In vitro Antidiabetic, anti-obesity and antioxidant proprities of Rosemary extracts

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ABSTRACT

Diabetes mellitus is a serious health problem worldwide that has adverse and long-lasting consequences for individuals, families, and communities. Hence, this study sought to investigate the inhibitory potential of rosemary extracts on key-enzymes related to diabetes such as α-amylase and pancreatic lipase activities, as well as to assess their antioxidant properties in vitro. The IC_{50} values of Rosemary essential oil, ethyl acetate and methanolic extracts against α-amylase were 28.36, 34.11 and 30.39 µg/mL respectively, and those against pancreatic lipase were 32.25, 36.64 and 34.07 µg/mL, suggesting strong anti-diabetic and anti-obesity effects of Rosemary. The methanolic extract was found to be the highest in levels of phenolic (282.98 µgGAE/mg extract) and flavonoids (161.05 µg QE /mg extract) contents as well as in the antioxidant activity (IC_{50} = 15.82 µg/mL) as compared to other extracts ethyl acetate (IC_{50} = 32.23 µg/mL) and essential oil (IC_{50} = 96.12 µg/mL).

Antioxidant efficacy of Rosemary extracts has been estimated in the stabilization of sunflower oil (SFO) at three different concentrations, i.e. 200 (SFO-200), 500 (SFO-500) and 1000 ppm (SFO-1000). Results showed the highest efficiency of SFO-1000.

The results obtained in this study demonstrated for the first time that Rosemary is a potent source of natural inhibitors of α-amylase and pancreatic lipase with powerful antioxidants proprieties that might be used in the food stabilization and the prevention of diabetes and obesity complications as a complementary pharmacological drug.

Keywords

Rosemary; diabetes; α-amylase; lipase; essential oil; oil stabilization

Academic Discipline And Sub-Disciplines

Chemistry

SUBJECT CLASSIFICATION

Plant extracts with antioxidant, antiobesity and antidiabetic activties

TYPE (METHOD/APPROACH)

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INTRODUCTION

Diabetes mellitus is a chronic disorder and considered as a major risk for developing several medical complications, including coronary vascular diseases and dyslipidemia [1]. One of the therapeutic approaches to treat the diabetes is to decrease the postprandial hyperglycemia by retarding absorption of glucose. Inhibition of carbohydrate-hydrolyzing enzymes, such as α-amylase, is considered a possible pathway because the enzyme plays a key role in digesting carbohydrates [2]. On the other side, diabetes mellitus is well known by higher risk association with hyperlipidemia [3]. In fact, pancreatic lipase is secreted from the pancreas and hydrolyzes non-absorbable triglycerides (TG) into absorbable glycerol and fatty acids by small intestine [4]. Thus, pancreatic lipase inhibitors may reduce postprandial plasma lipids level through delaying TG hydrolysis in the gastrointestinal tract and therefore, free fatty acids absorption. Hence, pancreatic lipase inhibitors are considered to be a valuable therapeutic tool for management of hyperlipidemia in humans [5]. Moreover, oxidative stress is highly correlated with a wide variety of inflammatory and metabolic disease states, including diabetes. Indeed, the increase of reactive oxygen species (ROS) leads to damage of β-cells through the induction of apoptosis and suppression of insulin biosynthesis [6]. On the other hand, Lipid peroxidation is responsible for the quality deterioration of vegetable oils, fats and other food systems [7]. It lowers the nutritive value of food and deteriorates its flavour   and taste [8]. It also causes aging, heart diseases, stoke, emphysema, mutagenesis and carcinogenesis [9]. The oil industry has to pay special attention in this context, as oils, fats and fatty foods suffer stability problems. Oil manufacturers aim at producing foods that maintain their shelf life and nutritional quality over a defined period. Thus, the use of antioxidants to minimize the oxidation of lipids in food materials is extensively practised.

In today's consumer perception of agriculture and food production, aspects like health, safety   and quality have become the key words. In these circumstances, research on development of safer natural antioxidants is the need of hour. Plant extracts provide phenolic antioxidants that might exhibit strong activity. Subjects of investigation have been various spice, herb and olive mill waste extracts, as well as fruits and vegetables [10, 11, 12]. In a previous study, we have used the polyphenolic extract from olive mill waste water and basil extract for the stabilization of refined oils [13,14].

Among natural herbs, rosemary has been widely accepted as one of the species with the highest antioxidant activity [15]. Rosemary (*Rosmarinus officinalis L.*, Lamiaceae) is an aromatic, evergreen, shrubby herb indigenous to most countries bordering on the Mediterranean sea, which is widely used in pharmaceutical products and folk medicine [16]. In Tunisia, it is traditionally used for the treatment of auricular diseases, liver diseases, asthma, dermatoses, bronchitis, cough, asthenia, gastralgia, otitis and cold. Rosemary bears linear, leathery dark green, needle like leaves and light, sky blue flowers. When dried, the leaves become rolled in appearance and dark green to brownish green in color [17].

The purpose of this work is to assess the inhibitory potential of Rosemary extracts against α-amylase and pancreatic lipase in vitro and to estimate the stabilizing efficiency of these extracts against oxidative deterioration. Refined, bleached and deodorized (RBD) sunflower oil was chosen to evaluate the antioxidant efficiency of rosemary extracts, because of its wide use among Tunisian population and due to its higher content of polyunsaturated fatty acids. Furthermore, the stabilization effect is more pronounced in sunflower oil [18].

EXPERIMENTAL

Materials

Sample of refined-bleached-deodorized (RBD) sunflower oil (SFO), without additives was received from a local commercial refining plant (Agrimed, Sfax). Fresh rosemary (*Rosmarinus officinalis L.*) leaves were harvested from Sfax province in the center of Tunisia in October 2012. All the chemicals and reagents used were of analytical reagent grade and were purchased from Fluka, or Sigma Chemical Co (St. Louis, MO, USA).

Solvent Extraction

Dried rosemary leaves (500 g) were chopped and extracted with organic solvents of increasing polarity: ethyl acetate and methanol. The extraction procedure is reported in our previous study [14] .

Extraction of essential oil

The fresh aerial part of rosemary was completely immersed in water and hydro-distilled for 4 hours in a Clevenger-type apparatus giving greenish-yellow oil. The essential oil was taken up in diethyl ether, dried over anhydrous sodium sulphate and stored in a dark glass bottle at 4 °C until tested and analyzed. The extraction yield was 0.83 % (w/w).

Liquid chromatography-mass spectrometry (LC-MS)

Electrospray ionisation mass spectroscopic (ESIeMS) analysis of phenolic compounds in MeOH was performed using an Applied Biosystems (LC/MSD TRAP _ CT) using the separation module (Knawer Analogy). Compounds were separated on C18 column (Zorbax, 2.6 ; 250 mm, 3.5 mm particle size) and a diode array detector (DAD) using a gradient system consisting of solvent A (water, 0.1% formic acid) and solvent B (Acetonitrile, 0.1% formic acid). Column temperature was maintained at 25 °C. A stepwise gradient system started from 90% A at 1 min, to 20% B at 4 min, 80% B at 30 min, 100% B at 32 min,100% B at 36 min and 20% B at 38 min was applied at a flow rate of 500 μl/min 6 min. Electrospray mass spectra data were recorded on a negative ionisation mode for a mass range 50 *m/z* to 1000 *m/z*. Mass spectra were achieved by electrospray ionisation in negative mode. Capillary voltage was set at 4500 v. The electrospray probe-flow was adjusted to 8 ml/min. Identification of the phenolic compounds was achieved by comparison with ESIeMS spectra comparisons with literature reports.

Determination of total flavonoid concentration

The content of total flavonoid was determined by colorimetric assays using the AlCl$_3$ method of Lamaison and Carnat [19] as described by Khadri et al. [20]. Briefly, 1 ml of extract solution was added to 1 ml of 2% aluminium trichloride (AlCl$_3$) methanolic solution. The absorbance was measured 10 min later at 415 nm. The total flavonoid content was calculated by a standard quercetin graph (treated in the same conditions) and the results expressed in µg of quercetin equivalents per mg of dry weight of extract. The assay was performed in triplicate for each extract.

Determination of total phenolic concentration

Total phenolic contents were assayed using the Folin–Ciocalteu reagent, following Singleton's method slightly modified by Oktay et al. [21] and gallic acid as a standard. The total phenolic content was calculated by a standard gallic acid graph, and the results expressed in µg of gallic acid equivalents per mg of dry weight of extract. The assay was performed in triplicate for each extract [14].

Determination of α-amylase activity in vitro

The in vitro α-amylase inhibition activity of all extracts was determined based on the spectrophotometric assay using acarbose as the reference compound [22]. The plant extract was dissolved in DMSO to give concentrations from 25, 50 and 100 μg/ml. The enzyme α-amylase solution was prepared by mixing 3.246 mg of α-amylase in 100 ml of 40 mM phosphate buffer, pH 6.9. Positive control, acarbose was obtained by dissolving 50 mg in 50 ml of phosphate buffer and diluted to get concentrations of 25, 50 and 100 μg/ml. The essays were conducted by mixing 80μl of plant extract, 20μl of α-amylase solution and 1 ml of 2-chloro-4-nitrophenol-α-D-maltotrioside (CNPG3). The mixture was incubated at 37°C for 5 minutes. The absorbance was measured at 405 nm. Similarly, a control reaction was carried out without the plant extract/acarbose. Percentage inhibition (PI) was calculated by the expression:

$$PI = \frac{Absorbance_{Control} - Absorbance_{test}}{Absorbance_{control}} \times 100$$

Pancreatic lipase in vitro assay

The method was modified from the assay reported by Nakai et al. [23], in which 4-methylumbelliferyl oleate (4-MU oleate) was used as a substrate to measure the pancreatic lipase inhibitory activity of all samples. Briefly, the assay was conducted by mixing 50 µL of the pancreatic lipase solution (2 unit/ml) in a buffer consisting of 50 mmol/L Tris HCl (pH 8.0), 100 µL of diluted sample solutions and 50 µL of 0.5 mmol/L 4-MU solution dissolved in the above buffer in the well of a 96 micro well plate to start the enzyme reaction. The plate was immediately placed in the 37°C pre-heating FL×800 micro plate fluorescence reader (Bio-Tek® Instruments, Inc., Winooski, VT) to measure the amount of 4-methylumbelliferone released by lipase every minute for 30 min at an excitation wavelength of 360 nm with a tolerance of ±40 nm and an emission wavelength of 455 nm with a tolerance of ±20 nm. The lipase inhibitive activity was determined by measuring the effect on the enzyme reaction rate after adding extracts, compared with the control. Fluvastatin was used as positive control.

$$PI = \frac{Absorbance_{Control} - Absorbance_{test}}{Absorbance_{control}} \times 100$$

DPPH radical scavenging assay

Determination of antioxidant activity of rosemary extracts and essential oil was accomplished using the 1,1-diphenyl-2-picrylhydrazyl (DPPH radical) scavenging method as employed by Fki et al. [13] using a Shimadzu UV-160A spectrophotometer, BHT was employed as reference [14].The antioxidant activity of each test sample and BHT was expressed in terms of concentration required to inhibit 50% DPPH radical formation (IC$_{50}$ µg/ml) and calculated from the log-dose inhibition curve.

Gas chromatography–mass spectrometry (GC–MS)

The analysis of the essential oil was performed on a GC–MS HP model 5975B inert MSD (Agilent Technologies, J&W Scientific Products, Palo Alto, CA, USA), equipped with an Agilent Technologies capillary DB-5MS column (30 m length; 0.25 mm i.d.; 0.25 mm film thickness), and coupled to a mass selective detector (MSD5975B, ionization voltage 70 eV; all Agilent, Santa Clara, CA). The carrier gas was He and was used at 1 ml min^{-1} flow rate. The oven temperature program was as follows: 1 min at 100 °C ramped from 100 to 260 °C at 4 °C min^{-1} and 10 min at 260 °C. The chromatograph was equipped with a split/splitless injector used in the split mode. The split ratio was 1:100. Identification of components was assigned by matching their mass spectra with Wiley and NIST library data, standards of the main components and comparing their Kovats Retention Indices (KRI) with reference libraries [24, 25] and from the literature. The component concentration was obtained by semi-quantification by peak area integration from GC peaks and by applying the correction factors.

Evaluation of thermal stability

Methanolic extract, exhibiting the powerful antioxidant activity, was used for further studies. Thermal stability of rosemary methanolic extract was evaluated by heating at 185 °C in an oven for a period of 120 min in separate crucibles. After each interval, a crucible was removed from the oven, cooled to room temperature and used for antioxidant activity determination following the DPPH radical scavenging assay.

Sample preparation for oxidative stability determination

The rosemary methanolic extract (RME) was applied to preheated RBD sunflower oil at different concentrations (200, 500 and 1000 ppm, based on extract weight), in a series of dark brown coloured bottles to examine its antioxidative activity. Synthetic antioxidant (BHT) was employed at its legal limit of 200 ppm [26] . The antioxidant enriched oil samples were evaporated in a vacuum evaporator below 40 °C to evaporate the solvent and then subjected to Rancimat assay for oil oxidation evaluation.

Evaluation of oil oxidation by the rancimat

Induction time to primary oxidation of lipid in sunflower oil was measured using a Rancimat apparatus (Metrohm, Herisau, Switzerland)]. Induction period (IP), the time elapsed from the beginning until the oil starts to become rancid, was measured by drawing tangents on both sides of the induction curve, the intercept of which meet the time axis.

Weight gain analysis

Analysis procedure is the same adopted in our previous study [14]. For weight gain analyses, 2.0 g of each sample (in triplicate) were placed in glass Petri dishes, which were kept in a vacuum oven overnight at 35 °C to remove any traces of moisture. The samples were reweighed and stored in the oven at 70 °C, along with other samples. The rate of oxidation, in terms of weight increase, was recorded at 24 h intervals up to 16 days. The time required for a 0.5% weight increase for oil was taken as the index of stability.

Statistical analysis

All analyses were performed in triplicate and data were reported as means ± standard deviation (SD). Differences between experiments were analyzed using Student's t-test in Microsoft Excel 2000 (Microsoft Corporation, USA). The confidence limits used in this study were based on 95% ($P < 0.05$).

RESULTS AND DISCUSSION

Extraction and antioxidant activity determination

Table 1 shows the percentage yield and antioxidant activity of rosemary extracts in different solvents as well as its essential oil. Range of extracts yields was 0.83-25.15%. It has been established that the extraction yield increases with increasing polarity of the extracting solvent [12]. In this study, highest yield was not obtained in methanol but in ethyl acetate extract and lowest is related to the essential oil. Hence rosemary is rich with medium polarity coumpounds,

Table 1 indicates that all rosmary extracts exhibited antiradical activity .Methanolic extract (IC50 = 15.82 µg/ml) is more active than BHT (IC_{50} = 25,62 µg/ml). Therefore, this extract was used for the stabilization of sunflower oil.

Table 1: Percentage yield and antioxidant activity of rosemary extracts

Sample	Yield (%)	IC50 (µg/ml)
Ethyl acetate extract	25.15 ± 0.26	32.23 ± 0.06
Methanolic extract	5.22± 0.28	15.82 ± 0.10
Essential oil	0.83± 0.04	96.12 ± 0.05
BHT	-	25.62 ± 0.06

Data are mean (n = 3) ± standard deviation (n = 3), (p < 0.05).

nd : not determined

GC/MS analysis

The chemical composition of *Rosmarinus officinalis L.* essential oil is presented in Table 2. The average yield in essential oil was 0.96 % (w/w). GC–MS analysis resulted in the identification of 20 compounds representing 96.05% of the total essential oil which contains 29.39% monoterpene hydrocarbons, 52.55% oxygenated monoterpenes, 13.16% sesquiterpene hydrocarbons and 0.95% oxygenated sesquiterpenes. Furthermore, the most abundant components (> 4%) of the Tunisian *Rosmarinus officinalis L* essential oil were α-pinene (14.05%), camphene (6.14%), camphor (15.32%), borneol (13.63%) and geraniol (16.32%). The identified compounds are known and were reported in previous studies.

However, the composition of the Tunisian rosemary essential oil is different from other Mediterranean rosemary oils [27, 28] . This could be related to rosemary variety, climatic conditions and period of plant collection.

Table 2 : Chemical composition of *Rosmarinus officinalis L.* essential oil

No.	Compound	KRI	Retention time (min)	Composition (%)
1	Tricyclene	1011	6.1	0.26
2	α−Pinene	1023	6.99	14.05
3	Camphene	1103	7.78	6.14
4	Verbenene	1121	8.01	0.77
5	Limonene	1206	8.29	3.48
6	β-Pinene	1109	8.54	2.61
7	γ−Terpinene	1251	8.58	2.08
8	Camphor	1514	8.66	15.32
9	Linalool	1538	8.87	3.32
10	α−Campholenal	1471	8.89	2.63
11	Borneol	1679	8.92	13.63
12	Pinocarvone	1548	9.06	1.33
13	α−Humulene	1657	9.20	2.42
14	Terpineol	1677	9.34	2.77
15	α− Geraniol	1828	9.56	16.32
16	γ-Curcumene	1738	9.89	3.04
17	Bornyl acetate	1579	9.99	3.81
18	Caryophyllene oxide	1977	10.12	0.53
19	α-Bisabolol	2022	10.34	0.42
20	Methyl eugenol	2032	10.52	1.12
Identification components (%)				96. 05 %
Monoterpene hydrocarbons				29.39 %
Oxygenated monoterpenes				52.55 %
Sesquiterpene hydrocarbons				13.16 %
Oxygenated sesquiterpenes				0.95 %
Oil Yield (%)				0.96 %

No: Numbers correspond to the peaks observed in the GC–MS chromatogram. KRI: Kovats Retention Index, retention index relative to n-alcane on DB-5MS Capillary column. w/fw: weight/fresh weight.

Polyphenols and flavonoids determination

polyphenols have received considerable attention because of their physiological function, including antioxidant, antidiabetic, antimutagenic and antitumour activities [29]. Phenolic compounds such as flavonoids, phenolic acids and tannins are widely distributed in plants, which have gained much attention, due to their antioxidant activities and free radical-scavenging abilities,with beneficial implications for human health [30].

Table 3 summarises the results from the quantitative determination of the flavonoids and total phenol contents of the different extracts. Except the essential oil, all extracts were found to be rich in flavonoids and polyphenols. The methanolic extract had high levels of phenolic (282.98 ± 5.63 µg GAE/mg extract) and flavonoid (161.05 ± 2.32 µg QE /mg extract) contents. The ethyl acetate extract contained less amounts of polyphenols (12.69 ± 2.51 µg GAE/mg extract) and flavonoids (72.94 ± 4.33 µg QE /mg extract) than methanolic extract. In a previous study, Erkan et al. [31] have determined the total phenol content of the methanolic extract from Greece rosemary (162 µg GAE/mg extract) which was lower than that found in our work. This can be explained by the period and region of plant harvest.

Table 3 : The contents of total flavonoid and phenolic compounds

Extract	Total flavonoids[a] (µg QE/mg extract)	Total phenols[b] (µg GAE/mg extract)
Ethyl Acetate extract	12.69 ± 4.33	44.69 ± 2.51
Methanol extract	161.05 ± 2.32	282.98 ± 5.63
Essential oil	-	-

a: Total flavonoids expressed as quercetin equivalents: micrograms quercetin per milligram (dry weight) extract.

b: Total phenols expressed as gallic acid equivalents: micrograms gallic acid per milligram (dry weight) extract

LC/MS analysis

Methanol extract of *R. officinalis* leaves extract was subjected to high performance liquid chromatography, coupled to photodiode-array and electrospray ionisation mass spectrometric analysis (LC/DAD/ELSD/ESI-MS^n) in order to obtain a tentative identification of his composition. Eight coumpounds were identified belonging to two representative classes of constituents: flavonoids and phenolic terpens. Three unknown compounds were also detected. Rosmanic acid was the only hydroxycinnamic derivative that was identified (Figure 1).

Because polyphenols contain one or more hydroxyl and/ or carboxylic acids groups, MS data were acquired in a negative ionization mode. The identification was based on chromatographic behaviour, mass spectra obtained under electron spray ionisation (ESI) conditions and comparison with reference compounds and scientific publications For the remaining compounds for which no standards were available, identification was based on accurate mass measurements of the pseudomolecular [M–H] ions and their fragmentation pattern, as has been documented in the literature [32]. Table 4 shows the retention times, mass spectrometric characteristics in negative mode as well as the name of the identified compounds.

Coumpound 3 gave a [M-H]$^-$ ion at m/z 305 attribueted to galocatechin a flavonoid previously reported in rosmary extracts [33]. Compound 6 gave a [M–H] ion at m/z 477 attributed to isorhamnetin-3-O-hexoside. Its MS/MS data showed the loss of a hexose moiety (162 amu) resulting in the fragment ions at m/z 315 corresponding to a deprotonated molecular ion of isorhamnetin in agreement with literature data [33]. Likewise, the MS$_2$ experiment for compound 7 with [M–H] at m/z 461 showed the loss of a hexose moiety confirming the presence of homoplantaginin, which has been previously reported in rosemary and extracts [34, 35]. The last flavonoid identified, compound 8, was the aglycon hispidulin (at m/z 299) eluting at 23, 7 min.

The peak with [M–H] at m/z 345, were found, i.e., isomer of rosmanol (compound 9). MS$_2$ analysis at m/z 345 gave the m/z 301, corresponding to the [M–H–CO2] fragments, in agreement with literature data [35, 36].

Coumpounds 10 and 11 are attribueted to carnosic acid and carnosol which were among the most frequently compounds reported in rosmary polar extracts (Herrero, plaza, Cfeuentes, &Ibáñez, 2010).
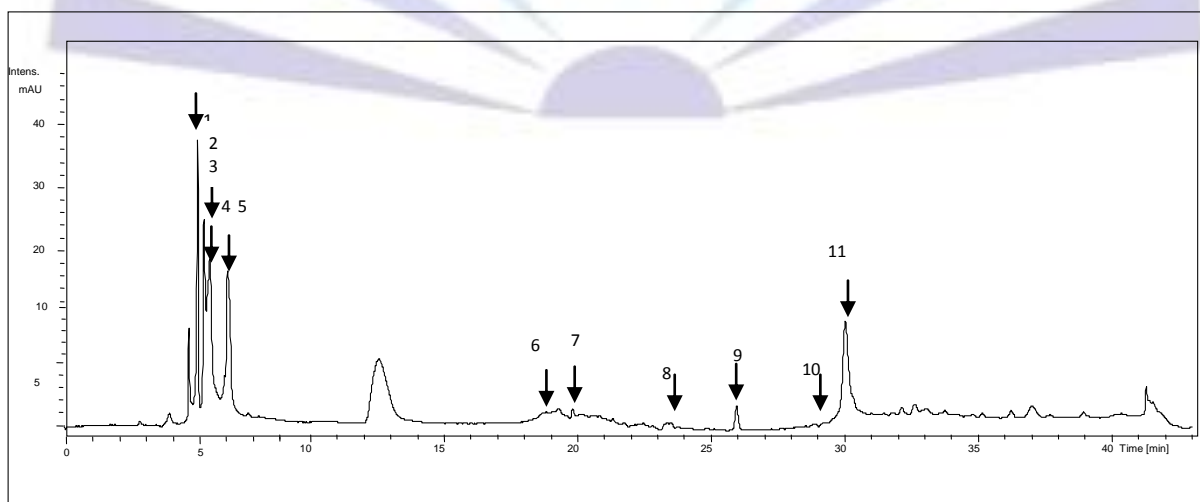

Fig 1: LC/MS chromatogram of *R.officinalis* leaves MeOH extract ; 1: unkown ; 2: unkown; 3: gallocatechin; 4: Rosmanic acid; 5: unkown; 6: rosmanol isomer; 7: carnosic acid; 8: carnosol; 9: rosmanol isomer ; 10: carnosic acid; 11: carnosol

Table 4: Peak assignments of MeOH rosemary leaves extract

Compounds	Rt (min)	[M-H]⁻ (m/z)	-MS²[M-H]⁻ (m/z) (%)	Compounds
1	4,8	165	144 (100)	Unknown
2	5,2	191	155 (100)	Unknown
3	5,3	305	226 (100)	gallocatechin
4	5,7	359	161 (100)	Rosmarinic acid
5	6,7	341	281 (100)	Unknown
6	18,6	477	315 (100)	Isorhamnetin-3-O-hexoside
7	19,5	461	299 (100)	Homoplantaginin
8	23,7	299	283 (100)	Hispidulin
9	25,7	345	301 (100)	Rosmanol isomer
10	29,3	331	287 (100)	Carnosic acid
11	30	329	285 (100)	Carnosol

Rt : retention times

[a] identification confirmed using commercial standards

Alpha-amylase inhibitory assay in vitro

This assay evaluated the ability of Rosemary extracts to inhibit the activity of α-amylase, a digestive enzyme secreted from the pancreas and salivary gland. Alpha-amylase is involved in important biological processes such as digestion of carbohydrates. Many crude drugs inhibit α-amylase activity [38]. Natural α-amylase inhibitors are beneficial in reducing post-prandial hyperglycemia by delaying the digestion of carbohydrates and consequently the absorption of glucose.

Table 5: Alpha-Amylase and Pancreatic Lipase inhibition assays of Rosemary extracts

Parameters		α-amylase activity		Pancreatic lipase activity	
Sample	Concentration (µg/ml)	% inhibition	IC₅₀ (µg/ml)	% inhibition	IC₅₀ (µg/ml)
Acarbose	25	84.00 ± 2.1	14.88	-	
	50	88.72 ± 2.07			
	100	90.35 ± 1.75			
Fluvastatin	25		-	74.58 ± 1.13	16.76
	50			86.78 ± 1.27	
	100			93.35 ± 1.25	
Ethyl acetate extract	50	73.31 ± 1.22	34.11	68.24 ± 1.34	36.63
	100	84.01 ± 1.57		78.49 ± 1.62	
	200	95.55 ± 1.33		84.21 ± 1.75	
methanolic extract	50	82.25 ± 1.73	30.39	73.36 ± 2.07	34.07
	100	85.55 ± 1.13		81.13 ± 1.69	
	200	98.68 ± 1.25		88.76 ± 1.97	
Essential oil	50	88.13 ± 1.37	28.36	77.53 ± 1.57	32.25
	100	96.65 ± 1.07		83.77 ± 1.83	
	200	98.33 ± 1.43		91.02 ± 2.13	

The data are expressed in mean ±S.E.M. n =3 in each group.

Table 5 indicated that each extract from Rosemary showed a potent inhibition of α-amylase enzyme. The IC_{50} values of Rosemary essential oil, ethyl acetate and methanolic extracts against α-amylase were 28.36, 34.11 and 30.39 µg/ml respectively. Moreover, α-amylase activity underwent a strong inhibition via Acarbose (IC_{50} = 14.88 µg/ml). It should be mentioned that Acarbose has been used for management of post-prandial hyperglycemia but it was reported that this agent was associated with several health side effects [27].

The potent α-amylase inhibitory activity of Rosmary ethyl acetate and methanolic extracts depended on their total phenolics and flavonoids contents . In fact, many phenolic compounds and specially flavonoids have been reported as potential antidiabetic agents because they exert a good inhibiting action of α-amylase and could have potential prevention in diabetes mellitus as part of a dietary strategy [28, 29]. Furthermore, the terpenes such as β-pinene which exist in rosemary essential oil might inhibited key enzymes related to type 2 diabetes principally α-amylase. It was reported that administration of terpenenes to diabetic exerts blood glucose lowering effect and high antioxidant activity in alloxan-induced diabetic rat [30].

Inhibitory activity of Rosemary extracts against pancreatic lipase

Pancreatic lipase is the most important enzyme responsible for the digestion of dietary triglycerides in which it hydrolyzes non-absorbable triglycerides into absorbable glycerol and fatty acids by small intestine [4], inhibition of this digestive enzyme is therefore beneficial in the treatment of obesity [31].

As shown in table 5, all the evaluated extracts interestingly suppressed the activity of pancreatic lipase. Thus the IC_{50} of essential oil, ethyl acetate and methanolic extracts against lipase activity were 32.25, 36.64 and 34.07 µg/ml, respectively, which indicated that Rosemary is endowed with a strong anti-obesity effect. It should be noted that fluvastatin, an anti-hyperlipidemic agent [32], showed more potent inhibition of pancreatic lipase (IC_{50} = 16.76 µg/ml) than the other evaluated extracts. Moreover, the inhibitive capacities of the different fractions against the lipase might perfectly coincident with their total phenolics compounds. It was reported that the inhibitory lipase activity might be derive from the phenolic compounds found in some medicinal plants such as gallic acid, catechin, epicatechin, ellagic acid, myricetin, quercetin, kaempferol, resveratrol, and anthocyanins [44,45].

Thermal stability of rosemary methanolic extracts

Fig. 2 shows the Effect of heating on methanolic extract from rosemary (at 185°C) for different intervals. antioxidant activity using DPPH radical scavenging method was employed for the Evaluation of thermally treated extract stability.

During 50 min heating time, Extract was almost stable. But a slight gradual decrease in antioxidant activity (increase in IC_{50} values) was observed around 55 min . The decrease in antioxidant activity become pronounced after 80 min heating time. extract lost considerably its antioxidant activity which becomes much weaker than BHT after 120 min heating time. this result is more interessant than that reported in our previous study using basil [14].

This loss of antioxidant activity can be explained by various chemical reactions leading to the formation of hydroperoxides, hydrolysis, polymerization and chemical decomposition after longer heating times at high temperatures, which lead to deterioration in oils and fats giving rancidity [46]. These results reveal rosemary to be a potential source of natural antioxidants, applicable in food systems even at high processing temperatures. In fact, Rosemary (*Rosmarinus officinalis L.*) is a rich source of di-and triterpenoids, phenolic acids, and flavonoids, especially Carnosic acid, carnosol and rosmarinic acid wchich are the main antioxidant compounds [32].
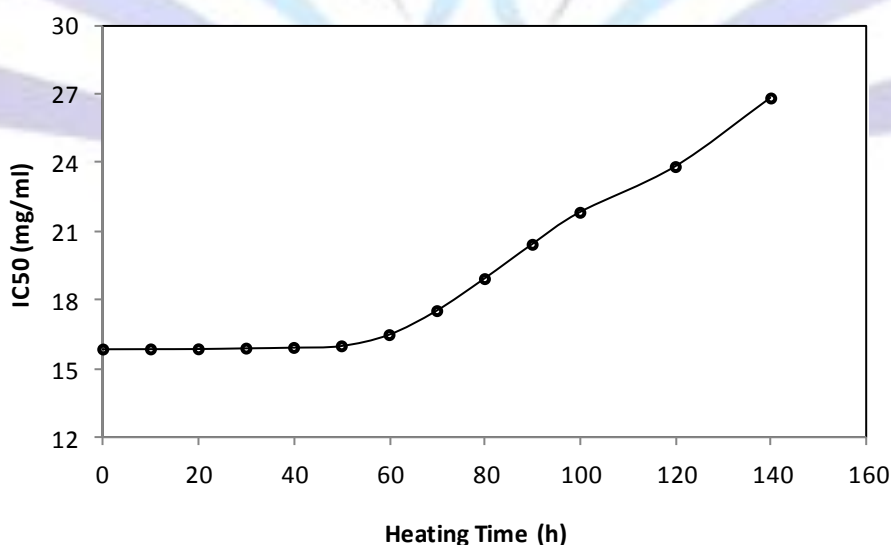

Fig 2: Antioxidant activity of thermally treated methanolic extracts of rosemary

Determination of induction period (IP) to primary oil oxidation with Rancimat method

Induction period (IP) provides direct evidence for trends in resistance to oxidative rancidity of vegetable oils. IPs were determined in all cases at 100 °C measured by Rancimat. The SFO oxidative resistance was greatly improved in the presence of rosemary methanolic extracts (Table 6). Indeed, the induction time of SFO increased significantly from 2.05 (control) to 3.52, 9.12 and 12.93 h by enrichment with 200, 500 and 1000 ppm of rosemary methanolic extract, respectively. Rosemary methanolic extract at 500 ppm was significantly more effective (9,12 h) than BHT at 200 ppm (4.72 h) or 500 ppm methanolic extract of garlic (2.79 h) methanolic extract of pomegranate peel (4.07 h) or basil (8,23 h) [47,48,14]. The highest stability of SFO supplemented with rosemary methanolic extract can be explained by its richness in components with powerful antioxidant activity, such as carnosic acid, carnosol, rosmarinic acid, rosmanol, epirosmanol, methyl carnosate, isorosmanol, 7-methylepirosmanol, rosmaridiphenol and rosmariquinone [15, 49, 50].

Table 6 : Antioxidant effects on sunflower oil measured by Rancimat under 100 °C.

Sample	Concentration (ppm)	Induction period (h)
Ctrl	0	2.05± 0.05
SFO + BHT	200	4.72 ± 0.04
SFO + RME	200	3.52 ± 0.01
SFO + RME	500	9.12± 0.03
SFO + RME	1000	12.93 ± 0.04

Ctrl: control; SFO: sunflower oil; RME: rosemary methanolic extract. Data are mean (n = 3) ± standard deviation (n = 3), ($p < 0.05$).

Weight gain (WG)

Weight gain (WG) is a technique generally employed for quantitative assessment of the amount of oxygen added to the unsaturated content of lipid molecules and formation of hydroperoxides during oxidation. This amount of oxygen is used as a good parameter for determining the induction period besides extent of oxidation and effect of antioxidants on the stability of oils. When there is no oxidation, oil samples weight remains practically constant. WG was measured for all the stabilized and control samples after 24 h intervals up to 16 days and results were calculated in percentage (Fig.3). Initially, WG was not appreciable but it increased very sharply for all the samples reaching a maximum value followed by a sharp decrease, after some time at maximum value, during the last days of storage. A significant increase in induction period of all the stabilized samples was observed compared to the control. Indeed, the time taken to achieve 0.5% increase in weight was 3.26, 4.28, 5.41, 8.1 and 11.2 days for Ctrl, SFO-200, SFO-BHT, SFO-500, and SFO-1000, respectively (Fig.2). These results show that the synthetic antioxidant, BHT has induction time between SFO-200 and SFO-500. It may be roughly that rosemary and basil methanolic extract at 300-400 ppm has the same effect as BHT at 200 ppm in SFO stabilisation.
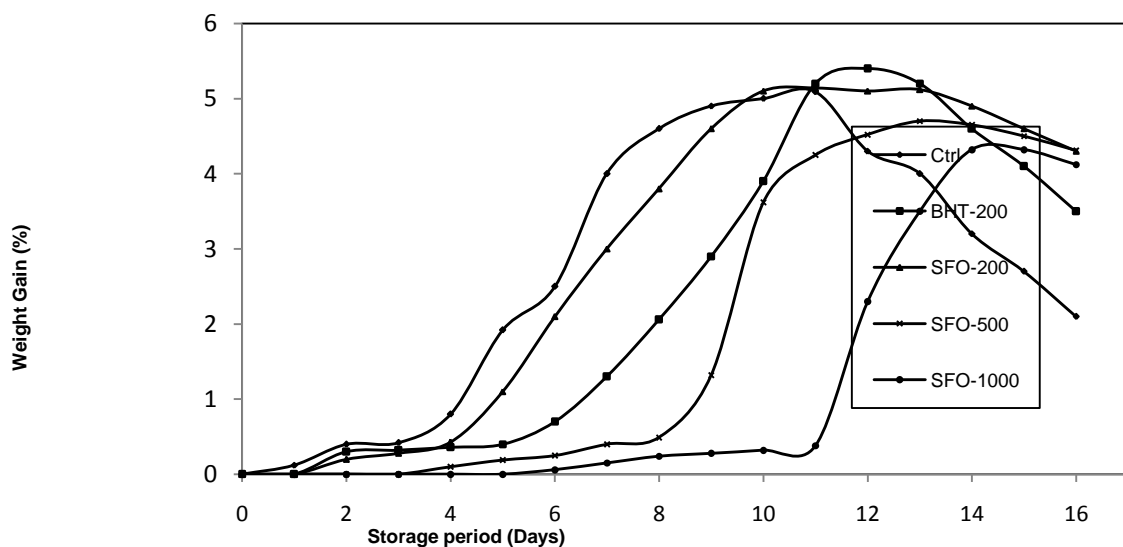

Fig 3: Increase in weight gain (WG) of control and stabilized sunflower oil samples with rosmary methanolic extract under accelerated storage

CONCLUSION

The results obtained in this study demonstrated for the first time that rosemary is a potent source of natural inhibitors of α-amylase and pancreatic lipase with powerful antioxidants proprieties that might be used in the food stabilization and the prevention of diabetes and obesity complications to be explored for new complementary pharmacological drug.

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