strength of some Mediterranean herbal plant infusions in reducing Fe^{3+} ions to Fe^{2+}. All the infusions acted as electron donors with the activity enhancing in a value depending behaviour. These scientists also found out that *P. lentiscus* cv. Chia., and *M. Communis* L., infusions offer the greatest capacity to chelate Fe^{2+} (86.64-96.14 at 1.6 mg. mL^{-1}) and were not shown any considerable discord between the chelation power of hot and cold infusions of each one (Gonçalves *et al*., 2013).

Table 2.1 Some novel sources of *anti-oxidant* components.

No	Extract name	Active elements and mechanism of actions
1	Grape seed	Including leucocyanidins: five folds superior to *anti-oxidant* potentials of *vitamin E,* increasing the influences of *vitamin C.*
2	Pine bark	Improves the body's *anti-oxidant* potentials, rich source of bio*flavonoids*, reduces most sorts of reactive oxygen or free radicals, induces creating of the cellular anti-oxidants species, superoxide dismutase and glutathione, extends the stability of *anti-oxidant vitamins E* and *C.* Pine bark infusions also contain alpha lipoic acid that reduce free radicals availability and improve the regeneration of interior *anti-oxidants*.
3	Green tea	Including the *polyphenol epigallocatechin gallate* (*EGCG*), suppresses reactive oxygen breeds and free radicals, passes the blood-brain hindrance and assists and enhances the protection procedure of brain in confront of the oxidative stress.
4	Curcuma	Including curcumin prevents lipid peroxidation and reduces superoxide and hydroxyl free radicals.
5	Glutathione	Behaviours such as a substrate of enzymes like glutathione peroxidise and omit radical peroxides, annihilate free radicals available, highly are condensed in most body tissues such as the eyes and the liver and prohibits oxidative *trauma*.
6	Mangos teen (*Garcinia mangostana* L.)	Including xanthones and diminish LDL oxidation reaction, decreases the application of other types of *anti-oxidants* such as tocopherols or *vitamin E*.
7	Pomegranate	Includes direct *anti-oxidant* potentials, improves protect levels of the internal *anti-oxidants* catalase, peroxidase and superoxide dismutase.
8	Vitaberry	Includes a potent *anti-oxidant* systems blending of fruit powders and extracts, so valuable source of *polyphenols*, *anthocyanins*, *proanthocyanin*, ellagic acid, chlorogenic acid, resveratrol and quinic acid.
9	Sea buckthorn (*Hippophae rhamnoides* L.)	Comprises *anti-oxidants* components, reduces the available superoxide radicals.
10	Resveratrol	Diminishes the generation of reactive oxygen breeds, annihilate superoxide radicals available in mitochondria and prevents lipid peroxidation.
11	Black rice	Encompasses *anthocyanin* cyanidin-3 Glucoside, diminishes age-relevant oxidative stress, health advantages in terms of medullary function.

Lipid peroxidation is an indicator of oxidative concussions to cells, generating derivatives such as *MDA* that is used to quantify the intensity of clashes on the experimented cells (Qingming *et al*., 2010). Free radicals are produced as a results of lipid peroxidation proceeding and are playing a critical role in the ruining of many neurodegenerative disorders (Oboh & Rocha, 2008). *Hydroxyl radicals* are generated when Fe^{2+} reacts with H_2O_2, that could participate in and boost lipid peroxidation reactions (Gutteridge & Halliwell, 1988). The capability of the infusions to deter hydroxyl radicals was determined in previous studies by controlling the reactions performed between *TBA* (*thiobarbituric acid*) and deoxyribose degradation products. Substantial relationship between the concentration of the infusions and inhibition rate was observed. At the minimal concentrations, the hot extracts of *P.lentiscus* cv. Chia and *L.viridis* L. showed the most highly potential in inhibition; the highest prohibition in the maximum concentration of the various examined hot extracts was obtained, about 50%. The findings of prior researches demonstrated, herbal plants, due to carrying components with reducing power, are potent and innate sources of *anti-oxidant* components which could be handled as herbal medicines with minimum negative adverse effects, unlike synthetic ones. Former researches have also demonstrated, solvents with high temperature might act in a drastic way to extract various combinations with heavy reducing properties; the deduction brought forward *polyphenols* and *flavonoids* as an effectual compounds in reducing reactions and stated, the most herbal plants are accounted as profitable medicines for treatment of diverse ailments relevant to oxidative reactions in the cells; furthermore, the observed conclusions have nominated organic solvents and hot aqueous infusions as methods with high efficiency for extraction of bioactive compound from natural resources with curative properties.

2.6. *Anti-carcinogenic* potential

Splay physiological and biochemical flows in the cells might generate side-products, such as reactive oxygen species like oxygen-centered free radicals that are considered as serious oxidant factors. Excessive generation of the free radicals might cause oxidative damage to ultrasensitive biomolecules, including *peptides*, *fatty acids* and *nucleotides*, thereupon leading to multifarious chronic disturbances, such as sclerosis, various *cancers*, diverse *diabetes*, aging and other degenerative

maladies in alive cells (Halliwell, 1994). The results of epidemiological research has produced a knowledge, proposing the most compositions with *anti-oxidant* potentials are probably *anti-inflammatory*, *anti-carcinogenic*, *anti-growth* (anti-tumour), *anti-mutagenic* and anti-sclerotic factors (Halliwell, 1994). On the base of scientific evidences obtained through experimental and clinical assays, consumption of beverages, such as tea and coffee as well as other products containing *anti-oxidant* compositions provide chemo-preventive shield against neurodegenerative, *cardiovascular*, various *cancers* and other disorders that are in growing interest nowadays in human cells. Researches on the procedure of the prohibition potentials of components widely available in beverages such as tea and coffee have proposed, the extracellular signals produced during cell proliferation are inhibited by *anti-oxidant* compounds such as *polyphenols* and *flavonoid* contents. This is offered based on the anti-inflammatory properties and inhibitory capability of *polyphenols* on the *NF-κB* activation pathways *in vivo* models of inflammation (Qingming *et al*., 2010). According to the results of performed researches ever, the ultimate aim of *anti-oxidants* in inhibition of cancer promotion is their *anti-inflammatory* potentials on spoiled cells, which occurs due to the inhibition activities of the bio-active compounds on the *NF-κB* activation causeway. At the microscopic and cellular area, copper and iron are active ions in creating free radicals; it means that specific free radical generation is prevented by the chelation of metal ions by bioactive compositions available in biological organs as such herbal plants, for instance *flavonoids*. Herbal plant infusions have achieved an extensive agreement for their medicinal activities in therapy of serious illnesses such as stomach ulcers, *cardiovascular*, prostate, colon and liver *cancers*, as important as digestive disturbances. The contents of *flavonoid* and *tannin* of the plants extracts as important as *tannin* and *polyphenol* contents in drinks like tea, might serve as good origins of *anti-carcinogenic* components (Huang *et al*., 2010) the mechanism and legs of such *anti-cancer* cytotoxicity is not well diagnosed. But the *anti-oxidant* behaviours of such those two bioactive compounds could not be relinquished. Among *polyphenols*, *epigallocatechin-3-gallate* or *tannin*, available in drinks such as tea proved a good *anti-oxidant* activity and induced apoptosis in spoiled gland cells (Chen *et al*., 2009). *Polyphenols* can cause an out of reach of the factors essential for cell-cycle improvement, and theses series of occurrence lead to retard or stoppage of cell cycle advancement and, eventually, pursuant apoptotic cell decease (Gosse *et al*., 2005).

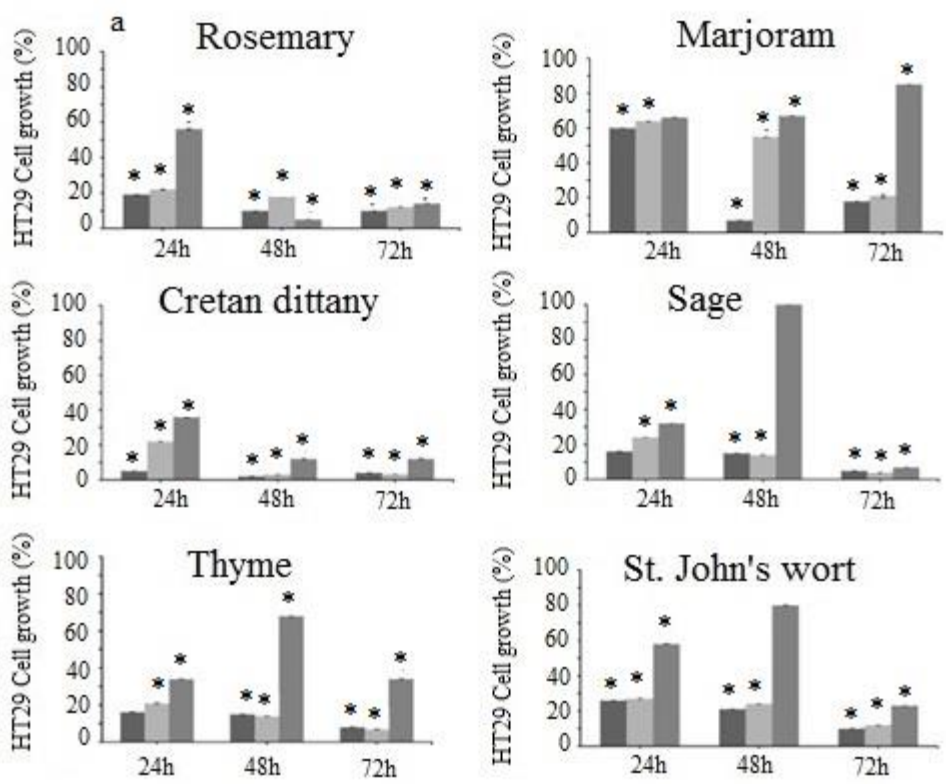
The results of diverse researches on *anti-carcinogenic* properties of bio-active compounds such as TPC and TFC have shown, these compounds involved in a splay variety, might be considered as constituents with *anti-carcinogenic* potentials. Therefore, geminating meals containing the bio-active compounds with regular foods is possibly able to diminish the rate of developing illnesses that threaten human life. According to performed researches on the *anti-carcinogenic* potentials of herbal plant infusions, *flavonoids* of diverse holdings were suggested, capable to adjust H_2O_2-induced *DNA* damages in some cell line models because of the scavenging system strength they are presenting (Cilla *et al*., 2009). As a summary, it was concluded that *flavonoids*, as good as *phenols*, might offer *anti-carcinogenic* potentials. However, it needs more researches to fully determine the mechanism of functions of *anti-carcinogenic* properties of these compounds. However, herbal infusions probably due to their capabilities in activation of cytotoxic activity and generation of cytokines have been showing *anti-carcinogenic* and health benefit properties.

2.6.1. HT29 and *PC3* culture conditions capacities

The *HT29* cell line was originally disassemble by Dr. Fogh in 1975, from a human carcinoma of the large intestinal systems. The *HT29* cells preserve certain specification of the native tissue, such as hormone recipient. Under various culture conditions, *HT29* cells tolerate a discrimination in polarized mono-lamella of mucus permeation (Fantini *et al*., 1986). The *HT29* cells, secrete various *peptides*, including factors that motivate the propagation of *fibroblasts* (Culouscou *et al*, 1987). *Human prostate cancer (PC3)* cells are classical experimental androgen-independent specimen of *prostate cancer* with high generation and metastatic capabilities (Nachshon-Kedmi *et al*., 2004). These two cells could be effective for researches on biochemical conversion of progressive phases of strict disturbances and in assessing the reflex of the decayed cells to synthetic remedies. Other teams of scientists have studied the *anti-carcinogenic* potential of herbal infusions from Greece, results have been presented in (Kaliora *et al*., 2014). Diverse concentrations and various time durations (24, 48 and 72 h) were examined, in the case of *HT29* (Fig. 2.2a) (Kaliora *et al*., 2014). The prevention of spoiled cell growth was considerable for *Cretan dittany* extract, achieving almost 95%. *Thyme* extracts expressed dose preventive potentials in extreme concentrations. Totally, *Marjoram*, *Sage* and *St*

john's wort did not show higher preventive potentials. Treating *PC3* (*prostate cancer cells*) with the same concentrations of herbal infusions for aforementioned time periods was examined (Kaliora *et al*., 2014).

The arrangement in anti-generation activity was accommodated as: *Thyme* < *St. john's wort* < *Rosemary* < *Sage* < *Marjoram* < *Cretan Dittany* (Kaliora *et al*., 2014). *Marjoram* has shown very powerful potential against *PC3* cell growth, but not against *HT29* cells. Unlike *Marjoram, thyme* has acted more effectually against *HT29* cells than versus *PC3* cell development. Also *Marjoram* presented the greatest quantum of *rosmarinic acid* content; moreover *carvacrol* concentration has been expressed the supreme value in both *thyme* and *dittany* species. Both *carvacrol* and *rosmarinic acid* have shown highly *anti-proliferative* properties versus colon cancer and human prostate cell (Encalada *et al*., 2011). In addition to cell development, as has previously been proposed, inflammation is a suffering factor in the succession of cancer cells. *IL-8* is a pro-inflammatory *cytokine* stablished and elevated in diverse classes of cancer, with numerous active impresses in tumour development and neutrophil chemotaxis (Vandercappellen et al., 2008). Scientists have inquired the effectiveness of a minimal condensation of herbal infusions on *IL-8* of aroused *HT29* and *PC3* cells, the results of which are presented in (Kaliora *et al*., 2014).



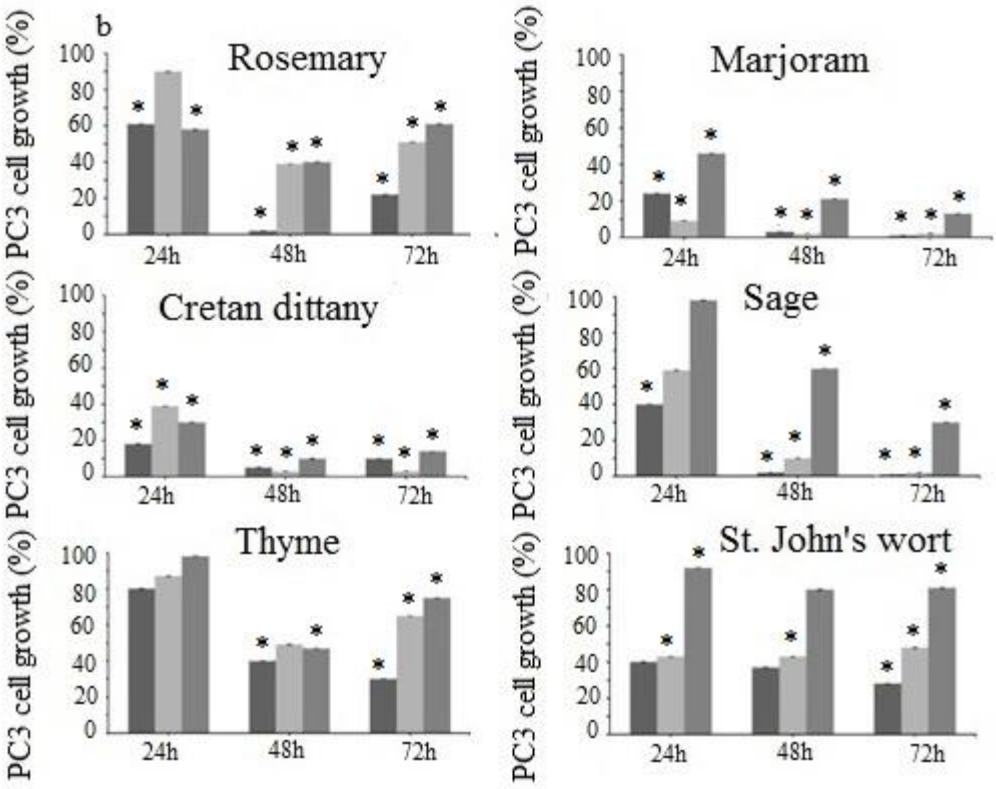


Figure 2.2 Time and dose dependent effect of herbal infusions on (a) *HT29* cell growth and (b) *PC3* cell growth. Results are expressed as percentage (%) (Mean ± *SD*) of cells in culture compared to untreated cells. Cell growth was evaluated by *MTT* assay. * Presents a statistically considerable diversity. Considerable level was adjusted at $p<0.05$. Each presented bar above indicates concentration of 1, 0.6 and 0.2 μg. μL^{-1} respectively from left to right (Kaliora *et al*., 2014).

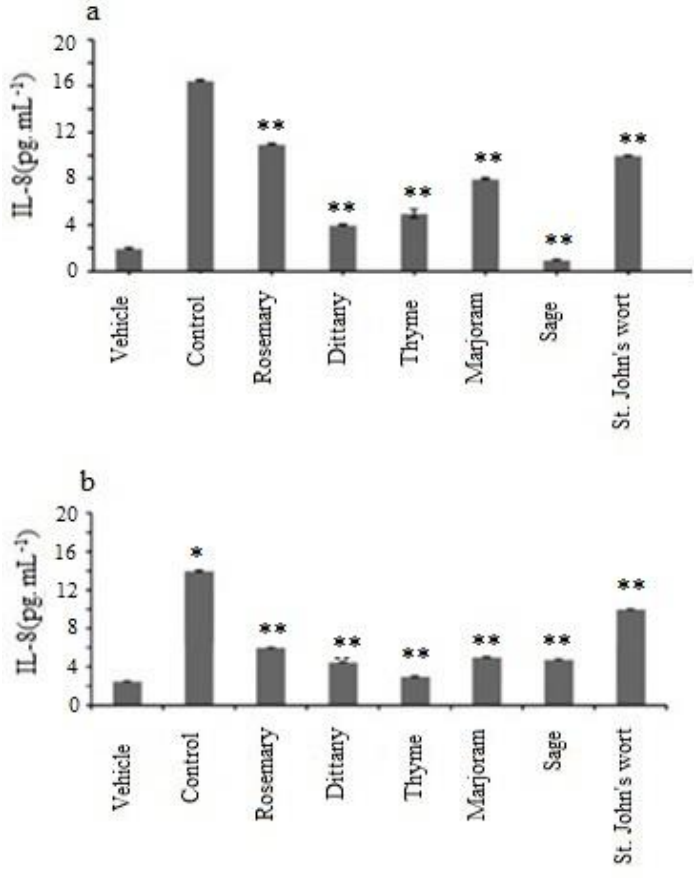


Figure 2.3 Effect of herbal infusions (in the lowest concentration 0.2 μg. μL^{-1} final in the well) on *IL-8* levels of (a) *HT29* and (b) *PC3*cells stimulated with *TNF-α*. Results are expressed as pg. mL^{-1} (mean ± *SD*). * Presents a statistically considerable diversity between vehicle and stimulated cells (control). ** Presents a statistically considerable diversity between control and cell culture treated with individual herbal infusion prior to stimulation. Substantial level was adjusted at $p<0.05$ (Kaliora *et al*., 2014).

Moreover the assumption that *NF-κB* is a molecule that mechanically concrete between cancer and inflammation is becoming more actual. In cancer, *NF-κB* activation might be due to exposure to pro-inflammatory stimulus in the promontory microenvironment, such as *TNF*-stimulation. The observations of some researchers about the chemo-suppressive activities of the assayed infusions were inquired by the diversity in phytochemicals and their additive, competitive and or antagonistic interactions at the molecular and cellular levels in spoiled cells. These researchers also attempted to correlate the existence specific *phenolic* compounds of the infusions with *anti-carcinogenic* properties; however, the results did not display any considerable clue of the infusions on preventing or suspension the growth of cancer cells.

The only exception was *rosmarinic acid* available in *Marjoram* which showed a decline of *NF-κb* in *PC3* cells ($p<0.05$) (Kaliora et al., 2014). This effect might be due to the existence of *phenols*, which proposed a considerable anti-generation effect on *colon* and *prostate cancer cells*. Among the *hydroxycinnamic acids*, *caffeic acid*, which is also available in higher amount in marjoram, is known to exhibit *anti-oxidant* activities through scavenging free radicals and reaction to oxygen species. Therefore it decreases the lipoperoxidation proceedings through the implantation of cell cycle detention and also excitation of apoptosis via prevention of nitrosation and nitration and signal conduction causeway of leg I enzymes or *acrylamide* (Huang et al., 2010). The consequence of herbal plant infusions on total *NF-κB-p65* levels of (a) *HT29* and (b) *PC3* cells aroused by *TNF-α* is presented in Fig. 2.4 (Kaliora et al., 2014). Analysing the observations of the former researches have provided that most *polyphenols* have positive attribute on prevention the advancement of cancer cells such as *rosmarinic acid* (as well as *carvacrol*) and *hydroxycynnamic acids*, like *caffeic acids* (known compounds in suppression of aroused cell lines by the relevant causeways). As a result, previous studies introduced several *polyphenols* as compounds

with good anti-proliferative potentials and *HT29* and *PC3* as aroused cells to research and evaluate the eventual *anti-carcinogenic* potentials of the known herbal plant infusions.

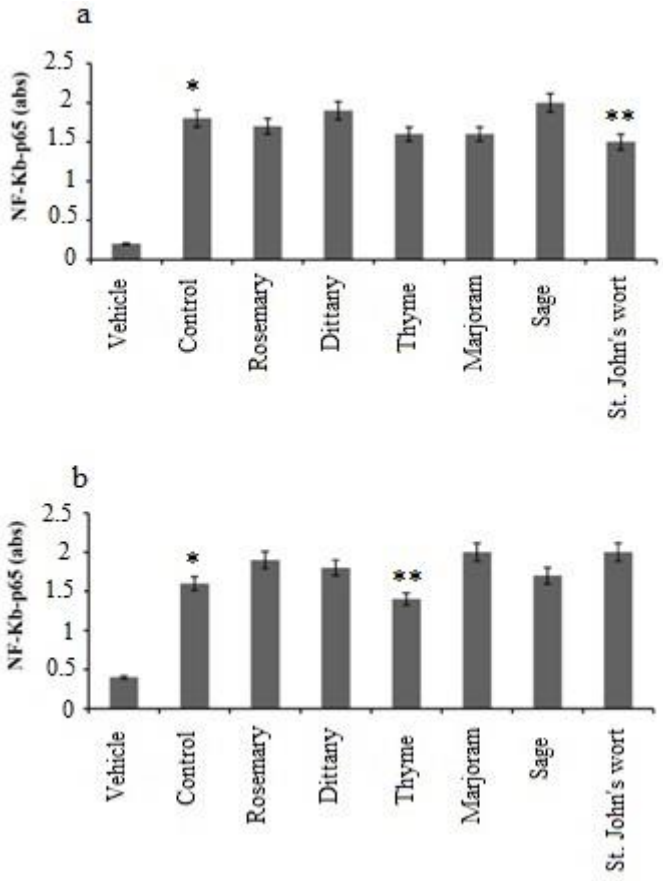


Figure 2.4 Effects of herbal infusions (in the lowest concentration 0.2 µg u-1l final in the well) on total *p65* levels of (a) *HT29* and (b) *PC3* cells stimulated with *TNF-α*. Results are expressed as optical density at 450 nm wavelength (mean ± *SD*). * Presents a statistically considerable diversity between vehicle and stimulated cells (control). ** Presents a statistically considerable diversity between control and cell culture treated with individual herbal infusion prior to stimulation. Considerable level was adjusted at $p<0.05$ (Kaliora et al., 2014).

2.6.2. Effect of Caco-2 cell lines (Intestinal cancer cells) and Fibroblast (responsible for tissue regeneracy)

The *caco cell line* is a persistent cell of incoherent epithelial mucous adenocarcinoma cells expanded by the Sloan-Kettering Institute for Cancer Research and carried out by *Dr. Joseph Fogh*. The *Caco-2* monolayer is widely served across the pharmaceutic professions as an experimental design of the human small intestinal mucosa to predict the absorbency of orally intaked

medicines. The correlation between the experimental definite permeance across *Caco-2* monolayers and clinical segment absorbed is well settled (Artursson & Karlsson, 1991). *Cutaneous fibroblasts* are the cells that exist within the dermis lamella and are responsible for generation crossbred tissue and allowing the skin to recover from *trauma*. Using organelles (specially the clumsy endoplasmic reticulum), *cutaneous fibroblasts* generate and preserve the copulative tissue, which unify *anti-septic* cell cortexes. Moreover, these cutaneous fibroblasts generate protein molecules, such as *laminin* and *fibronectin* that embrace the extracellular matrix. In one research, after incubation the cells with the herbal plant infusions, the contents of *MTT formazan* generated was determined. This conveys metabolic operation of the examined cells and, as such, their livability. In other study, the obtained outcome explained, at the prime three hours of contingence, an increase in the biological activity of the cells upon contact with the infusions of both examined sections of the plants was obtained (leaves and flowers of *Erica australis* L., the effect enhanced in a concentration depending behaviour). After six hours of consecutive exposure there was still an enhancement for both selected sectors, but in lower intensity. After a more extended collision, diversity was perceived in biological potential. This exposure showed an intense reduction in metabolic actuality, which eventuated in a major decline in cell livability, compared to that of the control. The supreme cytotoxic effect was obtained for the flower infusions at a determined concentration of 2 mg. mL^{-1}, followed by the leaf extract at 1 mg. mL^{-1}, which showed a very strong potentials (Nunes *et al*., 2014). The observed results of another research performed on *Q. obtusata*, *Q. grisea* and *Q. laeta* offered higher custody of the extract versus indomethacin *trauma*. Scientists have stated that apple peel bioactive compounds present highly potent preservation versus indomethacin-induced *trauma* in *Caco-2 cells*. They attributed the existence of protection potential and other bioactivity to the accessibility of *querecetin* and its *anti-oxidant* nature as proved by the *deoxy-D-ribose* assay (Carrasco-Pozo *et al*., 2011). The obtained results expressed, at the primer three and six hour incubation procedures, serious enhancement in the metabolic activity were observed, which demonstrates the impressive potentials of the applied extract. The observed positive properties on biological activity after six hours of incubation were considerably superior to those after three hours ($p<0.05$). Unlike that observed outcome for tested *Caco-2 cell lines*, the extract got started to present diverse trends

after six hour incubation procedure. The obtained results also demonstrated the protective effects of infusions containing bioactive compounds, such as *quercetin* and *ellagic acid*, versus the advancement of disturbances. It might be due to, the quantity of the bioactive compound in the flowers of *Erica australis* L., are higher than in the leaves or other parts. It is caused probably by receiving more sunlight and this phenomenon could explain sunlight's dominant potential for generations more useful compounds with bioactive potentials (Nunes *et al*., 2014). To reach to assured results, more researches on the *anti-oxidant* power of different herbal plant infusions and analogy of the bioactive compounds available in diverse sectors are required.

2.7. *Anti-microbial* potential

The difficulty regarding application of chemosynthetic *anti-biotics*, including *anti-microbial* resistance, environmental *anxiety*, *cancerogenity*, have reinforced a tendency for remodelling synthetic discovered *anti-microbial* factors with intrinsic alternative factors. The *anti-microbial* specification of herbal plant infusions and also essential oils are affirmed for so long period of time from human habitant and extensive researches are performed to assign the *anti-microbial* characteristics of those components which have been expressing repressive potential on various examined non-pathogenic and pathogenic agents (Shahidi Bonjar, 2004). Different suggested modes of functions are involved in the *anti-microbial* potentials of essential oils available in herbal infusions. Because of the availability of various compounds and extensive chemical profiles of the extracts and essential oils components, it is likely that *anti-microbial* potent is not just caused by one solitary mechanism; several circumstances of functions at cellular level are considered. Briefly, it has been proposed some major various mechanism functions of *anti-microbial* activities of infusions: (1) Decomposition of cytoplasmic membrane; (2) accretion with proteins placed on membrane (for example *ATPase*); (3) Disturbance or inactivation of the operation of outer membrane of gr^- bacteria by abandoning lipo-polysaccharides; (4) Fluctuation of the proton engine force of the cells with permeance of ions; (5) Coagulation of cell inner contents; and finally (6) Prevention of enzyme generation (Djilani & Dicko, 2012). The functions of herbal plant extracts and essential oil activities are mainly on the structures and cellular membrane. It has been shown that the permeability of the cell septums or membranes are impacted by bioactive compounds available in infusions, leading the

intracellular enclosures outside of the cell, that might be followed by the infraction in electron transfer systems, enzyme activities and nutrient adsorption capability of the cells (Amensour *et al*., 2010). It was indicated that essential oils, including *eugenol*, *α-terpinol* and *γ-terpinene*, have expressed an *anti-bacterial* potentials versus all species of bacteria with diverse membrane structures by infraction of the cell septum; therefore, it is stated that gr^- bacteria show more persistence to bioactive compounds than gr^+ (Taheri *et al*., 2013), due to gr^- bacteria containing an outer septum unified by a complex crust which reduce the penetration rate of hydrophobic compounds including essential oils, and compounds with *anti-microbial* potentials. Moreover, it has also been expressed that *flavonoids* have abilities to form collections with extracellular soluble protein and bacterial cells (Cushnie & Lamb, 2005). *Catechins* also posed *anti-bacterial* potential via *DNA gyrase* prohibition operations. In principle, particular connection was offered for the N-terminal fragment of *gyrase subunit B* availability (Gradisar *et al*., 2007). Also it was cited, *catechins* could enhance the sensitivity of bacteria's *anti-biotic* resistance to other kinds of *anti-biotics*, such as *tetracycline* and *beta-lactam*, by rehabilitating the sensitivity of the repressors (Stapleton *et al*., 2004).

Management of fungal infections poses many troubles because of limitation in the accessibility of *anti-fungal* factors with high inhibition potentials, resistance properties and low cost. Therefore, detection of noble *anti-fungal* factors, such as extracts of medicinal plants, with high potentials is required to be applied in suppression of the strains which express persistence to the available *anti-fungal* medicines. The extract's *anti-fungal* potentials might be ascribed to essential oils and *polyphenols* known causing cell membrane damage by amplifying leakage of interior cellular materials outside, and ultimately, microorganism death (Jurd *et al*., 1971). The *anti-fungal* properties of some herbal plant infusions were imputed to the existence of *polyphenols* and oxygenated mono *terpenes* (Aleksic & Knezevic, 2014). The modes of *anti-fungal* actions are similar to those presented previously for bacteria, including irreversible *trauma* to the cell septum, exuding and coagulation of cellular inner contents. Moreover, there are two further phenomena in yeast inhibition flow; first the infraction of pH deflection across the cytoplasmic membrane and second, the obstruction of yeast's energy production, which results in disruption of the cell septums. *Anti-viral* properties of herbal extracts are demonstrated by their traces on the viral structural protein and denaturation

(Djilani & Dicko, 2012), which are proposed by two mechanisms: (1) Interfering with the virus envelope by inhibition of specific processes happening during the virus replications; (2) Prevention the absorption of essential compounds into destination cells by chelating them (Saddi *et al*., 2007). In some occasions, due to synergistic phenomena, the blend of various essential oils and infusions might present highly *anti-viral* potential than individual ones. The best-distinguished *anti-viral* syntax generated by various herbal plant species is named *α-caryophyllene* (Djilani & Dicko, 2012). However, few researches have compared extensive items of oils and infusions that are directly got engaged in inhibition procedures. Plant oils are handled for extensive variety of aims since human settlements, from the application of cedar wood and also rosewood in aromatization to seasoning drinks with lime, juniper berry or fennel for extending or enhancement of the shelf life of accumulated food crops in traditional or industrial storehouses (Mishra & Dubey, 1994). Other scientists have studied microbial inhibition potential of the herbal infusions of the *Quercus* species. The plant materials were assessed versus an immense confine of bacteria and yeast species. Aqueous infusions of *Q. resinosa* and *Q. grisea* exhibited substantial prohibition in generation of tested micro-organisms. *K. pneumoniae* (ATCC 13883) expressed sensibility versus all obtained aqueous infusions of selected oak. A team of scientists has studied the *anti-bacterial* potential of leaves' infusions by application various solvents, such as, essential oils, *MeOH, $CHCl_3$, n-BuOH, EtOAc* extracts, as well as aqueous fractions at the concentration of about 2000 µg. mL^{-1} of *Callistemon viminalis. EtOAc* fraction has expressed the highest activity versus the extension of *E. coli* and the minimal inhibition potentials for *S. marcescens*. Furthermore, the mentioned fraction of *C. viminalis* subsp. ergnewasis leaves has expressed a considerable activity versus the expansion of other target bacteria specieis. Essential oils showed towering potentials against bacteria's increment and the obtained data approved that, *EtOAc* and *MeOH* infusions have acted as potent *anti-bacterial* factors against the tested bacteria's increment whilst *$CHCl_3$* and *n-BuOH* fractions have not expressed considerable potentials versus the selected bacteria strains (Salem *et al*., 2013). Almost 250,000 to 500,000 plant species have been estimated, and just one-tenth of which has been explored to date, and thousands of compounds have been disassembled from these plants and claimed as *anti-microbial* factors. The bioactive value of plants is classified based on the quantity of bioactive and chemical compounds that are

presenting an accurate function on the *anti-microbial*, chemical and sensorial properties of food products, and those components cited previously, are classified into various main categories, including *polyphenols*, *polypeptides*, *terpenoids*, *flavonoids*, *isothiocyanates*, *tannins*, *lectins*, and *alkaloids*, or also oxygen substituted formatives (Edeoga *et al*., 2005). Despite the many researches performed on the mechanism of functions of *anti-microbial* ingredients of herbal extracts, the principle of those procedures of compounds is still not fully recognized and need designing more researches. Table 2.2 presents some *anti-microbial* compound mechanisms. The marker and proposed compound's quantity available in herbal infusions, are modified by the variety of plant, geographical origin that plant is growing on, plant part used, age, methods of the extraction or drying, preparation, wrapping and keeping conditions of products. Identification and assessing of natural compounds for the determination of pathogens, and ensuring to consumers safety, nutritive food supplies and wholesome, is a challenge of much more researches. The difficulty of microbial resistance is growing and the perspectives of application chemical *anti-microbial* agents in the future's food products are still unknown. Plants contain thousands of constituents and precious sources of novel and biologically active molecules are offering considerable *anti-microbial* potentials.

Former studies performed on regular preservative and *anti-microbial* compounds, untied only a small doorway to plants containing combinations with *anti-microbial* potentials. As a result *flavonoids*, *polyphenols*, essential oils and other bioactive compounds accessible in herbal plants might also get considered authoritative for these activities. In some studies, it has been proven that *anti-bacterial* suppressive potentials of herbal plants are caused by *scopoletin*, *herniarin*, *xanthotoxin*, with various aqueous and organic solvents, it is approved that extraction with organic solvent, diffuses infusions with highly *anti-microbial* potentials, may be due to organic solvents such as acetone, methanol and ethanol are able to extract much more essential oils and other non-polar extremity component contents than water, therefore could act strongly against spoiling agents.

Furthermore, the *phenolic* compounds mentioned formerly are diagnosed as common *anti-microbial* factors available in plenty of examined herbal plants. It was reported that gr^+ bacteria are more sensitive to herbal infusions and essential oils than gr^- bacteria. As mentioned previously; the weak *anti-bacterial* activity of herbal infusions against the gr^-bacteria was attributed to the existence of one further

Table 2.2 Some known *anti-microbial* compounds in herbal plants with their mechanism of actions.

No	Compounds name	Mechanism of action	References
1 **2**	*Terpenoids* *Phenolics*	Efficacy on membrane infraction (1-2)	(Cowan, 1999) (1-2)
3 **4**	*Phenols* *Flavonoids*	Metal chelating (3-4)	(Cowan, 1999) (3-4)
5 **6**	*Coumarin* *Alkaloids*	Efficacy on genetic affairs (5-6)	(Cowan, 1999) (5-6)
7 **8**	*Thymol*, *Eugenol*	Membrane infraction, prohibition of *ATPase* actuality and deliverance of intracellular ATP and other constituents of microorganisms (7-8)	(Burt, 2004) (7-8)
9 **10**	*Carvacrol* *Cinnamaldehyde*	Decline in the intracellular ATP by *ATPase* activation without obvious conversion on the cell velum (9-10)	(Holley & Patel, 2005) (9-10)

outer septum that makes hydrophilic polysaccharide bonds as barrier in entering hydrophobic essential oils into cells. However, the results of some scientists have shown similar capacity for both gr^- and gr^+ bacterial species. The organic extracts, such as *EtOAc* and *MeOH* have stated higher *anti-microbial* potentials as compared to aqueous infusions. As being organic, they dissolve much more bioactive compounds, resulting in the dissemination of greater level of active *anti-microbial* factors (Salem *et al*., 2013). In particular, the *anti-microbial* activity of plant oils and infusions has created the foundation of plenty applications, including intermittent medicine, processed and crude food preservation, pharmaceutic and natural remedies.

2.8. *Anti-diabetic* potential

Diabetes as a very serious chronic disturbance, is becoming the one of the most fatal and noxious, disability for humankind, after *cerebrovascular* and *cardiovascular cancers*, due to its highly outbreak, malady and fatality. *Diabetic* is a chronic metabolic irregularity, mainly specified by disruption in proteins, fat and particularly carbohydrates metabolism caused by the thorough or relative inadequacy of insulin exudation or functions. About 366 million people are expected to become *diabetic* by the year 2030, due to it has not been discovered any *anti-diabetic* chemical medicine to provide *long-term glycaemic* overseer without causing any adverse negative side effects. Herbal plants partially could be prescribed for treatment of disorders relevant to *diabetes*. Also herbal plants are traditionally prescribed to modify the *diabetes* before the detection of insulin. It has been proposed that almost 1200 herbal plants in nature are capable to effectivity diminish or hold constant the blood glucose content. Herbal plants with *anti-diabetic* potentials may effectively inhibit insulin resistance and oxidative stress; in fact, the *anti-diabetic* properties of herbal plants might be due to chemical interactions occurre among effective compounds available in infusions with the diverse biochemical molecules involved in *diabetes*. *α-Glucosidase* and *α-Amylase* inhibitors demonstrate an effective mechanism to reduce the content of postprandial hyperglycemia and prevent the digestion of carbohydrates (Perez-Gutierrez & Damian-Guzman, 2012). Meanwhile, medicinal plants that are showing impressive potentials in adjusting plasma glucose level with minimal negative side effects are commonly being used in modern countries as alternative remedies. *α-Glucosidase* and *α-Amylase* repressor restrict postprandial glucose uprising by diminishing the rate of carbohydrate's absorption and disintegration. These repressors have been proposed efficacious in recuperating *type II diabetes* (Gholamhoseinian *et al*., 2009). Obtained results by a group of scientists have revealed the vulnerary potentials of medicinal plants on ruined blood glucose, lipids, *AChE* and *anti-oxidant* balance in *streptozotocin* (*STZ*)-*diabetic mice*, the symptom could be justified by the inhibitory potentials of herbal infusions on glucose transporters, *α-Glucosidase*, *α-Amylase*, lipase and also strong *anti-oxidant* potential of the extracts (Ponnusamy *et al*., 2011). Numerous functions of *hypoglycaemic* features of herbal infusions are declared. Some hypothesises affiliate their efficacy on pancreatic beta cells via the enhancement of insulin sensibility or insulin-like actuality of the herbal plant infusions, thereby increasing

the circumferential exploitation of glucose, inhibition or diminishing intestinal glucose absorbency, reduction of *glycaemic* indicator of carbohydrates and the decrement potentials of glutathione (Bnouham *et al*., 2006). This research discusses selected plant infusions in qualification of their *anti-diabetic* potentials and mechanism of functions. In a short term research, aqueous extracts generated significant *anti-hyperglycaemic* potentials at a particular value of body mass proportions in *diabetic* treated rats. As a result of that, aqueous extracts have shown a substantial antihyperglycemic property by improvement of the glucose endurance in normoglycemic rats or by diminishing the glucose balance in blood of 54% in *STZ*-persuaded-*diabetic rats*. The possible functions of *anti-diabetic* potentials are dependent on various pathways, as presented in Table 2.3. One of the prominent mechanisms might be enhancement of the pancreatic leakage of insulin from the attainable beta cells, by the redemption from the constrained configuration. Plant infusions containing *flavonoid* and *tannin* components are found out that act as the most potent origin for treatment of *diabetes*; over 150 plant extracts include *flavonoids* ascribed as *anti-diabetic* agents. Furthermore, plants containing other groups of *polyphenolics* demonstrate *anti-diabetic* properties as well. The insulin analogous activity of these bioactive compounds is probably authoritative for exposing this potential (Chikhi *et al*., 2014). As it was mentioned, the numeral of people attacked by *diabetes* has been boosting constantly over the back two decades; this high accession merits express consideration unto the therapy of *diabetes*. A few accurate management options available are costly and often associated with negative side effects; hereupon, the application of native medicinal plants confects a superior substitute, which is usually less virulent and more affordable. Additionally, regarding the customary application of potent herbal plants in most aggregations and great outbreak of *diabetes* mellitus, more *in vivo* or clinical researches should be advanced in order to accredit the *anti-diabetic* potentials of the identified plants, as is professed by customary healers.

2.9. Conclusions

Up the back 100 years, the development and mass production of chemically synthesized medicines has been used to boost health surveillance in various parts of the world. However, extensive proportions of the crowd in underdeveloped communities still rely on customary practitioners and herbal medicines for their initial

carefulness. Regarding the performed research by the National Centre for Complementary and Alternative Medicine (Barnes *et al*., 2008), herbaceous therapy, or the usage of natural products other than *vitamins* and minerals, has most commonly been handled. Due to maturing lot in the bioactive compounds accessible in herbal plants and their nutraceutical application, as well as their noticeable potential in therapy and ameliorating of various disturbances such as *diabetes* and *cancers*, herbal plant's cytotoxicity potentials as well as *anti-microbial* properties exploited extensively in nutraceutical and pharmaceutical industries, allocated more regards, nowadays. The most popular proofs for utilization of customary medicine are that: affordability; rather intimately correspond to the patient's outlook; allays apprehensions about the adverse negative adverse effects of chemical or synthetic medicines; assured an ambition for more personalized health care; and authorizes vast universal accessibility to safety awareness. The original acquisition of herbaceous medicines is for health advancement and remedy against persistent irregularity that are life-threatening estate. However, usage of traditional remedies enhances when conventional medicine is inconclusive in the treatment of those disturbances, as with progressive cancer, and in confront of new infectious disturbances. Irregardless of the cause, those people consuming herbaceous medicines should be assured that those products they are purchasing are safe and contain what they are assumed to. Consumers should also be given theoretical-supported notification on quantity, concentration and effectiveness. To reach this, a universal appropriateness of legislation is required to conduct the relevant production and marketing of herbal medicines. If sufficient scientific records of advantages are accessible for one herb, the legislation should allow application of the herbs for public, therefore the benefits could be perceived appropriately for the promotion of public health and the treatment of disturbances.

Table 2.3 Effective compounds in herbal plants and mechanism of actions.

No	Botanical name	Mechanism of actions	References
1	*Aegle marmelos* L. (Rutaceae)	Because of availability of *allyl propyl disulfide*, *S-methyl cycteine sulfoxide*, *Allii Sativi Bulbus* (Liliaceae), *Allicin* (*diallyldisulfide- oxide*) and *S-allyl cysteine sulfoxide* (the forerunner of *allicin* and garlic oil) by motivation insulin leakage from regional cells of pancreatic and enhancement serum insulin rate, glucose endurance and liver glycogen compound (1-3)	(Asaduzzaman *et al*., 2010; Kook *et al*., 2009) (1-3)
2	*Agrimonia pilosa* (Rosaceae)		
3	*Allii Cepa Bulbus* (Liliaceae)		
4	*Andrographis paniculata*	Due to availability of *Alkaloids vindoline, vindolinine*, *alkaloid* and *1-ephedrine* and by modification atrophied pancreatic islets and exudation of insulin (4-8)	(Mandlik *et al*., 2008) (4-8)
5	*Anisodus tanguticus* (Maxim) Pascher (Solanaceae)		
6	*Catharanthus roseus* (L.) G. Don (Apocynaceae)		
7	*Ephedra sinica Stapf*		
8	*Ephedra distachya* L. (Ephedraceae)		
9	*Euonyrnus alatus (*Thunb.) Sieb. (Celastraceae)	By enhancement *GLUT4 mRNA* and its protein declaration by increasing propagation of pancreatic islets and boosting postprandial leakage of insulin and therefore precipitating the glucose transportation (9-10)	(Singh, 2011) (9-10)
10	*Fructus Coini Sieb* (Cornaceae)		
11	*Acacia Arabica* (Leguminosae)	Persuading permeation of insulin by pancreatic beta cells	(Bhushan *et al*., 2010) (11)
12	*Aegle marmelos* L. (Rutaceae)	By enhancement exploitation of glucose or by direct instigation of glucose absorbance via improving insulin leakage	(Bhushan *et al*., 2010) (12)
13	*Abies pindrow*	By insulin secretory activation	(Bhushan *et al*., 2010) (13-17)
14	*Acacia arabica*	By instigation delivering of insulin from pancreas	
15	*Agrimony eupatoria*	By instigation delivering of insulin from pancreas and acts like insulin (insulin mimetic)	
16	*Vinca rosea*	Modification, regeneration and instigation of beta cell	
17	*Ipomoea batata* (L.) Lam.	Declining insulin persistence and blood glucose	

2.10. References

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CHAPTER 3

Release kinetics and *encapsulation efficiency* of bioactive compounds through encapsulated *Olea europaea* L. var. sylvestris., leaves' infusion

In this chapter the authors discussed encapsulation of extracts obtained from Portugues Olea europaea L. var. sylvestris., *leaves and modelled the extraction of bioactive compounds by Response Surface Methodology (RSM)*

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Release kinetics and *encapsulation efficiency* of bioactive compounds through encapsulated *Olea europaea* L. var. sylvestris., leaves' infusion

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Under review

Abstract

Context: The current manuscript discusses the optimization of the encapsulation process of Portugues *Olea europaea* L. var. sylvestris., leaves' infusion. This plant's extract has demonstrated several medicinal properties in different studies. Encapsulation is performed with various methods in nutraceutical and pharmaceutical industries, among which sodium alginate method is used in this research as a simple and quick approach.

Objective: The aim of this study is optimisation of the release kinetics and *encapsulation efficiency* of bioactive compounds of encapsulated *Olea europaea* L. var. sylvestris., aqueous infusions regarding the percentages of applied starch in the capsules' structure and duration of extraction for application in nutraceutical and pharmaceutical products.

Methodology: The extraction of bioactive compounds was carried out in an ordinary microwave oven; the hydrogel beads were prepared by ionic gelation, obtaining a calcium alginate matrix. The efficiency of encapsulation on Total *phenolic* compounds; Total *anti-oxidant* activity and Ferric reducing *anti-oxidant* power were detected using *spectrophotometric* methods with *Folin-Ciocalteu* reagent; phosphomolybdenum and *2, 2-diphenyl-1-picryl-hydrazyl* respectively. A Face-Centred *Central Composite Design* of *Response Surface Methodology* was exploited. The experimental design and statistical analysis were accomplished using *JMP®Pro*, Version 11.

Results: The optimised points of the selected independent variables were achieved. The existing strong *correlation coefficient*s between the experimental and predicted values of the detected variables expressed the reliability of the selected

model for handling a central control design in the optimisation of the encapsulation process.

Discussion and conclusions: Starch addition not only affects the *anti-oxidant* capabilities of encapsulated *Olea europaea* L. var. sylvestris., of hydrogel capsules, but also affects their optical morphology or physical characteristics.

Keywords: Microwave assisted extraction; *O.europaea* L., leaves*;* design of experiment; encapsulation

3.1. Introduction

Application of hydrogels acquired from polysaccharides in nutraceutical and pharmaceutical industries is continuously increasing, due to the high demand for bioactive natural substances (Farris *et al*., 2009). In this flow, hydrogels are used as carriers of bioactive compounds such as natural *anti-oxidants*, cells, unsaturated oils, medicines, etc. (Deladino *et al*, 2008; Gbassi *et al*., 2009; Goh *et al*., 2012; Pongjanyakul *et al*., 2010).

Sodium alginate is soluble in water and is capable of creating hydrogel beads by dropping the aqueous solution within a divalent or polyvalent cation solution (Draget *et al*., 1998). Although this is a simple and quick procedure of affording encapsulating systems, the method has a significant limitation in terms of dissipation within bead providing. The loss of active compound is intensified by, firstly, the time it takes for the cation to get distributed into the beads and, secondly, the compound condensation gradient between the beads and the periphery solution. Moreover, the existence of macrospores in the alginate matrix facilitates the diffusion of hydrophile molecules (George *et al*., 2006; Gouin, 2004). However, some researchers have been able to overcome this by mingling alginate with other polymers, such as starch, chitosan, cellulose and pectin, among others. In most cases, the mechanical and physical characteristics of the beads were improved (Chan *et al*., 2011; Santagapita *et al*., 2012). Other scientists have exploited ionic gelation to encapsulate bioactive compounds by other modes, such as interior gelation or absorbency assays (Chan *et al*., 2010). A promising substitute was offered by encapsulating an herbal aqueous infusion in calcium alginate beads using the absorbency mode, and it was found out that this technique afforded a two to six fold superior encapsulation performance than the straight extrusion mode. The incorporation of a filler substance into alginate matrix is a strategy for reducing the disadvantages. Starch is also used to boost the

probiotic bacteria entrapment (Sultana *et al*., 2000). In another procedure, scientists applied electrostatic extrusion and progressed the encapsulation loading of thyme infusion in comparison to the absorbency procedure (Stojanovic *et al*., 2011). They eliminated the driving force for diffusion by preserving the same concentration of infusions in the gelling solution as within the jelly matrix. Modelling is one of the reliable designs used commonly these days in different fields as well as food and medicinal chemistry (Dolatabadi *et al*., 2016; Farzaneh *et al*., 2016; Rostami *et al*., 2014).

Within Mediterranean countries, olive (*Olea europaea* L. var. sylvestris.) leaf has been greatly applied in folk medicine for a long period of time (Dolatabadi *et al*., 2016; Farzaneh *et al*., 2016; Rostami *et al*., 2014). Historically, olive leaf is used as a therapy for malaria and associated fever (Benavente-García *et al*., 2000). The main bioactive substances of olive leaf have been recognised as follows: *hydroxytyrosol*, *tyrosol*, *p-coumaric verbascoside*, *apigenin-7-glucosid*, *caffeic acid*, *luteolin-7-glocoside* and *diosmetin-7-glucoside* (Bianco *et al*., 2000; Tasioula-margari *et al*., 2001). Various reports have been published on olive leaf and have shown that olive leaves offer the capability of reducing blood pressure and raising blood circulation in the coronary arteries (Khayyal *et al*., 2002). The infusions of olive leaves present *anti-oxidative* capabilities (Somova *et al*., 2003; Škerget *et al*., 2005) as well as germicidal potentials versus *Campylobacter jejuni*, *Helicobacter pylori* or *Staphylococcus aureus* (Sudjana *et al*., 2009). Furthermore, a group of scientists found that olive leaves comprise substantial quantities of *oleuropein* and *phenols*, the most effectual substances for *anti-oxidant* capability (Lee *et al*., 2009). In addition (Benavente-García *et al*., 2000) found that olive leaf infusion demonstrated a synergistic manner with specifications of high dosage of *oleuropein* and further accessible bioactive substance contents.

Despite the availability of various reports on olive leaves and their *phenolic* compounds, no similar published researches on the application of encapsulated olive leaf infusion using ca-alginate matrix and starch of potato have been found. Additionally, the demand for olive leaf infusions for application in nutraceutical, pharmaceutical and cosmetic product has been increasing. Furthermore scientists reviewed the mechanism of actions of different health benefits of medicinal plants (Farzaneh and Carvalho, 2015). It should be mentioned that *encapsulation efficiency* has been calculated based the release *phenols* in the solvent to the *phenolic*

compounds contents of the original extract. Therefore, the objective of this study is the optimisation of the *encapsulation efficiency* based on the released *phenols* of encapsulated *Olea europaea* L. var. sylvestris., aqueous infusions regarding percentages of applied starch in the capsules' structure and duration of extraction.

3.2. Materials and methods

3.2.1. Plant materials

Olea europaea L. var. sylvestris., leaves were collected in June 2014 from the countryside of Faro, Algarve area, Portugal. The plants were identified by Mr. Rosa Pinto (University of Algarve) and a voucher specimen number (13493) was deposited at the Herbarium of the Faculty of Science and Technology, University of Algarve. Leaves were ground using a handling grinder (*Cetigo*-Portugal), then sieved and capped into individual vials and preserved under -20 ºC at the time of extraction.

3.2.2. Chemicals and reagents

Alginic acid sodium salt was provided by *Alfa Aesar* (A Johnson Matthey Company - Karlsruhe, Germany), calcium chloride 2-hydrate ($CaCl_2.2H_2o$) and starch from potato were purchased from *Panreac* (Barcelona, Spain), sodium carbonate anhydrous (Na_2CO_3), acetic acid (glacial) and sulphuric acid (95-97%), were shopped from *Merck* (*Darmstadt*, Germany). Citric acid anhydrous was bought from *Merck* (Hohenbrunn, Germany). Sodium phosphate dibasic anhydrous (Na_2HPO_4) (Purity >99.0%) was provided from *G-Bioscience* company (*USA*). Sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$) was procured from *VWR* (Haasrode, Belgium) and phosphate buffer solution was bought from *VWR* (England). *Folin-Ciocalteu* phenol reagent (*F-C* reagent), hydrochloric acid min. 37% (HCL), *2, 2-diphenyl-1-picryl-hydrazyl* (*TPTZ*) and ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$) were purchased from *Sigma-Aldrich*, (Switzerland-Germany). Iron (III) chloride ($Cl_3Fe.6H_2O$) was purchased from *VWR* international (EC, Leuven, Belgium).

3.2.3. Apparatus

Spectrophotometer (T70+Vis, PG Instrument Ltd, United Kingdom); evaporator (*Nahita* series 503, Navarra, Spain); *Hettich* centrifuge (*universal-320*, Germany); stirrer (*VWR*, 720 advanced, *USA*); disposable cuvettes (*VWR*-Leuven, Belgium); vortex mixer (*Stuart*, UL-Bibby Sterilin Ltd); grinders (*Philips*, Brazil)

and (*Cetigo*-Portugal); microwave oven (Samsung type 1713, Microwave output 800 W); shaker-incubator ES-20 (230 V, 50-60 Hz, 150 W).

3.2.4. Experimental design and statistical analysis

The extraction procedure was carried out in 10 runs. Experimental data were achieved as mean. A Face-Centred *Central Composite Design* is exploited when at least a pair of samples vary (p<0.05); in other words, when the *p*-value from *ANOVA* was lower than 0.05 value, differences among obtained data of various samples were determined using one-way analysis of variance (*ANOVA*) by application of SPSS software package, version 22.0 (*IBM* SPSS Statistics 22, licensed by Algarve University). The experimental design and statistical analysis were accomplished using *JMP® Pro*, Version 11. Copyright, *SAS* Institute (Inc. *SAS* and all other *SAS* Institute Inc., Cary, NC, *USA*).

The extraction of bioactive compounds from capsules was applied as a function of starch percentages in capsules (X_1) and period of sampling of supernatant of the beads (X_2) and obtained data were analysed using A *Face-Centred Central Composite Design* (*FCCCD*) with two replications in the centre of the pilot domain. The pilot domain was specified taking into account the outcomes captured in preliminary examinations. The confining independent variables studied were:X_1: percentages of the applied starch (0.5, 1 and 1.5%) and X_2: sampling time (60, 120 and 180 min) as shown in Table 3.1. Each of the independent variables was encoded at three levels (-1, 0, +1), corresponding to low, mid and high level.

Encoding of the variables was accomplished based on equation 3.1:

$$xi = ((xi\text{-}xc)/\Delta xi) \text{ and } i = 1,2,\dots.z \quad (3.1$$

Where xi presents the dimensionless use of an absolute variable, xi indicates the experimental value of an independent variable, xc expresses the experimental value of an independent variable at the centre point, and Δxi is the leg conversion of the actual value of the variable with i corresponding to a variation of a unit for the dimensionless value of the variable i.

The number of experiments (N) required for the extension of *central composite design* (*CCD*) with two centre points was determined as 10. The central points are applied to detect experimental errors. The efficiency of the process was assessed by analysing the responses (Y), which are dependent on the input factors X_1 andX_2.

Table 3.1 Independent variables and their coded and actual values used for optimization of *encapsulation efficiency (EE).*

Independent variables	Units	Symbol	Code levels		
			-1	0	1
Starch of patato	%	X_1	0.5	1	1.5
Extraction time	min	X_2	60	120	180

The relationship between the response and the input process parameters is described by equation 3.2:

$$Y = f(X1, X2, \dots) + e \qquad (3.2$$

Where f is the actual response function of an unknown format and e represents the error which expresses the difference. The behaviour of the response surface was studied for the response function (Y) using second-order multinomial equation.

The generalised response surface design is given in equation 3.3:

$$Y_n = \beta_0 + \Sigma_{i=1}^{n}\beta_i X_i + \Sigma_{\substack{i=1 \\ j>1}}^{n-1}\Sigma_{j=2}^{n}\beta_{ij} X_i X_j + \Sigma_{i=1}^{n}\beta_{ii} X_i^2 \qquad (3.3$$

Or in a simple form with two independent variables:

$$Y_n = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2$$

Where Y_n represents the response to be designed, β_0 is a constant, β_1 and β_2 are the coefficients of the linear modes, β_{12} expresses the coefficient of the interaction between the independent factors, and β_{11} and β_{22} indicate the coefficients of the quadratic modes of the input variables. The design adequacy was detected by evaluation of the model and lack of fit significances as well as the coefficient of determination (R^2) and the Fisher test values (F-value). Optimised independent variables (X_1 and X_2) for maximised responses of TPC, TAA and FRAP values were identified by desirability graphs.

A design matrix comprising of 10 various experimental runs is summarised in Table 3.2. All the trials were performed in triplicate. The ultimate release kinetics procedure of bioactive compounds, using optimised statuses, was justified based on the predictions of the developed design.

3.2.5. Olea europaea L. var. Sylvestris., extract

Microwave-assisted extraction (*MAE*) of bioactive compounds was carried out in an ordinary microwave oven (Samsung type 1713, Microwave output 800 W) at an effective frequency of 2450 MHz with adjustable microwave power and time. Extraction statuses were applied according to the obtained results of preliminary tests. About 2 g of ground and sieved sample was weighted and placed into a 500 mL Pyrex beaker containing 73 mL deionised water. The mixture was blended with a magnet stirrer for 5 min at 200 rpm to give adequate penetration of water. The beaker was situated in the middle of the oven over a rotational plate and was heated by exposing it to microwave radiation (300 W for 120 sec). The mixture in the Pyrex beaker was left to cool down to room temperature, then centrifuged within 10 min at 5000 rpm, and finally refined and filtered through *Sartorius stedim biograde 388* filter paper. When the extraction process was completed, water was vaporised using a rotary evaporator (*Nahita* series 503, Navarra, Spain) and the remaining substances were re-suspended in a particular volume of deionised water of adequate definitive concentration for the experiments. Infusions were transferred to Eppendorf tubes and were preserved under -20 °C until the analysis day. On the day of the experiments, infusions were placed in darkness until defrosting.

3.2.6. Determination of total phenolic contents (TPC)

The concentration of total *phenols* in the beads and samples was detected using *Folin-Ciocalteu* reagent and exterior calibration with gallic acid regarding the assays of (Huang *et al*., 2006), briefly 500 µL of 10% (v/v) *F-C* reagent blended with 100 µL of each supernatant and infusion (for beads and original extracts, respectively) and the contents rigorously blended. Following stirring, 400 µL of 7.5% Na_2CO_3 in water solution was added and, after shaking afresh, the solution was left to stand for 30 min in darkness at room temperature. The absorbance was recorded at 765 nm applying a T70+ UV/Vis Spectrophotometer (PG Instruments Ltd, United Kingdom) versus a blank comprising the same solution with an exception, solvent (citrate sodium and deionised water) instead of supernatants and diluted extracts, respectively, in equal volume. The concentrations of TPC in the original and encapsulated extracts were estimated by a calibration curve constructed by gallic acid as a standard in a designated confine and were expressed as mg of gallic acid equivalent per gram dry weight of each plant and bead, respectively ($mg_{(GAE).}\ g^{-1}{}_{dw}$).

3.2.7. Determination of total anti-oxidant activity (TAA)

In the present research, total *anti-oxidant* activity was detected using phosphomolybdenum method as previously mentioned (Prieto *et al*., 1999). Precisely 100 µL of each supernatant and infusion (for beads and original extracts, respectively) was mixed with 1000 µL reagent solution, including 0.6 M sulphuric acid, 28 mM anhydrous sodium phosphate and 4 mM ammonium molybdate. After stirring vigorously, it was placed into a water bath at 95 °C for 90 min. Then, after cooling down the samples to room temperature, the absorbance was recorded versus a blank comprising the same solution with an exception, solvent (citrate sodium and deionised water) instead of supernatants and diluted extracts, respectively, in equal volume. The *anti-oxidant* activity of the original and encapsulated extracts was computed on the basis of the calibration curve of *ascorbic acid* equivalent constructed in the designated confine and were expressed as mg *ascorbic acid* per gram dry weight of the plants and beads, respectively ($mg_{(AAE)}. g^{-1}_{dw}$).

3.2.8. FRAP (Ferric reducing anti-oxidant power) assay

The FRAP test was carried out according to the previously cited literature by (Benzie and Strain, 1999). Briefly, the FRAP reagent was prepared by 300 mM acetate buffer (pH=3.6), 10 mM *TPTZ* solution in 40 mM HCL (hydrochloric acid) as a solvent and 20 mM iron (ΠΙ) chloride in a voluminosity ratio of 10:1:1, respectively. The FRAP solution was freshly prepared daily and warmed up to 37 °C immediately before handling. 100 µL of supernatant and/or diluted plant infusions (for beads and samples, respectively) was added to 900 µL of the FRAP solution previously prepared. The mixtures were then blended intensively using a Vortex mixer (*Stuart*, UL-Bibby Sterilin Ltd) and then placed in darkness at room temperature for 30 min. After 30 min, absorbance was recorded by a Spectrophotometer (T70+Vis, PG Instruments Ltd, United Kingdom), versus a blank comprising the same solution with an exception, solvent (citrate sodium and deionised water) instead of supernatants and diluted extracts, respectively, in equal volume. The ultimate outcomes for original and encapsulated extracts were computed using a calibration curve constructed by $FeSO_4$ as a standard, and expressed as mg *Trolox equivalent* (*TE*) per each gram dry weight of the plants and beads, respectively ($mg_{(TE).} g^{-1}_{dw}$).

3.2.9. Prepared solutions

3.2.9.1. Acetate-acetic buffer (pH5.6)

2.86 mL glacial acetic acid was added to deionised water and diluted to 250 mL (acetic acid 0.2 M) and then about 25 mL acetate sodium 0.2 M was added. Finally, 250 mL of deionised water was added to the prepared solution and the pH adjusted to 5.6.

3.2.9.2. Sodium citrate (5 g. 100 mL^{-1})

7.434g citric acid was added to 6.159 g sodium carbonate and then 200 mL of deionised water was added.

3.2.9.3. Calcium chloride (0.05 M)

7.351g of $CaCl_2.2H_2o$ was dissolved in deionised water, and then diluted to 1000 mL.

3.2.10. Preparation of alginate hydrogels beads

The hydrogel beads were prepared by ionic gelation, obtaining a calcium alginate matrix according to the method previously explained by (López Córdoba *et al*., 2013). *Olea europaea* L. var. sylvestris., leaf infusion was well dissolved in a sodium alginate solution. In order to prepare a final solution with 3 g. 100 mL^{-1} concentration of infusions and 2% sodium alginate concentration, three different quantities of potato starch were added to prepare solutions with three concentrations (0.5, 1 and 1.5%) of potato starch according to our design. Once homogenised, the solution was transferred into a syringe (diameter: 1 mm) for dropping into a calcium chloride solution (0.05 M). The beads were preserved into the calcium chloride solution to harden for 15 min and were then filtered and washed well with an acetic-acetate buffer solution (pH=5.5). The prepared beads containing bioactive compounds and starch with three concentrations will be referred to as *CAS* (*Calcium Alginate Starch hydrogels*).

3.2.11. Hydrogels characterization

3.2.11.1. Encapsulation efficiency of different obtained values

The *encapsulation efficiency* of the various obtained values of hydrogels beads (%*EE*) was computed according to equation 3.4:

$$\%EE = (m_c/m_e) \times 100 \qquad (3.4$$

Where m_c represents the number of detected values (TPC, TAA and FRAP) of beads using sodium citrate solution as a solvent and m_e expresses the amount of the

same values of the crude original infusion used in bead formulation. The value of m_c was detected by adding 20 mg of beads to 2 mL sodium citrate (5g . 100 mL^{-1}) solution and then the container with the solution was placed in a shaker-incubator at 37 ºC at 180 rpm for 60, 120 and 180 min in accordance with our design. After filtration of the supernatant through *Sartorius stedim biograde 388* filter paper, the TPC, TAA and FRAP values were determined using the methods described in sections 3.2.6, 3.2.7 and 3.2.8, respectively.

3.3. Results and discussion

3.3.1. Visual characteristics of Calcium Alginate Starch (CAS) hydrogels beads

Optical and wizen micrographs of *Calcium Alginate Starch* (*CAS*) hydrogel beads (with 0.5, 1 and 1.5% starch concentrations) with *Olea europaea* L. var. sylvestris., aqueous infusion are shown in Fig. 3.1. The micrographs of each group show a homogenous size distribution.

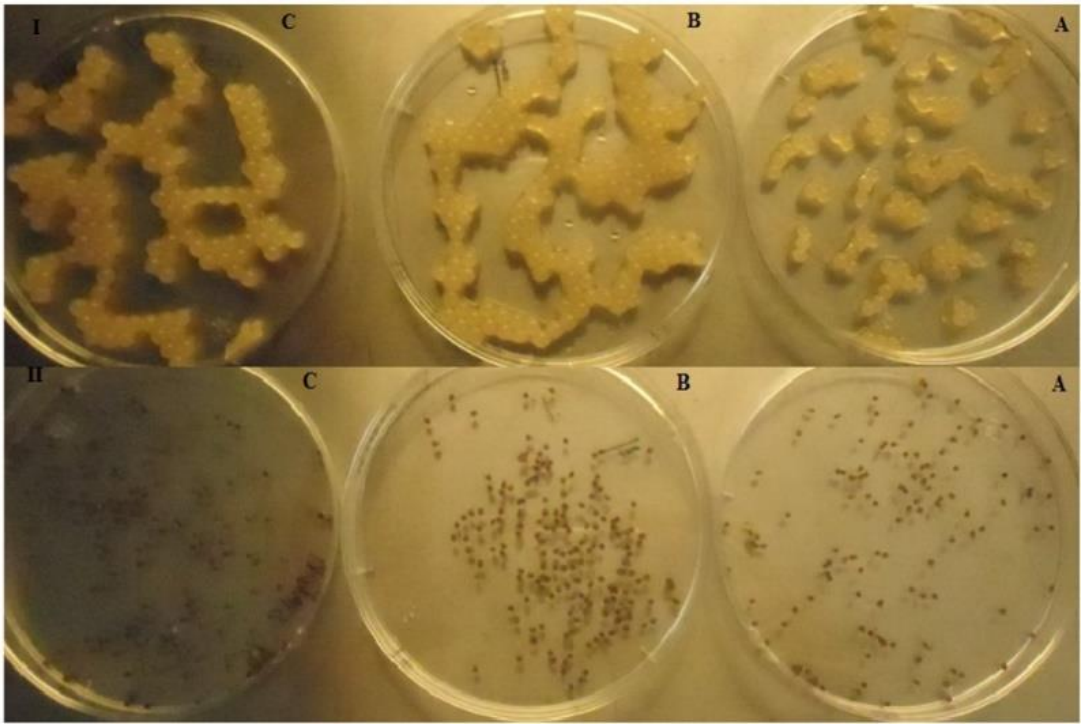

Figure 3.1 *Olea europaea* L. var. sylvestris., starch filled calcium alginate beads, in three different concentrations, a (0.5%), b (1%) and c (1.5%) in optical (I) and wizen (II) micrographs.

As shown in Fig. 3.1, starch addition affected the optical properties, such as the diameter and colour of the created capsules. An enhancement in the quantity of applied starch in encapsulation of *Olea europaea* L. var. sylvestris., aqueous infusion

enhanced the size and created darker capsules. This finding is not in agreement with the finding of (López Córdoba *et al*., 2013). However, other researchers have observed that the physico-mechanical properties of beads might be improved by increasing the quantity of applied starch in the capsule structures at a particular concentration (Chan *et al*., 2011; Santagapita *et al*., 2012).

3.3.2. EE of TPC

Table 3.2 shows the experimental and predicted encapsulation efficiencies of *CAS* systems, including different concentrations of starch filler (0.5, 1 and 1.5%) at different extraction times (60, 120 and 180 min). The highest yield of encapsulation was observed in run 9 with 1.5% starch concentration within a 120 min extraction time, and the lowest value was obtained in run 1 under the following condition, where X_1=0.5% and X_2=60 min. Fig. 3.2 illustrates the three-dimensional response surface plot of the predicted *encapsulation efficiency* as affected by different starch filler concentrations and different time extractions with the contour plot above. The results show that capsules with the starch concentration ranged almost 1.4-1.5 and within an extraction time of between 80-120 min presented the higher *encapsulation efficiency* for TPC value (Fig. 3.2).

Table 3.2 *Central composite design* matrixes with observed and predicted values of response.

Run	Coded variable levels		*Encapsulation efficiency* of TPC (%)		*Encapsulation efficiency* of TAA (%)		*Encapsulation efficiency* of FRAP (%)	
	X_1	X_2	EXP	Pred	EXP	Pred	EXP	Pred
1	0.5	60	**33.562**	32.668	**57.695**	57.267	2.609	2.505
2	0.5	120	34.193	35.373	62.789	63.093	**2.388**	2.409
3	0.5	180	36.334	36.046	64.303	64.426	2.688	2.770
4	1	60	34.185	35.324	60.745	61.943	2.843	3.003
5	1	120	37.43	36.815	68.115	67.404	2.688	2.726
6	1	120	37.268	36.815	67.988	67.404	2.713	2.726
7	1	180	36.347	36.273	**68.274**	68.372	3.119	2.907
8	1.5	60	39.893	39.646	61.444	60.672	**3.841**	3.784
9	1.5	120	**40.037**	39.922	64.775	65.767	3.401	3.327
10	1.5	180	37.806	38.166	66.590	66.370	3.198	3.327

As the obtained data showed, maximum desirability was observed in a starch concentration of 1.5% (X_1) and extraction time of 98.537 min (X_2). The effectiveness of adding starch to improve the *encapsulation efficiency* of calcium

alginate hydrogels was published in a previous study by (López Córdoba *et al*., 2013; Rassis *et al*., 2002; Sultana *et al*., 2000). This phenomenon might explain how potato starch is able to absorb and bind with bioactive compounds available in infusions within capsule formation inside a calcium chloride solution (0.05 M) for 15 min.

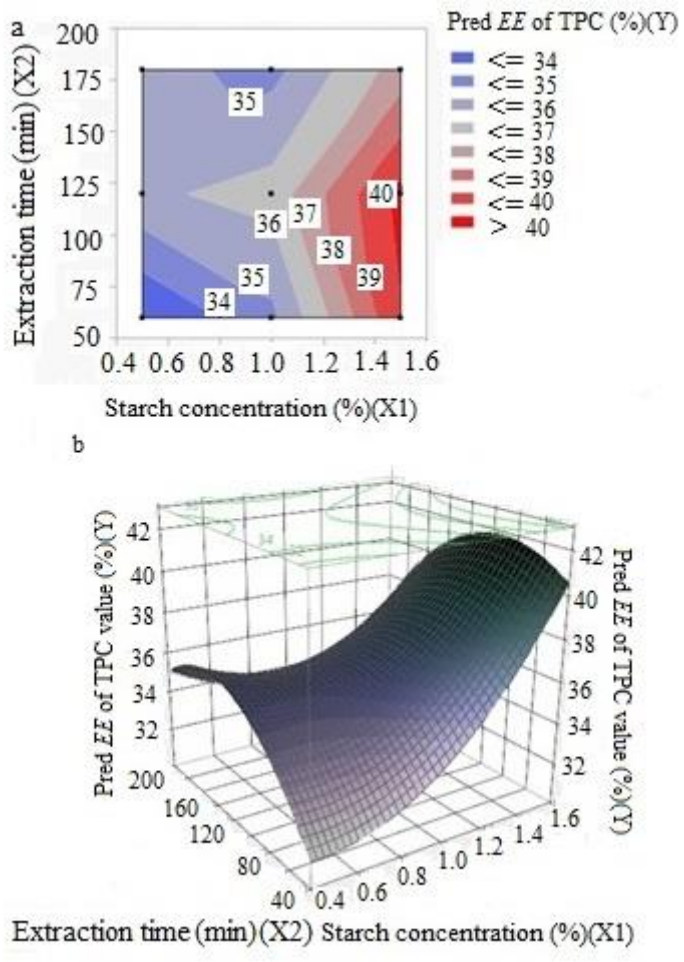

Figure 3.2 Contour (a) and response surface plots (b) of *encapsulation efficiency* of TPC value, (Pred Formula *Y*) as a function of X_1 (Starch concentration (%)) and X_2 (Extraction time (min)).

However, capsules with starch concentration of less than 1.5%, have shown less content of infusions, which may be due to a deficit in absorption and preserving the infusions within the capsule formation process. In other words, they lose more extracted contents into the calcium chloride solution because of the concentration gradients of bioactive compounds between the capsules' gel and the calcium chloride solution. It should be considered that some non-*phenols*, such as organic acids and sugars, could influence the determination of the total *phenolic* contents by *Folin-Ciocalteu*; therefore, this phenomenon might lead to an overvaluation of total

phenolic contents. Moreover, various *phenols* could present different behaviours to absorbance, unlike *gallic acid* and *rutin* (*rutoside*), which present similar behaviours, some *flavonoids* demonstrate low absorbance, which leads to an underestimation of various compounds (Kyoung Chun and Kim, 2004; Medina-Remón *et al*., 2009; Roura *et al*., 2006). Therefore, determination of the efficiency of encapsulation by TPC measurements is not the only reliable assay and, hence, TAA and FRAP determination are considered as well in this research.

3.3.3. EE of TAA value

Experimental and predicted values for TAA are illustrated in Table 3.2. The confine starts at 57.695% in run 1 (X_1=0.5% and X_2=60 min) and extends to 68.274% in run 7, under the conditions where X_1=1% and X_2=180 min. Fig. 3.3 shows the surface plot with the contour plot above. As was determined, the optimum condition for obtaining maximum TAA efficiency value is a starch concentration ranging 0.8-1.25% with an extraction time of 140-180 min. As shown, starch concentration in the range of 0.8-1.25% might act as a good absorber of bioactive compounds with the optimum releasing time ranging between 140-180 min. Starch concentrations of about <0.8% or >1.25% have not indicated any stronger association with bioactive compounds. Therefore, obtained data in this research show, in terms of TAA value, encapsulated infusions in the aforementioned boundaries of applied concentration of potato starch are able to stay in the human body or food products with greater resistance and are released gradually under a controlled manner dependent on the pH and temperature of the media situated.

3.3.4. EE of FRAP

Observed experimental and predicted results using *CCD* are given in Table 3.2. As shown, the lowest value was observed in run 2 under a starch concentration of 0.5% with an extraction time of 120 min, and the highest value was observed in run 8 under the following conditions, starch concentration of 1.5% and extraction time of 60 min. Fig. 3.4, shows the predicted three dimensional surface plot (b) with the contour plot (a) of response value (FRAP) above. As was observed, in the narrow range of starch concentration (1.4-1.5%) within an extraction time of between 60-80 min, the predicted *EE* of FRAP demonstrates the highest value. The response value in that range is >3.6%. This phenomenon also confirms the finding that starch

particles could react with bioactive compounds with reducing power accessible in the obtained infusions and maintain them into capsule structures.

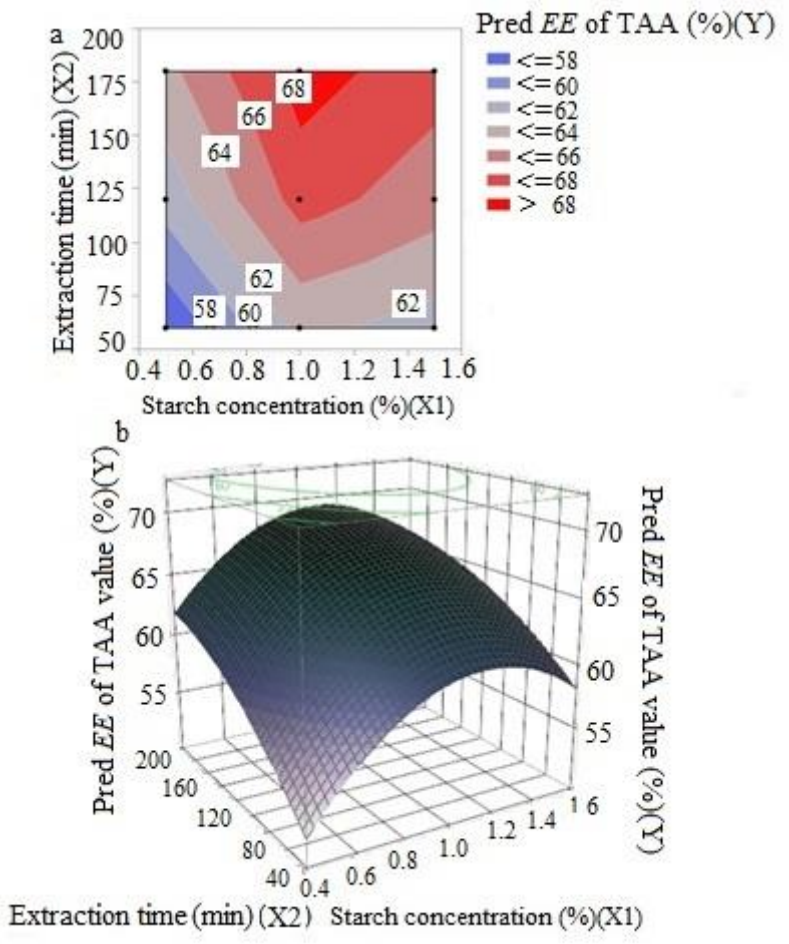

Figure 3.3 Contour (a) and response surface plots (b) of *encapsulation efficiency* of TAA value, (Pred Formula *Y*) as a fusnction of X_1 (Starch concentration (%)) and X_2 (Extraction time (min)).

Therefore, the availability of starch in the encapsulation process increases the response value; in other words, application of starch in capsule structures reduces the loss of bioactive compounds of extracts during the capsule formation period and prepares the conditions to release the compounds in a controlled manner, dependent on the chemical and physical properties of the target media. Table 3.3 also shows the *regression coefficient* results of FRAP's *encapsulation efficiency* (*EE*) value of obtained infusions of Portugues *Olea europaea* L. var. sylvestris., leaves. The obtained outcomes in Table 3.3 show a good fitness of the predicted model for FRAP value. Furthermore, the *correlation coefficient* between observed actual and predicted FRAP values is 0.932, which is a strong value and confirms the fitness of the selected model.

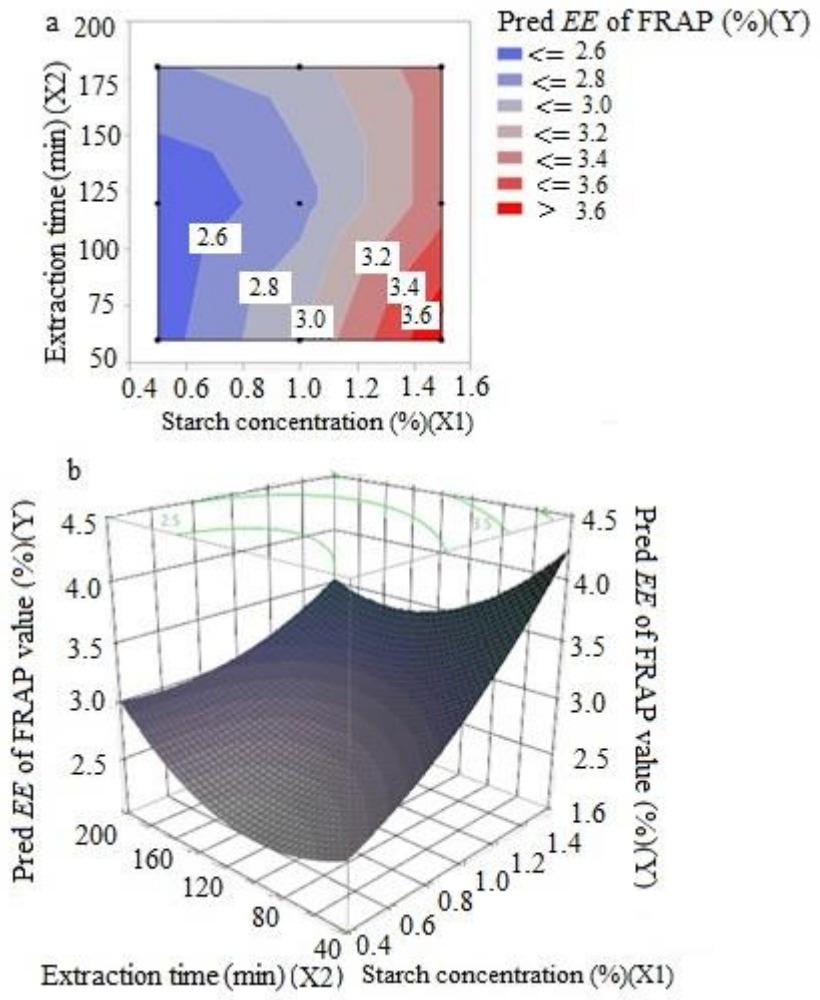

Figure 3.4 Contour (a) and response surface plots (b) of *encapsulation efficiency* of FRAP value, (Pred Formula *Y*) as a function of X_1 (Starch concentration (%)) and X_2 (Extraction time (min)).

3.3.5. Analysis of the model for encapsulation efficiency

Table 3.3 presents the outcomes of fitting the quadratic model of data. The *ANOVA* analysis of *encapsulation efficiency* of all three selected responses indicates non-significant lack of fit value (p_{value}>0.05) and significant value for the selected model (p_{value}<0.05) for the *encapsulation efficiency* of all three selected responses. This means that the selected model in this research is fit for all the responses as well. Also, the existence of a strong *correlation coefficient* (0.905, 0.963 and 0.932) between experimental and predicted values, respectively, for the *EE* of TPC, TAA and FRAP values shows that this performance is fit to explain more than 90.5%, 96.3% and 93.2% of the calculated model in *encapsulation efficiency* for TPA, TAA and FRAP values, respectively. The obtained R^2 values indicate that plotted points group around the diagonal line, expressing good adaptability with minimum

dissipations. The outcomes indicate that the model could act well for the prediction of those selected responses.

The optimisation of release kinetics of *Olea europaea* L. var. sylvestris'., aqueous infusions in citrate sodium (5 g. 100 mL^{-1}) was based on maximising the released bioactive compounds of capsules and/or all selected responses. The effect of starch concentration (X_1) (0.5. 1 and 1.5%) and time of extraction (X_2) (60, 120 and 180 min) of beads on the selected responses have been studied. The obtained second order equation models for dependent variables are given in Table 3.4. The linear effects of both independent variables on TAA response were both positive and significant. The means that, if all the extraction conditions are preserved constant, with an increase in starch concentration and/or time of extraction individually, the *encapsulation efficiency* of TAA value will show significant enhancement. Fig. 3.5 shows the individual impact of each independent value on the responses as clearly as possible. Furthermore, the quadratic effect of starch concentration and time of extraction show negative significant effect on this response. Table 3.5 also shows the optimised conditions of the variables on the selected responses. Moreover, achieving linear graphs in Fig. 3.5, confirms the controlled release or constant diffusion in a controlled manner of the bioactive compounds of the beads in this research.

3.4. Conclusions

Starch addition not only affects the *anti-oxidant* capabilities of encapsulated *Olea europaea* L. var. sylvestris'., infusion in hydrogel capsules, but also affects the optical morphology or physical characteristics. The release of bioactive compounds in citrate sodium (5g. 100 mL^{-1}) and the starch medias of encapsulation systems were managed mainly by constant diffusion. The application of starch in capsule structures creates the formation of darker and larger capsules. The findings in this research demonstrate that the encapsulation process in a starch concentration of ≥1.091% has the greatest effect on the efficiency of the extraction of bioactive compounds with total *anti-oxidant* activity. Regarding the obtained data, it could be concluded that the applied model for this research is suitable and could be applied in further prediction studies.

Table 3.3 *ANOVA* results for the effect of X_1 (starch concentration (%)) and X_2 time of extraction (min) on response variables (p<0.05).

Source	*Encapsulation efficiency* of TPC (%)				*Encapsulation efficiency* of TAA (%)				*Encapsulation efficiency* of FRAP (%)			
	DF	Sum of Squares	*F* Ratio	Prob>*F*	DF	Sum of Squares	*F* Ratio	Prob>*F*	DF	Sum of Squares	*F* Ratio	Prob>*F*
X_1	1	0.234	0.215	0.666	1	24.297	23.064	0.009*	1			
X_2					1	20.834	19.777	0.011*	1			
X_1^2					1	20.633	19.586	0.011*	1			
X_2^2					1	11.772	11.175	0.028*	1			
Model	5	41.759	7.652	0.035*	5	111.918	21.248	0.006*	5	1.608	11.138	0.0183*
Lack of fit	3	4.352	110.567	0.069	3	4.205	173.795	0.055	3	0.115	122.924	0.066
Error	4	41.759			4	4.213			4	0.115		
C. Total	9	46.125			9	116.132			9	1.724		
		R^2= 0.905				R^2= 0.963				R^2= 0.932		
		Adj, R^2= 0.787				Adj, R^2= 0.918				Adj, R^2= 0.849		

Table 3.4 *Regression coefficient*, standard error, and student's t-test results of response surface of the determined parameter ($p<0.05$).

Source	*Encapsulation efficiency* of TPC (%)				*Encapsulation efficiency* of TAA (%)				*Encapsulation efficiency* of FRAP (%)			
	Estimate	Std. error	*t* Ratio	Prob > |*t*|	Estimate	Std. error	*t* Ratio	Prob > |*t*|	Estimate	Std. error	*t* Ratio	Prob > |*t*|
Intercept	25.724	3.865	6.65	<0.003*	35.960	3.797	9.47	0.0007*	2.664	0.628	4.24	0.0133*
X_1					27.925	5.814	4.80	0.0086*				
X_2					0.215	0.048	4.45	0.011*				
X_1^2					-11.894	2.687	-4.43	0.011*				
X_2^2					-0.0006	0.0001	-3.34	0.028*				

Table 3.5 Optimum conditions of the variables on the selected responses.

Parameter	Starch of potato, concentration (%)	Time of extraction (min)	*Encapsulation efficiency* (%)	Desirability
TPC	1.5	98.537	40.057	0.866
TAA	1.091	161.620	68.651	0.888
FRAP	1.5	60	3.784	0.862

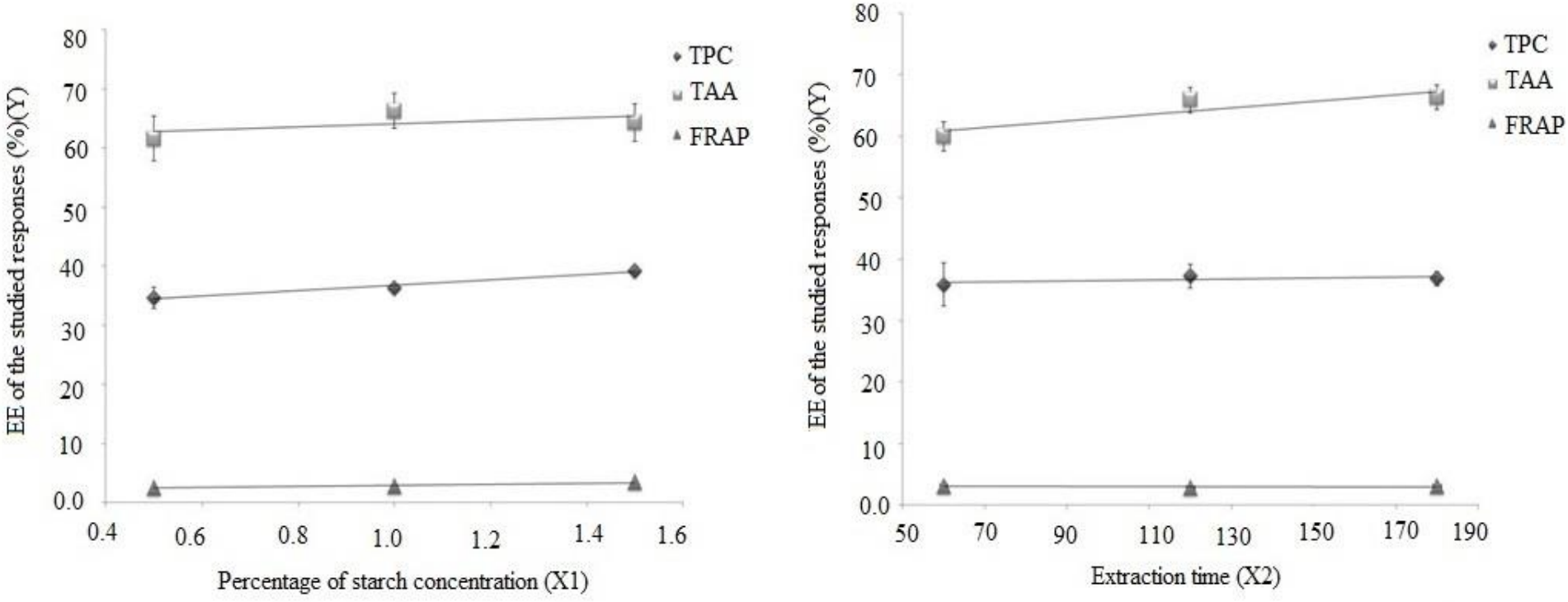

Figure 3.5 Graph of individual impact of each independent value on the responses.

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CHAPTER 4

Modelling of the extraction and screening of bioactive compounds of anise infusion using *GC-MS* analysis

This chapter expresses the optimal conditions of the extraction of bioactive compounds with anti-oxidant activities from Portugues Pimpinella anisum L.*, seeds and describes the profile of compounds of the infusions detected by GC-MS analysis*

Modelling of the extraction and screening of bioactive compounds of anise infusion using *GC-MS* analysis

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Abstract

Variables, including the time and temperature of the extraction of bioactive compounds of anise were optimised for the modelling of some selected *anti-oxidant* parameters. On *face Central Composite Design* (*FCCC*), comprising of 10 pilot runs with two replications at central points, was used. The optimised statuses were determined for the responses with higher desirability (≥81.8%). The *p* values obtained for the model fitness and lack of fit, and the existence of higher *correlation coefficient* values between experimental and predicted values of the responses, indicated adaptability of the selected design to the modelling of the extraction of bioactive components. The achieved results of *GC-MS* analysis indicated that the most predominant bioactive compounds are *fatty acids* including *linoleic*, *oleic* and *palmitic acids*; *triterpenoids* such as *lupeol*, *betulinic* and *ursolic acids*, the *phytosterols*, *fructose* and *coumarin*. The detected compounds presented anise as an eventual potential origin for the treatment of a wide confine of diseases.

Keywords: Bioactivity; *DOE*; *P. anisum* L.; *GC-MS*

4.1. Introduction

Herbal remedies are applied in the treatment of patients with various neurological disorders such as those who are *anti-epileptic*. Free radicals as well as *reactive oxygen species* (*ROS*) cause horror reactions in the human body as well as in deteriorations of food products [1]. Various researches are performed to acquire novel native and effectual bioactive origins. *Pimpinella anisum* L., (Anise) is one of the archaic identified and is extensively handled spice herbages in the treatment of a vast variety of disorders. This herb is aboriginal to the oriental Mediterranean lands and is a valuable origin in bioactive oil compounds. Various potential therapeutic uses including those for digestive irregularities, *anti-convulsant, dyspnoea* and also gynaecological uses with *anti-asthma* are portrayed for *Pimpinella anisum* L., seeds in classic pharmaceutical volumes. In addition, [1], demonstrated that aqueous and ethanol extracts of *Pimpinella anisum* L., seeds contain compounds with bioactivity and *anti-bacterial* potentials. Recently it was documented [2], that aqueous suspensions of anise possess wound healing and cyto-protective functions versus chemically induced gastrointestinal wounds in treated rats. Furthermore, the anti-convulsant potential of essential oils in the seeds of *Pimpinella anisum* L., was detected. Moreover it is expressed that this plant's oil boosts glucose absorbency and diminishes urine genesis, in other words it showed hypoglycemia potential [3]. Due to *Pimpinella anisum* L., being a highly applied herb in the folk medical profession, a research on the optimisation of the aqueous extraction and characterisation of bioactive compounds was deemed required and informative. No any similar published researches on optimisation of the extraction and identification of bioactive compounds of Portugues *Pimpinella anisum* L., seeds have been found.

4.2. Materials and methods

4.2.1. Plant materials

The aerial portions of *Pimpinella anisum* L., were picked in June 2014 from the exurbia of Faro-Algarve, Portugal. The plants were identified by Mr Rosa Pinto, (University of Algarve) before picking and an invoice instance was guaranteed at the Herbarium of the Faculty of Science and Technology of University of Algarve (voucher name: Registered as FSL2014). The whole of the aerial parts were dried in shade, separated manually and ground using an electrical grinder (Cetigo-Portugal).

Ground seeds were then shifted into particular vials; sealed and preserved under -20 ºC by the extraction day.

4.2.2. Chemicals and reagents

All chemicals and reagents required for the experiments and *GC-MS* analysis were provided in an analytical grade.

4.2.3. Apparatus

Spectrophotometer (T70+Vis, PG Instrument Ltd, United Kingdom); evaporator (*Nahita* series 503, Navarra, Spain); centrifuge *Hettich* (*universal-320*, Germany); hot plate-stirrer (*VWR*, 720 advanced, *USA*); vortex mixer (*Stuart*, UL-Bibby Sterilin Ltd); grinders (*Philips*-Brazil and *Cetigo*-Portugal).

4.2.4. Preparation of plant infusions

Initial extraction was encouraged to exploit distilled water. For each run, 1 g of the ground and sieved sample was dissolved in distilled water of different temperatures and terms regarding our design using a hot plate-magnet stirrer (*VWR*, 720 advanced, *USA*). Next, they were transferred to centrifuge vials and centrifuged in room temperature at 5000 rpm for 10 min using centrifuge *Hettich* (*universal-320*, Germany). The supernatant was filtered through *Sartorius stedim bio grade 388* filter papers. Once extraction flow was completed, solvents were vaporised by the rotational evaporator (*Nahita* series 503, Navarra, Spain) and the remaining materials were re-suspended in particular the volume of distilled water for the appropriate final concentrations of the assays. Infusions were shifted to Eppendorf tubes and preserved under -20 °C until the day of analysis. On the day of tests, the infusions were placed in darkness until defrosting.

4.2.5. Preparation of samples for GC-MS analysis

Soxhlet extraction is performed for *GC-MS* analysis. Concisely, 2 g of the ground and sieved *Pimpinella anisum* L., seeds were shifted into thimbles and extracted by dichloromethane (100 mL) overnight, which was sufficient time for discoloration. Afterwards, solvent was recovered using the rotary evaporator (*Qlabo*-Portugal) under the following conditions (500 mbar pressure, with the rotation value of 41 rpm under 40 ºC temperature). Obtained extracts were further dried at 40 ºC under the nitrogen atmosphere (to prevent the oxidation of the bioactive compounds) until extracts free of any solvent were obtained. Prior to the ultimate preparations of

samples, the obtained samples were shifted into a vacuum oven (35 ºC at 150 mbar for 48 h) to remove the eventual remaining humidity. Afterward, those obtained extracts were passed for *GC-MS* analysis.

4.2.6. Pilot layout and actuarial probing

The extraction procedure is accomplished in 10 runs. The pilot layout and actuarial probing were performed using *JMP® Pro* software, Version 11. Copyright, *SAS* Institute (Inc. *SAS* and all other *SAS* Institute Inc. product or service names are registered trademarks or trademarks of *SAS* Institute Inc., Cary, NC, *USA*). Pilot data was used as the mean. *On face CCD* (*Central Composite Design*) is applied when at least a pair of runs vary (p_{value}<0.05), and highlights when the obtained p-value by *ANOVA* becomes lower than 0.05. Differences between runs were recorded by *one-way ANOVA* followed by Tukey HSD *post-hoc*-test using the *SPSS* program, version 22.0 (*IBM SPSS* Statistics 22, licensed University of Algarve).

The extraction of bioactive components as a function of solvent temperature (X_1) and time-processing (X_2) was explored using on face *CCD* with two replications in the centre of the pilot domain (runs 5, 6). The pilot confine was specified taking into account outcomes obtained in the preliminary tests and the applied range for independent variables were selected as follows: X_1 (40-90 ºC) and X_2 (5-40 min) that were expressed in Table 4.1. Another of the selected independent variables were codified at three different levels (-1, 0, +1) demonstrating low, mid and high levels of the selected design.

The generalised response surface model was considered regarding the following equation 4.1:

$$Y_n = \beta_0 + \Sigma_{i=1}^{n}\beta_i X_i + \Sigma_{\substack{i=1 \\ j>1}}^{n-1}\Sigma_{j=2}^{n}\beta_{ij} X_i X_j + \Sigma_{i=1}^{n}\beta_{ii} X_i^2 \qquad (4.1$$

Where Y_n indicates the response selected to be modelled. β_0 Presents a constant, β_1 and β_2 express the coefficient of the linear modes, β_{12} is the coefficient of the interaction between the studied independent variables, and β_{11} and β_{22} demonstrate the coefficient of the quadratic modes of the independent variables. The model sufficiency was determined by the obtained *Pvalue* of the *Fisher test* for lack of fit and model in the confidence level of 0.05, as well as the *correlation coefficient* between the experimental and predicted values (R^2). Optimal extraction conditions for magnifying responses including TPC, TFC, TMA, TAA, FRAP and RP as well as

the minimisation of responses including $DPPH^{\cdot-}$ and $ABTS^{\cdot+}$ (IC_{50}) were tracked using the constructed graphs and collected data.

Table 4.1 Independent variables and their levels employed in on face *Central Composite Design* (*FCCC*) for optimization of *Pimpinella anisum* L., natural *anti-oxidant*.

Independent variable	Unit	Label	Coded levels		
			-1	0	+1
Time of extraction	min	X_1	5	22.5	40
Temperature of extraction	ºC	X_2	40	65	90

The layout matrix, comprising 10 runs, are summarised in Table 4.2. All the experiments are performed in three replications. The ultimate extraction conditions, using optimised statuses were validated based on the predictions of the developed model.

4.2.7. Determination of total phenolic contents (TPC)

The concentration of the total *phenols* in aqueous extracts was determined using *Folin-Ciocalteu* reagent and external gradation with *gallic acid* according to the explained assays of [4], and presented as $mg_{(GAE).}\ g^{-1}{}_{dw}$.

4.2.8. Measurement of total flavonoids (TFC)

Total flavonoid contents were monitored using a spectrophotometric assay with aluminium chloride hexahydrate, as described by [5] and presented as $mg_{(QE).}\ g^{-1}{}_{dw}$.

4.2.9. Total monomeric anthocyanin (TMA)

TMA content was recorded regarding the pH-differential assay reported by [6] and presented as mg. L^{-1}.

4.2.10. Determination of total anti-oxidant activity (TAA)

In on-going research, the total *anti-oxidant* potential was detected using phosphomolybdenum assay, as narrated by [7] and presented as $mg_{(AAE)}.\ g^{-1}{}_{dw}$.

Table 4.2 CCD with the observed responses for TPC, TFC, TMA, TAA, RP, FRAP, $ABTS^{\cdot+}$ and $DPPH^{\cdot-}$

Run	Coded variable levels		TPC ($mg_{(GAE)}. g^{-1}{}_{dw}$)		TFC ($mg_{(QE)}. g^{-1}{}_{dw}$)		TMA ($mg. L^{-1}$)		TAA ($mg_{(AAE)}. g^{-1}{}_{dw}$)	
	X_1	X_2	Exp	Pred	Exp	Pred	Exp	Pred	Exp	Pred
1	40	5	82.083	77.065	5.750	5.561	16.921	15.503	136.852	138.986
2	40	22.5	83.287	89.614	5.910	6.109	13.359	15.564	160.554	156.996
3	40	40	**80.143**	78.833	6.512	6.499	**4.453**	3.665	**130.101**	131.524
4	65	5	102.702	100.979	7.322	7.449	50.319	53.192	185.928	178.549
5	65	22.5	115.445	122.283	7.412	7.494	**69.467**	61.881	192.271	199.724
6	65	22.5	118.26	122.283	7.48	7.494	58.78	61.881	193.843	199.724
7	65	40	129.399	120.258	**7.613**	7.382	56.998	58.61	183.373	177.416
8	90	5	132.36	139.100	6.360	6.419	32.061	30.605	165.787	171.031
9	90	22.5	**186.35**	169.160	6.264	5.962	45.643	47.922	**205.147**	195.369
10	90	40	165.441	175.890	**5.112**	5.347	54.104	53.279	171.692	176.225

Bold numbers demonstrate maximum and minimum values for experimental values of each response. The statistical significance between the obtained data for each run have been detected ($p<0.05$).

Table 4.2 Continued

Run	Coded variable levels		RP ($mg_{(TE).}\ g^{-1}_{dw}$)		FRAP ($mg_{(TE).}\ g^{-1}_{dw}$)		$ABTS^{\cdot+}$ (IC_{50}(µg. mL^{-1}))		$DPPH^{\cdot-}$(IC_{50}(µg. mL^{-1}))	
	X_1	X_2	Exp	Pred	Exp	Pred	Exp	Pred	Exp	Pred
1	40	5	82.083	85.277	**118.809**	120.220	**498.904**	502.106	**376.236**	369.287
2	40	22.5	83.287	87.818	134.456	133.362	453.686	440.188	250.739	250.039
3	40	40	**80.143**	72.417	123.201	122.883	453.672	463.966	196.808	204.456
4	65	5	134.314	109.400	265.352	251.669	286.103	303.305	120.994	148.122
5	65	22.5	115.445	130.846	248.843	262.121	267.947	256.132	123.368	98.930
6	65	22.5	118.26	130.846	251.493	262.121	264.539	256.132	99.556	98.930
7	65	40	137.424	134.350	259.178	248.953	291.637	294.655	125.467	123.402
8	90	5	91.761	113.480	275.828	288.099	299.034	278.628	98.47	78.290
9	90	22.5	**186.35**	153.831	**318.676**	295.862	**212.483**	246.201	**73.39**	99.153
10	90	40	165.441	176.240	269.462	280.004	312.784	299.470	199.264	193.680

Bold numbers demonstrate maximum and minimum values for experimental values of each response. The statistical significance between the obtained data for each run have been detected (p<0.05).

4.2.11. Reducing Power (RP)

In order to detect the reducing power of each run, the experiments were performed regarding the described methods [8] and results were expressed as mg $_{(TE)}.g^{-1}_{dw}$.

4.2.12. FRAP (Ferric reducing anti-oxidant power) assay

The FRAP value was fulfilled according to the literature presented previously [9]. The obtained results were presented as $mg_{(TE).}\ g^{-1}_{dw}$.

4.2.13. $ABTS^{\cdot+}$ inhibition activity

Anti-oxidant activities of each run were also analysed by determination of their capabilities to inhibit $ABTS^{\cdot+}$ using an improved methodology stated previously [10], and IC_{50} values were expressed as µg. mL^{-1}.

4.2.14. Determination of $DPPH^{\cdot+}$ scavenging activity

The $DPPH^{\cdot+}$ scavenging abilities of the extracts was detected by adapting the method described [11] and IC_{50} values were indicated as µg. mL^{-1}.

4.2.15. Gas chromatography-mass spectrometry (GC-MS) analysis

The constituents were analysed with an Agilent 5975C inert *mass selective detector* (*MSD*) with Triple-Axis Detector coupled to an Agilent 7890A *gas chromatography* system, equipped with a *ZB-5HT* inferno capillary *GC* column (30 m × 0.025 mm × 0.1 µm film thickness) with temperature limits of (min=-60 to max= 400-430 ºC) (made in *USA*). Helium (flow rate, 1 mL. min^{-1}) was used as the carrier gas. The injector temperature was 280 ºC. The oven temperature programme was 100 ºC for 1 minute, increased by the rate of 10 ºC. min^{-1} to 370 ºC and was maintained for 1 minute, then increased by the same rate (10 ºC. min^{-1}) to 380 ºC and held for 5 min. Therefore the term of the whole run was reached on 35 min. The mass spectrometer was applied in EI mode and the injected sample was scanned from 40 to 950 *amu*. The compounds were identified using different databases by comparing their spectral data to those from the mass spectral libraries and with the spectral data of standard compounds.

4.3. Results and discussion

4.3.1. Analysis of the model

The optimisation of bioactive compounds' extraction of *Pimpinella anisum* L., seeds was performed based on the desirability of each response regarding the unit, including the maximisation of the TPC, TFC, TMA, TAA, FRAP and RP values, as well as the minimisation of $ABTS^{\cdot+}$ and $DPPH^{\cdot-}$ values. In order to diminish the number of parameters (independent variables), multiple parameters were formerly examined in an immense confine prior optimisation [12].

The effects of temperature (40-90 ºC) and the time of extraction (5-40 min) on the studied responses were studied. A higher linear *correlation coefficient* was obtained, 0.903, 0.954, 0.978 0.937, 0.971, 0.941, 0.970, and 0.966 between the experimental and predicted values of TPC, TFC, TMA, TAA, RP, FRAP, $ABTS^{\cdot+}$ and $DPPH^{\cdot-}$, respectively (see Tables 4.2 and 4.3).

The regression models of the intercept, linear and quadratic modes of the temperature of extraction, as well as the interactive effects between two independent variables, are showing a significant effect on the TFC value. In the TAA case, the linear and quadratic effects of the temperature of extraction, as well as the quadratic effects of time of the extraction, are presenting noticeable efficacies.

In TMA value, the intercept beside the linear and quadratic effects of temperature, as well as the interactive effects between the two independent variables have displayed considerable effects on the response. However, the intercept and quadratic modes of temperature are indicating noticeable efficacy on the FRAP value. The intercept and linear modes of both independent variables, as well as the quadratic mode of temperature of extraction, are showing significant effects on the $ABTS^{\cdot+}$ inhibition value, and finally, in $DPPH^{\cdot-}$, the scavenging activity of all modes have been expressing noticeable effects on response, with an exception in the quadratic mode of time of extraction. In RP and TPC responses, none of the modes are showing considerable efficacies (see Table 4.4).

Table 4.3 presents the outcomes of the fitting quadratic model of data. The *ANOVA* analysis stated appropriate model proficiencies with higher *correlation coefficients* (R^2>90%). The statistical analysis has defined substantial levels, attesting the fitness of the design in all responses in the confidence level of p_{value}=0.05. The outcomes illustrated that the model could work well for all the responses in this research.

Table 4.3 *ANOVA* results for the effect of temperature and time of extraction on response variables (p<0.05)**.**

Source	TPC ($mg_{(GAE)}. g^{-1}_{dw}$)				TFC ($mg_{(QE)}. g^{-1}_{dw}$)				TAA ($mg_{(AAE)}. g^{-1}_{dw}$)				TMA ($mg. L^{-1}$)			
	DF	Sum of Squares	*F* Ratio	Prob>*F*	DF	Sum of Squares	*F* Ratio	Prob>*F*	DF	Sum of Squares	*F* Ratio	Prob>*F*	DF	Sum of Squares	*F* Ratio	Prob>*F*
X_1					1	5.468	72.943	0.001*	1	1554.46	18.130	0.013*	1	2151.429	92.035	0.0007*
X_2																
$X_1 \times X_2$					1	1.010	13.472	0.021*								
X_1^2					1	4.964	66.215	0.001*	1	1293.122	15.082	0.017*				
X_2^2									1	1102.935	12.864	0.023*				
Model	5	2194.835	438.967	0.036*	5	6.261	16.704	0.008*	5	5124.676	11.954	0.016*	5	4322.223	36.979	0.002*
Lack of fit	3	231.796	77.265	0.137	3	0.297	40.465	0.114	3	341.713	113.905	0.076	3	36.398	0.212	0.881
Error	4	234.520			4	0.299			4	342.949			4	93.504		
Total model	9	2429.356			9	6.561			9	5467.625			9	4415.729		
			R^2= 0.903				R^2= 0.954				R^2= 0.937				R^2= 0.978	
			Adj, R^2= 0.782				Adj, R^2= 0.897				Adj, R^2= 0.857				Adj, R^2= 0.952	

*Indicates significant effect of each mode (p<0.05).

Table 4.3 Continued

Source	RP ($mg_{(TE).}\ g^{-1}_{dw}$)				FRAP ($mg_{(TE).}\ g^{-1}_{dw}$)				$ABTS^{\cdot+}$ (IC_{50}(ug. mL^{-1}))				$DPPH^{\cdot-}$(IC_{50}(ug. mL^{-1}))			
	DF	Sum of Squares	*F* Ratio	Prob>*F*	DF	Sum of Squares	*F* Ratio	Prob>*F*	DF	Sum of Squares	*F* Ratio	Prob>*F*	DF	Sum of Squares	*F* Ratio	Prob>*F*
X_1					1	9044.882	26.475	0.006*	1	26576.348	41.782	0.003*	1	25677.549	40.322	0.0032*
X_2					1				1	4980.563	7.830	0.048*	1	17833.083	28.0039	0.0061*
$X_1 \times X_2$					1				1				1	19631.092	30.827	0.0051*
X_1^2					1	5266.673	15.416	0.017*	1	17686.442	27.806	0.006*	1	13359.211	20.978	0.0102*
X_2^2					1				1	4283.914	6.735	0.060	1			
Model	5	10736.873	12.887	0.001*	5	45850.176	26.841	0.003*	5	83010.829	26.101	0.003*	5	73924.098	23.217	0.0047*
Lack of fit	3	662.550	55.740	0.098	3	1363.026	129.396	0.064*	3	2538.453	145.706	0.060	3	2263.724	2.661	0.416
Error	4	666.513			4	1366.537			4	2544.261						
Total model	9	11403.386			9	47216.713			9	85555.09						
		R^2= 0.941				R^2= 0.971				R^2= 0.970				R^2= 0.966		
		Adj, R^2= 0.868				Adj, R^2= 0.934				Adj, R^2= 0.933				Adj, R^2= 0.925		

*Indicates significant effect of each mode (p<0.05).

The *p* values obtained for model fitness and the lack of fit, as well as the existence of higher *correlation coefficients* between experimental and predicted values in all responses, confirmed the adaptability of the designed model in this study.

4.3.2. Analysis of responses

4.3.2.1. Optimisation of TPC extraction

The obtained experimental and predicted values for TPC are presented in Table 4.2 and expressed as mg $_{(GAE).}$ g^{-1}_{dw}. The TPC value was confined from 80.143 up to 186.35 mg $_{(GAE).}$ g^{-1}_{dw}, the minimum value for TPC in run 3 and 9 are respectively the highest and lowest values (see Table 4.2). Enhancement in temperature increased the extraction of bioactive compounds and this detection is in agreement with the obtained outcomes of [13, 14]. In Table 4.3, the analysis of variance (*ANOVA*) for TPC was stated. The relationship between the tentative and predicted values (R^2=0.903) showed good adaptability between those two variables with lower dispersion. Fig. 4.1a displays the contour plot of the predicted TPC value. The outcomes suggest that: time in the confine of (0-17 min) and temperature in the range of (85-90 ºC) lead to higher TPC values.

As Table 4.5 presents, the maximum desirability (0.820) was observed in X_1=90 ºC and X_2=11.724 min, the adequate response value for TPC in the maximum desirability was acquired as 120.088 ($mg_{(GAE).}$ g^{-1}_{dw}). This phenomenon could be justified by stating that in higher temperatures, *polyphenolic* compounds might be decomposed to further components if they have been impressed for a long term. This event also might be justified by releasing adhesive compounds such as polysaccharides from the cells under higher temperatures within longer period of extraction time [15]. Extraction under superior temperatures raises the proficiency of extraction by enhancing both the solubility of the solute (bioactive compounds) and diffusion modulus. Heating softens the herb's tissues and weakens phenol-protein and phenol–polysaccharide interactions, thus leading to further *polyphenols* diffusion into the solvent [16]. However, heating cannot augment the bioactive extraction indefinitely. In temperatures other than 50 ºC for a long term extraction, the resistance of bioactive compounds are reduced with dramatic effect on their bioactivity [17].

Table 4.4 *Regression coefficient*, standard error, and student's t-test result of response surface of the determined parameters (p<0.05).

Source	TFC ($mg_{(QE)}. g^{-1}_{dw}$)				TAA ($mg_{(AAE)}. g^{-1}_{dw}$)				TMA ($mg. L^{-1}$)			
	Estimate	Std. error	*t* Ratio	Prob > |*t*|	Estimate	Std. error	*t* Ratio	Prob > |*t*|	Estimate	Std. error	*t* Ratio	Prob > |*t*|
Intercept	-3.941	1.227	-3.21	0.032*					-168.441	21.679	-7.77	0.0015*
X_1	0.326	0.038	8.54	0.001*	5.501	1.292	4.26	0.013*	6.472	0.674	9.59	0.0007*
X_2												
$X_1 \times X_2$	-0.001	0.0003	-3.67	0.021*					0.019	0.005	3.57	0.023*
X_1^2	-0.002	0.0002	-8.14	0.001*	-0.037	0.009	-3.88	0.017*	-0.048	0.005	-9.52	0.0007*
X_2^2					-0.070	0.019	-3.59	0.023*				

*Indicates significant effect of each mode (p<0.05).

Table 4.4 Continued

Source	FRAP ($mg_{(TE)}. g^{-1}_{dw}$)				$ABTS^{\cdot+}$ (IC_{50} (μg. mL^{-1}))				$DPPH^{\cdot-}$(IC_{50} (μg. mL^{-1}))			
	Estimate	Std. error	*t* Ratio	Prob > |*t*|	Estimate	Std. error	*t* Ratio	Prob > |*t*|	Estimate	Std. error	t Ratio	Prob > |t|
Intercept	-297.06	82.88	-3.58	0.0231*	1222.542	113.089	10.81	0.0004*	1117.549	113.155	9.88	0.0006*
X_1					-22.747	3.519	-6.46	0.003*	-22.359	3.521	-6.35	0.0032*
X_2					-8.733	3.121	-2.8	0.048*	-16.526	3.123	-5.29	0.0061*
$X_1 \times X_2$									0.160	0.028	5.55	0.0051*
X_1^2	-0.076	0.019	-3.93	0.0172*	0.139	0.026	5.27	0.006*	0.121	0.026	4.58	0.0102*
X_2^2												

*Indicates significant effect of each mode (p<0.05).

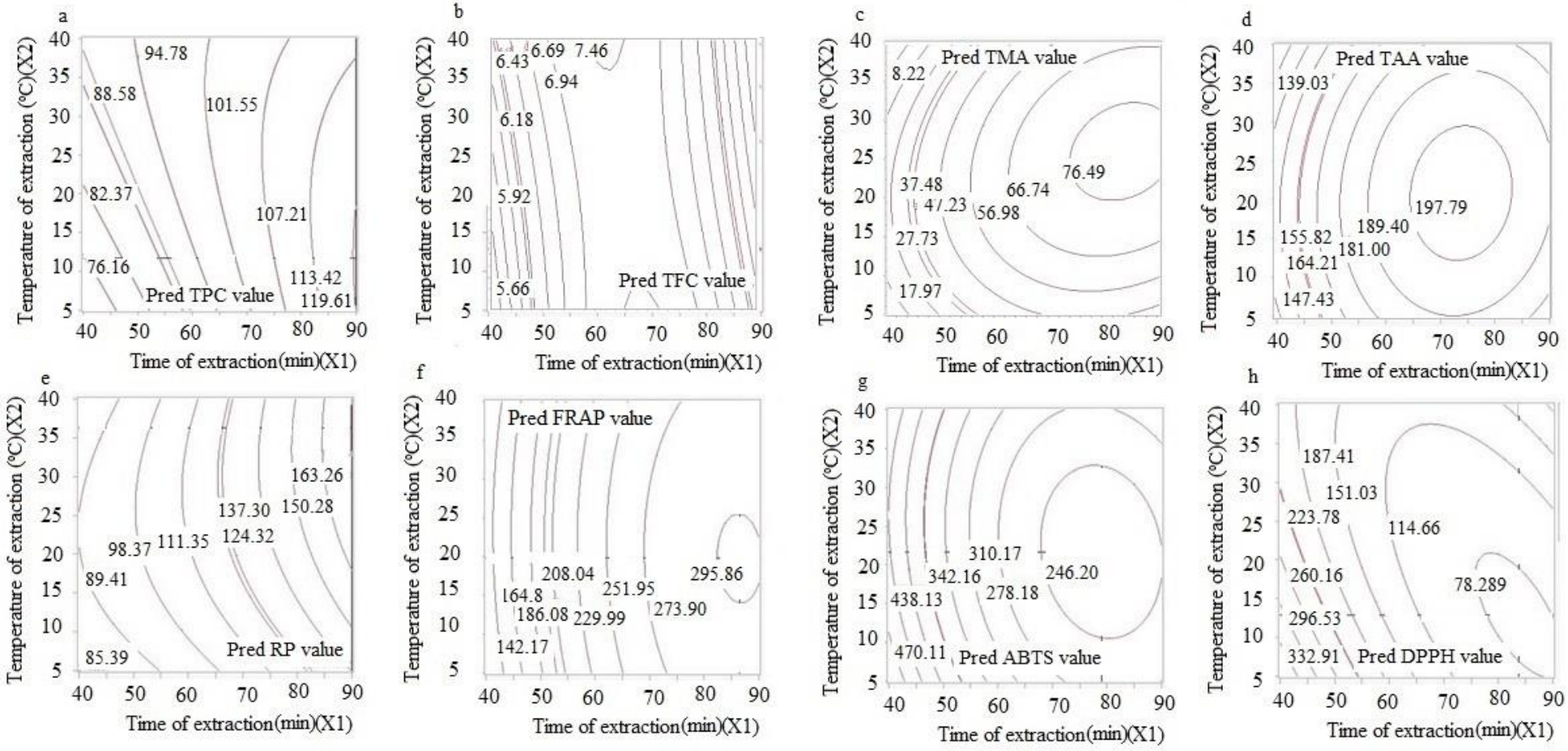


Figure 4.1 Contour plots of TPC (a), TFC (b), TMA (c), TAA (d), RP (e), FRAP (f), *ABTS* (g) and *DPPH* (h) values (Predicted formula) as a function of extraction temperature (X_1) and time (X_2).

4.3.2.2. Optimisation of the extraction of TFC

The considerable effects of temperature on TFC values are proven by various studies [18-20]. Experimental and predicted TFC values are shown in Table 4.2. As it is presented, the confine of the value initiates of 5.112 to 7.613 ($mg_{(QE)}. g^{-1}_{dw}$), the maximum and minimum values were obtained respectively in runs 7 and 10. The data obtained has indicated the circumstances of temperature and time on flavonoid contents in the obtained infusions of *P. anisum* L., seeds. Extraction was accomplished at various given temperatures and past times based on our design (Table 4.1). Fig. 4.1b shows that the output of the extraction of total *flavonoids* was boosted gradually with an increment of temperature. After reaching a peak at 65.057 ºC until 90 ºC, TFC value started dropping. A substantial *correlation coefficient* between the obtained experimental and predicted TFC values was observed (R^2=0.954). Fig. 4.1b presents the predicted contour plot with saddle form. The saddle shape of the graph represents the higher points of a curve on two opposite sides. It is therefore found that the temperature in the confine of (X_1= 75-90 °C) and time in the span of (X_2 = 5-20 min) lead to premium TFC extraction.

As shown in Table 4.5, the maximum desirability (0.819) was obtained at X_1= 65.057 °C and X_2= 19.56 min, and the corresponding response value for the predicted TFC in the maximum desirability is observed 7.497 ($mg_{(QE)}. g^{-1}_{dw}$). This phenomenon might be explained by the fact that solvent viscosity was declined and the movement of molecules was accelerated by an increment in temperature, and as a result, bioactive compounds could be released from cells.

However much higher temperatures boost the decay of some thermo-sensitive bioactive compounds [19, 21]. According to the wooden, rigid and tight construction of the *P. anisum* L., seeds, the solvent requires more time to penetrate the cells while on the other hand; a medium temperature is required to disintegrate the cells. It could be justified that a higher temperature in the widespread confine of time is able to release viscous or adhesive compounds of the cells that are able to pull down the extraction proficiency of TFC. Table 4.4 demonstrates the *regression coefficient* of the obtained data explained previously in section 3.2.1.

4.3.2.3. Optimisation of TMA extraction

The experiential and predicted values of TMA (mg. L^{-1}) obtained for *P. anisum* L., seeds were presented in Table 4.2. As is obvious for experimented TMA

values the supreme value (69.467) was obtained in run 5 and the lowest value (4.453) ($mg_{(cyanidin\text{-}3\text{-}glucoside)}$. L^{-1}) was observed in run 3. The analyses of the variance results of data were displayed in Table 4.3. As reported in Table 4.3, a potent relationship was gained (R^2=0.978) between the experimental and predicted values. Fig. 4.1c expresses the contour plot of the TMA (with peak response surface plot (not shown)) as affected by independent variables. As it is shown, the desirable range for the predicted TMA was observed in a given temperature in the confine of 70-75 ºC and the extraction time spent in the confine of 25-35 min. The optimal point for the TMA extraction of *P. anisum* L., was observed under the statuses of temperature (X_1=73.387 °C) and time (X_2=30.699 min). Maximum desirability (0.915) was observed in the optimal condition and corresponding response values for the predicted TMA value in the maximum desirability was detected as 65.229 ($mg_{(cyanidin\text{-}3\text{-}glucoside)}$. L^{-1}) (see Table 4.5). The proposed optimal conditions therefore for the extraction of TMA is introduced for temperatures in the confine of 70-75 ºC and the extraction time in the range of 25-35 min. This phenomenon could be illustrated by the fact that in the conditions lower than the declared, the solvent is not able to penetrate inside plants' cells to extract TMA.

Moreover, it is possible that the temperature and time higher than the optimal conditions cited formerly, could possibly decompose *anthocyanin* compounds or release some viscid compounds that diminish the extraction efficiency of *anthocyanins* - this is in line with the findings of [22], that stated temperature is one of *anthocyanins'* main degradation agents. Degradation kinetics is triggered by the exploiting of higher temperatures within an extraction time of anything other than 30.699 min.

The graphical portraits of the regression equation, the three-dimensional response and two-dimensional response surfaces are so adequate to present the relationship between affiliate variables and also interactive between them. Linear modes of temperature of extraction, as well as interactive modes between both independent variables, are demonstrating positive significant effects on the response, unlike the quadratic mode of temperature which is presenting a negative, noticeable effect on TMA value.

Table 4.5 Prediction profiler desirability and optimum conditions of the extraction.

Response	Factor		Correspondent response value	Desirability
	X_1 (Temperature (º C))	X_2 (Time (min))		
TPC($mg_{(GAE)}$. g^{-1}_{dw})	90	11.724	120.088	0.820
TFC($mg_{(QE)}$. g^{-1}_{dw})	65.057	19.56	7.497	0.819
TMA(mg. L^{-1})	73.387	30.699	65.229	0.915
TAA ($mg_{(AAE)}$. g^{-1}_{dw})	75.215	22.792	203.98	0.913
RP ($mg_{(TE)}$. g^{-1}_{dw})	90	36.298	176.412	0.818
FRAP ($mg_{(TE)}$. g^{-1}_{dw})	86.487	19.781	297.144	0.861
$ABTS^{\cdot+}$(IC_{50} (µg. mL^{-1}))	79.023	21.694	229.028	0.901
$DPPH^{\cdot-}$(IC_{50} (µg. mL^{-1}))	83.796	12.923	73.951	0.915

4.3.2.4. Response surface analysis of TAA

Experimental and predicted TAA values were expressed in Table 4.2. The confine is from 130.101 in run 3 to 205.147 ($mg_{(AAE)}$. $g^{-1}{}_{dw}$) in run 9, under defined conditions (Table 4.2). Fig. 4.1d, shows the contour plot (peak response surface plot was not shown). As detected; the optimal condition for achieving maximum TAA value is heating under the confine of a temperature of almost 70-80 ºC for 25-35 min. As the results obtained for the regression analysis for TAA in Table 4.4 showed, the linear mode of temperature of extraction demonstrates a positive significant effect on response, unlike the quadratic effects of both independent variables which indicate negative significant efficacies on TAA. Optimal conditions for TAA, as expressed in Table 4.5, are observed at temperatures of 75.215 ºC for 22.792 min extraction time, the obtained TAA value for this condition was 203.98 ($mg_{(AAE)}$. $g^{-1}{}_{.dw}$) with the higher desirability value equal to 0.913. As the results obtained by scientists have shown, increasing the temperature might boost the extraction efficiency of bioactive compounds that are responsible for offering *anti-oxidant* potentials, and the findings of this work are in agreement with the outcomes of [13, 14]. On the other hand; boosting the temperature over 75.215 ºC reduces TAA value, and furthermore higher temperatures for a long extraction time presented a decline in TAA value, which is probably due to the susceptibility of bioactive compounds when confronted with higher temperatures, and probably trigger the viscos compounds from cells with respect to the findings of [17, 19, 21].

4.3.2.5. Response surface analysis of RP

The experimental and predicted RP values are presented in Table 4.2, and as it is proposed in Table 4.3, there is a higher relationship (R^2=0.941) between the experimental and predicted RP values. The reduction of power activity confined from 80.143 up to 186.35 ($mg_{(TE)}$. $g^{-1}{}_{dw}$) under the defined conditions in table 4.2. In Table 4.4, an analysis of variance (*ANOVA*) for the response value is presented.

Fig. 4.1e, presents the contour plot of RP values (rising ridge response surface plot was not presented). The supreme predicted response value was observed in temperatures ranging from 85-90 ºC within an extraction time of 30-40 min. Table 4.5 indicates the maximum value for reducing the power of *P. anisum* L., seeds infusion. It shows that the optimal value has been proven at a temperature of 90 ºC for 36.298 min of extraction time. Desirability at this point is 0.818 and the

corresponding RP value at the optimal point was observed as 176.412 ($mg_{(TE).}\ g^{-1}{}_{dw}$). This phenomenon has proposed that the optimal conditions for the extraction of bioactive compounds are similar in extracts with higher reducing value. Therefore, it might be concluded that a collection of diverse compounds are possibly able to offer reducing activities.

4.3.2.6. Response surface results of FRAP

The experimental and predicted results obtained by *CCD* are presented in Table 4.2, and observed that the lowest value was obtained in run 1 and the highest value proved in run 9. Fig. 4.1f, demonstrates the predicted peak contour plot, as it has been realised, in a confine of temperatures of around 80-90 ºC and an extraction time of 15-25 min. The predicted FRAP value represents the higher values, and as has been shown in Table 4.5, the predicted optimal condition to obtain infusions with the highest FRAP value, is 86.487 ºC for 19.781 min extraction time. The achieved value for FRAP at this point was obtained at 297.144 ($mg_{(TE).}\ g^{-1}{}_{dw}$), the desirable optimal point was observed at 0.861. The values obtained for the *regression coefficient* of FRAP value demonstrates that the linear effects of both independent variables, unlike interactive effects between these two variables and quadratic effects of them, show a positive effect on the response, and among those cited modes, only the quadratic effect of temperature of extraction shows a significant effect on FRAP value. Obtained *p* values for the model and lack of fit, as well as a strong *correlation coefficient* between actual and predicted values (R^2=0.971) confirm the adaptability of the predicted model.

4.3.2.7. Response surface analysis of $ABTS^{\cdot+}$ inhibition activity

The outcomes of the predicted and actual $ABTS^{\cdot+}$ inhibition values obtained demonstrate the solid relationship (0.970) between them. Fig. 4.1g presents the contour plot, (with depth response surface plot not shown). As is observed, the optimal statuses for the extraction of infusions with higher $ABTS^{\cdot+}$ inhibition potentials (the lowest IC_{50}) are in the confine of temperatures of 75-85 ºC and an extraction time of 20-25 min. The results obtained in Table 4.5, show the optimum conditions for $ABTS^{\cdot+}$ inhibition potentials value of *P. anisum* L., seeds' infusions as follows: 79.023 ºC extraction temperature within 21.694 min extraction time. The corresponding response value at this point is observed at 229.02 µg. mL^{-1} and desirability for this point is achieved at 0.901. The results obtained in Table 4.3

demonstrate the appropriate *p* values of lack of fit ($p>0.05$) and model ($p<0.05$) as well as the excellent *correlation coefficient* between the experimental and predicted values ($R^2=0.970$) illustrate the good fit of the selected model for the response. Moreover, as presented in Table 4.4, the linear modes of both the independent variables, negatively, and the quadratic mode of temperature of extraction, positively, have noticeable effects on the response. Similar findings reported previously by [23] not only in this response but also for most of the responses found, which stated that, "the more linear modes, the less magnitude of interaction effects on responses and contrariwise".

4.3.2.8. Results of central composite design on $DPPH^{\cdot-}$ scavenging ability

The results obtained for both the actual and predicted values were expressed in Table 4.2. The confine initiates were from 73.39 in run 9 to 376.236 (µg. mL^{-1}) in run 1, and this phenomenon shows the stability of the components engaged in offering radical scavenging potentials versus great temperature (83 °C) by about a 13 min period of extraction. As Fig. 4.1h indicates, the optimal confine for scavenging $DPPH^{\cdot-}$ initiated from 80-90 ºC within 5-15 min extraction time. This finding is in line with the findings of [13, 14, 17, 19, 21]. The optimal point was determined at a temperature of 83.796 ºC for about 12.923 min extraction time; at which that point the $DPPH^{\cdot-}$ scavenging potential was found at 73.951 (µg. mL^{-1}) with 0.915 desirability value. As the data obtained and presented in Table 4.4, the linear modes of both the independent variables, negatively, interactive mode between them and quadratic mode of temperature of extraction positively have been showing substantial effects on response value. Table 4.3 represents the *p* values obtained for the model and lack of fit, thus the *p* values obtained for these two variables, as well as the higher *correlation coefficient* between the actual and predicted values of *DPPH* ($R^2=0.966$) present the suitability of the selected design model for this response.

4.3.2.9. Determination and experimental validation of the optimal conditions

The optimal conditions were detected by maximising the desirable exploiting *central composite design* prediction profiler. In order to specify the predictive precision of the model, the outcomes of the optimal conditions obtained were applied for the extraction of the bioactive compounds; the results obtained proved the accuracy of the achieved values by the model. The detected optimal conditions to

obtain infusions with the supreme values of TPC, TFC and TMA as well as TAA, FRAP, RP and the minimum values for (IC_{50}) of both $ABTS^{\cdot+}$ and $DPPH^{\cdot-}$ have been expressed in Table 4.5. As shown, the minimum temperature for the extraction of the bioactive compounds was suggested as 65.057 ºC. The maximum temperature for the extraction of these compounds was offered at 90 ºC for just 11.724 min of extraction time. As has been recognised, various bioactivity parameters have shown diverse optimal conditions in the confine of temperatures from 75.215-90 ºC for 12.923-36.298 min extraction time. It demonstrates the role of a wide range of bioactive compounds in presenting *anti-oxidant* potentials, such as several *alkaloids*; *fatty acids*; *triterpenoids*; *carbohydrates*, *monoacylglycerols*; *Ketons*, *aldehydes*, *sterols*, *phenols*, *anthocyanins* and *flavonoids* etc.

4.3.2.10. $ABTS^{\cdot+}$ inhibition and $DPPH^{\cdot-}$ scavenging potentials

Reactive oxygen species (*ROS*), or free radicals, are produced by some carcinogenic compounds, such as aromatic hydrocarbons, preservatives and betel nut (*Areca catechu*) infusions, and play an impressive role in the initiation, promotion and even growth of cancer agents in living cells [3, 24-26]. Furthermore, *ROS* is imagined as being most likely to be able to spoil tumour suppressor genes, leading to an acceleration of cancer cell growth, involving a *hydroxyl radical species* ($OH^{\cdot}$), generated as by-products in the process of cell metabolism [27, 28]. Therefore, bioactive components mainly inhibit *cancers* by effectively eliminating additional free radicals to diminish the gene transformation, accelerating the immunity function, and protecting tumour suppressor genes. $ABTS^{\cdot+}$ inhibition and $DPPH^{\cdot-}$ scavenging assays were intensively applied to evaluate bioactivity potentials due to their good segregation capabilities within the visible-light range's wavelength [29]. IC_{50} range for inhibition of $ABTS^{\cdot+}$ radicals initiates from 498.904 in run 1 to 212.483 (µg. mL^{-1}) in run 9 and for the scavenging of $DPPH^{\cdot-}$, starts from 376.236 in run 1 to 73.39 (µg. mL^{-1}) in run 8.

4.3.3. Detected compounds profile by GC-MS

The qualitative contents of the bioactive compounds from *P. anisum* L., seeds achieved by the soxhlet extraction technique by dichloromethane as solvent and *GC-MS* analysis technique are presented in Table 4.6. The relative proportions of the studied compounds could be useful in the approximation of absolute content in the selected extract. There was a fraction of unidentified compounds (24.4%) (See table

4.6). Bioactive compounds, including *fatty acids* such as *9,12-Octadecadienoic acid* (*linoleic acid*)+*oleic acid*, *hexadecanoic acid trimethylsilyl ester* (*palmitic* acid), *6,7-Dihydroxycoumarin di TMS* (*esculetin*) and *β-sitosterol TMS ether* (*sterol*), demonstrating 25.39, 10.19, 5.88 and 5.31% respectively, express the maximum values in *P. anisum* L., seed extraction. *Fatty acids* might interfere in the transcellular transferring system by affecting different cellular transporters. Additionally, *fatty acids* might act against the gene expression of cancer cell lines, and as a result might prevent the generation of carcinogenic cells. As shown in Table 4.6, along with *linoleic* and *oleic acids*, *palmitic acid* is found in substantial quantities in anise. *Conjugated linoleic acid* is detected as an effective suppressor in the lifecycle of carcinogenic cells including initiation, progress and metastasis along with angiogenesis and neovascularisation. Furthermore, (*CLA*) isomers indicated the substantial inhibitory potential on induced tumour cells of colon, mammary gland and the metastasis of inoculated cancer cells.

Oleic acid showed an enhancement in the absorption of mitoxantrone (*MXR*) and therefore an increase in the gene expression of *BCRP* in *CACO-2 cells* - in other words, it might prevent the generation of carcinogenic cells. *Palmitic acid*, along with *linoleic acid*, proves to be very effective in the reduction of blood cholesterol levels, and as a result it might diminish the precipitation of the remaining cholesterol in the inner wall of veins. *Esculetin* (*coumarin*), *β-sitosterol* (*phytosterols*) and *terpenoids* including *Ursolic acid*, *lupeol* and *β-Amyrin* are detected in substantial quantities in anise. Among natural compounds, *coumarin* such as *esculetin* is attracting interest due to its noticeable health benefits. Several health benefits are attributed to this compound, including *anti-inflammatory*, *anti-oxidative* and anti-tumour potentials. Additionally, most of the potential health benefits of *coumarin,* such as *hyperglycaemic*, *anti-inflammatory*, *anti-oxidative*, *anti-proliferative* and *anti-tumour*; are attributed to its higher activity [30].

Esculetin might lower plasma glucose levels through improved glucose utilisation in *type I diabetes*. *Phytosterols* (*PSE*) such as *β-sitosterol* detected in this research might improve *cardiovascular* protection. These compounds might increase the expression of *ABCG 5* and *8*, which are carriers involved in the reverse transport of *cholesterol* from enterocyte to the intestinal lumen. Additionally, *PSE* might reduce the activity of *Acetyl-coenzyme A acetyl-transferase* (*ACAT*), an enzyme that reesterifies *cholesterol* for its incorporation into the *chylomicrons*. Particularly, *β-*

sitosterol is a major plant sterol which arranges *cholesterol* levels in blood and is considered as an *anti-atherogenic*, *hypoglycaemia* and *anti-asthmatic* agent. *β-sitosterol* was also found to be effective in suppressing breast (*ATCC*), colon (*HT-29*) and prostate (*LNCaP*) cancer cell lines. On the other hand, *triterpenoids*, including *lupeol*; *β-amyrtin*; *betulinic* and *ursolic* acids were detected in Portugues *P. anisum* L., expressed as 4.09, 2.72, 1.77 and 1.19% respectively Also, further *triterpenoids* such as *oleanolic acids*, were determined in lower quantities. Many potential health benefits are attributed to *triterpenoids*. *Lupeol* presents a wide confine of health benefits including benefits against inflammation, *cancer, arthritis, diabetes, heart disease, renal* and *hepatic toxicity*. *Lupeol* and its derivatives such as *acetate, palmitate* and *linoleate*, exhibited higher *anti-inflammatory* activity than commonly used chemical drugs like *indomethacin*. This valuable compound also showed wound-improving potential and diminished *Benzo (a) pyrene* levels, recognised as eventual mutagen-induced *genotoxicity*. In summary, lupeol's eventual potential health effects are categorised as follows: *anti-microbial*; *anti-protozoal*; *anti-cancerous*, *hypoglycemia*; *cardioprotective*; *anti-inflammatory*; *skin protective*; *hepatoprotective* and *nephroprotective*.

β-Amyrtin has demonstrated *anti-inflammatory, gastro-protective* and anti-pruritic effects. Additionally, this compound might show *hypoglycemia*, *anti-nociceptive* activities. *Betulinic acid (BA),* along with *betulin*, has exhibited a wide range of potential health benefits. *Anti-HIV*; *anti-cancer*; *anti-tumour* and *anti-inflammatory* activities are attributed to this compound. *Ursolic acids*, as other *triterpenes*, were detected in anise, possessing many biological properties such as being *anti-tumour*, *anti-inflammatory* and *anti-oxidant*. Its administration is able to produce neuropharmacological effects including the improvement of the *cognitive deficits*, *neuroprotective*, *anti-septic* and *anti-depressant*-like activities.

Table 4.6 Detected compounds profile of extracts of *P. anisum* L., (seeds) obtained by *GC-MS* analysis.

Peak	Constituent	Area (%)	Method of identification
	Sugars	2.06%	
1	Sugar C5	0.33	a, b
2	Sugar C6	0.48	a, b
3	Fructose	1.25	a, b
	Fatty acids	47.68%	
4	*Decanoic acid, trimethylsilyl ester*	0.08	a, b
5	*Dodecanoic acid, trimethylsilyl ester*	0.15	a, b
6	*n-Pentadecanoic acid, trimethylsilyl ester*	0.34	a, b
7	*Fatty acid C16:1*	0.17	a, b
8	***Hexadecanoic acid, trimethylsilyl ester (Palmitic acid)***	10.19	a, b
9	*Fatty acid (ND)*	0.1	a, b
10	*Fatty acid (ND)*	0.68	a, b
11	*Heptadecanoic acid, trimethylsilyl ester*	0.38	a, b
12	*Linoleic acid, trimethylsilyl ester*	0.12	a, b
13	***9,12-Octadecadienoic acid (linoleic acid) + Oleic acid***	25.39	a, b
14	***Octadecanoic acid, trimethylsilyl ester***	**2.78**	a, b
15	*Nonadecanoic acid, TMS*	0.09	a, b
16	*Fatty acid (ND)*	1.12	a, b
17	***Octadecadienoic acid,TMS***	**2.28**	a, b
18	***Eicosanoic (Arachidic) acid, TMS***	0.7	a, b
19	*Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester*	0.75	a, b
20	***Docosanoic (behenic) acid, TMS***	**1.12**	a, b
21	*Octadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester*	0.29	a, b
22	*Tetracosanoic acid, trimethylsilyl ester*	0.5	a, b
23	*Hexacosanoic acid TMS; C:26*	0.18	a, b
24	*Fatty acid C28*	0.27	a, b
	Dicarboxylic acids	**0.98%**	
25	*Malic acid, tris (trimethylsilyl) ester*	0.1	a, b
26	*2-Isopropyl malic acid TMS*	0.02	a, b
27	*2-Butenedioic acid TMS*	0.05	a, b
28	*Heptanedioic acid TMS*	0.04	a, b
29	*Tartaric acid, TMS*	0.08	a, b
30	*Octanedioic acid*	0.69	a, b
	Aromatics	**0.59%**	
31	*Benzaldehyde, 3-methoxy-4-[(trimethylsilyl)oxy]-*	0.1	a, b
32	*Benzeneacetic acid, 4-[(trimethylsilyl)oxy]-, trimethylsilyl ester*	0.1	a, b

Table 4.6 Continued

33	*Benzaldehyde, 3,5-dimethoxy-4-[(trimethylsilyl)oxy]-*	0.08	a, b
34	*Benzoic acid, 3-methoxy-4-[(trimethylsilyl)oxy]-, trimethylsilyl ester*	0.05	a, b
35	*Azelaic acid, bis (trimethylsilyl) ester*	0.26	a, b
	Alkanes	1.79%	a, b
36	*Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-, scyllo-*	0.06	a, b
	Fatty alcohol	0.57%	
37	*Steryl alcohol (fatty alcohols)*	0.57	a, b
	Hydroxy fatty acids	1.1%	
38	*Hydroxy acid C:28*	0.48	a, b
39	*Hydroxy acid C:30*	0.62	a, b
	Sterol	7.29%	
40	*Campesterol TMS*	0.28	a, b
41	*Stigmasterol trimethylsilyl ether*	0.98	a, b
42	*β-sitosterol trimethylsilyl ether*	5.31	a, b
43	*Sitosterol – glucopyranoside*	0.72	a, b
	Triterpenoids	15.56	
44	***β-Amyrin TMS***	2.72	a, b
45-47	***Lupeol***	8.63	a, b
48	*Betulinic acid*	0.57	a, b
49	*Oleanolic acid TMS*	0.68	a, b
50	***Betulinic acid TMS***	1.77	a, b
51	***Ursolic acid***	1.19	a, b
	Others	1.34%	
52	*Octadec-9Z-enol TMS ether*	0.65	a, b
53	*6,7-dihydroxycoumarin -beta- d-glucopyranose*	0.69	a, b
	Not identified		
54	ND	24.4%	a, b

*Retention time obtained by chromatogram, ND: presents not detected, a= Retention Index; b= MS (*GC-MS*) Library.

Fig. 4.2 demonstrates the chromatogram obtained by *GC-MS* as well. Interestingly, *fructose* was found in substantial quantities in this plant. Several potential health effects are attributed to this *monosaccharides*, and *fructose* consumption is related positively to fasting blood sugar, *triglycerides* and *systolic* blood pressure and diminished *HDL cholesterol* [31]. A wide confine of bioactive compounds comprising of *fatty acids*; *sterols*; *triterpenoids*; *phenolic* compounds as well as *carbohydrates* are detected in different quantities. The chemical structures of the most dominant absolute detected compounds are presented in Fig. 4.3. These

findings demonstrated the substantial role of *fatty acids*; *triterpenoids* and *sterols* in demonstrating the health benefits in *P. anisum* L.

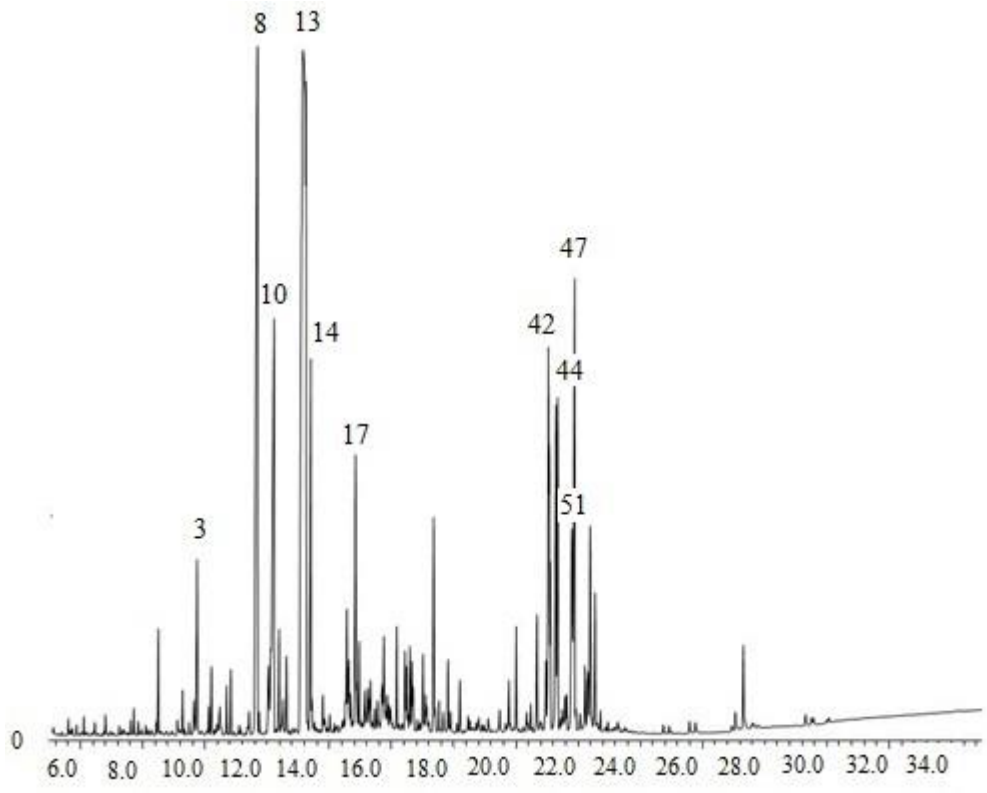
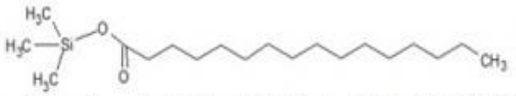


Figure 4.2 *GC* chromatogram of *P. anisum* L., seeds picked in southern Portugal.

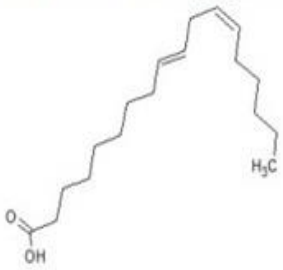
4.4. Conclusions

The current research provides an insight into the optimised extraction conditions of *P. anisum* L., seeds for achieving the maximum desirability of the selected response values. Optimal points for each response were presented in Table 4.5. Regarding the obtained outcomes, it is observed, that both temperature and time influence the bioactive compound extractions as well as *anti-oxidant* potentials with various intensities. The mass shift from cells to solvent is dependent on the time and temperature of extraction, which increases with time until the maximum extraction efficiency is achieved, while the temperature also improves the dissemination. The application of a higher extraction status than the recorded optimal points in this research (90 ºC beyond 11.724 min (TPC), 65.057 ºC beyond 19.56 min (TFC) and 73.387 ºC beyond 30.699 (TMA)), did not illustrate any substantial progressions in the extraction of bioactive compounds.

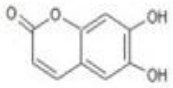


Palmitic acid (Hexadecanoic acid, trimethylsilyl ester) $C_{12}H_{42}O_2Si$ (328.60 g. mol-^{1})

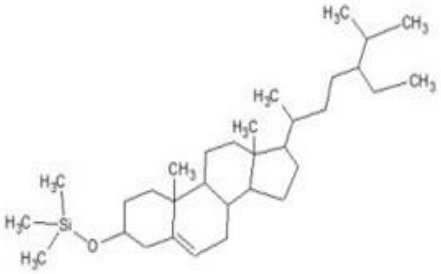
Oleic acid, Cis-Octadecenoic acid, $C_{18}H_{34}O_2$ (282.46 g.mol^{-1})



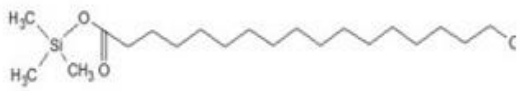
Linoleic acid (9,12-Octadecadienoic acid) $C_{18}H_{32}O_2$ (280.44 g.mol^{-1})



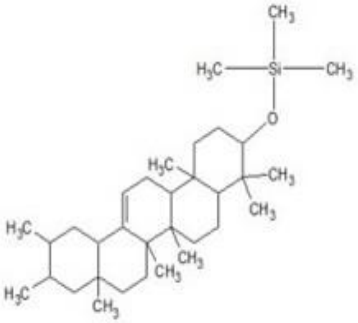
6,7-Dihydroxycoumarin, di-TMS, $C_6H_9O_4$ (356.65 g.mol^{-1})



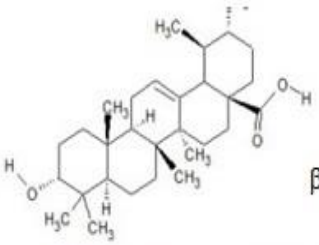
β-Sitosterol trimethylsilyl ether (β-Sitosterol TMS) $C_{32}H_{58}OSi$ (486.88 g. mol^{-1})



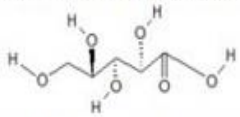
Stearic acid (Octadecanoic acid, trimethyl ester) $C_{21}H_{44}O_2Si$ (356.65 g. mol^{-1})



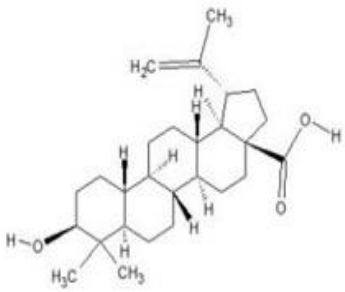
β-Amyrin TMS (Silane, trimethyl (olean-12-en-3β-yloxy)-$C_{33}H_{58}OSi$ (498.89 g. mol^{-1}))



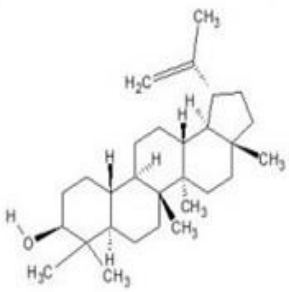
Ursolic acid, 3 β-Hydroxy 12-en-28-oic acid $C_{30}H_{48}O_3$ (456.70 g. mol^{-1})



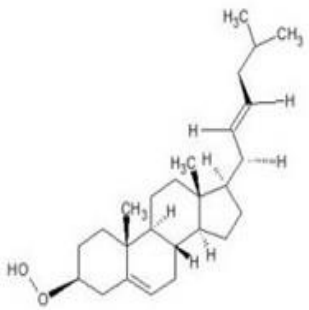
Fructose, D(-)Fructose (3S, 4R, 5R)-1, 2, 4, 6-pentahydroxyhexan-2-one $C_6H_{12}O_6$ (180.15 g. mol^{-1})



Betulinic acid



Lupeol



Stigmasterol

Figure 4.3 Chemical structures of the major compounds detected by *GC-MS* in Portuguese *Pimpinella anisum* L., seeds' infusion (Structure are constructed by *ChemSketch* released 2012).

This might be attributed to firstly, the thermal decomposition of susceptible bioactive components at higher temperatures that was intensified by long-term extraction; and secondly, the eventual triggering of the available cohesive compounds from cells that reduce extraction performance. The achieved results of the *GC-MS* analysis indicated that the most predominant effects of the three *fatty acids* (≥35.58%) including *linoleic*; *oleic* and *palmitic acids*, present the potential health benefits of *P. anisum* L., seeds. *Triterpenoids* such as *lupeol*; *ursolic* and *betulinic acids*, along with *esculetin* (*coumarin*); *β-sitosterol* (*phytosterols*) and carbohydrates (*fructose*) were also detected, demonstrating the substantial roles of the selected compounds in exhibition of bioactivity of anise. Therefore, anise might possess different potential health benefits such as being *anti-HIV, hypoglycaemia, anti-tumour, anti-inflammatory, anti-carcinogenic, anti-radical, anti-Alzheimer's* etc. regarding the nature of the detected compounds.

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CHAPTER 5

Modelling of the release kinetic and *encapsulation efficiency* of *phenolic* compounds in *simulated gastric fluid* (*SGF*) using *RSM* and *ANN*

In this chapter the release kinetic of the encapsulated Alisma plantago-aquatica L. subsp. orientale (Sam.)Sam., leaves extracts in simulated gastric fluid has been studied. Three independent variables have been designed to optimize the extraction process.

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MODELLING OF THE RELEASE KINETIC AND *ENCAPSULATION EFFICIENCY* OF *PHENOLIC* COMPOUNDS IN *SIMULATED GASTRIC FLUID* (*SGF*) USING *RSM* AND *ANN*

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Submitted (Got revision)

ABSTRACT

Three independent factors consisting of the extract concentration (X_1) and starch (X_2) in the prepared capsules with sodium alginate and the extraction time of capsules (X_3) were designed in this research. Three various levels for each factor, including extract concentration (20, 30 and 40 mg. mL^{-1}), starch concentration (0.75, 1.5 and 2.25%) and extraction time (60, 120 and 180 min) were studied. The specified optimised statuses for the maximum desirability of response value were obtained as follows: X_1=32.012 mg. mL^{-1}, X_2=0.934% and X_3=180 min. The corresponding response value and desirability in the optimised point were obtained as 32.112% and 0.960, respectively. The obtained statistical values authorised the compatibility of the selected design (*CCD*). The identical design was also applied to acquire a training anthology for *ANN*. *ANN* has shown superior prediction performance compared to *RSM*. Moreover, the obtained results showed that starch application in beads' structures might cause some alterations in the physical properties of the produced beads.

Keyword: Release kinetics; sodium alginate; potato starch; encapsulation; *phenols*; physical properties

PRACTICAL APPLICATION

Today, due to the high demand for natural products in the human diet, there is an increased focus on methods for enhancing the efficiency of the extraction of bioactive compounds from natural products, including medicinal plants. As a consequence, the detection of optimised conditions for extraction with minimum waste is an important area of research. In this research the extracted bioactive compounds have been encapsulated by sodium alginate combined with potato starch to optimise and study the release kinetics of bioactive compounds and some visual characteristics of the prepared beads. Prediction methods including *RSM* and *ANN* have been applied to optimise the release kinetics process in *SGF*. The results achieved by *ANN* and *RSM* were compared. Three independent variables have been optimised for the maximum efficiency of the encapsulation of *phenolic* compounds. The results obtained using root mean squared error (*RMSE)* and R^2 reveal the *ANN* model as being more accurate than *RSM*.

5.1. INTRODUCTION

Pa ghazeh (Persian name) or *Gaz ayaghi* (Turkish name), or Tachagem da água (Portugues name) with the methodical appellation of *Alisma plantago-aquatica* L. subsp. orientale (Sam.)Sam., and the prevalent name of "water plantain" is exploited widely as a common medicament in Iran, as well as other parts of the world. This precious herb is also named the "Frog's spoon" in central Europe. Some scholars of antiquity, such as Pietro Andrea Mattioli (1560) and Theodor Zwinger (1696), introduced the application of rhizomes and the atmospheric sectors of *Alisma plantago-aquatica* L., to tackle the three - and four-day fevers which came with malaria incurred by plasmodium vivax Grassi and Feletti (three days) and Plasmodium malariae Feletti and Grassi (four days) (Adams *et al*., 2011). The rhizomes of the sub-species of *Alisma plantago-aquatica* L. subsp. orientale (Sam.)Sam., (Synonym: *Alisma orientale* (Sam) Juzep.) are known in the Chinese medical profession as *ze xie,* and are highly regarded in the treatment of *diabetes* and *inflammation* (Nakajima *et al*., 1994). The plant is also used in China to facilitate the delivery and treatment of *leprosy*. It is also used in Iran due to its beneficial effects against *gonorrhoea, diabetes* and kidney disorders. Furthermore, this plant is astringent and could stimulate the production of breastmilk. A wide range of

medicinal plants and their mechanisms of action have been mentioned in many studies, such as Farzaneh and Carvalho, (2016).

Recently, *Response Surface Methodology* (*RSM*) and *Artificial Neural Network* (*ANN*) have been used for both the modelling and optimisation of the extraction procedures (Dolatabadi *et al*., 2016; Ghaffari *et al*., 2011; Rostami *et al*., 2014), no similar publications on the comparing the modelling of the release kinetics and *encapsulation efficiency* of *phenols* from encapsulated herbal infusions using *RSM* with *ANN* have been found. Although some researches have been performed on the optimisation of the extraction of bioactive compounds from different sources (not particularly *Alisma plantago-aquatica* L., leaves) (Brazinha *et al*., 2014; Ilaiyaraja *et al*., 2015), the authors have nevertheless found no researches that model the *encapsulation efficiency* and release kinetics of bioactive compounds with the three independent variables selected in this research, *using simulated gastric fluid* (*SGF*) as a solvent. In addition to identifying the optimised conditions for encapsulation, making it convenient, fast and economic for researchers and industry owners to screen the production procedures, this research has also compared the most reputed models, including *RSM* and *ANN*, and finally determines the most reliable model for wide-scale application in the nutraceutical and pharmaceutical industries, as these models make considerable cost and time reductions for the related industries as well. Therefore, in the current study two-level, three-factor (2^3) full factorial *central composite-orthogonal blocks* (*CCD-Orthogonal Blocks*) in *RSM* and *ANN*-based models were expanded to predict the optimised statuses of the concentration of extract (X_1) and starch (X_2) in the jelly and the time of extractions of the beads (capsules) (X_3) using *simulated gastric fluid* (*SGF*) as a solvent on the total *phenolic* compounds (TPC) (response variable (Y)). It should be demonstrated that in the current research, along with the release kinetics of *phenols* from encapsulated extracts, the *encapsulation efficiency* of the TPC value has been detected and is presented in the related table, since 3D graphs have been constructed based on *encapsulation efficiency* (*EE*) as the response. The release kinetic modes for each independent variable have been individually presented in a separate figure. The *encapsulation efficiency* of each run was detected based on the total *phenolic* compound values of the original infusion extracted using the *microwave-assisted method* (*MAE*). Finally, the outcomes obtained by *RSM* and *ANN* were statistically verified by the modulus of designation (R^2), *root mean squared error* (*RMSE*) and

the *absolute average deviation* (*AAD*) based on the validation data anthology. The aim of this research is to optimise the encapsulation process of *Alisma plantago-aquatica* L., extract with sodium alginate enriched with potato starch using *RSM* and *ANN*, and determination of the *in vitro* release kinetics and *encapsulation efficiency* (*EE*) of the fabricated particles and comparison in terms of visual morphology, such as colour and appearance as well as diameters. As it is found, this research is the first report studying *Alisma plantago-aquatica* L., leaves' extract using the current approach.

5.2. MATERIALS AND METHODS

5.2.1. Chemicals and reagents

Folin-Ciocalteu's phenol reagent (*F-C* reagent) was purchased from *Sigma-Aldrich*, (Switzerland-Germany). Alginic acid sodium salt was provided from *Alfa Aesar* (*A Johnson Matthey Company*-Karlsruhe, Germany). Calcium chloride 2-hydrate ($CaCl_2.2H_2o$) and starch from potatoes were obtained from *Panreac* (Barcelona-Spain). The hydrochloric acid min. 37% (HCL) was purchased from *Sigma-Aldrich* hydrochloric (Switzerland-Germany).

5.2.2. Plant materials

Dried plants were provided by the *Zarrin Giah greenhouse company*, located in Urmia-Iran, in February 2014 (Voucher number: ZGC139211). Once the plants had been moved to the laboratory, they were preserved in a dark and dry place until grinding. Selected plant materials were ground by two grinders respectively according to the power; afterwards, ground samples were sieved and stored in sealed containers at -20 °C until extraction.

5.2.3. Apparatus

A spectrophotometer (*T70+Vis*, PG Instrument Ltd, United Kingdom), evaporator (*Nahita* series 503, *Navarra*, Spain), centrifuge *Hettich* (*universal-320*, Germany), Plate-stirrer (*VWR*, 720 advanced, *USA*) were used. Disposable cuvettes were provided from *VWR* (Leuven, Belgium). Vortex mixer (*Stuart*, UL-Bibby Sterilin Ltd), grinders (*Philips*-Brasil and *Cetigo*-Portugal), microwave oven (*Samsung* type 1713, Microwave output 800 W) and shaker-incubator ES-20 (230 V, 50-60 Hz, 150 W).

5.2.4. Alisma plantago-aquatica L. subsp. orientale (Sam.)Sam., *(Pa ghazeh) leaf extract*

Microwave-assisted extraction (*MAE*) of bioactive compounds was carried out using an ordinary household microwave oven (*Samsung* type 1713, Microwave output 800 W) at a working frequency of 2450 MHz, with adjustable microwave power and time. Approximately 2 g of sample powder was weighed and placed into a 500 mL Pyrex beaker, with 73 mL distilled water added and blended thoroughly with a stirrer bar for 5 min at 200 rpm to afford the adequate penetration of the solvent. The beaker was situated in the middle of the oven over a rotational saucer and was exposed to microwave radiation power of 300 W for 120 s. The applied microwave power and extraction time of the *Alisma plantago-aquatica* L., leaves were adjusted according to our previous research (Farzaneh and Carvalho, 2016-Unpublished). After *microwave-assisted extraction* (*MAE*), the mixture in the Pyrex beaker was left to cool down to room temperature and centrifuged for 10 min at 5000 rpm, then filtered through *Sartorius stedim bio grade 388* filter paper. Once the extraction process was completed, water was vaporised using the rotary evaporator (*Nahita* series 503, Navarra, Spain) and the remaining materials were re-suspended in specific volume deionised water for defined ultimate concentration for the experiments. Infusions were transmitted to Eppendorf tubes and were kept at -20 °C until analysis. On the day of encapsulation and characterisation, extracts were placed in darkness until defrosting.

5.2.5. Experimental design and statistical analysis

RSM was applied to characterise the optimised conditions for the extraction of *polyphenols* of the encapsulated *Alisma plantago-aquatica* L. The experimental design and actuarial analysis for both the *response surface method* (*RSM*) and the *Artificial Neural Network* (*ANN*) models were performed using *JMP® Pro*[1]. Differences in the obtained data were confirmed using a one-way *analysis of variance* (*ANOVA*), which is a required stage of modelling. In regard to a lack of substantial differences in the data, the optimisation is meaningless.

Two-level, three-factor *central composite-orthogonal blocks* (*CCD-Orthogonal blocks*) were occupied to accomplish the extraction trial. *CCD-*

[1] *JMP® Pro*[1], Version 11. Copyright, *SAS* Institute (Inc. *SAS* and all other *SAS* Institute Inc. product or service names are registered trademarks or trademarks of *SAS* Institute Inc., Cary, NC, *USA*)

Orthogonal blocks is very adequate for providing a volume of knowledge on probation variable effectiveness and overall tentative faults within a minimal volume of runs (Aghakhani *et al*., 2011). Selected models were used to assess the composed efficacies of three independent variables, consisting of the concentration of the extract (mg. mL^{-1}) and starch (%) of the jelly and extraction period of beads (capsules) (min), which were termed as X_1, X_2 and X_3, respectively in different levels (Table 5.1). The extract concentrations at 13.68, 20, 30, 40 and 46.32 (mg. mL^{-1}), starch concentrations of 0.276, 0.75, 1.5, 2.25 and 2.724% and extraction time of beads at 22.08, 60, 120, 180 and 217.92 (min) were set for the trial as the layout. The complete design of 20 combinations, including 6 replications of the centre point (Table 5.2), and the response function *(Y)* was partitioned into linear, quadratic, and interactive components.

TABLE 5.1 EXPERIMENTAL CONFINE AND LEVEL OF INDEPENDENT PROCESS VARIABLES.

Independent variables	Units	Symbol	Range and levels				
			-α	-1	0	1	+α
Extract concentration	mg. mL^{-1}		13.68	20	30	40	46.32
Starch concentration	%		0.276	0.75	1.5	2.25	2.724
Extraction time of beads	min		22.08	60	120	180	217.92

$$Y = \beta_0 + \sum_{i=1}^{3} B_i X_i + \sum_{i=1}^{3} B_{ii} X_i^2 + \sum_{\substack{i=1 \\ i<j}}^{2} \sum_{j=i+1}^{3} B_{ij} X_i X_j \quad (5.1$$

Where *Y* indicates the response; β_0 denotes the model intercept; B_i, B_{ii} and B_{ij} represent the coefficients of the linear, quadratic, and interactive modes, respectively. X_i and X_j are the coded absolute variables; and equal to the number of examined factors (*F*=3). The outcomes obtained from the *analysis of the variance* (*ANOVA*), and *regression coefficients* of the individual linear, quadratic, and interactive terms, are provided in Tables 5.3 and 5.5. The significance of each term was assessed statistically by computing the *F*-value at the probability of (*p*=0.05).

5.2.6. Artificial Neural Network (ANN)

Recently, *ANN* has been recognised as a towering gadget for the simulation and optimisation of the extraction process (Cavas *et al*., 2011). *Artificial Neural Networks* (*ANN*s) are mathematical designs that approximate the appendage of biological neural networks. A *multilayer perceptron* (*MLP*) *neural network* is a feed-forward *ANN* that includes three or more layers of nodes, with two layers of nodes representing the inputs and outputs. Each of the nodes in the first layer (input) is connected to one or more layers of hidden neurons that consist of one or more of the three various activation functions, including *Hyperbolic tangent* (*TanH*), *linear* and *Gaussian*. These neurons are in turn connected to ultimate level output neurons and, by exploiting learning algorithms, the respective authority of each input neuron and their intricate interplays on the obtained outcomes can be calculated. A *Multi-Layer Perception* (*MLP*) was expanded in *JMP® Pro*, Version 11. Copyright, *SAS* Institute, with three input neurons expressing the infusion concentrations (mg. mL^{-1}) (X_1) and starch concentrations (%) (X_2) in the jelly and also the extraction period of the beads or capsules (min) (X_3), and a single hidden layer of neurons, including eight nodes of *Hyperbolic tangent* (*TanH*) activation function and eight nodes of *Linear* activation function with the arrangement of *3:16:1*, and an output layer representing the *encapsulation efficiency* of *polyphenolic compounds* or response value (TPC) (*Y*). The numeral of nodes in the hidden layer needs to be designed to construct an effectual *ANN* model. An *ANN* model with extra hidden neurons might prove to have lower training errors, but higher extension error due to over-fitting (Cevoli *et al*., 2011), therefore, the issue of hidden neurons was resolved by training various feed-forward networks of various topologies such as *3:14:1*, *3:14:14:1*, *3:12:12:1*, *3:16:16:1* etc. and nominating the optimum one i.e. (*3:16:1*) to rely on the minimisation of the r*oot mean squared error* (*RMSE*) and the maximisation of the *correlation coefficient* (R^2) (Table 5.5). The selection of one hidden layer is generally adequate for the objective of ongoing nonlinear functions, as additional hidden layers might cause over-fitting (Hush and Horne, 1993), therefore, a solitary hidden layer network with the arrangement of *3:16:1* was selected in this research (Figure 5.4). The numeral of nodes in the hidden layer was specified by trial and error to minimise the deviance of predictions from experiential outcomes. It should be noted that in each of the arrangements featuring one or two hidden layers, each layer should contain nodes belonging to all or one of the three various activation functions, including *TanH*, *Linear* or *Gaussian*. To avoid the over-fitting of the *ANN* model to

TABLE. 5.2 *CENTRAL COMPOSIT-ORTHOGONALBLOCKS (CCD-ORTHOGONAL BLOCK)* DESIGN MATRIX WITH OBSERVED AND EDICTED VALUES.

Run no	Extract concentration (mg. mL^{-1}) (X_1)	Starch concentration (%) (X_2)	Extraction time of beads (min) (X_3)	Released *phenolic* compounds of the capsules ($mg_{(GAE)}. g^{-1}_{dw}$)	*Encapsulation efficiency* of TPC (%)		
					Experimental*	Predicted response by *RSM*	Most likely response by *ANN*
1	20(-1)	0.75(-1)	60(-1)	7.115 ± 0.507	19.232 ± 1.371	19.551	19.232
2	20(-1)	2.25(1)	180(1)	6.428 ± 0.395	17.375 ± 1.068	17.575	17.375
3	30(0)	1.5(0)	120(0)	10.052 ± 0.552	27.169 ± 1.494	28.116	27.925
4	30(0)	1.5(0)	120(0)	10.460 ± 0.548	28.271 ± 1.478	28.116	27.925
5	40(1)	0.75(-1)	180(1)	11.514 ± 0.382	31.121 ± 1.032	30.187	31.121
6	20(-1)	2.25(1)	60(-1)	7.976 ± 0.645	21.558 ± 1.739	21.179	21.558
7	20(-1)	0.75(-1)	180(1)	10.514 ± 0.906	28.417 ± 2.443	28.332	28.417
8	20(-1)	2.25(1)	60(-1)	5.581 ± 0.296	15.085 ± 0.798	15.555	15.085
9	30(0)	1.5(0)	120(0)	10.322 ± 0.949	27.899 ± 2.558	28.105	27.925
10	30(0)	1.5(0)	120(0)	10.083 ± 0.464	27.253 ± 1.252	28.105	27.925
11	40(1)	0.75(-1)	60(-1)	7.492 ± 0.457	20.249 ± 1.232	19.585	20.249
12	40(1)	2.25(1)	180(1)	9.530 ± 0.457	25.757 ± 1.234	24.975	25.757
13	13.68(-1.632)	1.5(0)	120(0)	5.915 ± 0.810	15.987 ± 2.184	15.212	15.987
14	30(0)	0.276(-1.632)	120(0)	8.581 ± 0.607	23.193 ± 1.638	23.807	23.193
15	30(0)	1.5(0)	22.08(-1.632)	7.587 ± 0.273	20.508 ± 0.738	20.442	20.508
16	30(0)	1.5(0)	120(0)	10.332 ± 8.816	27.925 ± 2.202	26.626	27.925
17	30(0)	1.5(0)	120(0)	10.141 ± 0.760	27.410 ± 2.050	26.626	27.925
18	30(0)	1.5(0)	217.92(1.632)	11.088 ± 0.905	29.97 ± 2.44	30.729	29.97
19	30(0)	2.724(1.632)	120(0)	6.004 ± 0.424	16.228 ± 1.145	16.307	16.228
20	46.32(1.632)	1.5(0)	120(0)	7.337 ± 0.181	19.832 ± 0.490	21.300	19.832

* The experimental encapsulation efficiency of each run has been detected regarding the released phenolic compounds in *SGF* of each run (released phenols) and based on the detected TPC value of the original extract (37.19 ($mg_{(GAE)}. g^{-1}_{dw}$)).

5.2.7. Determination of total phenolic contents (TPC)

The concentration of total *phenolics* in the samples was estimated using the *Folin-Ciocalteu* reagent and exterior calibration with *gallic acid*, according to the assays of (Huang *et al*., 2006) and expressed as ($mg_{(GAE).}\ g^{-1}{}_{dw}$).

5.2.8. Prepared solutions

5.*2.8.1. Acetate-acetic buffer (pH=5.6)*

2.86 mL glacial acetic acid was diluted by deionised water and diluted to 250 mL (Acetic acid 0.2 M), after which approximately 25 mL acetate sodium 0.2 M was added; finally, 250 mL of deionised water was added to the prepared solution.

5.2.8.2. Calcium chloride (0.05 M)

7.351g of $CaCl_2.2H_2o$ was dissolved in deionised water and diluted to 1000 mL.

5.2.8.3. HCL solution (pH=2)

82.5 µL HCL 37% (12.1 M) was dissolved in deionised water and diluted to 100 mL (HCL=1 N).

5.2.9. Preparation of alginate hydrogels beads

The hydrogel beads were prepared by ionic gelation assay to obtain a calcium alginate matrix, a method presented by (López Córdoba *et al*., 2013) with some modifications. *Alisma plantago-aquatica* L., leaves' infusion with various proportions was dissolved in a sodium alginate solution (2%), in order to achieve a definitive solution with 13.68, 20, 30, 40 and 46.32 mg. mL^{-1} infusion, followed by the addition of five diverse quantities of potato starch to achieve the ultimate solution with five different concentrations regarding our design (0.276, 0.75, 1.5, 2.25 and 2.724%). Once completed, the homogeneous mixture was shifted into a syringe (diameter: 1 mm), to be transferred into the calcium chloride solution with a concentration of (0.05 mol. L^{-1}). The beads were immersed in the gelling bath ($CaCl_2$ solution: 0.05 M) to harden for 15 min. Afterwards, they were filtered and washed with a buffer solution (acetic-acetate, pH=5.5). The prepared beads, comprising of infusion and starch, would be referred as *CAS* (*Calcium Alginate Starch hydrogels*). The same flow was accomplished for the validation data to prepare capsules with the

infusion concentrations of 17, 25, 35 (mg. mL^{-1}) and starch concentrations of 0.5, 1.2, 2%.

5.2.10. *Determination of Release kinetics of polyphenols and encapsulation efficiency*

For *in vitro* release kinetics of TPC studies, a reclaimed method, "Delayed release dosage forms. Method B" of US Pharmacopeia, was performed (USP-(34)., 2011). The solution (pH=2) was prepared with a mixture of a calculated volume of HCL with distilled water, as described in Section 5.2.8.3. The solution was adjusted to pH=2 of *simulated gastric fluid* (*SGF*).

An Erlenmeyer flask containing 20 mg of the prepared beads (with various concentrations of extract and starch, according to our design), and 2 mL of *SGF* was situated in a shaker incubator at 37 ºC (simulated human body temperature) at 180 rpm for various determined times according to our model. All of the supernatant was pipetted at various times, i.e. 60, 120 and 180 min for the *central composite orthogonal block design* (*CCD-Orthogonal block*) and 45, 100 and 150 min for the validation data anthology, then centrifuged at 5000 rpm (Eppendorf 5415R), and preserved at 5 °C until the day of the analysis. The extraction process of capsules for each run was carried out separately. On the day of the analyses, a particular volume of the supernatant was used. The TPC value in the supernatant or the release kinetic for each run was detected following the method described in Section 5.2.7 and the *encapsulation efficiency* was determined according to equation 5.2:

$$\%EE = (m_c/m_e) \times 100 \quad (5.2$$

Where m_c represents the quantity of the determined TPC value extracted from the beads using *simulated gastric fluids* (*SGF*) solution (released *phenols*) and m_e indicates the TPC value of the original infusion extracted by microwave employed in beads formulation. The TPC value of the original extract was found to be equal to 37.19 $mg_{(GAE)}. g^{-1}_{dw.}$

5.3. RESULTS AND DISCUSSION

5.3.1. Visual characteristics of Calcium Alginate Starch (CAS) hydrogels beads

Optical and Dried illustration of *Calcium Alginate Starch* (*CAS*) hydrogel beads (with 0.75, 1.5 and 2.25% starch concentrations) with *Alisma plantago-aquatica* L., *microwave-assisted extraction* (*MAE*) are shown in Figure 5.1. The illustrations of each group express a homogenous size distribution. As shown in

Figure 5.1, starch addition affected the optical properties such as the diameter and colour of the produced capsules. An enhancement in the quantity of applied starch in the encapsulation of *Alisma plantago-aquatica* L., aqueous infusion enhanced the size and produced darker capsules. This finding conflicts with that of (López Córdoba *et al*., 2013). As presented in Figure 5.1, increasing the starch percentages from 0.75 to 2.25 in the beads structures increases the size from 1.6 mm to almost 2 mm. According to the data collected by (López Córdoba *et al*., 2013) starch addition does not affect the sphericity factor, bulk density, moisture content or water activity. Some researchers have observed that the physico-mechanical properties of beads might be improved by increasing the quantity of applied starch in the structures of the capsules up to a particular amount (Chan *et al*., 2011; Santagapita *et al*., 2012). Based on the observed visual characteristics (diameter, colour, sphericity of the beads) and obtained results of the models in terms of the *in vitro* release kinetics and *encapsulation efficiency*, a starch concentration equal to 0.932% combined with other optimised independent variables was detected for the best formulation in the formation of capsules.

5.3.2. Release kinetic

The release outcomes were evaluated individually for each independent variable and the results showed that the linear mode of the selected independent variables has an effect on the response (released *phenolic* compounds in *SGF*). As can be observed from Table 5.5 and Figure 5.2, the extract concentration in the beads, as well as the extraction time of the beads, demonstrates a significant positive effect on the released *phenols*, while the starch concentration in the beads' structure expresses a significant negative effect. As Table 5.2 and Figure 5.2 demonstrate, an extraction concentration of 40 mg. mL^{-1}, a starch concentration of beads equal to 0.75% and an extraction time of 180 min presented the highest quantity individually on the released *phenols*. As has been shown, an enhancement in the starch concentration in the beads' profile of up to 0.934% could improve and control the release kinetic efficiency. However, with greater enhancement in the starch concentration, the bioactive compounds could become entrapped strictly by the starch molecules; thus, the delivery of the target compounds decreases within the restricted time (López Córdoba *et al*., 2013). The *in vitro* release of Pa ghazeh from *CAS* beads presented an initial burst after 180 min, which might be attributed to the

molecules (possibly potato starch) being loosely connected with the surface or embedded in the surface layer (Natarajan *et al.*, 2011). As more compounds are released from the *CAS* beads, more channels might be produced, contributing to faster delivery rates. The fabrication approach seems to have no significant effect on the diffusion rate or release profile of the loaded samples (Yesil-Celiktas and Cetin-Uyanikgil, 2012).

5.3.3. RSM model

The experimental and predicted values, obtained by performing the extraction of encapsulated extracts (beads) using the *central composite orthogonal-block* (*CCD-Orthogonal block*) matrix, are presented in Table 5.2.

The *analysis of variance* (*ANOVA*) is considered to be useful for testing the statistical significance of the response surface quadratic model. The *ANOVA* outcomes (Table 5.3) of the quadratic model suggest that the model is significant (p<0.05), as is evident from Fisher's *F-value* of 35.572, with a low probability (p<0.0001). The reliability of the model was further assured by the observed *correlation coefficient* (R^2) between the experimental and predicted values of the response variables. A fairly high R^2 value of 0.979 implies that the regression model was statistically significant and just 2.21% of the total variations were not described by the model. Altogether, the *ANOVA* analysis expresses the applicability of the model for the simulation of the TPC extraction from the encapsulated *Alisma plantago-aquatica* L., leaves' infusions within the bounds of the experimental factors. A regression analysis of the model equation (Table 5.5) demonstrates that the linear modes of the three independent variables, as well as the interactive modes between the extract concentration-starch concentration and the starch concentration-extraction time of the beads and also the quadratic modes of both the extract and starch concentrations have expressed substantial effectiveness in terms of the response value (*encapsulation efficiency* of TPC) with a confidence level of 0.05 (p<0.05). It should be considered that the results of the interactive mode between the starch concentration-extraction time of the beads and the quadratic modes of both the starch and extract concentration, as well as the *linear* effect of starch concentration, indicated significant negative effects (p<0.05). The interactive mode between the extract concentration-time extractions of beads has shown a non-significant but

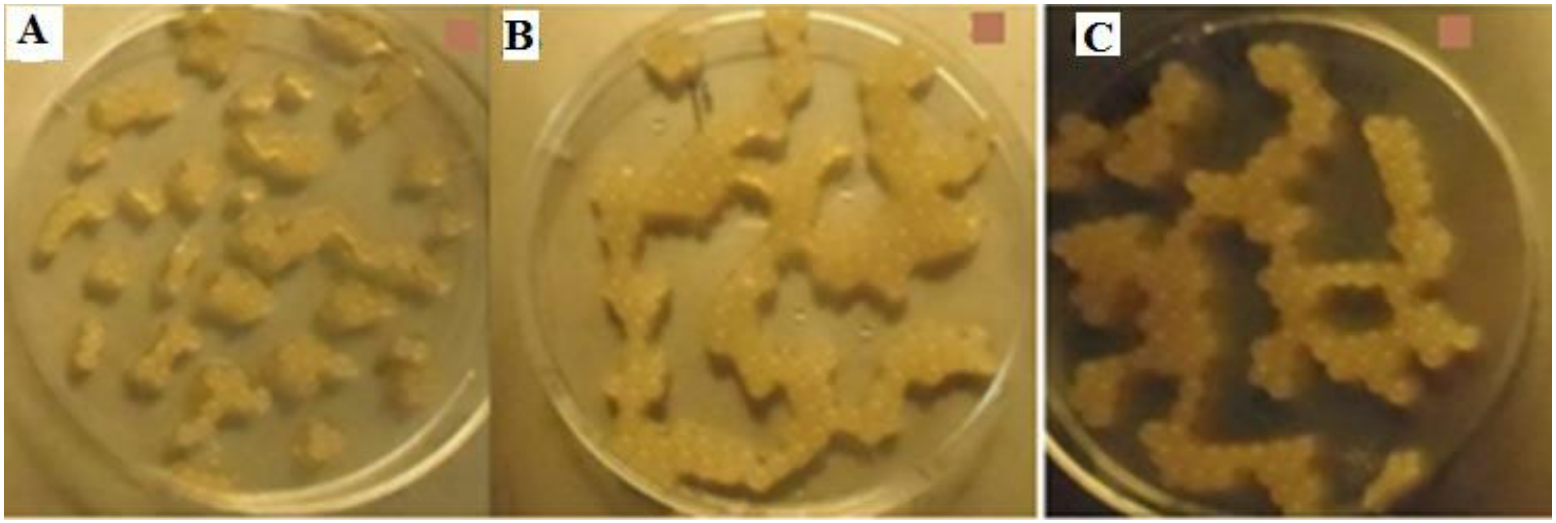

FIG. 5.1. *ALISMA PLANTAGO-AQUATICA* L., STARCH FILLED CALIUM ALGINATE BEADS, IN THREE DIFFERENT CONCENTRATIONS, A (0.75%), B (1.5%) AND C (2.25%).

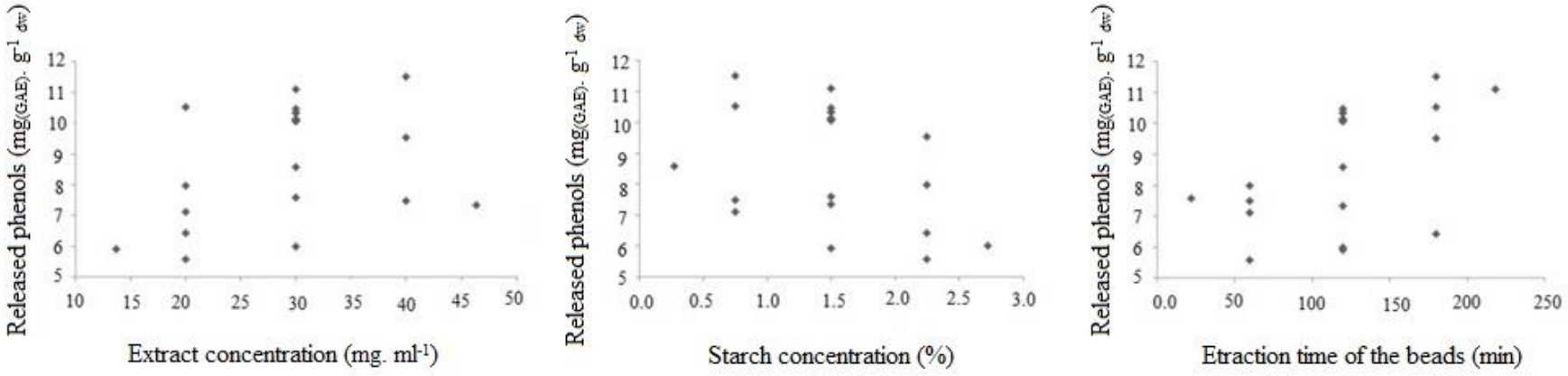

FIG. 5.2. RELEASE PROFILES OF TPC FROM CALCIUM ALGINATE BEADS BASED ON THE SELECTED INDEPENDENT VARIABLES INDIVIDUALLY IN *SIMULATED GASTRIC FLUID* (*SGF*) (pH=2) AT 37 ºC (EACH EXPERIMENT HAS BEEN PERFORMED WITHIN THREE REPLICATIONS).

TABLE. 5.3 *ANOVA* FOR THE RESPONSE SURFACE QUADRATIC MODELS FOR *SIMULATED GASTRIC FLUIDS* (*SGF*) EXTRACTION OF TPC FROM ENCAPSULAED EXTRACTS OF *ALISMA PLANTAGO AQUATICA* L., LEAVES.

Source	Sum of squares	Degree of freedom (DF)	Mean square	F-Ratio	Probablity>F
Model	496.295	11	45.117	35.572	<0.0001*
Lack of fit	9.198	5	1.839	5.818	0.089*
Pure error	0.948	3	0.316		
Total error	10.146	8			

positive effect on the response. Moreover, the quadratic mode of the time of extraction presented both non-significant and negative effects on the response.

In order to investigate the interactions between the various independent variables and their corresponding consequence on the response (*encapsulation efficiency* (*EE*)), surface plots were constructed (Figure. 5.3). A surface plot is a three-dimensional response surface as a function of two independent variables, propping all further variables at a constant level. These plots might be effectual in analysing both the main and interaction modes of the independent variables on the response (Jain *et al*., 2011).

The combined efficacies of infusion concentration (X_1) and starch concentration (X_2) on the response function are shown in the response plot in Figure 5.3A. The yield of TPC extraction of encapsulated *Alisma plantago-aquatica* L., leaves' infusion (response) was increased with an enhancement in extract concentration by 32.012 (mg. mL^{-1}) as well as in starch concentration up to 0.934%; that response value in this point was obtained at 32.112%. This phenomenon could be explained by the fact that with an enhancement in starch concentration by 0.934%, starch molecules could preserve bioactive compounds into beads' structures and release them into *SGF*, but in a concentration higher than the optimised quantity (>0.934%), because of the trapping of bioactive compounds by starch molecules, they are unable to be released into the solution within the given extraction time (López Córdoba *et al*., 2013). As Table 5.5 demonstrates, the linear modes of extract concentrations, as well as the interactive modes between extract and starch concentration, show a positive effect on the response value in which the interaction between them demonstrates a significant effect. The linear mode of starch

concentration and the quadratic modes of starch and extract concentrations, meanwhile, presented significant negative effects.

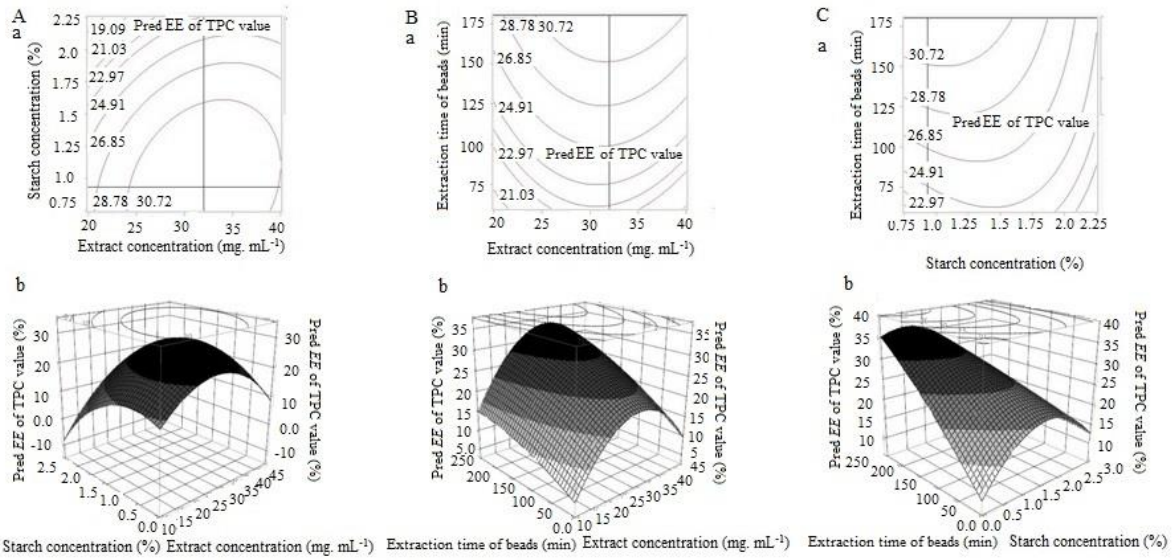

FIG. 5.3 CONTOUR PROFILER (a) AND RESPONSE SURFACE PLOTS (b) OF THREE INDEPENDENT VARIABLES INCLUDING EXTRACT CONCENTRATION (X_1), STARCH CONCENTRATION (X_2) AND EXTRACTION TIME (X_3) ON *ENCAPSULATION EFFICIENCY* (*EE*) OF TPC AS A RESPONSE (*Y*).

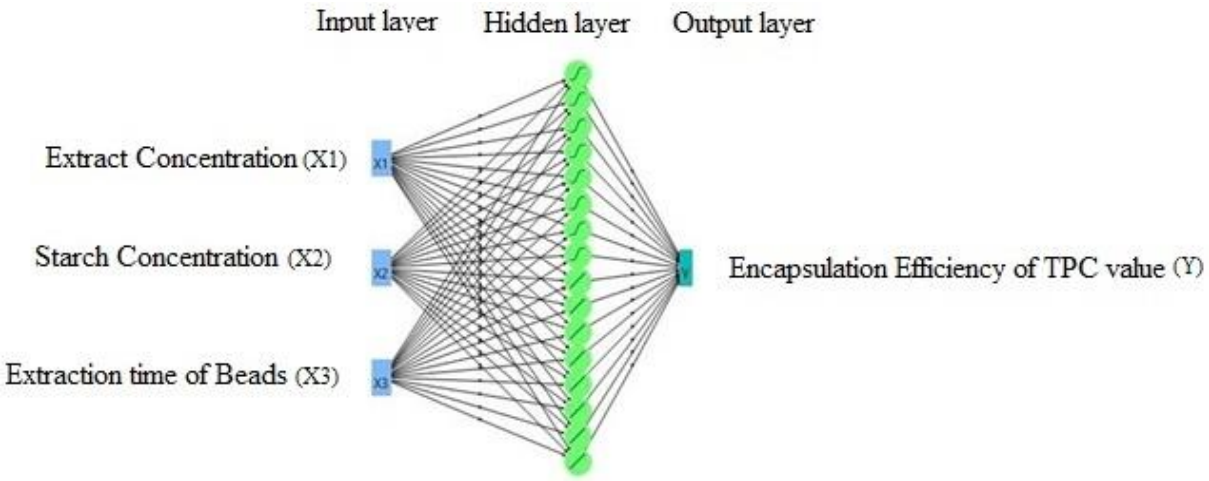

FIG. 5.4 TOPOLOGY OF *3:16:1* MODEL OF *ANN* ARCHITECTURE.

TABLE. 5.4 COMPARISON OF *RSM* AND *ANN* MODELS IN *CENTRAL COMPOSIT ORTHOGONAL MODEL* (*CCD-Orthogonal block*).

Parameter	*RSM*	*ANN*
RMSE	1.126	0.470
R^2	0.979	0.990

TABLE. 5.5 REGRESSION ANALYSIS USING THE 2^3 FACTORIAL C*ENTRAL COMPOSIT ORTHOGONAL BLOCKS* (*CCD-Orthogonal design*).

Model term	Coefficient estimate	Standard error	*T*-ratio	*P*-value
X_1	1.864	0.308	6.04	0.0003*
X_2	-2.296	0.308	-7.45	<0.0001*
X_3	3.149	0.308	10.21	<0.0001*
$X_1 \times X_2$	1.391	0.398	3.5	0.0081*
$X_1 \times X_3$	0.449	0.398	1.13	0.291
$X_2 \times X_3$	-1.696	0.398	-4.26	0.0028*
X_1^2	-3.138	0.309	-10.13	<0.0001*
X_2^2	-2.463	0.309	-7.95	<0.0001*
X_3^2	-0.39	0.309	-1.26	0.243

* Presents significant difference

Figure 5.3B also presents a response surface plot of the efficacies of infusion concentration and extraction time. As can be observed in Table 5.5 and Figure 5.3B, both linear efficacies of the extract concentration and extraction time have a positive effect, in that the effect of extraction time is more prominent than the extract concentration. Also, the interactive mode between these two independent variables is positive, but not significant. As can be seen in Figure 5.3B, by increasing the extract concentration by 32.012 (mg. mL^{-1}) within an extraction time of 180 min, the maximum (optimised) response value was obtained at 32.112%. Therefore, the optimal values (X_1= 32.012 (mg. mL^{-1}) and X_3=180 (min)) are recognised for the maximum response value.

The surface plot of Figure 5.3C, as well as Table 5.5, suggests that both the linear modes of starch concentration and extraction time, as well as the interactive effects between those two variables, have significant effects on the response values, amongst which the linear effect of extraction time is positive. Furthermore, the

quadratic modes of starch concentration and extraction time demonstrate negative effects on the response in which the quadratic mode of starch concentration is significant. As can be observed in Figure 5.3C, with an enhancement in starch concentration of 0.934% and extraction time of 180 min, the response value has increased; therefore, the maximum response value has been observed as 32.112% in the X_2=0.934% and X_3=180 min.

As shown in Table 5.6, the optimised conditions obtained using the *central composite orthogonal design* (*CCD-Orthogonal block*) is presented as follows: X_1=32.012 mg. mL^{-1}, X_2=0.934% and X_3=180 min. The corresponding response value was observed as 32.112%, and the desirability in the optimised point was observed as 0.960.

Table 5.6 PREDICTION PROFILER DESIRABILITY AND OPTIMIZED CONDITIONS OF THE EXTRACTION.

Response	Factor			Correspondent response value	Desirability
	X_1	X_2	X_3		
Encapsulation efficiency of TPC value (%)	32.012	0.934	180	32.112	0.960

X_1; X_2 and X_3 demonstrates Extract concentration (mg. mL^{-1}), Starch concentration (%) and Extraction time (min) respectively.

5.3.4. Artificial Neural Network (ANN) model

Since its invention in recent years, *ANN* has been considered an effectual tool in the simulation and prediction of the extraction process (Ghaffari Moghaddam and Khajeh, 2011; Sinha *et al*., 2012). In the present research, therefore, an *ANN*-based design was also broadened for exhibiting the *simulated gastric fluid* (*SGF*) extraction of TPC from the encapsulated infusions (beads) of *Alisma plantago-aquatica* L., leaves. A three-layer *ANN* design with the arrangement of *3:16:1*, as illustrated in Figure 5.4, was applied for this aim. The hidden layer generated eight nodes of *Hyperbolic tangent* (*TanH*) and eight nodes of linear activation functions; the mentioned arrangement has offered the best results with the highest *correlation coefficient* (R^2) and the lowest *root mean square error* (*RMSE*) in comparison to other possible arrangements. The data generated from the experimental design

planned through the *central composite-orthogonal block* (*CCD-Orthogonal block*) (Table 5.2) was exploited to determine the optimal architecture of *ANN*. The partitioning of the data into *training*, *validation* and *test subsets* was accomplished to estimate the proficiency of the *Artificial Neural Network model* for the prediction of unseen data that were not exploited for training. In this way, the popularisation capability of the *ANN* model might be assessed.

As has been mentioned, in this study the numeral of hidden layers and nodes was nominated by training various feed-forward networks of different topologies and selecting the optimal one relying on the minimisation of the performance function-*mean square error* (*MSE*). The optimal architecture (topology) of the *ANN* model for the current research, comprising of a *feed-forward neural network* with three inputs, one hidden layer with 16 nodes and one output layer (including one node (the response)) has been accomplished. Therefore, the *feed-forward neural network* topology is defined as a *multi-layer perceptron*, *MLP* (*3:16:1*), assigning to the numeral of inputs and the number of nodes in the hidden and output layers, respectively. The training of the perceptron neural network with *acquirement algorithm back propagation error* alongside the *K_Fold validation* method was employed, where the *numeral of the folds* and *learning rate* were assumed to be 5 and 0.1, respectively.

The reliability of fitness between the experimental and the most likely response obtained by the *ANN* model has demonstrated (data not shown) a *correlation coefficient* (R^2) of 0.990. The high *correlation coefficient* indicates better results from the expanded *ANN* model compared to the *RSM* model.

5.3.5. Comparison of RSM and ANN models

In this research *RSM* and *ANN* designs were applied for the modelling, prediction and optimisation of the release kinetics of TPC from an encapsulated *microwave-assisted extraction* of Iranian *Alisma plantago-aquatica* L., leaves in *simulated gastric fluid* (*SGF*). In order to examine the validity of *RSM* and *ANN* results, experiments were conducted for nine novel trials, comprising of the composition of experimental factors, which do not belong to the training data collection. Figure 5.5 shows the dispensations of residuals of the two applied designs for comparison as well. The fluctuation of the residuals is rather small and regular for the *ANN* model compared to the *RSM*. The results from the *RSM* model present a

major deviation from those of the *ANN* model (data for actual and predicted values have not been shown).

The proficiency of the applied *ANN* and *RSM* designs was also statistically determined by the *root mean squared error* (*RMSE*) and the *mean of absolute deviation* (*MAD*) or *absolute average deviation* (*AAD*), as follows (Geyikçi *et al*., 2012):

$$RMSE = \sqrt{\left(\frac{1}{N}\sum_{i=1}^{n}(T_{TPC,predic} - T_{TPC,exp})^2\right)} \qquad (5.3$$

$$AAD = \left(\frac{1}{N}\sum_{i=1}^{n}\left((T_{TPC,predict} - T_{TPC,exp})/T_{TPC,exp}\right)\right) \times 100 \qquad (5.4$$

Where *n* indicates the numeral of runs, $T_{TPC,\ predict}$ is the predicted value and $T_{TPC,\ exp}$ indicates the actual value for the response. Both the *RSM* and *ANN* models provided appropriate quality prediction in this research; however, the *ANN* demonstrated a superiority over *RSM* in terms of both the data fitting and estimation capabilities.

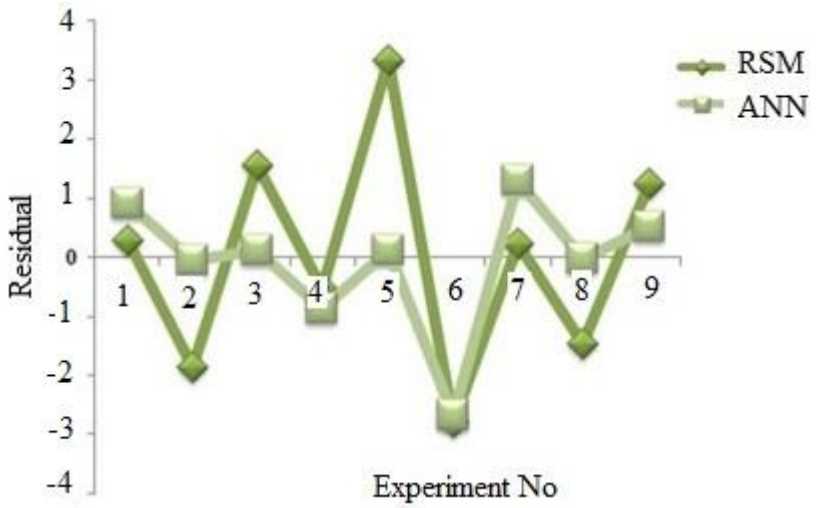

FIG. 5.5 DISTRIBUTION OF RESIDUALS OF THE VALIDATION DATA OF BOTH *RSM* AND *ANN* DESIGNS.

It should be considered that the *RSM* has the advantage of providing a regression equation for the prediction of the effectiveness of experimental factors and their interactions and responses in comparison with *ANN*. *ANN* does not require a standard experimental design to construct a model (Geyikçi *et al*., 2012). The

Artificial Neural Network (*ANN*) approach is flexible and permits the addition of new experiential data to create a reliable model. Thus, the interpretation of encapsulated *MAE* total *phenolic* infusion data through the process of *ANN* architecture would be more rational and reliable.

5.4. CONCLUSIONS

Recently, there have been substantial considerations in exploiting medicinal plants in various industries. This is mainly due to the effectual roles of *polyphenols* against some human disorders such as *cancers* and *diabetes*. In this research, the encapsulated *MAE* of *Alisma plantago-aquatica* L., leaves were developed. Not only does starch addition affect the *anti-oxidant* capabilities of encapsulated *Alisma plantago-aquatica* L., of hydrogel capsules, but it also affects the optical morphology and eventually the physical characteristics; this requires further research. With the enhancement of the applied potato starch from 0.75 to 2.25 the diameter of the prepared capsules increased from 1.6 mm to almost 2 mm. Furthermore, the application of starch in the beads' structure produced darker beads. Aside from the visual characteristics of the produced beads, the release kinetic of *phenols* in *SGF* was screened, demonstrating the significant impact of the linear modes of the three independent variables. On the other hand, the effectiveness of the infusion concentration (X_1), starch concentration (X_2) in the hydrogel beads and also the extraction time (X_3) of beads by *SGF* were studied using *RSM* and *ANN* tools regarding the encapsulation proficiency of the TPC value. The optimised conditions, consisting of X_1=32.012 (mg. mL^{-1}), X_2=0.934% and X_3=180 min, were recognised. The corresponding response value in the optimised conditions was obtained at 32.112%. Desirability was also observed at 0.960. The critical stage of the encapsulation process of the obtained extracts seems to be the hardening of the jelly (capsules) into the $CaCl_2$ (0.05 M) solution. Regarding the existence of a deep gradient of extract concentration between the jellies (forming capsules) and $CaCl_2$ solution, a considerable quantity of the *phenols* might be diffused from jellies into calcium chloride solution. On the other hand, the application of higher quantities of potato starch up to 0.934% in the beads' structures could diminish the diffusion of infusions in the environment through the over-entrapment of the compounds. The *root mean squared error* (*RMSE*), *coefficient of determination* (R^2) and *absolute average deviation* (*AAD*) were used together to compare the performances of the

RSM and *ANN* models. The *ANN* model was found to have higher predictive capabilities than the *RSM* model in both the *training* data and also in the case of the *validation* data for confirmation (Table 5.4). The achieved optimised results of the designs were applied and the obtained data confirmed the outcomes. Based on the results, this research might be used as a reference for the prediction of the best conditions for the encapsulation of bioactive compounds for application in the nutraceutical and pharmaceutical industries. The authors have declared that for the determination of the micrographs of the prepared beads and for further research into the physico-mechanical properties, *FTRI*, *DSC* and *X-ray* analysis should be performed in future researches.

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CHAPTER 6

Screening of the bioactivity potential of some reputed plants using a *GC-MS* analysis

In this chapter the different bioactive compounds and anti-oxidant parameters of hot and cold infusions of a group of Iranian plants were detected and GC-MS analysis was applied to characterize the profile of bioactive compounds of the plants.

ORIGINAL ARTICLE

Screening of the bioactivity potential of some reputed plants using a *GC-MS* analysis

Submitted

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Abstract

Context: The current manuscript discusses the *anti-oxidant* activities obtained with various parameters of 10 selected Iranian herbs reputed with several health potentials. The extractions of bioactive compounds of the herbs have been performing with different methods that among which aqueous infusions detected as the most healthy, fastest and cheapest approach.

Objective: The aim of this research is to characterize and compare the natural product profile of the infusions using *GC-MS* analysis obtained with two different methods exploiting hot and cold-water extraction. The potential *anti-radical*, reducing power and further *anti-oxidant* potentials of a wide range of selected plants including three leaves, three seeds and four flowers were achieved and compared with each other. The main compounds attributed in revealing health benefit potentials of the selected plants were specified.

Materials and Methods: The extraction of bioactive compounds was carried out using hot and cold water within different times. Total *phenolic compounds*; Total *anti-oxidant activity* and Ferric reducing *anti-oxidant power* of the hot and cold infusions were detected using *spectrophotometric* methods with *Folin-Ciocalteu reagent*; *phosphomolybdenum* and *2, 2-diphenyl-1-picryl-hydrazyl* respectively. Total *flavonoid* content was determined using *aluminium chloride hexahydrate* and *ABTS* inhibition activity was detected. Finally bioactive compounds profiles of some of the extracts were characterized using *GC-MS* analysis.

Results: Selected leaves presented the highest *anti-oxidant* potentials. *Triterpenoids* and *fatty acids* were detected as the most predominant compounds in leaves and seeds respectively.

Discussion and conclusions: The tested plants might have the potential to act as *hypoglycaemia, anti-carcinogenic, anti-Alzheimer's, anti-Parkinson's* and *anti-microbial* agents.

Keywords: Iranian herbs; *anti-oxidant* potential; *anti-radical* agent; *GC-MS*

Practical application

Regarding the existence of many adverse effects of the chemical medicines in the market and lack of existence of medicines with natural origins it has been found out that finding novel origins of bioactive compound for application in different products including nutraceutical and pharmaceutical products have considerable role in the health of human. With concern to these 10 different plants of Iran have been selected in this study for detection of their bioactivity potentials. Therefore different extraction methods using water as a healthy solvent have been performed; finally the compounds profiles of these plants have been characterized by *GC-MS* analysis. This research approved the substantial effects of the plants selected in this study revealing their higher potentials in treatment of different diseases.

6.1. Introduction

Medicinal plants and their infusions containing crude compounds as well as secondary metabolite constituents have been used by the earliest habitation of humans on earth up to modern western medicines, generating one of the fundamentals for healthcare across the world. It was identified that reactive oxygen sorts engage in the etiology of many disturbances, such as *aging*, *cancer*, *asthma*, *atherosclerosis*, *diabetes*, *coronary heart disorders* and *rhinitis* (Barnham *et al*., 2004; Eberhardt, 2000; Finkel and Holbrook, 2000). Application of crude infusions of medicinal plants as an origin of bioactive compounds in different products is increasing due to their potential to slow the oxidative reactions of lipids. As a result, they could improve the characteristics of different products in the nutraceutical, pharmaceutical and cosmetic industries. In this research three different leaves, seeds species as well as four flowers species were studied. All of the selected plants are traditionally used amongst people in the Middle East, especially Iran.

As presented in Table 6.1, the selected plants are reputed among native populations to present a widespread confine of health benefits such as having *anti-microbial* effects recorded in *T. vulgaris* L., *R. officinalis* L., *E. angustifolia* DC., *S. officinalis* L., *anti-inflammation* potential in *A. officinalis* L., *M. chamomilla* L. Moreover, among the selected plants, some are applied for the treatment of sore throat (*T. vulgaris* L., *P. anisum* L.), heals (*T. vulgaris* L.), the treatment of respiratory diseases (*S. officinalis* L., *E. angustifolia* DC., *A. officinalis* L.), as a digestive aid (*R. officinalis* L., *E. angustifolia* L., *M. chamomilla* L., *C. sativum* L., and *P. anisum* L.), *anti-depressant* (*R. officinalis* L., *E. angustifolia* DC.), and diuretic agents (*T. vulgaris* L., *L. officinale* W. D. J. Koch.)) etc. (Table 6.1) (Atal and Kapur, 1982; Bernath, 1993; Bruneton, 1995; Hobbs, 1994; Stary and Jirasek, 1978).

Bioactive compounds with bioactivity might delay oxidation reactions using different pathways, including: (1) scavenging of the species initiate peroxidation, (2) chelating metal ions which generate reactive species and therefore decompose lipid peroxides (3) reducing accessible oxygen concentration in the environment (4) interfering in out-oxidative reactions and (5) quenching free radicals and protecting them from the generation of peroxides.

In the current study, *in vitro anti-oxidant* parameters of the plants were determined. The aim of this research is to identify the optimal method of extracting the bioactive compounds of plants, specifications and introducing plants with the highest possible number of potential health benefits and characterisation of the bioactive compounds in the selected plants using *GC-MS* analysis. This study might open novel perspectives for researchers on the health potential of the examined plants.

6.2. Materials and Methods

6.2.1. Chemicals and reagents

All required chemicals and reagents for the experiments and *GC-MS* analysis have been purchased in an analytical grade.

6.2.2. Plant materials

Dried plants were purchased from the *Zarrin Giah green house company*, located in Urmia, Iran, in February 2014. Plants were shipped to the lab at the University of Algarve, Portugal, kept in darkness and in dry conditions until

grinding. Afterwards they were ground, sieved and preserved into capped containers and warehoused at -20 °C until extraction. The specifications of the used herbs are presented in Table 6.1.

6.2.3. Preparation of plant infusions

Extraction was carried out using two pathways handling hot (90 °C) and cold (RT) distilled water. 1 g of each sample was dissolved into 20 mL of solvent.

6.2.4. Preparation of samples for GC-MS analysis

Soxhlet extraction was performed for *GC-MS* analysis. Concisely, 2 g of ground and sieved samples were shifted into thimbles and extracted by dichloromethane (100 mL) overnight (almost 20 hours). Afterwards, the solvent was vaporised using a rotary evaporator (*Qlabo*-Portugal) under the following conditions, (500 mbar pressure, with a rotation value of 41 rpm at a 40 ºC temperature). The misella solvent was recovered at 40 ºC under the nitrogen atmosphere (to minimise the oxidation rate of the extracted compounds) to form a pure extract. Prior to the ultimate preparation of the samples, the obtained samples were shifted into a vacuum oven (35 ºC temperatures under the pressure of 150 mbar for almost 48 hours) to remove the remaining solvent and humidity content. Finally, the samples obtained were used for the preceding *GC-MS* analysis.

6.2.5. Chemical analysis

6.2.5.1. Colour analysis

The direct measurements of the absorbance (*Abs*) of the extracts at different wavelengths were accomplished using the method described previously by (Glories, 1984).

6.2.5.2. Determination of chlorophyll (a, b and total) contents (TCC)

The determination of the *chlorophyll* content of plants was performed using a combination method described previously by (Porra *et al*., 1989) with some modifications and using *DMSO* as solvent. The outcomes obtained were expressed as mg. $cm^{-2}_{(plant\ part)}$.

Table 6.1 Selected Iranian plants, botanical, common, local, Portuguese and family names and medicinal properties.

No	Botanical name (Voucher number)	Common name	Local name	Family	Part used	Medicinal potentials	Reference
1	*Thymus vulgaris.* L (ZGC139201)	Garden thyme	Avishan baghi	Lamiaceae (Mint family)	Leaves plus flowers	*Anti-bacterial*, *anti-fungal* , Treatment for cough and sore throat, (Portugues name: Tomilho)	(Bernath, 1993; Bruneton, 1995; Duke, 1982)
2	*Rosmarinus officinalis* L. (ZGC139202)	Rosemary	Aklile kohi(Ros mary)	Lamiaceae (Mint family)	Leaves	Treatment for rheumatism and migraines, Gout and muscle aches, tremors and Paralysis of hands and feet, disinfectant, diuretic, digestive, Booster nerves, *anti-bile* secretion and healer, *Anti-depressant* (Portugues name: Rosmaninho-comum)	(Bernath, 1993)
3	*Salvia officinalis* L. (ZGC139203)	Kitchen sage	Maryam goli	Lamiaceae (Mint family)	Leaves	Treatment for pharynx and larynx disorders, Mouthwashes, *Anti-bacterial* (Portugues name: Salva) (Portuguese name: Salva-espanola)	(Atal and Kapur, 1982; Bernath, 1993)
4	*Echium amoenum Fisch. C.A. Mey*. (ZGC139204)	Common borage	Gavzabane Irani	Boraginaceae	Flower	Treatment for respiratory disorders, Immune system booster *Anti-depressant* (Portuguese name: Borragem)	(Atal and Kapur, 1982)
5	*Echinacea angustifolia* DC. (ZGC139205)	Blacksamson echinacea	Sar khar gol	Asteraceae (Aster family)	Flower	Treatment for cough, sore mouth and gums, for the treatment of cold, *anti-gonorrhea* , suppressing of infectious diseases and respiratory diseases digestive, *Anti-bacterial*, immune system booster (Portuguese name: Camomila-alema)	(Bruneton, 1995; Hobbs, 1994)
6	*Althaea officinalis* L. (ZGC139206)	Common marshamallow	Khatmi darouie	Malvaceae (Mallow family)	Flower	*Anti-cough*, Softener respiratory tract, Phlegm propulsive *Anti-inflammation* (specially eye's inflammation), *anti-mucous* membrane irritation, Treatment for *trachea*, *bronchi* and lung (Portuguese name: Malvaisco)	(Hornok, 1978; Stary and Jirasek, 1978)
7	*Matricaria chamomilla* L. (Syn. M. Recutita L.). (ZGC139207)	German chamomile	German Baboneh	Asteraceae (Aster family)	Flowers	*Anti-inflammation*, appetizer, digestive, *Anti-microbial*, and Treatment for abdominal pain, (Portuguese name: Camomila-alema)	(Bruneton, 1995; Homak, 1978; Hornok, 1978)
8	*Coriandrum sativum* L (ZGC139208).	Coriander	Geshgniz	Apiaceae (Carrot family)	Seeds	Flavor agent, carminative, *Anti floating*, digestive (Portuguese name: Ceandro)	(Bruneton, 1995; Chevallier, 1996)
9	*Pimpinella anisum* L. (ZGC139209)	Anise burnet saxifrage	Badian roomi (anison)	Apiaceae (Carrot family)	Fruits(seeds)	Appetizer, digestive and Treatment for abdominal pain, Sore throat (Portuguese name: Anise)	(Atal and Kapur, 1982; Bernath, 1993; Bhat and Pandita, 1982; Bruneton, 1995)
10	*Levisticum officinale* W. D. J. Koch. (ZGC139210)	Garden lovage	Anjedane roomi	Apiaceae (Carrot family)	Seeds	Diuretic, Kidney cleansing, Treatment for kidney stones (Portuguese name: Ligustico)	(Bernath, 1993; Bruneton, 1995; Atal and Kapur, 1982; Bruneton, 1995)

6.2.5.3. Definition of total phenols (TPC)

The concentration of total *phenols* in the infusions was determined using a *Folin-Ciocalteu* reagent and external determination with *gallic acid* in relation to the assay of (Huang *et al*., 2006), and the results obtained are presented as ($mg_{(GAE).}\ g^{-1}_{dw}$).

6.2.5.4. Measurement of total flavonoids (TFC)

The total flavonoid contents were monitored using a spectrophotometer with aluminium chloride hexahydrate, as described by (Quettier-Deleu *et al*., 2000) and the results obtained are presented as ($mg_{(QE).}\ g^{-1}_{dw}$).

6.2.5.5. FRAP (Ferric reducing anti-oxidant power) assay

The FRAP was accomplished in relation to literature presented by (Benzie and Strain, 1999) and presented as $mg_{(TE).}\ g^{-1}_{dw}$.

6.2.5.6. Reducing Power (RP)

To determine the diminishing power of different infusions, the described method by (Barros *et al*., 2010) was applied and the achieved results are presented as $mg_{(TE).}\ g^{-1}_{dw}$.

6.2.5.7. $\boldsymbol{ABTS}^{\cdot+}$ Inhibition activity

The *anti-oxidant* potentials of the samples were also analysed by studying their potential to inhibit $ABTS^{\cdot+}$ using a modified methodology previously reported by (Re *et al*., 1999) and the obtained *IC50* values are presented as µg. mL^{-1}.

6.2.5.8. Determination of total anti-oxidant activity (TAA)

The total *anti-oxidant* activity was detected using the phosphomolybdenum method, as described by (Prieto *et al*., 1999) and presented as $mg_{(AAE)}.\ g^{-1}_{dw}$.

6.2.5.9. Expression of data and statistical analysis

The experimental results were expressed as the mean ± *SD* of three parallel measurements and were analysed by using the *SPSS* program, version 22.0 (*IBM SPSS* Statistics 22, licensed University of Algarve). Differences between the means of two groups of samples (hot and cold infusions) and between the individual samples of hot and cold infusions were determined using one way *ANOVA* followed by *Tukey* (*HSD*) with a confidence level of 5%. Relationships between the

parameters were obtained by using the *Pearson correlation coefficient* option on *Microsoft Office Excel 2007* and graphs were constructed by *Microsoft Office Excel 2007*.

6.2.6. *Gas chromatography-mass spectrometry* (*GC-MS*) analysis

The constituents were analysed with an Agilent 5975C inert *mass selective detector* (*MSD*) with a *Triple-Axis Detector* coupled to an Agilent 7890A *gas chromatography* system, equipped with a *ZB-5HT* inferno capillary *GC* column (30 m×0.025 mm×0.1 um film thickness) with temperature limits of (min=-60 to max=400-430 ºC) (made in *USA*). Helium (flow rate, 1 mL. min^{-1}) was used as the carrier gas. The injector temperature was 280 ºC. The oven temperature programme was set at 100 ºC for 1 minute, increased by a rate of 10 ºC. min^{-1} to 370 ºC and was maintained for 1 minute and then enhanced with the same rate (10 ºC. min^{-1}) to 380 ºC held constant for 5 minutes. Therefore, the term of the whole run reached 35 minutes. The mass spectrometer was used in *EI* mode and the injected samples were scanned from 40 to 950 *amu*. The compounds were identified by different databases by comparing their spectral data to those from mass spectral libraries and with spectral data of the standard compounds.

6.3. Results and discussion

The extracts obtained showed that cold infusions are darker than hot infusions, (Fig. 6.1), and significant differences between the colour parameters of hot and cold aqueous infusions confirmed this phenomenon (Table 6.2).

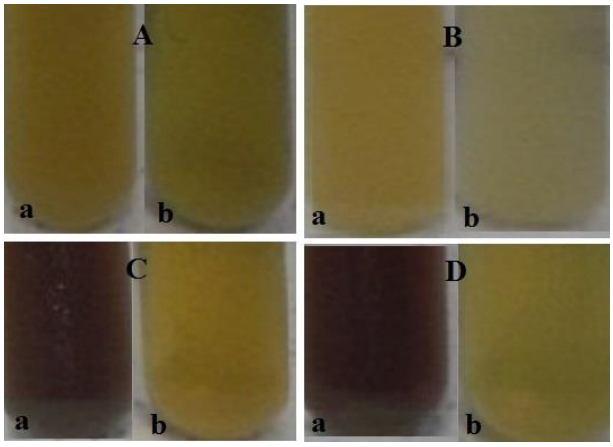

Figure 6.1 Differences in colour of the obtained infusions (cold (a) and hot (b)), A (*M. chamomilla* L.), B (*C. sativum* L.), C (*S. officinalis* L.) and D (*R. officinalis* L.).

It states that higher temperatures might not positively affect the physico-chemical properties of the pigments. On the other hand, regarding the osmosis or diffusion rule, it is stated that most pigments, especially *anthocyanins* in this study, might be unstable at higher temperatures and are decomposed to colourless compounds. This phenomenon is in line with the findings of, (Jenshi roobha *et al*., 2011), in regards to the heat susceptibility of pigments (≥20 °C).

As may have been expected, leaves contain a higher TCC value (total *chlorophyll* contents), followed by most flowers and seeds respectively (Table 6.3). It indicates the dominant impact of sunlight in *chlorophyll* generation. In most cases higher relationships were observed between the determined TCC and *anti-oxidant* parameters (Table 6.4).

The determined TPC value in hot infusions is significantly higher (p<0.05). The highest and lowest value for TPC belongs to leaf and seed species respectively in both hot and cold infusions (Table 6.3). In hot infusions, after leaves, the TPC values of most of the flowers are the highest. The TPC value of the seeds are considerably lower (p<0.05) when compared to leaves and flowers with an exception in the cold infusion of *P. anisum* L., (seed). Furthermore in *L. officinale* W. D. J. Koch., and *P. anisum* L., (seeds), no significant differences were observed between the hot and cold infusions' value (p<0.05).

This might be justified by the wooden and tough structures of the cells in the seeds where *phenolic* compounds could not be extracted within just five minutes, even by hot water. Additionally, it should be noted that some *non-phenols*, such as organic acids and sugars detected in the plants of this research (Table 6.5) could influence the estimation of TPC by the *Folin-Ciocalteu* pathway. On the other hand, different *phenolic* compounds present adverse behaviours to absorbency; some *flavonoids* demonstrate low absorbency, leading to a depreciation of various compounds (Kyoung Chun and Kim, 2004; Medina-Remón *et al*., 2009; Roura *et al*., 2006).

The *phenolic* compounds might trap *lipid alkoxyl radicals* generated within peroxidation. Different *phenolic* compounds show different *anti-oxidant* potential regarding the structure of the molecules, and number and position of the carboxylic groups (Milić *et al*., 1998).

Flavonoids are identified to present *anti-oxidant* potential and substantial effectiveness for human nutrition and health issues. In hot infusions, *S. officinalis* L.,

(leaf) and *E. angustifolia* DC., (flower) possessed the highest and lowest values, respectively. In cold infusion, *S. officinalis* L., showed the maximum value while in *C. sativum* L., (seed) and *E. angustifolia* DC., (flower) jointly, no *flavonoids* were detected. In hot infusions, TFC values are significantly higher than in cold infusions, except *A. officinalis* L., and *R. officinalis* L., (p<0.05) (Table 6.3). This might demonstrate the soft and frangible tissue of those plants based on the possibility that cold water could extract the most TFC within applied term.

The *anti-oxidant* potential of the extracts depends on not only the category of available bioactive compounds but also the test biological systems. This could be generated through consecutive pathways, and could not be entirely estimated by one or single assay, therefore, the determination of various *in vitro* tests, including *anti-radical* and diminishing power and etc. are required (Wong *et al*., 2006).

The *anti-oxidant* potential mechanisms of *flavonoids* might be based on scavenging or chelating procedures, and the obtained data by *ABTS* assay in this research strongly confirmed these findings. Further *in vitro* studies showed that *flavonoids* have the potential to scavenge the molecular species of active oxygen including *hydrogen peroxide* (H_2O_2), *hydroxyl radicals*, *-superoxide* ($.O_2^-$), *single oxygen* ($^1O_2$) as well as *hydroxyl radicals* ($^{\cdot}OH$). Therefore their *anti-oxidant* potential was based on their ability in the donation of hydrogen atoms and/or electrons (Arti Arora *et a*l., 1998; Bartosz, 1997; Khan *et al*., 2000; Rice-Evans *et al*., 1997; Sakihama *et al*., 2000). As it is clearly demonstrated, hot infusions of *E. amoenum* Fisch & C.A. Mey., (flower) show more similar behaviours to the leaves in the TFC and *ABTS* values, while the cold infusions do not observe these qualities (Tables 6.3, 6.4). For instance, heavy metals such as cadmium generate oxidative reactions through different pathways such as interfering in the *anti-oxidant* defence reactions, the disruption in the electron transfer process within the lifecycle of the cells as well as the induction of peroxidation reactions such as through activation lipoxygenase in the lipid peroxidation process (Smeets *et al*., 2005). Therefore, the chelating potential of the *flavonoids* proposes to prevent the circumstance of the abovementioned reactions. On the other hand (Arora *et al*., 2000) showed *flavonoids* can change peroxidation kinetics by modifications in the lipid packing order. Interestingly, *phenolic* compounds have shown higher *anti-oxidant* activities than other natural *anti-oxidants* such as *vitamins C* and *E*, therefore the importance of *phenolic* compounds is more obvious.

Table 6. 2 Colour parameters of hot and cold infusions of the selected plants (The values are expressed as means ± *SD* of triplicate assay).

Parameter	Colour Intensity		Tint		Ye%		Rd%		Bl%	
	Infusion type									
	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold
T. vulgaris L.	2.317 ± 0.05^{Ab}	4.165 ± 0.05^{Bb}	1.72 ± 0.039^{Ab}	0.97 ± 0.004^{Ba}	49.73 ± 086^{Ae}	25.80 ± 0.35^{Bb}	28.90 ± 016^{Ac}	26.33 ± 0.25^{Bae}	21.35 ± 0.70	47.860 ± 0.60^{Bc}
R.officinalis L.	1.608 ± 0.04^{Ae}	3.62 ± 0.116^{Bad}	2.45 ± 0.036^{Ad*}	0.98 ± 0.004^{Ba}	60.11 ± 0.45^{Ag*}	30.04 ± 0.53^{Bcd}	24.52 ± 0.17^{Ade}	30.63 ± 0.474^{Bd}	15.36 ± 0.275^{Ae}	39.32 ± 1.003^{Bb}
S. officinalis L.	1.81 ± 0.02^{Ace}	3.67 ± 0.07^{Bad}	2.37 ± 0.016^{Ad}	0.98 ± 0.003^{Ba}	60.07 ± 0.21^{Ag}	28.82 ± 0.38^{Bac}	25.262± 0.11^{Ae}	29.37 ± 0.496^{Bb}	14.65 ± 0.18^{Ae}	41.8 ± 0.87^{Bf}
E. amoenum Fisch.	2.950 ± 0.08^{Ag}	3.42 ± 0.11^{Bd}	1.14 ± 0.013^{Aa}	0.97± 0.005^{Ba}	36.87 ± 0.77^{Ad}	30.98 ± 0.63^{Bd}	**32.14 ± 0.33^{Af*}**	**31.62 ± 0.558^{Ad}**	30.98 ± 1.101^{Af}	37.39 ± 1.18^{Bb}
E. angustifolia DC.	3.439 ± 0.07^{Aa}	4.142 ± 0.01^{Bb}	1.08 ± 0.002^{Aa}	0.988 ± 0.001^{Ba}	33.56 ± 0.32^{Ac}	25.62 ± 0.06^{Bb}	30.87 ± 0.23^{Ab}	25.91 ± 0.089^{Bef}	35.55± 0.560^{Ab}	48.45 ± 0.14^{Bce}
A. officinalis L.	1.15 ± 0.09^{Ad}	2.374 ± 0.25^{Ba}	2.38 ± 0.137^{Ad}	1.34 ± 0.062^{Bc*}	56.4 ± 1.87^{Af}	40.04 ± 1.24^{Bf*}	23.70 ± 0.57^{Ad}	29.78 ± 0.518^{Bb}	19.89± 1.33^{Acd}	30.17± 0.86^{Bd}
M. chamomilla L.	2.024 ± 0.04^{Ac}	2.838 ± 0.07^{Bc}	1.95 ± 0.091^{Ac}	1.151 ± 0.015^{Bb}	54.065 ± 1.44^{Af}	37.95 ± 0.62^{Be}	27.62 ± 0.56^{Aa}	32.95 ± 0.097^{Bc*}	18.312 ± 0.881^{Ad}	29.083 ± 0.71^{Bd}
C. sativum L.	3.836 ± 0.05^{Af*}	4.591 ± 0.09^{Be*}	1.02 ± 0.008^{Aa}	0.97± 0.005^{Ba}	29.29 ± 0.07^{Aa}	24.22 ± 0.05^{Bb}	28.48 ± 0.24^{Aac}	24.97 ± 0.152^{Bf}	42.22 ± 0.28^{Aa*}	50.79 ± 0.17^{Be*}
P. anisum L.	3.418 ± 0.12^{Aa}	3.754 ± 0.01^{Ba}	1.09 ± 0.005^{Aa}	1.007 ± 0.002^{Ba}	32.89 ± 0.72^{Abc}	29.48 ± 0.19^{Bacd}	**30.13 ± 0.54^{Ab}**	**29.27 ± 0.18^{Ab}**	36.97 ± 1.26^{Ab}	41.23 ± 0.36^{Bbf}
L. officinale W.D. J.	3.596 ± 0.10^{Aa}	3.872 ± 0.08^{Bab}	1.08 ± 0.006^{Aa}	1.018 ± 0.008^{Ba}	30.97 ± 0.44^{Aab}	27.96 ± 0.87^{Ba}	28.65 ± 0.25^{Aac}	27.46 ± 0.62^{Ba}	40.37 ± 0.69^{Aa}	44.56 ± 1.49^{Ba}

Different small letters in each column demonstrate significant difference (p_{value}<0.05). Different bold letters in each row for each parameter individually, express significant difference (p_{value}<0.05).

Table 6.3 Determined *anti-oxidative* parameters of the selected plants (The values are expressed as means ± *SD* of triplicate assay).

Plant species	TPC ($mg_{(GAE)}. g^{-1}_{dw}$)		TFC ($mg_{(QE)}. g^{-1}_{dw}$)		FRAP ($mg_{(TE)}. g^{-1}_{dw}$)		RP ($mg_{(TE)}. g^{-1}_{dw}$)	
	Infusion types							
	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold
T. vulgaris L.	479.154 ± 31.923^{Da}	220.567 ± 29.550^{Db}	14.186 ± 0.491^{Fa}	4.614 ± 2.148^{CDEb}	**741.723 ± 35.124^{Ea}**	**181.106 ± 3.145^{Eb}**	**240.049 ± 5.50^{Aa}**	**108.069 ± 2.224^{Fb}**
R. officinalis L.	398.602 ± 3.840^{Ca}	182.645 ± 3.960^{Cb}	**6.432 ± 0.451^{CDa}**	**5.086 ± 1.932^{CDEa}**	730.220 ± 26.759^{Ea}	178.472 ± 5.143^{Eb}	230.320 ± 4.97^{Ca}	77.564 ± 1.487^{Eb}
S. officinalis L.	381.393 ± 29.267^{Ca}	209.786 ± 7.237^{CDb}	16.755 ± 0.375^{Fa}	7.501 ± 2.441^{Eb}	515.819 ± 7.220^{Da}	152.289 ± 2.367^{Eb}	230.130 ± 31.247^{Ca}	107.641 ± 11.354^{Fb}
E. amoenum Fisch.	119.606 ± 6.334^{ABa}	56.291 ± 0.527^{Ab}	6.248 ± 0.303^{Ca}	0.449 ± 0.052^{ABb}	237.795 ± 23.237^{Ca}	53.951 ± 0.260^{Cb}	74.524 ± 1.484^{Ba}	31.375 ± 4.214^{BCb}
E. angustifolia DC.	111.056 ± 0.653^{ABa}	53.472 ± 1.710^{Ab}	0.347 ± 0.166^{Aa}	ND*^{Ab}	103.979 ± 15.418^{Aa}	49.360 ± 1.325^{BCb}	**34.409 ± 2.926^{Aa}**	19.571 ± 2.706^{ABb}
A. officinalis L.	97.078 ± 1.542^{Aa}	62.691 ± 1.117^{Ab}	**7.708 ± 1.038^{DEa}**	**6.185 ± 1.828^{DEa}**	**55.580 ± 3.961^{Aa}**	40.841 ± 5.377^{ABb}	50.387 ± 8.208^{ABa}	28.885 ± 1.359^{BCb}
M. chamomilla L.	158.804 ± 23.615^{Ba}	107.193 ± 12.508^{Bb}	15.068 ± 0.383^{Fa}	3.458 ± 0.142^{ABCDb}	155.776 ± 5.288^{Ba}	41.675 ± 7.075^{ABb}	60.261 ± 8.819^{ABa}	34.607 ± 4.496^{Cb}
C. sativum L.	99.877 ± 16.396^{Aa}	43.317 ± 1.685^{Ab}	3.356 ± 0.340^{Ba}	ND^{Ab}	88.883 ± 1.694^{Aa}	**31.941 ± 2.344^{Ab}**	43.469 ± 3.979^{ABa}	**12.617 ± 1.195^{Ab}**
P. anisum L.	**91.506 ± 3.452^{Aa}**	**98.331 ± 3.868^{Ba}**	2.363 ± 0.217^{Ba}	1.944 ± 0.089^{ABCb}	104.332 ± 2.567^{Aa}	92.614 ± 2.869^{Db}	**51.752 ± 2.665^{ABa}**	**57.776 ± 1.42^{Db}**
L. officinale W. D. J.	**78.777 ± 14.208^{Aa}**	**60.487 ± 2.295^{Aa}**	7.995 ± 0.125^{Ea}	4.257 ± 0.078^{BCDEb}	69.936 ± 10.370^{Aa}	35.518 ± 0.953^{Ab}	**34.588 ± 1.468^{Aa}**	**37.602 ± 2.456^{Cb}**

Different small letters in each row demonstrate significant difference (p_{value}<0.05). Different bold letters in each column for each parameter individually, express significant difference (p_{value}<0.05).
* ND indicates not detected.

Table 6.3 Continued

Plant species	ABTS (µg. mL^{-1})		TAA mg$_{(AAE)}$. g$^{-1}_{dw}$		*Chlorophyll* contents (mg. cm$^{-2}_{(plant\ part)}$)		
	Infusion types						
	Hot	Cold	Hot	Cold	*Chlorophyll* a	*Chlorophyll* b	Total *Chlorophyll*
T. vulgaris L.	126.016 ± 8.538^{Aa}	267.036 ± 25.816^{Ab}	$270.258 \pm 21.394^{C^a}$	111.176 ± 5.102^{Bb}	$0.038 \pm 0.00004^{C*}$	$0.013 \pm 0.000077^{C*}$	$0.051 \pm 0.00007^{C*}$
R. officinalis L.	104.022 ± 13.544^{Aa}	343.712 ± 89.440^{ABb}	$320.743 \pm 15.920^{D^a}$	172.616 ± 9.474^{Eb}	$0.035 \pm 0.00006^{F*}$	$0.011 \pm 0.000085^{F*}$	$0.046 \pm 0.00009^{F*}$
S. officinalis L.	155.393 ± 13.435^{Aa}	330.968 ± 20.462^{ABb}	$226.420 \pm 7.950^{BC^a}$	143.409 ± 20.321^{CDb}	$0.065 \pm 0.00040^{I*}$	$0.019 \pm 0.000185^{H*}$	$0.084 \pm 0.00057^{H*}$
E. amoenum Fisch .	135.3162 ± 5.934^{Aa}	1184.043 ± 58.755^{Db}	$183.219 \pm 7.211^{B^a}$	164.982 ± 4.52^{DEb}	0.008 ± 0.00033^{H}	$0.025 \pm 0.000354^{G*}$	0.034 ± 0.00069^{G}
E. angustifolia DC.	**791.698 ± 75.875^{Ca}**	**910.170 ± 74.095^{Ca}**	$103.334 \pm 3.086^{A^a}$	46.127 ± 2.151^{Ab}	0.003 ± 0.00002^{G}	0.002 ± 0.000088^{AE}	0.005 ± 0.00001^{A}
A. officinalis L.	364.263 ± 3.746^{Ba}	904.621 ± 10.025^{Cb}	$80.963 \pm 6.685^{A^a}$	54.736 ± 3.451^{Ab}	0.006 ± 0.00002^{E}	0.002 ± 0.000008^{E}	0.008 ± 0.00001^{E}
M. chamomilla L.	**398.306 ± 69.129^{Ba}**	**452.215 ± 21.309^{Ba}**	$80.092 \pm 3.346^{A^a}$	58.240 ± 1.411^{Ab}	0.014 ± 0.00002^{D}	0.004 ± 0.000008^{D}	0.018 ± 0.00001^{D}
C. sativum L.	746.328 ± 50.035^{Ca}	1413.899 ± 89.923^{Eb}	$80.008 \pm 5.670^{A^a}$	53.972 ± 2.367^{Ab}	0.002 ± 0.00004^{B}	0.001 ± 0.000015^{B}	0.003 ± 0.00002^{B}
P. anisum L.	**796.524 ± 60.803^{Ca}**	**598.621 ± 37.895^{Ca}**	**$113.769 \pm 3.632^{A^a}$**	**117.518 ± 3.858^{BCa}**	0.002 ± 0.00002^{B}	0.0009 ± 0.000008^{B}	0.003 ± 0.00001^{B}
L. officinale W. D. J.	**998.289 ± 112.710^{Da}**	**992.584 ± 82.529^{Ca}**	**$113.789 \pm 6.851^{A^a}$**	**$136.890 \pm 19.161^{BCDa}$**	0.005 ± 0.00002^{A}	0.001 ± 0.000008^{A}	0.006 ± 0.00001^{A}

Different small letters in each row demonstrate significant difference (p_{value}<0.05). Different bold letters in each column for each parameter individually express significant difference (p_{value}<0.05).

Table 6.4 *Pearson correlation coefficient* (R^2) detected between different obtained parameters of the hot and cold infusions.

Parameter	TAA		TPC		TFC		RP		FRAP		*ABTS*		TCC	
	Infusion types													
	Hot	**Cold**	**Hot**	**Cold**	**Hot**	**Cold**	**Hot**	**Cold**	**Hot**	**Cold**	**Hot**	**Cold**	**Hot**	**Cold**
TAA	1	1												
TPC	0.878	0.451	1	1										
TFC	0.314	0.292	0.597	0.65822	1	1								
RP	**0.913***	0.549	**0.983***	**0.966***	0.579	0.668	1	1						
FRAP	**0.955***	0.561	**0.975***	**0.943***	0.483	0.521	**0.973***	**0.929***	1	1				
ABTS	-0.702	-0.294	-0.721	**-0.884***	-0.569	-0.656	-0.753	-0.830	-0.745	-0.792	1	1		
TCC	0.774	0.579	0.847	0.850	0.701	0.604	**0.897***	0.846	0.816	0.784	-0.782	-0.629	1	1

* Indicates the high values.

Table 6.5 Lipophilic bioactive components identified by *GC-MS* (as methyl esters and TMS derivates) in the dichloromethane extracts of some selected plants.

No	Compounds and constituent	*Examined Plant species*				
		***P. anisum* L.**	***R. officinalis* L.**	***S. officinalis* L.**	***T. vulgaris* L.**	***C. sativum* L.**
	Sugars	**2.14**	**0.09**	**0.57**	**7.61**	**0.72**
1	*Sugar in C_5*	0.28	-	0.07	0.51	0.33
2	*Sugar in C_6*	0.67	0.09	-	0.24	0.32
3	*Fructose*	0.48	-	-	-	0.07
4	*Pentitol*	0.25	-	-	-	-
5	*Turanose*	0.21	-	-	0.69	-
6	*Erythrodiol*	-	-	0.5	-	-
7	*NI sugar dimer*	0.25	-	-	6.17	-
8	***Alkanes***	**1.08**	**5.27**	**2.36**	**5.51**	**0.15**
	Fatty acids	**49.04**	**2.44**	**8.97**	**16.78**	**80.22**
9	*Nonanoic acid* (C9:0)	-	-	-	-	0.13
10	*Decanoic acid* (C10:0)	0.05	0.05	-	0.11	0.14
11	*Dodecanoic acid* (C12:0)	-	-	-	-	0.13
12	*Tridecanoic acid* (C13:0)	-	-	-	-	0.12
13	*Tetradecanoic acid* (C14:0)	-	0.11	-	-	2.38
14	*Pentadecanoic acid* (C15:0)	0.21	-	0.07	0.14	0.22
15	*Hexadecanoic acid* (C16:0)	5.93	0.56	2.14	2.92	9.03
16	*Heptadecanoic acid* (C17:0)	0.11	-	-	-	0.19
17	*Octadecanoic acid* (C:18:0)	1.4	0.26	0.64	1.04	2.33
18	*Nonadecanoic acid* (C:19:0)	-	-	-	-	0.04
19	*Eicosanoic acid* (C20:0)	0.31	-	0.2	0.44	0.55
20	*Docosanoic acid* (C22:0)	0.34	-	-	0.43	0.25
21	*Tetracosanoic acid* (C24:0)	0.27	0.03	0.06	0.19	0.25
22	*Hexacosanoic acid* (C26:0)	-	-	0.07	-	0.13
23	*Hexadecenoic acid* C(16:1)	0.09	-	-	-	0.81
24	*Octadecenoic acid* (C18:1)	0.44	1.28	4.98	11.51	62.46
25	*Heptadecanoic acid* (C17:1)	-	-	-	-	0.14

Table 6.5 Continued

26	*9,12-Octadecadienoic acid* (C18:2)	39.89	0.15	0.81	-	0.42
27	*Eicosenoic acid* (C22:1)	-	-	-	-	0.5
	α-Hydroxy *fatty acids*	**0.62**	**-**	**0.02**	**-**	-
28	*α-Hydroxy acid in C_8*	-	-	0.02	-	-
29	*α-Hydroxy acid in C_{16}*	0.18	-	-	-	-
30	*α-Hydroxy acid in C_{28}*	0.29	-	-	-	-
31	*α-Hydroxy acid in C_{30}*	0.15	-	-	-	-
	Fatty alcohols	**0.64**	**0.14**	**0.44**	**0.64**	**-**
32	*1-octadecanol*	0.29	-	0.27	0.37	-
33	*Octadec-9-enol*	0.35	0.09	-	-	-
34	*1-hexadecanol*	-	0.05	0.17	0.27	-
	Di-Acids	**0.43**	**0.03**	**0.11**	**0.64**	**0.13**
35	*Malic acid*	0.18	-	0.04	0.24	-
36	*Azelaic acid*	0.25	0.03	0.07	0.08	0.13
37	*Citric acid*	-	-	-	0.32	-
	Waxes	**1.79**	**0.06**	**0.15**	**1.65**	**6.4**
38	*Wax C_{12}*	-		0.14	-	-
39	*Wax C_{13}*	-	0.06	0.01	-	-
40	*Wax C_{16}*	-	-	-	0.6	-
41	*Wax C_{28}*	-	-	-	-	2.25
42	*Wax C_{34}*	1.79	-	-	1.05	-
43	*Wax C_{36}*	-	-	-	-	4.15
	Sterols* and *Triterpenoids	**2.07**	**63.42**	**47.64**	**33.51**	**4.57**
44	*Campesterol*	0.1	-	0.34	-	0.23
45	*Stigmasterol*	0.48	-	-	-	0.39
46	*β-Sitosterol*	0.61	0.7	1.51	-	0.81
47	*β-Amyrin*	0.30	0.9	0.25	-	-
48	*Betulinic acid*	0.08	13.48	2.01	1.66	3.00

Table 6.5 Continued

49	*Oleanolic acid*	0.38	16.65	14.14	15.27	0.14
50	*Ursolic acid*	0.12	21.86	22.34	16.00	-
51	*Lupeol*	-	-	0.5	-	-
52	*Squalene*	-	0.06	0.16	0.36	-
53	*Olean*-12-ene, 3-one	-	1.33	-	-	-
54	*Sterol* NI	-	1.95	4.57	-	-
55	*Maslinic acid*	-	6.38	0.31	-	-
56	*Corosolic acid*	-	-	0.35	-	-
57	*Borneol*	-	-	1.13	-	-
58	*Caryophyllene*	-	0.06	0.03	0.05	-
59	*Caryophyllene oxide*	-	0.05	-	0.17	-
	Aromatics	**1.08**	**0.57**	**0.12**	**6.41**	**0.22**
60	*Benzoic acid, 2-hydroxy*	-	-	0.04	-	-
61	*Benzoic acid, 4-methoxy*	0.28	-	-	-	0.04
62	*Benzoic acid, 3-methoxy-4-hydroxy*	-	0.19	-	-	-
63	*Benzaldehyde, 3-methoxy-4-hydroxy*	-	-	-	-	0.02
64	*Vanil Ethanediol*	0.19	-	-	6.3	-
65	*Benzeneacetic acid*	0.61	0.08	0.08	-	-
66	*2,6- Dihydroxyacetophenone*	-	-	-	0.11	-
67	*Benzeneacetic acid, 2-methoxy-α-hydroxy*	-	-	-	-	0.16
68	*tert-Butylhydroquinone*	-	0.14	-	-	-
69	*Syringic acid*	-	0.06	-	-	-
70	*P-coumaryl alcohol*	-	0.1	-	-	-
	Monoacylglycerols	**2.16**				**1.34**
71	*1-Monoglycerol in C16*	-	-	-	-	0.11
72	*1-Monoglycerol in C16:1*	-	-	-	-	1.13
73	*1-Monoglycerol in C18*	1.86	-	-	-	0.1

Table 6.5 Continued

74	*1-Monoglycerol in C21*	0.3	-	-	-	-
	Others		**0.22**		**0.14**	**0.27**
75	*Quinic acid*	-	0.06	-	0.14	-
76	*Xylonic acid*	-	-	-	-	0.05
77	*Azulene*	-	-	-	-	0.22
78	*3-Trimethylsilyloxystearic acid*	-	0.16	-	-	-
	Identified	**61.1**	**72.2**	**60.4**	**72.9**	**94**
	Unidentified	**39.0**	**27.8**	**39.6**	**27.1**	**6.0**

The ferric diminishing the *anti-oxidant* power of the selected herbal plants presented a major confine in both hot and cold infusions (Table 6.3). As it is shown *T. vulgaris* L., (leaf), both hot and cold infusions contain the highest compounds with FRAP. *A. officinalis* L., (flower) and *C. sativum* L., (seed) in hot and cold infusions respectively demonstrating the lowest quantity of compounds with FRAP. Three selected leaves demonstrate higher FRAP values. This might be attributed to the higher quantity of accessible TPC, TFC and further bioactive detected compounds (Table 6.5) in most leaves compared to the other plants. The existence of bioactive compounds with diminishing activity might prevent the peroxidation process by reducing free radicals such as *peroxyl* or *alkoxyl* to *hydroperoxides* or *alkoxides*, respectively (Rice-Evans *et al*., 1997). Table 6.4 shows a higher *correlation coefficient* (R^2) between FRAP and *ABTS* values, 74.5 and 79.2% respectively for hot and cold aqueous infusions.

As is presented in Table 6.3, the diminishing power in hot infusions is significantly (p<0.05) higher than cold infusions with two exceptions in *L. officinale* W. D. J. Koch., and *P. anisum* L., (seeds), in which the diminishing power in cold infusions is significantly (p<0.05) higher, probably for their wooden and strict structures of the selected seeds, that application of hot water with 90 °C for 5 minutes might not be sufficient to penetrate into plant cells to extract compounds with diminishing power. *T. vulgaris* L., in both hot and cold infusions, presented the highest value for RP. *E. angustifolia* Fisch & C.A. Mey., (flower) and *C. sativum* L., (seed) in hot and cold infusions respectively presented the lowest values. Leaves demonstrate the higher values for RP. This method is the appropriate assay for the estimation of *low-molecular-weight anti-oxidants* (*LMWAs*) (Pohanka *et al*., 2009). This assay is also a criterion to report the diminishing power of plasma.

The excessive existence of free radicals such as *ROS* and *RNS* (*Reactive Oxygen* and *Nitrogen Species*, respectively) are involved in neural damage, including *central nervous system* (*CNS*) disorders or a brain stroke. The healing of *CNS* occurs immediately after ischemic. The accumulation of free radicals, including (*ROS*) and (*RNS*), leading to earlier brain ischemic (Liu *et al*., 2011), and generated neuronal apoptosis (Broughton *et al*., 2009). *ROS* involves *hydrogen peroxide* (H_2O_2), *superoxide anion* (O_2^-) as well as *hydroxyl radical* ($^{\cdot}OH$), on the other hand *RNS* includes highly toxic *peroxynitrite* ($ONOO^-$) and *nitric oxide radical* ($NO^{\cdot}$) (Iadecola and Anrather, 2011). The outcomes obtained from some researches presented that

pro-inflammatory mediators, including free radicals, *interleukin-1β* (*IL-1β*), *cyclooxygenase-2* (*COX-2*) as well as the *tumour necrosis factor-α* (*TNS-α*) have a substantial impact on brain damage (Amantea *et al*., 2009). $ABTS^{\cdot+}$ assay is an electron shift, end-point examination. Various *anti-oxidant* compounds with diverse bioactivity potential, release one or two electrons to those available cation radicals to reduce free radicals and make them consistent. This assay is a precise method to determine the *anti-radical* activity of plant infusions. As might have been expected in respect of the previous experiments, IC_{50} values in cold infusions are significantly higher than hot infusions with some exceptions in two seeds, while significant differences were not observed ($p<0.05$). This phenomenon might be due to the hard and wooden structures of the seeds in which hot water is not able to extract the bioactive components of the cells in the short term. In *M. chamomilla* L., (flower) and *E. angustifolia* DC., (flower) terms despite the lack of significant difference ($p<0.05$) between the *ABTS* values of hot and cold infusions, however, hot aqueous infusions presented slightly higher $ABTS^{\cdot+}$ inhibition activity (Table 6.3). It could be justified; that the cold water extraction method for two hours might extract an almost equal value of compounds with electron donation activity as the hot water extraction method extracts within five minutes in both flowers.

The highest values of TAA in both hot and cold infusions were obtained in leaves. The lowest values meanwhile were obtained in *C. sativum* L., (seed) and *E. angustifolia* DC., (flower) in hot and cold infusions respectively (Table 6.3). It should be considered that *E. amoenum* Fisch & C.A. Mey., (flower) presents higher *ABTS* and TAA values than other flowers.

Concerning the behaviours of the values obtained values from the cold and hot infusions of *P. anisum* L., and *L. officinale* W. D. J. Koch., (seeds with tough tissues) for TPC, *ABTS*, RP and TAA, it could be demonstrated that most bioactive compounds with electron donation and diminishing activities might not be extracted in such short time through hot water.

As is shown (Table 6.4), strong relationships between the determined parameters of hot and cold infusions are observed. The stronger relationships in both hot and cold infusions were achieved in RP-TPC, followed by FRAP-TPC and FRAP-RP respectively. As is evident, the most diminishing activities are generated by TPC, followed by TCC in both hot and cold infusions, respectively. The results obtained showed that TPC and TCC might be the main contributor of *TEAC* (*ABTS*),

RP, FRAP and TAA compared to TFC, these results are in respect to some of the previous researches performed by (Cai *et al*., 2004; Guleria *et al*., 2013; Li *et al*, 2008). It is observed in Table 6.4 that in most of the parameters, the *correlation coefficient* (R^2) in hot infusions is higher than cold extracts; the mentioned difference between hot and cold infusions is the lowest in TPC-RP cases with almost 1.7% and is the highest in *ABTS*-TAA with almost 58.1%. The above-mentioned data shows that in hot infusions, the most predominant compounds presenting bioactivity are TPC, TCC and further characterised bioactive compounds such as *fatty acids* and *triterpenoids* rather than TFC.

6.3.1. Detected compounds profile with GC-MS

The outcomes achieved by various researches have indicated the substantial roles of phytochemicals in diminishing cancer risk by up to 20%, especially against common and widespread *cancers* such as colon, breast and prostate cancer (Bradford and Awad, 2007; Liby *et al*., 2007; Setzer and Setzer, 2003). The compounds profile of five selected plant infusions, including three leaves and two seeds, are presented in Table 6.5. In *Coriandrum sativum* L., *octadecenoic acid* (*oleic acid*) is detected as the predominant *fatty acid*, followed by *hexadecanoic acid* (*Palmitic acid*), both demonstrating almost 71.49% of the total detected compounds. *Fatty acids* might interfere in the transcellular transfer system of cellular contents by interfering with the performance of different cellular transporters in the intestinal epithelium. Therefore, an enhanced transportation system of particular hydrophilic components accessible in surroundings is generated (Aspenstrom-Fagerlund *et al*., 2012). Concerning the findings of scientists, *oleic acid* was indicated to enhance the absorption of *mitoxantrone* (*MXR*) and as a result, increases the gene expression of *BCRP* in *CACO-2 cells* (Aspenstrom-Fagerlund *et al*., 2012). Therefore *fatty acids* could interfere in the gene expression of cancer cell lines and prevent the generation of *carcinogenic cells*. *Betulinic acid* is a *triterpenoid* detected in a considerable proportion compared to further *triterpenoids* in *C. sativum* L., *Anti-inflammatory* activity is attributed to this compound (Mullauer *et al*., 2010). This potential is relevant to the modulation mode of *betulinic acid* on the expression of *NADPH oxidase* and *nitric oxide synthase* (*NOS*) isoforms genes. Moreover, *betulinic acid* presented *HIV-1 anti-viral* potential and the mechanism is established by its potential in the modification of the carboxylic acid group at position C28, recorded to inhibit

HIV-1 entry cells (Holz-Smith *et al*., 2001; Labrosse *et al*., 1997; Mayaux *et al*., 1994).

In *Pimpinella anisum* L., seeds 9, 12-*octadecadienoic acid* (*linoleic acid*), *hexadecanoic acid* (*palmitic acid*) as well as *octadecanoic acid* (*stearic acid*) comprise of 47.22% of the total detected compounds by *GC-MS*. *Conjugated linoleic acid* (*CLA*) is recognised as an effectual suppressor through all lifecycles of carcinogenesis cells, including initiation, progress and metastasis, as well as angiogenesis and/or neovascularisation. Various performed researches on the health potential effects of *CLA* isomers expressed its substantial inhibitory potential on induced tumour cells of colon (Bassaganya-Riera *et al*., 2012; Kelley *et al*., 2007; Rosberg-Cody *et al*., 2007), mammary gland (Lau and Archer, 2009; Rakib *et al*., 2013) and the metastasis of inoculated cancer cells. Moreover this reputed fatty acid acts against *obesity*, *hyperglycaemia* and *hypertension* (Koba and Yanagita, 2014) within particular pathways on the lifecycles of the spoiled cells. Figure 6.2 presents the obtained chromatograms for the two seeds.

To summarise, with respect to the abovementioned outcomes, it might be stated that *P. anisum* L., and *C. sativum* L., could act as active agents against *carcinogenic cells* and the *HIV-1 virus* as well as *hyperglycaemia*, *hypertension* and *obesity*.

In *R. officinalis* L., *sterols* and *triterpenoids* including *ursolic*, *oleanolic*, *betulinic*, and *maslinic acids*, are detected extensively in the extract (58.37%). The *plants' sterol* (*PSE*) is detected as being the most effectual against *cardiovascular* disorders. In one research, *PSE* increased the inverse transport of *cholesterol* from the *enterocyte* to the *intestinal lumen* and reduced the chylomicrons rate by decreasing *ACAT* (an essential enzyme in the esterification of cholesterol) level and activity (García-Llatas and Rodríguez-Estrada, 2011).

Ursolic acid has expressed a wide range of health benefits such as being *anti-inflammatory*, *anti-tumour* and *anti-oxidant* (Lee *et al*., 2007; Sultana and Saify, 2012). Furthermore, it has established an improvement in cognitive deficits (*anti-Alzheimer's* effects) and *neuroprotective* (*anti-Parkinson's*), *anti-septic*, *anti-depressant*, *hepatoprotective* (Jin *et al*., 2012; Saravanan *et al*., 2006), *immunomodulatory* and *anti-inflammatory*, *hypoglycaemic* (*anti-diabetic*), *anti-bacterial*, *anti-viral* (Kong *et al*., 2013) and *anti-ulcer* (Ishikawa *et al*., 2008) potential. The multifunctional *anti-cancer* activity of *ursolic acid* (*UA)* is attributed

to its potential to induce *cancer cell apoptosis* followed by the suppression of cancer proliferation. *Oleanolic acid* presents *anti-viral* activity by preventing the replication of the *HCV genotype 1b* replicon and *HCV genotype 2a JFH 1* virus. Moreover, *oleanolic acid* and *ursolic acid* exhibited *anti-HCV* activity (Kong *et al*., 2013). Additionally, further biological and potential health benefits, including *anti-inflammatory*, *anti-tumour*, *hepatoprotective*, *hypoglycaemic* and *anti-HIV* activities are attributed to this *terpenoid*.

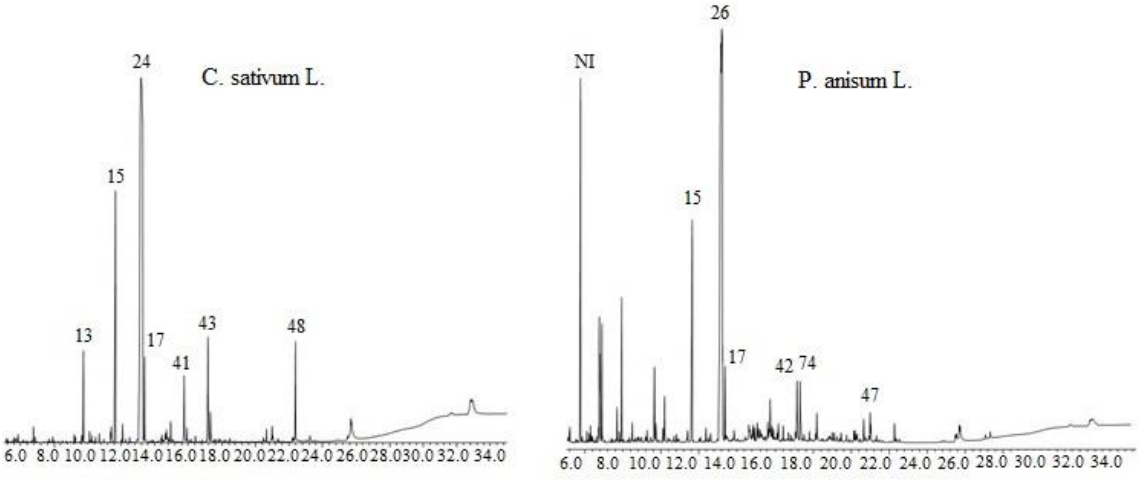

Figure 6.2 Obtained chromatograms of some selected seeds (NI: Not identified).

Another detected bioactive compound in *R. officinalis* L., leaves is *maslinic acid* (*MA*). A wide confine of therapeutic effects, including *anti-oxidant*, *anti-inflammatory*, *hypotensive*, *anti-viral* and *anti-tumour* (Reyes *et al*., 2006) were reported as belonging to this compound.

In *Salvia officinalis* L., leaves, *triterpenoids* including *ursolic*, *oleanolic*, and *betulinic acids*, (almost 38.49%) and *fatty acids* such as *oleic* acid (*Octadecenoic acid*) and *palmitic acid* (*Hexadecanoic acid*) (7.12%) in higher values were detected. Besides the detected *terpenes*, *borneol* (*BO*) is another terpene found in *Salvia officinalis* L., infusion with the availability of 1.13%. This compound is reputed as a treatment of *cardiovascular* and *cerebrovascular* disorders. This exclusive compound is also known as an upper guiding agent which could transfer other medicines to reach the organs in the upper parts of the human body, particularly in the *central nervous system*, or in other words, *BO* could improve the accumulation of medicines in the *central nervous system* and enhance the susceptibility of the brain to medicines including *tetramethylpyrazine phosphate*, *geniposide* and *puerarin* (Cai *et al*., 2008; Gao *et al*., 2010; Yan-Yu *et al*., 2007). Moreover, *anti-radical* and *anti-*

inflammatory activities, along with the protective effectiveness of *BO* against cerebral ischemia and/or reperfusion injury were reported (Liu *et al*., 2011).

In *Thymus vulgaris* L., as shown in Table 6.5, *sterols* and *triterpenoids* are detected as major compounds, followed by *fatty acids*, sugars and aromatic compounds demonstrating a total of 64.31% of the whole detected compounds by *GC-MS*. *Oleanolic* and *ursolic acids*, as well as *betulinic acid* are the predominantly detected *triterpenoids*. Moreover, *oleic* and *palmitic acids* are the main detected *fatty acids* in this plant. Therefore, regarding the aforementioned potential of the detected compound in *T. vulgaris* L., this plant has the potential for different treatments such as *anti-inflammatory, anti-carcinogenic, anti-HIV, anti-oxidant, anti-Alzheimer's, anti-Parkinson's, hypoglycaemic, anti-ulcer, anti-bacterial, anti-viral, anti-tumour* etc. treatments (Fig. 6.3).

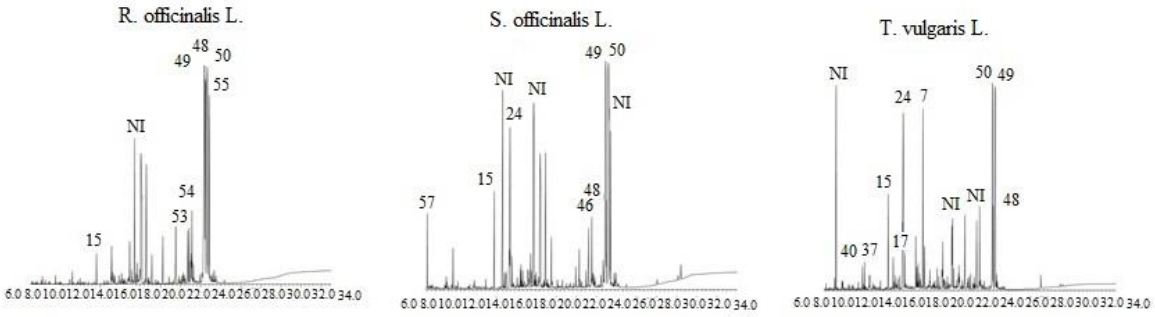

Figure 6.3 Obtained chromatograms of the selected leaves (NI: Not identified)

β-sitosterol is another compound detected along with others in this research. The *anti-asthmatic*, *anti-diabetic* and *anti-atherogenic* (Rosenblat *et al*., 2013) properties are attributed to this compound. The researches carried out on breast cancer cell lines demonstrated that *β-sitosterol* induces apoptosis through a *Fas signalling pathway*. Moreover, this compound was discovered as being effective in suppressing colon (*HT-29*) and *prostate cancer cell lines* (*LNCaP*). It is reported that this compound acts against cancer cell lines with ignorable adverse effects. Interestingly this compound has shown *neuroprotective* potential which might be effective in the treatment of *Alzheimer's disease* (Hamedi *et al*., 2014).

As shown in Table 6.5, *squalene* is another *triterpene* characterised in trace content in the selected leaves' infusions. This compound has expressed highly effectual scavenging activity against *reactive oxygen species* (*ROS*) and reduced intra

cellular oxidant stress. This might be a reason behind why *Thymus vulgaris* L., and other leaves present higher *anti-radical* activities in this research. This compound is not detected in the selected seeds. This compound, due to it offering *single oxygen quenching potential* might protect skin cells against lipid peroxidation and could be considered as an *anti-aging* agent. *Squalene's cardio-protective* effect is attributed to its potential in blocking lipid peroxidation (Lou-Bonafonte *et al*., 2012; Sabeena Farvin *et al*., 2004).

β-amyrin is identified in trace quantity in some plants in this study. The *anti-inflammatory*, *gastro-protective* and *anti-pruritic* potentials of this compound were previously reported in several studies. *β-amyrin*, along with *α-amyrin* improved periodontal inflammation *in vivo* models and exhibited *anti-nociceptive* and *anti-inflammatory* properties. This compound also presented *hypoglycaemic* potential exploiting the models of *STZ*-induced *diabetic rats* (Singh *et al*., 2009).

The synergistic effects of the different bioactive compounds have been studied in various studies. In one research performed by (Sidhu *et al*., 2009), individual and combined plant infusions extracted by methanol were assessed. The obtained outcomes revealed that the combined extracts of different plants have considerable synergistic *anti-toxin* and *anti-fungal* efficacies when compared to those infusions individually. It is reported, that the natural bioactive compounds could have synergistic effects, such as *flavonoids*, which might increase their own bioactivity potential in the presence of other bioactive compounds (Williamson, 2001). The synergistic effectiveness of bioactive compounds *in vivo* experiments was previously proven in the *eczema* treatment effects of *Chinese* plants (Williamson, 2001).

As shown in Table 6.5, *sterols* and *triterpenoids* in the leaves, demonstrating 63.42, 47.64 and 33.51% of all contents of the detected bioactive compounds respectively in *Rosmarinus officinalis* L., *Salvia officinalis* L., and *Thymus vulgaris* L., in this research are the principal compounds, but *fatty acids* with contents of 80.22 and 49.04% respectively in *Coriandrum sativum* L., and *Pimpinella anisum* L., seeds are the major compounds. Fig. 6.4 demonstrated the 2D chemical structures of some of the most detected compounds of the plants. Therefore, in relation to the obtained data by the determined *anti-oxidant* parameters of the plants and detected compounds' contents with the *GC-MS* analysis of the leaves and two seeds species, it could be stated that *sterols* and *triterpenoids* in the leaves and *fatty acids* in the seeds in the presence of *phenolic* compounds might be the major agents in demonstrating

bioactivity potential of the selected leaves and seeds species respectively in this research.

To summarise, *anti-inflammatory* and *anti-HIV* potential, as well as effects against *obesity*, *hyperglycaemia* and *hypertension* and spoiled cells by the seeds and *anti-cardiovascular*, *anti-inflammatory*, *anti-tumours*, *anti-oxidant*, *anti-Alzheimer's*, *anti-Parkinson's*, *anti-septic*, *anti-diabetic* etc. potential of leaves might be generated.

6.4. Conclusions

In summary, strong evidence has been found in this research based on the important impact of detected compounds from different categories, including *fatty acids*, *sterols*, *triterpenoid*, *sugars* etc. in generating bioactivity potentials. Consequently, *triterpenoids* including *betulinic*, *maslinic*, *oleanolic* and *ursolic acids* in the leaves, and *fatty acids*, including *palmitic acid* (*hexadecanoic acid*), *oleic acid* (*octadecenoic acid*) and *linoleic acid* (*9,12-octadecadienoic acid*) in the seeds and trace presence of further bioactive compounds are detected by *GC-MS* analysis as the major compounds in generating potential health benefits of the selected plants. On the other hand, all the specified categories of compounds in the examined plants including *sugars*, *alkanes*, *fatty acids*, *fatty alcohols*, *di-acids*, *waxes*, *sterols*, *terpenes*, *aromatics* and *monoacylglycerols* as well as further unrecognised available compounds might possess synergistic effects in generating potential health benefits. The authors suggest strongly that the selected plants in this study could potentially have *anti-carcinogenic*, *anti-diabetic*, *anti-Alzheimer's*, *anti-Parkinson's* and *anti-microbial* agents, which requires further studies.

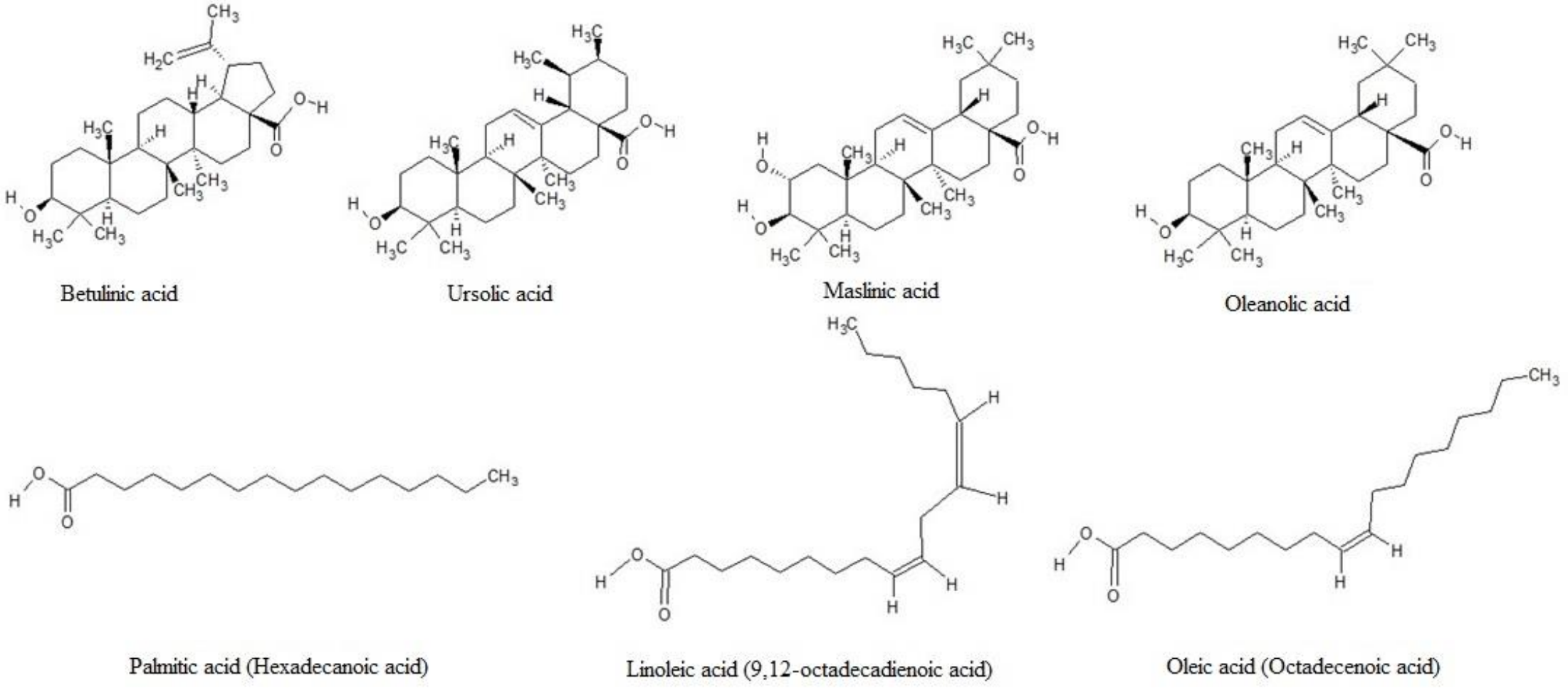

Figure 6.4 2D chemical structures of some major *triterpenoid* and *fatty acid* compounds in this research (Drawn by *ChemSketch* version 2012).

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CHAPTER 7

Medicinal chemistry, phytochemical profiles and potential health benefits of a selected group of plants

In the current chapter, the inhibitive effects of the selected plants against enzymes attributed in revealing diabetic and Alzheimer's diseases as well as some infectious and pathogenic bacterial species have been examined. Finally the profiles of bioactive compounds of the plants were recognized by GC-MS analyses and interpreted.

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Medicinal chemistry, phytochemical profiles and potential health benefits of a selected group of plants

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Abstract

BACKGROUND: 10 different species of Iranian plants including leaves, flowers and seeds have been selected for extraction using water as solvent. Hot aqueous extracts were screened for *hypoglycaemic*, *anti-cognitive* and *anti-bacterial* properties to screen their bioactivity and *GC-MS* analysis was applied for characterization of the compounds profiles. The aim of this research is discovering the plants with higher activities and identifying of their predominant bioactive compounds associated with health potentials.

RESULTS: The plants species demonstrated considerable activity against enzymes, associated with *diabetes* and *Alzheimer's* disease. *M. chamomilla* L., (flower) with 9.336, *A. officinalis* L., (seed) with 10.5441 and *S. officinalis* L., (leaf) with 9.038 (mg. mL^{-1}) *IC50* values were observed to have the highest activities against *α-Glucosidase*, *α-Amylase* and *Acetylcholinesterase* (*AChE*), respectively. *E. faecalis* showed the lowest inhibitive concentration compared to other bacterial species against the infusions.

CONCLUSIONS: The *GC-MS* analyses showed that *sterols* with *triterpenoids* and *fatty acids* are the predominant compounds in the leaves and seeds respectively. It assumes that *triterpenoids* in combine of free fatty acids and further bioactive compounds might expose synergistic effects in presenting potential health benefits. The obtained results demonstrated that the selected plants could be distributed as potential sources of relief in treatment of some chronic disorders.

Keywords: Plants; health benefits; enzyme inhibition; *anti-bacterial* activity; *GC-MS*.

7.1. Introduction

Iranian Traditional Medicine (*ITM*) is one of the most substantial and oldest origins with more than 1,000 years of history. Most medicinal plants contain a wide variety of secondary metabolites including *tannins*, *phenols*, *flavonoids*, alkaloids, *terpenoids* and *quinones* [1].

Type I diabetes is caused by a deficit in *pancreatic β-cells*, resulting in a lack of insulin secretion. This type of diabetes can be hereditary and found at any age. A deficit in *pancreatic β-cells* might be due to an autoimmune abnormality. While *type II diabetes* might be caused by a derangement in the metabolism of carbohydrate, protein and/or fat content [2]. *Aging*, *gestational*, lack of exercise and/or obesity might be related to the generation of *diabetes*. The mechanism of the actions of medicinal plants in *hypoglycaemia* could be described by the following reasons, inducing: insulin secretion from *pancreatic β-cells* and protective effects for releasing insulin against degradative conditions, decreasing the insulin resistance of cells, inducing the regeneration of *pancreatic β-cells* with an enhancement in cells' size and quantity, as well as the stimulation of *glycogenesis* and *hepatic glycolysis*. Another pathway is the protective impact of bioactive compounds against *pancreatic β-cells*, and their eventual inhibition effectiveness against *β-galactosidase*, *α-Glucosidase* and *α-Amylase* enzymes. The protective effects of the plants on *pancreatic β-cells* might be due to the *anti-oxidant* effects of the plants, which protect the cells against oxidative stress and decomposition. *Reactive oxygen* (*ROS*), *nitrogen* (*RNS*) and *chlorine* (*RCS*) species, as well as oxidative stress, presented an enhancement in both types of *diabetes*. Since the enhancement of free radicals' generation, along with reduced *anti-oxidant* systems might ruin enzymes, as well as cellular organelles and as a result cause chronic diseases [3], the plants therefore present *anti-oxidant* potential along with *hypoglycaemic* properties which could be more effective than current medicines prescribed solely for the treatment of *hyperglycaemia*.

Dramatic increases in cognitive related diseases such *Alzheimer's disease* (*AD*) prevalence has increased the importance of researches performed on *AD*. *Anti-oxidant*s are suggested as being effective agents in the delay or prevention of *AD*. Direct correlations were found between cholesterol content and *AD*. The use of

cholesterol in lowering medicine decreased the prevalence of *AD*. The *anti-Alzheimer's* properties of some bioactive compounds such as gugulipids might be attributed to their *Acetylcholinesterase* inhibition, cholesterol content lowering and/or radicals scavenging properties. *Cholinergic* hypothesis is the most accepted among other *AD* eventual causes because of its performance in signal transferring pathways in the nerves. A wide range of compounds including *polyphenols*, *flavonoids*, *fatty acids*, *tannins*, *lignans*, *sterols* as well as *triterpenes* have shown widespread health benefit potential with different intensities such as *anti-cholinesterase*, *hypolipidemic*, *anti-amyloidogenic* and *anti-oxidant* effects.

Infectious disorders suggest a substantial cause of death, particularly in developing communities. Therefore the requirements of pharmaceutical and nutraceutical companies to natural compounds with considerable *anti-microbial* potentials are elevated. Nowadays, bacterial species due to the achieving ability in generation of species with resistance against current *anti-microbial* agents have alarmed scientists, pushing them to detect new and natural compounds with substantial *anti-microbial* properties. The *anti-microbial* mechanisms of natural compounds might be related to several pathways including: destructive efficacy against cells' membrane; interfering in the electron flow and proton motive force as well as the coagulation of the cell contents [4]. For instance, the *anti-microbial* effectiveness of essential oils is attributed to their hydrophobic structures, interfering in cell membrane permeability and preparing them to be non-selective. The stimulating effects of essential oils on the growth of pseudo mycelia are proving their interference on the enzymes' activity involving the production of cellular structural components. The authors did not find any similar studies performed on such a collection of reputed Middle Eastern plants on this scale.

In the current research, 10 plants used traditionally in *Persian lands* were selected. The aim of this study is to establish and identify the compounds involved in revealing eventual *hypoglycaemic*, *anti-amnesia* and *anti-bacterial* properties.

7.2. Materials and methods

7.2.1. Plant materials and selection

Plants were cultivated by the *Zarrin Giah Green House Company* in Uremia, Iran, in February 2014. The nominated plants were then dried in shade and shifted to the lab where they were protected from light, heat and humidity until grinding. The

selected plant materials were ground, sieved and sealed into containers; and preserved at -20 °C until extraction. The botanical, common, local, English, Portuguese and family names plus the part used, as well as some general detected health benefits of each plant are presented in Table 7.1.

7.2.2. Materials and chemicals

All required chemicals and reagents for the experiments and *GC-MS* analysis were purchased in an analytical grade.

7.2.3. Apparatus

Spectrophotometer (T70+Vis, PG Instrument Ltd, United Kingdom); rotary evaporator (*Nahita* series 503, *Navarra*, Spain); centrifuge *Hettich* (*universal-320*, Germany); hot plate-stirrer (*VWR*, 720 advanced, *USA*); disposable cuvettes (*VWR*, Leuven, Belgium); vortex mixer (*Stuart*, UL-Bibby Sterilin Ltd); grinders (*Philips*- Brazil and *Cetigo*- Portugal) are briefly used equipment in this research.

7.2.4. Enzyme inhibition assays

7.*2.4.1. Preparation of plant extracts for enzyme experiment*

The extraction procedure was carried out by handling hot (90 °C) distilled water. 2 g of each sample (ground and sieved) was heated up for 5 minutes in 20 mL of water while being stirred by a hot plate-magnet stirrer (*VWR*, 720 advanced, *USA*). After cooling down, they were then centrifuged at room temperature at 5000 rpm for 10 minutes, using centrifuge *Hettich* (*universal-320*, Germany). The supernatant was refined across *sartorius stedim bio grade 388* filter papers. When the extraction proceedings were completed, the solvent was evaporated using a rotary evaporator (*Nahita* series 503, *Navarra*, Spain) and the remaining materials were re-suspended in the *milli Q water*, frozen and preserved at -20 ºC until the day of analysis.

7.*2.4.2. α-Glucosidase inhibition assay*

The *α-Glucosidase* inhibitory assay was performed in a spectrophotometer (T70+Vis, PG Instrument Ltd, United Kingdom) according to [5, 6] with slight modifications. Concisely, 150 µL of sample solutions in a 0.1 M phosphate buffer were blended with 150 µL of enzyme solution containing 1U. mL^{-1} (1 mg=14 U) and incubated at 37 ± 1 °C for 10 minutes. After incubation, 150 µL of the substrate *p-nitrophenyl α-D-glucopyranoside (pNPG)* (1 mM concentration in 0.1 M phosphate

Table 7.1 Selected Iranian plants, botanical, common, local, Portuguese and family names and medicinal properties.

No	Botanical name (Voucher number)	Common name	Local name	Family	Part used	Medicinal potentials	Reference
1	*Thymus vulgaris* L. (ZGC139201)	Garden thyme	Avishan baghi	Lamiaceae (Mint family)	Leaves plus flowers	*Anti-bacterial*, *anti-fungal* , Treatment for cough and sore throat, (Portugues name: Tomilho)	7-9
2	*Rosmarinus officinalis* L. (ZGC139202)	Rosemary	Aklile kohi(Ros mary)	Lamiaceae (Mint family)	Leaves	Treatment for rheumatism and migraines, Gout and muscle aches, tremors and Paralysis of hands and feet, disinfectant, diuretic, digestive, Booster nerves, *anti-bile* secretion and healer, *anti-depressant* (Portugues name: Rosmaninho-comum)	7
3	*Salvia officinali* L. (ZGC139203)	Kitchen sage	Maryam goli	Lamiaceae (Mint family)	Leaves	Treatment for pharynx and larynx disorders, Mouthwashes, *anti-bacterial* (Portugues name: Salva) (Portuguese name: Salva-espanola)	7, 10
4	*Echium amoenum* Fisch. Et Mey. (ZGC139204)	Common borage	Gavzabane Irani	Boraginaceae	Flower	Treatment for respiratory disorders, Immune system booster *anti-depressant* (Portuguese name: Borragem)	10
5	*Echinacea angustifolia* DC. (ZGC139205)	Blacksamson echinacea	Sar khar gol	Asteraceae (Aster family)	Flower	Treatment for cough, sore mouth and gums, treatment for colds, *anti-gonorrhea* , infectious diseases and respiratory diseases digestive, *anti-bacterial*, immune system booster (Portuguese name: Camomila-alema)	8, 11
6	*Althaea officinalis* L. (ZGC139206)	Common marshamallow	Khatmi darouie	Malvaceae (Mallow family)	Flower	*Anti-cough*, softener respiratory tract, Phlegm propulsive *anti-inflammation* (specially eye's inflammation), *anti-mucous* membrane irritation, Treatment for *trachea*, *bronchi* and lung (Portuguese name: Malvaisco)	12
7	*Matricaria chamomilla* L. (ZGC139207)	German chamomile	German Baboneh	Asteraceae (Aster family)	Flowers	*anti-inflammation*, appetizer, digestive, *anti-microbial*, and treatment for abdominal pain, (Portuguese name: Camomila-alema)	8, 12, 13
8	*Coriandrum sativum* L. (ZGC139208)	Coriander	Geshgniz	Apiaceae (Carrot family)	Seeds	Flavor agent, carminative, *anti-floating*, digestive (Portuguese name: Ceandro)	8, 14
9	*Pimpinella anisum* L. (ZGC139209)	Anise burnet saxifrage	Badian roomi (anison)	Apiaceae (Carrot family)	Fruits(seeds)	Appetizer, digestive and treatment for abdominal pain, Sore throat (Portuguese name: Anise)	7, 8, 10, 16
10	*Levisticum officinale* W. D. J. Koch. (ZGC139210)	Garden lovage	Anjedane roomi	Apiaceae (Carrot family)	Seeds	Diuretic, Kidney cleansing, Treatment for kidney stones (Portuguese name: Ligustico)	7, 8, 10, 16

buffer, pH 6.8) was added to the mixture, allowed to incubate at 37 ± 1 °C for 30 minutes; then the reaction was interrupted by the addition of 600 µL of a 0.4 M sodium carbonate solution. The absorbance of the solution was detected at a 405 nm wavelength.

The percentage of inhibition activity (*IC*%) of the enzyme is computed in comparison to the control regarding equation 7.1 and expressed as mean ± *SD*. The assay was performed in triplicate within at least four independent experiments.

$$Inhibition\ activity\ (\%) = \left(\frac{A_{Control} - A_{\ Sample}}{A_{control}}\right) \times 100 \qquad (7.1$$

The *inhibition concentration* percentage (IC_{50}) values were obtained from concentration versus percentage of inhibition curves (µg. ml^{-1}) (*Acarbose* as positive control).

7.2.4.3. α-Amylase inhibition assay

The assay was performed in spectrophotometer (T70+Vis, PG Instrument Ltd, United Kingdom) as described by [17-19], with some modifications. Briefly, 100 uL of starch solution (1%) (in 0.02 M phosphate buffer, pH 6.9, containing 0.006 M NaCl) and 50 µL of sample solution were mixed and incubated at 20 ± 1 °C for 5 minutes. After incubation, 100 µL of enzyme solution (1U. mL^{-1} in 0.02 M phosphate buffer (pH 6.9, containing 0.006 M NaCl)) was added and incubated at 20 ± 1 °C for 3 minutes afresh. Then the reaction was concluded by the addition of 100 µL of *DNS* (*Dinitrosalicylic acid*) colour reagent (96 mM *DNS* was blended in sodium potassium tartrate ($KNaC_4H_4O_6.4H_2O$, in 2 N NaOH)), and immediately shifted into a boiling water bath for 15 minutes, followed by the addition of 900 µL distilled water; afterwards the absorbance of the final solution was recorded at a 540 nm wavelength.

The percentage of inhibition activity (*IC*%) of the enzyme is achieved in comparison with the control regarding equation 7.2 and expressed as mean ± *SD*. The assay was performed in triplicate within at least four independent experiments.

$$Inhibition\ activity\ (\%) = \left(\frac{A_{Control} - A_{Sample}}{A_{control}}\right) \times 100 \qquad (7.2$$

Inhibition concentration (IC_{50}) values were calculated from concentration versus percentage inhibition curves (*Acarbose* as positive control).

7.2.4.4. AChE inhibition assay

AChE inhibition activity was evaluated by spectrophotometer (T70+Vis, PG Instrument Ltd, United Kingdom) regarding the method reported previously [20, 21] with minor modifications. Accordingly, 100 µL *AChE* solution (0.03 U. mL^{-1}) (1 mg= 149 unit), 50 µL test sample (dissolved in 30% MeOH) and 900 µL Tris-HCl buffer (50 mM, pH 8) were mixed vigorously and incubated at 4 °C for 30 minutes. Then, in the reaction mixture, 100 µL *DTNB* (*5,5'-dithiobis-2-nitrobenzoic acid*) (0.3 mM) and 100 µL *ATCI* (*Acetylthiocholine iodide*) (1.8 mM) were added and incubated at 37 °C for 20 min, followed by the detection of absorbance at 412 nm wavelength.

Percentage of inhibition activity (*IC*%) of the enzyme is computed in comparison with the control regarding the follow equation and expressed as mean ± SD. The assay was performed in triplicate within at least four independent experiments.

$$Inhibition\ activity\ (\%) = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100 \qquad (7.3$$

The *inhibition concentration* (IC_{50}) values were calculated from the concentration versus the percentage of the inhibition curves (µg. ml^{-1}) (*Tacrine* as positive control).

7.2.5. Microbial tests

7.2.5.1. Bacterial strains culture preparation

Bacterial species were preserved by freezing them at -20 ˚C in broth, including 15%, v/v glycerine. Before the experiments for activation of each bacterial species, the stock cultures of each microorganism were inseminated into sterile tubes containing 5 mL Luria broth. The inseminated tubes were stirred drastically and incubated at 37 ˚C for 24 hours and then a minor quantity of the bacterial solution was shifted and streaked into a petri dish containing 20 mL dry *plate count agar medium* (*PCA*) which had been previously prepared and stored in the fridge at least one day before examination. Afterwards, cultivated plates were capped and covered loosely by para film and placed in an incubator and the bacterial species left to grow overnight at an appropriate temperature.

7.*2.5.2. Standardized bacterial colony numbers*

The concentration of bacterial species in a ringer solution was adjusted in regards to the method described previously [22].

7.*2.5.3. Preparation of resazurin solution*

The resazurin solution was accumulated by dissolving a ratio of 270 mg in 40 mL of sterilised, distilled water. A vortex mixer was applied to certify an integrated and well-dissolved solution.

7.*2.5.4. Disc diffusion assay*

For the determination of the susceptibility of the selected bacterial species versus infusions and the diameter of the inhibition zone, an assay similar to the pathway of [23] with some modifications (infusions instead of *anti-biotics* in a standard concentration of 10 mg. mL^{-1}) was accomplished, and the results obtained were expressed as a mean of three determinations of the discs (Mean ± *SD*).

7.*2.5.5. Micro-dilution assay using 96 well Eliza plate*

Plates were accumulated under aseptic condition using the resazurin solution as an indicator, following the assay described by scientists [24]. The lowest concentration of each infusion was considered as the *MIC* value of which colour alteration arose. An average of two replications were computed and reported as the *Minimum Inhibitory Concentration* (*MIC*) of each infusion versus each bacterial species.

7.2.6. Gas chromatography-mass spectrometry (GC-MS) analysis

The bioactive constituents were analysed with an Agilent 5975C inert *mass selective detector* (*MSD*) with a Triple-Axis Detector coupled to an Agilent 7890A *gas chromatography* system, equipped with a *ZB-5HT* inferno capillary *GC* column (30 m × 0.025 mm × 0.1 µm film thickness) with temperature limits of (min=-60 to max=400-430 ºC) (made in the *USA*). Helium (flow rate, 1 mL. min^{-1}) was used as the carrier gas. The injector temperature was 280 ºC. The oven temperature programme was 100 ºC for 1 minute, increased by the rate of 10 ºC. min^{-1} to 370 ºC and was maintained for 1 minute, then enhanced at the same rate (10 ºC. min^{-1}) to 380 ºC and kept constant for an extra 5 minutes, therefore the term of whole run reached to 35 minute. The mass spectrometer was applied in *EI* mode and the injected samples were scanned from 40 to 950 amu. The bioactive compounds were

identified by chromatograms using different database and comparing their spectral data to those from mass spectral libraries and with spectral data of standard compounds.

7.2.7. Expression of data and statistical analysis

Experimental outcomes were expressed as the mean ± *SD* of three parallel records and analysed using the SPSS software package, version 22.0 (IBM SPSS Statistics 22, licensed University of Algarve). Differences between the data obtained from the various plants were identified by one way *ANOVA* pursued by *Duncan post hoc* test with a confidence level of 5% (p<0.05). Statistical graphs were drawn using *Microsoft Office Excel* 2010.

7.3. Results and discussion

7.3.1. Enzyme inhibitory activities of hydrophilic infusions

A limited number of herbs showed *hypoglycaemic* properties using various pathways. For instance, they could induce the delivery of insulin from pancreatic *β-cell lines* without any detected negative adverse effects. Herbal plants' infusion might induce the delivery of insulin from *BRIN-BDII* pancreatic *β-cell* lines, glucose oxidation, converting the glucose content to glycogen and the enhancement of *2-deoxy-glucose* transporter contents [25], providing the required ions for reactions including zinc, magnesium, manganese and copper. Other pathways for plants containing *AGS-IV* (a newly detected *glycoside* of *cycloartane*-type *triterpene*) might enhance the activity of the glutathione peroxidase in nerves suppressing the accumulation of the advanced glycation end products in the nerves and erythrocytes. Moreover, *glycosides* might elevate Na^+/K^+-ATPase activity in both the nerves and erythrocytes [26]. However, the inhibition activities of the bioactive compounds against *α-Glucosidase* and *α-Amylase* have attracted much more attention in presenting their *hypoglycaemic* activities.

7.3.2. Suppression of enzymes

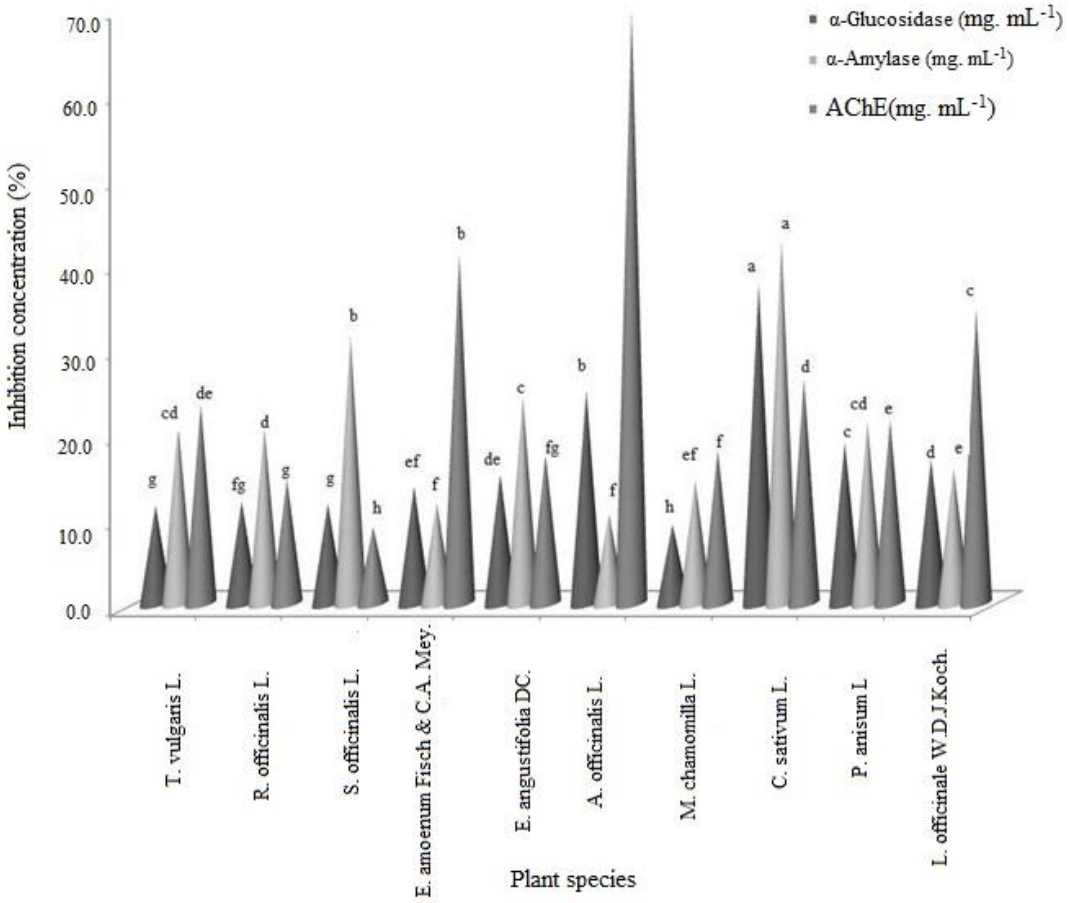
As is shown Table 7.2, selected plants demonstrated a widespread range of inhibitors against *α-Glucosidase* and *α-Amylase. M. chamomilla* L., demonstrated the highest activity against *α-Glucosidase* followed by leaves. *C. sativum* L., showed the lowest inhibitory activity. However, *A. officinalis* L., expressed the strongest activity against *α-Amylase* followed by *E. amoenum* Fisch & C.A. Mey., and *M. chamomilla*

L., respectively. *C. sativum* L., showed the weakest activity against *α-Amylase* activities. As is clear from Fig. 7.1, *C. sativum* L., demonstrated the lowest activity against both *α-Glucosidase* and *α-Amylase* in comparison to other plants tested. *S. officinalis* L., showed the highest activity against *AChE* followed by *R. officinalis* L. *A. officinalis* L., showed the lowest activity against *AChE*. Most of the leaves, along with *M. chamomilla* L., showed stronger effectiveness against *α-Glucosidase.* As is shown in Table 7.6, leaves have not presented higher values in *fatty acid* contents in comparison to seeds; however the leaves presented higher values in *triterpenoid* when combined with *sterol* contents. *C. sativum* L., showed the lowest activity against both of the *diabetes*-related enzymes (Fig. 7.1). As is demonstrated, this seed showed the highest amount of *fatty acids* (≥80.22%) and the lowest amounts in *sterols* and *triterpenoids* as well as aromatics with presenting 4.57% and 0.22% respectively (Table 7.6). The outcomes obtained might justify the most predominant impact of *triterpenoids* in presenting *hypoglycaemic* activities. Overall, flowers presented good inhibition activities against *α-Glucosidase* and *α-Amylase* enzymes, confirming their effects against diabetes along with other plants.

Choline might be attributed to the transferring system of nerves; therefore the *cholinergic* hypothesis is the most accepted justification of *AD* causes. Amongst plants, two leaves, including *S. officinalis* L., and *R. officinalis* L., demonstrated the highest eventual *anti-Alzheimer's* properties respectively and two flowers, *A. officinalis* L., and *E. amoenum* Fisch & C.A. Mey., respectively expressed the lowest inhibition activity against *AChE* activity. Regarding the obtained data of the *GC-MS* analysis of the five examined plants, the substantial effects of *triterpenoids* and *fatty acids* in the prevention of *AChE* activity might be indicated. As is observed in Fig. 7.1, *C. sativum* L., (seed) demonstrates the weakest inhibiting activities against enzymes to affect in *diabetes*; however it showed higher inhibition activity against *AChE* compared to *α-Glucosidase* and *α-Amylase* enzymes.

Tested leaves exhibited considerable *anti-diabetic* activity including flowers such as flowers *A. officinalis* L., and *M. chamomilla* L. The strongest activity against *α-Glucosidase* and *α-Amylase* was achieved in the flowers of *M. chamomilla* L., and *A. officinalis* L., respectively. Interestingly, *C. sativum* L., (seed) suggested the minimum inhibition against *diabetes*. Unlike *α-Amylase*, *A. officinalis* L., presented the weakest activity against *AChE*. As is clear in Fig. 7.1 and mentioned in Table 7.2, *S. officinalis* L., showed the highest inhibition potential against *AChE*, or put in

another way, *Salvia officinalis* L., might show the highest activity against *Alzheimer's disease (AD).* The *anti-Alzheimer's* disease mechanism of *S. officinalis*



L., might be attributed to *triterpenoid* compound contents in a combination of *fatty acids*, along with eventual *galanthamine* and *alkaloid* contents. Table 7.3 presented some main bioactive compounds detected in plants by scientists with *anti-Alzheimer's diseases* activities.

Figure 7.1 Comparison of the inhibition activities of the selected plants against three tested enzymes.

7.3.3. Anti-bacterial properties

The *anti-bacterial* potential of the studied plants were tested on both hot and cold aqueous infusions. The obtained results using the disc diffusion and microdilution methods are shown in Tables 7.4 and 7.5 respectively. Figs. 7.2 and 7.3 show the results obtained of the *MIC* for some plants. In most plants cases and bacterial species, significant differences were not found between hot and cold infusions with two exceptions, *P. anisum* L., and *T. vulgaris* L., against *L. monocytogenes* and *E. faecium* bacterial species respectively, in which the first case

Table 7.2 Obtained *α-Glucosidase*, *α-amylase* and *acetylcholinesterase* inhibition potentials of ten examined plants with the positive controls. The results indicate the mean ± *SD* of three independent triplicate experiments.

Scientific name	***Inhibition Concentration* (IC_{50} (mg. mL^{-1}))**		
	α-Glucosidase	***α-Amylase***	***Acetylcholinesterase***
T. vulgaris L.	11.531 ± 0.374 ^{Bg}	21.0665 ± 0.702 ^{Acd}	23.358 ± 1.890 ^{Ade}
S. officinalis L.	11.763 ± 0.64 ^{Bg}	31.518 ± 1.243 ^{Ab}	**9.038 ± 2.46** $^{\mathbf{Bh,*}}$
R. officinalis L.	11.995 ± 0.92 ^{Bfg}	20.5865 ± 2.112 ^{Ad}	14.574 ± 0.426 ^{Bg}
P. anisum. L.	18.898 ± 0.836 ^{Ac}	21.2095 ± 0.555 ^{Acd}	21.592 ± 2.595 ^{Ae}
C. sativum L.	**37.494 ± 1.298** $^{\mathbf{Ba}}$	**42.774 ± 2.483** $^{\mathbf{Aa}}$	26.411 ± 1.394 ^{Cd}
L. officinale W. D. J. Koch.	16.942 ± 0.328 ^{Bd}	15.912 ± 2.356 ^{Be}	34.523 ± 1.204 ^{Ac}
M. chamomilla L.	**9.336 ± 0.85** $^{\mathbf{Ch,*}}$	14.438 ± 2.063 ^{Bef}	18.076 ± 0.949 ^{Af}
E. amoenum Fisch & C.A. Mey .	13.787 ± 1.187 ^{Bef}	11.846 ± 0.801 ^{Bf}	40.92067 ± 3.034 ^{Ab}
A. officinalis L.	25.047 ± 0.63 ^{Bb}	**10.5441 ± 0.240** $^{\mathbf{Cf,*}}$	**69.744 ± 2.800** $^{\mathbf{Aa}}$
E. angustifolia DC.	15.223 ± 1.014 ^{Bde}	24.246 ± 0.602 ^{Ac}	17.579 ± 1.055 ^{Bfg}
Positive control	0.57 ± 0.04^{A}	0.68 ± 0.06^{B}	0.89 ± 0.05^{C}

Values have been presented as a mean of two separate experiments using duplicate samples. *ND represents none detected in ≤10 mg. mL^{-1}concentration. (Different small letters in each column and bold letters in each row demonstrate significant difference). * Expresses the strongest plant.

cold infusion showed higher *anti-bacterial* properties, probably due to the wooden and tight structure of *P. anisum* L., (seeds), showing that hot infusion is not able to extract compounds with *anti-bacterial* within properties within just 5 minutes, and in the second plant, might have been expected, hot infusions confirmed the hypothesis in which hot water within just 5 minutes could extract *anti-bacterial* compounds from soft cells (leaves) higher than cold water even for a 2-hour extraction term. Moreover, among bacterial species, *E. faecalis* has shown more susceptibility. As it was expected, the gram-positive bacterial species such as *E. faecalis*, are not as

Table 7.3 Some categorized *anti-Alzheimer's* disease recorded ingredients in medicinal plants.

No	**Classification**	**Components**	**References**
1	*Alkaloids*	*Tetrandrine* *Berberine* *Huperzine A*	27 28 27
2	*Flavonoids*	*Icariin* *Breviscapine*	29 30
3	*Ketons*	*Tanshinone II A* *Turmerone*	31
4	*Polyphenols*	*Resveratrol* *Paeonol* *Salidorside*	32 33 34
5	*Polysaccharides*	*Fucoidan*	35
6	*Saponins*	*Caltrop saponins* *Seed saponin* *Gypenosides*	36 37 38
7	Others	*Inulicin* *Ginkgolides*	39 40, 41

resistant as the gram negative bacterial species such as *E. coli* in this study. The achieved results by microdilution assay are presented in Table 7.5, which are in line of *disc diffusion assays'* results. *E. faecalis* appeared to be the most susceptible to infusions (*MIC<0*.156 mg. mL^{-1}), except for *E. angustifolia* DC., (*MIC*=ND or >10 mg. mL^{-1}). Hot infusions of *R. officinalis* L., and *M. chamomilla* L., showed *minimum inhibitory concentrations* which are equal with 5<*MIC*≤10 against *E. coli*.

Interestingly, these infusions did not present inhibitory activity against most of the gram positives bacteria (except in *E. faecalis*). Moreover, hot infusions of *R. officinalis* L., and *T. vulgaris* L., expressed *MIC*=10 mg. mL^{-1} against *E. faecium.* Gram-negative bacteria, as a result of it having one more external wall, might restrict the penetration of hydrophobic compounds with *anti-bacterial* properties such as essential oils including *fatty acids*, *triterpenoids*, *phenols*, *flavonoids*, *sterols* etc. with detected *anti-microbial* activities. In other words, gram-negative bacteria might due to mocopolysaccharides and protein content in cell membranes involving higher amounts of phospholipids than gram-positive bacteria, leading to a decrease in

Table 7.4 *Disc propagation assay* (inhibition zone (diameter mm) against five selected bacteria species responsible for foodborne and wounds infections).

Plant species	**Extraction**	**Microorganisms**				
		S. aureus	***L. monocytogenes***	***E. faecium***	***E. coli***	***E. faecalis***
L. officinale W. D. J. Koch.	Hot Infusion	5.58 ± 0.144[a]	5.333 ± 0.321[a]	5.333 ± 0.152[a]	5.666 ± 0.288[a]	6.366 ± 0.230[a]
	Cold Infusion	5.5 ± 0.100[a]	5.566 ± 0.404[a]	5.533 ± 0.057[a]	5.833 ± 0.288[a]	7.5 ± 1.322[a]
P. anisum L.	Hot Infusion	5.75 ± 0.25[a]	**5.133 ± 0.057[a]**	6.166 ± 0.288[a]	5.866 ± 0.230[a]	6.416 ± 0.144[a]
	Cold Infusion	5.916 ± 0.144[a]	**5.733 ± 0.251[b]**	5.666 ± 0.381[a]	5.833 ± 0.152[a]	6.416 ± 0.381[a]
C. sativum L.	Hot Infusion	5.566 ± 0.404[a]	5.633 ± 0321[a]	5.5 ± 0.100[a]	5.833 ± 0.288[a]	8 ± 1.322[a]
	Cold Infusion	5.633 ± 0.321[a]	5.666 ± 0.288[a]	5.16 ± 0.288[a]	5.816 ± 0.317[a]	6.583 ± 0.381[a]
M. chamomilla L.	Hot Infusion	5.66 ± 0.288[a]	5.5 ± 0.500[a]	5.2 ± 0.100[a]	5.666 ± 0.288[a]	5.933 ± 0.115[a]
	Cold Infusion	5.666 ± 0.288[a]	5.5 ± 0.500[a]	5.083 ± 0.144[b]	5.666 ± 0.305[a]	5.7 ± 0.264[a]
A. officinalis L.	Hot Infusion	5.466 ± 0.057[a]	5.666 ± 0.305[a]	5.583 ± 0.381[a]	5.666 ± 0.305[a]	6.5 ± 0.0.866[a]
	Cold Infusion	5.75 ± 0.661[a]	5.416 ± 0.144[a]	5.75 ± 0.250[a]	5.5 ± 0.100[a]	6.65 ± 0.917[a]
E. angustifolia DC.	Hot Infusion	5.666 ± 0.288[a]	5.166 ± 0.115[a]	5.53 ± 0.057[a]	5.833 ± 0.288[a]	6.366 ± 0.230[a]
	Cold Infusion	5.666 ± 0.381[a]	5.416 ± 0.144[a]	5.73 ± 0.251[a]	5.333 ± 0.152[a]	7.5 ± 1.322[a]
E. amoenum Fisch & C.A. Mey.	Hot Infusion	5.5 ± 0.300[a]	5.2 ± 0.100[a]	5.4 ± 0.100[a]	5.83 ± 0.288[a]	7.366 ± 0.321[a]
	Cold Infusion	5.633 ± 0.321[a]	5.566 ± 0.404[a]	5.666 ± 0.288[a]	5.433 ± 0.208[a]	6.75 ± 0.433[a]
S. officinalis L.	Hot Infusion	5.25 ± 0.250[a]	5.8 ± 0.100[a]	5.7 ± 0.300[a]	5.5 ± 0.100[a]	8.066 ± 1.677[a]
	Cold Infusion	5.4 ± 0.264[a]	5.75 ± 0.250[a]	5.966 ± 0.057[a]	5.833 ± 0.288[a]	6.566 ± 1.289[a]
R. officinalis L.	Hot Infusion	5.666 ± 0.288[a]	6.083 ± 0.144[a]	5.7 ± 0.264[a]	5.5 ± 0.100[a]	6.75 ± 0.661[a]
	Cold Infusion	5.366 ± 0.152[a]	5.5 ± 0.435[a]	6.166 ± 0.288[a]	5.583 ± 0.144[a]	7.25 ± 1.089[a]
T. vulgaris L.	Hot Infusion	5.66 ± 0.305[a]	5.666 ± 0.288[a]	**5.533 ± 0.057[a]**	5.866 ± 0.115[a]	6.833 ± 0.288[a]
	Cold Infusion	5.666 ± 0.288[a]	5.333 ± 0.577[a]	**5.2 ± 0.100[b]**	5.866 ± 0.230[a]	6.083 ± 0.629[a]

Values represent the mean ± *SD* of three separate experiments using triplicate samples. The different letters in each column for each bacteria specieis are demonstrating the significant difference ($p<0.05$) between results of hot and cold infusions of each plant infusion against each bacterial species.

Table 7.5 Results of *Minimum inhibition concentration* (*MIC*) of the selected herbal plants infusions (The tested range of ≤10 mg. mL^{-1} concentration of the extracts) against five selected bacterial species responsible for foodborne and wounds infections (mg. mL^{-1}).

Plant species	**Extraction**	**Microorganisms**				
		S. aureus	***L. monocytogenes***	***E. faecium***	***E. coli***	***E. faecalis***
L. officinale W. D. J. Koch.	Hot Infusion	ND or >10	ND or >10	ND or >10	ND or >10	<0.156
	Cold Infusion	ND or >10	ND or >10	ND or >10	ND or >10	<0.156
P. anisum L.	Hot Infusion	ND or >10	ND or >10	ND or >10	ND or >10	<0.156
	Cold Infusion	ND or >10	ND or >10	ND or >10	ND or >10	<0.156
C. sativum L.	Hot Infusion	ND or >10	ND or >10	ND or >10	ND or >10	<0.156
	Cold Infusion	≥10	ND or >10	ND or >10	ND or >10	<0.156
M. chamomilla L.	Hot Infusion	ND or >10	ND or >10	ND or >10	**5<MIC ≤10**	<0.156
	Cold Infusion	ND or >10	ND or >10	ND or >10	ND or >10	<0.156
A. officinalis L.	Hot Infusion	ND or >10	ND or >10	ND or >10	ND or >10	<0.156
	Cold Infusion	ND or >10	ND or >10	ND or >10	ND or >10	<0.156
E. angustifolia DC.	Hot Infusion	ND or >10	ND or >10	ND or >10	ND or >10	**ND or >10**
	Cold Infusion	ND or >10	ND or >10	ND or >10	ND or >10	**ND or >10**
E. amoenum Fisch & C.A. Mey.	Hot Infusion	ND or >10	ND or >10	ND or >10	ND or >10	<0.156
	Cold Infusion	ND or >10	ND or >10	>10	ND or >10	<0.156
S. officinalis L.	Hot Infusion	ND or >10	ND or >10	ND or >10	ND or >10	<0.156
	Cold Infusion	ND or >10	ND or >10	ND or >10	ND or >10	<0.156
R. officinalis L.	Hot Infusion	ND or >10	ND or >10	**=10**	**5< MIC ≤10**	<0.156
	Cold Infusion	ND or >10	ND or >10	ND or >10	ND or >10	<0.156
T. vulgaris L.	Hot Infusion	ND or >10	ND or >10	**=10**	ND or >10	<0.156
	Cold Infusion	ND or >10	ND or >10	ND or >10	ND or >10	<0.156

Values have been presented as a mean of two separate experiments using duplicate samples. *ND represents not detected in ≤10 mg. mL^{-1}concentration.

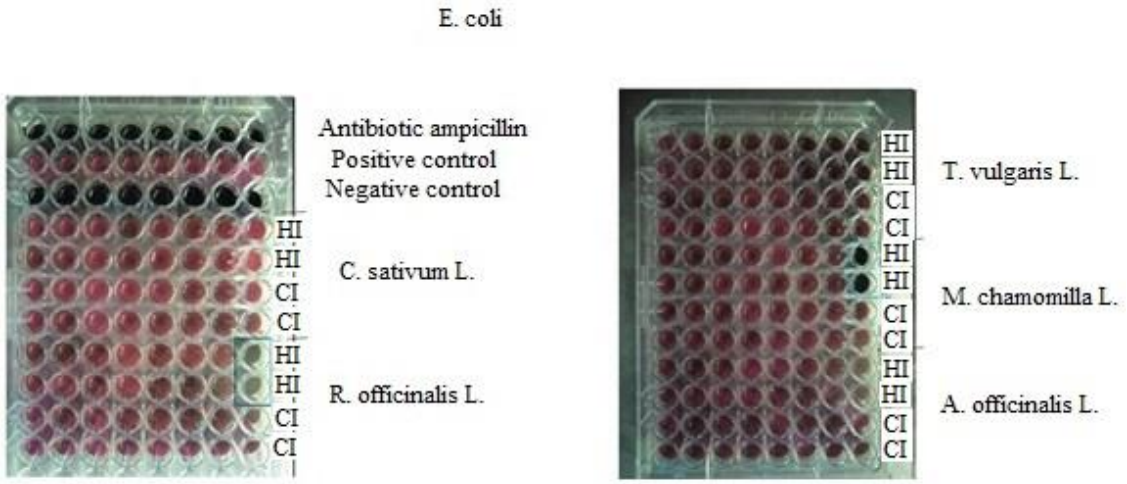


Figure 7.2 *Microdilution assay* results of some plants infusions against *E. coli.*

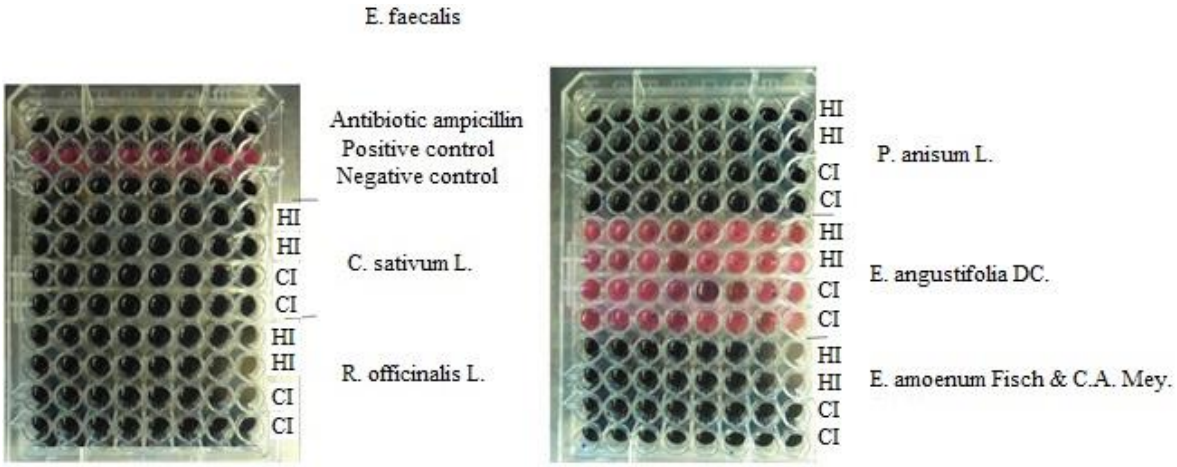


Figure 7.3 *Microdilution assay* results of some plants infusions against *E. faecalis.*

permeability, entrance and reaction of *anti-biotic* and *anti-microbial* compounds with cytoplasmic contents.

Bioactive compounds with various mechanisms showed *anti-microbial* effects such as (1) the destruction of cell membranes (2) association with membrane proteins like *ATPase* (3) interfering in the performance of the cell membrane, particularly in gram negative bacteria through the release of membrane lipo-polysaccharides and (4) interfering in enzyme generation, proton and electron flow through the membrane and coagulation of cells' internal contents. *R. officinalis* L., and *T. vulgaris* L., as well as *M. chamomilla* L., infusions, revealed restricted inhibitory activities in the standard concentration range ($\leq$10 mg. mL^{-1}). The widespread range of characterised compounds in leaves and seeds might declare the eventual synergistic and or antagonistic activities between compounds in presenting *anti-bacterial* properties. To summarise, the tested plants might be used in the standard range concentration and/or

higher in nutraceutical and pharmaceutical products as natural valuable preservatives or additives.

7.3.4. GC-MS analysis

The chromatograms obtained are presented in Figs. 7.4, 7.5. The outcomes obtained by scientists have stated that phytochemicals have the potential to prevent or delay different chronic disorders. For instance, *mono acylglycerols* (*MAG*), which were found in considerable quantity in the seeds in this study (see Table 7.6), are active *anti-microbial* and chemotherapy agents. For instance, these potential health benefits involve *anti-tumour* activity by triggering death in various human *leukemic cell lines*. Health benefits were not reported from *triacylglycerol* (*TAG*) and *diacylglycerol* (*DAG*). Many health potential effects are attributed to *octadecadienoic acid* or *conjugated linoleic acid* (*CLA*) as are found in this research; including *anti-diabetic* and *anti-obesity* properties, *anti-carcinogenesis*, *anti-atherosclerosis*, *anti-inflammation* as well as growth promoting properties such as bone formation-promoting properties [42].

CLA isomers and either c9, *t11-CLA* or *t10*, *c12-CLA* isomers in special concentrations exhibited inhibition against induced tumours, including mammary glands; colon; forestomach neoplasia and the metastasis of inoculated cancer cells. The *anti-diabetic* impact of *CLA* is attributed to the *PPATγ* activation pathway, which could enhance the adiponectin level in plasma, and finally leads to ameliorate *hyperinsulinemia*. *Maslinic acid* (*MA*), as a main compound of *R. officinalis* L., infusion, demonstrated *in vivo anti-diabetic* potential in mice [45].

The use of *maslinic acid* in *streptozotocin (STZ)-induced hyperglycaemic mice* diminished the expression of glucose transporters such as *SGLT1* and *GLUT2*, this pathway is similarly found in further hypoglycaemia medicines used commonly today including insulin and metformin which contributed to diminishing plasma glucose level [43]. Regarding the findings of previous researches, plants rich in *maslinic acid* (*R. officinalis* L.) might be full of advantages in the treatment of diseases such as diabetes. *β-sitosterol* found in trace quantity in the analysed plants using *GC-MS* analysis in this research, except *T. vulgaris* L., has indicated *anti-diabetic* potential, along with *anti-atherogenic* and *anti-asthmatic* properties. This compound also presented selective toxic effects against cancer cells although causing no damage to normal cells.

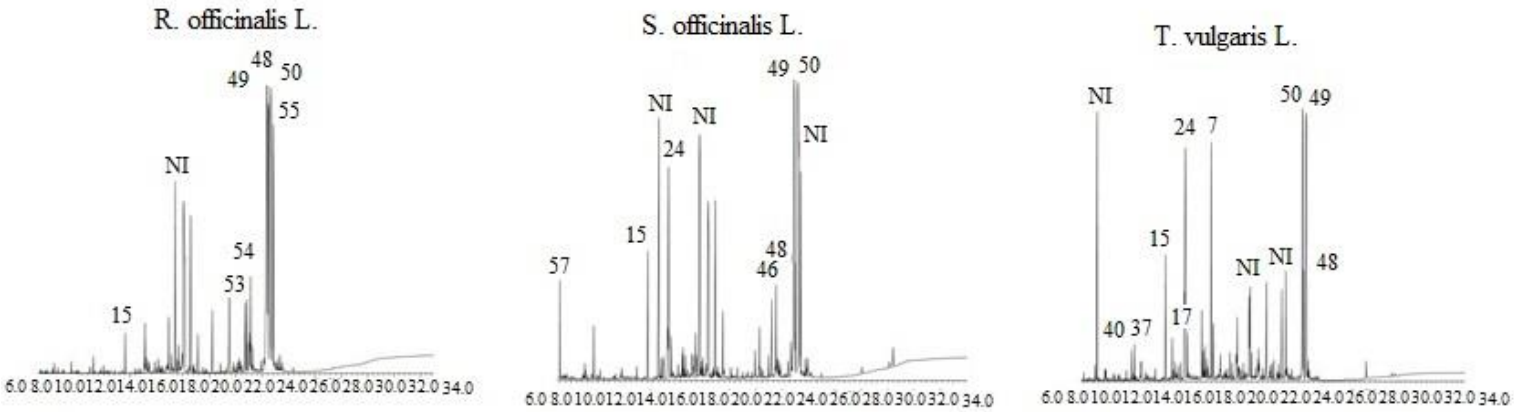


Figure 7.4 Obtained chromatograms of the leaves (NI: Not identified).

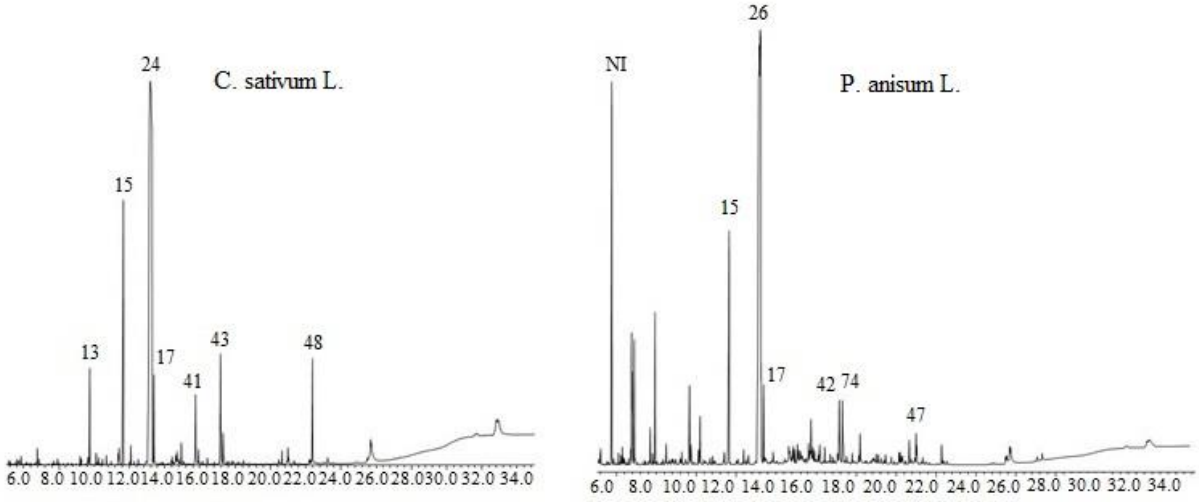


Figure 7.5 Obtained chromatograms of two seeds (NI: Not identified).

Table 7.6 Chemical composition of extracts of some selected plants (three leaves and two seeds species) obtained by *GC-MS*.

No	Compounds and constituent	*Tested Plant species*				
		***P. anisum* L.**	***R. officinalis* L.**	***S. officinalis* L.**	***T. vulgaris* L.**	***C. sativum* L.**
	Sugars	**2.14**	**0.09**	**0.57**	**7.61**	**0.72**
1	*Sugar in C_5*	0.28	-	0.07	0.51	0.33
2	*Sugar in C_6*	0.67	0.09	-	0.24	0.32
3	*Fructose*	0.48	-	-	-	0.07
4	*Pentitol*	0.25	-	-	-	-
5	*Turanose*	0.21	-	-	0.69	-
6	*Erythrodiol*	-	-	0.5	-	-
7	*NI sugar dimer*	0.25	-	-	6.17	-
8	***Alkanes***	**1.08**	**5.27**	**2.36**	**5.51**	**0.15**
	Fatty acids	**49.04**	**2.44**	**8.97**	**16.78**	**80.22**
9	*Nonanoic acid* (C9:0)	-	-	-	-	0.13
10	*Decanoic acid* (C10:0)	0.05	0.05	-	0.11	0.14
11	*Dodecanoic acid* (C12:0)	-	-	-	-	0.13
12	*Tridecanoic acid* (C13:0)	-	-	-	-	0.12
13	*Tetradecanoic acid* (C14:0)	-	0.11	-	-	2.38
14	*Pentadecanoic acid* (C15:0)	0.21	-	0.07	0.14	0.22
15	*Hexadecanoic acid* (C16:0)	5.93	0.56	2.14	2.92	9.03
16	*Heptadecanoic acid* (C17:0)	0.11	-	-	-	0.19
17	*Octadecanoic acid* (C:18:0)	1.4	0.26	0.64	1.04	2.33
18	*Nonadecanoic acid* (C:19:0)	-	-	-	-	0.04
19	*Eicosanoic acid* (C20:0)	0.31	-	0.2	0.44	0.55
20	*Docosanoic acid* (C22:0)	0.34	-	-	0.43	0.25
21	*Tetracosanoic acid* (C24:0)	0.27	0.03	0.06	0.19	0.25
22	*Hexacosanoic acid* (C26:0)	-	-	0.07	-	0.13
23	*Hexadecenoic acid* C(16:1)	0.09	-	-	-	0.81
24	*Octadecenoic acid* (C18:1)	0.44	1.28	4.98	11.51	62.46
25	*Heptadecanoic acid* (C17:1)	-	-	-	-	0.14

Table 7.6 Continued

26	*9,12-Octadecadienoic acid* (C18:2)	39.89	0.15	0.81	-	0.42
27	*Eicosenoic acid* (C22:1)	-	-	-	-	0.5
	α-Hydroxy fatty acids	**0.62**	**-**	**0.02**	**-**	-
28	*α-Hydroxy acid in C_8*	-	-	0.02	-	-
29	*α-Hydroxy acid in C_{16}*	0.18	-	-	-	-
30	*α-Hydroxy acid in C_{28}*	0.29	-	-	-	-
31	*α-Hydroxy acid in C_{30}*	0.15	-	-	-	-
	Fatty alcohols	**0.64**	**0.14**	**0.44**	**0.64**	**-**
32	*1-octadecanol*	0.29	-	0.27	0.37	-
33	*Octadec-9-enol*	0.35	0.09	-	-	-
34	*1-hexadecanol*	-	0.05	0.17	0.27	-
	Di-Acids	**0.43**	**0.03**	**0.11**	**0.64**	**0.13**
35	*Malic acid*	0.18	-	0.04	0.24	-
36	*Azelaic acid*	0.25	0.03	0.07	0.08	0.13
37	*Citric acid*	-	-	-	0.32	-
	Waxes	**1.79**	**0.06**	**0.15**	**1.65**	**6.4**
38	*Wax C_{12}*	-		0.14	-	-
39	*Wax C_{13}*	-	0.06	0.01	-	-
40	*Wax C_{16}*	-	-	-	0.6	-
41	*Wax C_{28}*	-	-	-	-	2.25
42	*Wax C_{34}*	1.79	-	-	1.05	-
43	*Wax C_{36}*	-	-	-	-	4.15
	Sterols and Triterpenoids	**2.07**	**63.42**	**47.64**	**33.51**	**4.57**
44	*Campesterol*	0.1	-	0.34	-	0.23
45	*Stigmasterol*	0.48	-	-	-	0.39
46	*β-Sitosterol*	0.61	0.7	1.51	-	0.81
47	*β-Amyrin*	0.30	0.9	0.25	-	-
48	*Betulinic acid*	0.08	13.48	2.01	1.66	3.00

Table 7.6 Continued

49	*Oleanolic acid*	0.38	16.65	14.14	15.27	0.14
50	*Ursolic acid*	0.12	21.86	22.34	16.00	-
51	*Lupeol*	-	-	0.5	-	-
52	*Squalene*	-	0.06	0.16	0.36	-
53	*Olean*-12-ene, 3-one	-	1.33	-	-	-
54	*Sterol* NI	-	1.95	4.57	-	-
55	*Maslinic acid*	-	6.38	0.31	-	-
56	*Corosolic acid*	-	-	0.35	-	-
57	*Borneol*	-	-	1.13	-	-
58	*Caryophyllene*	-	0.06	0.03	0.05	-
59	*Caryophyllene oxide*	-	0.05	-	0.17	-
	Aromatics	**1.08**	**0.57**	**0.12**	**6.41**	**0.22**
60	*Benzoic acid, 2-hydroxy*	-	-	0.04	-	-
61	*Benzoic acid, 4-methoxy*	0.28	-	-	-	0.04
62	*Benzoic acid, 3-methoxy-4-hydroxy*	-	0.19	-	-	-
63	*Benzaldehyde, 3-methoxy-4-hydroxy*	-	-	-	-	0.02
64	*Vanil Ethanediol*	0.19	-	-	6.3	-
65	*Benzeneacetic acid*	0.61	0.08	0.08	-	-
66	*2,6- Dihydroxyacetophenone*	-	-	-	0.11	-
67	*Benzeneacetic acid, 2-methoxy-α-hydroxy*	-	-	-	-	0.16
68	*tert-Butylhydroquinone*	-	0.14	-	-	-
69	*Syringic acid*	-	0.06	-	-	-
70	*P-coumaryl alcohol*	-	0.1	-	-	-
	Monoacylglycerols	**2.16**				**1.34**
71	*1-Monoglycerol in C16*	-	-	-	-	0.11
72	*1-Monoglycerol in C16:1*	-	-	-	-	1.13
73	*1-Monoglycerol in C18*	1.86	-	-	-	0.1

Table 7.6 Continued

74	*1-Monoglycerol in C21*	0.3	-	-	-	-
	Others		**0.22**		**0.14**	**0.27**
75	*Quinic acid*	-	0.06	-	0.14	-
76	*Xylonic acid*	-	-	-	-	0.05
77	*Azulene*	-	-	-	-	0.22
78	*3-Trimethylsilyloxystearic acid*	-	0.16	-	-	-
	Identified	**61.1**	**72.2**	**60.4**	**72.9**	**94**
	Unidentified	**39.0**	**27.8**	**39.6**	**27.1**	**6.0**

Among the wide range of characterised bioactive compounds of the five selected plants by *GC-MS* analysis (Table 7.6), *fatty acids* and *triterpenoids*, with *sterols*, were found in higher amounts. The detected *fatty acid* content in the seeds (*C. sativum* L., and *P. anisum* L.) were found in higher quantities than leaves, while the *sterol* and *triterpenoid* contents in the leaves were detected in higher quantities. Among the detected free *fatty acids* in the plants, *palmitic* (*hexadecanoic acid* (C16:0)); *stearic acid* (*octadecanoic acid* (C18:0)); *oleic acid* (*octadecenoic acid* (C18:1)); and *linoleic acid* (*9, 12-Octadecadienoic acid* (C18:2)) were the main compounds regarding plants species. Among the detected *sterols* and *triterpenoids β-sitosterol*; *betulinic acid*; *oleanolic acid*; *ursolic acid*; *maslinic acid;* and *borneol* were found in considerable amounts compared to the other detected *triterpenoids* (Table 7.6). Most of those specified compounds have recorded many health benefits.

Most of the potential health benefits of coriander or *C. sativum* L., are attributed to its *free fatty* acid contents. As is observed in Table 7.6, *C. sativum* L., demonstrated the highest contents of *fatty acids* compared to further examined plants (80.22%). of which *octadecenoic* (*oleic acid*: 62.46%); *hexadecanoic* (*palmitic acid*: 9.03%); and *octadecanoic* (*stearic acid*: 2.33%), are the most predominant *free fatty acids* respectively. *C. sativum* L., however has demonstrated the lowest activity against diabetes in this study. Several potential health benefits might be caused by *fatty acids*. It is recorded that *oleic acid* increases the absorption of *mitoxantrone* (*MXR* "is a model substance for *BCRP* transport"), causing an enhanced gene expression of *BCRP* in *Caco-2 cancer cell lines*. Moreover, *oleic acid* plays a substantial role in lowering *low-density lipoprotein* (*LDL*) content and blood pressure. Along with the aforementioned advantages, coriander was reported to contain the *fatty acid* desaturase, which could promote saturated *fatty acids* to *monounsaturated fatty acids* (*MUFA*) or lead to the production of di and/or *polyunsaturated fatty acids*. Therefore, this crop with decreasing fat contents and increased *mono* and *poly unsaturated fatty acids* (*PUFA*) contents may lead to higher insulin sensitivity within the cells.

Unlike *C. sativum* L., *P. anisum* L., is also higher in *9, 12-octadecadienoic* (*linoleic acid*) content, of which a wide spread of potential health benefits are associated with this free *fatty acid*. Coriander contains noticeable amounts of *betulinic acid* (*BA*: 3%). *Betulinic acid,* along with *betulin,* presented a substantial

role in the prevention of *HIV*; *cancers* and *inflammation*. Among the different derivatives of *betulinic acid*, "*bevirimat 3-O-(3', 3'-dimethylsuccinyl)*" exhibited remarkable *anti-HIV* properties [44].

Different types of phytochemicals, particularly *triterpenoids* and further secondary metabolites could present *anti-diabetic* activity. On the other hand, *R. officinalis* L., contains the lowest fatty acid content and is the highest in *triterpenoids* and *sterols*. *S. officinalis* L., and *T. vulgaris* L., contain 22.34% and 16% *ursolic acid* (*UA*) content of the whole lipophilic compound content respectively. *UA* is a substantial *terpenoid* and has presented a wide range of health advantages including *anti-diabetic*; *hepatoprotective*; immunomodulatory; *anti-inflammatory*; *anti-bacterial*; *anti-viral* as well as *anti-cancer* and *anti-ulcer* properties. The multifunctional *anti-cancer* activities of *UA* have attracted increasing attention. *UA*, due to its abundant natural supply of common resources and inexpensive accessibility, might be considered as an attractive source in the treatment of diabetes. *Oleanolic acid* (*OA*), with contents of 15.27 and 14.14% respectively in *T. vulgaris* L., and *S. officinalis* L., is the other highest detected *triterpenoid*. Along with *UA* and *OA*, further *triterpenoids* including *betulinic*, *18β-glycyrrhetinic*, *maslinic* and *corosolic* acids are detected with *anti-diabetic* potential in previous researches. *OA*, due to having a protective impact on *mitochondrial ROS* production and oxidative stress, is proposed to possibly act as an *anti-diabetic* agent. *ROS* injures mitochondria using *DNA* fragmentation, cross linking with protein content, peroxidation of membrane phospholipids and final stress pathways, resulting in insulin resistance, ruined pancreatic *β-cells* and *diabetic* compilation. As is shown, *R. officinalis* L., along with *T. vulgaris* L., and *S. officinalis* L., contains higher quantities of *oleanolic* and *ursolic acids*, also *R. officinalis* L., showed a considerable amount of *betulinic* and *maslinic acids* (13.48 and 6.38% respectively), unlike *T. vulgaris* L., and *S. officinalis* L., leaves (Table 7.6). However, it contains lower quantities of *fatty acids* compared to other leaves. The substantial role of *triterpenoids* in demonstrating enzyme inhibition therefore is revealed. The *in vivo hypoglycaemia* potential of *maslinic acid* is established by [45], however the mechanism of action is not well known. Along with the *anti-diabetic* activity of *maslinic acid*, the *anti-tumour* and *anti-oxidant* potential of it have attracted much more attention. *In vivo* tests have revealed that *maslinic acid* might decrease lipid profile levels of serum with a significant reduction in total *cholesterol*, *triglycerides*,

LDL-cholesterol levels and the enhancement of *HDL-cholesterol* content. Moreover, the *hypoglycaemic* properties of *betulinic acid* and *β-sitosterol* are accepted. Regarding the detection of the highest amount of *triterpenoids* and the minimum value of *fatty acids* in *R. officinalis* L., and its inhibitive effects on *α-Amylase* and *α-Glucosidase* as well as *AChE*, the hypothesis based on the synergistic effects of *triterpenoids* and *fatty acids* contents in demonstrating health potentials could be highlighted. The tested seeds show *C. sativum* L., and *P. anisum* L., indicated 80.22 and 49.04% *fatty acid* contents of all lipophilic characterised compounds. In *C. sativum* L., *oleic* (*octadecenoic acid (C18:1)*), *palmitic* (*hexadecanoic acid* (C16:0)) and *stearic* (*octadecanoic acid (C18:0)*) showing 62.46, 9.03 and 2.38%, and in *P. Anisum* L., *linoleic acid* (*9, 12-octadecadienoic acid (C18:2)*) and *palmitic acid* (*hexadecanoic acid (C16:0)*) demonstrating 39.89 and 5.93% of the whole identified lipophilic compounds, respectively, are the most dominant compounds. Coriander (*C. sativum* L.) seeds demonstrate an extensive confine of potential health benefits including *anti-oxidant*; *anti-Alzheimer's*; *antibiotic*; *hypoglycaemia*; blood cholesterol lowering; *anti-carcinogenic* etc. Coriander showed the lowest quantity of aromatic compounds (0.22%), and the existence of aromatic compounds, whilst enhancing the bioactivity of the plants; it does however make those plants inappropriate for application in nutraceutical products. Unlike coriander, *T. vulgaris* L., presented the highest value for aromatic compounds (6.41%). The most common use of coriander is as a *bactericidal*, *fungicidal* agent in folk medicines. However, coriander has demonstrated the weakest *α-Amylase* and *α-Glucosidase* inhibitive potential in comparison to other tested plants. *P. anisum* L., contains the highest value of *linoleic acid* (*CLA*). It is effective against an extensive range of disorders including *atherosclerosis*; *inflammation*; *obesity* and *diabetes* [42]. *CLA* is useful in improving syndromes by exerting *anti-obesity*; *anti-diabetic* and *anti-hypertensive* effects. Among *sterols*, *β-sitosterol* was detected in trace amounts in most of the tested plants. The *hypoglycaemic* properties of *β-sitosterol* might be attributed to its stimulation of glycogen-synthase and the reduction of phosphorylase activities, or it could be due to its inhibitive potential to affect renal glucose absorption, which reduces the sugar content of blood. In relation to the treatment of patients suffering from *diabetes*, scientists discovered that the *anti-oxidant* concentration in the blood of patients treated with natural *anti-diabetic* medicines was reduced, indicating a substantial correlation between the *anti-oxidant* and hypoglycaemia activities of

plants [46]. It should be cited that the bioactive compounds of *S. officinalis* L., could block the release of glucose from the liver. On the other hand, there is a direct relationship between the *cholesterol* content of the blood and *AD*. *S. officinalis* L., along with demonstrating the highest inhibitive activity against *AChE,* also expressed higher inhibitive activity against *α-Glucosidase*. *S. officinalis* L., might considerably lower the glucose and cholesterol levels of blood. It could be observed that this valuable plant might prevent *type II diabetes* and reduce fasting glucose levels in *diabetic* patients, and is also an appropriate *AChE* inhibitor. *Sterols* and *triterpenoids* comprise the main profile of bioactive compounds in sage, demonstrating 47.64% of all detected bioactive compounds, followed by *fatty acids* with almost 8.97%. *R. officinalis* L., involves the highest quantity of *sterols* and *triterpenoids* and the lowest value of *fatty acids* compared to further studied plants demonstrating 63.42% and 2.44% respectively. In this study, this plant presented higher activity against eventual enzymes contributing to the generation of diabetic and *Alzheimer's diseases*. It is established that the bioactive compounds available in rosemary infusion may have *anti-inflammatory* activity, along with enhanced blood circulation and immunity as well as *free radical-suppressing* activities. It can also be suggested that *R. officinalis* L., improves the cognitive performance of the *central nervous system*. *Carnosic acid,* which has been detected in other researches as the main compound in rosemary, might have a substantial role against ageing. Moreover, *anti-coagulant, AChE* inhibitory and diuretic potential are attributed to this plant. Among selected leaves, *R. officinalis* L, presented the highest inhibitive properties against *α-Amylase*. *T. vulgaris* L., is another leaf in this research which presented high inhibition activity against the three applied enzymes which contribute to the development of *diabetes* and *Alzheimer's diseases*. This plant demonstrates a reduction in the resistance of different bacterial species against common used *anti-biotics* such as penicillin. Moreover, the *anti-acne* properties of thyme are attributed to its effects against bacterial species which cause acne. Interestingly, thyme proposed higher activity than *benzoyl peroxide* in standard concentrations against bacterial species. Scientists recorded that thymol has *anti-microbial* potential which 25-fold higher than *phenols*, although being less toxic [47].

E. angustifolia DC., presented the weakest activity against *E. faecalis,* not only among flowers but also among the all tested plants. *M. chamomilla* L., also showed behaviour similar to *R. officinalis.* L against *E. coli* but stronger than of the rest of

plants in this study. *M. chamomilla* L., was reported to contain a wide range of bioactive compounds categorised in various classes including *flavonoids*, *coumarins*, and *polyacetylenes*. Several *flavonoids* and *phenolic* compounds are detected in *M. chamomilla* L., flowers. Major *flavonoids* found in *M. chamomilla* L., flowers include *apigenin, quercetin, patuletin* and *luteolin* in order of largest quantity [48]. *Cinnamic acid* derivatives, *ferulic* and *caffeic acids,* along with further unidentified *phenolic* compounds were determined in the flowers of this plant [49]. The essential oils produced from *M. chamomilla* L., exhibited inhibitive properties against some bacterial species, fungus and viruses. *M. chamomilla* L., oils presented slightly higher inhibitive activity against different gram positive and gram negative bacterial species than *Chamaemelum nobile* L. *(*Roman chamomile*),* moreover, the *anti-fungal* activity of this plants' infusion is higher than other infusions achieved from similar plants species. Other potential health benefits of *M. chamomilla* L., were reported, including *anti-platelet*; chemo-preventive; *anti-inflammatory*; *anti-genotoxic*; *hypocholesterolemic*; *cardiac*; *gastrointestinal*; *hepatic* and *central nervous system* protective properties. The detected bioactive compounds in *M. chamomilla* L., such as *flavonoids* in a special dose reduced the accumulated ceramide contents in the liver generated by ageing. The ethanoic extract of *M. chamomilla* L., presented *hypoglycaemic* activity in controlling the blood's glucose level. The results obtained in this research confirmed that this plant could present higher activity against enzymes attributed to revealing disorders such as *diabetes* and even *Alzheimer's*. *Reactive oxygen radicals* (*ROS*), as well as further free radicals are attributed to brain injuries that finally result in *AD*. The delivery of free radicals in cells' environment causes damage to cell membranes, enzymes, *DNA*, lipids and proteins structures, and as a result, impairs their functions. The obtained data from our previous research showed that the aqueous infusion of *M. chamomilla* L., indicating *IC50* equals with 452.215 ± 21.309 µg. mL^{-1} shows the highest capability in the inhibition of *ABTS* free radicals compared to the other flowers tested in this study. This phenomenon is in line with the gathered data for the *AChE* inhibition of the same plants. Moreover, *ROS* might not only cause cognitive issues, but also leads to further disorders including *diabetes*, *atherosclerosis*, *cardiovascular*, *neurological* diseases as well as *cancers*.

Most of the potential health benefits of herbs might belong to the *anti-radical* activities of compounds that they consist of Harmful free radicals, including *reactive*

oxygen species (*ROS*), *nitrogen* (*RNS*) and *chlorine* (*RCS*) are attributed to the damaging of cell membranes and interfering in normal cells' metabolism. These free radicals might attach to enzymes participating in cell metabolism and/or bind to enzyme receptors, and change their 3D structures leading to a pause in the required reactions of the cells. Nowadays, free radicals get more attention from scientists due to their eventual role in providing appropriate conditions for the growth of *carcinogenic cells*, *diabetes* and *Alzheimer's disease*, *DNA* nicking and mutation as well as further neural disorders such as *Parkinson's* in health cells. Fig. 7.6 shows 2D structures of some of the major detected compounds of the analysed plants.

7.4. Conclusions

In this study, which included a wide range of performed tests on the selected plants' species, inhibition activities against eventual enzymes attributed to *diabetes* and *Alzheimer's diseases*, as well as *anti-bacterial* effects, were shown. The eventual *anti-diabetic* activity was found in the tested plants, of which *C. sativum* L., (seed) showed the weakest inhibitive effect. All of the plants also presented eventual *anti-Alzheimer's* activities, in which *A. officinalis* L., (flower) showed the lowest activity. Regarding the obtained data from the *GC-MS* analysis performed on half of the samples, it is found that a mix of *sterols* with *triterpenoids* might show higher activities against tested enzymes than free *fatty acids*. Interestingly, although the seeds contain higher essential oil content, they did not show significant *anti-bacterial* activities compared to leaves and flowers in the standard range of concentration. These facts could be justified through not only one or two categories of compounds together but also a mix of all available compounds in plants that might have synergistic or even antagonistic effects on each other in presenting potential health benefits. Except *T. vulgaris* L., all characterised plant infusions showed no considerable quantities of aromatic compounds, showing their potentials for use in functional foods with minimum alterations in organoleptic properties.

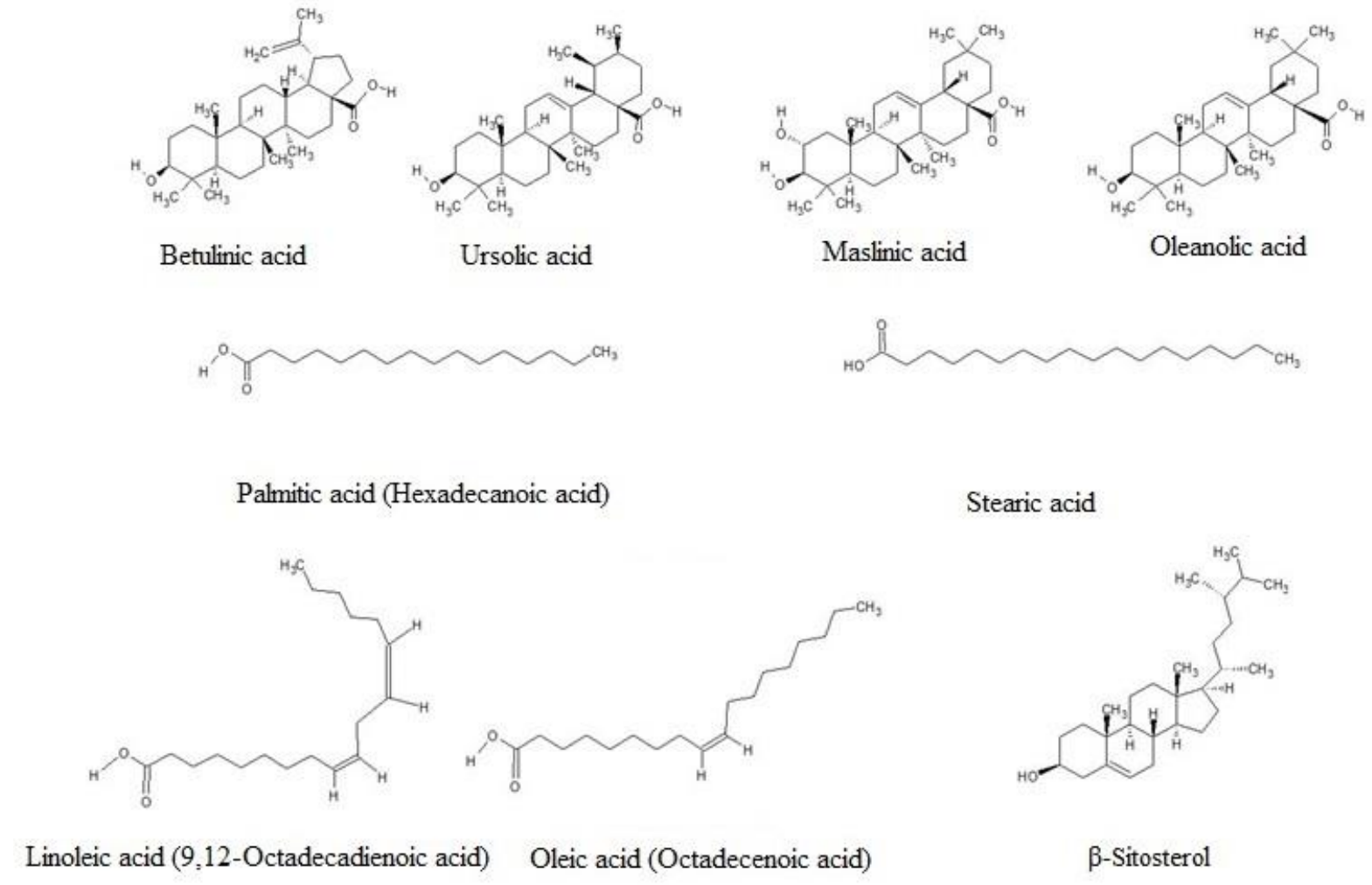


Figure 7.6 2D chemical structures of some compounds detected in this research (Drawn by *ChemSketch* software version 2012).

7.5.References

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CHAPTER 8

Modelling of *Microwave Assisted Extraction* (*MAE*[2]) of *Anthocyanin*s (TMA[3])

In the present chapter the optimized conditions of microwave-assisted extraction (MAE) of total monomeric anthocyanins (TMA) has been modelled by Box-Behnken design (BBD) with three independent variables. The optimized values for this extraction with the effect of each mode on the responses have been revealed.

[2] *Microwave Assisted Extraction*
[3] Total Monomeric *Anthocyanin*s

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Modelling of *Microwave Assisted Extraction* (*MAE*[4]) of *Anthocyanin*s (TMA[5])

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Submitted

Abstract

Box Behnken design (*BBD*) was applied to investigate the effectiveness of three independent variables. The quadratic effects of the independent variables on total monomeric *anthocyanin* and the quadratic modes of irradiation time and solvent/sample proportion on total *anti-oxidant* activity were identified as being negatively significant. Also, the interactive effect between microwave power and solvent/sample proportion was diagnosed negatively significant (p<0.05) on total *anti-oxidant* activity value. The highest Total monomeric *anthocyanins* (273.284 (mg. L^{-1})) value was obtained along the proportion of solvent/sample value equals to 30.321 (mL. g^{-1}), at microwave power of 464.876 W, within 114.281 s, the desirability was recorded as 0.878 at the optimum point. Meanwhile, the optimised status for total *anti-oxidant* activity (369.734 ($mg_{(AAE)}$. $g^{-1}{}_{dw}$)) was recorded along a 34.807 (mL. g^{-1}) proportion of solvent/sample, under microwave power of 300 W within 107.339 s. Desirability was obtained at 0.952. Achieving the higher *correlation coefficient* (R^2) between the experimental and predicted values of the responses confirmed the validity of the predicted design.

Keywords: *L. pedunculata* L. subsp. pedunculata.; *Box-Behnken design* (*BBD*); Monomeric *anthocyanins*; optimization

[4] *Microwave Assisted Extraction*
[5] Total Monomeric *Anthocyanins*

Practical application

Today, due to the high demand for crops to be used extensively in the human diet, enhancements in the efficiency of the processing are getting more attention. In this way, finding and/or the determination of the optimal conditions for processing with minimum waste looks very substantial. Therefore, the use of prediction methods in food processing is considered to be a tool for improving the efficiency and the quality of the produced products. In this regard, *Response Surface Methodology* (*RSM*) as a novel prediction tool, along with the *fuzzy logic design* and *Artificial Neural Network* (*ANN*) are applied extensively. *RSM* is optimized to predict the extraction conditions. Therefore predicting the extraction conditions of this crop against different selected parameters can improve the quality and performance of the products with the minimum wastes during very short time.

8.1. Introduction

Today, the trend for plant-derived compounds in the nutraceutical, pharmaceutical and cosmetic industries has enhanced, due to their perception by consumers as being relatively safe and healthy compared to those synthetic counterpoint components. The *Lavandula* species (*Lamiacease*) is one of the most reputable aromatic herbages and is extensively used in the food, perfume and pharmaceutical industries (Boelens, 1995; Kim and Lee, 2002). Various *Lavandula* species develop compounds with *anti-microbial* (Hanamanthagouda *et al*., 2010; Zuzarte *et al*., 2009), *anti-oxidant, anti-diabetic* and *anti-cholinesterase* (*anti-Alzheimer's*) potential (Costa *et al*., 2011; Costa *et al*., 2012; Matos *et al*., 2009). The mechanism of actions of the abovementioned health benefits have been described previously (Farzaneh and Carvalho, 2015). Aboriginal bioactive compounds could effectually protect cells against oxidative stress, due to their capabilities in acting as *free radical scavengers* and/or inhibitors, reducing agents, hydrogen donators and conduction metal chelators (Dai and Mumper, 2010). *Lavandula pedunculata* L. subsp. pedunculata., is an aromatic prevalent herb in the Iberian Peninsula and customarily applied in *Portugues* remedies and as a decorative herbage (Franco, 1984). Three subspecies for *Lavandula pedunculata* L. subsp. pedunculata., in northwest Portugal, subsp. Lusitanica in central and south Portugal and subsp. Sampaiana in north and central Portugal are used commonly in traditional medicines.

Infusions extracted from flowered aerial parts are customarily expended to treat *anxiety*, *insomnia*, *anorexia*, *bronchitis* and *coughs* (Proenca da Cunha *et al*., 2003).

Anthocyanin pigment content has a decretive impress in the hue evidence of many pristine and processed fruits, groceries as well as herbage products. Various aboriginal colourants in herbage and nutraceutical output products are *anthocyanin*-derived compounds (e.g., grape-peel, red-cabbage, purple-carrot and further herb infusions). In addition, there is a drastic concern around the *anthocyanin* content of food and nutraceuticals because of several conceivable known health advantages, such as declining coronary heart maladies (Bridle and Timberlake, 1997), improving visual acuity (Timberlake and Henry, 1988), being *anti-oxidant* (Tamura and Yamagami, 1994; Wang *et al*., 1997) and *anti-carcinogenic* (Kamei *et al*., 1995; Karaivanova *et al*., 1990).

Anthocyanins, which were vastly found in the roots, leaves, as well as flowers and fruits, or the whole of aerial sectors of tall plants, are exploited as a successor for combinatorial pigments due to their marvellous and physiological functionality (Mazza and Miniati, 1993). *Anthocyanins* have also demonstrated identifiable pharmacologic potentials and are consumed for various therapeutic intentions (Francis, 1989). A developing number of studies have expressed that *anthocyanins* feature the ability to protect cells against numerous disturbances, such as *liver dysfunction*, *eyesight disorders*, *hypertension*, *microbial putrefaction*, and *diarrhoea* (Giuseppe Mazza and Kay, 2008; Smith *et al*., 2000).

Generally, the conventional solvent extraction of *anthocyanin* is time consuming, solvent-wasting as well as having low yield (Sun *et al*., 2007). Moreover, calorific extraction over an eminent period of time could cause the decomposition of *anthocyanins* and diminish the bioactivity of the obtained infusions (Camel, 2000; Lapornik *et al*., 2005). *Anthocyanin* might also undergo denaturalisation when extracted from a natural origin. The infusion process involves a lessening in colour encouraged by the formation of brownish degradation products and insoluble compounds (Castillo-Sánchez *et al*., 2006). *Microwave-assisted extraction* (*MAE*) uses the power of microwaves to stimulate the molecular motion and spin of liquids with a constant dipole. It has superiority over conventional extraction techniques, such as modified proficiency, deducted extraction period, lower solvent expenditure, and a great level of automation (Buldini *et al*., 2002; Sparr Eskilsson and Björklund, 2000). Lately, different studies have been performed on the utilisation of natural

products, such as *artemisin* (Hao *et al*., 2002), *ginseng saponins* (Kwon *et al*., 2003), and *anthocyanin* (Sun *et al*., 2007). However, no further publications were met by the authors on the exploitation of MAE to obtaining the *anthocyanin* in *Lavandula pedunculata* L. subsp. pedunculata.

Response Surface Methodology (*RSM*) is an impressive statistic method for optimising assembled proceedings. It is immensely applied to optimise the various variables. Upon research, the basic theoretical and fundamental aspects of *RSM*, colour was found to be one of the most effectual attributes of natural colourants (Farooq Anjum *et al*., 1997; Liyana-Pathirana and Shahidi, 2005). Moreover *Response Surface Methodology* (*RSM*) along with *Artificial Neural Network* (*ANN*) has been used in many fields of food technology ((Dolatabadi *et al*., 2016; Rostami *et al*., 2014)). In this research, *MAE* parameters, such as microwave irradiation power, irradiation time and the ratio of solvent/sample were optimised by *RSM* to obtain the optimal extraction proficiency of *anthocyanin* from *Lavandula pedunculata* L. subsp. pedunculata. The achieved results of this research demonstrates the optimum conditions of the extraction of *anthocyanins* from not only this plant but also many other plants with similar texture, with the least wastes in chemicals and saved time.

8.2. Materials and Methods

8.2.1. Plant materials

Lavandula pedunculata L. subsp. pedunculata., was acquired in June 2014 from the countryside of Faro city, located in the Algarve. The plants have been identified by Mr Rosa Pinto (University of Algarve) and a voucher instance was entrusted to the Herbarium of the MeditBio institute, *Faculty of Science and Technology* (*FCT*) of University of Algarve (Voucher number: FSL2014). After drying the whole aerial parts including flowers, leaves and younger branches were disassembled attentively and ground using a grinder (*Cetigo*-Portugal). Ground samples were sieved (mash size of 8 mm) and capped into particular vials and preserved at -20 ºC until the day of extraction.

8.2.2. Chemicals and reagents

Ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$), was purchased from *Sigma-Aldrich* (Switzerland-Germany). Sulphuric acid (H_2SO_4) (95-97%, GR for analysis) and potassium chloride (KCL), were obtained from *Merck* (*Darmstadt*-

Germany). Sodium phosphate dibasic anhydrous (Na_2HPO_4) (Purity>99.0%) was provided from the *G-Bioscience* company (*USA*) and sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$) was purchased from *VWR* (*Haasrode*-Belgium).

8.2.3. Apparatus

Spectrophotometer (T70+Vis, PG Instrument Ltd, United Kingdom), evaporator (*Nahita* series 503, Navarra, Spain), Centrifuge *Hettich* (*universal-320*, Germany), Hot plate-stirrer (*VWR*, 720 advanced, *USA*), Disposable cuvettes were provided from *VWR* (Leuven, Belgium). Vortex mixer (*Stuart*, UL-Bibby Sterilin Ltd), grinder (*Philips*- Brasil) and (*Cetigo*-Portugal) and microwave oven (*Samsung* type 1713, Microwave output 800 W).

8.2.4. Extraction Total Monomeric Anthocyanin (TMA) from aerial parts of Lavandula pedunculata L. subsp. pedunculata., species

MAE of TMA content was performed in an ordinary household microwave oven (*Samsung* type 1713, Microwave output 800 W) at a working frequency of 2450 MHz with adjustable microwave power and time. Around 1 g of sample powder was weighed and situated into a 250 mL Pyrex beaker and various volumes of deionized water (20, 30 and 40 mL) were poured and mixed well with a stirrer bar for a period time of 5 minutes to give an adequate penetration of water. Regarding the applied experimental design (Table 8.1), the beaker with diverse proportions of solvent/samples were situated in the middle of the oven over a rotational plate and were exposed to microwave radiation at three diverse selected powers (300, 450, 600 W) for three diverse characterised irradiation times (60, 120, 180 s). The containers containing the suspension have been capped during microwave emission to prevent of missing of the solvent through evaporation. After microwave streaming, the mixture in the Pyrex beaker was left to cool down until reaching to room temperature and centrifuged within 10 minutes at 5000 rpm, then refined using *Sartorius stedim bio grade 388* filter paper. Once the extraction process was terminated, water was vaporised using a rotary evaporator (*Nahita* series 503, Navarra, Spain), and the remaining materials were re-suspended in a specified volume of distilled water for adequate definitive concentrations for the assays. Infusions were shifted to *Eppendorf* tubes and were preserved at -20 °C until analysis. On the day of the experiments, the infusions were kept in darkness until defrosting.

Table 8.1 Independent variables and their coded and actual values used for optimization.

Independent variables	Units	Symbol	Code levels		
			-1	0	1
Microwave irradiation power	W		300	450	600
Irradiation time	s		60	120	180
Solvent to sample proportion	mL. g^{-1}		20	30	40

8.2.5. Experimental design and statistical analysis

RSM was applied to specify the optimal status for the extraction of the maximum quantity of *anthocyanin* compounds from the *Lavandula pedunculata* L. subsp. pedunculata., species. The pilot design and actuarial analysis were performed exploiting *JMP® Pro*, Version 11. Copyright, *SAS* Institute (Inc. *SAS* and all other *SAS* Institute Inc. product or service names are registered trademarks or trademarks of *SAS* Institute Inc., Cary, NC, *USA*). Diversity among experimental data has been confirmed using a *one-way analysis of variance* (*ANOVA*).

A three-level three-factor *Box-Behnken design* (*BBD*) was chosen to assess the composed effectiveness of the three independent variables. Microwave irradiation power, irradiation time and solvent/sample ratio were termed as X_1, X_2 and X_3, respectively. The minimum and maximum values for the independent variables were set as follows, 300 and 600 W for microwave irradiation power, 60 and 180 s for irradiation time and 20 and 40 mL. g^{-1} for solvent/sample ratios (see Table 8.1). The entire layout of 15 combinations, comprising of three iterations of the centre point (Table 8.2), and the response function *(Y)* were broken up into linear, quadratic, and interactive components as follows:

$$Y = \beta_0 + \sum_{i=1}^{3} B_i X_i + \sum_{i=1}^{3} B_{ii} X_i^2 + \sum_{\substack{i=1 \\ i<j}}^{2} \sum_{j=i+1}^{3} B_{ij} X_i X_j \qquad (8.1$$

Where Y stands for responses; β_0 marks the model intercept; B_i, B_{ii} and B_{ij} represent the modulus of the linear, quadratic, and interactive consequences, respectively; X_i and X_j are the codified independent variables; and equal to the numeral of examined factors (X_1, X_2 and X_3).

Table 8.2 *Box-Behnken experiment design* (*BBD*) matrix with observed and predicted values.

Run order	Microwave power (W) (X_1)	Irradiation time (s) (X_2)	Solvent/sample ratio (mL. g^{-1}) (X_3)	Total monomeric *anthocyanin* contents (TMA) ($mg_{(cyanidin\text{-}3\text{-}glucoside)}$. L^{-1})		Total *anti-oxidant* activity (TAA) ($mg_{(AAE)}$. g^{-1} $_{dw}$)	
				Experimental	Predicted	Experimental	Predicted
1	300(-1)	60(-1)	30(0)	86.277	86.520	313.671	324.236
2	300(-1)	120(0)	20(-1)	91.008	72.639	288.631	269.459
3	300(-1)	120(0)	40(1)	81.546	97.340	**350.675**	352.873
4	300(-1)	180(1)	30(0)	**67.63**	69.961	299.161	305.568
5	450(0)	60(-1)	20(-1)	77.649	95.774	**204.215**	212.821
6	450(0)	60(-1)	40(1)	143.888	127.850	297.297	284.532
7	450(0)	120(0)	30(0)	280.3	271.578	328.33	334.966
8	450(0)	120(0)	30(0)	256.12	271.578	330.56	334.966
9	450(0)	120(0)	30(0)	**278.314**	271.578	346.01	334.966
10	450(0)	180(1)	20(-1)	69.022	85.059	227.994	240.758
11	450(0)	180(1)	40(1)	91.565	73.439	244.848	236.242
12	600(1)	60(-1)	30(0)	138.322	135.991	300.61	294.202
13	600(1)	120(0)	20(-1)	136.374	120.579	299.933	297.734
14	600(1)	120(0)	40(1)	97.966	116.334	262.343	281.514
15	600(1)	180(1)	30(0)	87.669	87.425	303.084	292.518

Bold numbers in each column demonstrate the minimum and maximum values.

The tables of the *analysis of variance* (*ANOVA*) were generated (Table 8.3), and the efficacy and *regression coefficients* (R^2) of the individual linear, quadratic and interactive terms were defined (Table 8.4). The conditions of the whole terms in the multinomial were diagnosed statistically by computing the *F-value* at the probability of (p=0.05).

Table 8.3 *Analysis of variance* for the *response surface* quadratic model of two responses (p<0.05)**.**

Source	TMA ($mg_{(cyanidin\text{-}3\text{-}glucoside)}$. L^{-1})				TAA ($mg_{(AAE)}$. g^{-1}_{dw})			
	DF	Sum of Squares	*F-ratio*	*Prob>F*	DF	Sum of Squares	*F-ratio*	*Prob>F*
Linear								
X_1								
X_2								
X_3								
Interaction								
$X_1 \times X_2$								
$X_1 \times X_3$					1	2481.733	7.258	0.0431*
$X_2 \times X3$					1			
Quadratic								
X_1^2	1	26806.07	49.3377	0.0009*	1			
X_2^2	1	30844.041	56.7698	0.0007*	1	7090.291	20.737	0.0061*
X_3^2	1	26457.058	48.6954	0.0009*	1	8350.804	24.737	0.0043*
Model	9	78428.237	16.039	0.0035*	9	23162.978	7.527	0.0194*
Lack of fit	3	2356.1926	4.3585	0.9956	3	1524.148	5.48	0.158
Error	5	2716.589			5	1709.568		
C. Total	14	81144.826			16	24872.545		
		R^2= 0.966				R^2= 0.931		
		R^2_{adj}= 0.906				R^2_{adj}= 0.807		

X_1: Microwave irradiation power, X_2: Irradiation time, X_3: Solvent/sample ratio.

Table 8.4 Test of significance for *regression coefficient* of two selected responses.

Source	TMA ($mg_{(cyanidin\text{-}3\text{-}glucoside)}. L^{-1}$)				TAA ($mg_{(AAE)}. g^{-1}_{dw}$)			
	Estimate	Std. error	*t* ratio	*Prob* > |*t*|	Estimate	Std. error	*t* ratio	*Prob* > |*t*|
Intercept	271.578	13.457	20.18	**<0.0001***	343.895	10.675	31.38	**<0.0001***
Linear								
X_1								
X_2								
X_3								
Interaction								
$X_1 \times X_2$								
$X_1 \times X_3$					-24.908	9.245	-2.69	**0.043***
$X_2 \times X3$								
Quadratic								
X_1^2	-85.205	12.130	-7.02	**0.0009***				
X_2^2	-91.398	12.130	-7.53	**0.0007***	-43.821	9.622	-4.55	**0.0061***
X_3^2	-84.649	12.130	-6.98	**0.0009***	-47.557	9.622	-4.94	**0.0043***

X_1: Microwave irradiation power, X_2: Irradiation time, X_3: Solvent/sample ratio.

8.2.6. Determination of the Total Monomeric Anthocyanin (TMA)

TMA was a diagnosed pursuant to the *pH*-differential assay defined by (Mónica Giusti and Wrolstad, 2001). Firstly, the adequate dilution factor for the sample was characterised by diluting the sample with 0.025 M potassium chloride buffer (*pH*=1.0) for a generic volume of 1 mL with the absorbency at a wavelength with maximum absorbance ($\lambda_{vis\ max}$) was less than 1.2. To characterise the $\lambda_{vis\text{-}max}$, a spectrum of the sample (260-710 nm) was accumulated. Two dilutions of the sample were prepared until a total volume of 1 mL was reached, one with a buffer *pH*=1.0 and the other with a 0.4 M sodium acetate buffer (*pH*=4.5). These dilutions were preserved consistently for 15-45 minutes to reach equilibrium. A blank of distilled water was applied to zero the spectrophotometer at all specified wavelengths. The ultimate absorbency of each dilution was recorded at $\lambda_{vis\text{-}max}$ and 700 nm. The monomeric *anthocyanin* pigment concentration in the original sample was reported by using the subsequent equation (equation 8.2):

$$\text{Monomeric } anthocyanin \text{ pigment } (mg.L^{-1}) = (A \times MW \times DF \times 1000)/(\varepsilon \times 1) \quad (8.2$$

Where:

$A = (A_{\lambda\, vis-\max -} A_{700})_{pH=1} - (A_{\lambda\, vis-\max -} A_{700})_{pH=4.5}$, *MW* is the molecular weight of cyanidin-3-glucoside expressed in mg, *DF* is the dilution factor and ε is the molar absorptivity. When the sample composition is unknown, the pigment quantity is expressed as *cyanidin-3-glucoside*.

8.2.7. Determination of total anti-oxidant activities (TAA)

In the current study, the total *anti-oxidant* activity was recorded using the phosphomolybdenum method as narrated by (Prieto *et al*., 1999). Concisely, 100 µL of each infusion was composed using 1000 µL reagent solution comprising 0.6 M sulfuric acid, 28 mM anhydrous sodium phosphate and 4 mM ammonium molybdate. After blending severely, the combined solutions were transferred into a water bath at 95 °C for 90 minutes, and after cooling down the mixtures to room temperature, the absorbency was recorded. *Anti-oxidant* power was calculated on the basis of the calibration curve of *ascorbic acid* constructed in the specified particular confine and was expressed as mg *ascorbic acid* per gram dry weight of sample ($mg_{(AAE)}. g^{-1}{}_{dw}$).

8.3. Results and discussion

8.3.1. Optimization of MAE condition on TMA

Acidified methanol, ethanol, and acetone are often used as organic solvents for *anthocyanin* extraction (Giusti and Jing, 2008). Nevertheless, extraction with organic solvents has economic and environmental disadvantages; and the intent of green chemistry is encouraging in the development and the use of less perilous proceedings and ingredients out of any reduction in the proficiency of responses (Chemat *et al*., 2012). Accordingly, solvent extraction of *anthocyanin* should be optimised for the maximum extraction of TMA using the least organic solvents. Therefore, water was selected for this research. The optimisation of *MAE* conditions was reported in many studies (Sparr Eskilsson and Björklund, 2000; Junlong Wang *et al*., 2010; Zheng *et al*., 2009). Many researchers have applied solitary factors; factorial and orthogonal arranged layouts to acquire optimum states. *RSM* optimisation contains more advantages than the customary solitary parameter optimisation in that, it accumulates time, space and crude materials - in fact, it is economic, reliable and fast. There are a total of 15 runs for optimising the three particular parameters within the *Box-*

Behnken layout. Table 8.2 shows the pilot status and the outcomes of extraction of TMA according to the *Box-Behnken design* (*BBD*). The maximum pilot values of TMA 278.314 ($mg_{(cyanidin\text{-}3\text{-}glucoside)}$. L^{-1}) were recorded under the experimental conditions of microwave power 450 W, irradiation time 120 s and solvent/sample ratio about 30 mL. g^{-1}, and the minimum value was observed as 67.63 ($mg_{(cyanidin\text{-}3\text{-}glucoside)}$. L^{-1}) in the subsequent conditions, the microwave power of 300 W, irradiation time of 180 s and a solvent/sample proportion of 30 mL. g^{-1}.

By applying multiple regression analysis on the experimental data, the mathematical model representing the extraction of TMA as a function of independent variables within the region under research could be obtained.

In general, the exploration and optimisation of a fitted response surface might create faint results unless the design exposes a desirable fit, which announces the original design sufficiency (Liyana-Pathirana and Shahidi, 2005). The *P*-values of the design for the extraction of TMA were observed as 0.0035 (Table 8.4), which indicated that the fitness of the model was acutely consequential. "Lack of fit's *F*-value" 0.995 showed that the lack of fit was not noticeable respective to pure error. The modulus of relationship (R^2) of determination is specified as the proportion of the explained conversion to the total conversion and is a determination of the dignity of fit (Nath and Chattopadhyay, 2007). The determined relationship (R^2= 0.966) expressed that just 3.44% of the total conversion was not illustrated by the design. The value of the adjusted relationship (R^2_{adj}= 0.906), affirmed that the model was extremely reliable.

The results of the *regression coefficient* of the three independent variables on the responses were also presented in Table 8.4. The *p*-value is used as a tool to examine the impacts of each mode between the conversions. It could be found from Table 8.4, that the quadratic terms of the three independent variables (X_1^2, X_2^2 and X_3^2) were extremely noticeable, with a negative effect on TMA response ($p \leq 0.001$).

The entire design-filled Table 8.4, created the 3-dimensional *response surface plot* to predict the relationships between the independent and dependent variables. The effectiveness of microwave power, irradiation time and the solvent/sample ratio on the recovery of TMA, as well as their interactions, are expressed in Fig. 8.1. The axiom of heating during microwave extraction is relying on the direct consequence of microwaves on molecules by ionic transmission and dipole circulation. Ionic transmission is the electrophoretic departure when electromagnetic amplitude is

used. The persistence of the solution against this flow of ions will terminate in friction, which will braise the solution (Song *et al*., 2009). The *3-D response surface* plot based on autonomic variables including microwave power and irradiation time was shown in Fig. 8.1A. While the solvent/sample ratio was kept permanent, an increase in the recovery of TMA could be seriously attained with the increases in irradiation time. It is clearly observed that the extraction of TMA was enhanced from 60 to about 114.281 s, meaning that further increases of irradiation time out of this range would not enlarge the extraction of TMA value any further. The extension of microwave time could terminate in a higher extraction recovery. However, the extraction of TMA decreased with the extension of exerted microwave time. When a greater microwave power is emitted, it could be stated that most pigments might be unstable at higher temperatures caused by exposure to microwave emissions and are decomposed and broken down to colourless compounds. This result is in line with finding of Roobha et al (Jenshi roobha *et al*., 2011).

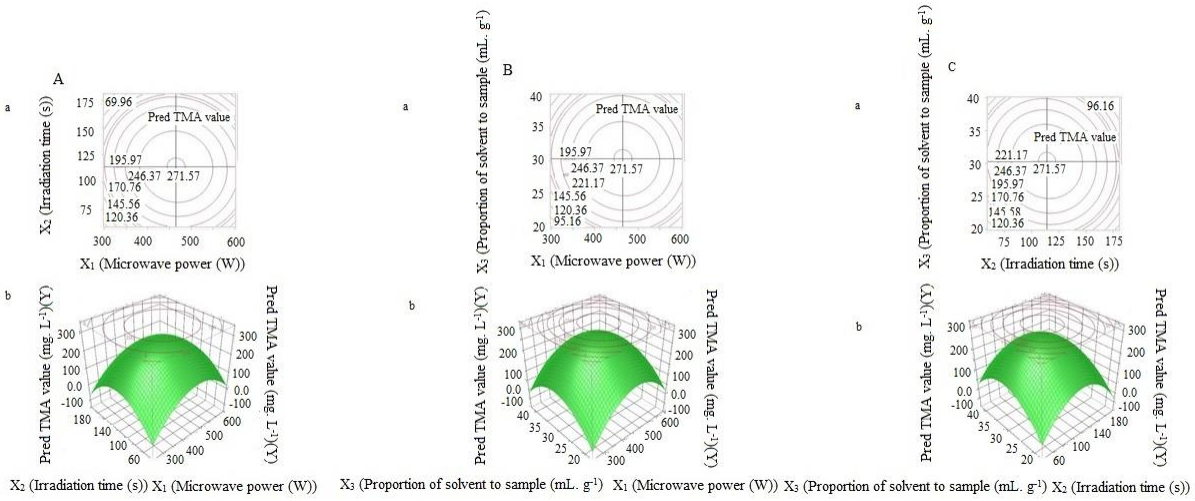

Figure 8.1 Contour profiler (a) and surface plot (b) of the TMA (mg. L^{-1}) (*Y*) as a function of X_1 (Microwave power (W)), X_2 (Irradiation time (S)) and X_3 (Proportion of solvent to sample (mL. g^{-1}).

The attainment of TMA contents, affected by various solvent/sample proportions and irradiation period is presented in Fig. 8.1C, when microwave power was preserved as constant (0 level). It is explicit that with an increase in the solvent/sample proportion from 20 to about 30.321 mL. g^{-1}, the extraction of TMA was increased. The solvability of *anthocyanin* might be boosted by using more proportion of solvent/sample (Jenshi roobha *et al*., 2011). It was observed that the maximum recovery of TMA could be achieved when irradiation time and the

proportion of solvent/sample developed from 60 to about 114.281 s and from 20 to about 30.231 mL. g^{-1}, respectively.

Using the predictions of a computing programme, the optimal conditions to obtain the highest recovery of TMA, were determined as follows: microwave power, 464.876 W; irradiation time, 114.281 s; and solvent/sample proportion of 30.321 mL. g^{-1}, that is presented in Table 8.5.

To summarise, the consequential negative quadratic effectiveness of microwave irradiation power, irradiation time and solvent/sample ratio on TMA value at the level of (p=0.05) have been observed (Table 8.4).

8.3.2. Response surface results of total anti-oxidant activity (TAA)

The obtained experimental values and predicted results using the *Box-Behnken design* (*BBD*) were demonstrated in Table 8.2. As it was presented, the lowest pilot value was achieved in run 5, under a microwave power of 450 W within an irradiation time of 60 s and solvent/sample proportion of 20 (mL. g^{-1}). The supreme value was perceived in run 3 under the subsequent statuses, a microwave power of 300 W, irradiation time 120 s and solvent/sample proportion of 40 (mL. g^{-1}). Fig. 8.2 demonstrates the predicted three-dimensional surface plots (b) with contour plots (a) of the response value (TAA) above.

The effects of microwave power (X_1) and irradiation time (X_2) on the TAA value of *Lavandula pedunculata* L. subsp. pedunculata., are presented in Fig. 8.2A. As the X_1 increased, the TAA gradually decreased, while as X_2 increased, reaching X_2 around 107.339 s increases TAA value. The obtained results demonstrated that an increase in the irradiation time (X_2) from 60 to about 107.339 s with a microwave power of 300 W, improved TAA value up to 369.734 ($mg_{(AAE)}$. $g^{-1}{}_{dw}$).

As shown in Fig. 8.2B, TAA value was increased with the enhancement of the solvent/sample proportion from 20 up to about 34.807 (mL. g^{-1}). Therefore, the increased solvent/sample proportion within the range of 20-34.807 (mL. g^{-1}) led to an enhancement in the TAA value. An increase in the microwave power from 300 to 600 W decreased the TAA value.

Fig. 8.2C, demonstrates the efficacies of X_2 (Irradiation time) versus solvent/sample proportion at a constant level of microwave power (X_1). As shown, an increase in X_2 from 60 up to about 107.339 s and X_3 from 20 to about 34.807 (mL. g^{-1}) boosted TAA value up to 369.734 ($mg_{(AAE)}$. $g^{-1}{}_{dw}$).

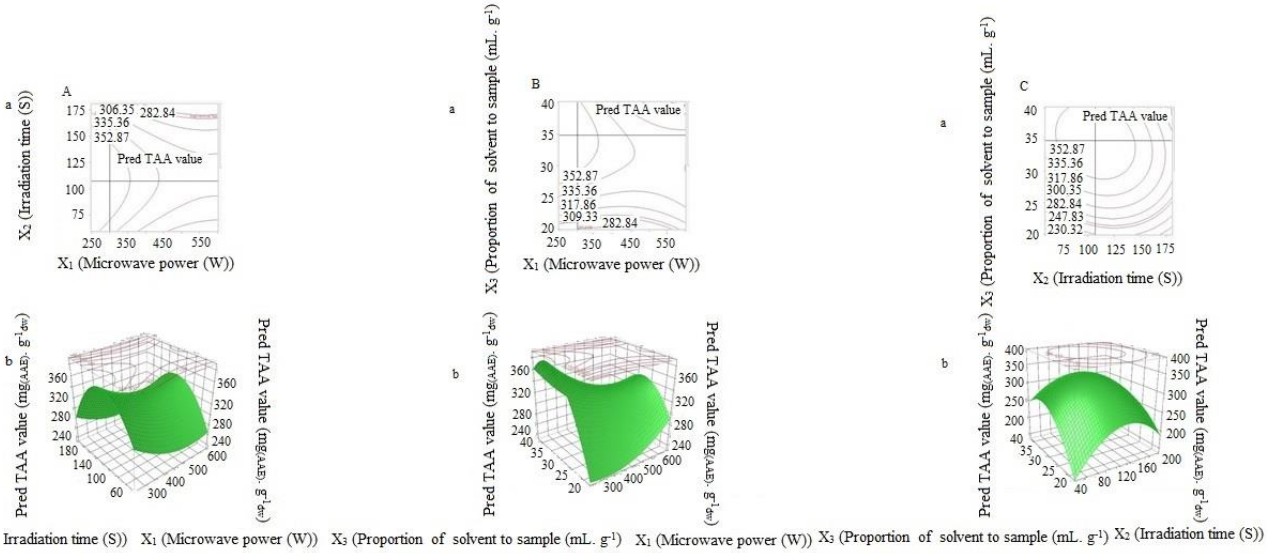

Figure 8.2 Contour profiler (a) and surface plot (b) of the TAA (mg. L^{-1}) (*Y*) as a function of X_1 (Microwave power (W)), X_2 (Irradiation time (S)) and X_3 (Proportion of solvent to sample (mL. g^{-1}).

As Table 8.5 shows, the maximum desirability (Desirability=0.952) for TAA value, was obtained in X_1=300 W, X_2=107.339 s and X_3=34.807 mL. g^{-1}. The corresponding response value for the predicted TAA in the maximum desirability is 369.734 ($mg_{(AAE)}$. $g^{-1}{}_{dw}$).

Table 8.5 Prediction profiler desirability and optimum conditions of the extraction.

Response	Factor			Correspondent response value	Desirability
	X_1 (W)	X_2 (s)	X_3 (mL. g^{-1})		
TMA ($mg_{(cyanidin\text{-}3\text{-}glucoside)}$. L^{-1})	464.876	114.281	30.321	273.284	0.878
TAA ($mg_{(AAE)}$. $g^{-1}{}_{dw}$)	300	107.339	34.807	369.734	0.952

X_1: Microwave irradiation power, X_2: Irradiation time, X_3: Solvent/sample ratio.

This phenomenon might be explained because solvent viscosity was declined and the movement of molecular accelerated with the increase of temperature modified by microwave emissions, and as a result leads to the release of bioactive compounds from the plant cells. Rather, the irradiation time of about 107.339 s with microwave power 3000 W in this research promoted the degradation of some

thermo-sensitive compounds with bioactivity potential (Prommuak *et al*., 2008; Trabelsi *et al*., 2010) such as *polyphenols*, also it could be considered that further microwave power in an extensive confine of time is authoritative to release highly viscous or cohesive syntaxes that could pull down the extraction performance of compounds with bioactive and *anti-oxidant* properties. The content of bioactive components as such other *flavonoids* (*flavones*, *flavanols*, *isoflavone*, *proanthocyanin* and etc.) and *phenols* in the obtained infusion is increased gradually under the microwaves emission and high ratio of solvent/sample. Regarding the construction of the herbages' cells, solvents require adequate periods to penetrate cells. On the other hand, medium microwave power is required to disintegrate the cells structures.

The outcomes of *ANOVA* and regression analysis for the quadratic model based on TAA are summarised in Tables 8.3 and 8.4, respectively. The statistical analysis stated that the model was adequate, possessing noticeable design ($p<0.05$) with admitted values of a relationship between the experimental and predicted values (R^2= 0.931). The *coefficient of determination* (R^2) indicates that the model accurately represents a good relationship between the parameters examined (Jing Wang *et al*., 2008).

Notable negative quadratic modes of irradiation time and solvent/sample proportion and the considerably negative interactive clues between microwave power and solvent/sample proportion on TAA value at the level of (p=0.05) were observed (Table 8.4). The insignificant negative interactive effect between irradiation time and solvent/sample proportions as well as insignificant positive interactive effects between microwave power and irradiation time were observed. Furthermore, the insignificant negative linear effect of microwave power and irradiation time as well as positive linear effect of solvent/sample proportion, at the level of (p=0.05) on TAA value was obtained (Table 8.5).

8.4. Conclusions

The existence of a great correlation of mathematical design stated that quadratic multinomial design could be used to optimise the *microwave extraction* process of TMA. From *response surface plots*, the quadratic effects of the whole three factors (microwave power (X_1), irradiation time (X_2) and solvent/sample ratio (X_3)) negatively have had a significant influence on the extraction of TMA. Furthermore, the quadratic effects of irradiation time (X_2) and solvent/sample

proportion (X_3) negatively, and also the interactive effect between microwave power and solvent/sample proportion (X_3) negatively have presented a significant effect on the TAA value of *Lavandula pedunculata* L. subsp. pedunculata., infusions. The optimal conditions for attaining the supreme extraction of TMA were characterized as 464.876 W, 114.281 s and 30.321 mL. g^{-1} and for TAA were specified 300 W, 107.339 s and 34.807 (mL. g^{-1}). *ANOVA* indicated that under the optimal statuses, the experimental values are in compromise with the predicted. Thus this methodology could provide a foundation for the model to demand a non-linear nature between independent variables and responses as part of a short-term examination. In addition, *L. pedunculata* L. subsp. pedunculata., infusion obtained exploiting *MAE*, displayed *anti-oxidant* potential for application in health-care, pharmaceutic and food industries. However, further researches concerning the nutritional and health benefits of *L. pedunculata* L. subsp. pedunculata., are required.

8.5. References

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CHAPTER 9

Screening of the *anti-oxidant* and enzyme inhibition potentials of Portugues *Pimpinella anisum* L., seeds by *GC-MS*

In the present chapter the different anti-oxidant parameters as well as enzymes inhibitive activities of Portugues P. anisum L., seeds have been screened. The bioactive compounds profile has been detected using GC-MS analysis and the results obtained were interpreted.

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Screening of the *anti-oxidant* and enzyme inhibition potentials of Portuguese *Pimpinella anisum* L., seeds by *GC-MS*

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Abstract

A bioactive compound profile of an infusion was performed using *GC-MS*. The outcomes obtained demonstrated that the infusion of this plant shows high values for total *phenols*, *flavonoids* and *monomeric anthocyanins*. Moreover, high *anti-oxidant* values were achieved for the infusion of *Pimpinella anisum* L., seeds. As might have been expected, this infusion demonstrated a stronger activity against *free radicals* than in the inhibition of enzymes, indicating the role of *free radicals* in generating a wide confine of disabilities. Among the detected compounds, *fatty acids*, representing 47.68%, are the highest, followed by *triterpenoids* and *sterols*, representing 15.56% and 7.29% of all detected bioactive compounds, respectively. The main compounds detected in this research, including *linoleic*, *oleic* and *palmitic acids* from the *fatty acids* category, along with *lupeol*, *β-Amyrin* and *betulinic acids* (*BAs*) from *triterpenoids*, as well as *sterols* such as *β-sitosterol* and *stigmasterol* demonstrated that the abovementioned compounds play the most substantial role in indicating the health benefits of *P. anisum* L., seeds.

Keywords: *Diabetes*; *Alzheimer's*; *anti-oxidant* potentials; *GC-MS*; anise

9.1. Introduction

Today, application of medicinal plants in food and nutrition has become more popular because of the potential health benefits that have been attributed to them. Therefore, medicinal plants in nutraceutical, pharmaceutical as well as cosmetic products and in place of synthetic medicines are developed; additives and preservatives in different industries are known substantial. *Pimpinella anisum* L., species is native to *Mediterranean* countries and contains valuable bioactive oils. This plant is known to be effective in the treatment of several acute and even *chronic* illnesses, including the treatment of digestive irregularities and dyspnoea, and it also acts as an *anti-convulsant* agent. Moreover, its application in the field of *gynaecology* is known within traditional medicine. The aqueous infusion of anise heals wounds and has *cyto-protective* potential against chemically stimulated wounds *in vivo* tests, and a positive effect on *hypoglycemia* has also been recorded. Along with *anti-diabetic* effects, *anti-viral* and *insecticidal* properties and potential as an expectorant, it has an application in flavouring medicines, chewing gum, ice cream, toothpaste and so on. Not only the seeds of *P. anisum* L., but also all its aerial parts including leaves and branches have been reported to be beneficial. An infusion of this plant extracted using the supercritical liquid method (CO_2) showed up many bioactive compounds including *anethole*, *γ-himachalene*, *p-anisaldehyde*, *methylchavicol*, *cis-pseudoisoeugenyl* 2-*methylbutyrate* and *trans-pseudoisoeugenyl* 2-*methylbutyrate* (Rodrigues et al., 2003). The scientists who found this also detected a novel *terpenoid* in this plant and named it neophytadiene. *Type I diabetes* is caused by a deficiency in *pancreatic β-cells*, probably due to an autoimmune abnormality, whereas *type II diabetes* might be generated by a disturbance in the metabolism of compounds, including proteins, fats and carbohydrates (Pathak and Pathak, 2012). The eventual mechanism of the *hypoglycaemic* action of medicinal plants might be described as: (1) the inducible effect of extracts on pancreatic *β cells* in the secretion of insulin and the protective effects on the released insulin against inappropriate environmental effects; (2) the boosting of insulin sensitivity of the cells and the regeneration of pancreatic *β cells* by the enhancement of a cell's size and the quantity of cells and (3) the stimulation of glycogenesis and hepatic glycolysis as well as the inhibiting effects against *β-galactosidase*, *α-Glucosidase* and *α-Amylase*. The *anti-Alzheimer* properties of bioactive compounds might be attributed to their inhibition of *Acetylcholinesterase* (*AChE*), the fact that they lower the *cholesterol* quantity of

blood as well as their suppression of *free radical* contents. A widespread range of compounds with bioactivity, including *polyphenols*, *flavonoids*, *fatty acids*, *tannins*, *lignans* and *sterols* as well as *triterpenoids* indicated many potential health benefits. Concerning the role of *free radicals*, including *reactive oxygen species* (*ROS*), *reactive nitrogen species* and *reactive chlorine species* (*RCS*) in the rise of *chronic* disorders such as *diabetes*, *Alzheimer's*, *Parkinson's* and other disorders related to the *central nervous system* (*CNS*), several mechanisms were reported. These include the following: (1) their binding to enzymes and/or enzyme receptors that participated in cell metabolism with the alteration of 3D structures, resulting in the pausing of essential required reactions within the cells and (2) the destruction of cell membranes and interference in the metabolism of normal cells.

The aim of this study is to discover the *anti-radical* activity of Portuguese *P. anisum* L., seeds and their potentials in the inhibition of the activities of *α-Amylase* and *AChE*, demonstrating the eventual capability of anise in delaying and/or preventing *chronic disorders* such as *diabetes* and *Alzheimer's disease*.

9.2. Materials and methods

9.2.1. Plants materials

In June 2014, the aerial parts of the *P. anisum* L., were picked from the countryside of Faro-Algarve, Portugal. The plants were identified by Mr Rosa Pinto, (University of Algarve) before pick up was arranged at the herbarium of the institute MeditBio, Faculty of Science and Technology, Algarve University. Each aerial part of the plant was dried in shade; then the seeds were removed manually and ground using an electrical grinder (*Cetigo*-Portugal). Henceforth, ground seeds were shifted into particular vials, sealed tightly and preserved under −20 °C until the extraction day.

9.2.2. Preparation of plant infusions

Extraction was carried out using hot (90 °C), distilled water for 5 minutes. About 3 g of each sample was dissolved into 30 mL of solvent (distilled water) while being stirred with a hot plate magnetic stirrer (*VWR*, 720 advanced, *USA*). After cooling down, the samples were centrifuged at room temperature at 5000 rpm for 10 minutes using a *Hettich* centrifuge (*universal-320*, Germany). The supernatant was refined across *Sartorius Stedim Biotech grade 388* filter papers. After extraction, the solvent was evaporated using a rotary evaporator (*Nahita series 503*, *Navarra*,

Spain), and the remaining materials were resuspended in *milli Q water*, were frozen and were preserved under −20 °C until the day of analysis.

9.2.3. Chemicals and reagents

All the chemicals and reagents required for the experiments and *GC-MS* analysis were of analytical grade.

9.2.4 Apparatus

Spectrophotometer (T70+Vis, PG Instrument Ltd, United Kingdom), evaporator (*Nahita series 503*, Navarra, Spain), *Hettich* centrifuge (*universal-320*, Germany), hot plate stirrer (*VWR*, 720 advanced, *USA*), vortex mixer (*Stuart*, UL-Bibby Sterilin Ltd), and grinders (*Philips*-Brazil) and (*Cetigo*-Portugal) were used in this study.

9.2.5. Preparation of samples for GC-MS analysis

Soxhlet extraction was performed for the *GC-MS* analysis. Briefly, 2 g of the ground and sieved *P. anisum* L., seeds were shifted into thimbles and extracted using dichloromethane (100 mL) overnight, which was a sufficient time for discoloration. Next, the solvent was recovered using a rotary evaporator (*Qlabo*-Portugal) under the following conditions: 500 mbar pressure, with a rotation value of 41 rpm at a temperature of 40 °C. The extracts thus obtained were further dried at 40 °C within a nitrogen atmosphere, until extracts free of any solvent were obtained. Prior to the final preparations of the samples, the obtained samples were shifted into a vacuum oven (35 °C at 150 mbar for 48 hours) to eliminate the remaining traces of humidity. Finally, the extracts obtained were passed for *GC-MS* analysis.

9.2.6. Chemical analysis

9.2.6.1. Bioactive compounds content

9.2.6.1.1. Definition of total phenols (TPC)

The concentration of total *phenols* (TPC) in the infusions was determined using *Folin–Ciocalteu reagent* and external determination using *gallic acid*. The obtained results were presented as $mg_{(GAE).}\ g^{-1}{}_{dw}$ (Huang et al., 2006).

9.2.6.1.2. Measurement of total flavonoids (TFC)

The total *flavonoid* (TFC) content was monitored using a spectrophotometer and the aluminium chloride hexahydrate method. The results obtained were presented as $mg_{(QE).}\ g^{-1}{}_{dw}$ (Quettier-Deleu et al., 2000).

9.2.6.1.3. Total monomeric anthocyanin (TMA)

Total monomeric *anthocyanin* (TMA) was determined in relation to the pH-differential assay, and the achieved results were presented as $mg_{(cyanidin\text{-}3\text{-}glucoside)}.\ L^{-1}$ (Giusti and Wrolstad, 2001).

9.2.6.*2. Anti-oxidant activities tests*

9.2.6.2.1. FRAP (Ferric reducing anti-oxidant power) assay

The ferric reducing *anti-oxidant* power (FRAP) value was taken from the previously established data in literature, and the results were presented as $mg_{(TE).}\ g^{-1}{}_{dw}$ (Benzie and Strain, 1999).

9.2.6.2.2. Reducing Power (RP)

To determine the reducing power (RP) value of different infusions, the method reported by a group of scientists was applied, and the results were expressed as $mg_{(TE).}\ g^{-1}{}_{dw}$ (Barros et al., 2010).

9.2.6.2.3. Determination of total anti-oxidant activity (TAA)

The total *anti-oxidant* activity (TAA) value was detected using the phosphomolybdenum method and was presented as $mg_{(AAE).}\ g^{-1}{}_{dw}$ (Prieto et al., 1999).

9.2.6.3. Determination of *anti-radical* activities

9.2.6.3.1. $ABTS^{\cdot+}$ Inhibition activity

P. anisum L., seeds were also analysed by studying their potential in the inhibition of $ABTS^{+}$, using a modified methodology, and the IC_{50} values obtained were presented as μg. mL^{-1} (Re et al., 1999).

9.2.6.3.2. Determination of $DPPH^{\cdot+}$ scavenging activity

The $DPPH^{+}$ scavenging abilities of the infusion were detected by adapting the method reported previously, and the IC_{50} values were presented as μg. mL^{-1} (Yen et al., 2000).

9.2.6.4. Enzyme inhibition assays

9.2.6.4.1. α-Amylase inhibition assay

The assay was performed using a spectrophotometer (T70+Vis, PG Instrument Ltd, United Kingdom) as described in previous studies (Conforti et al., 2005; Kim et al., 2014; Sancheti et al., 2013), with some modifications. Briefly, 100 µL of starch solution (1%) (in 0.02 M phosphate buffer (pH 6.9, containing 0.006 M NaCl) and 50 µL of the sample solution were mixed and incubated at 20 ± 1 °C for 5 minutes. After incubation, 100 µL of the enzyme solution (1 U. mL^{-1} in 0.02 M phosphate buffer (pH 6.9, containing 0.006 M NaCl)) was added and incubated at 20 ± 1 °C for 3 minutes afresh. Next, the reaction was terminated by the addition of 100 µL of *DNS* (*Di-nitro salicylic acid*) colour reagent (96 mM *DNS* was blended in sodium potassium tartrate ($KNaC_4H_4O_6{\cdot}4H_2O$, in 2 N NaOH)) and was immediately shifted into a boiling water bath for 15 minutes, followed by the addition of 900 µL of distilled water; afterwards, the absorbance of the final solution was recorded at 540 nm wavelength.

The percentage of inhibition activity (*IC*%) of the enzyme is achieved in comparison with the control, according to equation 9.1 and expressed as the mean ± *standard deviation* (*SD*). The assay was performed in triplicate within at least four independent experiments.

$$Inhibition\ activity\ (\%) = \left(\frac{A_{Control} - A_{Sample}}{A_{control}}\right) \times 100 \qquad (9.1$$

Inhibition concentration (IC_{50}) values were calculated from concentration versus percentage inhibition curves.

9.2.6.4.2. AChE inhibition assay

AChE inhibition activity was evaluated by a spectrophotometer (T70+Vis, PG Instrument Ltd, United Kingdom) according to the method reported previously, (Ellman et al., 1961; Sancheti et al., 2010) with minor modifications. Accordingly, 100 µL of *AChE* solution (0.03 U. mL^{-1}) (1 mg = 149 units), 50 µL of test sample (dissolved in 30% MeOH) and 900 µL of Tris-HCl buffer (50 mM, pH 8) were mixed vigorously and incubated at 4 °C for 30 minutes. Then, in the reaction mixture, 100 µL of *DTNB* (*5,5'-dithiobis-2-nitrobenzoic acid*) (0.3 mM) and 100 µL of *ATCI* (*Acetylthiocholine iodide*) (1.8 mM) were added and incubated at 37 °C for 20 minutes. Absorbance was detected at a 412 nm wavelength.

The *IC*% of the enzyme is computed in comparison with the control, according to the following *equation* and expressed as the mean ± *SD*. The assay was performed in triplicate within at least four independent experiments.

$$Inhibition\ activity\ (\%) = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100 \qquad (9.2$$

Inhibition concentration (IC_{50}) values were calculated from concentration versus percentage inhibition curves.

9.3. Results and discussion

9.3.1. Bioactive compound contents

In this study, considerable values of bioactive compounds, including TPC, TFC and TMA were detected for the infusion of *P. anisum* L., seeds. Phytochemicals were determined that could considerably diminish the risk of *cancer* by up to 20%, especially against the outbreak of common *cancers* including colon, breast and prostate. Several pathways were attributed to the health benefits of these compounds, including the presence of phytochemicals that modulate the host systems and potentially prepare more robust *anti-tumour* responses such as enhancing the immunity recognition of tumour cells; changing the hormone-dependent growth of the endocrine tumour and, finally, modulating *sterol* biosynthesis (Bradford and Awad, 2007). An extensive range of compounds, including *polyphenols*, *flavonoids*, *tannins*, *flavones*, *lignans*, *sterols* and *triterpenoids*, have shown different potential health benefits such as having *anti-oxidant*, *anti-amyloidogenic*, *anti-cholinesterase* as well as hypolipidemic properties, at different intensities. For instance, *hypolipidemic* properties of bioactive compounds might be generated by the antagonistic properties of the bile acid receptor, *farnesoid X receptor* (Cui et al., 2003). Moreover, simple *phenols* and *phenolic acids* including *caffeic acid*, *catechin*, *eugenol*, *quinones* such as *quinone* and *hypericin*, *flavones* and *flavonoids* including *flavone*, *catechin* and chrysin, *tannins*, *coumarins* including *coumarin*, *warfarin* and 7-*hydroxycoumarin* as well as *terpenoids* have shown substantial potential health benefits. *Anthocyanins* are implicated in the prevention of neural diseases, *cardiovascular* illnesses, *diabetes*, *inflammation* as well as *cancers* and many other such diseases. *Anthocyanins*, as a *flavonoid*, contain two benzene rings joined by a linear three-carbon chain. As is the case for other *flavonoids* and *phenols*, *anthocyanins* exhibit strong *anti-oxidant* qualities that act against neural damage,

cardiovascular illnesses, *cancer*, *diabetes* along with *inflammation* and further disorders. These categories of compounds are reported to be appropriate in the treatment of *hypos*, as well as for human nutrition, and these compounds can suppress tumour cell growth by arresting them between the *S* and *G2* phases of the cell cycle (Nichenametla et al., 2006). *Anthocyanins* can modulate blood pressure in vessels and preserve the integrity of the endothelial cells that line the blood vessel walls. For instance, strawberries (a rich source of *anthocyanins*) have shown inhibiting qualities in the case of esophageal *cancer* and diminished the neuronal ageing in treated animals. Further potential health benefits include allergy relief, a healthy heart and eyesight, ulcer treatment and improved cognitive functions that are all attributed to *anthocyanins*. They have also been shown to have positive effects in the treatment of various diseases. Their positive effects in preventing *cholesterol*-induced atherosclerosis and inhibiting platelet aggregation as well as in the treatment of diseases resulting from capillary fragility have also been shown.

9.3.2. Anti-oxidant activities

The *anti-oxidant* potential of the extracts depends on the type and category of the available bioactive compounds; therefore, in this study, it is necessary to determine the *anti-oxidant* qualities. In other words, the *anti-oxidant* qualities of infusions cannot be detected by individual tests; therefore, it is necessary to perform various tests, including *anti-radical* and reduction powers, along with the tests for total *anti-oxidant* activity. The detected values for TAA, RP, FRAP as well as *IC50* for $ABTS^{+}$ inhibition and $DPPH^{-}$ scavenging activities showed high *anti-oxidant* activities (Table 9.1). The *anti-oxidant* properties of bioactive compounds, particularly *flavonoid* in this research, are attributed to their scavenging and/or chelating potentials. *Flavonoids* might show *anti-oxidant* activities with the scavenging of the molecular species of *active oxygen*, including *ROS*, *hydroxyl radicals* ($^{\cdot}OH$) and -*superoxide* ($.O_2^-$) along with *single oxygen* ($^1O_2$).

The potential donation of hydrogen atoms and/or electrons for the bioactive compound plays a substantial role in demonstrating the *anti-oxidant* activities (Khan et al., 2000). Figure 9.1 shows a significant difference in the obtained *IC50* values of *ABTS* and *DPPH* with the achieved *IC50* of the infusion against *α-Amylase* and *AchE* enzymes.

Table 9.1 Different detected parameters indicating health benefit potentials of *P. anisum* L., seeds.

No	Parameter name (unit)	Detected value
1	TPC ($mg_{(GAE).}\ g^{-1}_{dw}$)	117.853 ± 7.547
2	TFC ($mg_{(QE).}\ g^{-1}_{dw}$)	6.158 ± 0.304
3	TMA ($mg.\ L^{-1}$)	36.737 ± 0.944
4	TAA ($mg_{(AAE).}\ g^{-1}_{dw}$)	165.787 ± 8.067
5	RP ($mg_{(TE).}\ g^{-1}_{dw}$)	132.360 ± 3.418
6	FRAP ($mg_{(TE).}\ g^{-1}_{dw}$)	275.828 ± 21.383
7	$ABTS^{\cdot+}$ inhibition (IC_{50} ($\mu g.\ mL^{-1}$))	299.034 ± 12.935
8	$DPPH^{\cdot-}$ scavenging (IC_{50} ($\mu g.\ mL^{-1}$))	98.470 ± 6.341
9	*α-Amylase* inhibition* (IC_{50} ($mg.\ mL^{-1}$))	31.931 ± 0.188
10	*AchE* inhibition* (IC_{50} ($mg.\ mL^{-1}$))	33.299 ± 0.419

Positive controls for *α-Amylase (Acarbose) and AchE (Tacrine) were obtained* 0.68 ± 0.06 ($mg.\ mL^{-1}$) and 0.89 ± 0.05 ($mg.\ mL^{-1}$), respectively.

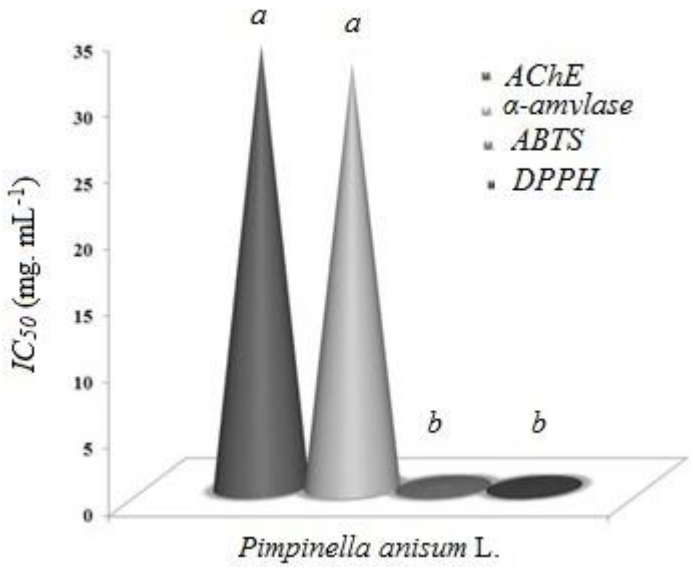


Figure 9.1 *IC50* value of *P. anisum* L., against some tested free radicals and enzymes.

9.3.3. Enzyme inhibition

Portuguese *P. anisum* L., has shown inhibiting activities against *α-Amylase* and *AchE*. As the outcomes obtained by *GC-MS* analysis show (Table 9.2), the seeds of this plant contain compounds with different health-promoting potential. *Cholinergic* deficit is one hypothesis for the cause of *Alzheimer's* disease. Enzymes, including *acety-l* and *butyrylcholinesterase*, have been shown to have a substantial impact on the degradation of main neurotransmitters such as choline. Moreover, the inhibiting activity of the extracts on the activity of *α-Amylase* suggests one pathway for preventing or inhibiting *diabetes*. Therefore, in the next section, the authors will attempt to interpret the role of detected bioactive compounds and the potential health benefits of *P. anisum* L.

9.3.4. GC-MS analysis

Figure 9.2 demonstrates the obtained chromatogram for *GC-MS* analysis of the *P. anisum* L., infusion. Table 9.2 expresses the detected compounds of the *P. anisum* L., seeds. As observed, *fatty acids* representing 47.68% of all the detected compounds in this research are predominant, followed by *triterpenoids* representing 15.56%. *Linoleic acid*, along with *oleic acid*, representing 27.67% is considered to be the predominant *fatty acid*, followed by *palmitic acid* and *octadecanoic acid* along with *octadecadienoic* (*linoleic*) and *docosanoic* (*behenic*) *acids*. Among the *triterpenoids*, *lupeol*, representing an eventual total content of 8.63%, is present in the highest quantities, followed by *β-Amyrin* with 2.72%. Furthermore, among the sugars, representing 2.06% of all the detected bioactive compounds, fructose, with 1.25%, is present in the highest quantity. Figure 9.3 presents the 2D structures of some detected compounds in anise infusion.

9.3.5. Health benefit potential of the detected compounds

Conjugated linoleic acid (*CLA*) was described as being effective against a wide range of health issues including atherosclerosis, *inflammation*, obesity, *diabetes*, carcinogenesis, as well as promoting growth and bone formation-promoting properties (Bergamo et al., 2014). This compound is considered to be an inhibitory factor through all stages of carcinogenesis, initiation, promotion and metastasis along with neovascularization or angiogenesis. On the other hand, *CLA* is described as potentially improving human metabolic syndromes by handling *anti-obesity*, *anti-diabetic* and *anti-hypertensive* properties. *Anti-obesity* and the mechanism for *hypolipidemic* effects of this compound are related to their capacity to lower hepatic *triglyceride* (*TG*) and *cholesterol* concomitant with several procedures. The *anti-diabetic* effect of *CLA* could be due to *PPATγ* activation, similar to the effect of *troglitazone*. *Palmitic acid* is another *fatty acid* component found in considerable quantities. Although *palmitic acid* increases the risk of *cardiovascular* diseases, it may, however, have many potential health benefits including being an *anti-oxidant* and *anti-atherosclerotic*, boosting the sensitivity of cells to insulin and containing *hypoglycaemia* properties that are attributed to it. *Oleic acid* has been proved to increase the absorption of *mitoxantrone* (*MXR*) and causes an increased gene expression of *BCRP* in *Caco-2 cell lines*. *Oleic acid* might diminish the risk of coronary *heart disease* by 20%–40%, mainly through low-density *lipoprotein* (*LDL*) *cholesterol* reduction; moreover, it could enhance *LDL* oxidative and insulin sensitiv-

Table 9.2 Detected compounds profile of extracts of *P. anisum* L., (seeds) obtained by *GC-MS* analysis.

Peak	Constituent	Area (%)	Method of identification
	Sugars	2.06%	
1	Sugar C5	0.33	a, b
2	Sugar C6	0.48	a, b
3	Fructose	1.25	a, b
	Fatty acids	47.68%	
4	*Decanoic acid, trimethylsilyl ester*	0.08	a, b
5	*Dodecanoic acid, trimethylsilyl ester*	0.15	a, b
6	*n-Pentadecanoic acid, trimethylsilyl ester*	0.34	a, b
7	*Fatty acid C16:1*	0.17	a, b
8	***Hexadecanoic acid, trimethylsilyl ester (Palmitic acid)***	10.19	a, b
9	*Fatty acid (ND)*	0.1	a, b
10	*Fatty acid (ND)*	0.68	a, b
11	*Heptadecanoic acid, trimethylsilyl ester*	0.38	a, b
12	*Linoleic acid, trimethylsilyl ester*	0.12	a, b
13	***9,12-Octadecadienoic acid (linoleic acid) + Oleic acid***	25.39	a, b
14	***Octadecanoic acid, trimethylsilyl ester***	**2.78**	a, b
15	*Nonadecanoic acid, TMS*	0.09	a, b
16	*Fatty acid (ND)*	1.12	a, b
17	***Octadecadienoic acid,TMS***	**2.28**	a, b
18	***Eicosanoic (Arachidic) acid, TMS***	0.7	a, b
19	*Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester*	0.75	a, b
20	***Docosanoic (behenic) acid, TMS***	**1.12**	a, b
21	*Octadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester*	0.29	a, b
22	*Tetracosanoic acid, trimethylsilyl ester*	0.5	a, b
23	*Hexacosanoic acid TMS; C:26*	0.18	a, b
24	*Fatty acid C28*	0.27	a, b
	Dicarboxylic acids	**0.98%**	
25	*Malic acid, tris (trimethylsilyl) ester*	0.1	a, b
26	*2-Isopropyl malic acid TMS*	0.02	a, b
27	*2-Butenedioic acid TMS*	0.05	a, b
28	*Heptanedioic acid TMS*	0.04	a, b
29	*Tartaric acid, TMS*	0.08	a, b
30	*Octanedioic acid*	0.69	a, b
	Aromatics	**0.59%**	
31	*Benzaldehyde, 3-methoxy-4-[(trimethylsilyl)oxy]-*	0.1	a, b
32	*Benzeneacetic acid, 4-[(trimethylsilyl)oxy]-, trimethylsilyl ester*	0.1	a, b

Table 9.2 Continued

33	*Benzaldehyde, 3,5-dimethoxy-4-[(trimethylsilyl)oxy]-*	0.08	a, b
34	*Benzoic acid, 3-methoxy-4-[(trimethylsilyl)oxy]-, trimethylsilyl ester*	0.05	a, b
35	*Azelaic acid, bis (trimethylsilyl) ester*	0.26	a, b
	Alkanes	1.79%	a, b
36	*Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-, scyllo-*	0.06	a, b
	Fatty alcohol	0.57%	
37	*Steryl alcohol (fatty alcohols)*	0.57	a, b
	Hydroxy fatty acids	1.1%	
38	*Hydroxy acid C:28*	0.48	a, b
39	*Hydroxy acid C:30*	0.62	a, b
	Sterol	7.29%	
40	*Campesterol TMS*	0.28	a, b
41	*Stigmasterol trimethylsilyl ether*	0.98	a, b
42	*β-sitosterol trimethylsilyl ether*	5.31	a, b
43	*Sitosterol – glucopyranoside*	0.72	a, b
	Triterpenoids	15.56	
44	***β-Amyrin TMS***	2.72	a, b
45-47	***Lupeol***	8.63	a, b
48	*Betulinic acid*	0.57	a, b
49	*Oleanolic acid TMS*	0.68	a, b
50	***Betulinic acid TMS***	1.77	a, b
51	***Ursolic acid***	1.19	a, b
	Others	1.34%	
52	*Octadec-9Z-enol TMS ether*	0.65	a, b
53	*6,7-dihydroxycoumarin -beta- d-glucopyranose*	0.69	a, b
	Not identified		
54	ND	24.4%	a, b

*Retention time obtained by chromatogram, ND: presents not detected, a= Retention Index; b= MS (*GC-MS*) Library.

-ity. Also, the use of an *MUFA*, like *oleic acid*, in dishes could restrict the intake of further saturated *fatty acids*. With the consumption of *oleic acid, TG* concentration decreases by 19% and the concentration of *high-density lipoprotein* (*HDL*) *cholesterol* is increased by 19%. Some potential health benefits attributed to *oleic acid* include: reducing blood pressure, burning fat content, preventing *ulcerative colitis*, alleviating *type II diabetes* and, finally, protecting against the effects of *free radicals* on membranes.

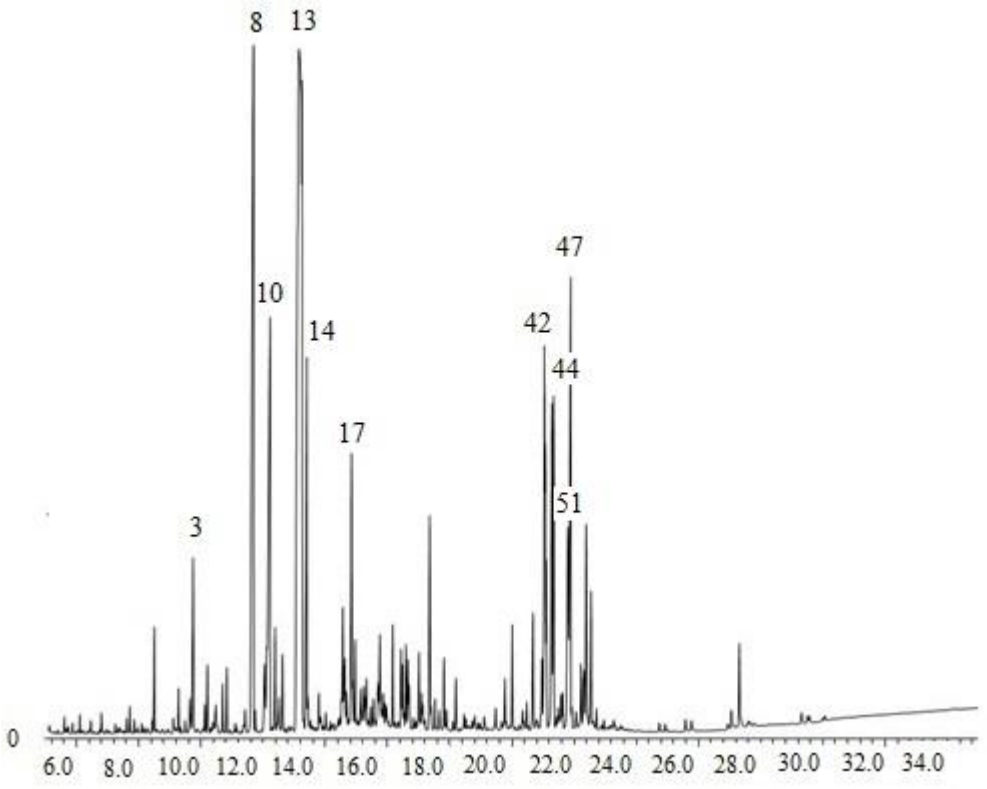
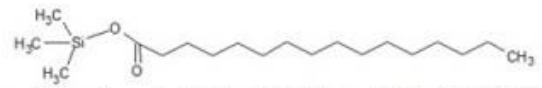


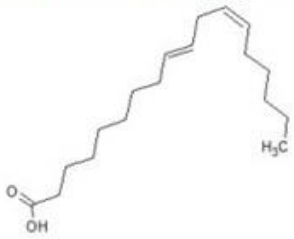
Figure 9.2 Obtained chromatogram of *P. anisum* L., seeds' infusion by *GC-MS* analysis.

Following *fatty acids*, *triterpenoids* were determined in high quantities in *P. anisum* L., *Lupeol*, *β-amyrin*, *BAs* and *ursolic acids* were also detected in high quantities in *P. anisum* L., The detected *triterpenoids* have been proved to have considerable potential health benefits. For instance, *lupeol* acts against several disorders, including arthritis, *diabetes, heart disease, renal toxicity* and *cancer*, along with *hepatic toxicity* with different pathways (Sudhahar et al., 2007). This compound and its derivatives, including *palmitate*, *acetate* and *linoleate*, exhibited higher *anti-inflammatory* properties than those commonly found in synthetic medicines. Its *anti-inflammatory* activity is attributed to its activity in the modulation of the immune system and the generation of *inflammatory* factors. *Lupeol* has not been proved to have any *anti-nociceptive* and *ulcerogenic* actions in arthritic animals; therefore, the *lupeol* mechanism of actions differs from that being used regularly, *anti-inflammatory* medicines (Preetha et al., 2006).

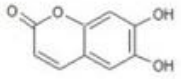


Palmitic acid (Hexadecanoic acid, trimethylsilyl ester) $C_{12}H_{42}O_2Si$ (328.60 g. mol^{-1})

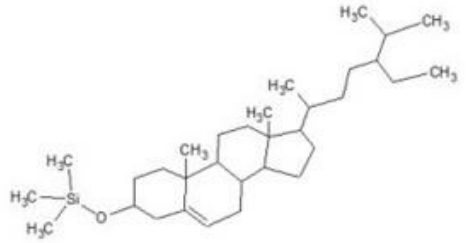
Oleic acid, Cis-Octadecenoic acid, $C_{18}H_{34}O_2$ (282.46 g.mol^{-1})



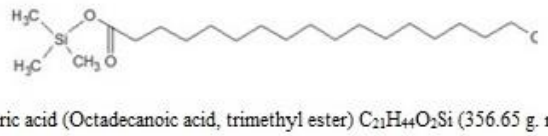
Linoleic acid (9,12-Octadecadienoic acid) $C_{18}H_{32}O_2$ (280.44 g.mol^{-1})



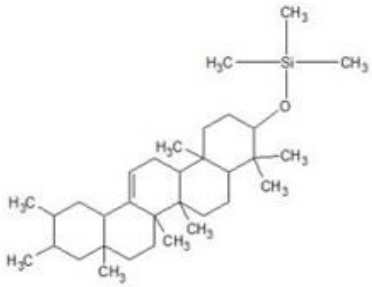
6,7-Dihydroxycoumarin, di-TMS, $C_6H_9O_4$ (356.65 g.mol^{-1})



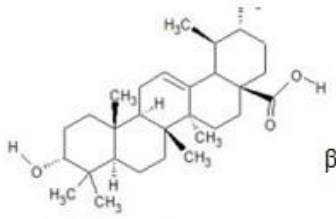
β-Sitosterol trimethylsilyl ether (β-Sitosterol TMS) $C_{32}H_{58}OSi$ (486.88 g. mol^{-1})



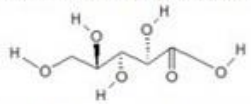
Stearic acid (Octadecanoic acid, trimethyl ester) $C_{21}H_{44}O_2Si$ (356.65 g. mol^{-1})



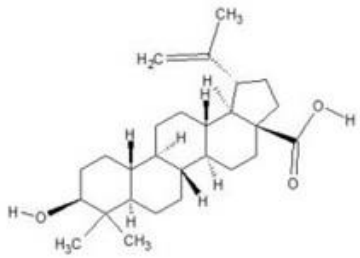
β-Amyrin TMS (Silane, trimethyl (olean-12-en-3β-yloxy)-$C_{33}H_{58}OSi$ (498.89 g. mol^{-1}))



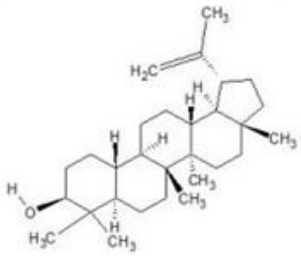
Ursolic acid, 3 β-Hydroxy 12-en-28-oic acid $C_{30}H_{48}O_3$ (456.70 g. mol^{-1})



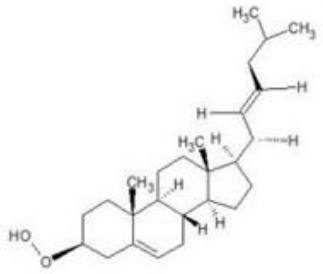
Fructose, D(-)Fructose (3S, 4R, 5R)-1, 2, 4, 6-pentahydroxyhexan-2-one $C_6H_{12}O_6$ (180.15 g. mol^{-1})



Betulinic acid



Lupeol



Stigmasterol

Figure. 9.3 2D structures of main detected compounds of *P. anisum* L., seeds' infusion by *GC-MS* analysis (Drawn by *ChemSketch* version 2012).

On the other hand, *lupeol* has demonstrated *anti-diabetic* potential in *in vivo* models. The mechanism of actions for the potential *anti-diabetic* properties of *lupeol* might be justified in the following way: this compound could suppress carbohydrate absorption using the inhibitory effects on enzymes that participate in glucose digestion; however, the complete mechanism of action of *lupeol* is not well known. It is possible that the targeting of *PTP1B*, *α-Amylase* and *α-Glucosidase* activities could be a potential mechanism (Ortiz-Andrade et al., 2007). In summary, many potential health benefits are attributed to *lupeol* including those that are *anti-microbial*, *anti-protozoal*, *anti-cancerous*, *anti-diabetic*, *cardio-protective*, *anti-inflammatory*, *skin protective*, *hepatoprotective*, *nephroprotective* and so on. *β-Amyrin* is another major *triterpenoid* component that is detected in this study, which demonstrates a wide range of potential health benefits, including *anti-inflammatory*, *gastro-protective* and *anti-pruritic* effects. *α-* and *β-Amyrin* have demonstrated *hypoglycemia* activity in *in vivo* models. The evidence obtained by different experiments has revealed that amyrin exhibits systemic *anti-nociceptive*, *anti-inflammatory*, *anti-pruritic*, *hepatoprotective* and *gastroprotective* properties which are all valid in *in vivo* models. *α-* and *β-Amyrin* are examples of pentacyclic *triterpene* alcohols that not only constitute secondary metabolites themselves but can also undergo oxidation reactions to yield other derivatives such as *BAs* and *ursolic* and *maslinic acids*. Another *terpenoid* detected in this study was *BAs*. *BAs,* along with *betulin*, exhibits diverse pharmacological properties including being *anti-HIV*, *anti-cancer* and *anti-inflammatory*. Scientists have hypothesised that *BAs* could be a potential source of *anti-tumour* and *anti-inflammatory* activities. Among *betulin* and *BAs* derivatives, recently *bevirimat* has been detected to exhibit remarkable *anti-HIV* properties against drug-resistant *HIV* isolates, representing a uniquely detected natural compound (Bedard and Krause, 2007). These pentacyclic *triterpenoids* spread broadly in different parts of the plants including the stem, root, leaves and even bark. The *anti-HIV* mechanism of the actions of *BAs* is interpreted as follows: *BAs* derivatives contain a modification in position C: 28 of the *carboxylic acid* group appearing to inhibit *HIV-1* entry cells, while compounds containing an alteration in position C: 3 of the carboxylic group appear to act at the later stages of the virus life cycle, such as assembly and maturation. *Ursolic acid* is another *terpenoid* found in anise infusion. Many biological effects, including being *anti-tumour*, *anti-inflammatory* and *anti-oxidant*, are attributed to *ursolic acid*. Other applications of

this compound include improvements in cognitive deficits caused by various deficiencies along with neuroprotective, *anti-septic* and *anti-depressant*-like effects (Colla et al., 2014). Moreover, further pharmacological potential benefits, including being *hepatoprotective*, *immunomodulatory*, *anti-inflammatory*, *anti-diabetic*, *anti-viral*, *anti-ulcer* as well as *anti-cancer*, have all been recorded for *ursolic acid*. The potential health benefits of *ursolic acid*, such as *anti-carcinogenic* effects, are attributed to its ability to induce *cancer* cell apoptosis, demonstrating *tumorigenesis* and the inhibition of *cancer* proliferation. A novel *terpene* hydrocarbon was detected in *P. anisum* L., and named neophytadiene. As has been demonstrated, *fatty acids* showed the highest quantity among others followed by *triterpenoids* and *sterols* in the infusion of anise seeds. In mice treated with anise infusion, a considerable difference was shown between the *cholesterol* and TG content of the bloods in tested mice, demonstrating the *hypoglycaemic* and *hypolipidemic* properties of anise seeds. *Sterols* are ranked third after *fatty acids* and *terpenoids* in quantity. *β-Sitosterol* is the main *sterol* compound detected in *P. anisum* L., Many potential health benefits are attributed to this compound. This compound is a major plant *sterol* that regulates blood *cholesterol* levels and is considered to be an *anti-atherogenic*, *anti-diabetic* and *anti-asthmatic* agent. This compound has shown protective effectiveness against colon (*HT-29*) and prostate (*LNCaP*) *cancer* cell lines. This compound appears to have neuroprotective properties. *Phytosterols* are also connected to the prevention of *cancer* cell growth, angiogenesis and apoptosis of *cancer* cells in organs including lungs, stomach, prostate, ovaries and breasts.

9.4. Conclusions

Several parameters are determined in an aqueous infusion of *P. anisum* L., seeds to evaluate the bioactivity of this plant. Anise was shown to be a rich source of *phenols* and *flavonoids*, and on the other hand, the considerable values for *TAA*, *FRAP* and *RP* demonstrated high *anti-oxidative* activities in the extracted infusion. Moreover, a high *anti-radical* activity for the infusion was obtained where significant differences were detected between the *IC50* values of *ABTS*, *DPPH* with *α-Amylase* and *AChE*, demonstrating that *free radicals* are not the only cause of different *chronic* disorders and that a wide range of risk factors might be attributed to revealing *chronic* disorders. The *IC50* value obtained for enzyme inhibitions for anise confirmed the potential of using anise infusions against *chronic* disorders such

as *diabetes* and *Alzheimer's* disease, as well as further *chronic* disorders such as *cancer*. The result of the *GC-MS* analysis showed that *fatty acids* play the most substantial role in promoting health benefits, followed by *terpenoids* and *sterols*.

It could be suggested that anise is a potentially beneficial substance for use against *chronic* disorders, and they suggest performing further *in vitro* and *in vivo* tests to establish more accurate findings based on the potential health benefits of anise.

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CHAPTER 10

Concluding Remarks

This chapter presents the main achievements and major conclusions of this thesis.

10.1. Concluding remarks

From the beginning to the end of this research, the authors' aim is to produce scientific information for use in different industries including nutraceutical, pharmaceutical and cosmetic to support consumers in having access to fresh and safe products. The major intention of this research was to investigate the wide range of Iranian and Portugues plants that have been identified traditionally as potential sources for the treatment of widespread disorders including chronic and even acute diseases, whilst the other objective was the characterisation of the profile of the main bioactive compounds or phytochemicals in the selected plants' extracts. Another aspect that the current research attempted to determine was the optimisation of the aqueous extraction process of plants within different conditions including temperature, time, microwaves, proportions of sample/solvent etc. Moreover, the extraction of the encapsulated bioactive compounds in *simulated gastric fluid* (*SGF*) was modeled in different concentrations of filling substances in the beads' matrix and extraction time. Despite studying the different plants species, the studied parts of the plants were selected according to their traditional applications. The achieved information is most considerable for consumers and patients who are seeking herbal medicines with higher impact and fewer adverse effects.

Medicinal plants have been considered as sources of bioactive compounds and enrichment source in different products. The presence of a wide range of bioactive compounds in the tested plants, including *anthocyanins*, *chlorophylls*, *phenols*, *flavonoids*, *terpenes*, *free fatty acids*, *fatty alcohols*, *aromatics*, *sterols*, *monoacylglycerols*, *waxes*, *di-acids*, *α-hydroxy fatty acids*, *alkanes* and sugars, etc., in principal are related to their nutritional values - a wide confine of potential health benefits against chronic and acute disorders and preservative effects in the enhancement of the shelf life of different products related to the nutraceutical and pharmaceutical industries. In the present thesis, new experiments have been performed on the plants which have not previously been studied on such a scale. The use of water in the extraction as the healthier extraction approach with minimum disadvantages and costs, alongside the use of different *in vitro* experiments for the bioactivity determination of the plants and analytical methodologies for the specification of their compounds' profiles were encouraged. Hot aqueous extracts, along with *microwave-assisted extraction (MAE)* were used to prove that these

methods could be considered as potential extraction approaches with fewer disadvantages compared to other organic solvents. According to the wide range of extraction methods of the bioactive compounds of the plants and beads (encapsulated compounds) with different methodologies and designed models, the main achievements of this thesis were:

With the designed model using *RSM* with three different variables in the optimization of *encapsulation efficiency* on TAA value using water as solvent, the optimal starch concentration of the beads (=1.091%) was determined with 68.651% efficiency to screen the release kinetics of the bioactive compounds *in vivo* models with the highest efficiency.

An optimised *simulated gastric fluid* (*SGF*) extraction of the encapsulated bioactive compounds (sodium alginate in combine of potato starch as a matrix) within different independent variables (three) was successfully designed using *Response Surface Methodology* (*RSM*) and *Artificial Neural Networks* (*ANN*). Starch concentration of the beads (0.934%) with the desirability of 0.96% was detected as the optimized value. *ANN* with demonstrating the lower *RMSE* and higher R^2 values compared to *RSM* model showed the highest reliability.

The sequence of different extraction methodologies to obtain aqueous extracts with the highest bioactivity, including the maximum quantity of *anthocyanins*, *phenols* and *flavonoids* demonstrated that hot aqueous extracts (90 ºC for 5 minutes), along with the *microwave-assisted extraction* (*MAE*) with microwave power in the range of 300-500 (W) could extract the bioactive compounds with maximum performance.

An optimal *microwave assisted extraction* (*MAE*) procedure using water as the solvent was performed successfully. This procedure demonstrated that it could be an efficient methodology for the extraction of water-soluble bioactive compounds. This procedure also revealed the optimal proportion of the solvent to sample needed in the range of 30-35 mL. g^{-1} in the extraction of the bioactive compounds.

Due to the low concentration of the bioactive compounds (milligram or microgram. 100 gr^{-1} of plants dependent on the plants species or the part used) in the extracts obtained from the samples (seeds, leaves, flowers and or aerial parts) and according to the toxicity of the available organic solvents commonly used for extraction, the optimisation of the aqueous extraction procedure seemed important on a commercial or industrial scale. Due to most of the plants studied in this thesis being

listed in the aromatic plants category, a *Gas Chromatography* (*GC*) system equipped with a *ZB-5HT* inferno capillary *GC* column with *Mass Detector* (*MS*) was applied. Concerning the detection and identification of the bioactive compounds in this thesis, the major achievements were as the following:

The suggested *GC-MS* method was applied to characterise and quantify the bioactive compounds' profiles. Five Iranian and one Portugues plants were selected for *GC-MS* analysis. The criteria of the selection were based on the bioactivity of those plants detected during the previous experiments.

A wide confine of the bioactive compounds, including *free fatty acids*, *fatty alcohols*, *monoacylglycerols*, *sugars*, *sterols*, *triterpenes*, *phenols*, aromatic compounds, *alkanes*, *α-Hydroxy fatty acids*, *di-acids* and *waxes* have been detected. The identified compounds by *GC-MS* analysis presented differences in the profiles and percentages of the detected compounds in the leaves compared to the seeds. The leaves, including *R. officinalis* L.; *S. officinalis* L., and *T. vulgaris* L., respectively, presented the higher percentages of the *triterpenoids* combined with *sterols* compared to seeds. Meanwhile, the analysed seeds contained higher quantities of fatty acids in comparison to other detected compounds. Moreover, the results did not prove any *monoacylglycerols* content in the leaves, while Iranian *P. anisum* L., and *C. sativum* L., demonstrated 2.16% and 1.34% of *monoacylglycerols* content respectively.

According to the identified compounds of the plants using the *GC-MS* analysis and their *in vitro anti-diabetic* and *anti-Alzheimer's* disease potential, *anti-bacterial* and *anti-oxidant* properties, different hypotheses could be considered - firstly, *triterpenoids* combined with *sterols* in the leaves and *fatty acids* in the seeds have the predominant activities in demonstrating potential health benefits; secondly, the wide range of the plants' advantages might not be attributed to the existence of just one or two categories of compounds, but synergistic activities among at least two or more categories of compounds with bioactivities could be considered in revealing potential health benefit potential.

Gas chromatography-mass spectrometry (*GC-MS*) analysis revealed the highest quantity of *triterpenoids* + *sterols* in the leaves as the following: *R. officinalis* L., *S. officinalis* L., and *T. vulgaris* L., with demonstrating 63.42%; 47.64% and 33.51% of all of the detected compounds respectively. Among the tested seeds, Portugues *P. anisum* L., demonstrated the higher percentage of *triterpenes* in

conjunction of *sterols* (22.85%) that were 10-fold higher than the same value in Iranian *P. anisum* L. (2.07%).

On the other hand, the characterised *fatty acid* contents using *Gas Chromatography-Mass Spectrometry (GC-MS)* revealed that seeds contain a higher quantity of *fatty acids* compared to leaves. *C. sativum* L., presented 80.22% *fatty acid* content compared to all detected compounds followed by Portugues and Iranian *P. anisum* L., respectively, demonstrating 49.04% and 47.68% of all detected compounds.

In relation to the various extraction methodologies performed on the samples, the infusions expressed different *anti-oxidant* and *anti-microbial* potential, among them the hot aqueous extracts demonstrated the higher activity in most of the extracts compared to the cold aqueous infusions. Among the Iranian plants in most of the determined parameters, the selected leaves demonstrated the higher activities followed by flowers and seeds based on the tested parameters. In the achieved results of the enzyme inhibition activities of the plants, all of the examined plants presented activity showing their eventual activities against *Alzheimer's disease* and *diabetes*. Most of the leaves expressed higher activity against *α-Glucosidase*; *α-Amylase* and *Acetylcholinesterase*. In the seeds and flowers, with respect to the species, different enzyme inhibition activities were recorded.

The inhibition activities of the plant extracts against five different bacterial species have been indicated some extract's potentials in suppressing of some foodborne and pathogenic bacterial species. However, most of the infusions might present considerable inhibition activity in higher concentrations than the detected standard range in this research (>10 mg. mL^{-1}).

A study on the optimisation of the release kinetics of the encapsulated bioactive compounds of Portugues *Olea europaea* L. var. sylvestris., leaves by *Face-Centred Central Composite Design* (FCCCD) revealed that the selected model could predict the optimised concentration of the potato starch of the beads or capsules since the higher desirability (≥0.862) for each of the optimised responses have been obtained. In other research on the modelling of the *encapsulation efficiency* of the *microwave-assisted extraction* (*MAE*) (300 W) of *phenols* of *Alisma plantago-aquatica* L., *(Pa ghazeh)* leaves, both of *central composite-orthogonal blocks (CCD-Orthogonal Blocks*) of the *Response Surface Methodology* (*RSM*) and *multilayer perceptron* (*MLP*) neural network design of *Artificial Neural Network* (*ANN*) model

have been applied and compared. The obtained values for both models confirmed the suitability of both; however the *ANN* model demonstrated lower *RMSE* value and higher R^2 value expressing higher reliability than the *RSM* design. These findings demonstrated the suitability of the applied statistical models in the prediction of the extraction processes.

The optimal conditions of the *microwave-assisted extraction* (*MAE*) of the total monomeric *anthocyanins* (TMA) have been modelled using the *Box-Behnken design (BBD)* with three independent variables. The quadratic modes of the three independent variables for TMA response and one of the interactive modes alongside two of the quadratic modes of the TAA responses were selected as being negatively significant ($p<0.05$). Regarding the obtained *p* values of the lack of fit and model, the *Box-Behnken design (BBD)* expressed good reliability in the optimisation, in which the predicted response values of TMA and TAA were obtained with the desirability values of 0.878 and 0.952 respectively.

The effect of geological conditions on the same species has been identified by the results obtained by Iranian and Portugues plants from the same species (*P. anisum* L.), since the values obtained for the different *anti-oxidant* properties of Portugues *P. anisum* L., are significantly higher. However, the inhibition activity against enzymes in the Iranian species was found to be significantly stronger than the Portugues one. In the identified bioactive compounds by *GC-MS*, Portugues *P. anisum* L., presented higher quantities of *triterpenoids* than the Iranian variety (15.56% versus 2.07%), and lower *fatty acid contents* (47.68% versus 49.04%), demonstrating the effectiveness of a wide range of detected and non-detected bioactive compounds in revealing potential health benefits. Therefore growing conditions including light intensity, soil quality, day length, available water, etc., have substantial roles in the bioactivity of plants, even of the same species.

In relation to the potential health benefits of the studied plants, plants species and the used parts (seeds, leaves, flowers and or whole aerial parts) have the greatest effectiveness on the bioactive compound content; their ratio and as a result in presenting potential health benefits. The extraction conditions of the compounds such as solvent's temperature, the presence of microwaves and stirring rate as well as the concentration of the filling substances in the prepared beads' matrix in the encapsulated extracts had very considerable impacts on the quantity of the released compounds in the solvents. A widespread range of the characterised compounds and

their proportions, as well as higher *correlation coefficients* (R^2) between the different *anti-oxidant* parameters achieved, revealed that the potential health benefits of the plants might be created by the synergistic effects of among different compounds.