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DISSERTATION

Titel der Dissertation

„Bioassay guided isolation of compounds with acetylcholinesterase inhibitory activity from selected medicinal plants used in Iranian Traditional Medicine“

Verfasser

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List of Abbreviations

µg	microgram
µl	microliter
µM	micromolar
AChE	acetylcholinesterase
APCI	atmospheric-pressure chemical ionization
ASE	accelerated solvent extraction
ATCI	acetylthiocholine iodide
BChE	butyrylcholinesterase
BSA	bovine serum albumin
BTCI	butyrylthiocholine iodide
CC	column chromatography
CCC	counter current chromatography
$CDCl_3$	deuterated chloroform
CH_2Cl_2	dichloromethane
$CHCl_3$	chloroform
cm	centimeter
COSY	correlation spectroscopy
DCM	dichloromethane
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
ESI	electrospray ionization
EtOAc	ethylacetate
FBS	Fast Blue B salt
g	gram
HMBC	heteronuclear multiple bond correlation
HPCCC	high performance counter current chromatography
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
HSQC	heteronuclear single-quantum correlation
IR	infrared spectroscopy

ITM	Iranian traditional medicine
M	molar
MeOH	methanol
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MS	mass spectrometry
nm	nanometer
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser effect spectroscopy
Q-TOF	quadrupole time-of-flight
rpm	rounds per minute
s	second
SEC	size exclusion chromatography
SPE	solid phase extraction
TEA	triethylamine
TLC	thin layer chromatography
TOCSY	total correlation spectroscopy
TOF	time-of-flight
UHPLC	ultra high performance liquid chromatography
UV	ultra violet
VLC	vacuum liquid chromatography

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Introduction:

Cognitive deficits are one of the most common causes of mental deterioration such as learning impairment, delayed amnesia and memory problems that are the debilitating consequences of aging in elderly people. The past three decades have witnessed a considerable research effort, directed towards discovering the cause of cognitive disorders with the ultimate hope of developing safe and effective pharmacological treatments (Corbett *et al*., 2012). An epidemiological study carried out by Ferri *et al*. (2005) revealed that 24.3 million people are involved with dementia with increase of 4.6 million new cases each year; so the number of affected people will be doubled every 20 years. The number of the people with dementia in 2010 was reported by Alzheimer's disease International with more than 35 million people worldwide (Anonymous a, 2011). Only in USA, an estimated 5.4 million Americans are suffering under Alzheimer's disease in 2012 (Anonymous b, 2012).

Most presenile dementia conditions caused by aging, such as Alzheimer's disease (AD) are characterized by common psychopathological phenomena (Brewer *et al*., 1998). Alzheimer's disease was identified for the first time about 100 years ago, but only since approximately three decades the research is focusing on its symptoms, causes, risk factors and treatment.

The etiology of Alzheimer's disease and similar illnesses has been partly defined and several factors have been suggested to be effective in reduction of the incidence of these diseases. Since the improved hypotheses and theories about the etiology of these neurological disorders are largely based on epidemiological studies, none of them has been completely accepted (Howes and Houghton, 2003). The pathological features that have been identified in the central nervous system (CNS) in Alzheimer's disease are senile plaques, neurofibrillary tangles and neurotransmitter disturbances (García-Sierra *et al*., 2012)

The pathophysiology of Alzheimer´s disease is complex and involves different biochemical pathways like a defective beta-amyloid metabolism, inflammatory and oxidative processes as well as disordered cholinergic neurotransmission (Welberg, 2011). It has been well indicated that a consistent neuropathological occurrence

associated with memory loss is a cholinergic deficit, which correlates with the severity of Alzheimer´s disease (Garcia-Alloza *et al*., 2006). Thus, attempts to restore cholinergic function have been considered as a rational target to improve therapeutics used to treat the symptoms of Alzheimer´s disease. The cholinergic function could be enhanced by stimulation of cholinergic receptors or prolonging the availability of the neurotransmitter acetylcholine (ACh) at cholinergic synapses (Fig. 1). The latter can be achieved by using acetylcholinesterase (AChE) inhibitors that block this key enzyme in the breakdown of acetylcholine (Howes and Houghton, 2003).

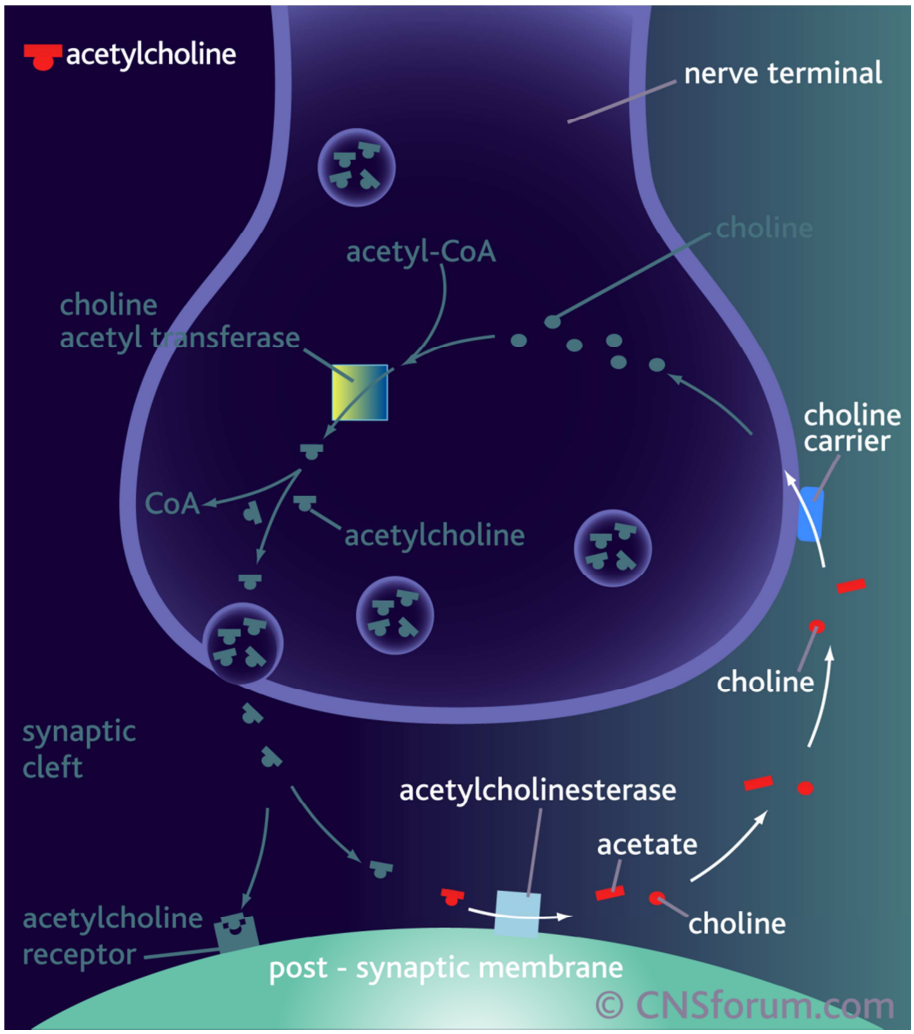

Fig. 1. The function of ACh and AChE in synaptic area (CNS forum.com)

Recent studies showed that the function of acetylcholinesterase is not limited to the termination of cholinergic neurotransmission. It is now clear that the cholinergic system is also involved in the regulation of some signal transduction pathways like beta-amyloid processing and modulation of regional cerebral blood flow. Concerning

this matter the cholinergic therapies as disease-improving agent can have potential as one strategy in addition to the classic cholinergic hypothesis, which is limited to symptomatic treatment of Alzheimer´s disease (Lane *et al*., 2004).

During the last two decades the use of complementary medicines, such as herbal medicinal substances in dementia therapy has been studied (Andrade *et al*., 2000). A potential source of acetylcholinesterase inhibitors is certainly provided by several plants used in traditional practices of medicine to enhance cognitive functions (Mukherjee *et al*., 2007). As many of people are relying and using the herbal preparations based on traditional medicines, the allopathic medicine is encouraged to profit and apply the knowledge of these important sources. Using this knowledge, e.g. *Ginkgo biloba* L. (ginkgo) with antioxidant and cholinergic activities has been studied in clinical trials and shown global benefit for patients with cognitive disorders. Memory-improving properties and cholinergic activities have also been showed for extracts from *Salvia officinalis* L. (sage) and *Melissa officinalis* L. (lemon balm) (Singhal *et al*., 2012). Secondly, studies for natural compounds with respective activity have resulted e.g. in the identification of galantamine from *Galanthus nivalis* L. (snowdrop) as a potent acetylcholinesterase inhibitor, which today is one therapeutic option in the treatment of Alzheimer´s disease (Hostettmann *et al*., 2006; Prvulovic *et al*., 2010).

Iran (formerly called Persia) is located in southwest Asia, within the Middle East region and covers a territory of 1,648,195 square kilometers. Iran's specific geographical position makes it unique in the world with its diverse climatic conditions. Among its 12 different geographic environments, Iran is divided into 5 major climate zones including Mediterranean, desert and half-desert, warm-humid, warm-dry, and mountainous areas (Fig. 2). Due to this variety of climate, more than 7500 plant species grow in Iran, and for about 1800 of those species a medicinal use is described (Adhami *et al*., 2007).

In addition, Iran is among those countries with the longest and richest history in traditional medicine (TM). The activities and authorships of famous Iranian physicians like Avicenna (author of The Canon of Medicine) and Rhazes (author of Al-Hawi) and many others, showed brilliantly this claim (Adhami *et al.,* 2007).

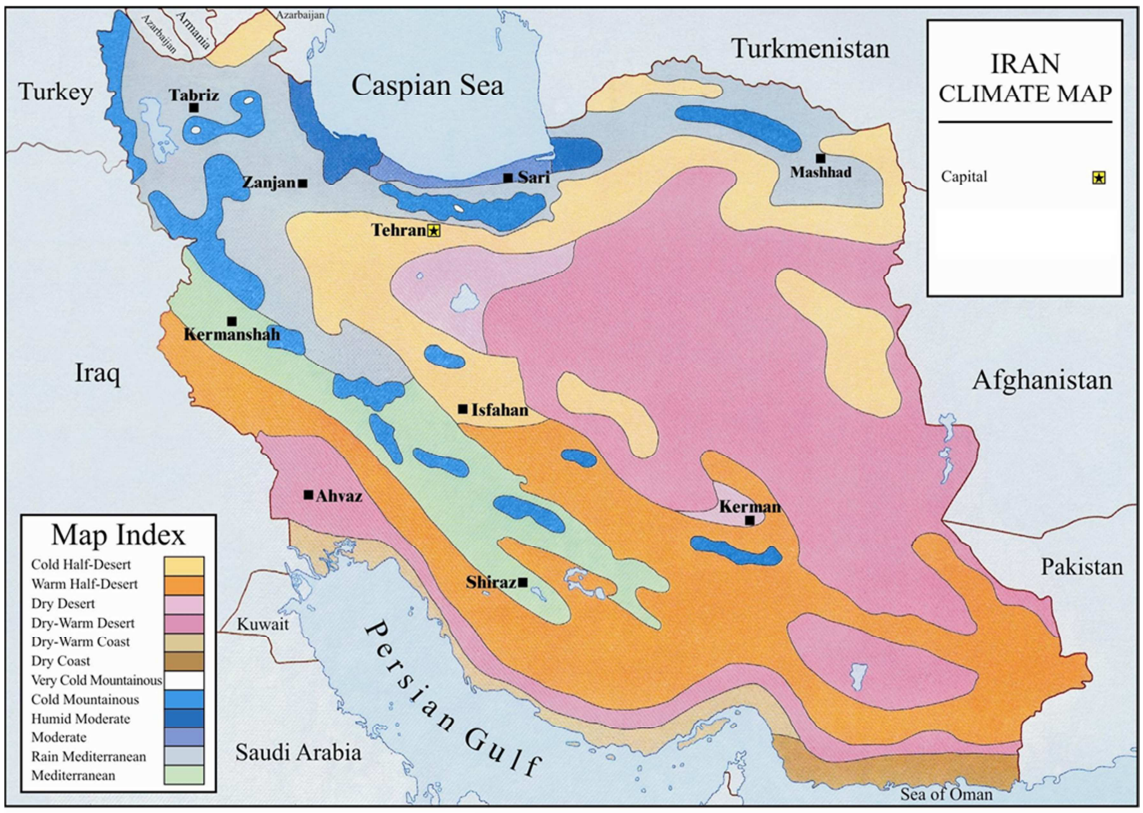

Fig. 2. Iran climate map (Adhami *et al*., 2007).

Some early Iranian physicians were already engaged in the study and treatment of cognitive disorders and successfully used different plants to treat these diseases (Sharafkandi, 2004).

To find new herbal compounds with AChE inhibitory effects, drugs which have been used in Iranian traditional medicine (ITM) for treatment of cognitive disorders seemed a promising source for an *in vitro* approach.

In this doctoral project, a bio-activity screening for AChE inhibition was carried out on herbal drugs which have been used in ITM for memory loss and enhancement of cognitive performance. Then the most active extracts were investigated in detail to elucidate the active compounds.

Methodology overview

The process of this doctoral project is divided into 2 sections:

1- Bio-activity screening for AChE inhibitory activity.

1-1- Selection of promising medicinal plants used for memory loss and enhancement of cognitive performance from several authoritative written documents of ITM. The respective herbal drugs were obtained from herbal shops and identified scientifically.

1-2- Extraction of 40 selected drugs with dichloromethane and methanol by accelerated solvent extraction (3 times with dichloromethane followed by 3 times with methanol). The extracts of each solvent were combined and evaporated under reduced pressure to obtain 80 extracts.

1-3- TLC bioautography screening of 80 extracts. Duplicated TLC was carried out for each extract. Bioautography assay was performed on one of the plates with 1-naphtyl acetate as reagent. The other one was derivatized with anisaldehyde-sulfuric acid or other sufficient reagents to determine the chemical composition of the extract. In this investigation 32 extracts showed activity for AChE inhibition.

1-4- Analyses of the active extracts by colorimetric assay. A quantitative microplate assay based on Ellman's method (Rhee et al., 2001; Adhami et al., 2012) was used to quantify the AChE inhibitory activities of the substances. The assay was performed in triplicate for every concentration and each experiment was repeated 5 times. The most active herbal drugs included the seeds from *Peganum harmala* L., the fruit resin from *Semecarpus anacardium* L., the gum-resin from *Dorema ammoniacum* D. Don and the oleo gum-resin from *Ferula gummosa* Boiss., which were selected for further studies.

2- Detailed investigation of the most active herbal drugs in 3 steps:

2-1- Isolation and structure elucidation of the active compounds. The active compounds of the four active drugs were identified and isolated using several chromatographic techniques such as thin layer chromatography (TLC), vacuum liquid chromatography (VLC), column chromatography

(CC), size exclusion chromatography (SEC), solid phase extraction (SPE), high performance liquid chromatography (HPLC), counter current chromatography (CCC) and automated high performance thin layer chromatography (HPTLC). The structures of the active components were characterized by one and two-dimensional ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry.

2-2- Determination of IC_{50} values for the active compounds. Quantitative microplate assay was carried out for different concentrations of each extract and active pure compound. Each assay was repeated 3 times.

2-3- Quantification of the active compounds in the total extracts. The content of the active compounds in the four different sources was determined by HPLC analyses with external standardization. Each analysis was performed in duplicate.

All methods are explained comprehensively in the respective articles.

Results and Conclusion

All results of the research carried out during this thesis are included in 4 articles:

1- Adhami HR, Farsam H, Krenn L. Screening of medicinal plants from Iranian traditional medicine for acetylcholinesterase inhibition. *Phytotherapy Research*. 2011, 25(8): 1148-1152.

This article contains the results of the screening of 80 extracts of 40 selected herbal drugs from ITM and the detailed investigation of the seeds from *Peganum harmala*. The screening includes both bioautography and microplate assay. This is the first report of AChE inhibition for *P. harmala* that showed the two alkaloids harmine and harmaline are the major AChE inhibitory compounds in the seeds of *Peganum harmala*. In this study, the pulverization of all the herbal drugs, extraction, bioautography assay, microplate assay as well as HPLC-quantification were performed by the author of the thesis.

2- Adhami HR, Linder T, Kaehlig H, Schuster D, Zehl M, Krenn L. Catechol alkenyls from *Semecarpus anacardium*: acetylcholinesterase inhibition and binding mode predictions. *Journal of Ethnopharmacology*. 2012, 139(1): 142-148.

This article contains detailed investigation of the fruit resin from *Semecarpus anacardium.* In this study, two catechol alkenyls from this resin were isolated and their AChE inhibitory activities were proved for the first time and their activities additionally confirmed by an *in silico* docking experiment. A comparison of their inhibitory capacity on AChE versus BChE showed that they are selective AChE inhibitors. For this study, extraction, bioautography assay, microplate assay, all the chromatographic processes to isolate the active compounds and HPLC-quantification were carried out by the first author.

3- Adhami HR, Kaehlig H, Zehl M, Krenn L. Spiro-sesquiterpenoidic chroman-diones from gum ammoniacum with acetylcholinesterase inhibitory activity.

The manuscript contains the detailed investigation of the gum-resin from *Dorema ammoniacum*. A new and a known spiro-sesquiterpenoidic

chromadione were proven in this investigation as AChE inhibitors for the first time. From the correlation of IC_{50} values of these two compounds in AChE inhibition with their concentrations in gum ammoniacum can be concluded that they are among the major AChE inhibitory substances in this drug. The manuscript will be submitted to *Phytochemistry Letters*. In detailed investigation of gum ammoniacum, extraction, bioautography assay, microplate assay, all the chromatographic steps for the isolation of the active components as well as HPLC-quantification were done by the author of the thesis.

4- Adhami HR, Scherer U, Kaehlig H, Hettich T, Schlotterbeck G, Reich E, Krenn L. Combination of bioautography with HPTLC-MS/NMR: a fast identification of acetylcholinesterase inhibitors from galbanum.

The manuscript contains the detailed investigation of the oleo gum-resin from *Ferula gummosa*. This study showed the advantage of TLC-MS/NMR as a fast method for dereplication in natural compound research. Two coumarin derivatives with AChE inhibitory activity were reported for the first time in *Ferula gummosa*. The manuscript will be submitted to the *Journal of Planar Chromatography*. In this investigation, extraction, bioautography assay, microplate assay, fractionation of the extract, automated HPTLC, extraction of the active zones from HPTLC plates into mass spectrometer and for NMR spectroscopy as well as HPLC-quantification were performed by the author of the thesis.

The achieved results confirmed for several known and new compounds that they considerably contribute to the effects of their respective drugs and underline the plausibility of their use in the treatment of cognitive deficits in Iranian traditional medicine. Nevertheless, their activities were not strong enough that they might serve as very promising new lead compounds for the development of new therapeutic AChE inhibitors. However, it has to be underlined that all herbal drugs investigated in this study, even such ones which did not show AChE inhibition might enhance the cognitive performance by different mechanisms.

PHYTOTHERAPY RESEARCH
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Screening of Medicinal Plants from Iranian Traditional Medicine for Acetylcholinesterase Inhibition

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To find new herbal compounds with an acetylcholinesterase (AChE) inhibitory effect, this study focused on herbal drugs and resins which have been used in Iranian traditional medicine for the treatment of cognitive disorders. Forty drugs were selected from authoritative written documents of Iranian traditional medicine. Each drug was extracted by accelerated solvent extraction using dichloromethane followed by methanol. The 80 extracts were screened for AChE inhibitory activity by a TLC bioautography method. The inhibiting effect of the 32 most active extracts was measured by a microplate colorimetric assay. Due to the best activity, the seeds of *Peganum harmala* L. were investigated in detail. From the TLC bioautography assay the alkaloids harmaline and harmine were identified as active compounds. This result was confirmed by means of HPLC-DAD. The IC_{50} values were 41.2 μg/mL for the methanol extract, 95.5 μg/mL for the dichloromethane extract, 8.4 μg/mL for harmaline and 10.9 μg/mL for harmine. The concentrations of active compounds in the extracts were determined by a fast and precise HPLC method. As the amounts of harmaline and harmine in the extracts were correlated with the IC_{50} values of the extracts, it can be concluded that these two alkaloids are responsible for the AChE inhibitory activity of *P. harmala*. **Copyright © 2011 John Wiley & Sons, Ltd.**

Keywords: acetylcholinesterase inhibition; Iranian traditional medicine; TLC bioautography; Peganum harmala; harmaline; harmine.

INTRODUCTION

Cognitive deficits are one of the most common causes of mental deterioration such as memory problems that are the debilitating consequences of aging in elderly people (Francis *et al.*, 1999). Most presenile dementia conditions caused by aging, such as Alzheimer's disease are characterized by common psychopathological phenomena (Brewer, 1998).

It has been well indicated that a cholinergic deficit correlates with the severity of Alzheimer's disease (Garcia-Alloza *et al.*, 2006). Thus, attempts to restore cholinergic function have been considered as a rational target to improve the symptoms of Alzheimer's disease. The cholinergic function could be enhanced by stimulation of cholinergic receptors or by prolonging the availability of the neurotransmitter acetylcholine at cholinergic synapses. The latter can be achieved by AChE inhibitors which block this key enzyme in the breakdown of acetylcholine (Howes and Houghton, 2003).

During the past decade the use of complementary medicines, such as herbal medicinal substances in dementia therapy, has been studied (Andrade *et al.*, 2000) based on traditional medicine, which has been practised in many parts of the world. The knowledge of these important sources could profitably apply to allopathic science. Using this knowledge, for example, galantamine from *Galanthus nivalis* L. (Snowdrop) has been identified as a potent acetylcholinesterase inhibitor, which today is one therapeutic option in the treatment of Alzheimer's disease (Hostettmann *et al.*, 2006).

Iran is among those countries with a long and rich history in traditional medicine, as shown by 'The Canon of Medicine' of Avicenna or 'The Continents' by Rhazes (Adhami *et al.*, 2007). Some early Iranian physicians were already engaged in the study and treatment of cognitive disorders and successfully used different plants to treat these diseases.

To find new herbal compounds with an AChE inhibitory effect, this study focused on drugs which have been used in Iranian traditional medicine for the treatment of cognitive disorders.

There are several authoritative written documents of Iranian traditional medicine such as Al-Qanun fi-Tibb, Al-Hawi fi-Tibb, Tuhfat al-Mu'minin, Makhzan al-adviyah, in which a number of chapters is related to cognitive disorders. Therefore, the herbal drugs for this study were selected from medicinal plants, the use of which in treatment of memory loss and enhancement of cognitive performance was described in the mentioned documents. Additionally, folkloric prescriptions were considered.

MATERIALS AND METHODS

Chemicals. Acetylcholinesterase (AChE) from electric eel, 1-naphthyl acetate, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), Tris-HCl, bovine serum albumin (BSA),

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Table 1. AChE inhibitory activity of medicinal plants from Iranian traditional medicine as determined by TLC bioautography assay

Scientific name	Part used	Common Persian name[a]	Voucher number	DCM extract	MeOH extract
Acorus calamus L. (Acoraceae)	Rhizome	Agir-e torki	PMP-201	+[b]	-[c]
Aloe littoralis Baker (Aloaceae)	Leaves extract	Sabr-e zard	PMP-401	-	-
Anethum graveolens L. (Apiaceae)	Fruit	Tokhm-e shevid	PMP-601	-	-
Asarum europaeum L. (Aristolochiaceae)	Rhizome	Āsāron	PMP-202	-	+
Astragalus arbusculinus Bornm. & Gauba (Fabaceae)	Gum-resin	Anzarout	PMP-801	-	-
Boswellia carterii Birdw. (Burseraceae)	Gum-resin	Kondor	PMP-802	-	-
Bunium persicum (Boiss.) B. Fedtsch (Apiaceae)	Fruit	Zire-e siyāh	PMP-602	-	-
Cannabis sativa L. (Cannabaceae)	Seed	Shāhdāne	PMP-701	+	+
Cinnamomum zeylanicum Nees (Lauraceae)	Stem bark	Dārchin	PMP-901	-	-
Commiphora mukul Engl. (Burseraceae)	Gum-resin	Moql	PMP-803	+	-
Coriandrum sativum L. (Apiaceae)	Fruit	Tokhm-e geshniz	PMP-603	-	-
Cuminum cyminum L. (Apiaceae)	Fruit	Zire-e sabz	PMP-604	-	-
Cyperus longus L. (Cyperaceae)	Tubular root	Moshk-e zamin	PMP-203	-	-
Dorema ammoniacum D. Don (Apiaceae)	Gum-resin	Vashā	PMP-804	+	-
Echinops cephalotes DC. (Asteraceae)	Manna	Shekar tighāl	PMP-805	+	-
Emblica officinalis Gaertn. (Euphorbiaceae)	Fruit	Āmele	PMP-605	+	-
Eugenia caryophyllata Thunb. (Myrtaceae)	Bud	Mikhak	PMP-501	+	+
Ferula assa-foetida L. (Apiaceae)	Oleo gum-resin	Ānghozeh	PMP-806	+	-
Ferula gummosa Boiss. (Apiaceae)	Oleo gum-resin	Bārije	PMP-807	+	-
Iris germanica L. (Iridaceae)	Rhizome	Rishe-e irisā	PMP-204	-	-
Nardostachys jatamansi DC. (Valerianaceae)	Hypocotyl	Sonboletib	PMP-606	+	+
Nepeta menthoides Boiss. & Buhse (Lamiaceae)	Flowering part	Ostokhodus	PMP-301	+	+
Nigella sativa L. (Ranunculaceae)	Seed	Siyāhdāne	PMP-702	+	-
Orchis latifolia L. (Orchidaceae)	Root	Tha'lab-e panjei	PMP-205	+	+
Paeonia officinalis L. (Paeoniaceae)	Tubular root	Oud-e salib	PMP-208	+	+
Peganum harmala L. (Zygophyllaceae)	Seed	Espand	PMP-703	+	+
Piper cubeba L. (Piperaceae)	Fruit	Kabābe	PMP-607	-	-
Piper longum L. (Piperaceae)	Fruit	Dārfelfel	PMP-608	+	-
Piper nigrum L. (Piperaceae)	Seed (peeled)	Felfel-e sepid	PMP-704	+	+
Pistacia atlantica Desf. (Anacardiaceae)	Oleo gum-resin	Saqqez	PMP-808	-	-
Pistacia lentiscus L. (Anacardiaceae)	Oleo gum-resin	Mastaki	PMP-809	+	-
Portulaca oleracea L. (Portulacaceae)	Seed	Tokhm-e khorfe	PMP-705	-	-
Semecarpus anacardium L. (Anacardiaceae)	Fruit	Belādor	PMP-609	+	+
Semecarpus anacardium L. (Anacardiaceae)	Fruit resin	Asal-e belādor	PMP-610	+	+
Sesamum indicum L. (Pedaliaceae)	Seed	Konjed	PMP-706	-	-
Terminalia bellirica (Gaertn.) Roxb. (Combretaceae)	Fruit	Balile	PMP-611	-	-
Terminalia chebula Retz. (Combretaceae)	Fruit (unmatured)	Halile-e siyāh	PMP-612	-	-
Terminalia chebula Retz. (Combretaceae)	Fruit (matured)	Halile-e Kāboli	PMP-613	+	-
Vitis vinifera L. (Vitaceae)	Fruit (dried)	Maviz	PMP-614	-	-
Zingiber officinale Rosc. (Zingiberaceae)	Rhizome	Zanjebil	PMP-206	-	-

[a] For some samples the Persian names are specific for used parts.
[b] +, active (bands more intense than the one of 24 ng physostigmine).
[c] -, nonactive.

physostigmine and harmaline hydrochloride dihydrate were purchased from Sigma (St Louis, USA). Acetylthiocholine iodide (ATCI) and chelidonine were obtained from Fluka (Buchs, Switzerland). Fast Blue B salt (FBS), triethylamine (TEA) and harmine hydrochloride were from Merck (Darmstadt, Germany). Two different buffer systems were used. Buffer A: 50 mM Tris-HCl, pH 7.9 containing 0.1% BSA; Buffer B: 50 mM Tris-HCl, pH 7.9 containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$.

General. A Tecan Genios micro plate reader (Salzburg, Austria) was used to measure the absorbance. Extraction was done with a Dionex accelerated solvent extractor (ASE) model 200 (Sunnyvale, USA). TLC plates silicagel 60 F254, were purchased from Merck (Darmstadt, Germany) and 96-well microplates PS F-bottom were obtained from Greiner Bio-One (Frickenhausen, Germany). HPLC was performed on a Shimadzu instrument with LC-20AD pump, SPD M20A diode array detector and SIL 20AC HT auto sampler (Kyoto, Japan).

Plant material. Forty drugs were purchased from herbal shops in different cities of Iran and were identified on

the basis of anatomical and morphological features by Dr Gholamreza Amin at the herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran (Amin, 2005; Ghahreman and Okhovvat, 2004; Zahedi, 1959; Issa Bey, 1930). Voucher specimens are kept in the mentioned herbarium (Table 1).

Extraction. Each drug was ground and then 8 g of each was extracted by accelerated solvent extraction three times with 20 mL dichloromethane (DCM), each, followed by three times with 20 mL methanol (MeOH) at 40 °C. The extracts of each solvent were combined and evaporated to dryness under reduced pressure at 40 °C. By this mode of extraction, polar and non-polar plant metabolites were enriched in the DCM and MeOH extracts, respectively. Additionally, by the very gentle conditions of extraction any decomposition of sensitive substances could be avoided. A total of 80 extracts was obtained.

TLC bioautography assay. All extracts were examined by TLC on silica plates by use of adequate mobile phases for compounds of different polarity. Detection was performed with anisaldehyde–sulfuric acid reagent to check the chemical composition of the extracts.

In addition, a screening assay for AChE inhibition was performed according to Marston *et al.* (2002). Both DCM and MeOH extracts were prepared for TLC at concentrations of 6 mg/mL. 25 µL of each sample was spotted on the TLC plate. After development in the mobile phase chloroform–ethylacetate–methanol (90 + 7 + 3) for DCM extracts and ethylacetate–methanol–water (100 + 13.5 + 10) for MeOH extracts, the plate was dried completely and then sprayed with enzyme solution (6.7 U/mL in buffer A) until saturation. Incubation of the plate at 40 °C for 20 min in a humid environment followed. To detect the enzyme activity, 5 mL of a freshly prepared solution of 13.4 mM 1-naphthyl acetate in ethanol and 20 mL of 7.4 mM FBS were used. After incubation, the TLC plate was sprayed with a mixture of two solutions and the purple color appeared after 1–2 min while the active fractions remained as white bands. Chelidonine and physostigmine were used as positive controls for comparison of the DCM and MeOH extracts, respectively.

In a semiquantitative TLC approach physostigmine was used as a standard at 6–42 ng for comparison of the activity with the different samples. The plates were developed in the mobile phase chloroform–ethylacetate–methanol (80 + 7 + 3). After detection, the intensity of the white bands in the different samples was compared with the standard bands of physostigmine. In this way all samples showing bands more intense than that of 24 ng physostigmine were identified as active (+).

Microplate assay. The AChE inhibitory activities of the samples were measured by a quantitative colorimetric assay based on Ellman's method (Rhee *et al.*, 2001). In a 96-well plate, 25 µL of 15 mM ACTI, 125 µL of 3 mM DTNB in buffer B, 50 µL of buffer A and 25 µL of sample (10 mg/mL in DMSO diluted with buffer A to a concentration of 1 mg/mL) were mixed and the absorbance was measured at 405 nm five times every 15 s. Then 25 µL of AChE (0.22 U/mL in buffer A) was added and the plate was incubated at 25 °C for 10 min. Then the absorbance was measured again eight times every 15 s. Each assay was repeated five times. To avoid any increase in absorbance due to the color of the extracts or spontaneous hydrolysis of substrate, the absorbance before addition of the enzyme was subtracted from the absorbance after adding the enzyme. The assay was validated by measurement of different concentrations of physostigmine as a positive control. The percentage of inhibition was calculated by comparing the absorbance of sample to blank (10% DMSO in buffer A).

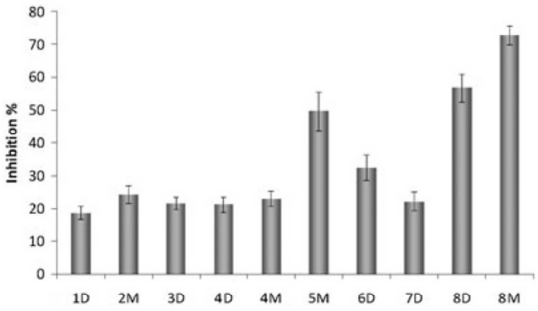

Figure 1. AChE inhibition of the most potent extracts at the concentration of 100 µg/mL. D, DCM extract; M, MeOH extract; 1, *Piper longum*; 2, *Nardostachys jatamansi*; 3, *Paeonia officinalis*; 4, *Piper nigrum*; 5, *Semecarpus anacardium*; 6, *Dorema ammoniacum*; 7, *Ferula gomusa*; 8, *Peganum harmala*.

HPLC. The DCM and the MeOH extracts of *Peganum harmala* were analysed by HPLC based on Wang's method (Wang *et al.*, 2008). HPLC was performed by isocratic elution on a Hypersil BDS-C18 column (250 × 4 mm i.d.) at 25 °C. The mobile phase was a solution of MeOH and 0.1% TEA (70:30 v/v) at a flow rate of 1 mL/min. The eluents were monitored at 320 nm.

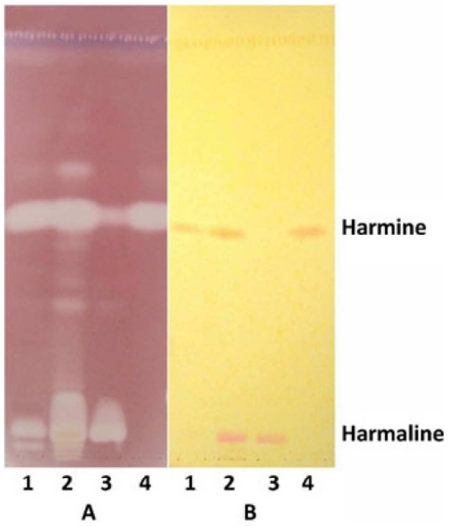

Figure 2. TLC of *Peganum harmala* in comparison with authentic alkaloids. 1, *Peganum harmala* (DCM extract); 2, *Peganum harmala* (MeOH extract); 3, harmaline; 4, harmine;. (A) Bioautography assay. (B) Detection with Dragendorff reagent. Mobile phase: chloroform–ethylacetate–methanol (90 + 7 + 3). This figure is available in colour online at wileyonlinelibrary.com/journal/ptr

The calibration curves for harmaline and harmine covered concentrations between 0.02 and 0.2 mg/mL.

RESULTS AND DISCUSSION

Forty drugs used in Iranian traditional medicine for the treatment of amnesia and memory improvement were selected for testing of AChE inhibitory activity. Based on the TLC screening of 40 DCM and 40 MeOH extracts, the most potent (21 DCM extracts and 11 MeOH extracts) were selected for a quantitative determination of their AChE inhibitory activities in a microplate assay (Table 1). Physostigmine served as a positive control and the IC_{50} value for this compound was determined as 0.8 μg/mL (2.9 μM) which is in accordance with published values (Carpinella *et al.*, 2010; Mukherjee *et al.*, 2007).

Due to the best AChE inhibitory activity in both the DCM and MeOH extracts (Fig. 1), the seeds of *Peganum harmala* L. were investigated in detail in this study. *P. harmala* is a wild-growing flowering plant from the Zygophyllaceae family. It is abundantly found in the Middle East and North Africa and the seeds have a long history in traditional medicine (Zargari, 1989; Adhami *et al.*, 2007). New research has shown many biological activities for this drug such as antibacterial (Shahverdi *et al.*, 2005), antifungal (Nenaah, 2010), MAO inhibitory (Herraiz *et al.*, 2010), antinociceptive (Monsef *et al.*, 2004), antiprotozoal (Arshad *et al.*, 2008), antitumour (Li *et al.*, 2007) and antioxidant (Moura *et al.*, 2007). Reduction of spermatogenesis (El-Dwairi and Banihani, 2007) was shown as well. The most important secondary metabolites of *Peganum harmala* are alkaloids (2% to 6% in seeds, Blaschek *et al.*, 2007).

From the TLC bioautography assay the alkaloids harmaline and harmine were identified as the most active compounds (Fig. 2). The AChE inhibitory effect of these alkaloids has recently been deduced (Zheng *et al.*, 2009) in a study of another *Peganum* species, namely *Peganum nigesllastrum* Bunge, from a TLC bioautography assay, only. The IC_{50} values of the extracts or the isolated alkaloids were not determined

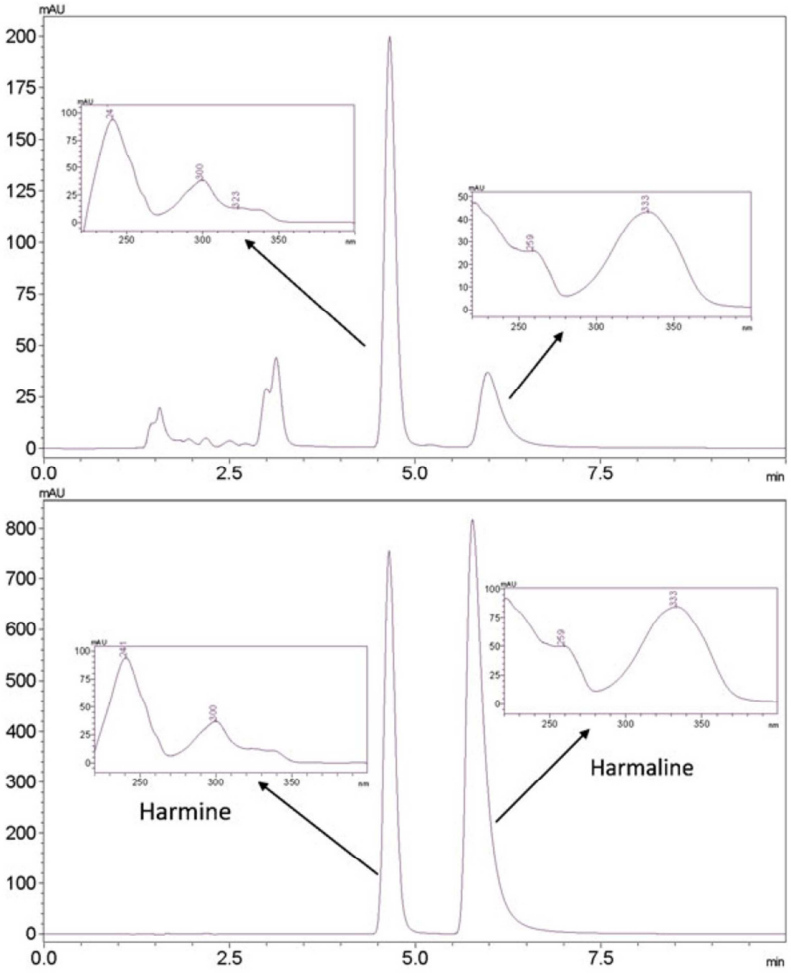

Figure 3. Comparison of the HPLC chromatograms of (upper image) MeOH extract of *Peganum harmala* and (lower image) harmaline and harmine. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr

in this investigation. In our study, the seeds of *P. harmala* were investigated by both TLC bioautography and microplate colorimetric assays. The IC_{50} values, as determined for the first time by a microplate assay, were 41.2 μg/mL for the methanol extract, 95.5 μg/mL for the dichloromethane extract, 8.4 μg/mL (39.2 μM) for harmaline and 10.9 μg/mL (51.6 μM) for harmine.

Additionally, the extracts were analysed by HPLC and the percentages of the active compounds in the extracts were determined by a fast and precise method, which was optimized and validated for the major alkaloids harmaline and harmine (Fig. 3). Due to the optimization of the sta-tionary phase it was possible to decrease the retention times of harmaline and harmine approximately to one half in comparison with the published method (Wang *et al.*, 2008). The content of harmaline and harmine was determined by external standardization. Over the selected range, peak areas of both analytes were linearly dependent on concentrations with correlation coefficients of $R^2 = 0.9930$ for harmaline and $R^2 = 0.9942$. The concentrations of harmaline in DCM and in the MeOH extract were 0.8% and 2.8%, respectively. Values of 4.0% and 14.6% harmine were determined in DCM and MeOH extract, respectively. The total amounts of these two alkaloids in the seeds were 0.41% harmaline and 2.04% harmine.

From the correlation of IC_{50} values of the extracts with the concentrations of harmaline and harmine in the respective extracts, it can be concluded that these two alkaloids are the major AChE inhibitory compounds in *Peganum harmala*.

Acknowledgement

H. R. Adhami is grateful to the University of Vienna for scholarship on this study in 2009. Acknowledgement is also due to Dr Gholamreza Amin for the identification of the herbal drugs.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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Supporting information for article 1

TLC bioautography for AChE inhibition was carried out according to Marston *et al.* (2002). Different mobile phases were used for DCM and MeOH extracts (Fig. 1-3).

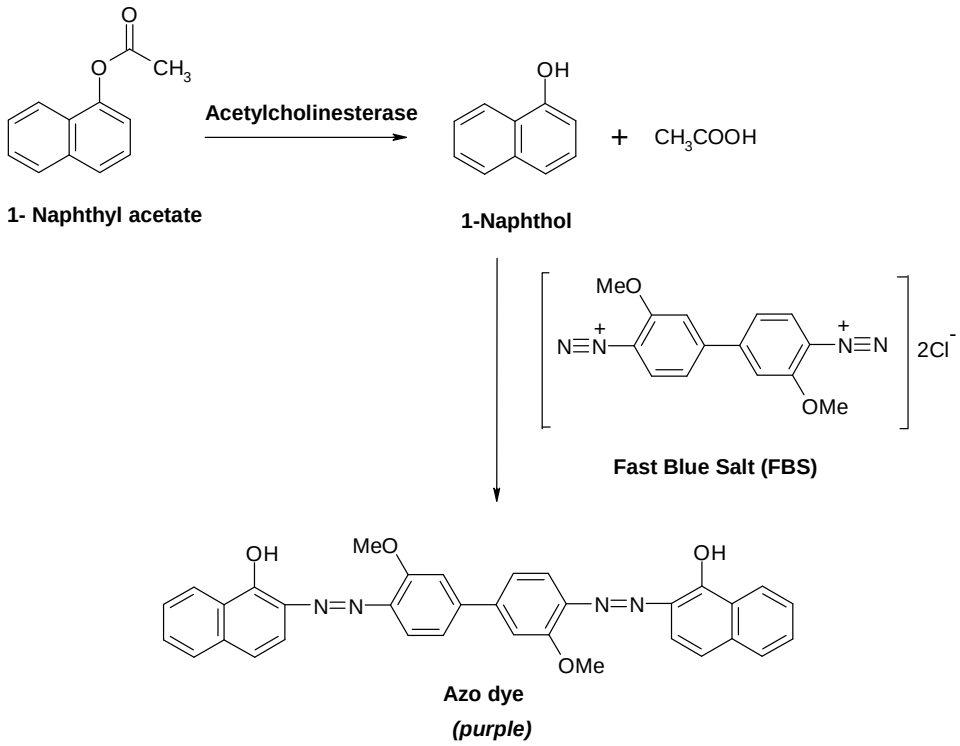

Fig. 1. The reaction mechanism of AChE inhibition used in bioautography.

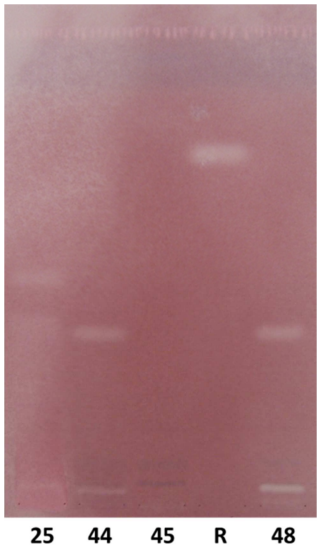

Fig. 2. Sample of bioautography for DCM extracts with the non-polar mobile phase $CHCl_3$-EtOAc-MeOH (90+7+3). 25: *Cuminum cyminum* L., 44: *Cannabis sativa* L., 45: *Coriandrum sativum* L., R: chelidonine (positive control in non-polar system), 48: *Sesamum indicum* L.

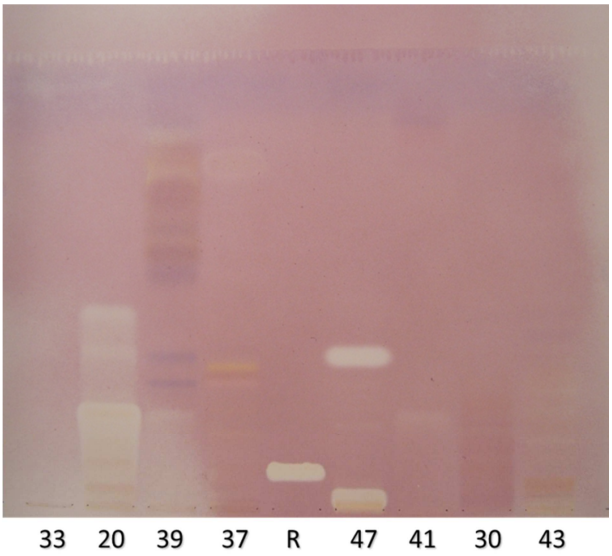

Fig. 3. Sample of bioautography for MeOH extracts with the polar mobile phase EtOAc-MeOH-H_2O (100+13.5+10). 33: *Boswellia carterii* Birdw., 20: *Asarum europaeum* L., 39: *Commiphora mukul* Engl., 37: *Aloe spp.*, R: physostigmine (positive control in polar system), 47: *Peganum harmala* L., 41: *Pistacia atlantica* Defs., 30: *Astragaus arbusculinus* Bornm. & Gauba, 43: *Anethum graveolens* L.

The AChE inhibitory activities of the substances were measured by a quantitative colorimetric assay based on Ellman's method (Rhee *et al.*, 2001) (Fig. 4).

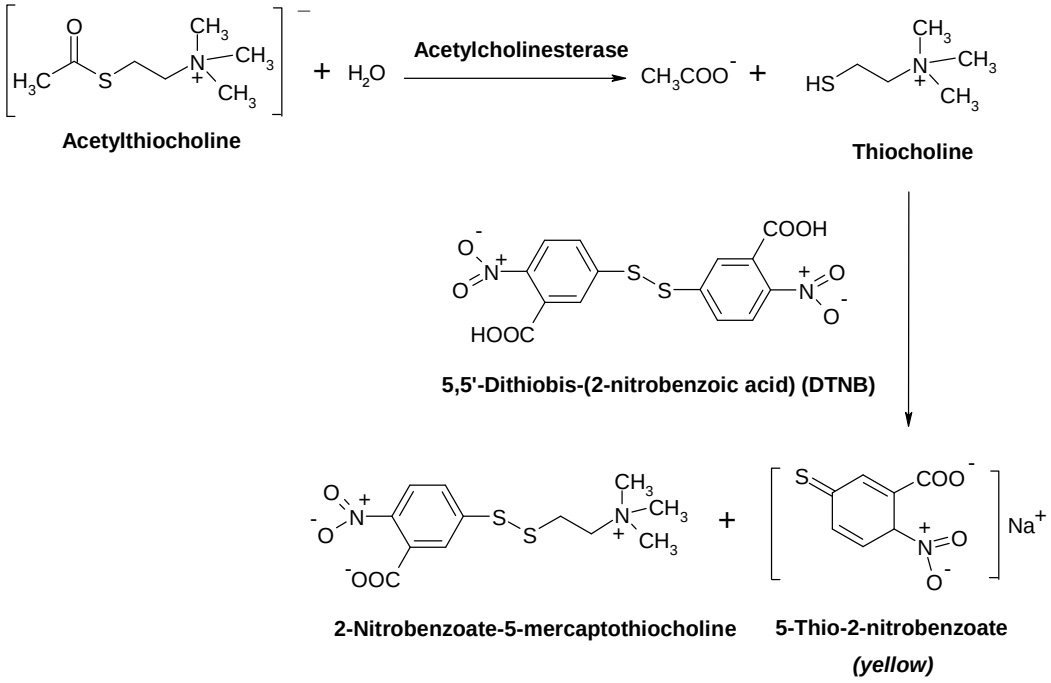

Fig. 4. The reaction mechanism of AChE inhibition used for microplate assay.

For calibration of the microplate assay 7 different concentrations of physostigmine (150, 75, 37.5, 18.75, 9.37, 4.69, 2.34 µg/ml) were used. After adding the enzyme, 6 different incubation times were examined. Based on the calibration curves, 10 minutes was considered as optimum incubation time (Fig. 5-6).

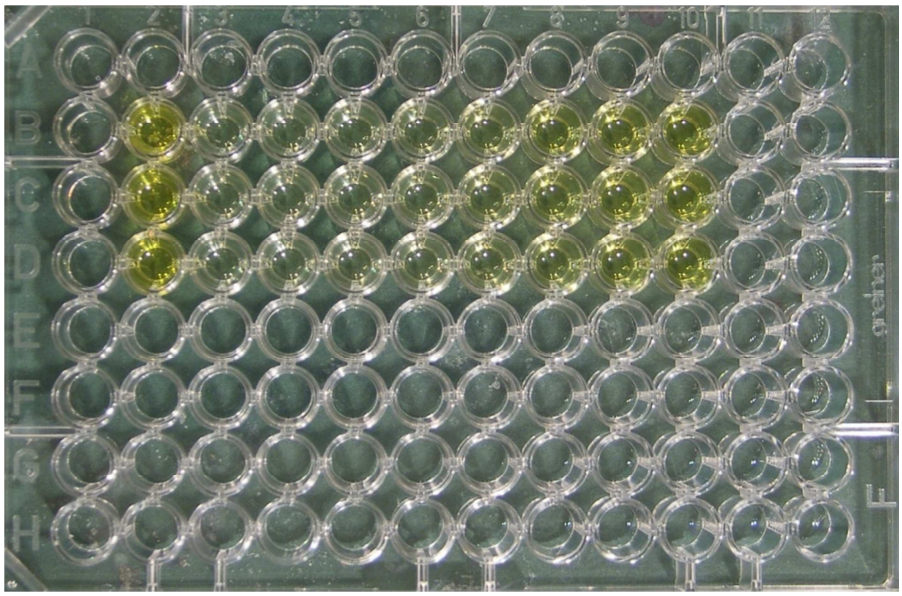

Fig. 5. AChE inhibition of physostigmine in a microplate assay. Columns No. 3-10 related to concentrations of 150-1.17 µg/ml. Column No. 2 is the negative control (10% DMSO).

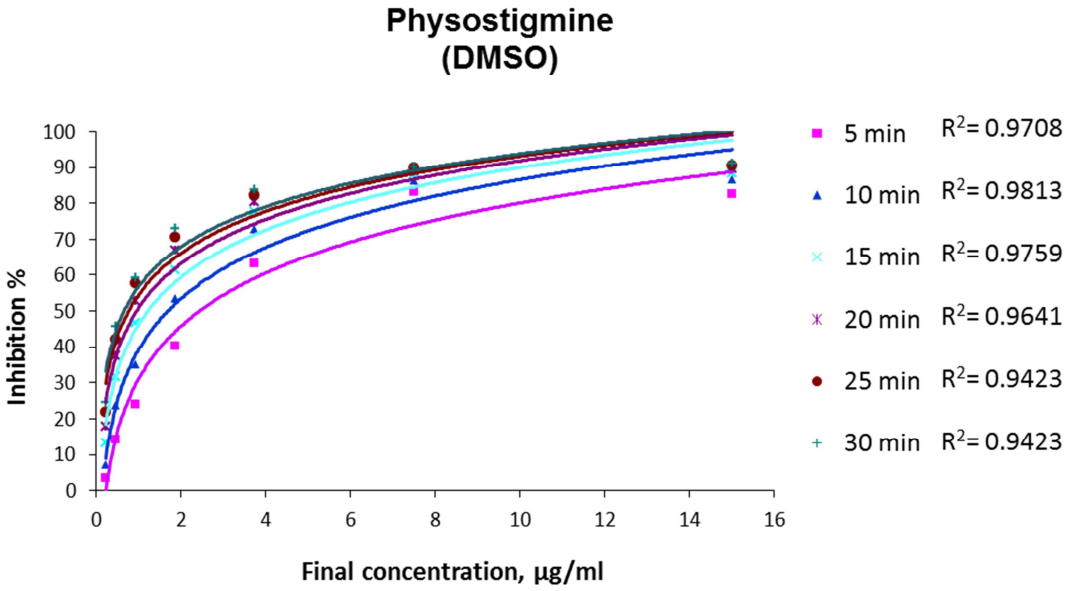

Fig. 6. Calibration curves for AChE inhibition of physostigmine after different incubation times.

Due to the best activity determined for the extracts from the seeds of *Peganum harmala* this drug was investigated in detail. The active compounds were identified as harmaline and harmine (Fig. 7-8).

Fig. 7. *Peganum harmala*. 1: shrub (http://crwma.co.crook.or.us), 2: flower (http://agri.nv.gov), 3: capsules (http://caliban.mpiz-koeln.mpg.de), 4: dried capsules (http://www.madrean.org), 5: seeds.

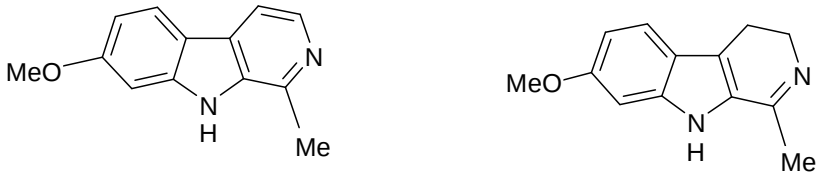

Fig. 8. Structures of harmine and harmaline, the most active compounds for AChE inhibition in seeds of *Peganum harmala*.

The IC_{50} values of the extracts and the two alkaloids for AChE inhibition were determined by microplate assay (Fig. 9).

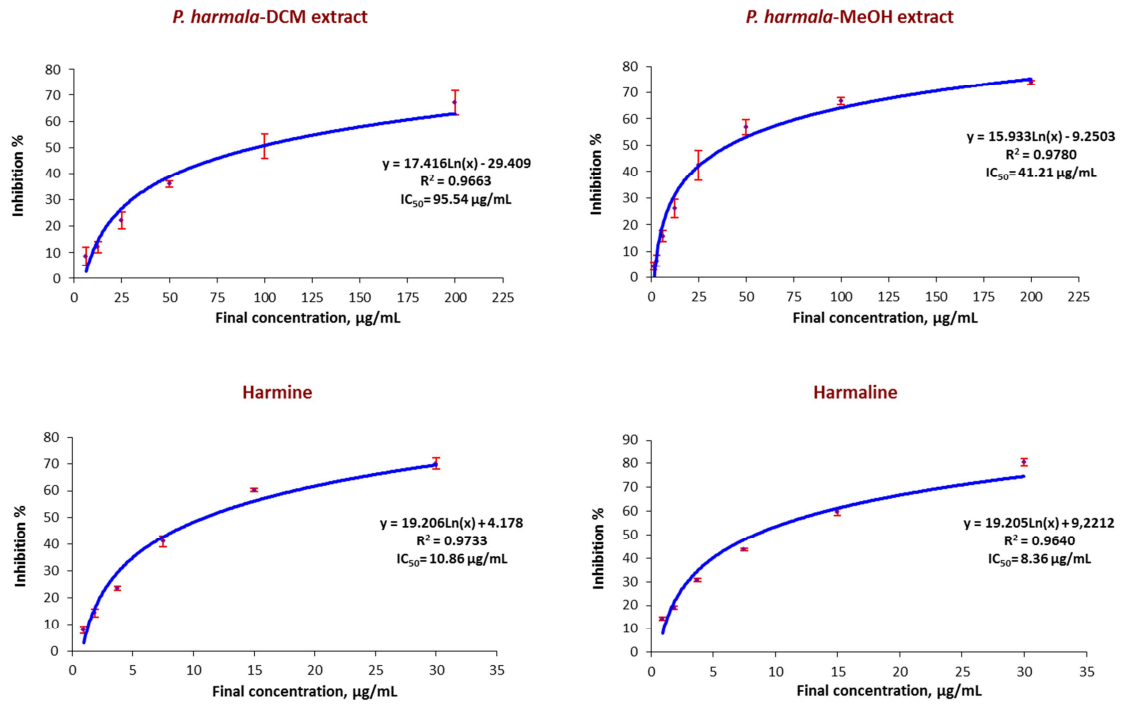

Fig. 9. AChE inhibition of DCM and MeOH extracts (200, 100, 50, 25, 12.5, 6.25, 3.13 µg/ml) and harmine and harmaline (30, 15, 7.5, 3.75, 1.87, 0.94 µg/ml).

The concentrations of active compounds in DCM and MeOH extracts were determined by HPLC analyses using external standardization (Fig. 10).

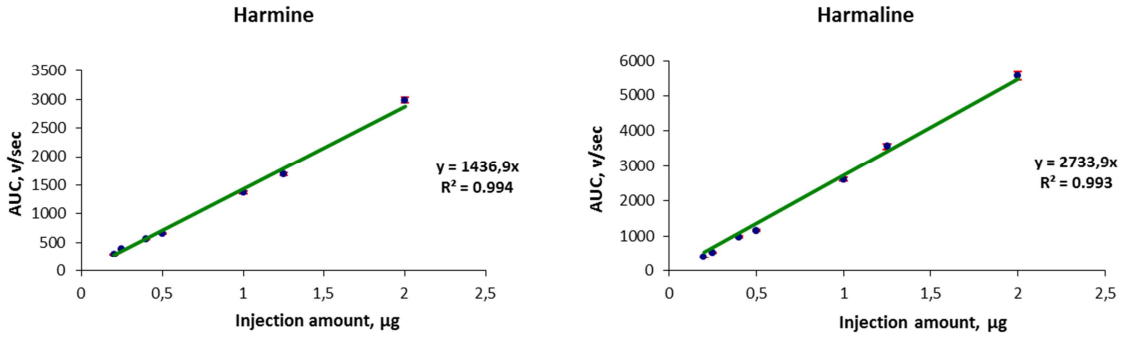

Fig. 10. Calibration curves for HPLC-quantification of harmine and harmaline.

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Catechol alkenyls from *Semecarpus anacardium*: Acetylcholinesterase inhibition and binding mode predictions

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ABSTRACT

Ethnopharmacological relevance: The fruits of *Semecarpus anacardium* L. f. (Anacardiaceae) are used in Ayurvedic medicine and also in Iranian Traditional Medicine for various indications, among those for retarding and treatment of dementia.
Aim of the study: The severity of Alzheimer's disease obviously correlates with a cholinergic deficit. In a screening for acetylcholinesterase (AChE) inhibitory activity, an extract from the fruit resin of *Semecarpus anacardium* was among the most active ones. Thus, the aim of this study was to isolate the active compounds and to investigate them in detail. Their binding mode to the active site of AChE was investigated by *in silico* docking experiments.
Materials and methods: From a dichloromethane extract in an activity-guided fractionation the active compounds were isolated under use of different chromatographic techniques. Their structures were unambiguously identified by one and two-dimensional ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry and their cholinesterase inhibitory activities were determined by a microplate assay. In order to compare the 3D active sites of AChE from *Torpedo californica* (TcAChE) and from *Electrophorus electricus* (EeAChE), three files from the Protein Data Bank (PDB) were used and for docking experiments, GOLD 3.1 software was employed. The concentrations of active compounds in the extract and the fruits were determined by HPLC analysis.
Results: The active compounds were determined as 1′,2′-dihydroxy-3′-pentadec-8-enylbenzene (A) and 1′,2′-dihydroxy-3′-pentadeca-8,11-dienylbenzene (B). Their IC_{50} values in an *in vitro* assay on AChE inhibition were determined as 12 and 34 μg/mL, respectively, while they were not active in the inhibition of butyrylcholinesterase (BChE). *In silico* docking experiments showed a similar bioactivity for compounds A and B. The concentration of compounds A and B in the fruits was 1.85% and 1.88%, respectively.
Conclusion: In the search for the active principle of the fruit resin of *Semecarpus anacardium*, compounds A and B were identified as two selective inhibitors for AChE versus BChE.

1. Introduction

Moderate degenerative conversions during ageing have been shown to occur in cholinergic neurons of the basal forebrain complex (Schliebs and Arendt, 2006). Alzheimer's disease (AD) is a neurodegenerative disorder known for memory impairment, emotional disturbance and personality changes (Bartolucci et al., 2001). The affection of cholinergic neurons in AD led to the cholinergic hypothesis, which associates AD symptoms to cholinergic deficiency (Greenblatt et al., 2004). A prolongation of the availability of acetylcholine, the most important cholinergic neurotransmitter, can be achieved by using inhibitors of acetylcholinesterase (AChE), the key enzyme in the breakdown of acetylcholine (Howes and Houghton, 2003).

Thus, natural compounds with AChE inhibitory effects such as galanthamine became an option in the supportive treatment of AD (Prvulovic et al., 2010). The identification of natural compounds with AChE inhibitory activity in plants from traditional medicine can be supported by virtual screening (Rollinger et al., 2004; Schuster et al., 2010). The use of molecular modeling techniques in the field of AChE inhibitors is facilitated by the availability of AChE X-ray crystal structures with and without co-crystallized inhibitors. AChE consists of about 540 amino acids and its active site is buried deep in the enzyme. A narrow gorge, consisting of 14 aromatic amino acids, connects the active site with the surface of AChE. This so-called active site gorge, which is approximately 20 Å

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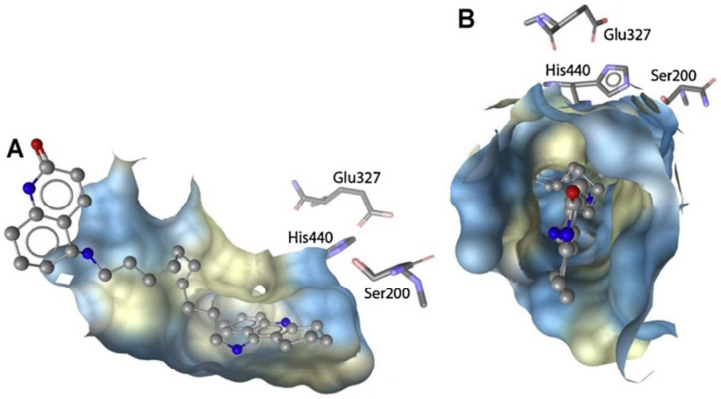

Fig. 1. The ligand binding pocket of *Tc*AChE (PDB: 1zgb). The catalytic triad Ser200-His440-Glu327 is shown in line style. The cocrystallized ligand of 1zgb, N-9-(1,2,3,4-tetrahydroacridinyl)-N′-5-[5,6,7,8-tetrahydro-2′(1′H)-quinolinonyl]-1,10-diaminodecane, a tacrine-derivative, is shown in ball-and-stick style. The ligand binding pocket surface is visualized as blue and yellow areas, where blue represents hydrophilic and yellow hydrophobic areas. A and B show two different perspectives of the ligand binding pocket. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

deep, plays a key role in enzyme activity. Substrates have to pass this tunnel in order to get metabolized. At the active site, the ester bond of acetylcholine is hydrolyzed by the catalytic triad Ser200-His440-Glu327 (Fig. 1) (Haviv et al., 2005; Rollinger et al., 2006).

In Ayurvedic medicine dementia is known as *Cittinasa* (loss of mind) in *Caraka Samhita*, the early Ayurvedic compendium on internal medicine. Several herbal drugs have been used in the *Materia Medica* of Ayurveda for the treatment of dementia for many centuries and are considered to be both safe and effective.

Semecarpus anacardium L. f. (Anacardiaceae) is a deciduous tree in the outer Himalayas, parts of India and Northern Australia. Due to its medicinal value, it is a well-known plant in the Ayurvedic and Siddha system of medicine (Adams et al., 2007; Semalty et al., 2010). Bhallataka (Indian name of *Semecarpus anacardium*) has been applied in several herbal mixtures, e.g. Brahmarasayana, for retarding and treatment of memory loss (Manyam, 1999). Additionally, in Iranian Traditional Medicine (ITM) *Semecarpus anacardium*, known as Belādor in Persian language, is an important component in many herbal preparations used for memory improvement or treatment of dementia (Sharafkandi, 2004). Pharmacological studies have shown several biological activities for *Semecarpus anacardium* such as anti-inflammatory (Bhitre et al., 2008), anti-atherogenic (Sharma et al., 1995), anti-oxidant (Pal et al., 2008), anti-microbial (Gajjar et al., 2009), hypoglycemic (Kothai et al., 2005), hypolipidemic (Jaya et al., 2010), anti-carcinogenic (Nair et al., 2009) and anti-spermatogenic effects (Sharma et al., 2003). Methanolic and dichloromethane (DCM) extracts from the fruit resin of *Semecarpus anacardium* were among the most potent hits for AChE inhibition in a screening of plants used for the enhancement of cognitive performance and treatment of memory loss in ITM (Adhami et al., 2011). In this study, an activity-guided approach was applied to isolate and characterize the components with AChE inhibitory activity. The activity of the isolated compounds to inhibit butyrylcholinesterase (BChE) was investigated as well. In *in silico* docking experiments their binding mode to the active site of AChE was studied.

2. Materials and methods

2.1. Chemicals and reagents

AChE from electric eel, BChE from equine serum, 1-naphthyl acetate, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), Tris–HCl, bovine serum albumin (BSA), lipophilic Sephadex LH20 and physostigmine were purchased from Sigma (St. Louis, USA). Acetylthiocholine iodide (ATCI), butyrylthiocholine iodide (BTCI) and chelidonine were obtained from Fluka (Buchs, Switzerland). Fast Blue B salt (FBS) and silica gel 60 were from Merck (Darmstadt, Germany). 1,2-Dihydroxy-3-pentadecylbenzene (urushiol) was obtained from Chromadex (Irvine, USA). Two different buffer systems were used (buffer A: 50 mM Tris–HCl, pH 7.9 containing 0.1% BSA; buffer B: 50 mM Tris–HCl, pH 7.9 containing 0.1 M NaCl and 0.02 M $MgCl_2 \cdot 6H_2O$).

2.2. General

A Tecan Genios microplate reader (Tecan, Salzburg, Austria) was used to measure the absorbance. Extraction was performed with a Dionex accelerated solvent extractor (ASE) model 200 (Dionex, Sunnyvale, USA). TLC plates Silicagel 60 F254 and HPTLC RP-18 were purchased from Merck (Darmstadt, Germany) and 96-well microplates PS F-bottom were obtained from Greiner Bio-One (Frickenhausen, Germany). HPLC was performed on a Shimadzu instrument with LC-20AD pump, SPD M20A diode array detector and SIL 20AC HT auto-sampler (Kyoto, Japan). For solid phase extraction (SPE) mega bond elut-C18 cartridges from Varian (Santa Clara, USA) were used. Direct infusion ESI-MS/MS analyses were performed with an HCT ion trap mass spectrometer equipped with an orthogonal ESI source (Bruker Daltonics, Bremen, Germany) and a syringe pump from kdScientific (Holliston, USA). The NMR spectrometer was a Bruker Avance DRX 600 operating at 600.13 MHz for ^{1}H and at 150.90 MHz for ^{13}C, respectively (Bruker BioSpin, Rheinstetten, Germany). All spectra were recorded at a temperature of 298 K using a triple resonance probe (^{1}H, ^{13}C, broad band) with triple axis pulsed field gradients.

2.3. Plant material

The fruits of *Semecarpus anacardium* were purchased from a herbal shop in Tehran, Iran, and identified on the basis of anatomical and morphological features by Dr. Gholamreza Amin at the herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran (voucher number PMP-609).

Table 1
^{1}H and ^{13}C NMR data of compounds A and B ($CDCl_3$, 600 MHz).

Position	A		B	
	δ_C (dept)	δ_H	δ_C (dept)	δ_H
1′	143.0 (s)	–	143.0 (s)	–
2′	141.9 (s)	–	141.9 (s)	–
3′	129.3 (s)	–	129.3 (s)	–
4′	122.1 (d)	6.71 (1H, m)	122.1 (d)	6.72 (1H, m)
5′	120.1 (d)	6.71 (1H, m)	120.1 (d)	6.71 (1H, m)
6′	112.8 (d)	6.71 (1H, m)	112.8 (d)	6.71 (1H, m)
1′	–	5.23 (1H, brs)	–	5.20 (1H, br s)
2′	–	5.18 (1H, s)	–	5.20 (1H, br s)
1	29.8 (t)	2.60 (2H, t, J = 7.9 Hz)	29.7 (t)	2.60 (2H, t, J = 7.7 Hz)
2	29.7 (t)	1.61 (2H, m)	29.7 (t)	1.61 (2H, quin, J = 7.7 Hz)
3	29.5 (t)	1.36 (2H, m)	29.6 (t)	1.34 (2H, m)
4	29.0 (t)[a]	1.29 (2H, m)[a]	29.5 (t)[e]	1.34 (2H, m)
5	29.2 (t)[a]	1.31 (2H, m)[a]	29.4 (t)[e]	1.34 (2H, m)
6	29.7 (t)[b]	1.33 (2H, m)	29.2 (t)[e]	1.34 (2H, m)
7	27.2 (t)[c]	2.01 (2H, m)	27.2 (t)	2.10 (2H, m)
8	129.9 (d)[d]	5.35 (1H, m)	130.1 (d)	5.39 (1H, dtt, J = 10.8, 6.8, 1.4 Hz)[g]
9	129.8 (d)[d]	5.35 (1H, m)	128.1 (d)[f]	5.33 (1H, dtt, J = 10.8, 6.9, 1.4 Hz)[f]
10	27.2 (t)[c]	2.01 (2H, m)	25.6 (t)	2.77 (2H, quin tt, J = 0.6, 1.2, 6.8 Hz)
11	29.7 (t)[b]	1.33 (2H, m)	128.0 (d)[f]	5.35 (1H, dtt, J = 10.8, 6.9, 1.4 Hz)[f]
12	29.4 (t)[a]	1.33 (2H, m)[a]	129.9 (d)	5.38 (1H, dtt, J = 10.8, 6.9, 1.4 Hz)[g]
13	31.8 (t)	1.27 (2H, m)	29.3 (t)	2.04 (2H, m)
14	22.7 (t)	1.29 (2H, m)	22.8 (t)	1.39 (2H, six, J = 7.4 Hz)
15	14.1 (q)	0.88 (3H, t, J = 7.1 Hz)	13.8 (q)	0.91

Signals with the same superscript are interchangeable.

2.4. Extraction

To obtain the compounds from the resin, the shells of 20 g fruits of *Semecarpus anacardium* were broken and extracted 2 times by sonification with 100 mL dichloromethane (DCM) at 40 °C. The extracts were combined and evaporated to dryness under reduced pressure at 40 °C resulting in 5.52 g of DCM extract.

2.5. TLC bioautography assay

The DCM extract of *Semecarpus anacardium* was examined by TLC on silica plates by use of the mobile phase chloroform–ethylacetate–methanol (90 + 7 + 3). Detection was performed with anisaldehyde–sulfuric acid reagent to determine the chemical composition of the extract. In addition, a TLC bioautography screening assay was performed for AChE inhibitory activity according to a published method under use of the same stationary and mobile phase and very thorough removal of the mobile phase under airstream before detection (Marston et al., 2002; Adhami et al., 2011). Chelidonine served as a positive control showing Rf 0.45 in this system.

2.6. Microplate assay

The AChE inhibitory activities of the substances were measured by a quantitative colorimetric assay based on Ellman's method (Rhee et al., 2001; Chaiyana et al., 2010; Adhami et al., 2011). In a 96-well plate, 25 μL of 15 mM ACTI, 125 μL of 3 mM DTNB in buffer B, 50 μL of buffer A and 25 μL of active compounds (from 10.9 μg/mL to 1.40 mg/mL in 10% DMSO) were mixed and the absorbance was measured at 405 nm every 15 s for 5 times. Then 25 μL of AChE (0.22 U/ml in buffer A) was added and the plate was incubated at 25 °C for 10 min. Then the absorbance was measured again 8 times every 15 s. A solution of 10% DMSO was used as negative control. To avoid any increase in absorbance due to the color of the extracts or spontaneous hydrolysis of substrate, the absorbance before addition of the enzyme was subtracted from the absorbance after adding the enzyme. The assay was performed with three repetitions for every concentration. For validation different concentrations of physostigmine (15–0.12 μL/mL as the final concentration) served as a positive control. Each experiment was carried out in triplicate.

2.7. Isolation and characterization of active compounds

The fractionation of 5.0 g DCM extract was performed by vacuum liquid chromatography (VLC) on a silica gel 60 column with chloroform as the mobile phase. The fractions were combined according to their chemical composition and the resulting 6 collective fractions (A1–A6), which were checked by TLC bioautography for their activity. The most active oily fraction A4 (2.146 g) was further purified by VLC on a silica gel column by gradient elution with 100-50% petroleum ether in chloroform resulting in 407 mg of an enriched active subfraction. After another purification step on lipophilic Sephadex LH-20 under elution with methanol, 262 mg of a mixture of two active compounds was detected by TLC on a RP-18 stationary phase. To isolate the single compounds, solid phase extraction was performed on a Megabond Elut-C18 cartridge. The mobile phase consisted of 70–90% methanol in water and a flow rate of 2.5 mL/min was applied resulting in the isolation of the two pure oily compounds A (24 mg) and B (10 mg). The structures of the active components were identified by one and two-dimensional ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry.

Compound A: ESI-MS (*m/z*, rel. intensity %): 317.2 $[M-H]^-$ (100); ESI-MS^2 (317.2→) (*m/z*, rel. intensity %): 317.1 (22), 259.0 (6), 190.9 (6), 134.8 (46), 121.8 (100). ^{1}H and ^{13}C NMR data are summarized in Table 1.

Compound B: ESI-MS (*m/z*, rel. intensity %): 315.2 $[M-H]^-$ (100); ESI-MS^2 (315.2→) (*m/z*, rel. intensity %): 315.1 (6), 173.9 (2), 147.8 (7), 134.8 (16), 121.8 (100). ^{1}H and ^{13}C NMR data are summarized in Table 1.

2.8. HPLC

The DCM extract of *Semecarpus anacardium* was analyzed by HPLC under gradient elution on a Hypersil BDS-C18 column (250 mm × 4 mm id) at 25 °C (Shin et al., 1999). The mobile phase consisted of acetonitrile (A) and water (B) at a flow rate of 0.75 mL/min. The elution started with 80% A + 20% B. After an

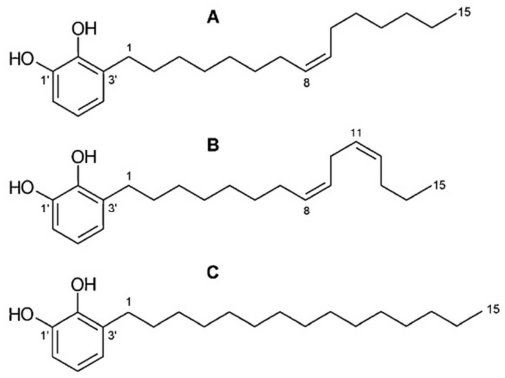

Fig. 2. Structures of compounds A, B and C.

isocratic step of 5 min the composition was changed to 93% A + 7% B within 10 min and this concentration was kept for further 10 min. The eluents were monitored at 280 nm.

2.9. Overlay of TcAChE/EeAChe active sites

In order to compare the 3D active sites of *Tc*AChE and *Ee*AChE, three PDB files, 1zgb (*Tc*AChE), 1w76 (*Tc*AChE), and 1c2b (*Ee*AChE), were downloaded from the PDB (http://www.pdb.org/) (Berman et al., 2003), and loaded into the LigandScout software (Wolber and Langer, 2005). Several amino acids from each active site were selected for alignment, which was performed within the alignment perspective. For each AChE crystal structure, only essential amino acid residues forming the ligand binding pocket were selected as "core molecules" (for 1c2b: Trp86, Glu202, Ser203, Tyr337, and His447; for 1zgb and 1w76: Trp84, Glu199, Ser200, Phe330, and His440) and exported to the alignment perspective.

2.10. Docking experiments

For docking experiments, GOLD 3.1 software was employed (www.ccdc.cam.ac.uk/products/life_sciences/gold), which uses a genetic algorithm for the generation of multiple docking solutions for each compound (Verdonk et al., 2003; Krovat et al., 2005). In general, the default parameters of GOLD were used. All cocrystallized water molecules were preserved.

Compounds A, B, and urushiol (compound C) were prepared for docking studies with Corina 3.0, which calculated a starting ligand geometry (www.molecular-networks.com/products/corina). Further ligand and protein preparation for docking was performed within GOLD.

3. Result and discussion

The fruit resin of *Semecarpus anacardium* has been used in Ayurvedic medicine and in ITM for the treatment of cognitive deficits in several pharmaceutical formulations, usually in combination with other plants, e.g. Brahmarasayana boluses (Manyam, 1999). In a previous screening of plants used for similar indications in ITM, we showed an AChE inhibitory activity for this resin (Adhami et al., 2011). Vinutha et al. (2007) reported AChE inhibitory activity for the stem bark from *Semecarpus anacardium*. Based on these results the active compounds from the fruit resin were isolated and characterized, and their binding mode to the active site of AChE was investigated.

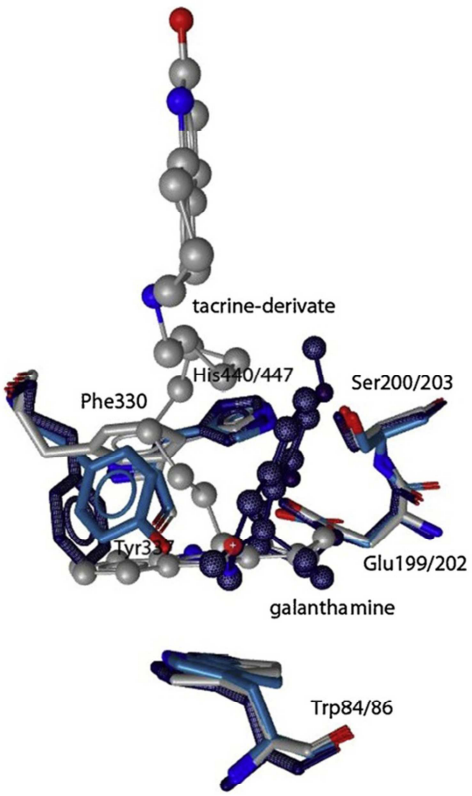

Fig. 3. Alignment of ligand binding domains of *Tc*AChE (PDBs: 1zgb, grey; 1w76, highlighted) and *Ee*AChE (1c2b, blue, line style) created with LigandScout. Ligand binding pockets show a high similarity except for Phe330 (TcAChE). which is replaced by Tyr337 in *Ee*AChE. The conformational flexibility of Phe330/Tyr337 allows the formation of an aromatic sandwich with flat, aromatic ligands such like tacrine derivatives. Example ligands (N-9-(1,2,3,4-tetrahydroacridinyl)-N′-5-[5,6,7,8-tetrahydro-2′(1′H)-quinolinonyl]-1,10-diaminodecane from 1zgb and galanthamine from 1w76, highlighted) are shown in ball-and-stick style. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.1. Extraction, isolation and identification of active compounds

In preliminary tests the resin had been extracted with DCM and methanol, to cover active compounds of low to high polarity (Adhami et al., 2011). By TLC the same active compounds had been detected in both extracts. Due to very high amounts of tannins in the methanolic extract (approx. 90% as determined in a detannification step) the DCM extract was selected for the isolation of these compounds. After VLC of the DCM extract, the active fraction A4 was submitted to gel permeation chromatography. This enrichment step resulted in an active fraction containing two very similar compounds, which could be separated by RP chromatography (for HLPC see Fig. 5). On HPTLC RP18 plates with acetonitrile–water (99.5 + 0.5) as the mobile phase Rf values of 0.31 for A and 0.42 for B were determined. A final isolation step was performed by solid phase extraction on an RP-18 stationary phase and led to the isolation of compounds A and B. Both compounds were oily substances. ESI-MS yielded molecular weights of 318.2 Da for compound A and 316.2 Da for compound B, which is in agreement to the molecular formulae of $C_{21}H_{34}O_2$ and $C_{21}H_{32}O_2$, respectively. In negative

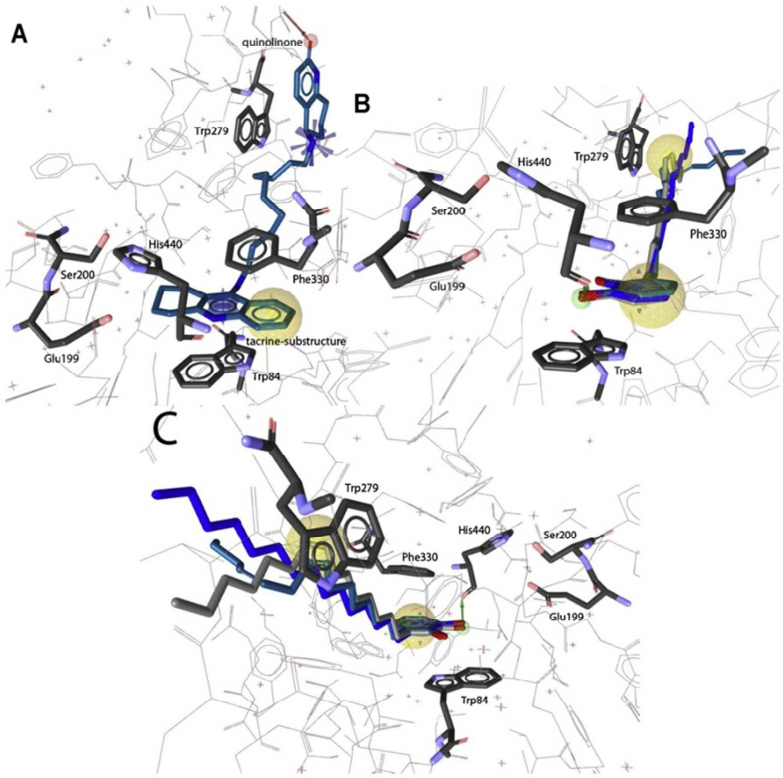

Fig. 4. N-9-(1,2,3,4-tetrahydroacridinyl)-N′-5-[5,6,7,8-tetrahydro-2′(1′H)-quinolinonyl]-1, 10-diaminodecane bound to *Tc*AChE from the PDB entry 1zgb (A) and two perspectives of compounds A (grey), B (cyan), and C (dark blue) docked into the ligand binding pocket of *Tc*AChE (B and C) revealing similar binding modes, visualized with LigandScout. Chemical interactions of compound B with the binding site are color-coded: blue circle, π–π interactions; yellow sphere, hydrophobic feature; red arrow, hydrogen bond acceptor; green arrow, hydrogen bond donor; blue star, positively ionizable group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

ion mode ESI-MS/MS spectra major fragment ions at *m/z* 134.8 and 121.8 were observed for both compounds showing fragmentation typical for alkenyl chatechols known from *Semecarpus anacardium* (Zhao et al., 2009). One- and two-dimensional NMR experiments were performed for unambiguous structure elucidation.

The comparison of the measured ^{1}H and ^{13}C NMR data proved them to be 1′,2′-dihydroxy-3′-pentadec-8-enylbenzene (A) and 1′,2′-dihydroxy-3′-pentadeca-8,11-dienylbenzene (B) (Fig. 2). These structures are largely in conformity with earlier reports, which have not given the detailed assignment of all ^{13}C shifts (Sargent et al., 1989; Nair et al., 2009). For compound B, anticancer activity has recently been shown (Nair et al., 2009).

3.2. Cholinesterase inhibition

The cholinesterase inhibitory activity of the DCM extract and the isolated compounds was determined for the first time in a microplate assay. The IC_{50} values for AChE inhibition were 24.12 μg/mL for the extract, 33.32 μg/mL for fraction A4, 12.62 ± 2.84 μg/mL (39.7 μM) for compound A and 34.15 ± 5.55 μg/mL (108 μM) for compound B. The BChE inhibition of compounds A and B at a final concentration of 200 μg/mL was 27.31% and 24.82%, respectively. Thus, compounds A and B could be considered as selective inhibitors for AChE versus BChE.

Compound A containing a double bond in position 8 of the aliphatic chain presented a stronger inhibitory activity in comparison to compound B with two double bonds in positions 8 and 11. To investigate the influence of double bonds in the carbon chain of such compounds on AChE inhibition, urushiol (=1,2-dihydroxy-3-pentadecylbenzene, compound C), which does not contain a double bond, was also tested in the microplate assay. For this compound, less than 10% AChE inhibition at a final concentration of 100 μg/mL was determined. These results suggested that the reduced conformational flexibility of the aliphatic chain imposed by the double bonds has an impact on the AChE inhibiting activity.

3.3. Evaluation and selection of X-ray structures for docking

In order to rationalize the observed bioactivity data, the binding mode of the compounds to the active site of AChE was investigated by application of the docking program GOLD3. First of all, a precise analysis of known AChE X-ray crystal structures was performed. The Protein Data Bank (PDB, www.pdb.org) (Berman et al., 2003) provides AChE structures from several source organisms. In order to obtain accurate docking solutions, the crystallized AChEs should not be aged, mutated or covalently bound to an inhibitor. Furthermore, the structure of a ligand-bound enzyme would be ideal. To date, three X-ray crystal structures of

Electrophorus electricus (electric eel) AChE (*Ee*AchE), the system used for the *in vitro* testing, are available in the PDB, but none of them represent a ligand-bound state. AChE structures containing co-crystallized inhibitors were collected. AChE structures from *Torpedo californica* (*Tc*AChE) were selected for docking studies. Enzyme structures of this organism can be divided into two classes. The first has tacrine or tacrine derivatives as ligands (PDB entry 1zgb), whereas the second exhibits a galanthamine-like ligand (PDB entry 1w76). Tacrine, a clinically used synthetic AChE inhibitor, exhibits a planar aromatic structure. Some tacrine derivatives with a long alkyl residue have also been used in crystallographic studies. In comparison, galanthamine has a ring system at an angle of about 90° to the remaining molecule (Fig. 3). The conformational flexibility of Phe330, which has a crucial effect on the size and shape of the ligand binding pocket, enables the binding of these different ligand types. In 1zgb, Phe330 and Trp84 are in a nearly parallel conformation, fixing the tacrine derivative N-9-(1,2,3,4-tetrahydroacridinyl)-N′-5-[5,6,7,8-tetrahydro-2′(1′H)-quinolinonyl]-1,10-diaminodecane in a sandwich-like manner, which forms ideal π–π stacking interactions. In comparison, Phe330 in 1w76 (containing galanthamine as ligand) is rotated by about 90°.

In order to validate whether docking results obtained with *Tc*AChE could be compared with *Ee*AchE, the ligand binding domains of the PDB entries 1zgb, 1w76 (*Tc* variants), and 1c2b (*Ee*AChE) were aligned by using the program LigandScout. The alignment of essential amino acid residues of the active site (*Tc*/*Ee*AChE: Trp84/86, Glu199/202, Ser200/203, Phe330/Tyr337, His440/447) resulted in a highly similar superimposition (RMS value of 0.227 for alignment of 1zgb to 1w76 and 0.453 for alignment of 1zgb to 1c2b). This indicates that the ligand binding pockets of *Tc*AChE and *Ee*AChE have nearly the same binding site geometry and are therefore expected to bind inhibitors in a very similar way. In *Ee*AChE, Phe330 of *Tc*AChE is replaced by Tyr337 (Fig. 3). However, as docking studies suggest, this exchange does not lead to crucial differences in ligand binding (Mizutani and Itai, 2004).

Before compounds A, B, and C were docked into the crystal structures, the ability of GOLD to reproduce accurate binding solutions for the cocrystallized 1zgb and 1w76 ligands was determined. The ligand of 1w76, galanthamine, was docked identically to the experimentally obtained binding position into the enzyme. The planar aromatic structure of the tacrine derivative N-9-(1,2,3,4-tetrahydroacridinyl)-N′-5-[5,6,7,8-tetrahydro-2′(1′H)-quinolinonyl]-1,10-diaminodecane of 1zgb also exhibits docking modes similar to the reported binding position. However, the long alkyl residue and the quinolinone structure, which reaches out of the binding pocket, show a certain aberration from the exact binding position. Since these parts of the molecule are located at the entrance of the binding site gorge and can therefore move rather freely, this deviation was tolerated. These redocking experiments confirmed the applicability of the GOLD docking algorithm for the experiments carried out.

3.4. Docking of compounds A, B, and C in TcAChE

The ligand binding site of the PDB entry 1zgb was selected for docking studies since the tacrine-derivative and compounds A, B, and C share a planar aromatic structure and a long alkyl residue suggesting similar binding modes. The interactions of the tacrine-derivative with AChE include π–π stacking with Trp84 and a hydrophobic interaction of the tacrine substructure in the active site, a positively ionizable feature at the exit of the hydrophobic gorge leading to the active site, and a hydrogen bond acceptor feature of the quinolinone substructure also at the exit (Fig. 4A). GOLD produced several docking solutions for the tested compounds A–C. The two major interaction patterns exhibit a π–π stacking either

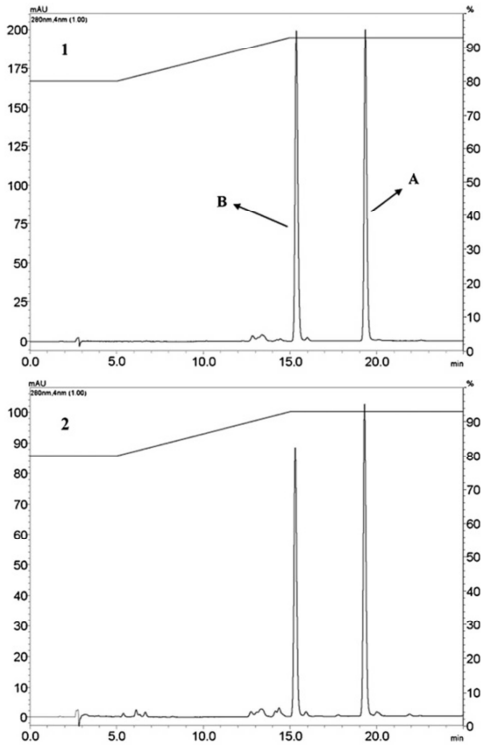

Fig. 5. Comparison of the HPLC chromatograms of (1) compounds A and B with (2) the DCM extract of *Semecarpus anacardium*.

to Trp84 and Phe330 or to Trp279. For compound C, all docking solutions are located in the Trp84/Phe330 sandwich. Comparison of the tacrine-derivative and compounds A, B, and C revealed the high analogy of their binding modes. The planar aromatic benzene-1,2-diol substructure of compounds A, B, and C perfectly squeezes between Trp84/Phe330, comparable to the tacrine-substructure. One hydroxyl function allows the formation of a hydrogen bond to the backbone carbonyl of His440. The long alkyl residues of compounds A, B, and C stretch into the hydrophobic channel that connects the active site with the surface of the enzyme. The same binding mode was observed for the long alkyl linker of the tacrine-derivative. Double bonds generated hydrophobic features near to Trp279 (Fig. 4B and C). The similarity of the binding orientation of the cocrystallized ligand and compounds A/B/C and the high GoldScores computed for the reported docking poses of compounds A/B/C (54.4892/50.2912/57.4973 versus 77.4428 for the tacrine-derivative) allow the assumption that compounds A, B, and C interact with the *Ee*AChE *in vitro* via the reported docking poses.

3.5. Structure–activity relationship

Based on the bioactivity data and the calculated binding poses, a structure–activity relationship can be proposed. Compounds A and B showed a similar bioactivity and similar computed GoldScores. In comparison to compound C, which is inactive *in vitro*, those compounds share a 8,9 double bond. This bond is suggested to direct the long, hydrophobic side chain into a more favorable binding conformation. Additionally, the double bond reduces the

flexibility of the compound, which is favorable for enthalpic ligand binding contributions.

3.6. HPLC quantification of compounds A and B

For the quantification of compounds A and B in the resin and in the tested extract, HPLC analyses were performed and the concentration of the two active compounds was determined by external standardization (Fig. 5).

A published method (Shin et al., 1999) was optimized and validated for compounds A and B. The peak areas of both components were linearly dependent on concentrations over the selected range with correlation coefficients of $R^2 = 0.9958$ for compound A and $R^2 = 0.9920$ for compound B. The concentration of compound A and compound B in the extract was 6.73% and 6.80%, respectively. Total amounts of these two compounds in the resin were 1.85% compound A and 1.88% compound B. By this method also the purity of compounds A and B was determined, being 88% and 85% for A and B, respectively. The purities were taken into account in the determination of the IC_{50} values of A and B. The IC_{50} values of the extract and the isolated compounds showed good correlations with the concentrations of the isolated compounds in the extract. Due to this correlation and the fact that no other AchE inhibiting compounds were detected by TLC bioautography in the DCM and methanol extracts can be concluded that A and B are the major AChE inhibitory compounds in the resin of *Semecarpus anacardium*.

4. Conclusion

This study showed that two catechol alkenyls, 1′,2′-dihydroxy-3′-pentadec-8-enylbenzene and 1′,2′-dihydroxy-3′-pentadeca-8,11-dienylbenzene, are responsible for the AChE inhibitory activity of the fruit resin from *Semecarpus anacardium* and thus might contribute to the effects of this drug in the treatment of cognitive deficits. Obviously, both compounds are selective AChE inhibitors as they showed a much lower activity on BChE. The study of the AChE inhibitory effect of urushiol and *in silico* experiments suggested that the 8,9 double bond is necessary for the better direction of the side chain into a favorable binding conformation at the receptor.

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Supporting information for article 2

The fruit resin from *Semecarpus anacardium* was the second drug investigated in detail (Fig. 1).

Fig. 1. *Semecarpus anacardium*. 1: tree (http://www.hear.org), 2: fruits (http://www.flickr.com/photos/dinesh_valke), 3: dried fruits, 4: fruits resin.

TLC bioautography was performed on the DCM extract of the fruit resin from *S. anacardium* (Fig. 2).

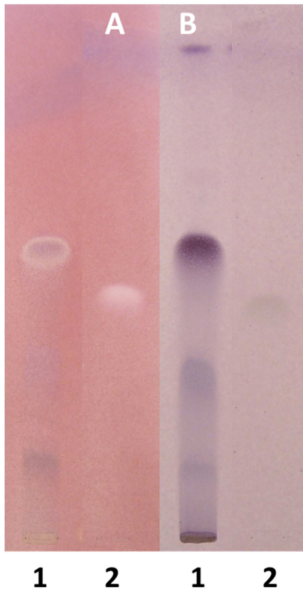

Fig. 2. TLC of DCM extract. A: bioautography assay, B: derivatization with anisaldehyde-sulphuric acid. 1: DCM extract of fruit resin from *S. anacardium,* 2: chelidonine (positive control). Mobile phase: $CHCl_3$-EtOAc-MeOH (90+7+3).

The active compounds were isolated and purified using several chromatographic techniques including VLC, SEC and SPE (Fig. 3-6).

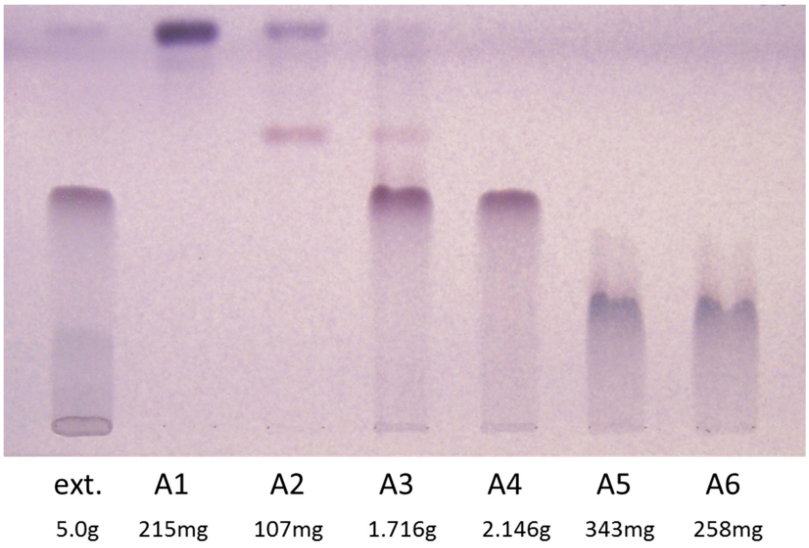

Fig. 3. TLC of fractions A1-A6 after VLC of DCM extract. Stationary phase: silica gel 60, mobile phase: $CHCl_3$ (250 ml/fraction). TLC Mobile phase: $CHCl_3$-EtOAc-MeOH (100+7+3).

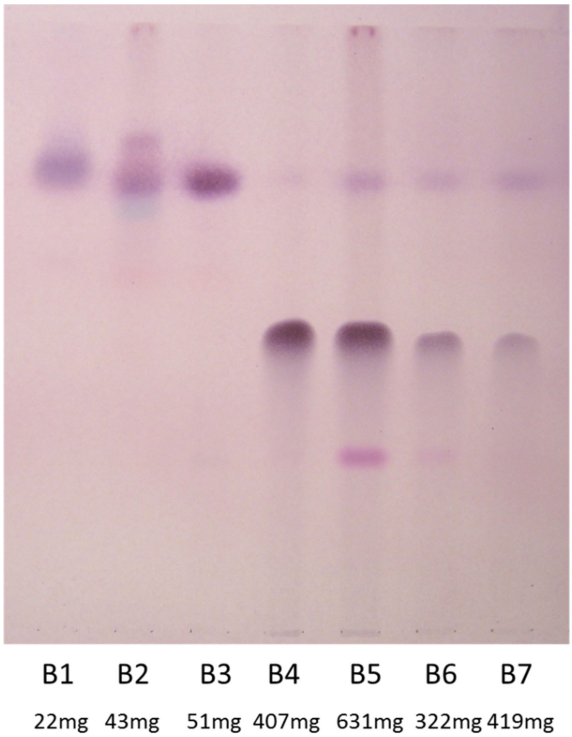

Fig. 4. TLC of fractions B1-B7 after VLC of fraction A4. Stationary phase: silica gel 60, mobile phase: 100-50% petroleum ether in $CHCl_3$ (100 ml/fraction). TLC Mobile phase: $CHCl_3$-EtOAc-MeOH (90+7+3).

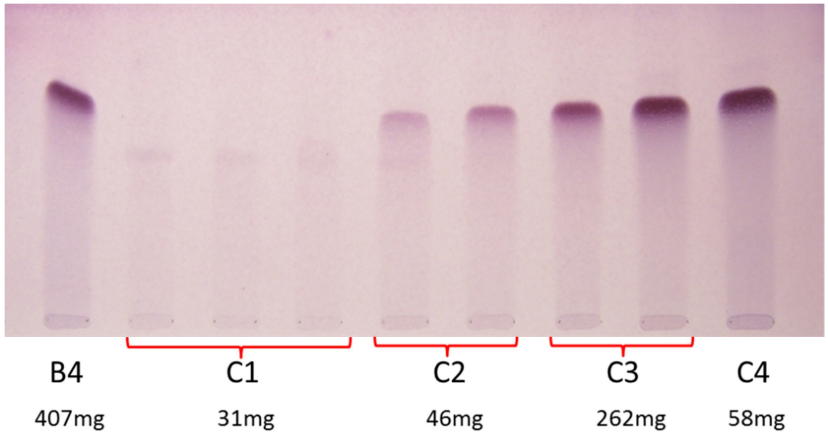

Fig. 5. TLC of fractions C1-C4 after SEC of fraction B4. Stationary phase: lipophilic Sephadex LH20, mobile phase: MeOH (3 ml/20 min). TLC Mobile phase: $CHCl_3$-EtOAc-MeOH (100+4+1).

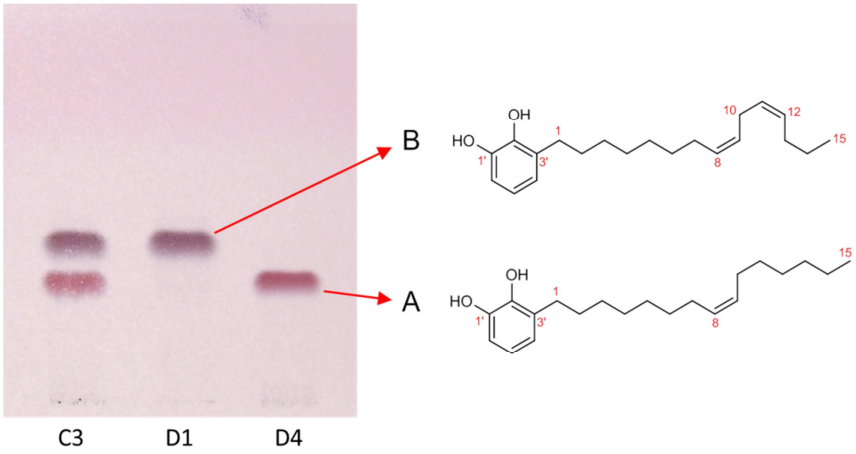

Fig. 6. TLC of compounds A and B after SPE of fraction C3. Stationary phase: Megaband Elut-C18, mobile phase: 70-90% MeOH in water (2.5 ml/min). TLC stationary phase: RP-18, mobile phase: MeOH.

The structures of the isolated components were characterized by one and two-dimensional ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry (Fig. 7-20).

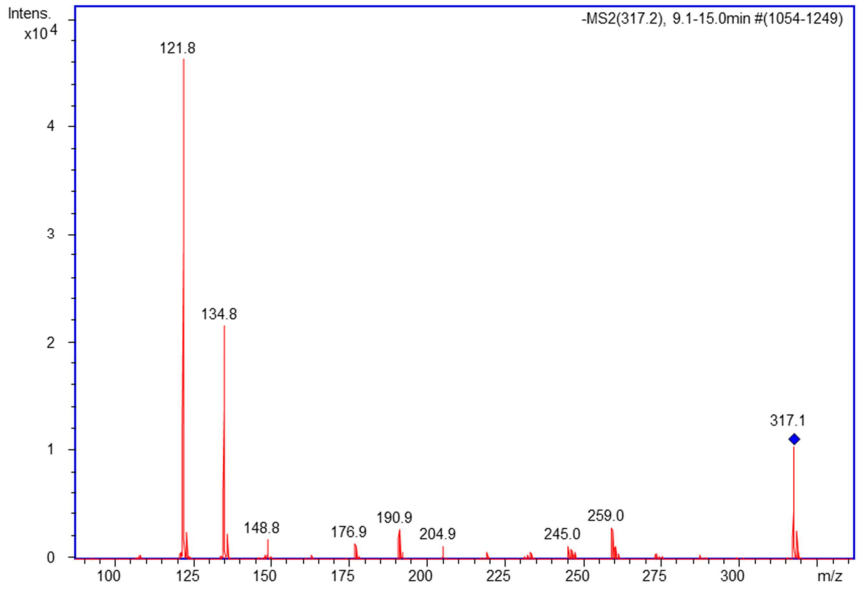

Fig. 7. MS spectrum of compound A.

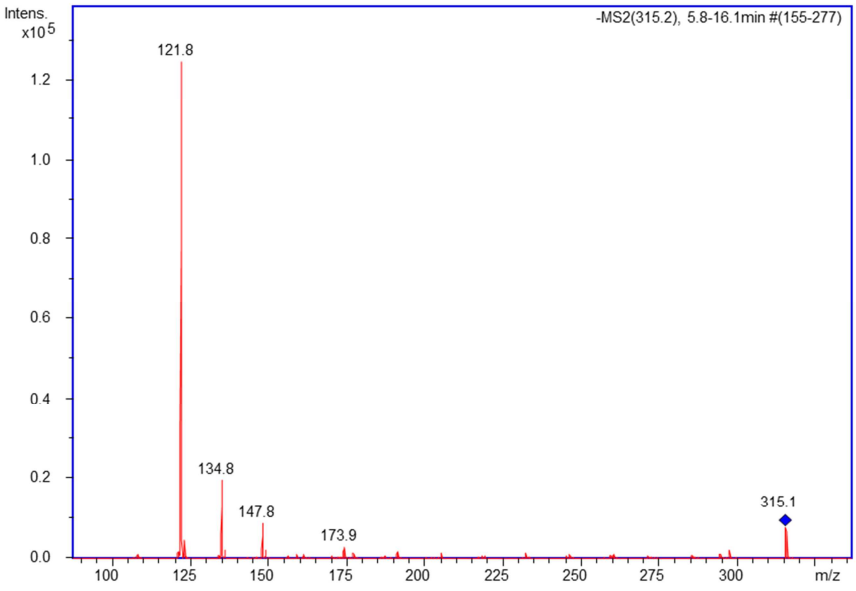

Fig. 8. MS spectrum of compound B.

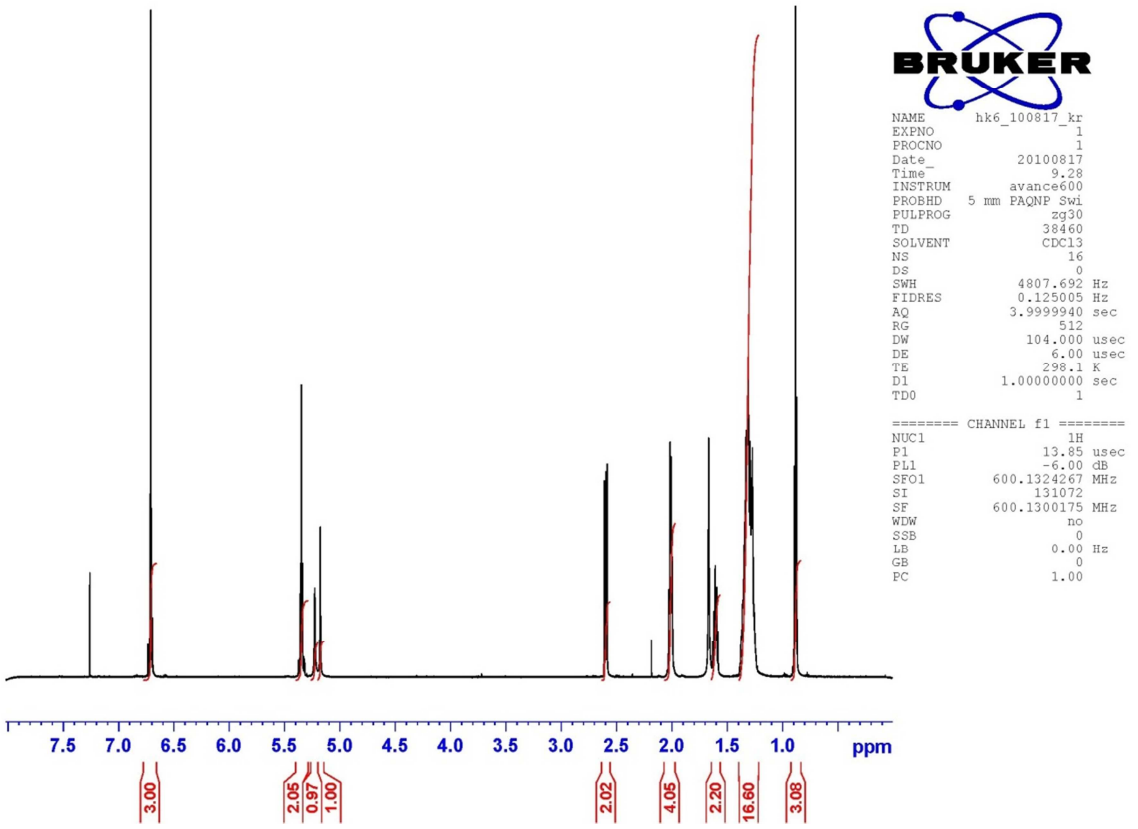

Fig. 9. ^{1}H NMR spectrum of compound A.

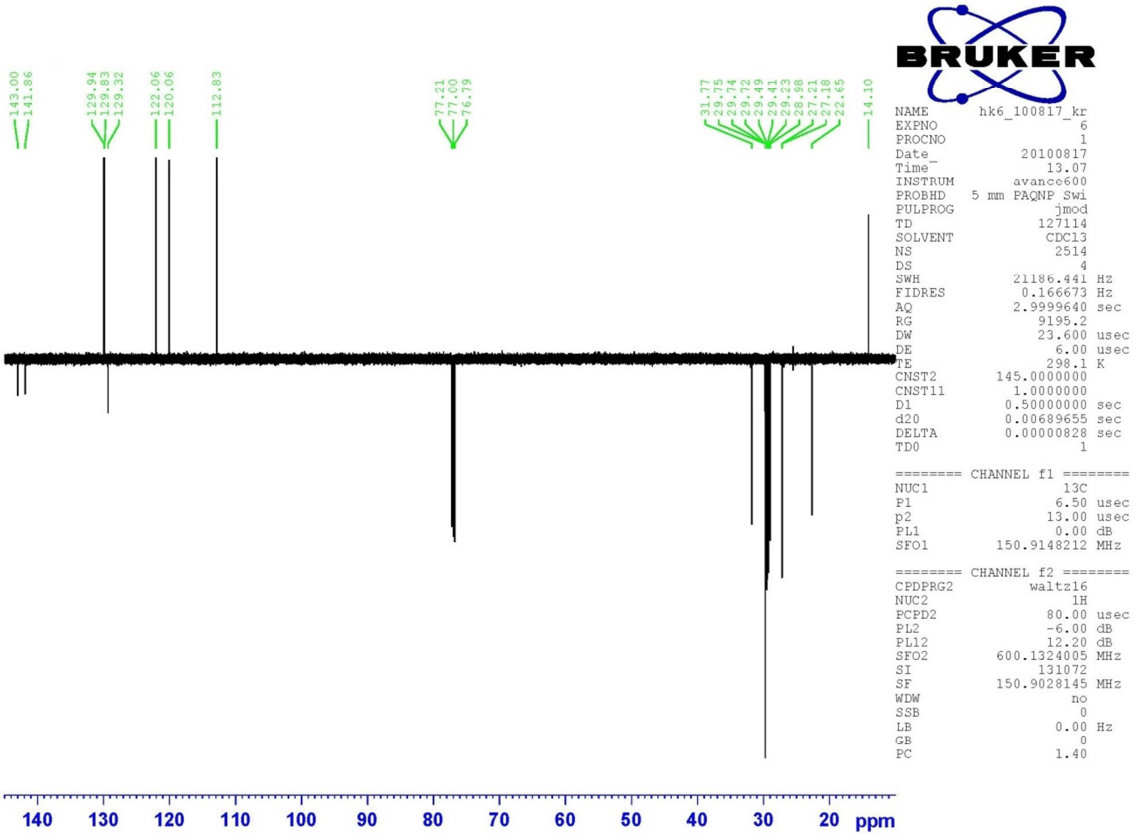

Fig. 10. ^{13}C NMR spectrum of compound A.

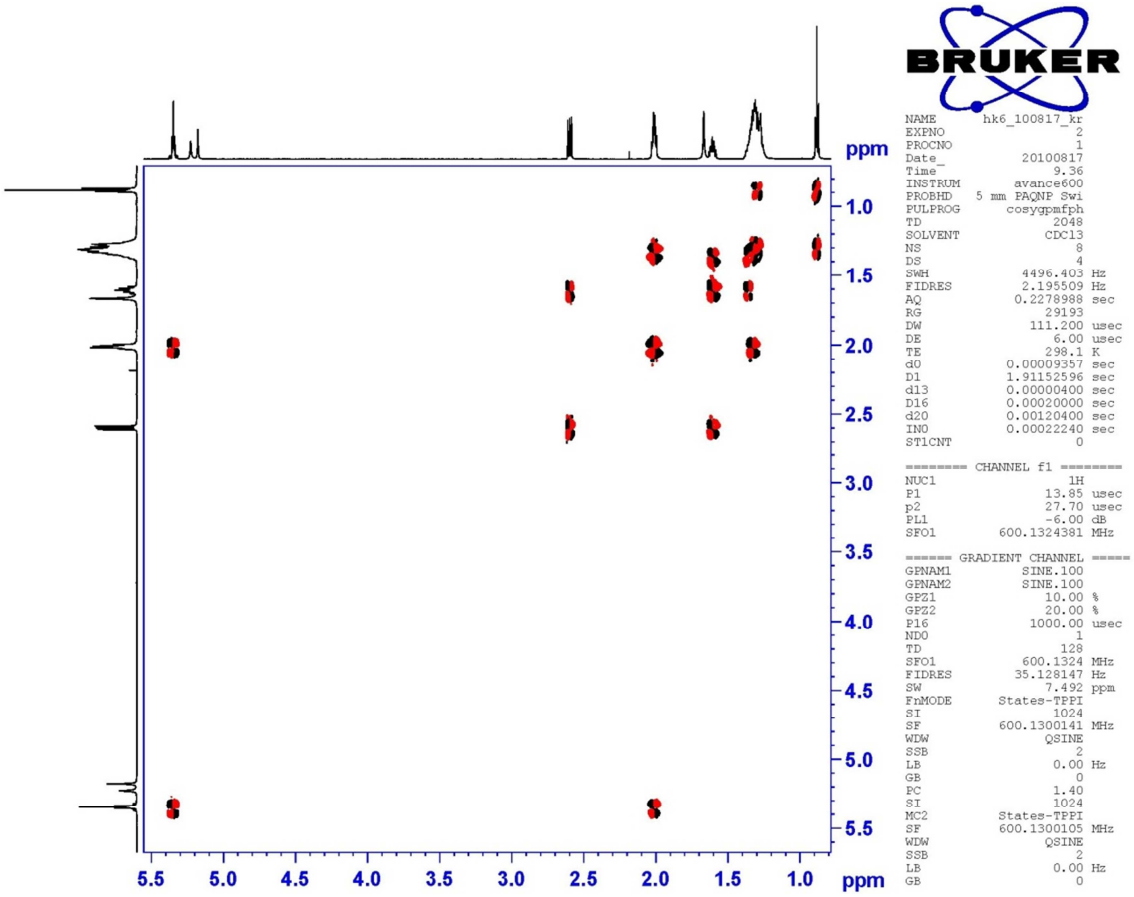

Fig. 11. COSY spectrum of compound A.

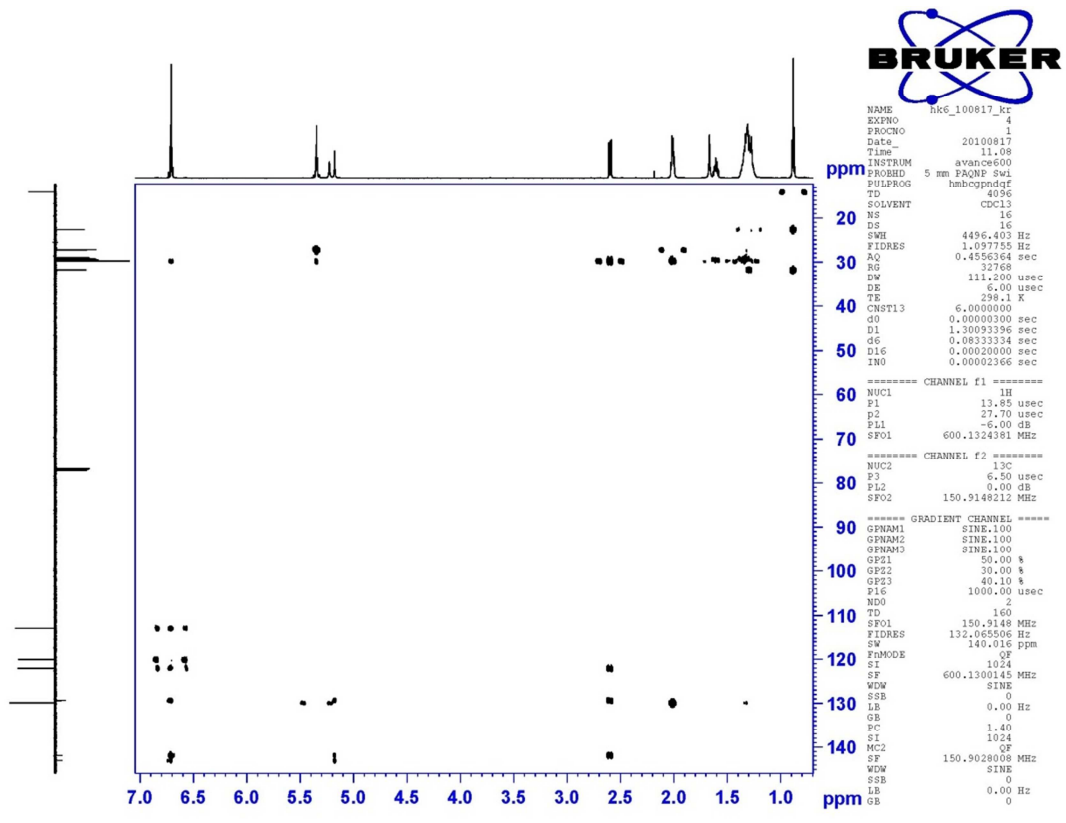

Fig. 12. HMBC spectrum of compound A.

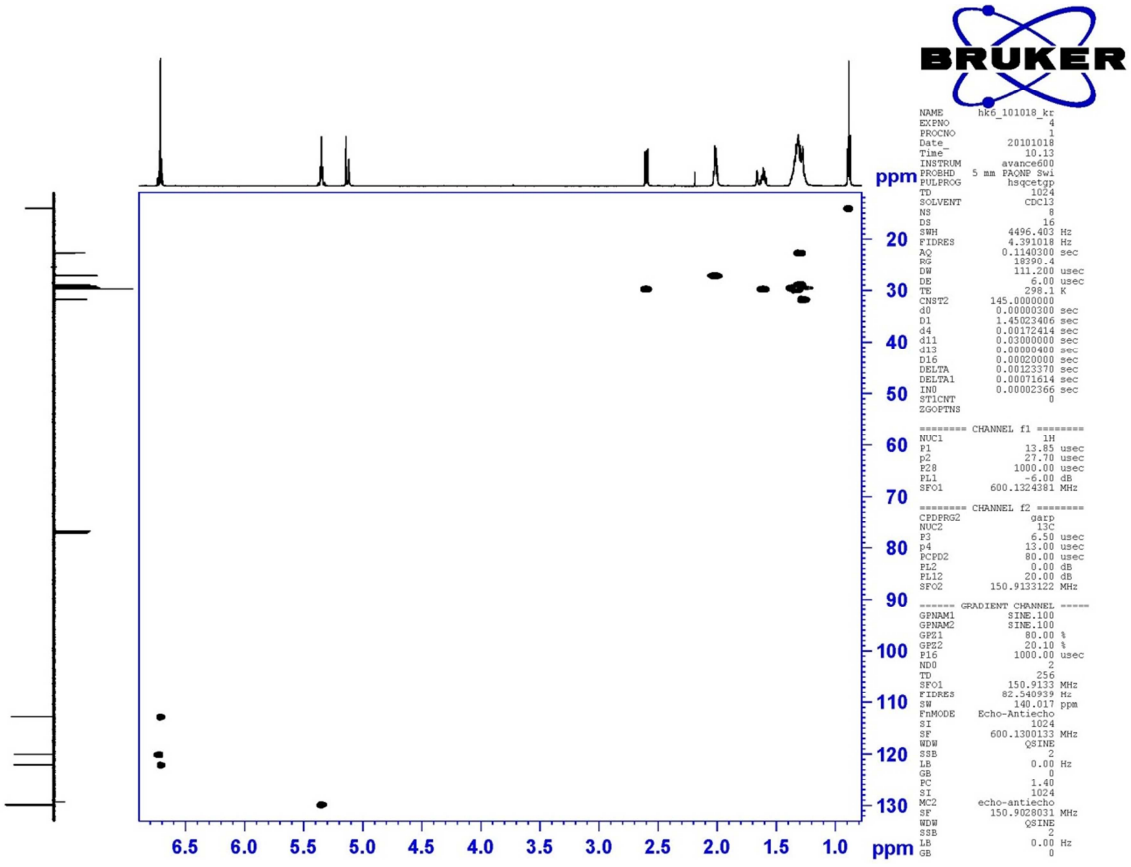

Fig. 13. HSQC spectrum of compound A.

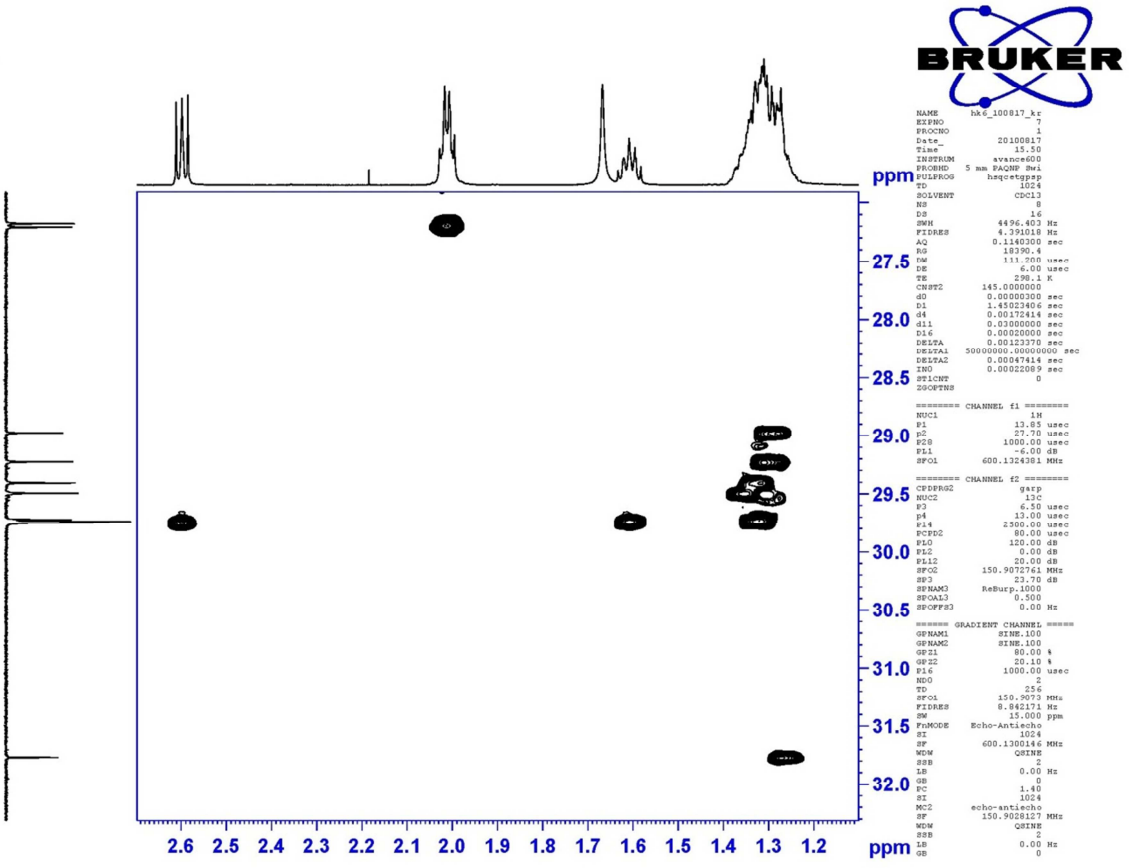

Fig. 14. Band selective HSQC spectrum of compound A.

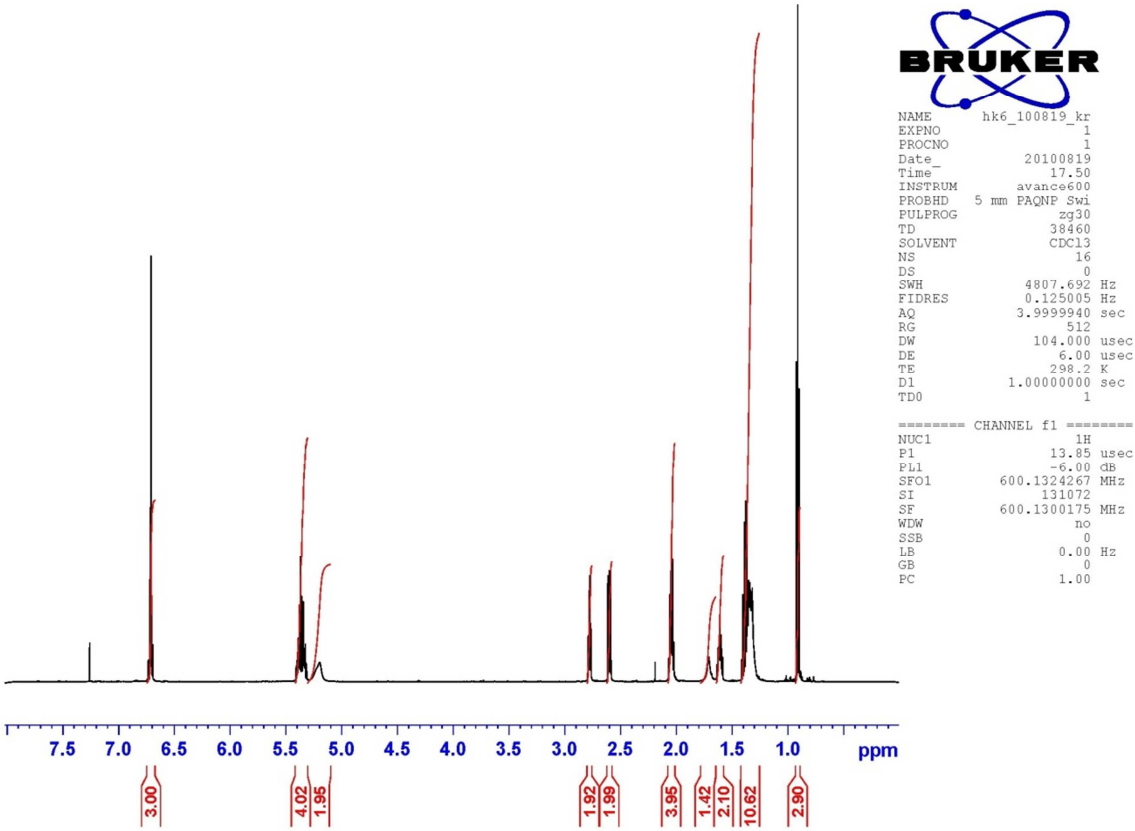

Fig. 15. ^{1}H NMR spectrum of compound B.

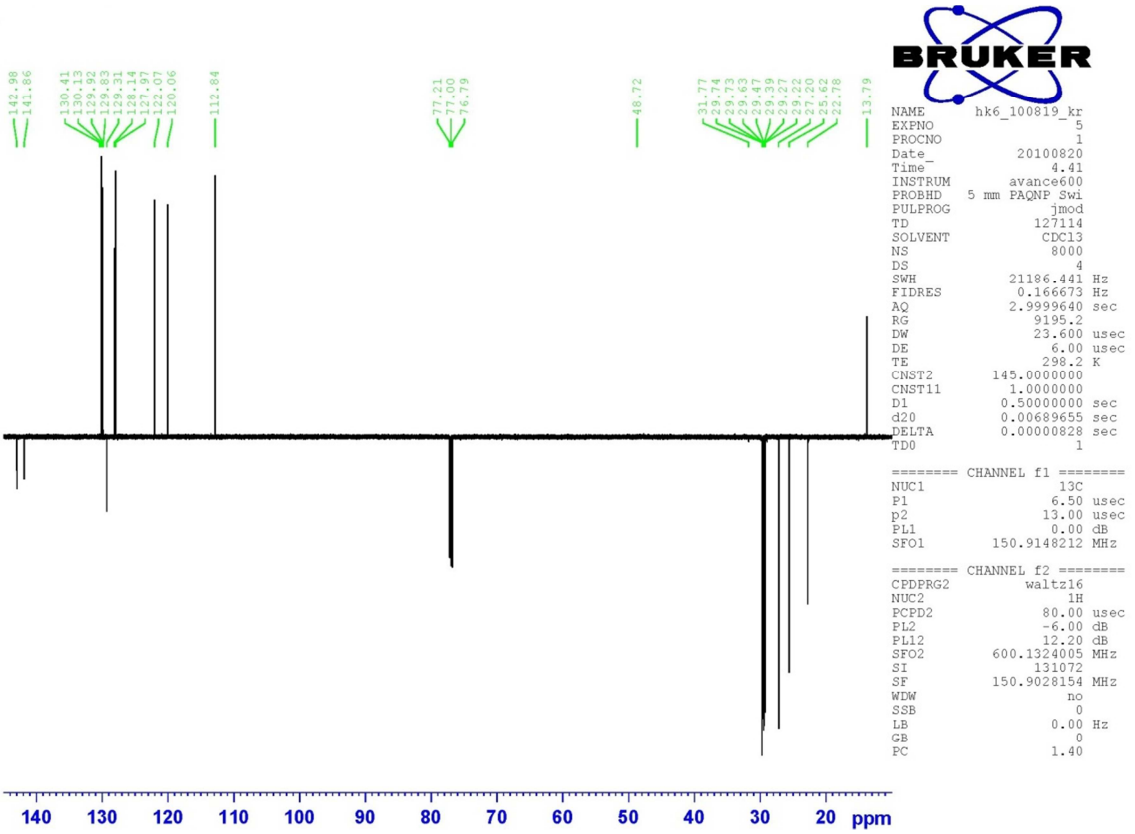

Fig. 16. ^{13}C NMR spectrum of compound B.

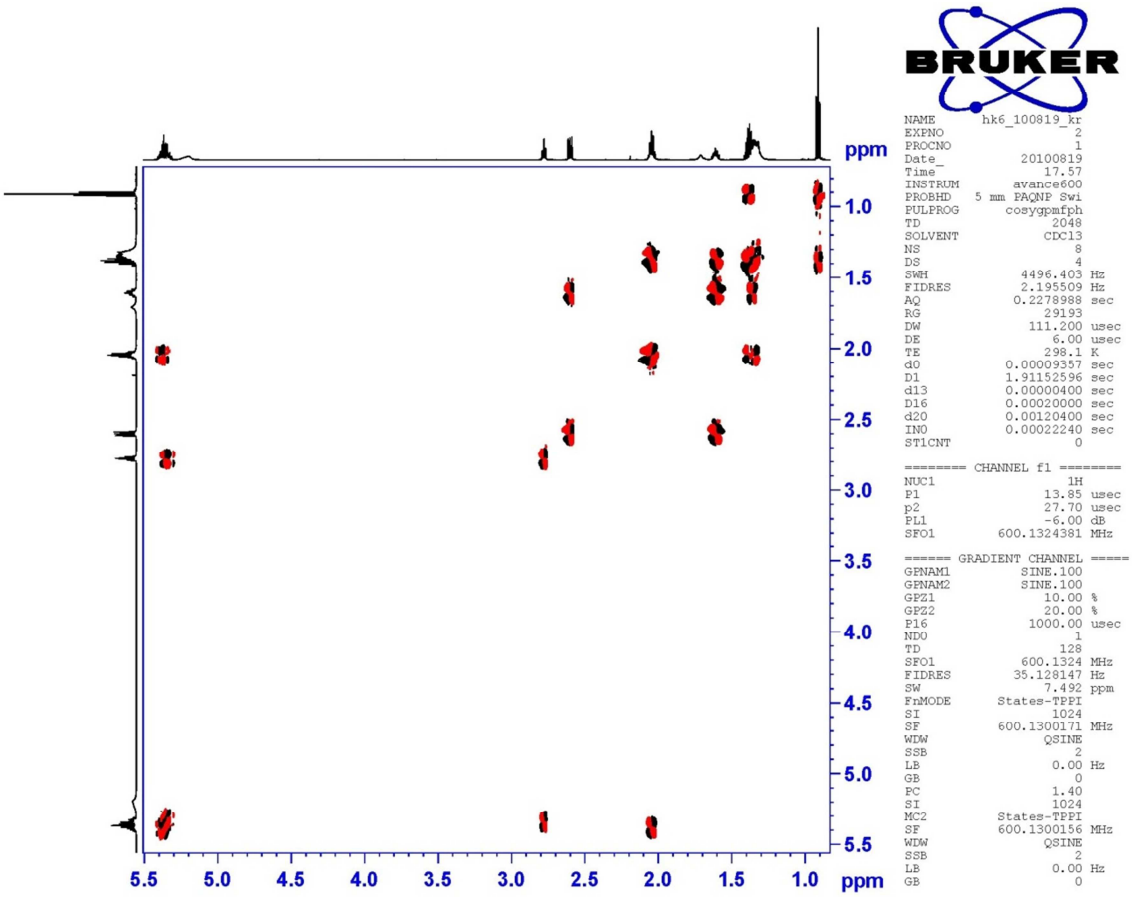

Fig. 17. COSY spectrum of compound B.

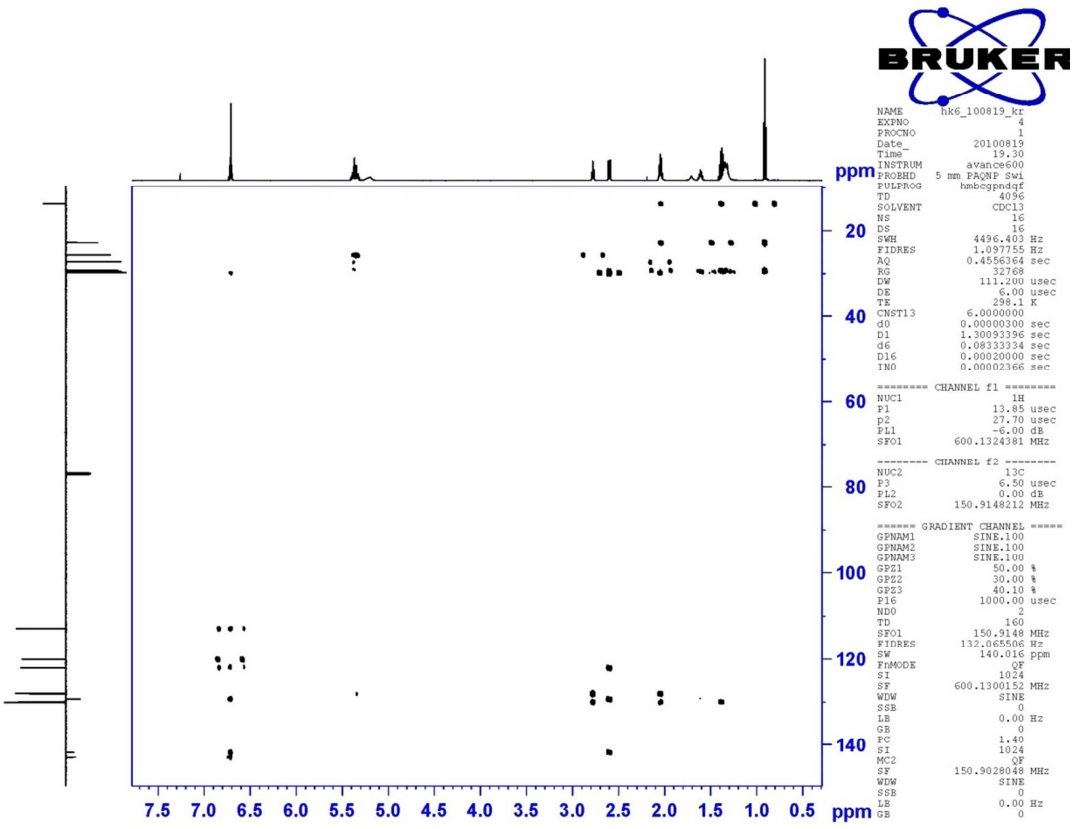

Fig. 18. HMBC spectrum of compound B.

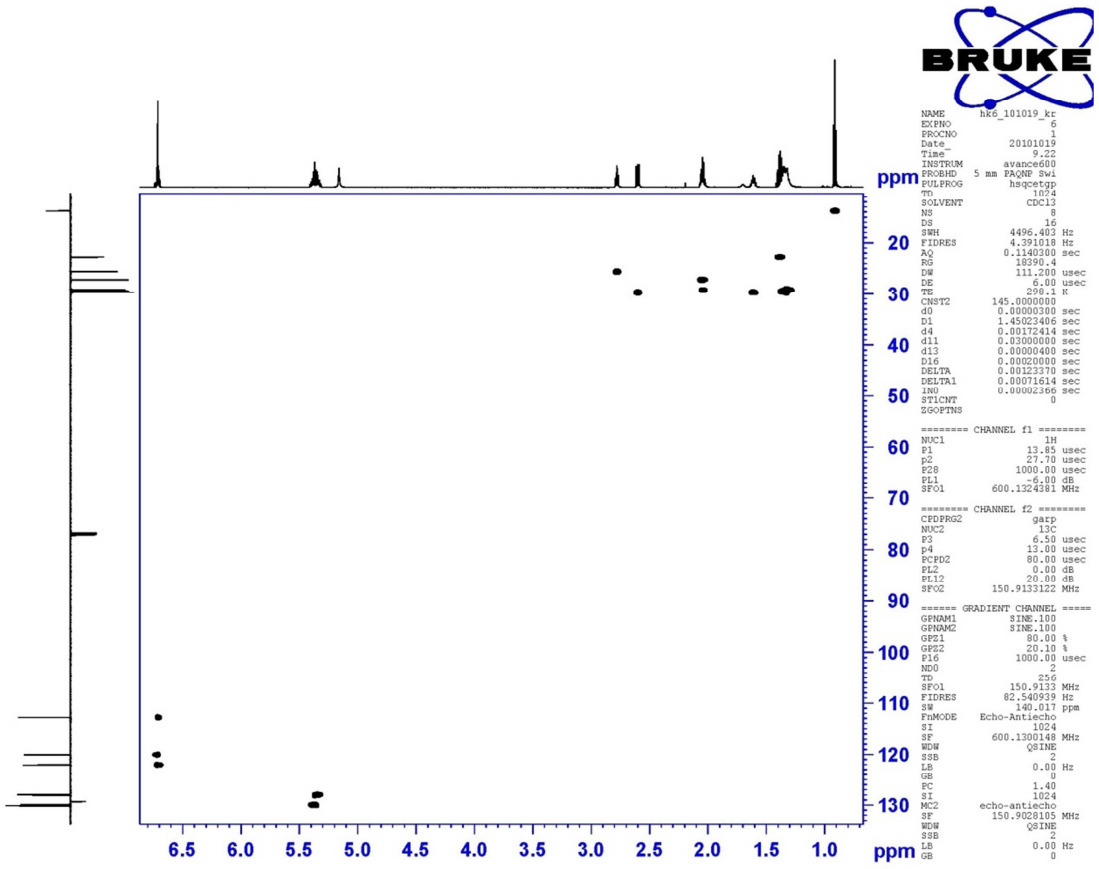

Fig. 19. HSQC spectrum of compound B.

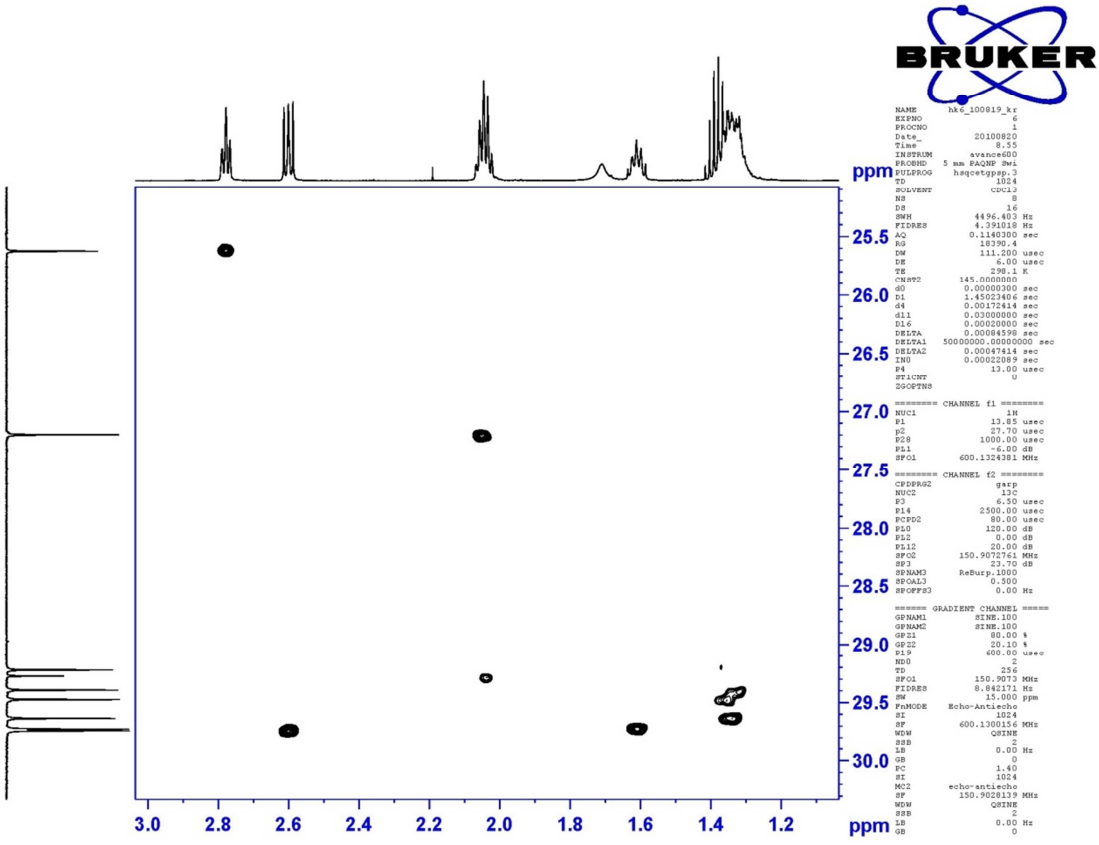

Fig. 20. Band selective HSQC spectrum of compound B.

The IC_{50} values for AChE inhibition were determined for DCM extract, fraction A4 and compounds A and B by microplate assay (Fig. 21-22).

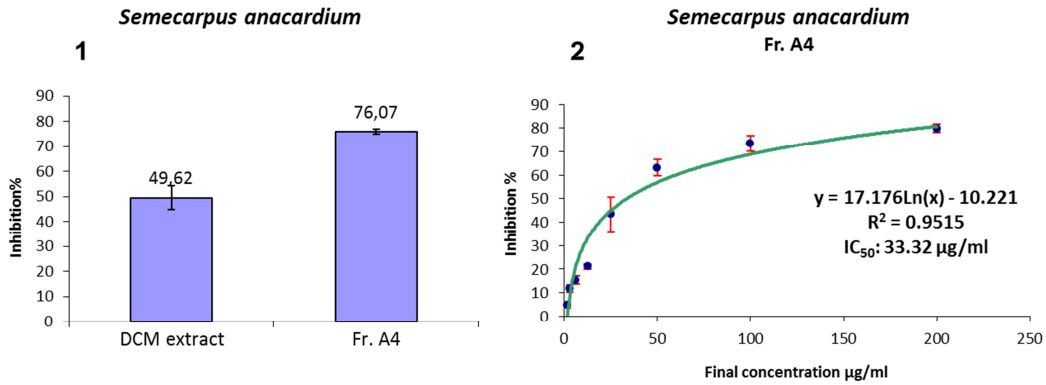

Fig.21. 1: Comparison of AChE inhibition of the DCM extract and the enriched fraction A4 at a final concentration of 100 µg/ml, 2: AChE inhibition of fraction A4 (200, 100, 50, 25, 12.5, 6.3, 3.1, 1.6 µg/ml).

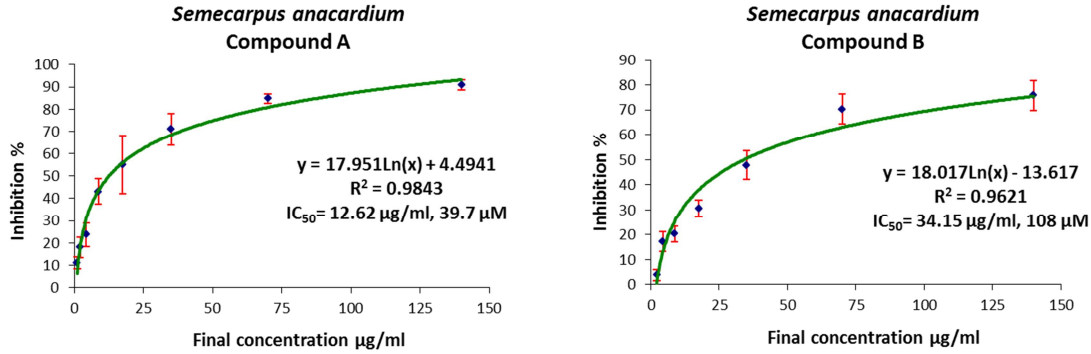

Fig. 22. AChE inhibition of compounds A and B (140, 70, 35, 17.5, 8.8, 4.4, 2.2, 1.1 µg/ml).

The concentrations of active compounds in the extract were determined by HPLC analyses using external standardization (Fig. 23).

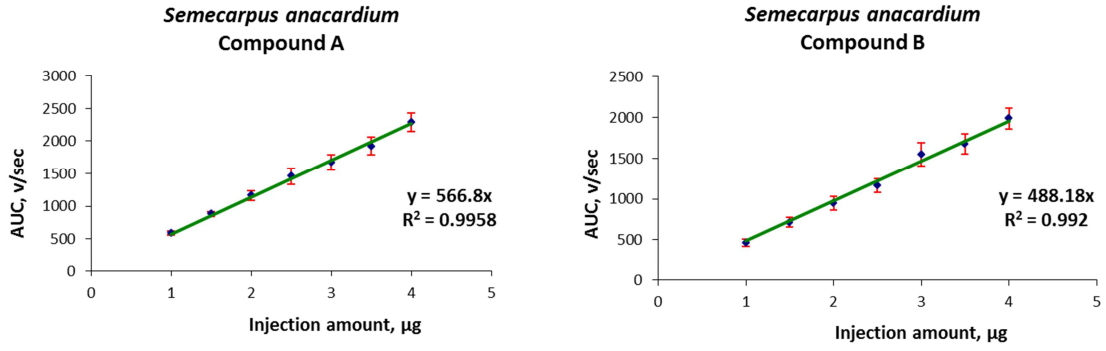

Fig. 23. The calibration curves for HPLC-quantification of compounds A and B.

Spiro-sesquiterpenoidic chromadiones from gum ammoniacum with acetylcholinesterase inhibitory activity

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Abstract

Attempts to restore cholinergic function have been considered as a rational target to improve the symptoms of Alzheimer's disease. One therapeutic option is the use of AChE inhibitors. During the last two decade the use of herbal medicinal preparations in dementia therapy has been studied based on traditional medicine. Gum ammoniacum is the gum-resin from *Dorema ammoniacum* D. Don which has been used in Unani and Iranian traditional medicine for several indications. A previous study had shown AChE inhibitory activity for a dichloromethane extract of this resin. The aim of this study was the isolation and characterization of the active compounds from gum ammoniacum. Extraction of the resin was performed by sonification with dichloromethane. The extract was investigated by a respective colorimetric microplate assay and the active zones were identified via TLC bioautography and isolated using several chromatographic techniques. The structures of the active components were characterized by one and two-dimensional ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry as (2'*S*,5'*S*)-2'-ethenyl-5'-(3-hydroxy-6-methyl-4-oxohept-5-en-2-yl)-7-methoxy-2'-methyl-4*H*-spiro[chromene-3,1'-cyclopentane]-2,4-dione and (2'*S*,5'*R*)-2'-ethenyl-5'-[(2*R*,4*R*)-4-hydroxy-6-methyl-3-oxohept-5-en-2-yl]-7-methoxy-2'-methyl-4*H*-spiro[chromene-3,1'-cyclopentane]-2,4-dione of which the first one is a new natural compound. Their IC_{50} values for AChE inhibitory activity were 77 and 100 ¯g/ml, respectively. Their concentrations in the resin were determined by HPLC analyses as 3.1% and 4.6%.

Keywords: Dorema ammoniacum, gum ammoniacum, acetylcholinesterase inhibition, spiro-sesquiterpenoidic chromadione.

1. Introduction

It is commonly accepted that a cholinergic deficit correlates with the severity of Alzheimer's disease (Garcia-Alloza *et al.*, 2006). Thus, attempts to restore cholinergic function have been considered as a rational target to slow down the progress of Alzheimer's disease. One therapeutic option is the use of AChE inhibitors which block this key enzyme in the breakdown of acetylcholine (Howes and Houghton, 2003).

During the last two decades the use of herbal medicinal substances in dementia therapy has been studied based on traditional medicine (Andrade *et al.*, 2000). Using this knowledge, e.g. galanthamine from *Galanthus nivalis* L. (snowdrop) has been identified as a potent acetylcholinesterase inhibitor, which today is one therapeutic option in the treatment of Alzheimer's disease (Hostettmann *et al.*, 2006).

Dorema ammoniacum D. Don (Apiaceae) is a perennial plant in Iran, Afghanistan and northern India. The gum-resin, commonly known as gum ammoniacum, which is secreted from damaged stems and roots has been traditionally used as an expectorant, stimulant and antispasmodic drug in the Unani system of medicine (Rajani *et al.*, 2002). It is also used as antihelminitic and for gastrointestinal disorders in Iranian traditional medicine (ITM) (Amin, 2005). Some biological activities such as antibacterial and vasodilatory effects have been reported for this resin (Rajani *et al.*, 2002; Leaman 2006). Recently, a low cytotoxic activity was shown for the essential oil from fruits of *D. ammoniacum* (Yousefzadi *et al.*, 2011). Additionally, it is listed in the British herbal pharmacopoeia as an antispasmodic and expectorant, and used occasionally for chronic bronchitis and persistent coughs (Langenheim, 2003; Anonymous, 1996).

A previous screening study on selected medicinal plants and plant products used in ITM showed AChE inhibitory activity for a dichloromethane (DCM) extract of gum ammoniacum (Adhami *et al.*, 2011). The aim of this study was the isolation and characterization of active compounds from this gum-resin.

2. Materials and methods

2.1- Chemicals and reagents

AChE from electric eel, 1-naphthyl acetate, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), Tris-HCl, bovine serum albumin (BSA) and physostigmine were purchased from

Sigma (St. Louis, USA). Acetylthiocholine iodide (ATCI) and chelidonine were obtained from Fluka (Buchs, Switzerland). Fast Blue B salt (FBS) and silica gel 60 were from Merck (Darmstadt, Germany). Two different buffer systems were used (buffer A: 50 mM Tris-HCl, pH 7.9 containing 0.1% BSA; buffer B: 50 mM Tris-HCl, pH 7.9 containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$).

2.2. General

A Genios microplate reader (Tecan, Salzburg, Austria) was used to measure the absorbance. Extraction was performed by sonification in a Branson 3150 ultrasonic bath (Dumbury, USA). TLC plates, silicagel 60 F_{254}, were obtained from Merck (Darmstadt, Germany) and 96-well microplates PS F-bottom from Greiner Bio-One (Frickenhausen, Germany). For LC-MS analyses, the separation was performed on an Acclaim 120 C18 column, 2.1 × 150 mm, 3 µm (Dionex, Germering, Germany) and mass spectroscopy was carried out on an UltiMate 3000 RSLC-series system (Dionex, Germering, Germany) coupled to a 3D quadrupole ion trap mass spectrometer equipped with an orthogonal ESI source (HCT, Bruker Daltonics, Bremen, Germany). All NMR spectra were recorded on a Bruker Avance DRX 600 spectrometer (Bruker BioSpin, Rheinstetten, Germany). A Spectrum HPCCC instrument (Dynamic Extractions, Berkshire, UK) was used for high performance counter current chromatography (HPCCC). HPLC was performed on a Shimadzu instrument with LC-20AD pump, SPD M20A diode array detector and SIL 20AC HT auto-sampler (Kyoto, Japan).

2.3. Plant material

The dry gum-resin of *D. ammoniacum* was purchased from a herbal shop in Tehran, Iran, and identified by Dr. Gholamreza Amin at the herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran (voucher number PMP-804).

2.4. Extraction

Twenty grams of the resin were ground and extracted twice by sonification with 200 ml DCM at 40°C for 1 hour. The extracts were combined and concentrated under reduced pressure at 40° C to yield 12.98 g of DCM extract.

2.5. TLC bioautography assay

The DCM extract of gum ammoniacum was examined by TLC on silica plates using the mobile phase chloroform-ethylacetate-methanol (90+7+3). Anisaldehyde-sulfuric acid was used as detection reagent to determine the chemical composition of the extract (Wagner and Blat, 2009). In parallel, a TLC bioautography assay was performed for AChE inhibitory activity according to Marston *et al*., 2002 and Adhami *et al*., 2011. The mobile phase was completely removed under airstream before detection. Chelidonine served as a positive control showing R_F 0.42 in this TLC system.

2.6. Microplate assay

A quantitative colorimetric assay based on Ellman's method (Rhee *et al*., 2001; Adhami *et al*., 2012) was used to measure the AChE inhibitory activities of the substances. Briefly, in a 96-well plate, 25 µl of 15 mM ATCI, 125 µl of 3 mM DTNB in buffer B, 50 µl of buffer A and 25 µl of the extract or the isolated substances (from 15.6 µg/ml to 2.0 mg/ml in 10% DMSO) were thoroughly mixed and the absorbance was read at 405 nm every 15 s for 5 times. Finally 25 µl of AChE (0.22 U/ml in buffer A) were added and the plate was incubated at 25°C for 10 minutes. Then the absorbance was measured again 8 times every 15 s. A 10% DMSO solution was used as negative control. To prevent any increase in absorbance due to the color of the extracts or spontaneous hydrolysis of substrate, the absorbance before addition of the enzyme was subtracted from the absorbance after adding the enzyme. The assay was repeated three times for every concentration. Different concentrations of physostigmine (0.12-15 µg/ml as the final concentration) served as a positive control for validation.

2.7. Isolation and characterization of active compounds

The first fractionation of 12.0 g DCM extract of gum ammoniacum was performed by vacuum liquid chromatography (VLC) with silica gel 60 and chloroform as the stationary and the mobile phases, respectively. Fractions with similar chemical composition were combined. Twelve collective fractions (A1-A12) were examined by TLC bioautography for their AChE inhibitory activity. Further purification of the active fraction A4 (4.25 g) by VLC on a silica gel column under gradient elution with 100-50 % petroleum ether in chloroform yielded 2.85 g of an active fraction B4. Two grams of

fraction B4 were loaded on a silica gel column and eluted with chloroform to obtain 478 mg of fraction C10. An HPCCC under normal phase elution was carried out on 280 mg of C10 to obtain two oily active compounds K10 (6.0 mg) and K13 (119 mg). The stationary and the mobile phase for HPCCC consisted of hexane, ethylacetate, methanol and water at a ratio of 5+1+5+1, respectively. The rotation speed was 1620 rpm and the fractions 6 ml/min of were collected.

The separation in LC-MS analysis was carried out at 25°C and a flow rate of 0.5 ml/min. 0.1% aqueous formic acid and acetonitrile were used as mobile phase A and B, respectively. The following gradient program was used: 40% B (0 min), 40% B (2 min), and 70% B (32 min). The eluent flow was split roughly 1:8 before the ESI ion source, which was operated as follows: capillary voltage: -3.7/+3.5 kV, nebulizer: 26 psi (N2), dry gas flow: 9 L/min (N2), and dry temperature: 340 °C. CID spectra were obtained in automated data-dependent acquisition (DDA) mode with helium as collision gas, an isolation window of 4 Th, and a fragmentation amplitude of 1.0 V.

Compound K10: (+)ESIMS *m/z* 427.0 $[M+H]^+$; $ESIMS^2$ (427.0 →) *m/z* 408.8 (100), 326.7 (14); $ESIMS^3$ (427.0 → 408.8 →) *m/z* 326.7 (100), 298.7 (12), 284.7 (51), 204.6 (19), 150.7 (13); (-)ESIMS *m/z* 425.0 $[M-H]^-$; $ESIMS^2$ (425.0 →) *m/z* 380.8 (11), 362.8 (16), 204.6 (100).

Compound K13: (+)ESIMS *m/z* 427.0 $[M+H]^+$; $ESIMS^2$ (427.0 →) *m/z* 408.8 (100), 258.7 (11); $ESIMS^3$ (427.0 → 408.8 →) *m/z* 390.8 (57), 352.7 (38), 334.7 (25), 326.7 (63), 310.6 (21), 298.7 (14), 284.7 (27), 270.6 (17), 258.6 (100), 240.6 (11), 230.7 (11), 218.6 (18), 216.7 (15), 204.6 (63), 192.7 (22), 174.8 (11), 150.7 (18); (-)ESIMS *m/z* 425.0 $[M-H]^-$; $ESIMS^2$ (425.0 →) *m/z* 380.8 (28), 362.8 (15), 256.7 (100), 166.6 (20), 150.7 (24).

All NMR spectra were recorded on a Bruker Avance DRX 600 NMR spectrometer using a 5mm switchable quadruple probe (QNP, ^1H, ^{13}C, ^{19}F, ^{31}P) with z axis gradients and automatic tuning and matching accessory. The resonance frequency for ^1H NMR was 600.13 MHz, for ^{13}C NMR 150.92 MHz. All measurements were performed for a solution in $CDCl_3$ at 298K. Standard 1D and gradient-enhanced (ge) 2D experiments, like double quantum filtered (DQF) COSY, TOCSY, NOESY, HSQC, and HMBC, were used as supplied by the manufacturer. Chemical shifts are referenced internally to the

residual, non-deuterated solvent signal for ^{1}H (δ = 7.26 ppm) or to the carbon signal of the solvent for ^{13}C (δ = 77.0 ppm). ^{1}H and ^{13}C NMR data are summarized in Table 1.

Table 1

^{1}H and ^{13}C NMR data of compounds K10 and K13 ($CDCl_3$, 600 MHz)

	K10		K13	
Position	δ_C (dept)	δ_H	δ_C (dept)	δ_H
2	169.8 (s)	---	168.4 (s)	---
3	70.8 (s)	---	72.1 (s)	---
4	191.4(s)	---	189.2 (s)	---
5	127.9 (d)	7.85 (1H, d, *J*= 8.8 Hz)	128.6 (d)	7.80 (1H, d, *J*= 8.8 Hz)
6	111.7 (d)	6.76 (1H, dd, *J*= 8.8, 2.4 Hz)	112.2 (d)	6.75 (1H, dd, *J*= 8.8, 2.4 Hz)
7	165.5 (s)	---	166.2 (s)	---
8	100.7 (d)	6.58 (1H, d, *J*= 2.4 Hz)	100.8 (d)	6.57 (1H, d, *J*= 2.4 Hz)
9	156.5 (s)	---	156.3 (s)	---
10	115.4 (s)	---	113.8 (s)	---
11	55.8 (q)	3.86 (3H, s)	55.6 (q)	3.88 (3H, s)
1'	112.9 (t)	5.07 (1H, dd, *J*= 17.3, 0.8 Hz) 5.13 (1H, dd, *J*= 10.8, 0.8 Hz)	115.9 (t)	4.90 (1H, dd, *J*= 10.8, 0.7 Hz) 4.89 (1H, dd, *J*= 17.3, 0.7 Hz)
2'	142.7 (d)	5.96 (1H, dd, *J*= 17.3, 10.8 Hz)	140.2 (d)	5.60 (1H, dd, *J*= 17.3, 10.8 Hz)
3'	56.1 (s)	---	56.2 (s)	---
4'	34.2 (t)	1.86 (2H, m)	35.9 (t)	2.36 (1H, m) 1.54 (1H, ddd, *J*= 12.9, 5.5, 2.7 Hz)
5'	29.6 (t)	2.21 (1H, m) 1.93 (1H, m)	28.4 (t)	2.25 (1H, m) 1.87 (1H, m)
6'	49.4 (d)	3.03 (1H, ddd, *J*= 9.0, 10.5, 10.5 Hz)	46.1 (d)	3.44 (1H, ddd, *J*= 8.0, 10.4, 10.4 Hz)
7'	39.7 (d)	1.93 (1H, m)	44.7 (d)	3.03 (1H, qd, *J*= 7.0, 10.4 Hz)
8'	79.8 (d)	3.90 (1H, dd, *J*= 7.8, 9.6 Hz) 2.77 (1H: OH, d, *J*= 7.8 Hz)	215.0 (s)	---
9'	201.4 (s)	---	73.9 (d)	4.69 (1H, br d, *J*= 9.8 Hz) 3.41 (1H: OH, br)
10'	122.7 (d)	6.01 (1H, sep, *J*= 1.1 Hz)	120.0 (d)	4.99 (1H, sep d, *J*= 1.4, 9.8 Hz)
11'	158.7 (s)	---	140.3 (s)	---
12'	28.1 (q)	1.90 (3H, d, *J*= 1.1 Hz)	26.0 (q)	1.80 (3H, d, *J*= 1.4 Hz)
13'	22.2 (q)	0.90 (3H, s)	23.2 (q)	0.98 (3H, d, *J*= 0.6 Hz)
14'	16.1 (q)	0.92 (3H, d, *J*= 6.9 Hz)	15.7 (q)	1.21 (3H, d, *J*= 7.0 Hz)
15'	21.4 (q)	2.10 (3H, d, *J*= 1.1 Hz)	18.5 (q)	2.10 (3H, d, *J*= 1.4 Hz)

2.8. HPLC

The DCM extract of *gum ammoniacum* and the isolated compounds were analyzed by HPLC under gradient elution on a Hypersil BDS-C18 column (250×4 mm id) at 35°C. The mobile phase consisted of acetonitrile (A) and water (B) at a flow rate of 0.75 ml/min. After an isocratic step at 50% A for 10 min the concentration of solvent A increased up to 80% in 30 min and the elution was kept at this concentration for further 5 min. The eluates were monitored at 238 nm.

3. Result and discussion

The gum-resin of *Dorema ammoniacum* has been used in ITM for centuries for different indications. There are several herbal mixtures for which a use for memory enhancement or treatment of memory loss is described in ITM. As most of these herbal preparations contain one or more gum-resins, gum ammoniacum had been included in a screening study for AChE inhibition (Adhami *et al.*, 2011). Based on a moderate activity of the DCM extract of gum ammoniacum in the mentioned study, two active compounds from this gum-resin were isolated and their structures were characterized.

3.1. Isolation of active compounds

By VLC a very fast enrichment of the active fractions from the extract was achieved. Subsequent column chromatography resulted in fraction C10 which contained two active compounds as monitored in TLC bioautography. For the final step of isolation, HPCCC under use of hexane, ethylacetate, methanol and water at a ratio of 5+1+5+1 in normal phase elution was applied which yielded two oily active compounds (K10 and K13). The distribution ratio of C10 for this solvent system was 0.68.

3.2. *Identification of active compounds*

ESI-MS resulted in a molecular weight of 426.0 Da for both compounds K10 and K13 which is in agreement with the molecular formula of $C_{25}H_{30}O_6$. In positive ion mode ESI-MS^3 spectra major fragment ions were determined at *m/z* 326.7 and 258.7 for K10 and K13, respectively. The ^{1}H- and ^{13}C NMR data proved K10 to be (2'*S*,5'*S*)-2'-ethenyl-5'-(3-hydroxy-6-methyl-4-oxohept-5-en-2-yl)-7-methoxy-2'-methyl-4*H*-spiro [chromene-3,1'-cyclopentane]-2,4-dione and K13 as (2'*S*,5'*R*)-2'-ethenyl-5'-[(2*R*,4*R*)-4-hydroxy-6-methyl-3-oxohept-5-en-2-yl]-7-methoxy-2'-methyl-4*H*-spiro[chromene-3,1'-cyclopentane]-2,4-dione (Doremon A). The structure of K13 is largely in agreement with earlier reports (Arnone *et al.*, 1991) while K10 is a new natural substance reported for the first time (Fig 1).

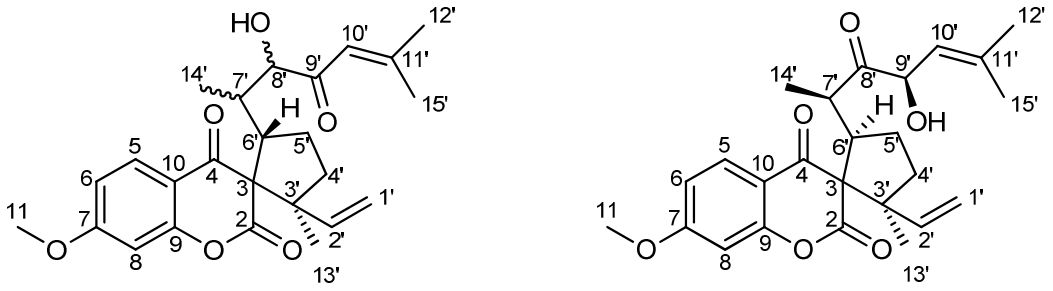

Fig.1. Structure of active compounds, left: K10, right: K13 (Doremon A)

The detailed ^{1}H and ^{13}C NMR analyses from compound K13 together with numerous connectivities derived from 2D NMR spectra like COSY, TOCSY NOESY, HSQC, es well as HMBC results in the structure of a spiro-sesquiterpenoidic chroman-2,4-dione derivative, which is known as doremone A in the literature (Arnone *et al.*, 1991). All ^{1}H as well as ^{13}C chemical shifts (Table 1) match exactly with the published data. The relative stereochemistry at carbon 3’ and 6’ could be confirmed by a NOESY crosspeak from H-6’ to the methyl group 13’. The configuration of the stereocentres 7’ and 9’ were proven by comparing the NMR data with those of the acetylated doremone A given by Appendino *et al*. (Appendino *et al.*, 1991), who in addition published a X-ray structure of the 3’ racemic mixture of acetyldoremone A.

The NMR spectra of K10 revealed a lot of similarities to that of K13. All ^{1}H and ^{13}C NMR signals for the 7-methoxy-chroman-2,4-dione part showed almost the same chemical shifts including the spirane carbon with its very characteristic downfield shift to about 72 ppm. All other NMR signals varied from K13, most predominantly in the sidechain. The ^{13}C ppm value of the ketone shifts from 215 ppm in doremon A for almost 14 ppm to 201.4 ppm in K10 was indicative for a conjugation with the double bond. The corresponding olefinic proton 10’ in K10 is 1 ppm deshielded in the ^{1}H NMR compared to K13 showing a septett multiplicity due to the allylic coupling with the two methyl groups only. The vicinal coupling as in K13 is lost. The methyl protons 14’ in K10 give a HMBC correlation to C-8’, which is a CH signal bearing an alcohol functionality due to a carbon shift of 79.8 ppm and a ^{1}H shift of the attached proton of 3.90 ppm. As a result, these differences in the NMR spectra of the two compounds prove that the ketone 8’ and the alcohol at 9’ in doremon A have switched their positions in K10, so that the carbonyl 9’ is now in conjugation with the double bond and the alcohol has

moved to 8’ (Fig. 1). The stereochemistry of these two centers remains undefined. The small shift variations in the cyclopentane part are not only due to the altered sidechain, a NOESY crosspeak between H-6’ and the olefinic H-2’ reveals a change in the relative configuration of the stereocentres 3’ and 6’ compared to doremone A (Fig. 1).

3.2. AChE inhibition

The AChE inhibitory activity of the DCM extract and the isolated compounds was determined for the first time in a microplate assay. The final tested concentrations of the samples ranged from 1.56 to 200 µg/ml. The IC_{50} values for AChE inhibition were 668 ± 3.12 µg/ml for the extract, 77.14 ± 3.75 µg/ml (181 µM) for K10 and 100.82 ± 5.14 µg/ml (236 µM) for K13.

3.3. Quantification of K10 and K13

HPLC analyses were performed for the quantification of K10 and K13 in the tested extract and gum ammoniacum. Several HPLC conditions under variation of the mobile phase, flow rate and elution gradient were examined to optimize the HPLC separation for K10, K13 and the DCM extract. The concentration of the two active compounds was determined by external standardization. The peak areas of both compounds were linearly dependent on concentrations over the selected range with correlation coefficients of R^2= 0.9869 for K10 and R^2= 0.9837 for K13. The concentrations of K10 and K13 in the DCM extract were determined as 5.8% and 8.4%, respectively. Total amounts of these two components in gum ammoniacum were 3.1% K10 and 4.6% K13.

Conclusion

A new and a known spiro-sesquiterpenoidic chromadione were isolated and their activity on AChE inhibition determined for the first time. From the correlation of their IC_{50} values for AChE inhibitory activity with their concentrations in gum ammoniacum can be concluded that they are among the major substances responsible for AChE inhibition in this drug. Due to these results the use of this drug in ITM in mixtures improving cognitive functions seems plausible.

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Supporting information for article 3

The gum-resin from *Dorema ammoniacum* (gum ammoniacum) was investigated in detail as the third active drug in this thesis (Fig. 1).

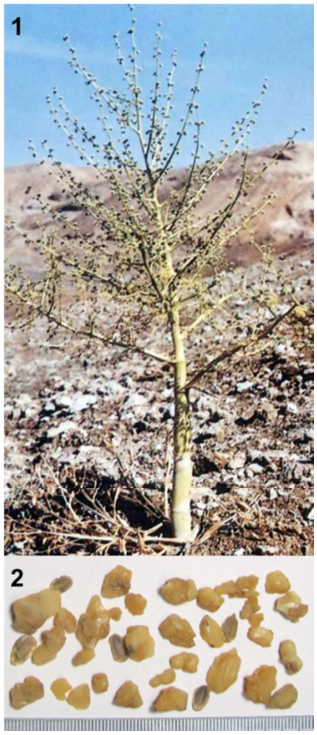

Fig. 1. *Dorema ammoniacum*. 1: shrub (http://davesgarden.com), 2: gum-resin.

The active zones were identified by a TLC bioautography of the DCM extract of gum ammoniacum (Fig. 2).

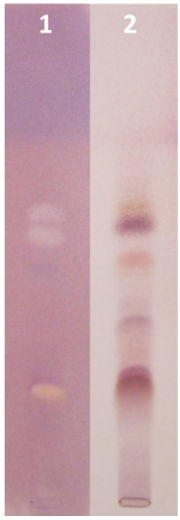

Fig. 2. TLC of DCM extract. 1: bioautography with 1-naphtyl acetate reagent, 2: derivatization with anisaldehyde-sufuric acid. Mobile phase $CHCl_3$-EtOAc-MeOH (90+7+3).

The active compounds were isolated using several chromatographic methods including VLC, CC, HPCCC (fig. 3-7).

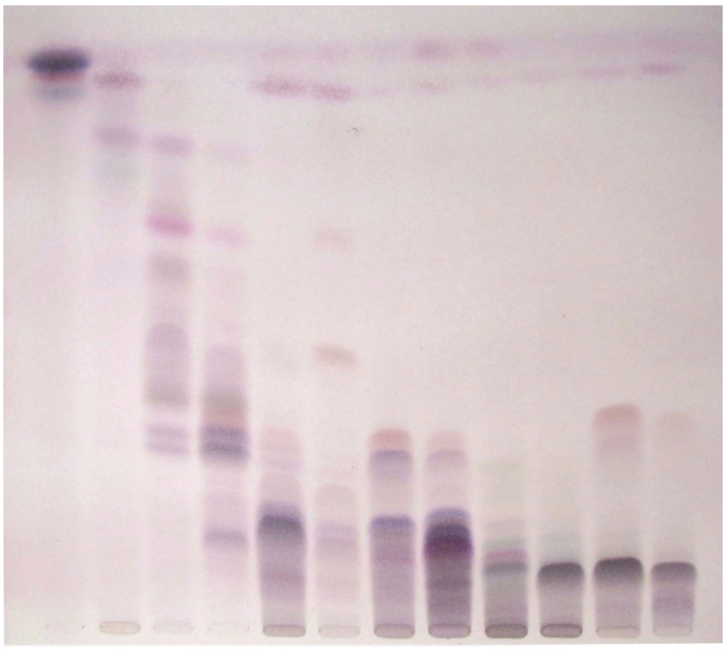

Fig. 3. TLC of fractions A1-A12 after VLC of DCM extract on a silicagel 60 column under elution with chloroform. TLC mobile phase: $CHCl_3$-EtOAc-MeOH (100+7+3). Reagent: anisaldehyde-sulfuric acid.

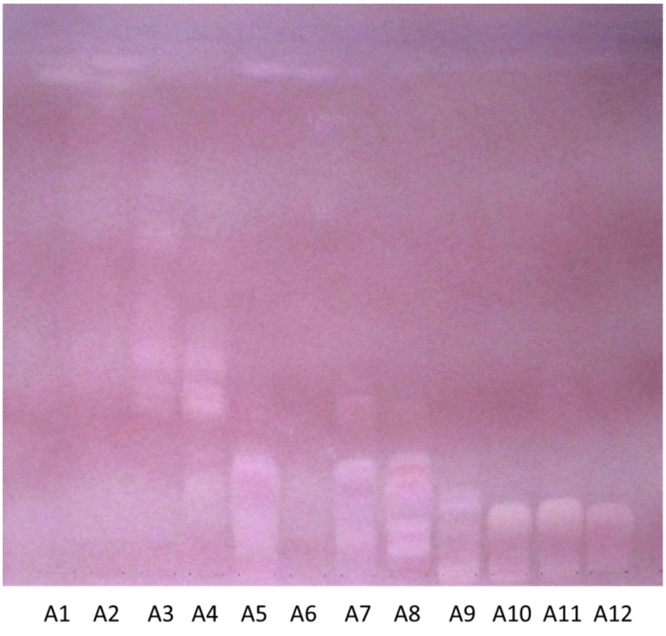

Fig. 4. TLC bioautography of fractions A1-A12. TLC mobile phase: $CHCl_3$-EtOAc-MeOH (100+7+3).

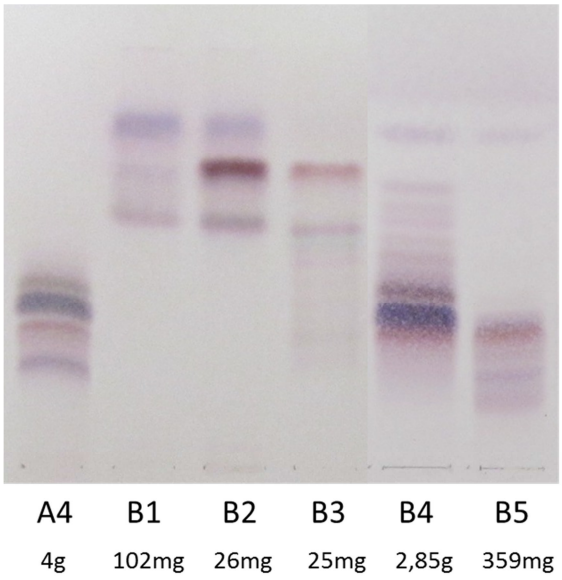

Fig. 5. TLC of fractions B1-B5 after VLC of fraction A4 under gradient elution with 100-50 % petroleum ether in chloroform. TLC mobile phase: $CHCl_3$-EtOAc-MeOH (100+3+1).

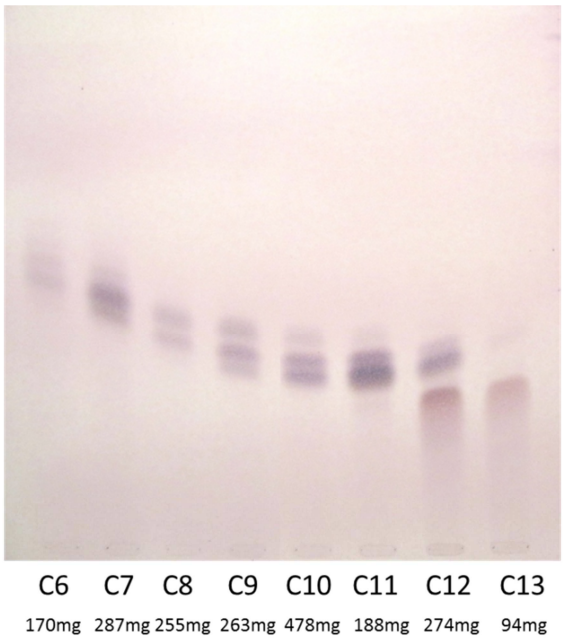

Fig. 6. TLC of fractions C6-C13 after CC of fraction B4 on silicagel 60 under elution with chloroform. TLC mobile phase: $CHCl_3$-EtOAc-MeOH (110+4+1).

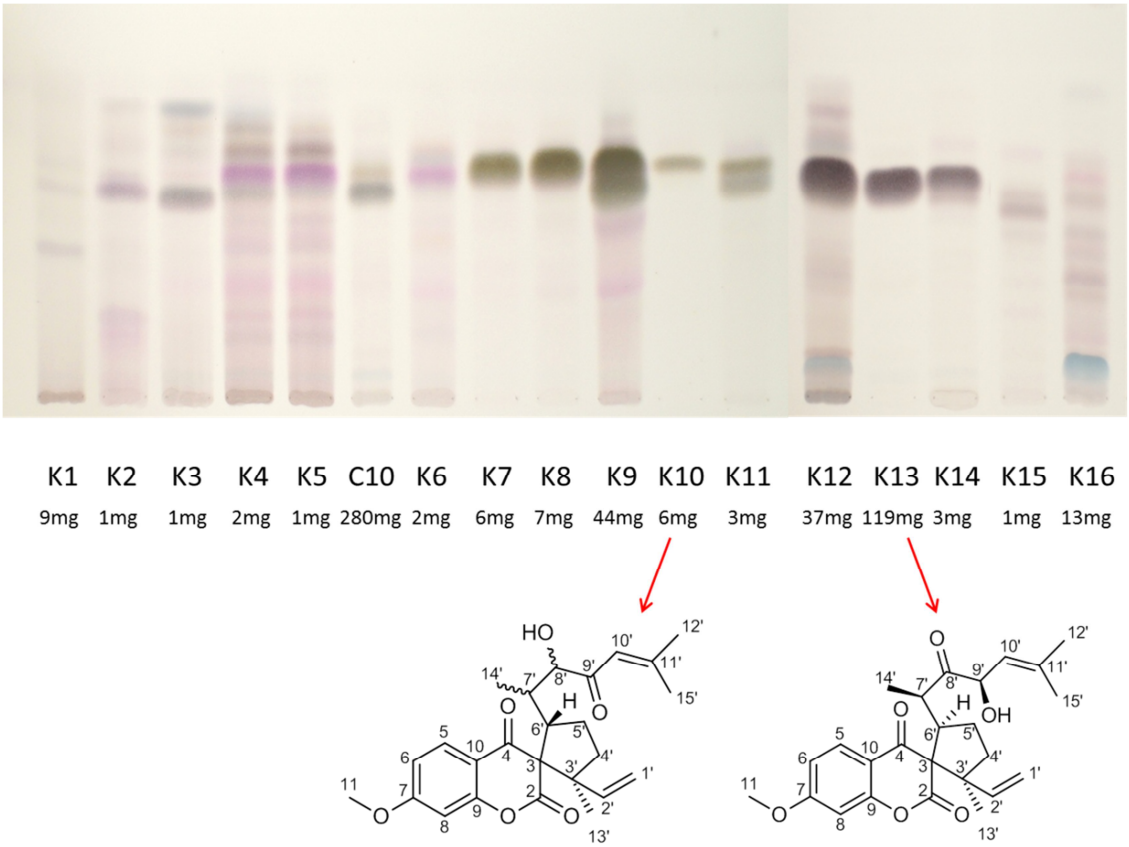

Fig. 7. TLC of the fractions K1-K16 after HPCCC of fraction C10 under normal phase elution with hexan-EtOAc-MeOH-H_2O (5+1+5+1). K10 and K13 were characterized as active compounds.

The structures of the active components were characterized by one and two-dimensional ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry (Fig. 8-21).

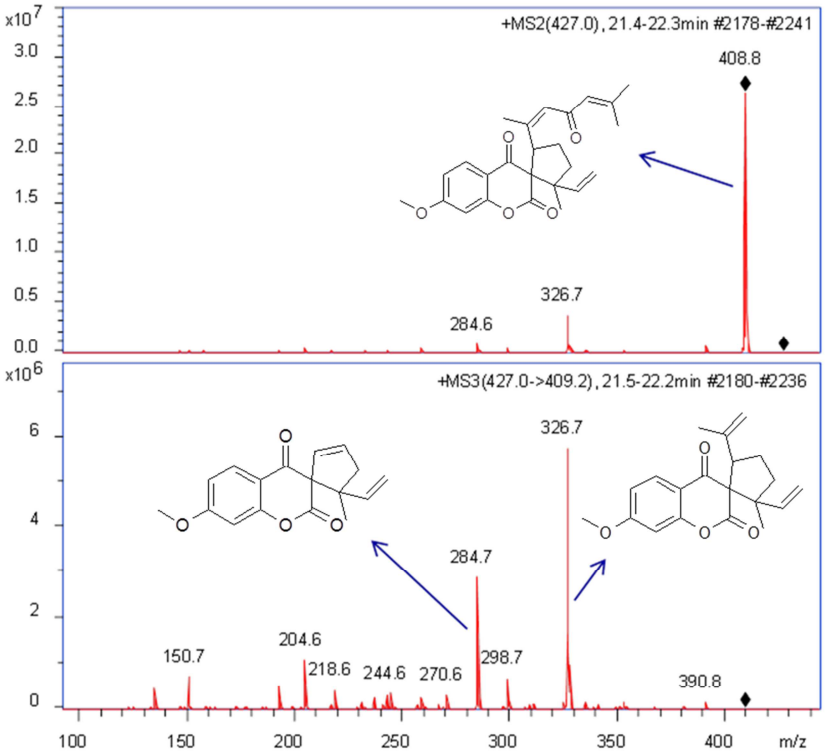

Fig. 8. MS spectrum of K10 with the structure of main fragments.

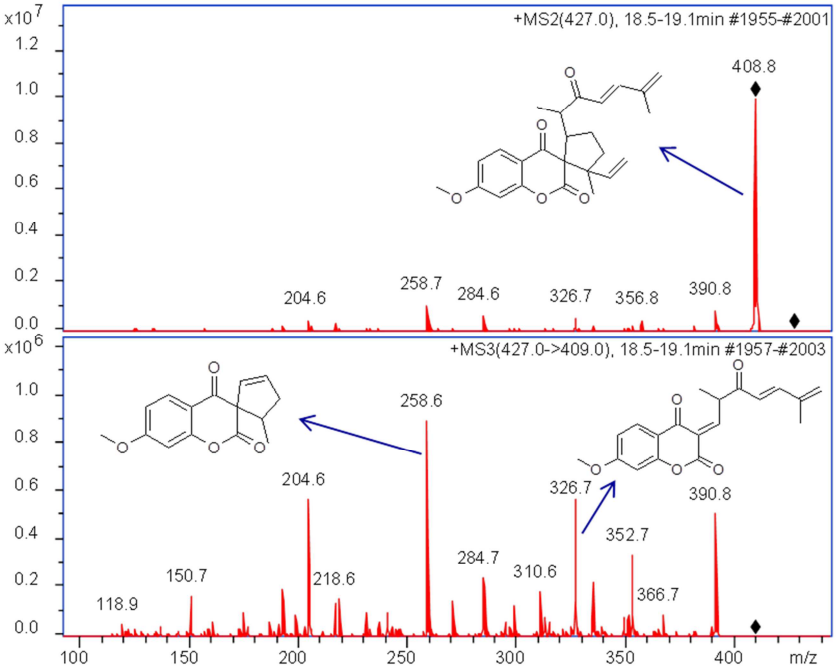

Fig. 9. MS spectrum of K13 with the structure of main fragments.

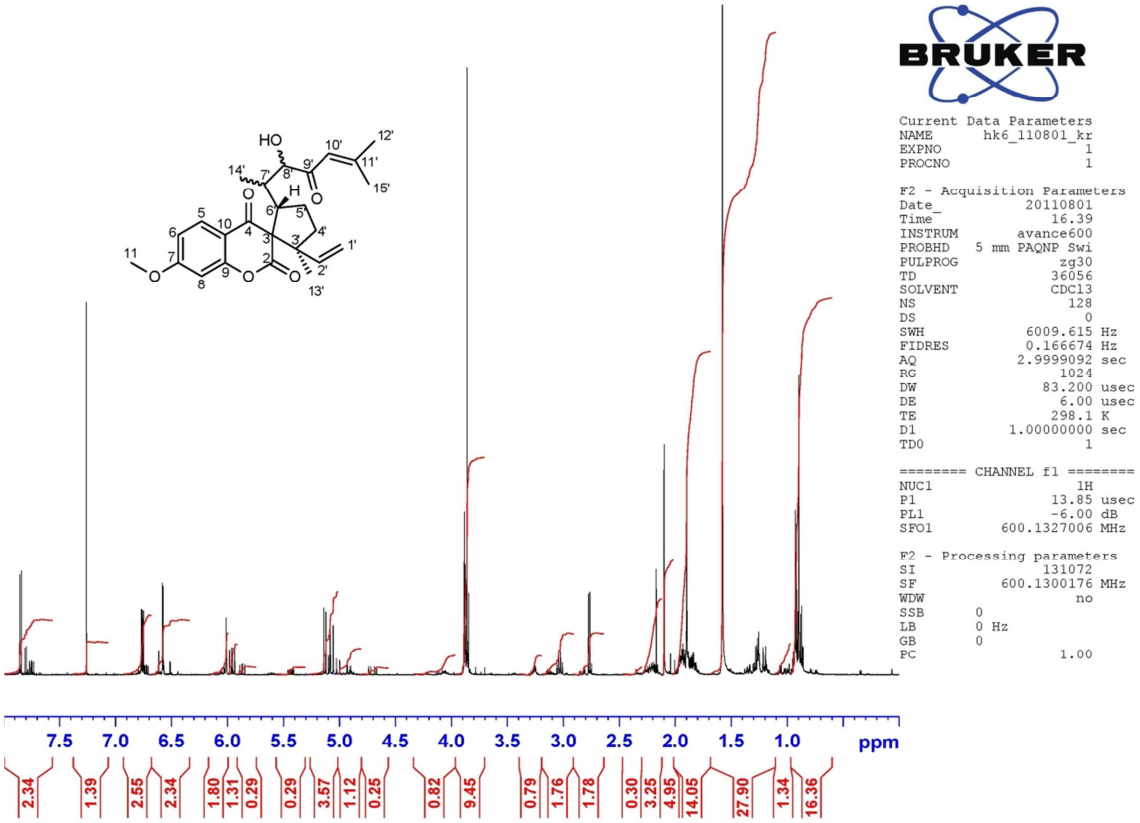

Fig. 10. ^{1}H NMR spectrum of K10.

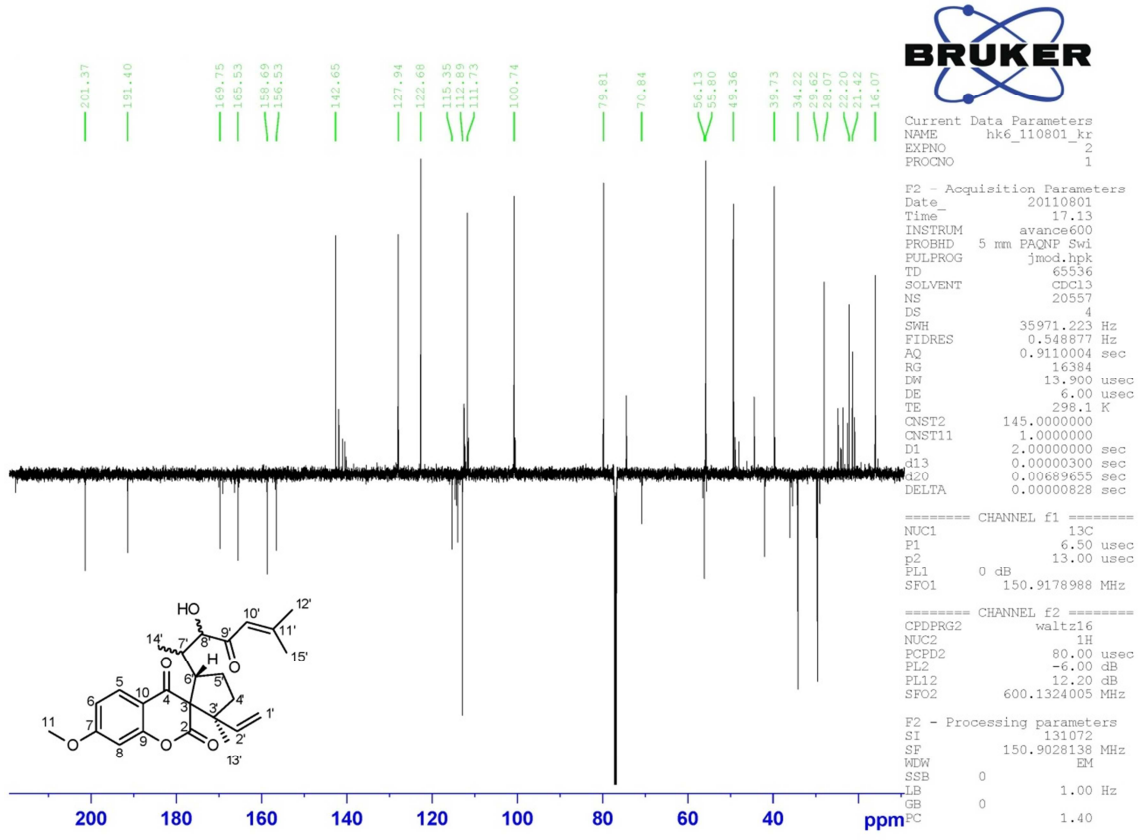

Fig. 11. ^{13}C NMR spectrum of K10.

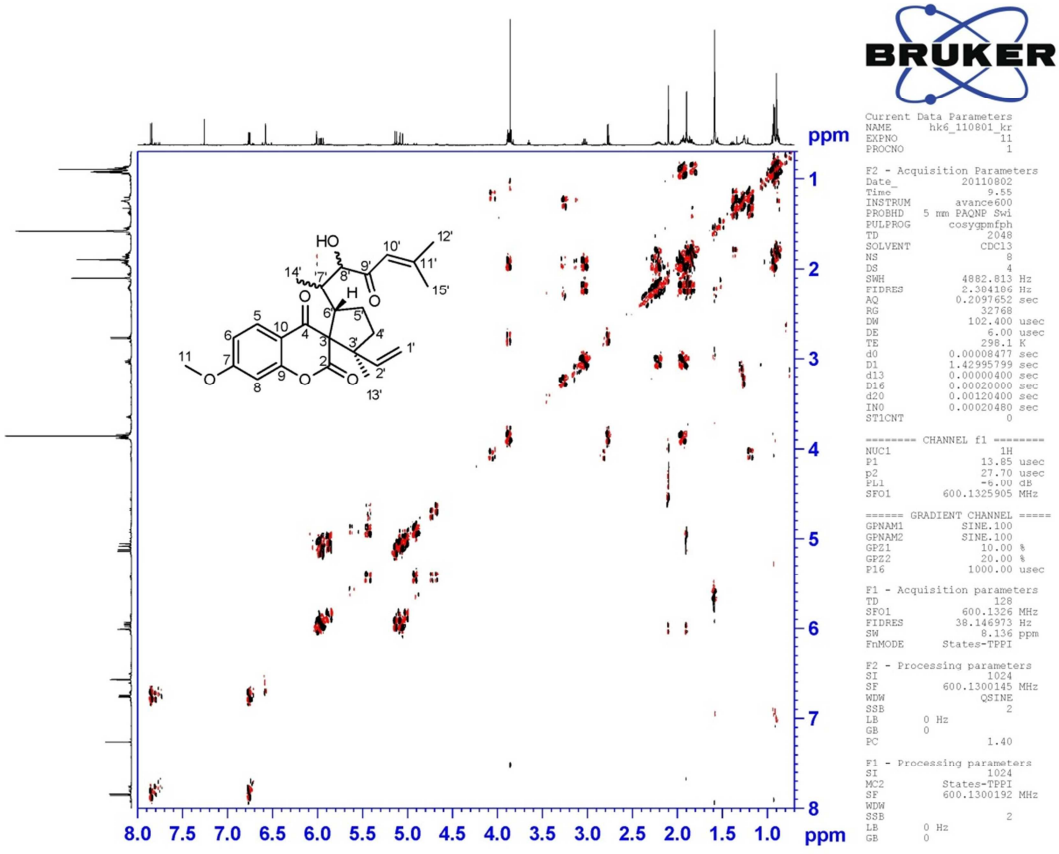

Fig. 12. COSY spectrum of K10.

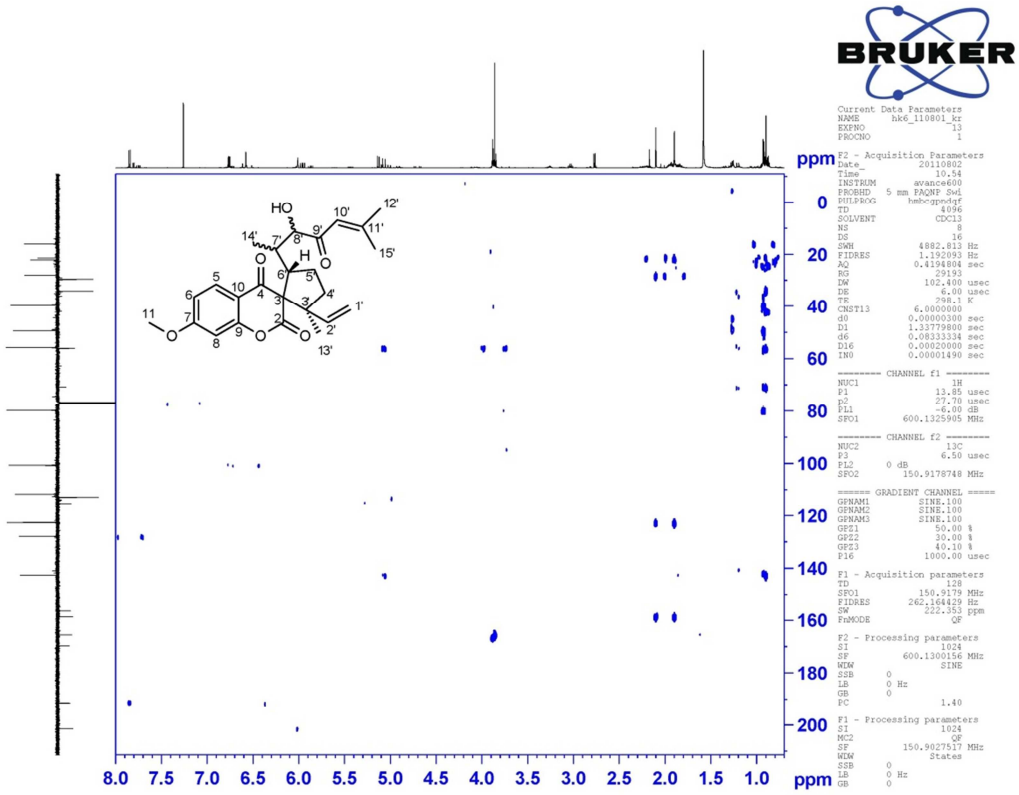

Fig. 13. HMBC spectrum of K10.

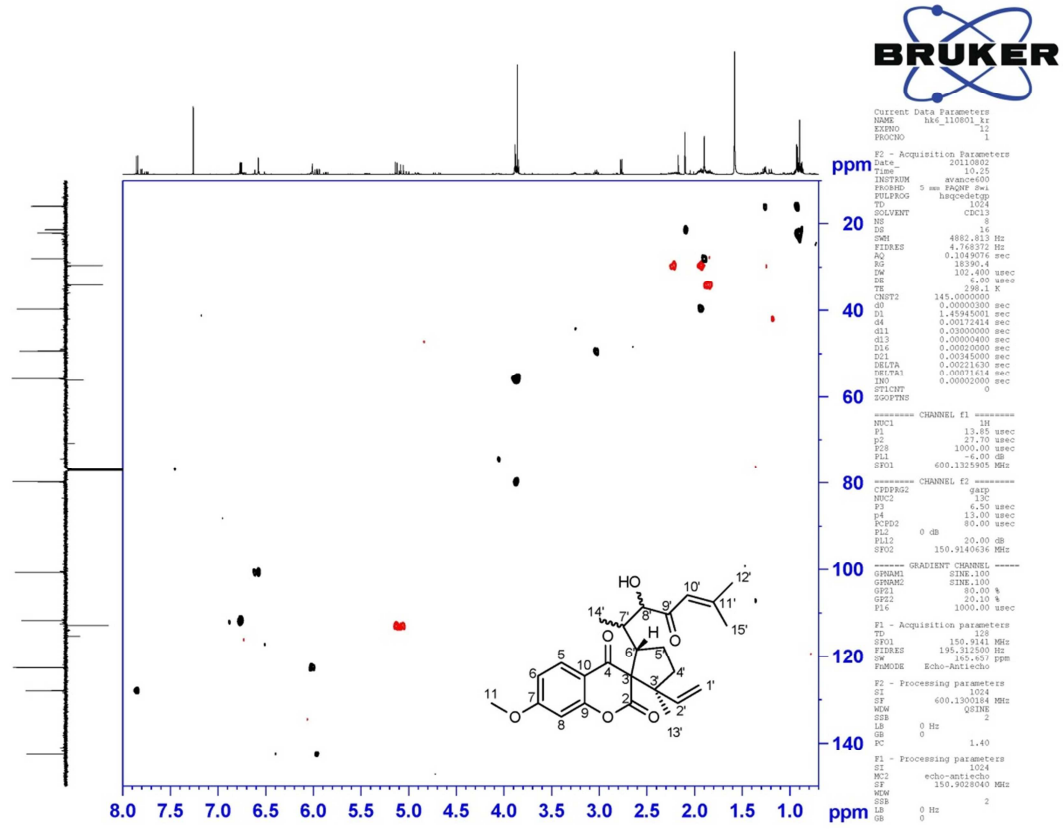

Fig. 14. HSQC spectrum of K10.

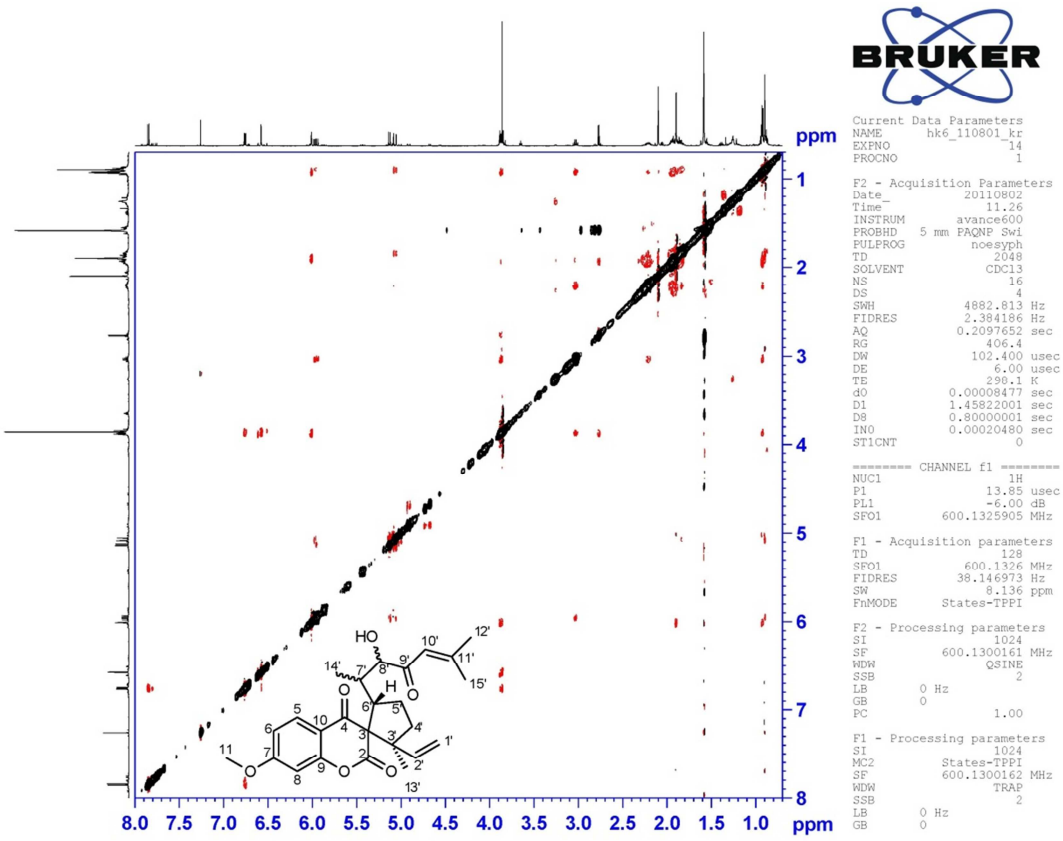

Fig. 15. NOESY spectrum of K10.

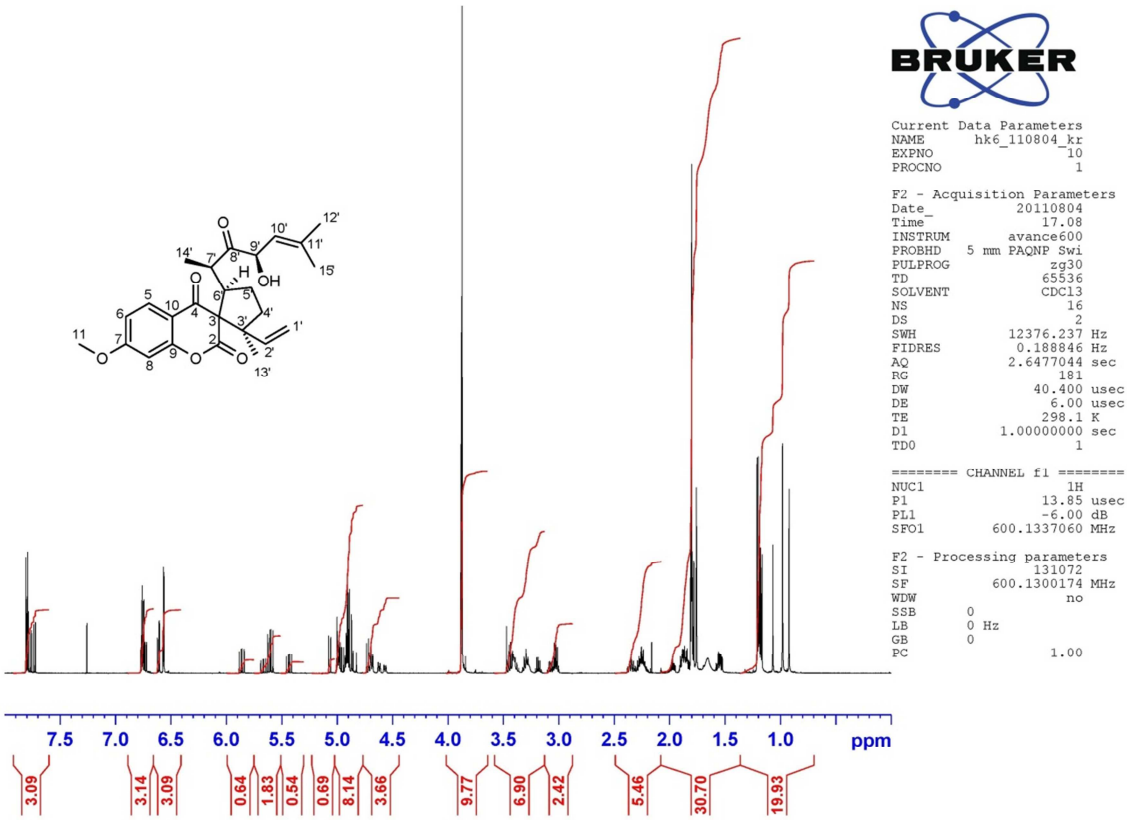

Fig. 16. ^{1}H NMR spectrum of K13.

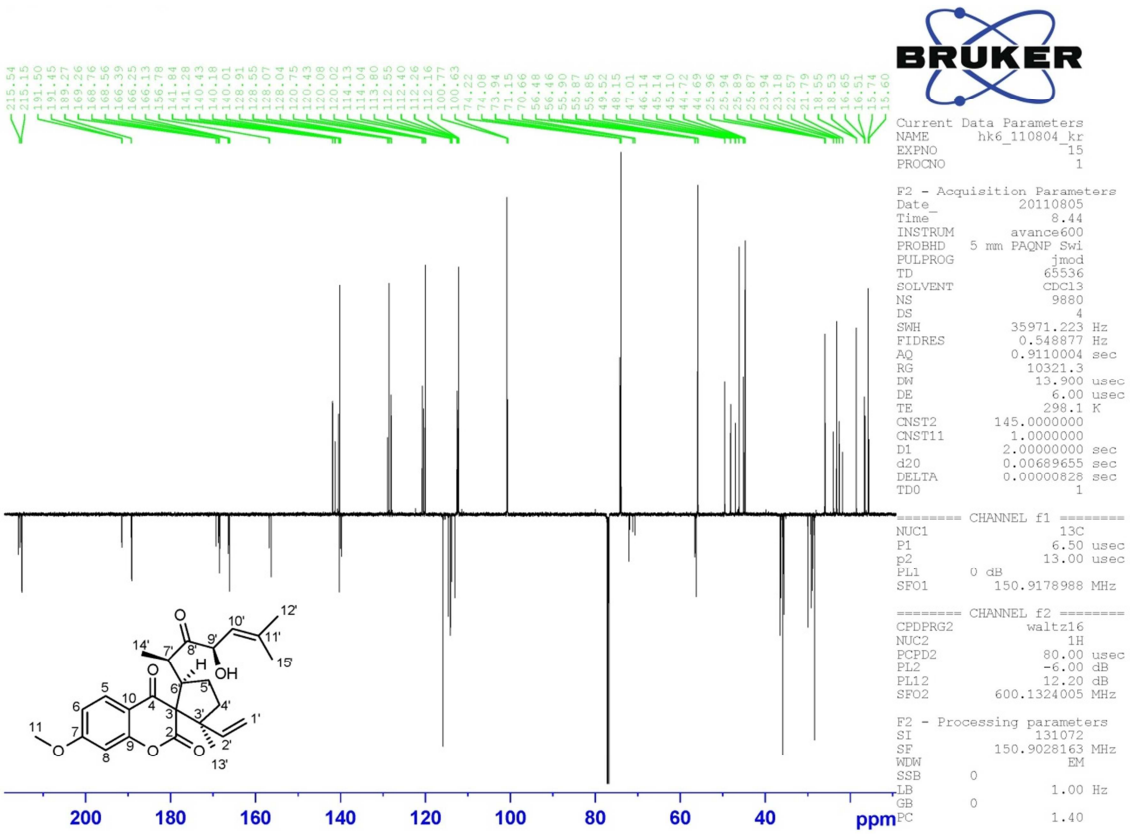

Fig. 17. ^{13}C NMR spectrum of K13.

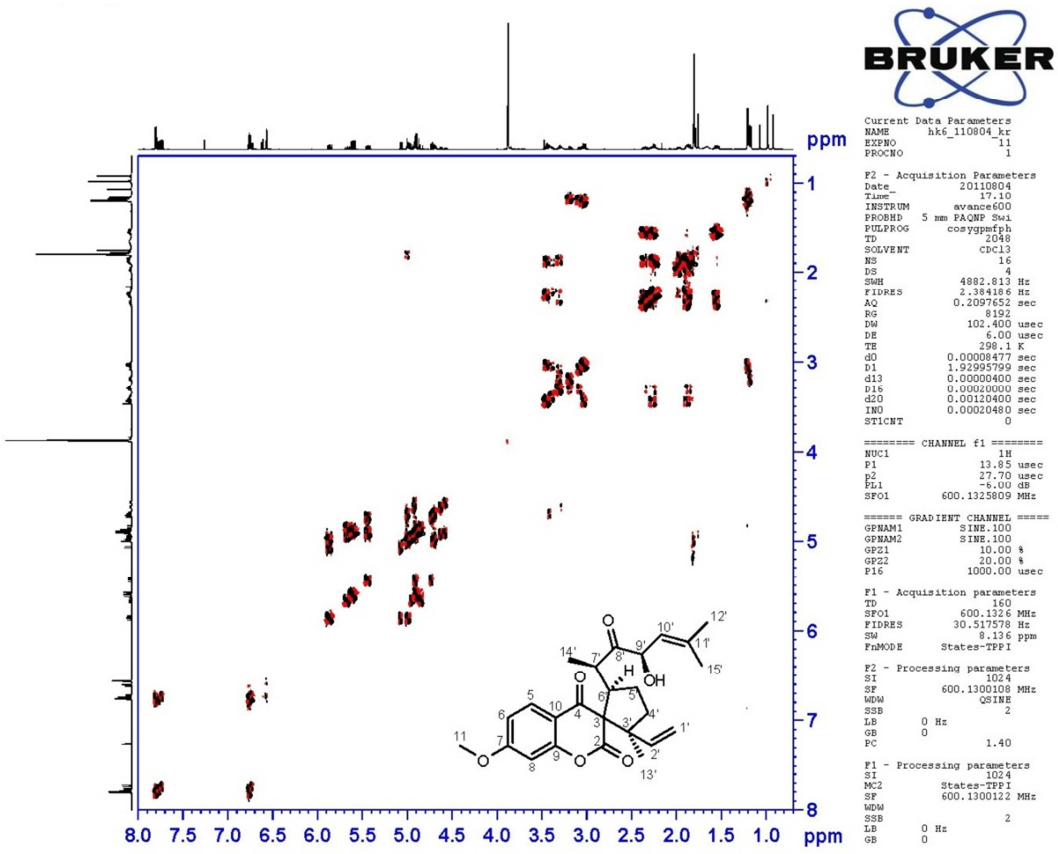

Fig. 18. COSY spectrum of K13.

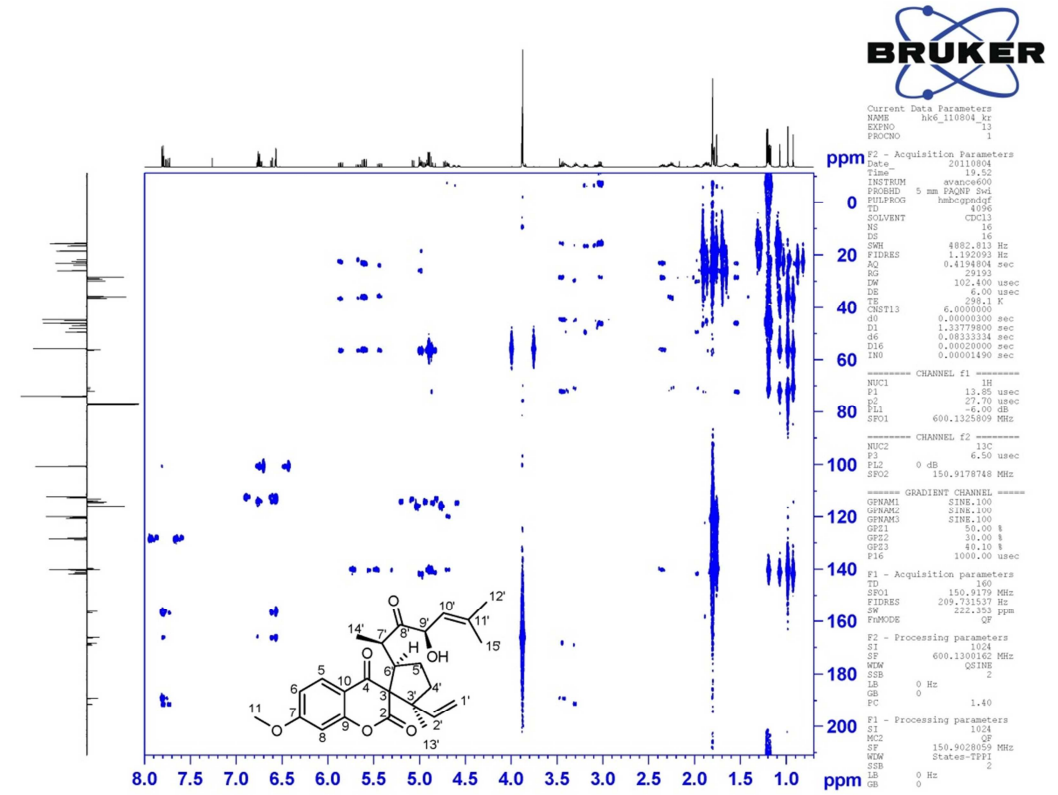

Fig. 19. HMBC spectrum of K13.

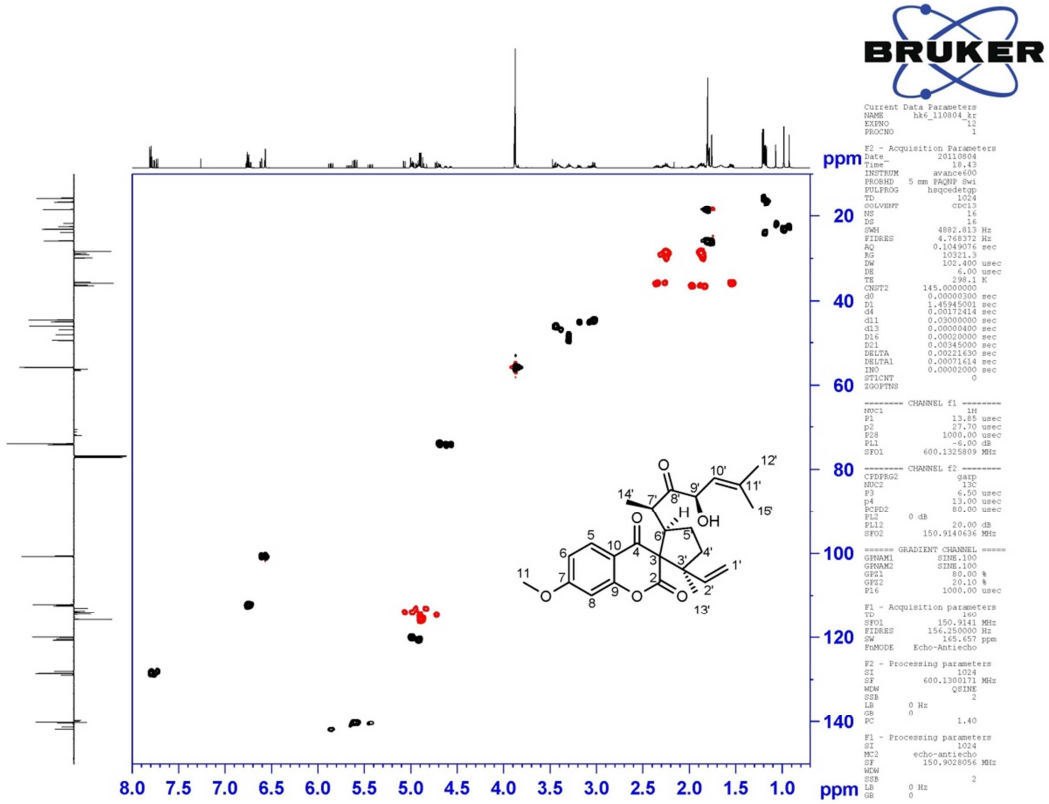

Fig. 20. HSQC spectrum of K13.

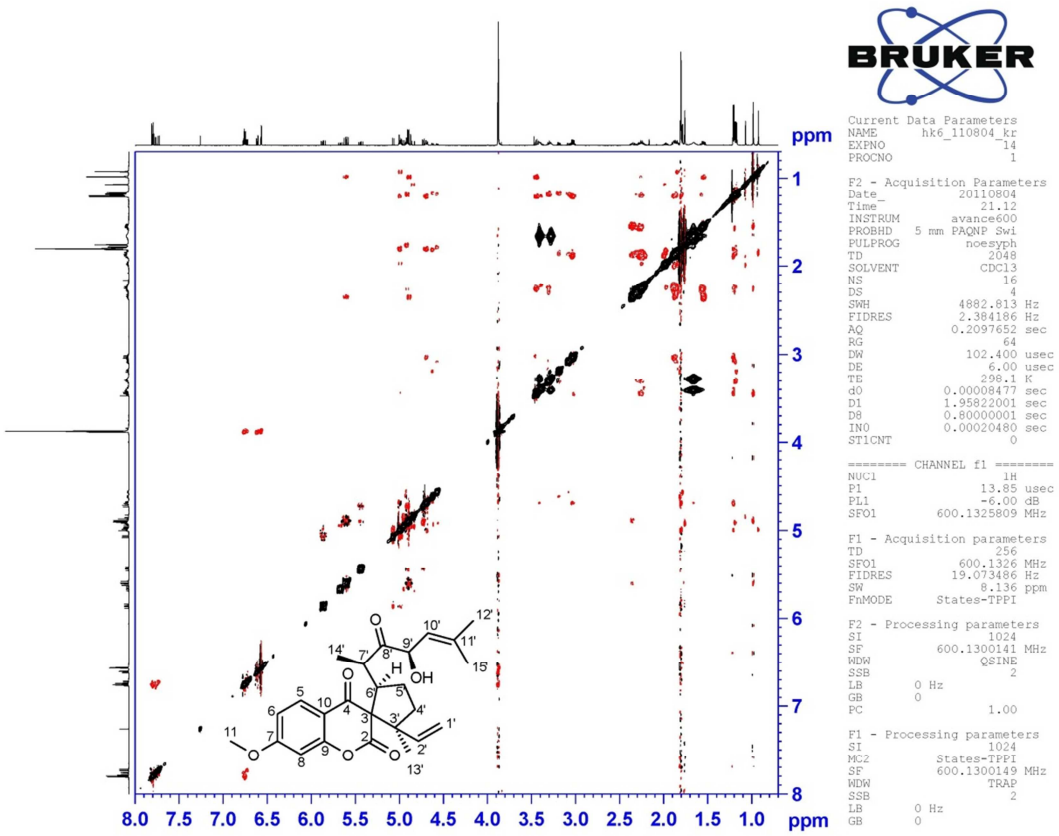

Fig. 21. NOESY spectrum of K13.

The IC_{50} values of DCM extract and the active compounds for AChE inhibition were determined in the microplate colorimetric assay (Fig. 22).

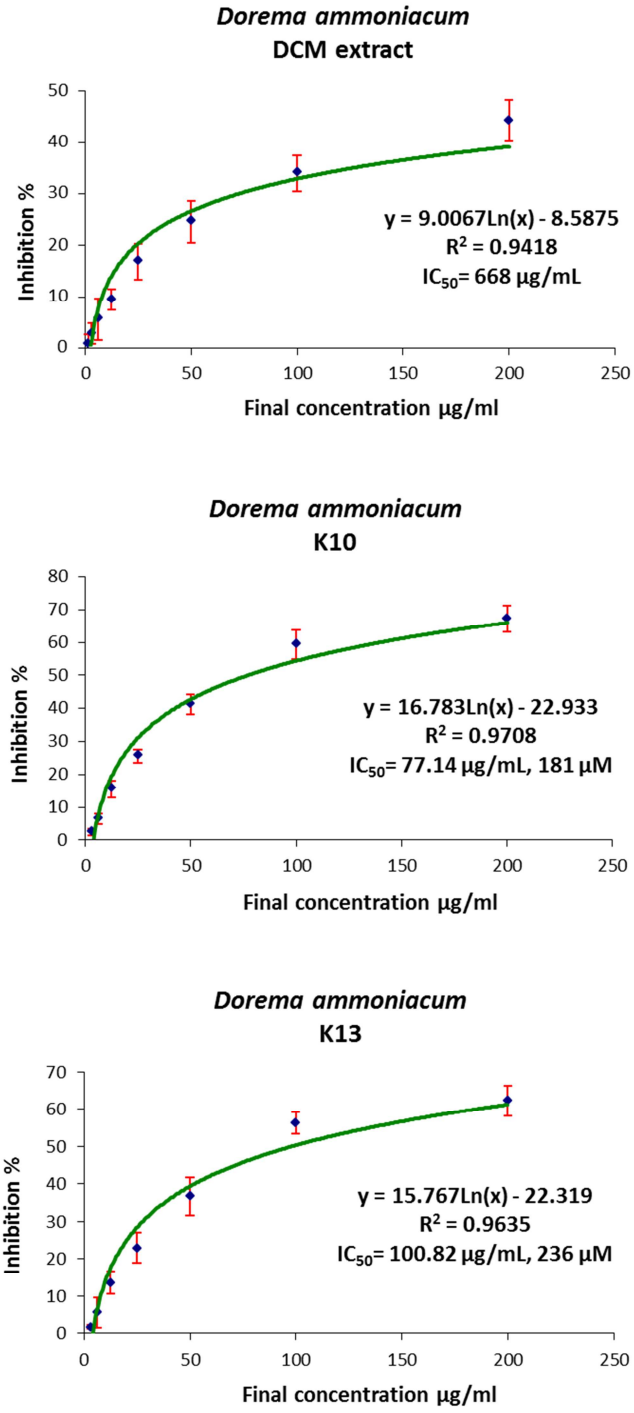

Fig. 22. AChE inhibition of DCM extract, K10 and K13 (200, 100, 50, 25, 12.5, 6.25, 3.12 µg/ml).

In addition, the concentrations of the active components in gum ammoniacum were determined by HPLC analyses using external standardization (Fig. 23-24)

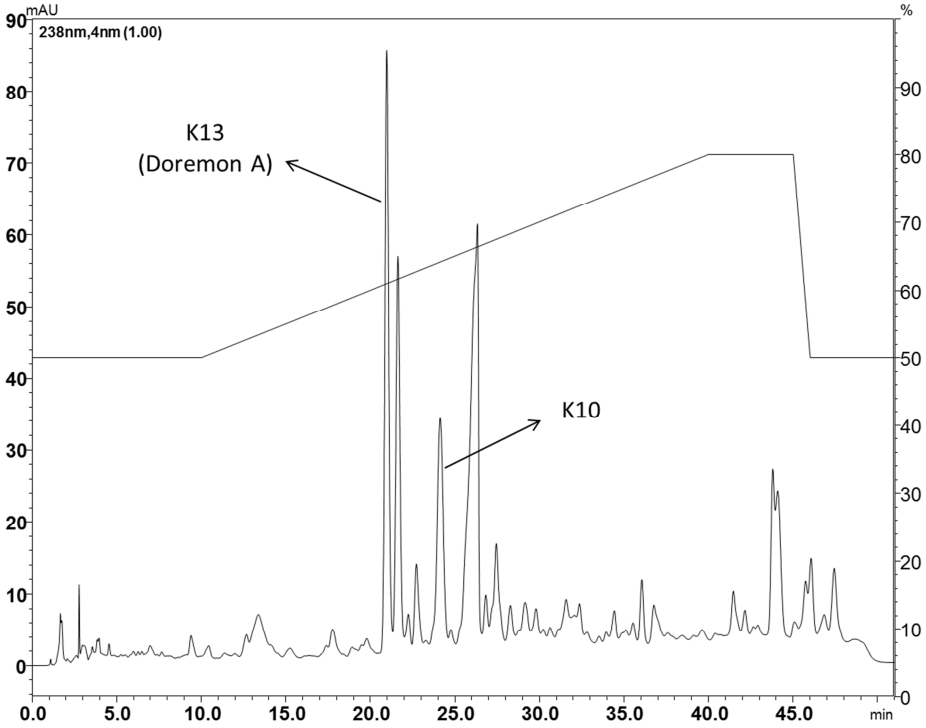

Fig. 23. HPLC chromatogram of the DCM extract from gum ammoniacum.

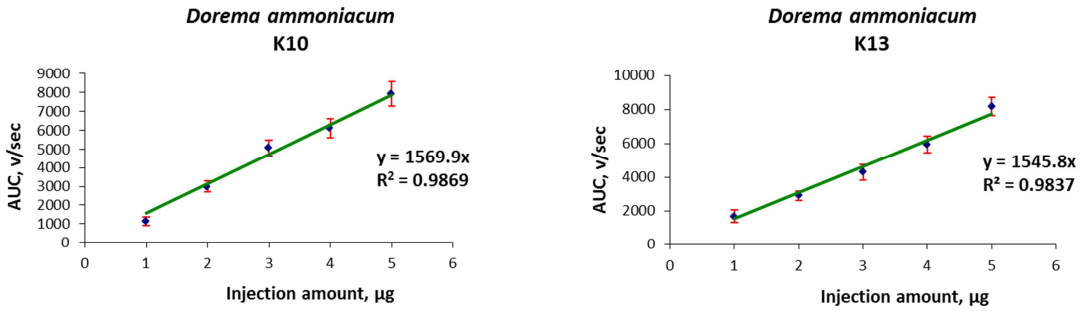

Fig. 24. Calibration curves for HPLC-quantification of K10 and K13.

Combination of bioautography with HPTLC-MS/NMR:

A fast identification of acetylcholinesterase inhibitors from galbanum

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Abstract

In the search for new natural compounds with acetylcholinesterase (AChE) inhibitory activity, this study focused on galbanum, the oleo gum-resin from *Ferula gummosa* Boiss. which had shown AChE inhibitory activity in a screening. The isolation of bio-active compounds from plant extracts usually is laborious and time-consuming. Thus, for fast identification of active compounds, HPTLC bioautography was combined with HPTLC-MS/NMR. After pre-fractionation of the dichloromethane extract by vacuum liquid chromatography, fractions were separated by automated HPTLC and active zones determined by bioautography. A TLC-MS interface was used to elute the single zones from the plates directly into the mass spectrometer. In addition, the two major active zones were extracted via the interface HPTLC plates for one and two-dimensional ^{1}H and ^{13}C NMR spectroscopy and Q-TOF mass spectrometry. The extracted compounds were identified as 7-{[(2E)-3,7-dimethylocta-2,6-dien-1-yl]oxy}-2H-chromen-2-one (auraptene) and 7-(((1R,4aR,6S,8aS)-6-hydroxy-5,5,8a-trimethyl-2-methylen edecahydronaphthalen-1-yl)methoxy)-2H-chromen-2-one (farnesiferol A). This is the first report of auraptene and farnesiferol A in *Ferula gummosa*. Their IC_{50} values for acetylcholinesterase inhibition were determined for the

first time as 47 and 17 µg/ml, and the concentrations of these compounds in galbanum were quantified by HPLC analyses as 3.5% and 7.9%, respectively.

Keywords: Galbanum, *Ferula gummosa* Boiss., acetylcholinesterase inhibition, HPTLC-MS.

1. Introduction

The correlation of cholinergic deficit with aggravation of Alzheimer's disease has been commonly accepted (Garcia-Alloza *et al.,* 2006). Thus, to slow down the symptoms of Alzheimer's disease, restoration of the cholinergic function has been considered as a treatment option. The use of AChE inhibitors is one therapeutic option which blocks this key enzyme in the breakdown the acetylcholine (Howes and Houghton, 2003).

Based on traditional medicine, the use of herbal medicinal substances for the treatment of dementia has been studied during the last two decades (Andrade *et al.,* 2000). Using this knowledge, e.g. galantamine from *Galanthus nivalis* L. (snowdrop) has been identified as a potent acetylcholinesterase inhibitor, which today is one therapeutic option in the treatment of Alzheimer's disease (Hostettmann *et al.,* 2006).

Galbanum is an oleo gum-resin from *Ferula gummosa* Boiss. (Apiaceae) which is a perennial plant indigenous to central Asia, growing in the northern and western parts of Iran. *F. gummosa* (Bārije in Persian) is an Iranian medicinal plant and has been used as a tonic, and in epilepsy and chorea (Zargari, 1997). Additionally it has been used for gastrointestinal disorders and as a wound-healing remedy in Iranian traditional medicine (ITM) (Amin, 2005). For extracts from different parts of *F. gummosa* several biological activities have been demonstrated in recent investigations including antibacterial (Abedi *et al.,* 2008), antioxidant (Nabavi *et al.,* 2010), anticonvulsant (Sayyah *et al.,* 2002), spasmolytic (Sadraei *et al.,* 2001) and antiepileptic activities (Sayyah *et al.,* 2001).

Extracts from medicinal plants are very complex, thus isolation and structural characterization of the active compounds is often an extremely laborious and time-

consuming process. Thus, finding a fast and reliable method for dereplication of active compounds is always one of the major targets in medicinal plant research.

A bioactivity-guided screening showed AChE inhibitory activity for a dichloro-methane (DCM) extract from galbanum (Adhami *et al.,* 2011). In the study presented here, HPTLC bioautography was combined with HPTLC-MS/NMR for the very fast dereplication of compounds active on AChE inhibition in galbanum.

2. Materials and Methods

2.1. Chemicals and reagents

AChE from electric eel, 1-naphthyl acetate, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), Tris-HCl, bovine serum albumin (BSA) and physostigmine were purchased from Sigma (St. Louis, USA). Acetylthiocholine iodide (ATCI) and chelidonine were obtained from Fluka (Buchs, Switzerland). Fast Blue B salt (FBS) and silica gel 60 were from Merck (Darmstadt, Germany). Two different buffer systems were used (buffer A: 50 mM Tris-HCl, pH 7.9 containing 0.1% BSA; buffer B: 50 mM Tris-HCl, pH 7.9 containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$).

2.2. General

Automatic TLC sampler (ATS 4) and automatic developing chamber (ADC 2) from Camag (Muttenz, Switzerland) were used for automated HPTLC. TLC visualizer, TLC scanner 4, chromatogram immersion device for derivatization, TLC plate heater and TLC-MS interface were also from Camag. The absorbance was measured in a Genios microplate reader (Tecan, Salzburg, Austria). Extraction was performed by sonification in a Branson 3150 ultrasonic bath (Dumbury, USA). TLC and HPTLC plate silicagel 60 F_{254} were obtained from Merck (Darmstadt, Germany) and 96-well microplates PS F-bottom from Greiner Bio-One (Frickenhausen, Germany). For TLC-MS, a pump of HPLC Agilent 1260 (Agilent Technologies, Santa Clara, USA) and a single quadrupole Agilent 6120 mass spectrometer were used. ESI Q-TOF experiments were performed on an Agilent Q-TOF 6540 mass spectrometer equipped with an Agilent UHPLC 1290 System. Two NMR spectrometers from Bruker (Bruker BioSpin, Rheinstetten, Germany) were used for NMR spectra; a 400MHz Bruker Avance II instrument, equipped with a 5mm BBO probe and controlled by TOPSPIN 2.1., and a Bruker Avance DRX 600

spectrometer operating at 600.13 and 150.90 MHz for ^{1}H and ^{13}C, respectively, at a temperature of 298 K using a triple resonance probe (^{1}H, ^{13}C, broad band) with triple axis pulsed field gradients. HPLC was performed on a Shimadzu instrument with LC-20AD pump, SPD M20A diode array detector and SIL 20AC HT auto-sampler (Kyoto, Japan). A EZ-2plus evaporator from Genevac (New York, USA) was used to evaporate the solvents.

2.3. Plant material

Galbanum was obtained from a herbal shop in Tehran, Iran, and identified by Dr. Gholamreza Amin at the herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran (voucher number PMP-807).

2.4. Extraction

Thirty grams of galbanum were stirred with 200 ml DCM at room temperature over the night, followed by sonification at 40°C for 30 min. The extract was concentrated under reduced pressure at 40°C to yield 17.34 g of a viscous residue.

2.5. Bioautography assay

Duplicated TLC was carried out on the DCM extract of galbanum using the mobile phase chloroform-ethylacetate-methanol (90+7+3). The solvent was completely removed under airstream. A bioautography assay was performed on one of the plates according to Marston *et al*., 2002 and Adhami *et al.,* 2011 to detect the zones active for AChE inhibition. The other one was derivatized with anisaldehyde-sulfuric acid. Chelidonine served as a positive control showing R_F 0.42 in this TLC system.

2.6. Microplate assay

The AChE inhibitory activities of the substances were measured by a quantitative colorimetric assay based on Ellman's method (Rhee *et al.,* 2001; Adhami *et al.,* 2012). In a 96-well plate, 25 µl of 7.5 mM ATCI, 125 µl of 1.5 mM DTNB in buffer B, 50 µl of buffer A and 25 µl of the extract or the isolated substances (from 7.8 µg/ml to 1.0 mg/ml in 10% DMSO) were thoroughly mixed and the absorbance was read at 405 nm 5 times every 15 s. After adding 25 µl of AChE (0.11 U/ml in buffer A) the plate was incubated at 25°C for 10 minutes and the absorbance was measured again every 15 s for 8 times. A solution including 10% DMSO was used as negative control. To prevent

any increase in absorbance due to the color of the substances or spontaneous hydrolysis of substrate, the absorbance before addition of the enzyme was subtracted from the absorbance after adding the enzyme. The assay was repeated three times for every concentration. The validation for a positive control was served by different concentrations of physostigmine (15-0.12 µl/ml as the final concentration).

2.7. Fractionation and optimization of HPTLC

The fractionation of 17 grams DCM extract of galbanum was started by vacuum liquid chromatography (VLC). Silica gel 60 was used as the stationary phase under gradient elution with chloroform and hexane (20-100%) as the mobile phase. Fractions with similar chemical composition were combined to obtain ten collective fractions (A1-A10). Automated HPTLC was used to optimize the separation of the fractions A5 and A6 which contained the most active zones.

The mobile phase was individually optimized for each fraction according to the published guideline (Reich and Schibli, 2006). In the first step each fraction was examined by HPTLC with single solvent mobile phases. Then multiple solvent mobile phases were prepared based on the solvent strength which affects the R_F value of the analyte and the selectivity which affects the relative position of the zones. The mobile phases were modified another time to obtain the optimum mobile phase (Fig. 1).

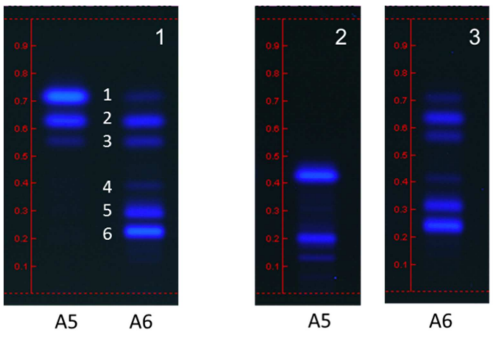

Fig.1. HPTLC of fractions A5 and A6. 1: HPTLC of A5 and A6 with the mobile phase $CHCl_3$-EtOAc-MeOH (100+10+2), 2: A5 with the optimum mobile phase $CHCl_3$-MeOH (99+1), 3: A6 with the optimum mobile phase $CHCl_3$-EtOAc-MeOH (95+10+2).

For detection of the fluorescent compounds a TLC scanner was used. Based on the optimized separation of the zones in fractions A5 and A6, the zones 1 to 6 which showed activity in the assay on AChE inhibition were selected for HPTLC-MS (Fig. 1).

2.8. HPTLC-MS

For HPTLC-MS, the interface was connected between a pump and the mass spectrometer. The round and oval extraction head were used for extraction of the different zones. Methanol as the solvent and APCI as the ionization method showed optimum spectra. Charging high voltage was 2000 V with a nebulizer pressure of 20 psig. The molecular weights of the zones are listed in table 1.

Table 1

The molecular weights of active zones obtained from HPTLC-MS

Fr. A5		Fr. A6	
Zone	MW (Da)	Zone	MW (Da)
1	298.3	1	298.3
2	296.2	2	296.2
		3	364.3
		4	364.3
		5	382.3
		6	382.3

2.9. Isolation and characterization of active compounds

To extract the active zones 1 to 6, the TLC-MS interface was used. The eluate of each zone was collected instead of analysis in the mass spectrometer. Methanol was the eluent with the flow rate of 0.4 ml/min. Each extraction was carried out in 40 seconds. The extracted solution of each zone was concentrated by a Genevac evaporator and the residues (from 200 to 400 μg) were used for HPTLC (Fig. 2).

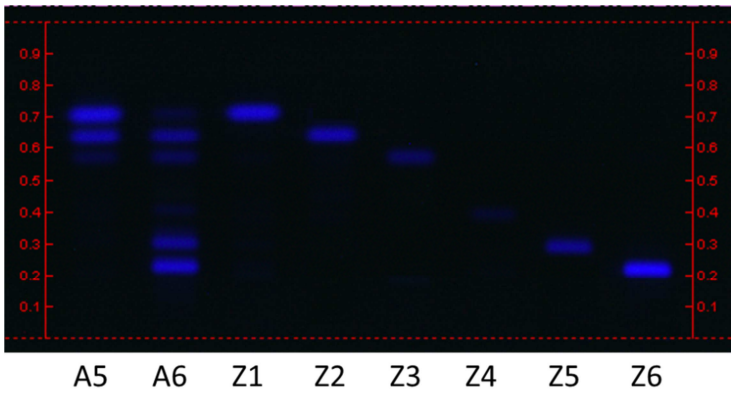

Fig. 2. HPTLC of isolated zones under UV 366 nm. Mobile phase: $CHCl_3$-EtOAc-MeOH (100+10+2).

Due to the highest yield and purity based on TLC scanning, zones 1 and 6 from the fractions A5 and A6, respectively, were selected for further investigation. The extraction of these two zones was continued to obtain sufficient amounts for NMR spectroscopy and determination of the biological activity. Finally 1.58 and 1.75 mg of the substances 1 and 6 were obtained, respectively. The structures of the active compounds were characterized by one and two-dimensional ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry. The accurate molecular weights of the two oily substances were identified by a UHPLC-QTOF MS. A flow injection analysis was performed with 0.3 µL sample solution and a flow rate of the mobile phase at 0.4 ml/min in an isocratic mode. The mobile phase consisted of acetonitrile-water (80:20 v/v) with 0.1% formic acid. The mass data were acquired in the positive mode, with targeted MS/MS at 4 scans. The Agilent Jet Stream ESI-Source was set at 300 °C with a drying gas flow at 8 l/min and a nebulizer pressure about 35 psi. The nitrogen sheath gas flow was kept at 8 l/min at 300°C. The capillary voltage was set about 3000 V with a nozzle voltage of 1000 V and the fragmentor voltage of 100 V. As collision gas nitrogen was used by collision energy of about 5 V.

Compound 1: (+)ESIMS *m/z* 299.1626 $[M+H]^{+}$; $ESIMS^{2}$ (299.1626 →) (*m/z*, rel. Intensity%): 299.16 (8), 163.04 (100), 137.13 (14).

Compound 6: (+)ESIMS *m/z* 383.2221 $[M+H]^{+}$; $ESIMS^{2}$ (383.2221 →) (*m/z*, rel. Intensity%): 383.22 (100), 365.21 (56), 203.18 (87), 163.04 (46), 147.12 (15).

For subsequent NMR measurement of compound 1 a 400MHz Bruker Avance II instrument, equipped with a 5mm BBO probe and controlled by TOPSPIN 2.1. was used. In ^{1}H spectra standard 1D proton spectra were acquired with a pulse angle of 30°. Exponential line broadening of 0.3 Hz was applied prior to Fourier transformation. A standard ^{13}C pulse program from the vendor's library was used for ^{13}C spectra. The acquired data were treated with an exponential window function and a line broadening of 1 Hz. To determine $^{1}H^{1}H$ COSY spectra, a standard pulse program for COSY experiments from the vendor's library was used with gradient, multiple quantum filter and no decoupling. The transmitter frequency offset was set to 3.75 ppm. A gradient echo-antiecho HSQC pulse sequence from the vendor's library was employed for $^{1}H^{13}C$ HSQC spectra. To obtain $^{1}H^{13}C$ HMBC spectra, a standard pulse program for

HMBC experiment from the vendor's library with gradient, low-pass J-filter, absolute value mode and no decoupling was used. The transmitter frequency offsets were set to 3.74 and 105 ppm for ^{1}H and ^{13}C, respectively. All the two-dimentional data were treated with a squared sinus window function in both dimensions.

All NMR spectra for compound 6 were recorded on a Bruker Avance DRX 600 NMR spectrometer using a 5mm switchable quadruple probe (QNP, ^{1}H, ^{13}C, ^{19}F, ^{31}P) with z axis gradients and automatic tuning and matching accessory. The resonance frequency for ^{1}H NMR was 600.13 MHz, for ^{13}C NMR 150.92 MHz. All measurements were performed for a solution in $CDCl_3$ at 298K. Standard 1D and gradient-enhanced (ge) 2D experiments, like double quantum filtered (DQF) COSY, TOCSY, NOESY, HSQC, and HMBC, were used as supplied by the manufacturer. Chemical shifts are referenced internally to the residual, non-deuterated solvent signal for ^{1}H (δ = 7.26 ppm) or to the carbon signal of the solvent for ^{13}C (δ = 77.0 ppm).

The NMR data of the two compounds are summarized in table 2.

2.10. HPLC

The DCM extract of galbanum and the isolated compounds 1 and 6 were analyzed by HPLC under gradient elution on a Hypersil BDS-C18 column (250×4 mm id) at 60°C. The mobile phase consisted of acetonitrile (A) and water (B) at a flow rate of 0.75 ml/min. After an isocratic step at 30% A for 5 min the concentration of solvent A increased up to 80% in 30 min and the elution was kept at this concentration for further 5 min. The eluents were monitored at 322 nm.

3. Result and discussion

A major challenge in medicinal plant research is the limitation of available time, especially in isolation and characterization of natural compounds which is a time consuming process. Combination of bioautography with HPTLC/MS is a fast method for the identification of compounds that show bio-activity via a respective assay. Compounds are separated on TLC plates and the respective zones are eluted via a special interface into the mass spectrometer or extracted for NMR spectroscopy or further analysis.

Table 2

^{1}H and ^{13}C NMR data of compounds 1 and 6.

Compound 1 ($CDCl_3$, 400 MHz)			Compound 6 ($CDCl_3$, 600 MHz)		
Position	δ_C (dept)	δ_H	Position	δ_C (dept)	δ_H
2	161.3 (s)	---	2	161.2 (s)	---
3	113.0 (d)	6.25 (1H, d, *J*= 9.3 Hz)	3	113.0 (d)	6.25 (1H, d, *J*= 9.4 Hz)
4	143.4(d)	7.76 (1H, d, *J*= 9.3 Hz)	4	143.4 (d)	7.63 (1H, d, *J*= 9.4Hz)
4a	124.4 (s)	---	4a	112.5 (s)	---
5	128.7 (d)	7.36 (1H, d, *J*= 8.6 Hz)	5	128.7 (d)	7.35 (1H, d, *J*= 2.5 Hz)
6	113.3 (d)	6.85 (1H, dd, *J*= 8.3, 2.3 Hz)	6	113.3 (d)	6.81 (1H, dd, *J*= 8.4, 2.4 Hz)
7	162.2 (s)	---	7	162.0 (s)	---
8	101.6 (d)	6.82 (1H, d, *J*= 2.5 Hz)	8	101.6 (d)	6.80 (1H, d, *J*= 2.4 Hz)
8a	155.9 (s)	---	8a	155.9 (s)	---
1'	65.5 (t)	4.61 (2H, d, *J*= 6.6 Hz)	1'	56.6 (t)	2.21 (1H, dd, *J*= 6.3, 5.8 Hz)
2’	118.4 (d)	5.47 (1H, qd, *J*= 6.6, 1.3 Hz)	2’	146.6 (s)	---
3’	142.4 (s)	---	3’eq ax	32.3 (t)	2.34 (1H, m) 2.07 (1H, m)
4’	39.5 (t)	2.10 (2H, m)	4’eq ax	23.0 (t)	1.73 (1H, m) 1.43 (1H, m)
5’	26.3 (t)	2.10 (2H, m)	4a’	46.2 (d)	1.31 (1H, dd, *J*= 12.6, 3.0 Hz)
6’	123.6 (d)	5.08 (1H, m)	5’	39.0 (s)	---
7’	132.0 (s)	---	6’	79.1 (d)	3.26 (1H, m)
8’	25.7 (q)	1.67 (3H, d, *J*= 1.0 Hz)	7’	27.6 (t)	1.70 (1H, m)
9’	16.8 (q)	1.76 (1H, s)	8’ax eq	34.8 (t)	1.63 (1H, m) 1.37 (1H, m)
10’	17.7 (q)	1.60 (1H, s)	8a’	37.7 (d)	---
			9’	68.0 (t)	4.29 (1H, dd, *J*= 9.5, 5.8 Hz) 4.02 (1H, dd, *J*= 9.8, 6.3 Hz)
			10’b a	111.3 (t)	4.82 (1H, dd, *J*= 2.1, 2.1 Hz) 4.73 (1H, dd, *J*= 2.1, 1.9 Hz)
			11’	15.7 (q)	0.82 (3H, s)
			12’	28.5 (q)	1.05 (3H, s)
			13’	22.1 (q)	1.00 (3H, d, *J*= 0.7 Hz)

Galbanum, which has been used for different indications in ITM, showed several zones active in AChE inhibition via bioautography assay. Thus, this study aimed to extract and identify the active compounds by HPTLC-MS/NMR.

3.1. Optimization of HPTLC

HPTLC optimization is a crucial step and was carried out by automated instrumentation. This way very distinct and accurate zones were achieved which allowed precise extraction of each single compound. In cases where the total extract shows a very complex pattern, a primary fractionation by other chromatographic methods might be required.

3.2. Structure elucidation

The active zones 1 and 6 from fractions A5 and A6, respectively, were extracted from HPTLC plates for structure elucidation. The fractions A5 and A6 were applied on HPTLC plates as 16 cm line with concentrations of 5.0 µg/mm. To obtain sufficient amounts for NMR and the *in vitro* assay, 10 HPTLC plates from each fraction were developped.

Q-TOF MS resulted in very accurate molecular weights of 299.1626 Da and 383.2221 Da for compounds 1 and 6, respectively, which are in agreement with the molecular formulae of $C_{19}H_{22}O_3$ and $C_{24}H_{30}O_4$. Comparison of all NMR data proved component 1 and 6 as 7-{[(2E)-3,7-dimethylocta-2,6-dien-1-yl]oxy}-2H-chromen-2-one (auraptene) and 7-(((1R,4aR,6S,8aS)-6-hydroxy-5,5,8a-trimethyl-2-methylenedeca hydronaphthalen-1-yl)methoxy)-2H-chromen-2-one (farnesiferol A), respectively (Fig. 3). This is the first report of aurapten and farnesiferol A in *Ferula gummosa*. The NMR data for auraptene are in agreement with previous reports (Abulrob *et al*., 2004). For farnesiferol A which is a very rare natural compound only known from few *Ferula* species there is only proton NMR available in literature which is largely in conformity with presented data (Hofer *et al.,* 1984). The assignments of all ^{13}C shifts were determined in detail.

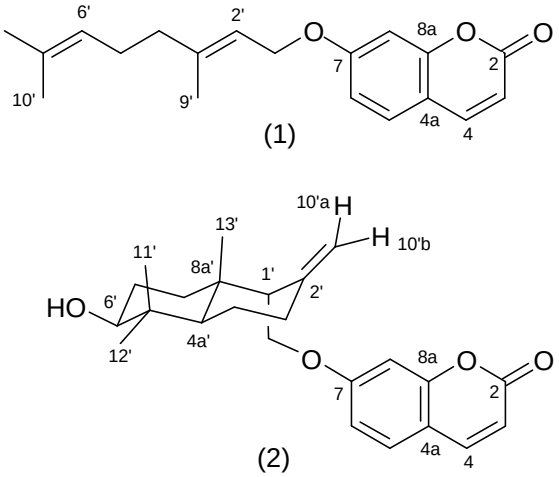

Fig. 3. The structures of auraptene (1) and farnesiferol A (2).

3.3. AChE inhibition

For the first time an *in vitro* examination of the AChE inhibitory effect of galbanum was performed. The IC_{50} value of the DCM extract was determined as 88.44 µg/ml. The major active compounds isolated in a bioactivity guided approach were auraptene and farnesiferol A showing IC_{50} values of 47.49 µg/ml (159 µM) and 17.63 (46.2 µM), respectively. Recently, low AChE inhibitory activities were reported for auraptene and farnesiferol A by Karimi *et al*. (2010), while this study showed much higer activity for them in AChE inhibition. This difference might be due to the different sources of AChE. In this study AChE was from electric eel, while Karimi *et.al*. (2010) had used human red blood cell AChE.

3.4. Quantification

The content of the active compounds auraptene and farnesiferol A in the extract was determined by HPLC analyses with external standardization (Fig. 4). Over the selected range peak areas of both analytes were linearly dependent on concentrations with correlation coefficients of R^2= 0.9951 for auraptene and R^2= 0.9978 for farnesiferol A. Their concentrations in DCM extract were 6.1% and 13.7%, respectively. The total amounts in galbanum were 3.5% auraptene and 7.9% farnesiferol A.

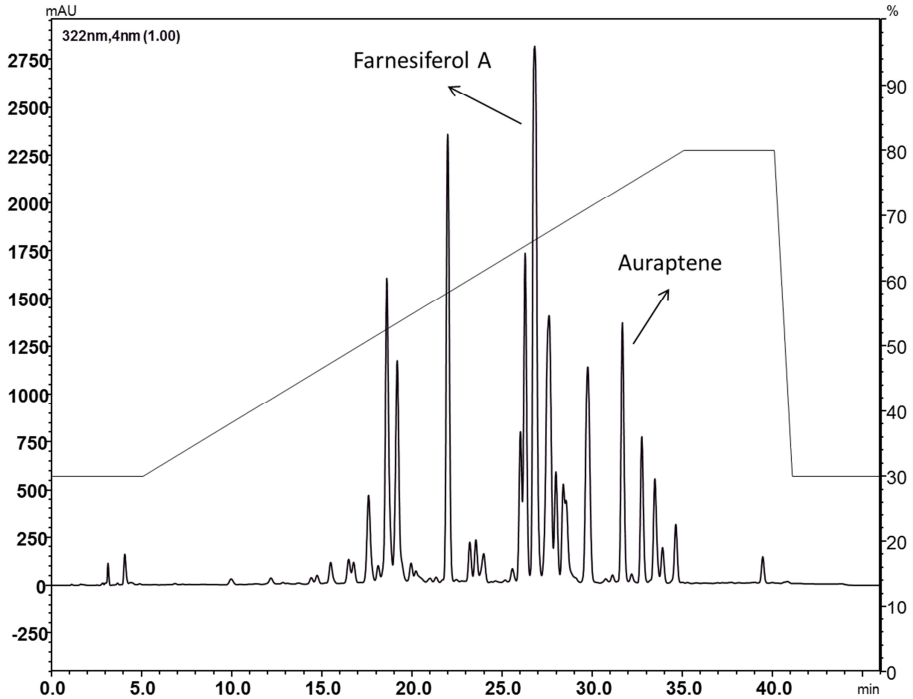

Fig. 4. HPLC chromatogram of the DCM extract from galbanum.

4. Conclusion

The study showed that HPTLC-MS/NMR can be considered as a fast method for dereplication of natural compounds, when an optimum separation on HPTLC plate is provided. From the correlation of the concentration of elucidated compounds and their IC_{50} values for AChE inhibition, it can be concluded that auraptene and farnesiferol A are of the major responsible compounds in galbanum for this bio-activity.

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Supporting information for article 4

Galbanum, the oleo gum-resin from *Ferula gummosa* was the fourth herbal drug investigated in detail (Fig. 1).

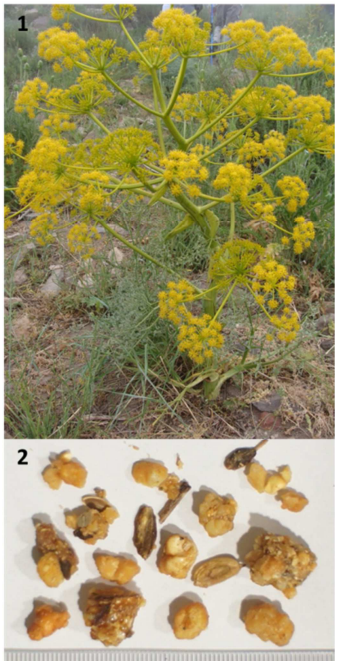

Fig. 1. *Ferula gummosa*. 1: shrub (http://yakhaar.ir), 2: oleo gum-resin.

A TLC bioautography assay for AChE inhibition on DCM extract of galbanum presented several active zones which showed strong UV absorbance in 366 nm (Fig. 2).

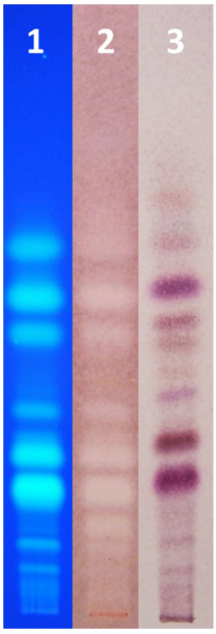

Fig. 2. TLC of the DCM extract. 1: UV 366 nm, 2: bioautography with 1-naphtyl acetate reagent, 3: derivatization with anisaldehyde-sufuric acid. Mobile phase: $CHCl_3$-EtOAc-MeOH (90+7+3).

As the DCM extract of galbanum was very complex, a primary fractionation was performed by VLC to obtain 10 collective fractions. Then the isolation of the fractions A5 and A6 which included the most active zones were optimized by automated HPTLC (Fig. 3-5).

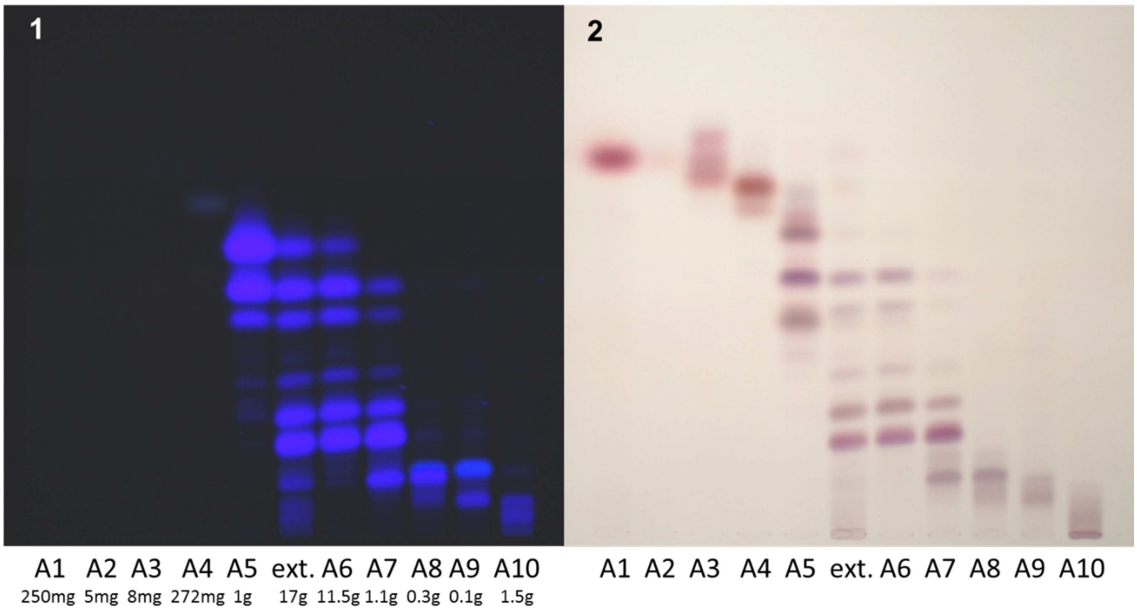

Fig. 3. TLC of fractions A1-A10 after VLC of DCM extract on a silicagel 60 column under elution with chloroform and hexane (20-100 %). 1: UV 366 nm, 2: derivatization with anisaldehyde-sufuric acid. TLC mobile phase: $CHCl_3$-EtOAc-MeOH (90+7+3).

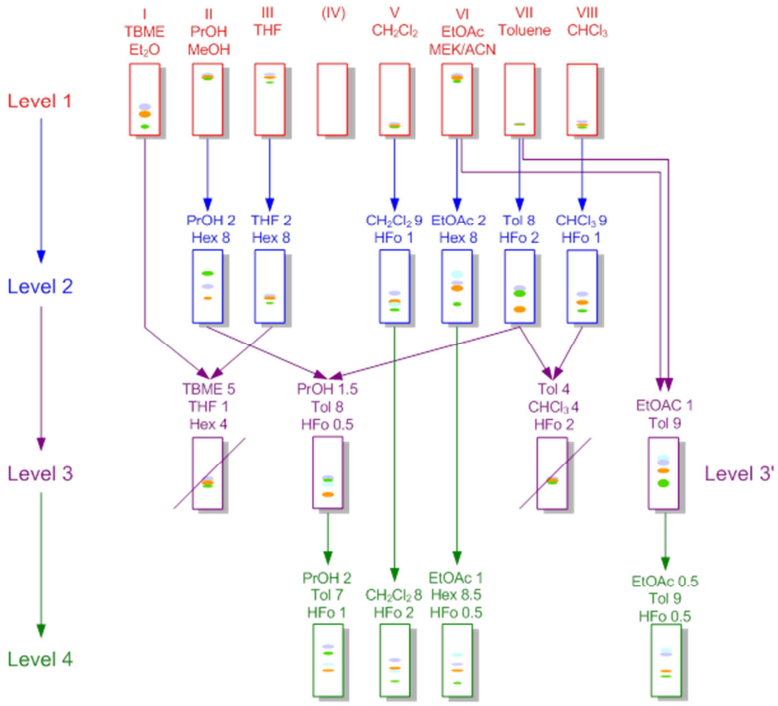

Fig. 4. General guideline for optimization of mobile phases in HPTLC (Reich and Schibli, 2006).

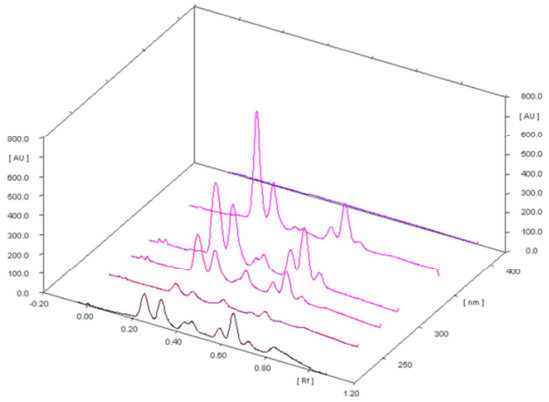

Fig. 5. HPTLC scanning of fraction A6 at different wavelengths (220, 254, 280, 310, 366, 420 nm).

The selected zones were extracted by a TLC-MS interface directly into the mass spectrometer and the molecular weights were determined (Fig. 6-12).

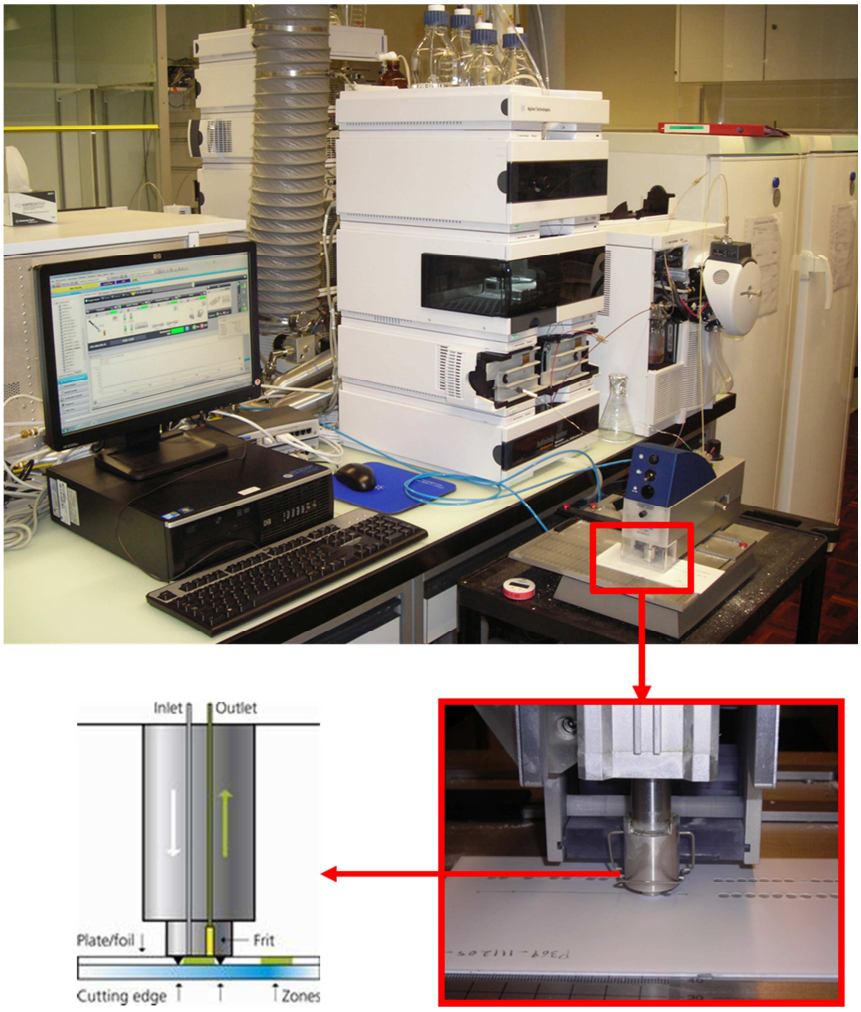

Fig. 6. HPTLC-MS. 1: interface between pump and mass spectrometer, 2: extraction head, 3: schematic function of the extraction head (www.camag.com).

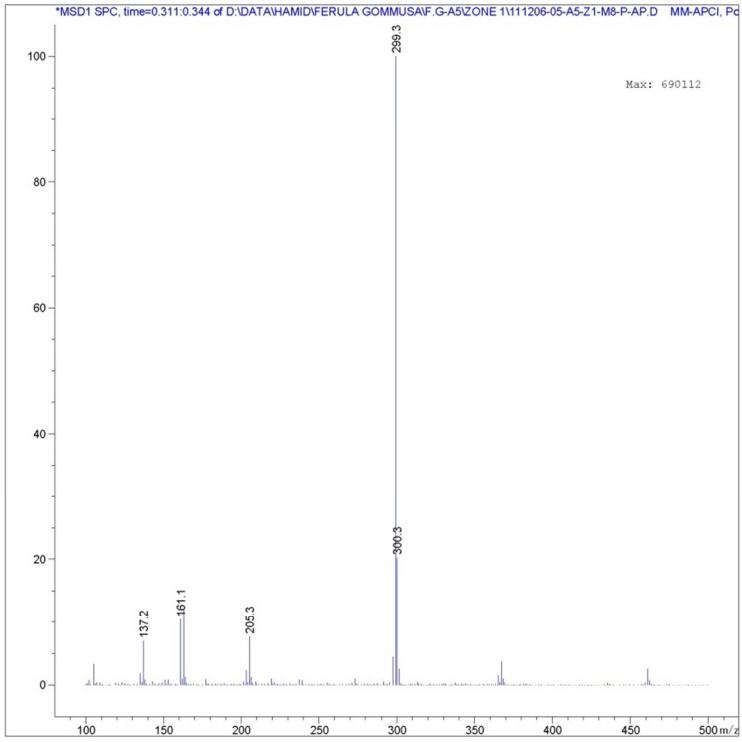

Fig. 7. Mass spectrum of zone 1.

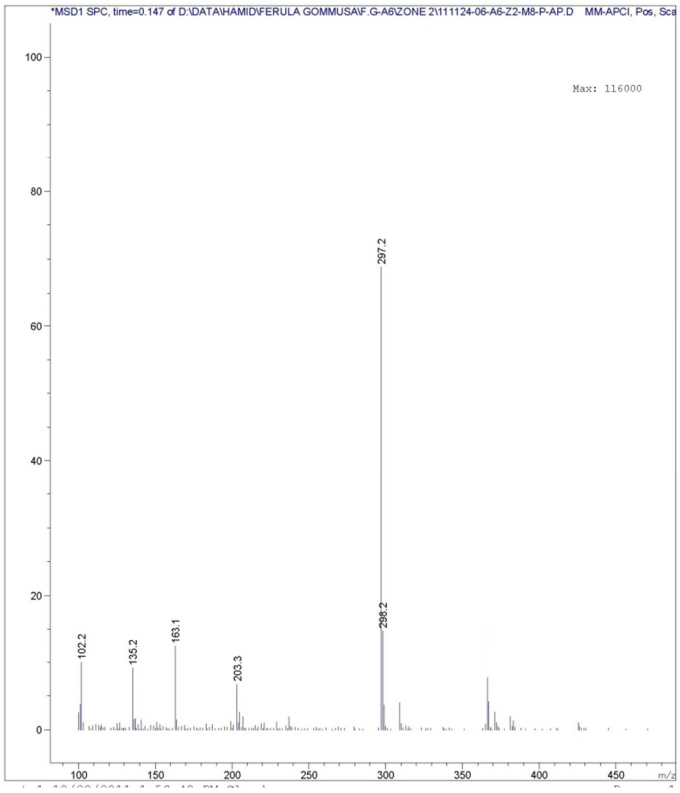

Fig. 8. Mass spectrum of zone 2.

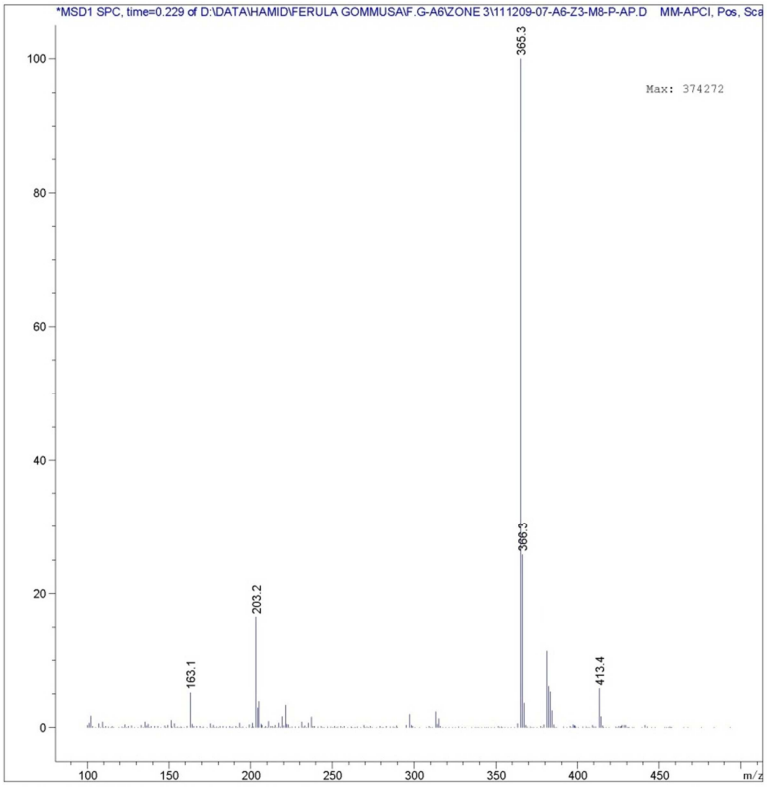

Fig. 9. Mass spectrum of zone 3.

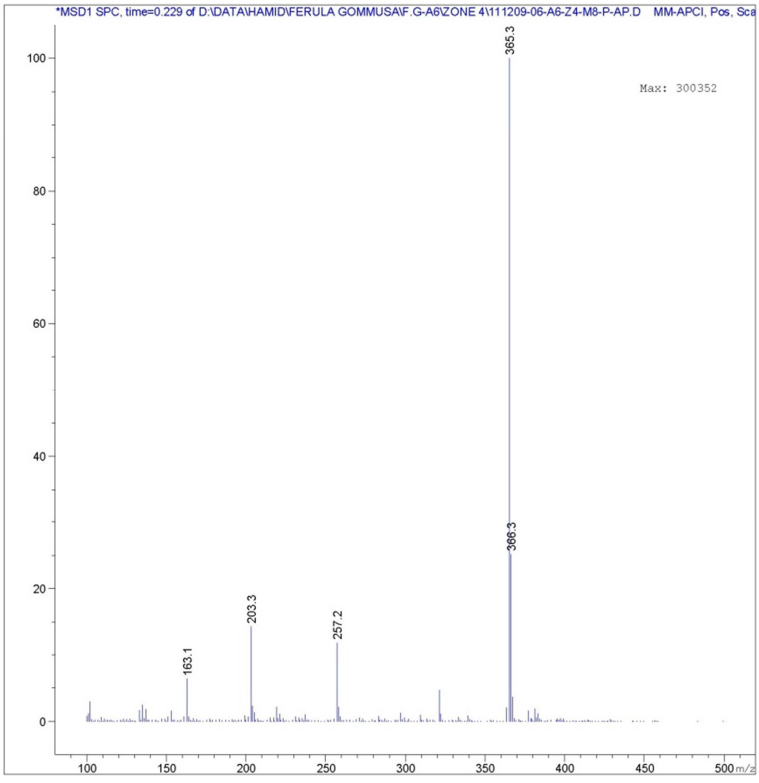

Fig. 10. Mass spectrum of zone 4.

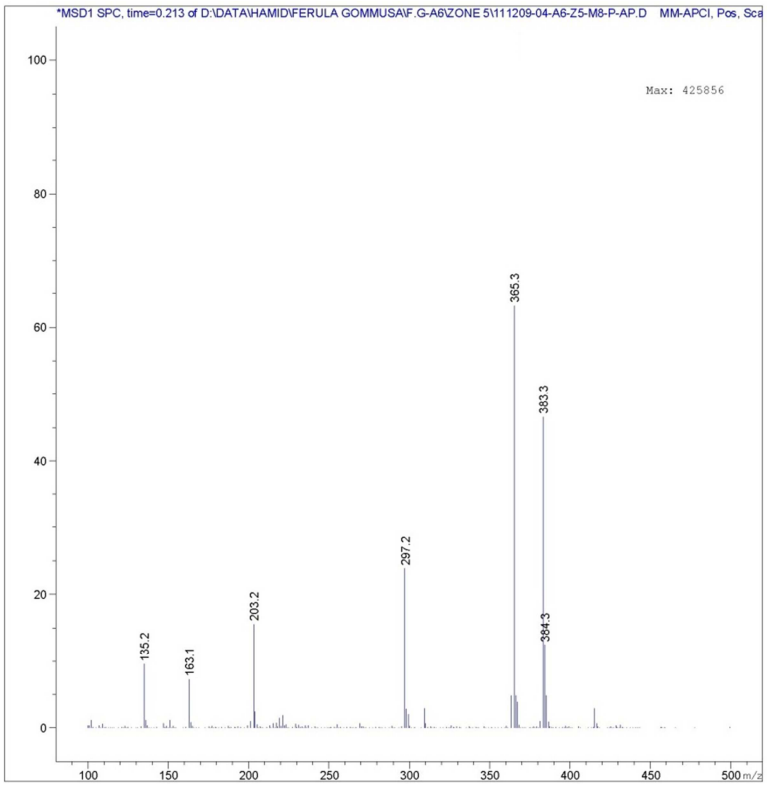

Fig. 11. Mass spectrum of zone 5.

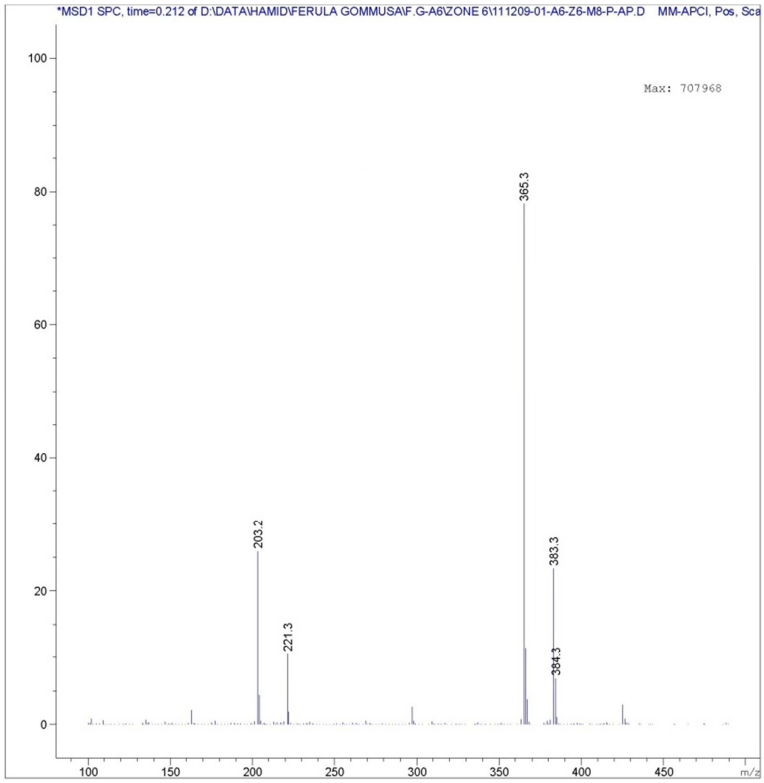

Fig. 12. Mass spectrum of zone 6.

The structures of compounds 1 and 6 were elucidated by one and two-dimensional ^{1}H and ^{13}C NMR spectroscopy and Q-TOF mass spectrometry (Fig. 13-28)

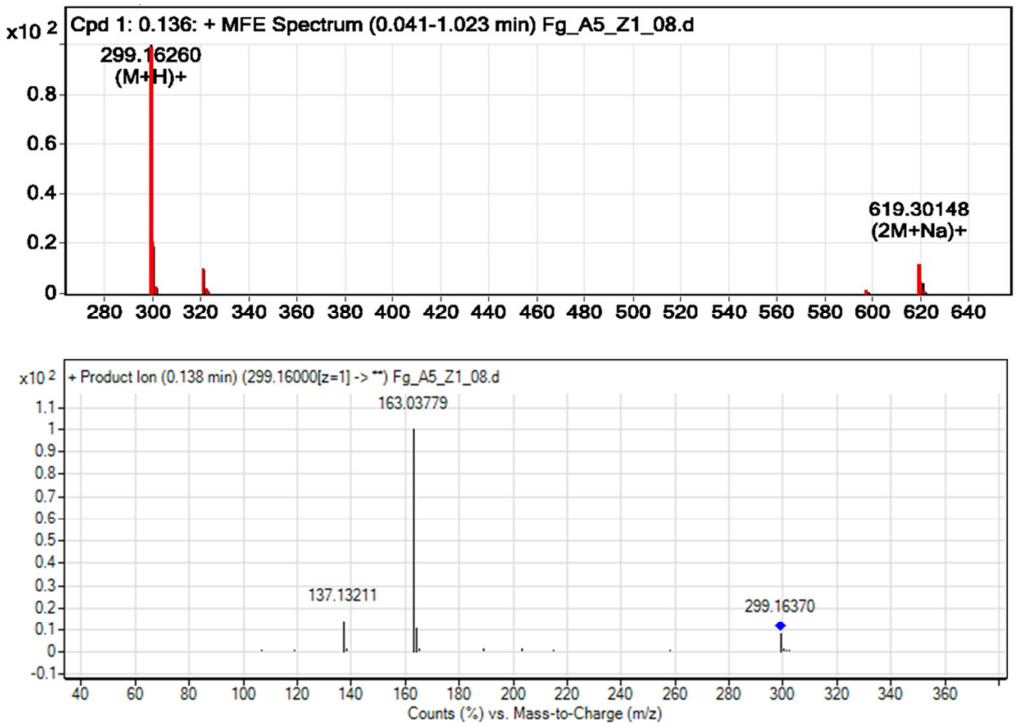

Fig. 13. Q-TOF mass spectrum of compound 1.

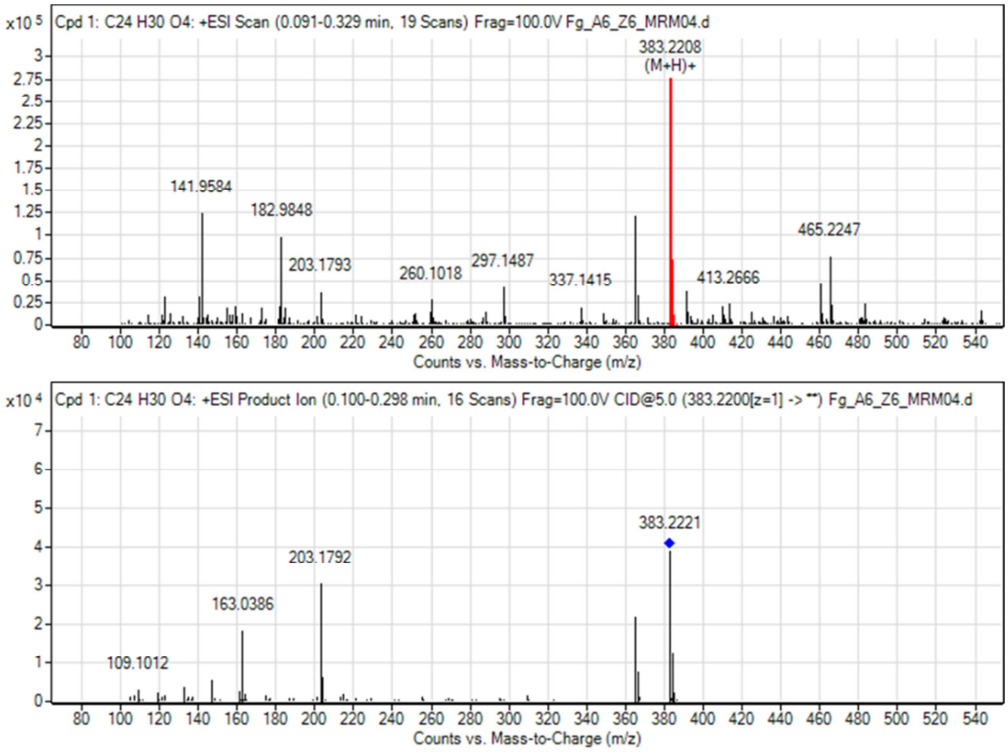

Fig. 14. Q-TOF mass spectrum of compound 6.

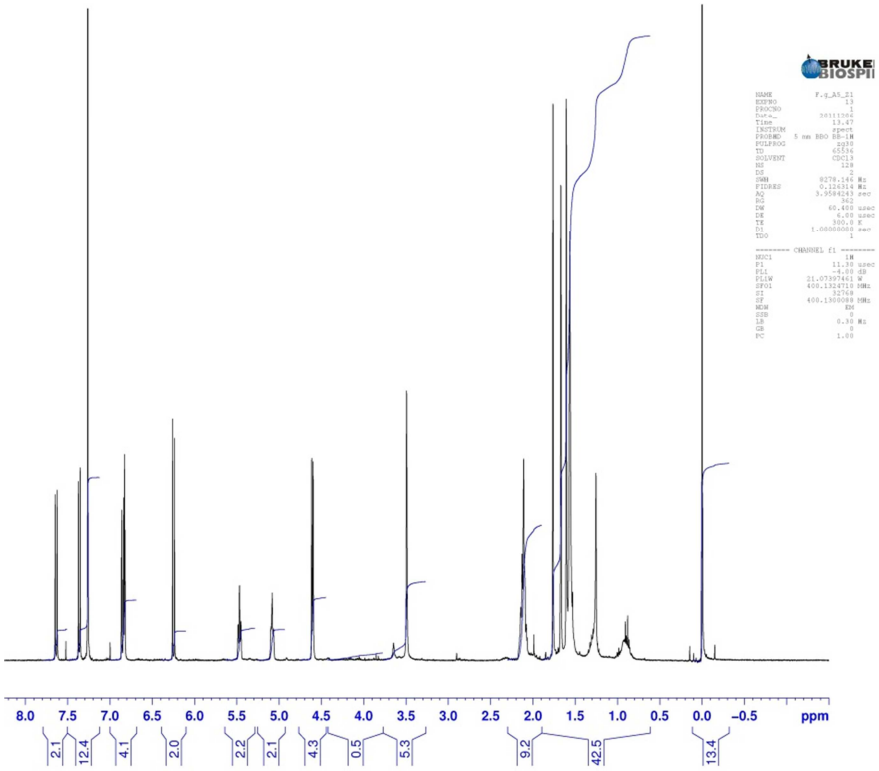

Fig. 15. ^{1}H NMR spectrum of compound 1.

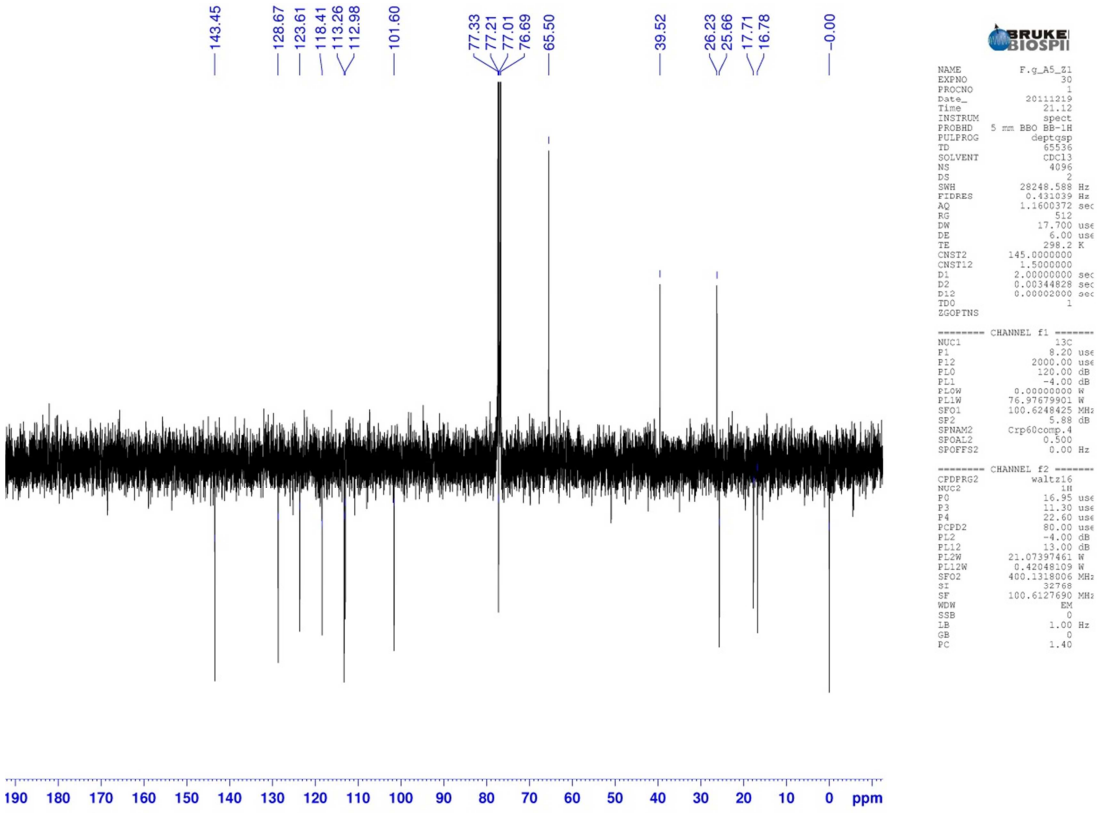

Fig. 16. ^{13}C NMR spectrum of compound 1.

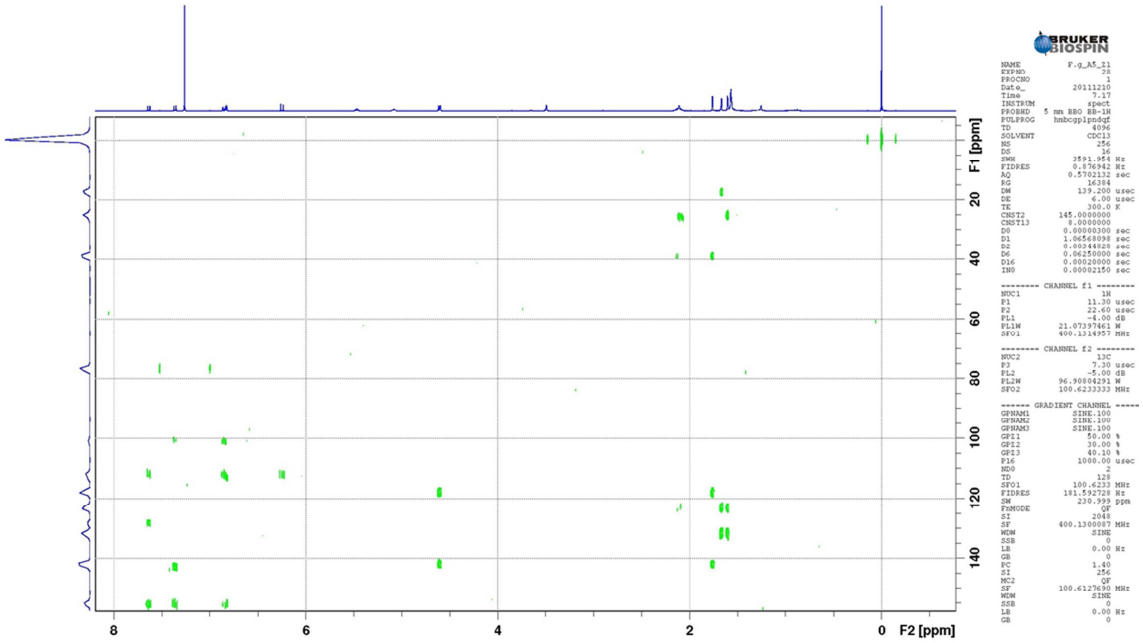

Fig. 17. HMBC spectrum of compound 1.

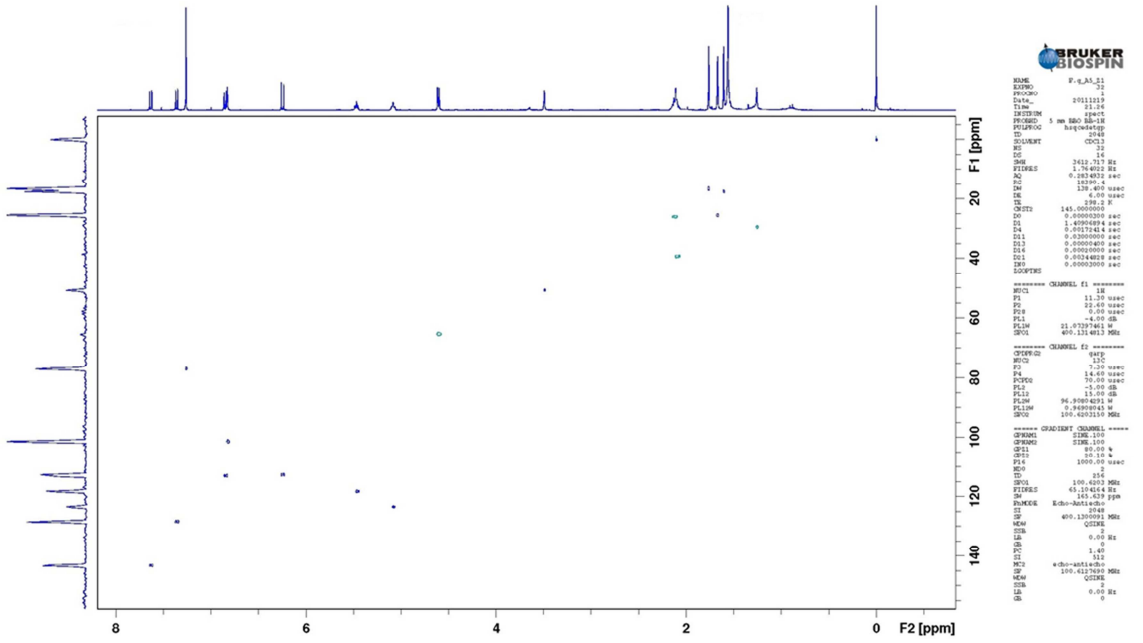

Fig. 18. HSQC spectrum of compound 1.

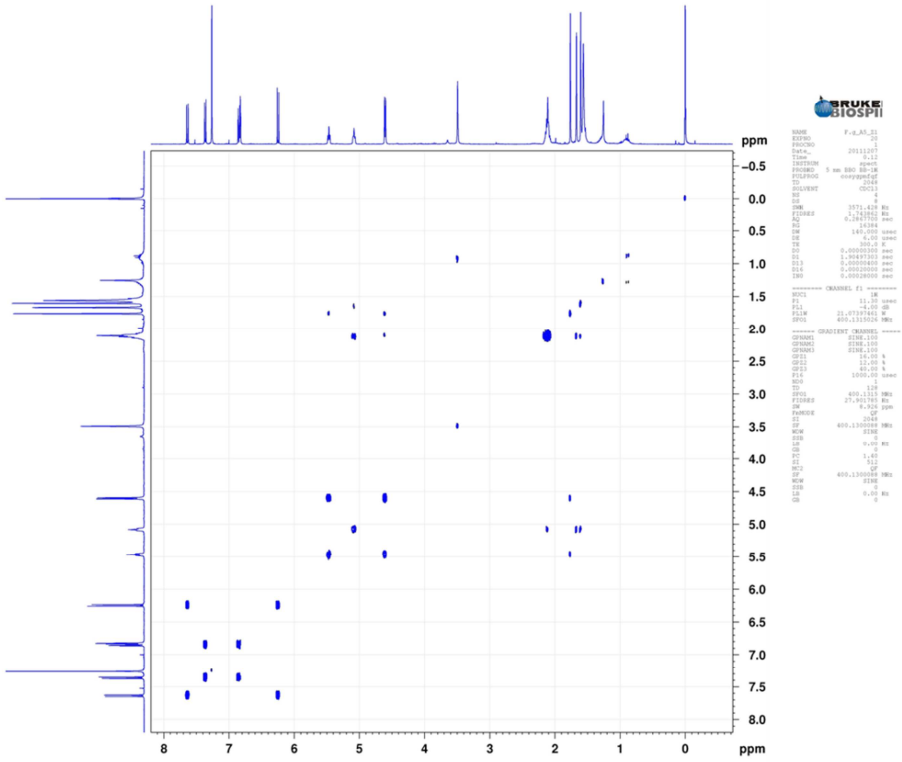

Fig. 19. COSY spectrum of compound 1.

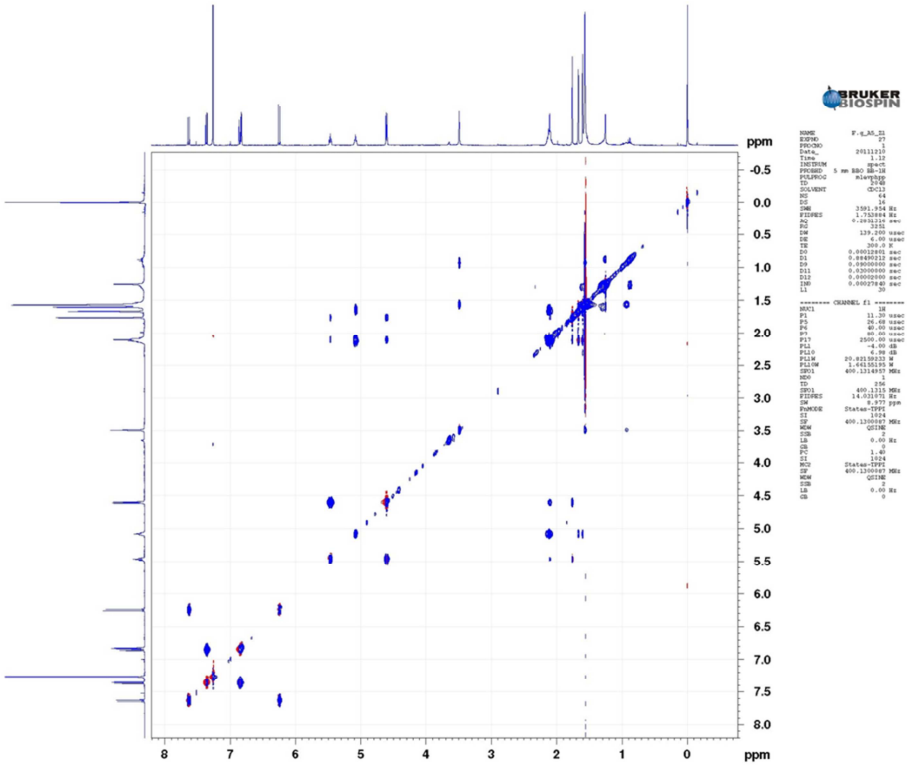

Fig. 20. TOCSY spectrum of compound 1.

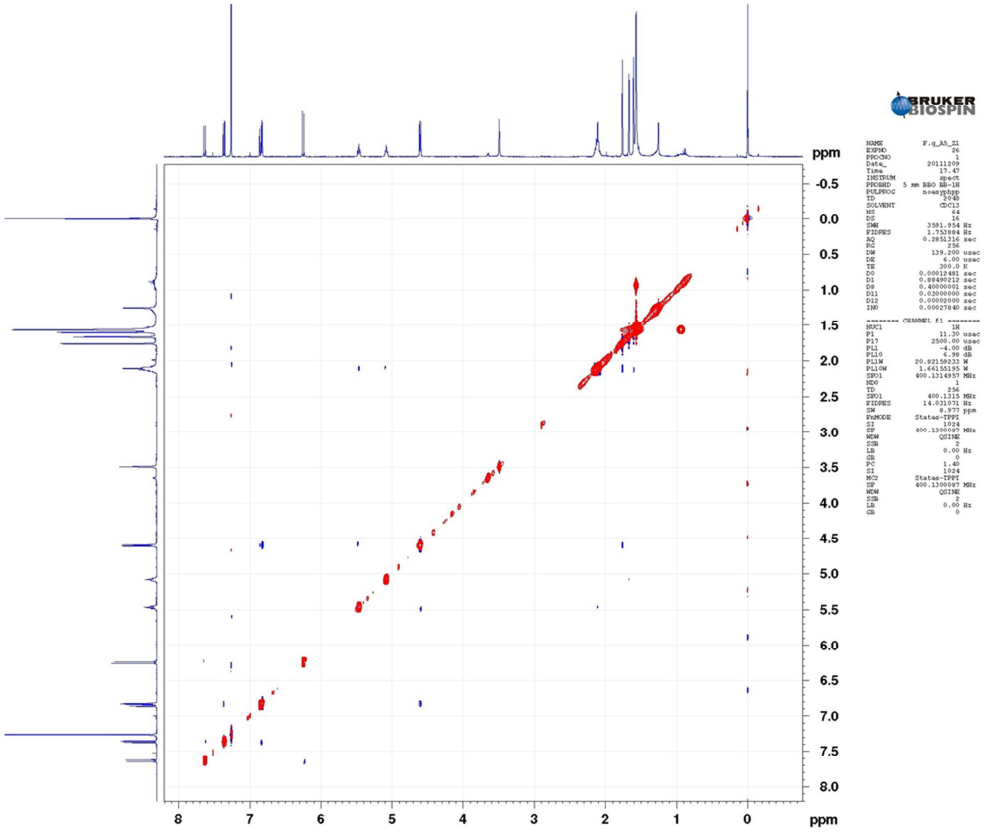

Fig. 21. NOESY spectrum of compound 1.

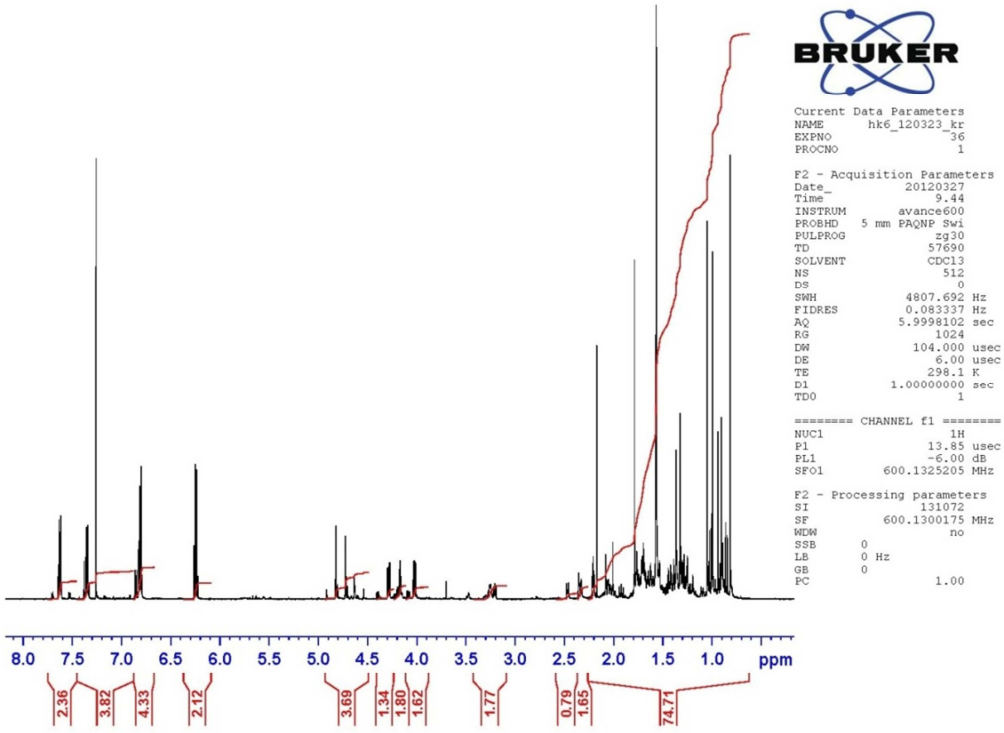

Fig. 22. ^{1}H NMR spectrum of compound 6.

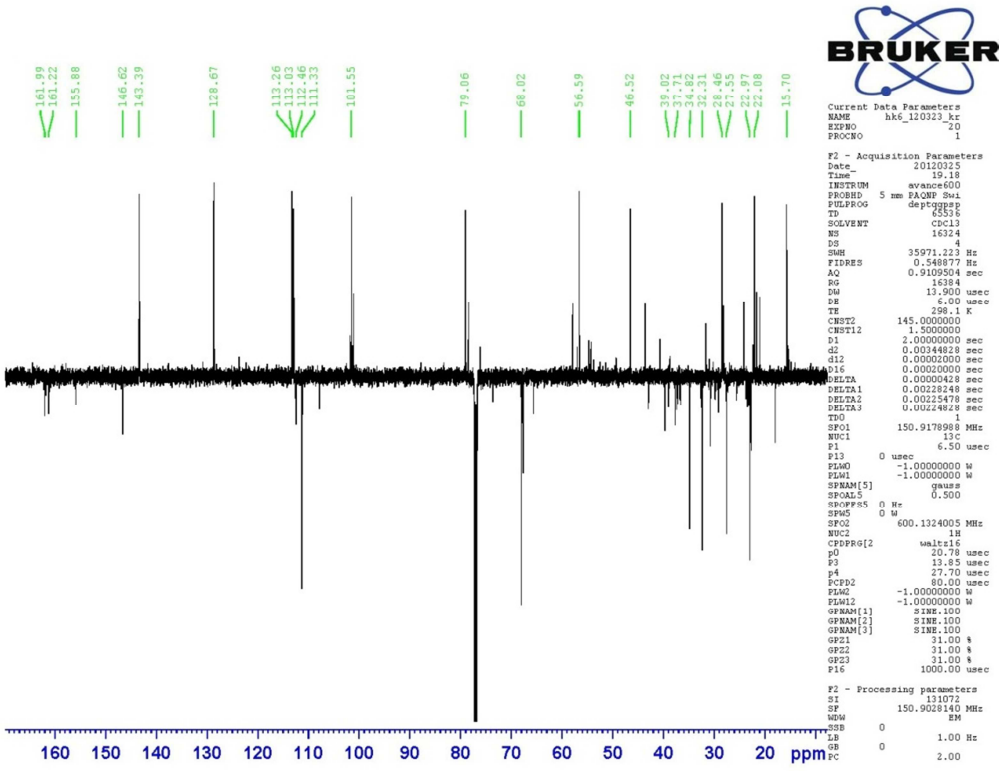

Fig. 23. ^{13}C NMR spectrum of compound 6.

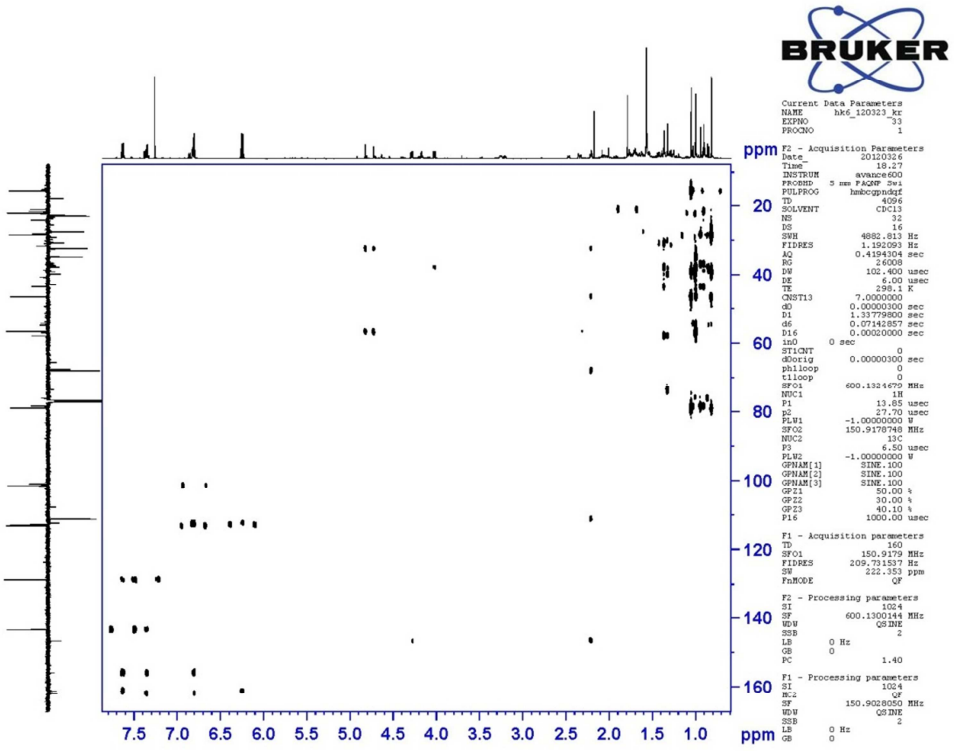

Fig. 24. HMBC spectrum of compound 6.

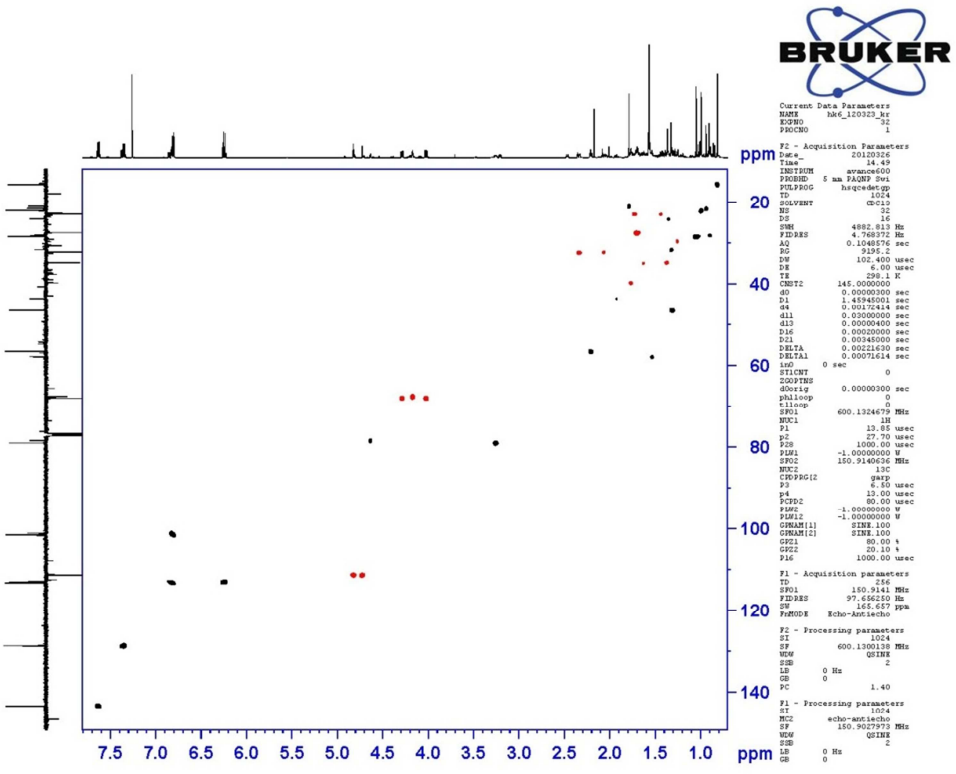

Fig. 25. HSQC spectrum of compound 6.

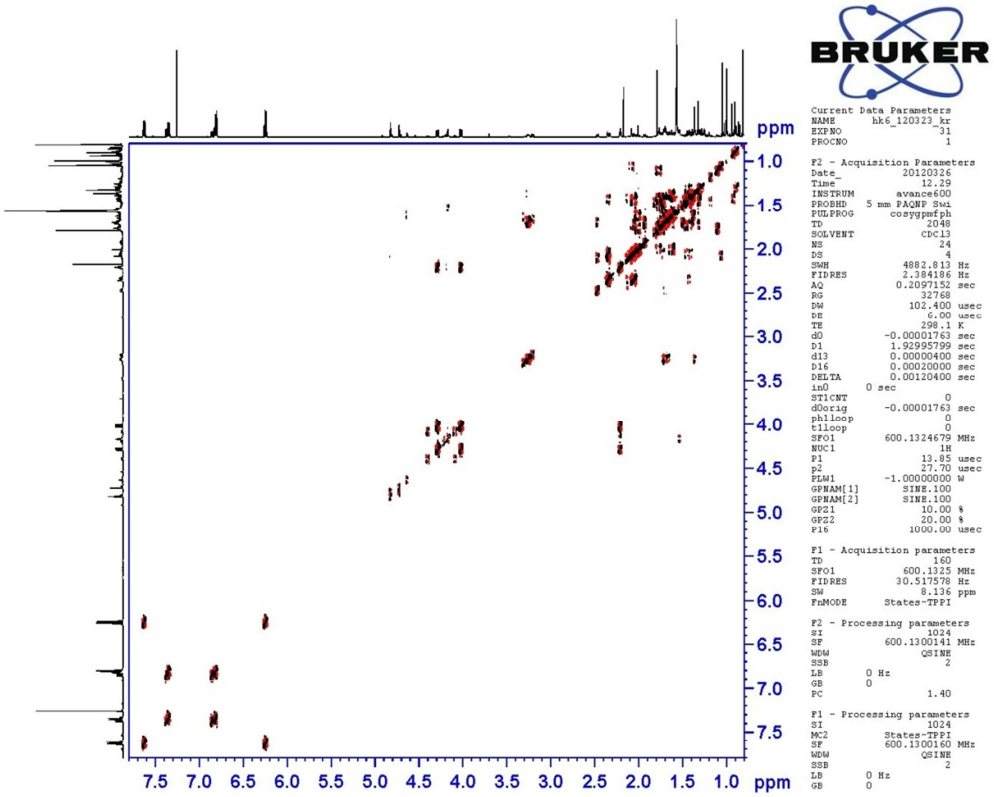

Fig. 26. COSY spectrum of compound 6.

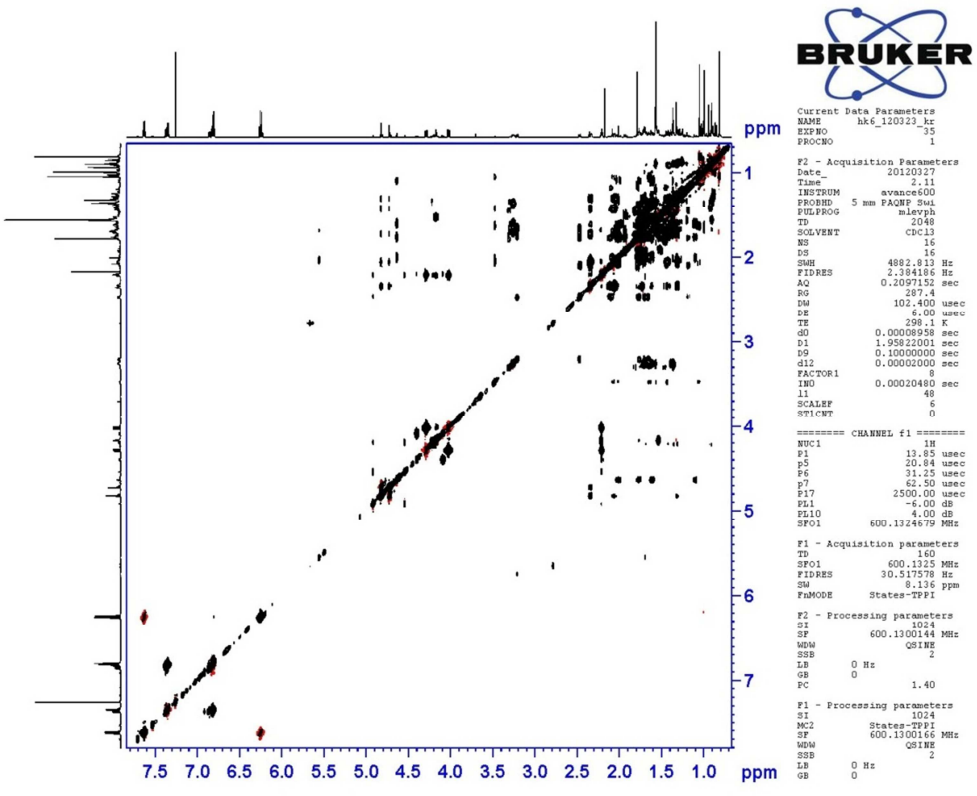

Fig. 27. TOCSY spectrum of compound 6.

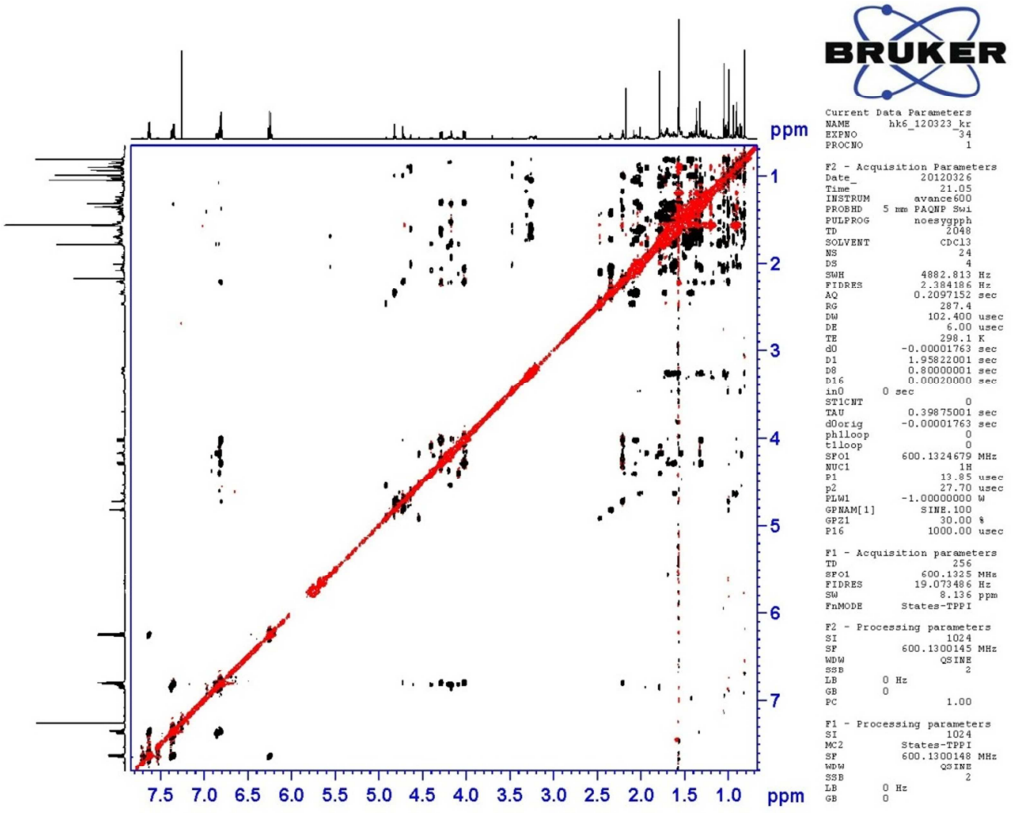

Fig. 28. NOESY spectrum of compound 6.

The IC_{50} values of DCM extract and compounds 1 and 6 for AChE inhibition were determined in the microplate colorimetric assay (Fig. 29).

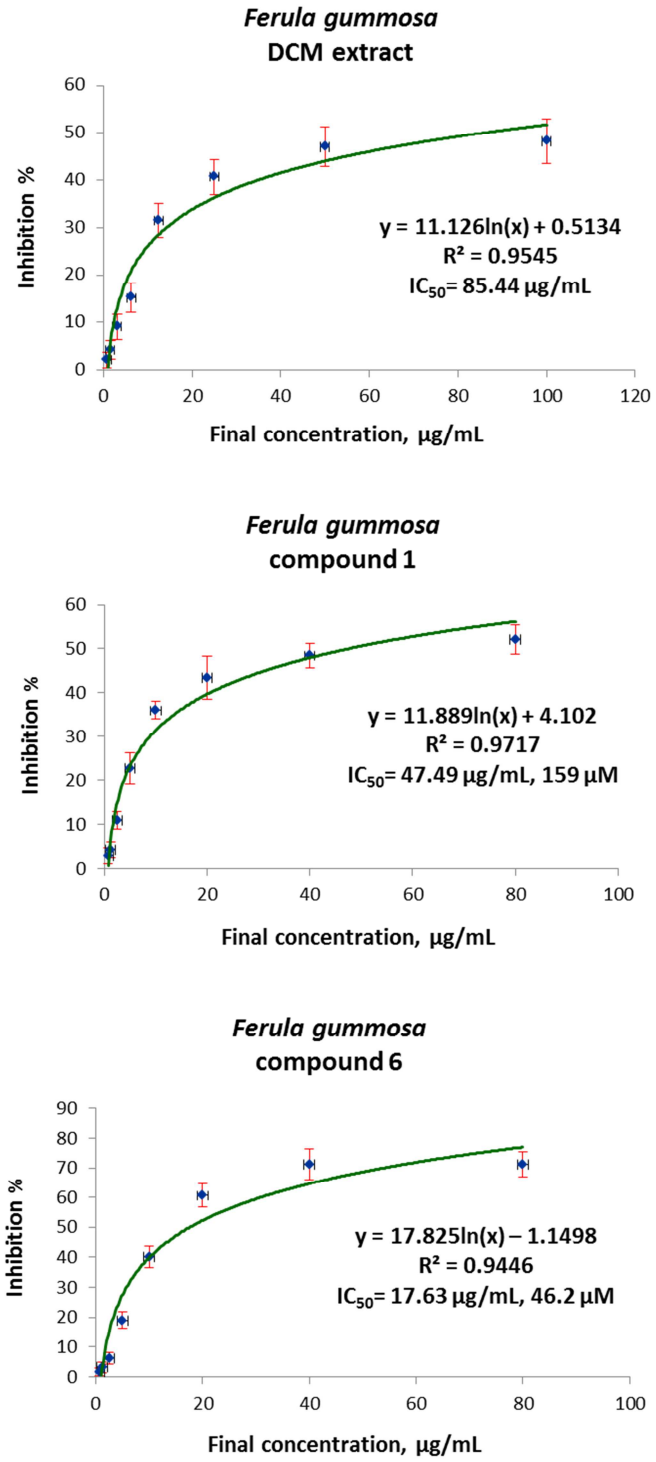

Fig. 29. AChE inhibition of DCM extract (100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 µg/ml), compounds 1 and 6 (80, 40, 20, 10, 5, 2.5, 1.25, 0.63 µg/ml).

Additionally, the amounts of these components in galbanum were determined by HPLC analyses using external standardization (Fig. 30).

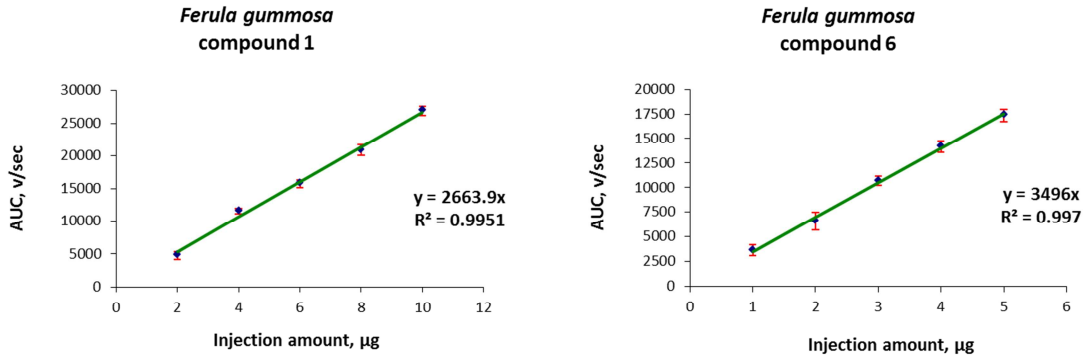

Fig. 30. Calibration curves for HPLC-quantification of compounds 1 and 6.

Summary

Cognitive deficits are one of the most common causes of mental deterioration such as learning impairment, delayed amnesia and memory problems e.g. in Alzheimer's disease. The enhancement of cholinergic function by inhibition of acetylcholinesterase (AChE) is considered as a rational approach for the treatment of neurological disorders such as Alzheimer´s disease and senile dementia. During the last two decades the use of herbal medicinal substances in dementia therapy has been studied well.

Iran is among those countries with the longest and richest history in Traditional Medicine and especially in the use of herbal medicinal preparations. In this thesis 40 herbal drugs or plant products that are reported in Iranian traditional medicine (ITM) or ethnomedicine as sources for the treatment of cognitive disorders were examined.

In the first step, the bioactivity of polar methanolic and non-polar dichloromethane extracts of all selected herbal samples from ITM was studied on AChE inhibition by TLC bioautography and in a microplate assay. The most active herbal drugs were the seeds from *Peganum harmala*, the fruit resin from *Semecarpus anacardium*, the gum-resin from *Dorema ammoniacum* and the oleo gum-resin from *Ferula gummosa*. Those were selected for further study.

In detailed investigations, the active compounds were identified and isolated using several chromatographic techniques such as thin layer chromatography, vacuum liquid chromatography, column chromatography, size exclusion chromatography, solid phase extraction, high performance liquid chromatography, counter current chromatography and automated high performance thin layer chromatography. The structures of the active components were characterized by one and two-dimensional ^{1}H and ^{13}C NMR spectroscopy (COSY, TOCSY, HSQC, HMBC, NOESY) and mass spectrometry. The IC_{50} values for active compounds were determined by a quantitative colorimetric assay. Additionally, the concentrations of active components in the four different sources were determined by HPLC analysis.

The active compounds in the seeds of *Peganum harmala* were identified as harmine and harmaline. From the correlation of IC_{50} values of the extracts in AChE

inhibition with the concentrations of harmaline and harmine in the respective extracts can be concluded that these two alkaloids are the major AChE inhibitory compounds in *Peganum harmala*.

The characterization of the active compounds in the fruit resin of *Semecarpus anacardium* resulted in 1',2'-dihydroxy-3'-pentadec-8-enylbenzene and 1',2'-dihydroxy-3'-pentadeca-8,11-dienylbenzene. In addition, an *in silico* study confirmed their AChE inhibitory effect. Further experiments showed their selective inhibitory activity for AChE versus BChE. The correlation of AChE inhibition with the percentage of the active compounds in the fruit resin, can explain their responsibility for the AChE inhibition of this resin.

In the gum-resin of *Dorema ammoniacum* the active components were identified as (2'*S*,5'*S*)-2'-ethenyl-5'-(3-hydroxy-6-methyl-4-oxohept-5-en-2-yl)-7-methoxy-2'-methyl-4*H*-spiro[chromene-3,1'-cyclopentane]-2,4-dione and (2'*S*,5'*R*)-2'-ethenyl-5'-[(2*R*,4*R*)-4-hydroxy-6-methyl-3-oxohept-5-en-2-yl]-7-methoxy-2'-methyl-4*H* spiro[chromene-3,1'-cyclopentane]-2,4-dione. The second compound is a new natural substance. From the high concentration of these two components in this gum-resin can be deduced they are of among the major compounds responsible for the AChE inhibitory activity of this drug.

The structure elucidation of the active substances in the oleo gum-resin of *Ferula gummosa* gave 7-{[(2E)-3,7-dimethylocta-2,6-dien-1-yl]oxy}-2H-chromen-2-one and 7-(((1R,4aR,6S,8aS)-6-hydroxy-5,5,8a-trimethyl-2-methylenedecahydronaphthalen-1-yl)methoxy)-2H-chromen-2-one. This is the first report of these two compounds in *F. gummosa* and from the result can be concluded that they are of importance for the activity. The study showed that HPTLC-MS/NMR can be considered as a fast method for dereplication of natural compounds, when an optimum separation on HPTLC plate is provided.

The AChE inhibition of the isolated compounds from the investigated herbal drugs and their IC_{50} values were reported for the first time in this thesis. The achieved results confirmed that the compounds considerably contribute to the effects of these drugs and underline the plausibility of their use in the treatment of cognitive deficits in ITM.

Zusammenfassung

Kognitive Störungen sind eine der Ursachen von mentalem Abbau, der von Symptomen wie Erinnerungsverlust und Gedächnisschwund z.b. im Rahmen der Alzheimer-Krankheit begleitet wird. Die Steigerung von cholinergen Funktionen durch Inhibierung von Acetylcholinesterase (AChE) stellt eine therapeutische Möglichkeit zur Behandlung von neurologischen Störungen wie Alzheimer-Krankheit und Demenz dar. In den letzten zwei Jahrzehnten rückte auch der Einsatz traditioneller Heilpflanzen zur Therapie von Demenz-Krankheiten in den Mittelpunkt des wissenschaftlichen Interesses.

Der Iran ist eine der Kulturen mit einer langen und reichen Geschichte der traditionellen Medizin, und speziell der Pflanzenheilkunde. In dieser Studie wurden 40 Heilpflanzen bzw. Pflanzenprodukte wie Harze, die in der Iranischen Traditionellen Medizin zur Behandlung von kognitiven Störungen eingesetzt wurden, untersucht.

Zuerst wurden polare methanolische und apolare Dichlormethan-Extrakteaus den selektierten Pflanzen hergestellt. Die Aktivität der Extrakte, AChE zu hemmen, wurde mittels Dünnschichtchromatographie und in einem Microplate Assay analysiert. Die stärkste Inhibierungs wurde für Extrakte aus den Samen von *Peganum harmala*und aus Harzen von *Semecarpus anacardium, Dorema ammoniacum* und *Ferula gummosa* festgestellt. Diese vier Ausgangsmaterialien wurden für die weiteren Analysen verwendet. Die aktiven Inhaltsstoffe wurden mittels chromatographischer Methoden wie Vakuumflüssigchromatographie, Säulenchromatographie, Gel-Permeations-Chromatographie, Festphasenextraktion, Gegenstromverteilungschromatographie und automatisierter HPTLC isoliert. Die Charakterisierung der isolierten bioaktiven Komponten erfolgte durch ein- und zwei dimensionale ^{1}H and ^{13}C NMR Spektroskopie (COSY, TOCSY, HSQC, HMBC, NOESY) und Massenpektrometrie. Die Hemmwirkung der aktiven Extrakte und isolierten Verbindungen auf AChE wurde durch die Bestimmung der IC_{50} Werte quantifiziert. Ausserdem wurden die Konzentrationen dieser Wirkkomponenten in den einzelnen Drogen mittels validierter HPLC-Verfahren bestimmt.

In Samen von *Peganum harmala* wurden die Alkaloide Harmine und Harmaline als die aktiven Komponente identifiziert. Die Korrelation zwischen den IC_{50} Werten der Extrakte und den Konzentrationen der aktiven Komponenten zeigte, dass die Hemmung der AChE durch *Peganum harmala* überwiegend auf diese zwei Alkaloide zurückzuführen ist.

Im Harz der Früchte von *Semecarpus anacardium* wurden 1',2'-dihydroxy-3'-pentadec-8-enylbenzene und 1',2'-dihydroxy-3'-pentadeca-8,11-dienylbenzene als Komponenten mit inhibitorischer Wirkung auf AchE identifiziert. Dieser Effekt konnte zusätzlich durch ein *in Silico* Experiment bestätigt werden. Weitere Experimente zeigten, dass diese Komponenten nur AChE inhibieren, während sie keine Hemmung der Butyrylcholinesterase (BchE) bewirkten. Zusätzlich konnte eine Korrelation zwischen der AchE Inhibierung und der Konzentration dieser Substanzen im Harz nachgewiesen werden. Diese Resultate deuten erstmalig darauf, dass diese zwei Substanzen für die AChE Inhibierung durch das Harz der Früchte von *Semecarpus anacardium* hauptverantwortlich sind.

Im Harz von *Dorema ammoniacum* wurden 2 Inhaltstoffe (2'*S*,5'*S*)-2'-ethenyl-5'-(3-hydroxy-6-methyl-4-oxohept-5-en-2-yl)-7-methoxy-2'-methyl-4*H*-spiro[chromene-3,1'-cyclopentane]-2,4-dione und (2'*S*,5'*R*)-2'-ethenyl-5'-[(2*R*,4*R*)-4-hydroxy-6-methyl-3-oxohept-5-en-2-yl]-7-methoxy-2'-methyl-4*H*-spiro[chromene-3,1'-cyclopentane]-2,4-dione mit AChE-hemmender Wirkung identifiziert. Der zweite Inhaltstoff ist ein neuer Naturstoff, der zum ersten Mal in dieser Arbeit identifiziert werden konnte. Beide Inhaltstoffe waren in relativ hoher Konzentration im Harz enthalten und die Korrelation zwischen den Konzentrationen und dem Inhibierungseffekt war wiederum ein starkes Indiz dafür, dass diese Inhaltstoffe für die AchE Inhibierung verantwortlich sind.

Aus dem Harz von *Ferula gummosa* wurden die Komponenten 7-{[(2E)-3,7-dimethylocta-2,6-dien-1-yl]oxy}-2H-chromen-2-one und 7-(((1R,4aR,6S,8aS)-6-hydroxy-5,5,8a-trimethyl-2-methylenedecahydronaphthalen-1-yl)methoxy)-2H-chromen-2-one isoliert und charakterisiert. Die relativ hohen Konzentrationen dieser Komponenten in Korrelation mit der AChE-Inhibierung lassen die Anwendung dieses Harzes in der Iranischen traditionellen Medizin plausibel erscheinen. Diese Studie zeigte ausserdem,

dass die HPTLC-MS/NMR als eine sehr effiziente Methode zur direkten und schnellen Identifizierung von Inhaltstoffen aus komplexen Pflanzenextrakten herangezogen werden kann, wobei eine optimierte Trennung in der HPTLC eine wichtige Voraussetzung ist.

Im Rahmen dieser Dissertation wurde zum ersten Mal die Aktivität der Inhaltstoffe von vier Heilpflanzen bzw. Pflanzenprodukten aus der Iranischen Traditionellen Medizin auf AChE-Hemmung nachgewiesen. Die Isolierung, Charakterisierung und Bestimmung der IC_{50} Werte dieser Inhaltstoffe wurde erfolgreich durchgeführt. Durch diese Arbeit konnte der Einsatz dieser Heilpflanzen in der Iranischen Traditionellen Medizin zur Behandlung von kognitiven Störungen wissenschaftlich belegt werden.

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Education

- PhD student: Since Oct 2008, Department of Pharmacognosy, University of Vienna.

  Thesis: Bioassay guided isolation of compounds with acetylcholinesterase inhibitory activity from selected medicinal plants used in Iranian traditional medicine.

  Supervisor: Ao Univ. Prof. Dr. Liselotte Krenn

- Post graduate in Homeopathy: 2003, American University of Hawaii, Tehran branch, Iran

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  Thesis: Synthesis of substituted 1-Arryl-2-(alkylthio) pyrrolo [2, 3-d] imidazole-5-carboxylic acid as angiotensin II receptor antagonists.

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Research Interests

- Phytochemistry: Isolation, characterization and purification of biological active secondary metabolites from natural sources.

Publications

Papers

1. **Adhami HR**, Linder T, Kaehlig H, Schuster D, Zehl M, Krenn L. Catechol alkenyls from *Semecarpus anacardium*: acetylcholinesterase inhibition and binding mode predictions. *Journal of Ethnopharmacology* 2012; 139(1): 142-148.
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Workshops and Trainings

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- Cellular and Molecular Methods in Medical Sciences Research; 2007, Tehran University of Medical Sciences, Tehran-Iran.
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- Scientific Writing; 2005, National Research Center for Medical Sciences of Iran, Tehran, Iran.
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Position Held

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4- Manager of Pharmaceutical office, Rey City Health Care Net, Tehran University of Medical Sciences, Tehran, Iran (2004)

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Work Experience

1- Lecturer in Homeopathic Pharmacy, Azad University (2004- 2010)

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Professional Memberships

1- American Society of Pharmacognosy (2009-present)

2- Society for Medicinal Plant and Natural Product Research (2009-present)

3- Young Researchers Club, Azad University, 2004- present

4- Iranian Medical Council, 2001-present

5- Iranian Pharmaceutical Society, 1997- present

6- Iranian Chemistry and Engineering Society, 1996- present

7- Iran Life Saving Federation, 1992- present

8- Iranian Young Researchers Club, 1991- present

Sport Activity

1- 3rd rank (badminton team of TUMS): The 4th Sport Olympiad of Medical Students, Rasht, Iran. 2000.

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