Pharmacognosy Journal 5 (2013) 41–45

Contents lists available at SciVerse ScienceDirect

Pharmacognosy Journal

journal homepage: www.elsevier.com/locate/phcgj

Original article

Computational analysis of bioactive phytochemicals as potential inhibitors for calcium activated potassium channel blocker, tamulotoxin from *Mesobuthus tamulus*

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ARTICLE INFO

Article history:
Received 3 January 2013
Accepted 8 February 2013
Available online 26 February 2013

Keywords:
Tamulotoxin
Virtual screening
Phytochemicals
Pharmacokinetics
Molecular docking

ABSTRACT

Several research works are being carried out on natural medicines because they are cost effective and play a vital role in providing a permanent remedy. Phytochemicals are pharmacologically important source of plant products which targets many diseases especially finding antidote for envenomation. Accidental toxin bite becomes highly lethal due to lack of proper treatment. The red Indian scorpion, *Mesobuthus tamulus* produces a highly toxic protein called tamulotoxin (TmTx). TmTx blocks the $Ca2^{+}$ activated K^{+} ion channels, which is responsible for cellular proliferation and migration. Several antidotes were used for neutralizing the action of scorpion bites because most of the synthetic drugs produce undesirable side effects. In this work, we have found some of the potential lead molecules (chemical compounds and their analogs) obtained from plant source and a series of bioinformatics based studies including computational analogs search, virtual screening, pharmacokinetic profiling and molecular interaction were executed. From this study, we suggest that some of the potential ligands having a therapeutic effect against TmTx protein. This work will help researchers to enhance their analysis toward designing a protocol for antidote based therapy against TmTx protein.

1. Introduction

Ion channels play a significant role in cellular mechanism of *Homo sapiens*. Each ion channel has a different function according to its transport across the membrane proteins.[1] Among several ion channels, calcium activated potassium channels are the fundamental regulators of neuronal excitability.[2,3] Venomous neurotoxins from various toxin species produce a unique toxin for targeting a particular species of biological target, especially ion channels. Malfunctioning of ion channel proteins is inhibited by various toxins like neurotoxin.[4] Neurotoxins mainly target K^{+} channels and collapse the signal transmission between neurons, which cause a series of neurological problems.[5]

According to recent studies among several toxin bites, scorpion bites are reported in maximum numbers in India.[6] Several scorpion species produce various toxins with diverse polypeptides, which modulate the vital functions of ion channels.[5] Scorpion toxins have low molecular mass. Hence they are categorized in several ways based on the targeted action like cardiotoxin, nephrotoxin, hemolytic toxin, etc. Some neurotoxin of scorpion venom is deadlier than neurotoxin of snake venom.[7] The LD50 value of some scorpion neurotoxins has been analyzed to be more potent (>10 fold) than cyanide.[8] Tamulus toxin (or) tamulotoxin (TmTx) is a novel 36 residues protein which is found in Indian scorpion of eastern region (*Mesobuthus tamulus*). It has a potential activity on potassium channel.[9,10] Tamulus toxin is a novel protein and shares no homology with other species of scorpion, although the positions of the six cysteine residues share the same structural folds.[11] In India, several cases of scorpion bites are being reported every year and most of the cases are in highly pathetic condition and we have only few a few antidotes for scorpion sting. But most of them show less precise in their targeted action and also common to its general action.[12–17]

Plants are good source of medication from many centuries and till date they have a maximum therapeutic index. More number of natural products like plant derivatives and analogs represent more than 50% of drugs for clinical purposes.[18,19] In this work, we suggested some of the plant based bioactive antitoxic compounds as a lead molecule for scorpion bites through computational bioinformatics analysis. In our work, we have taken the structural analogs from plant species of *Ocimum sanctum*, *Ocimum basillicum*, *Andrographis paniculata*, *Achyranthes aspera*, *Atropa belladona*, *Argemone*

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0975-3575/$ – see front matter
http://dx.doi.org/10.1016/j.phcgj.2013.02.001

ochroleuca and *Martynia annua* through several chemical databases and various drug-likeliness properties are screened. Then all filtered compounds were allowed for pharmacokinetic profiling and molecular docking studies using several bioinformatics tools. Further compounds were retrieved on the basis of their molecular interaction with active site amino acids of tamulotoxin protein.[20,21]

2. Materials and methods

2.1. Bioactive compound selection

Bioactive plants and their phytochemicals having antitoxin properties were studied from several literature. Chemical structures of the extracted compounds and their analogs were searched in chemical databases like PubChem (http://pubchem.ncbi.nlm.nih.gov/), Drug Bank (http://www.drugbank.ca/), KEGG Ligand (http://www.genome.jp/kegg/ligand.html) and Zinc (http://zinc.docking.org/).Some structures were drawn manually in ACD ChemSketch 10.0. Suitable ligands were searched and filtered on the basis of their drug-likeliness.

2.2. Pharmacological based ligand screening

Structural analogs were searched for the selected structures of plant compounds and their derivatives. All selected compounds are further allowed for screening to analyze the level of their drug-likeliness. In this method, we have used Lipinski's rule and tanimoto score as a measure of drug-likeliness. As a result of pharmacological based ligand screening, we have obtained analogs with better drug like properties. Selected structural analogs of compounds were downloaded for further studies.

2.3. Pharmacokinetic profiling

The selected analogs were allowed for further *in silico* based validation studies by analyzing their pharmacokinetic activities. In this study we used, ADMET (Absorption, distribution, metabolism, elimination and toxicity) descriptors of Accelrys Discovery Studio (ADS) 2.0 as an analytical tool. All selected compounds were analyzed based on the default threshold values of each descriptor like blood brain barrier (BBB), solubility, hepatotoxicity, Cytochrome P450 oxidase enzyme (CYP2D6) activity and plasma protein binding (PPB).[22]

Table 1
Bioactive plants and their derivatives and number of retrieved analog compounds.

Bioactive phytochemicals	Name of plant derivatives	No. of analogs retrieved
Achyranthus aspera	Betaine	68
Martynia annua	Chlorogenic acid	507
	Terpenoid	73
	Anthraquinones	254
	Tannins	586
Argemone ochroleuca	Glycosides	80
	Flavanol	3128
	Berberine	1035
	Linoleic acid	1661
	Oleic acid	1653
	Palmitic acid	787
	Chelerytherine	457
	Myristic acid	646
	Optisine	680
Atropa belladonna	Atropine	485
Ocimum sanctum	Eugenol	1217
Ocimum basillicum	Linalool	113
	Eugenol	1217
	1,8-cineole	100
	Bergamotene	93
Andrographis paniculata	Andrographolide	148
	Deoxygrapholide	100
	Neographolide	

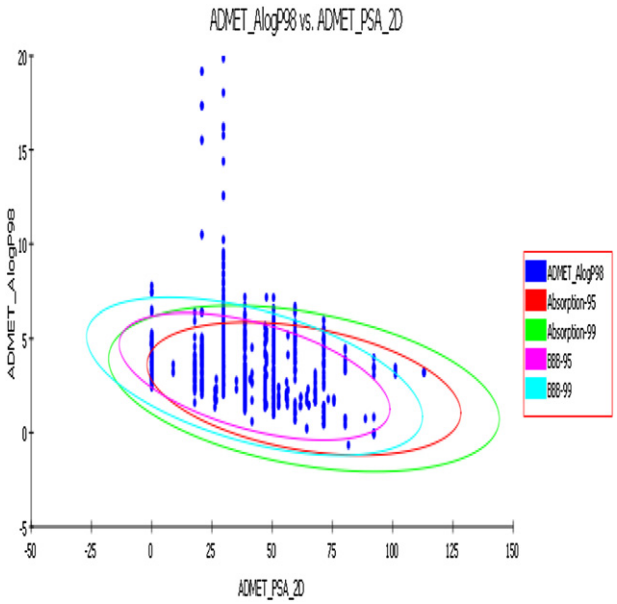

Fig. 1. Analogs passed in all levels of ADMET descriptor analysis.

2.4. Target protein selection and active site prediction

The three dimensional structure of target protein (TmTx) was unavailable in macromolecular structural databases. Hence, we

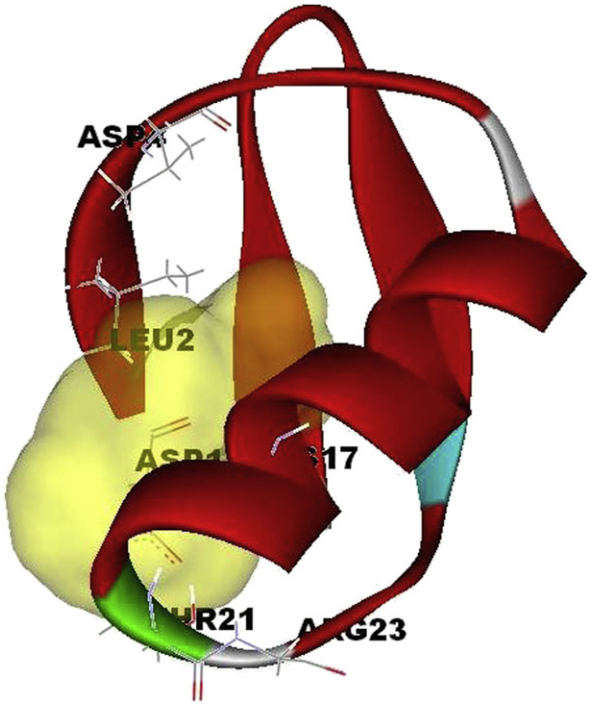

Fig. 2. Predicted active site pocket in surface view along with entire structure of tamulotoxin (TmTx) in ribbon view.

have predicted the three dimensional structure using homology modeling method in our previous work by selecting the suitable template (PDB ID: 1M2S) with a sequence identity of 77.1% between template and target. The final structure was validated in Ramachandran plot with 87.5% of residues in favorable region, 9.4% of residues in additionally allowed regions, 3.1% of residues in generously allowed region and none of the residues were found in disallowed region.[23] Energy minimization and stabilization process were done for overcoming the steric hindrance of the macromolecule by applying CHARMM force field.[24] Active site of target protein, TmTx was predicted using Q-site finder and verified with ADS 2.0.[25]

2.5. Molecular interaction studies of drug like compounds

Molecular interaction studies were done for understanding the strength of interactions like intermolecular bonding and electrostatic forces using AutoDock 4.0[26,27] and confirmation of results were analyzed using ADS-Ligand-Fit 2.0.[28] Except a few set of analogs, most of them produced better interactions with maximum dock score and H-bonding with the active site amino acids of TmTx protein.

3. Results and discussion

3.1. Bioactive plants and their analogs selection and validation

From literature search, we have identified that seven plants (*O. sanctum*, *O. basilicum*, *A. paniculata*, *A. aspera*, *A. belladona*, *A. ochroleuca* and *M. annua*) are having a potential therapeutic value along with devenomic properties against several toxin bites like scorpion and snakes bite.[21,29] Based on this information, we have identified suitable analogs of compounds through similarity based structure search and substructure search in chemical databases. Analogs were used for further virtual screening and the selection was based on the threshold value of tanimoto score. In order to achieve this, we have chosen analog compounds by setting tanimoto score into 0.2. Hence compounds of all selected plants (23 bioactive compounds) and their retrieved analogs (16,755 analogs) obtained from chemical databases are tabulated in Table 1.

Table 2
ADMET descriptors analysis and molecular interactions of selected best analog compounds.

Analogs compound ID	ADMET analysis results						Molecular docking and H-Bonding interactions		
	BBB	ABS	SOL	HEPTOX	CYP2D6	PPB	AutoDock binding energy (Kcal/mol)	DS-LigandFit dock score	H-bonding amino acids and its Distance(Å)
A. paniculata									
44393882	3	0	3	0	0	0	−5.93	39.10	L2, C17 1.99, 3.15
25121240	3	0	3	0	0	0	−4.18	38.61	L2 2.2
10293055	2	0	3	0	0	0	−4.00	37.39	L2, D4 1.43, 2.89
O. basillicum									
10820923	3	0	5	0	0	0	−4.82	61.52	C17, (2)T21 2.89, 2.95, 2.07
18409046	3	0	5	0	0	0	−0.34	54.38	L2, T21 1.92, 2.72
17834762	3	0	5	0	0	0	1.54	54.37	D1, D4 3.13, 2.31
M. annua									
5281400	0	3	1	0	2	1	−4.71	49.06	L2, D4 2.23, 2.31
115721	1	0	3	0	0	0	−1.58	43.78	(2)L2 2.39, 2.23
102235	1	0	3	0	0	0	−3.78	41.50	D1 3.07
A. aspera									
174174	3	1	3	0	0	0	3.21	34.81	T21 2.47
15238096	3	0	3	0	0	1	1.54	25.58	D4, C17 2.75, 2.48
81911	2	0	4	0	0	1	5.74	22.10	C17 2.79
A. ochroleuca									
445639	0	1	3	0	0	2	−2.74	40.03	C17 2.82
240630	0	1	2	0	0	2	−2.54	37.71	D4 2.25
31211	4	2	2	0	0	2	−1.82	37.48	D4 2.32
O. santum									
5280450	3	1	2	0	0	0	2.65	35.66	C17 3.14
53796082	3	0	5	0	0	0	3.52	32.40	D4 2.48
542421	3	0	5	0	0	0	2.35	31.04	T21 2.85

BBB—Blood brain barrier level; ABS—Absorption level; SOL—Solubility level; HEPTOX—Hepatotoxicity level; CYP2D6—Cytochrome oxidase enzyme activity level; PPB—Plasma protein binding level.

3.2. Pharmacokinetics studies of plant analog compounds

All screened compounds were allowed for the next level pharmacokinetic study to prevent the failures involved in inhibitor analysis. Compounds were removed based on violations in ADMET descriptors. In this study, all descriptors were set as default and resultant compounds were chosen for further study. As a result of this screening, 9062 analogs were eliminated due to its failure in the confidence levels (low solubility, high penetration levels in blood brain barrier level, low binding nature with plasma protein and high toxicity nature of compounds) of ADMET descriptor analysis. Hence, only 7701 analogs were considered by cross validating with all confidence levels of ADMET screening. From the result of screening, we have found that many compounds were poor either in PPB or BBB. As a result of this study, we found only 996 analogs were passed in all levels of ADMET screening and the detailed result of ADMET representation is given in Fig. 1. Analogs with strong pharmacokinetic strength were taken for next level. Finally, the selected compounds were allowed for *in silico* molecular docking analysis.

3.3. Binding site and molecular docking analysis

Active site was identified using Q-Site finder (http://www.modelling.leeds.ac.uk/qsitefinder/) and confirmed using ADS-binding site prediction program. From the active site search, we found ASP 1, LEU 2, ASP 4, CYS 17, THR 21 and ARG 23 were present in an active site and responsible for inhibitory mechanism on ion channel (Ca^{2+} activated K^{+} channel) protein. The detailed active site residues along with the secondary structure of TmTx protein were given in Fig. 2. Identified active site residues were used for molecular docking studies with selected ligand set. Molecular docking was performed in Ligand-Fit program of Discovery Studio 2.0. Using this program, TmTx were docked against a list of selected plant compounds and its analogs. Each compound generates different kinds of interactions with maximum docking score. The docking results were analyzed with five statistical functions (Ligand score 1 & 2, PLP (Piecewise Linear Potential) 1 & 2, PMF (potentials of mean force) and Jain score), dock scores along with H-bonds formed between ligands and active site amino acids were also considered. When we analyzed the docked complexes of natural bioactive compounds for each plant species, we have received some of the amazing results. Most of the bioactive compounds had a maximum binding affinity with TmTx toxin protein.

Among the selected plant species, *A. paniculata* was used as an ancient medicine based on its pharmacological properties against toxin bites.[30–32] *A. paniculata* and their analog compounds showed significant results in *in silico* studies of molecular interaction (both bonded and non-bonded interaction) with target protein. At the same time bioactive compounds and analogs of *O. basillicum* showed good results in pharmacokinetic profiling and non-bonding interactions with vital residues involved in blocking the action of ion channels. Several compounds were selected through a series of studies based on computational biology and we have obtained three top scoring compounds from each plant species through *in silico* analysis and the detailed results of docking analysis along with the details of hydrogen bonding are tabulated in Table 2. Validation studies for selecting best compounds were carried out by analyzing the binding energy of the docked complex. The ligands with best dock score were given in Table 2 and their lead molecules are given in Fig. 3. From the overall interactions studies, most of the selected ligand binds with amino acids (Asp1, Leu2, Asp4, Cys17 and Thr21) which are important for the crucial activity of tamulotoxin. Most of the active site residues of tamulotoxin (Asp1, Leu2, Asp4 and Cys17) are responsible for blocking ionic pores, which are vital for ionic transport located between S5–S6 segments of ion channel. Moreover Thr21 and Arg23 were showed as important with TmTx protein because they give more stability to the toxin structure. Among plant analogs, five compounds showed better activities and the best active compounds are CID 44393882 ([(3S,4E)-4-[2-[(1S,2S,4aS,5R,6R,8aR)-6-hydroxy-5-(hydroxymethyl)-5,8a-dimethylspiro[3,4,4a,6,7,8-hexahydro-1H-naphthalene-2,2′-oxirane]-1-yl] ethylidene]-5-oxooxolan-3-yl] acetate) analog of *A. paniculata*,

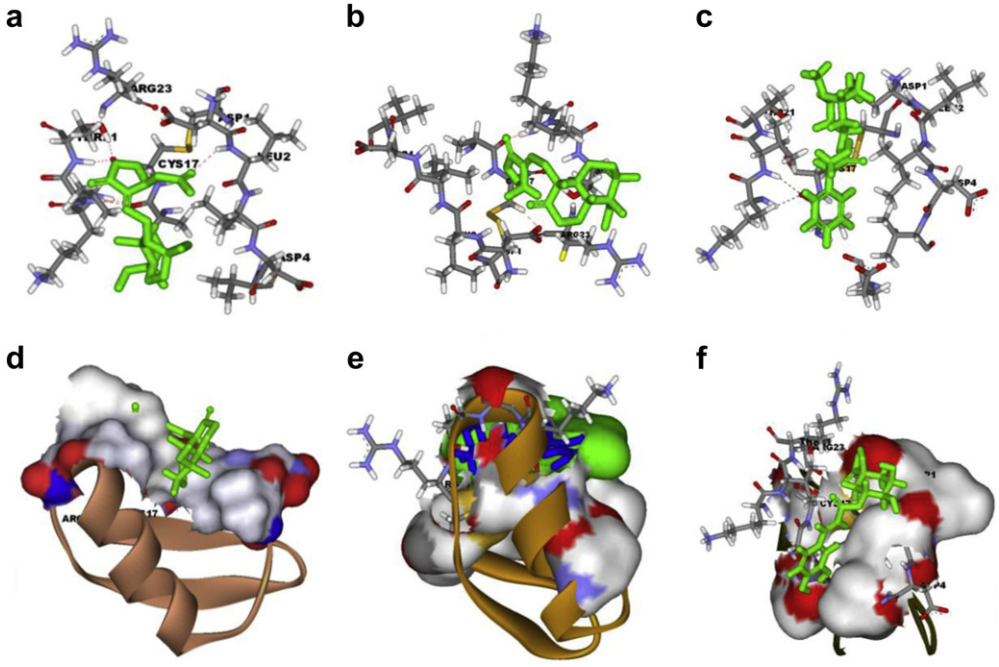

Fig. 3. Atom level interactions of selected best analogs of (a) *A. paniculata*; (b) *O. basillicum;* (c) *A. aspera* and the corresponding analogs bound at the binding site of TmTx protein represented in surface view (d) CID 44393882 (*A. paniculata*); (e) CID10820923 (*O. basillicum*); (f) CID174174 (*A. aspera*).

CID10820923 ((1R,5R)-6,6-dimethyl-4-propylbicyclo[3.1.1]hept-3-ene) from *O. basillicum*, CID174174 ([(1R,5S)-8-methyl-8-azabicyclo[3.2.1]octan-3-yl] 3-hydroxy-2-phenylpropanoate) from *A. aspera*.

4. Conclusion

Phytochemicals of plant source with pharmacological importance is an alternative approach for the modern medication against several toxic envenomations. According to recent studies, usage of phytochemicals are reported as the most reliable and efficient compounds with therapeutic significance. *In silico* studies provide an insight for developing novel inhibitors against tamulotoxin using plant based bioactive chemicals with their analogs. The inhibitory efficiency of selected compounds were computed and analyzed through number of hydrogen bonds formed between the target protein and the ligand molecule, electrostatic interactions and dock score. In this study we found the selected analogs of *A. paniculata* and *O. basillicum* were efficient against TmTx protein. Computational virtual screening, pharmacokinetic and molecular interaction studies will help researchers to design novel inhibitors for neutralizing the action of tamulotoxin.

Conflicts of interest

All authors have none to declare.

Acknowledgment

The authors thank the management of Sathyabama University for the computational facilities in the Department of Bioinformatics at the Cluster Computing Laboratory. Authors will be thankful to the anonymous reviewers for their valuable comments and suggestions.

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Pharmacognosy Journal 5 (2013) 56–59

Contents lists available at SciVerse ScienceDirect

Pharmacognosy Journal

journal homepage: www.elsevier.com/locate/phcgj

Original article

Acetylcholinesterase inhibitory activity of didehydrostemofoline, stemofoline alkaloids and extracts from *Stemona collinsiae* Craib roots

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ARTICLE INFO

Article history:
Received 5 January 2013
Accepted 17 February 2013
Available online 24 February 2013

Keywords:
Acetylcholinesterase inhibition
Didehydrostemofoline
Stemofoline
Stemona collinsiae
Stemonaceae

ABSTRACT

Introduction: Acetylcholinesterase (AChE) inhibition is one of insect toxicity mechanisms found in many plants. *Stemona collinsiae* Craib, a traditional insecticidal plant, has been of interest for its AChE inhibitory activity.
Methods: Powdered roots of *S. collinsiae* were successively extracted with hexane, dichloromethane, and methanol. The contents of major active insecticidal components, didehydrostemofoline and stemofoline alkaloids, were analyzed by HPLC. Anti-AChE activity was evaluated using Ellman's colorimetric method and TLC-bioautography.
Results: The contents of didehydrostemofoline and stemofoline alkaloids in the hexane, dichloromethane, and methanol extracts were 3.59 and 0.18, 40.78 and 0.74, and 1.43 and 0.09% w/w, respectively. TLC fingerprints of each extract showed major spots of didehydrostemofoline and stemofoline of which TLC bioassays indicated active AChE inhibitory activity. IC_{50} values on anti-AChE activities of hexane, dichloromethane and methanol extracts were 126.72, 73.78, and >1000 μg/ml, respectively while those of didehydrostemofoline, stemofoline, and standard galanthamine were 131.3, 102.1, and 1.30 μM, respectively.
Conclusion: The AChE inhibitory activity of hexane, dichloromethane and methanol extracts from *S. collinsiae* roots and their major alkaloids, didehydrostemofoline and stemofoline, were determined. The data support the traditional utilization of this plant as a natural insecticide.

1. Introduction

Acetylcholinesterase is one of the most important enzymes in many living organisms. Inhibition of this enzyme can promote sudden death, thus this mechanism can be used for killing insects and mosquitoes.[1,2] Currently, many AChE inhibitors have been isolated from plants and a lot of them are alkaloids.[3] Since AChE inhibition is one of insect toxicity mechanisms,[4] some plants build their own defensive system against insects by producing these alkaloids. Therefore, many potential insecticidal plants have been investigated for their acetylcholinesterase inhibitory activities.

Stemona collinsiae Craib is a remarkable species of *Stemona* genus (Family Stemonaceae) that possesses insecticidal activity.[5–7] Its roots contain characteristic alkaloids, didehydrostemofoline (asparagamine A) and stemofoline (Fig. 1) as major constituents.[8–10] In the present work, AChE inhibitory potential of *S. collinsiae* root extracts prepared from hexane, dichloromethane and methanol, and their purified major alkaloids, didehydrostemofoline and stemofoline, were evaluated using Ellman's AChE inhibition bioassay.[11] The contents of didehydrostemofoline and stemofoline in each extract were analyzed by HPLC–UV. This study supports the traditional use of *S. collinsiae* roots as a natural insecticide and provides information for further pharmaceutical and agricultural product development.

2. Materials and methods

2.1. Plant materials

The root sample of *S. collinsiae* (voucher specimen number WSCL040210) was collected from Sriracha, Chonburi province in the eastern part of Thailand in February 2010. The sample was identified by Dr. Srunya Vajrodaya, Faculty of Botany, Kasetsart University, Thailand and the voucher specimen was deposited at

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0975-3575/$ – see front matter
http://dx.doi.org/10.1016/j.phcgj.2013.02.004

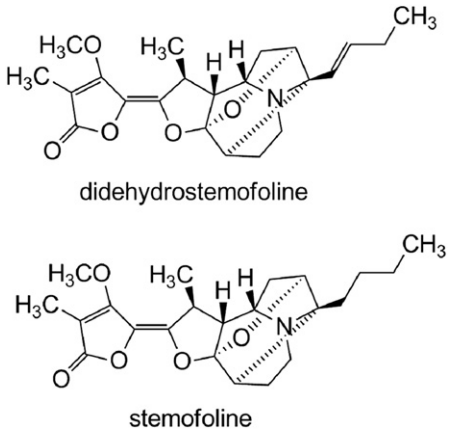

Fig. 1. Structures of didehydrostemofoline and stemofoline.

Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. The root sample was washed thoroughly with tap water, cut into small pieces and dried in a hot air oven at 50 °C for 72 h. The dried sample was ground into powder, passed through a sieve (60 mesh) and stored in an air-tight container at room temperature (28–30 °C) away from light until used.

2.2. Chemicals

Acetylthiocholine iodide (ATCI), bovine serum albumin (BSA), 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB) and AChE from electric eel (Type V-S) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Galanthamine hydrobromide standard was purchased from TCI (Japan). Tris–HCl (50 mM, pH 8.0) was used as buffer in this experiment. ATCI was dissolved in deionized water while DTNB was dissolved in buffer containing 0.1 M sodium chloride (NaCl) and 0.02 M magnesium chloride ($MgCl_2$). AChE enzyme was prepared in 0.1% BSA in buffer and stored in −80 °C until used.

HPLC grade methanol was purchased from Fisher Scientific (UK). Deionized water was purified by Water Pro PS (Labconco, Missouri, USA). Ammonium acetate was purchased from Sigma–Aldrich. All reagents were of analytical grade. Didehydrostemofoline and stemofoline were isolated from *S. collinsiae* roots and identified in our previous study.[10,12]

2.3. Preparation of plant extracts

Fifty grams of dried powdered roots were successively extracted by maceration at ambient temperature with hexane, dichloromethane, and methanol (300 ml × 3 times), in that order. Each extract was filtered and evaporated to dryness under reduced pressure using a rotary evaporator (45 °C). Each dried extract was then weighed and kept in a tight container protected from light until used.

2.4. HPLC quantitative analysis for didehydrostemofoline and stemofoline contents in S. collinsiae root extracts

The sample solutions for HPLC analysis were prepared by dissolving each extract of *S. collinsiae* roots in methanol to obtain the final concentration of 2000 μg/ml for hexane and methanol extracts and 500 μg/ml for dichloromethane extract. Validated high performance liquid chromatographic (HPLC) method[12] was used for quantitative analysis in this study. The analysis was performed on Shimadzu SPD-10A (Japan) HPLC equipped with a quaternary solvent delivery system with model LC-10AD pump, a DGU-10A degasser, UV–VIS detector SPD-10AV and auto-injector SIL-10AD. The separation was carried out on a Hypersil BDS C-18 column (4.6 × 150 mm i.d., 5 μm) with a C-18 guard column. The mobile phase was methanol: 1 mM ammonium acetate solution (55:45) at a flow rate of 1.0 ml/min. The column was washed out with methanol for 10 min and re-equilibrated with the mobile phase for 10 min before next injection. The detection was monitored at 295 nm and the injection volume was 10 μl. Prior to the injection, all solutions were filtered through a 0.2 μm nylon membrane filter.

2.5. Assay for AChE inhibitory activity of S. collinsiae root extracts, didehydrostemofoline and stemofoline alkaloids

Hexane, dichloromethane, and methanol extracts from *S. collinsiae* roots were assayed for AChE inhibitory activity and compared to those of pure major alkaloids, didehydrostemofoline and stemofoline, and standard galanthamine. Sample solutions were prepared by dissolving each extract in methanol to give the concentrations of 10,000, 5,000, 2,500, 1,250, 625, 312.5, and 156.3 μg/ml. Didehydrostemofoline, stemofoline and galanthamine hydrobromide were prepared in the same manner to give the concentrations of 1,000, 500, 250, 125, 62.5, 31.25 and 15.63 μg/ml for didehydrostemofoline and stemofoline, and 20, 10, 5, 2.5, 1.25, 0.625 and 0.313 μg/ml for galanthamine hydrobromide. The AChE activity assay was done using 96-well microplate reader.[11,13] Briefly, 125 μl of 3 mM DTNB, 25 μl of 15 mM ATCI, and 50 μl of buffer were mixed with 25 μl of each sample or standard solution, followed by the addition of 25 μl of 0.28 U/ml AChE. The absorbance at 405 nm was chosen to detect every 2 min for 10 min by microplate reader. The velocity of the reaction was calculated from regression line. Activity of the enzyme was calculated as percentage of velocity compared to that of the assay using buffer without any inhibitor.

2.6. Thin layer chromatography (TLC) with bioautographic assay for AChE inhibitory activity

The TLC with bioassay detection for AChE inhibition was modified from Rhee et al[14] TLC 0.2 mm thickness precoated with

Table 1
Yields of extracts, contents of active constituents and acetylcholinesterase inhibitory effects of *S. collinsiae* extracts and their purified active constituents.

Extract	Yield (% dry weight)	Content of active constituent[a] (% w/w)		IC_{50} (μg/ml)
		Didehydrostemofoline	Stemofoline	
Hexane extract	0.30	3.59 ± 0.09	0.177 ± 0.003	126.72
Dichloromethane extract	0.60	40.78 ± 0.49	0.736 ± 0.006	73.78
Methanol extract	31.89	1.43 ± 0.04	0.088 ± 0.006	>1000
Didehydrostemofoline				50.55 (131.3 μM)
Stemofoline				39.53 (102.1 μM)
Galanthamine				0.48 (1.30 μM)

[a] Expressed as mean ± SD ($n = 3$).

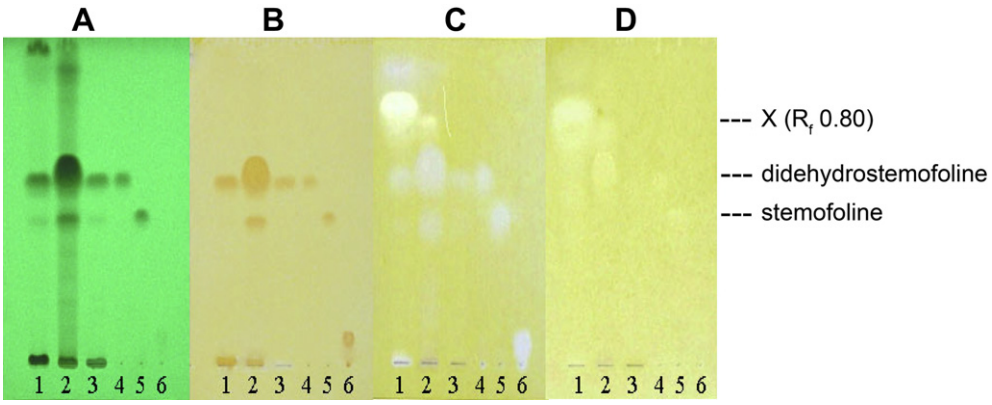

Fig. 2. TLC fingerprints of *S. collinsiae* Craib extracts (Silica gel F_{254}, dichloromethane:ethyl acetate:methanol, 70:25:5, v/v/v); track 1: hexane extract, track: 2 dichloromethane extract, track 3: methanol extract, tract 4: didehydrostemofoline, tract 5: stemofoline, track 6: galanthamine standard. (A) observed under UV 254 nm, (B) sprayed with Dragendorff's spraying reagent, (C) AChE-bioautography assay, (D) false-positive AChE-bioautography assay. X = unknown compound at R_f 0.80 showing a false-positive spot.

silica gel 60 F_{254}, cat. No. 1.05554.0001 (Merck, Darmstadt, Germany) was used as a stationary phase. The solutions of plant extracts, didehydrostemofoline and stemofoline, and galanthamine were spotted on the TLC plate and developed using a mobile phase, dichloromethane:ethyl acetate:methanol (70:25:5, v/v/v). The plate was dried at room temperature, sprayed with 30 mM ATCI solution followed by 20 mM DTNB solution, then dried again at room temperature for 15 min, and sprayed with AChE solution (5 U/ml). Within 15 min, a colorless positive spot on the yellow background was indicative of the AChE inhibitory activity.

A false-positive test was carried out in order to confirm true enzyme inhibition using a modified method from Rhee et al[15] Briefly, thiocholine spraying reagent was prepared by incubating 30 mM ATCI solution with 2 U/ml AChE for 20 min at room temperature (28–30 °C). After the TLC plate was developed and dried, it was sprayed with 20 mM of solutions of DTNB and thiocholine. Colorless spots on a yellow background were recorded and the results were compared with the results of the TLC-AChE bioassay.

3. Results

Yields of hexane, dichloromethane, and methanol extracts of dry powdered *S. collinsiae* roots were 0.30, 0.60, and 31.89 %w/w, respectively. The results shown in Table 1 indicated that dichloromethane was the solvent that yielded the highest contents of didehydrostemofoline and stemofoline.

AChE inhibitory activities of *S. collinsiae* root extracts were determined by Ellman's colorimetric method. IC_{50} values of hexane and dichloromethane extracts were 126.72 and 73.78 μg/ml, respectively while methanol extract possessed very low activity (>1000 μg/ml). Purified didehydrostemofoline and stemofoline inhibited AChE with the IC_{50} of 50.55 and 39.53 μg/ml (equivalent to 131.3 and 102.1 μM), respectively, compared to 0.48 μg/ml (equivalent to 1.30 μM) of positive control galanthamine hydrobromide (Table 1).

The AChE inhibitory activities of the extracts and their two major alkaloids were also evaluated by TLC bioautographic assay and the positive results are shown in Fig. 2(C). White spots on a yellow background showed AChE inhibitory activities. Hexane and dichloromethane extracts of *S. collinsiae* roots demonstrated AChE inhibitory activities, and didehydrostemofoline (R_f value = 0.55) and stemofoline (R_f value = 0.45) were the major compounds with AChE inhibitory activities (Fig. 2(A)–(C)). Galanthamine, as a positive control, also exhibited white spot on a yellow background. The comparison with the false-positive assay (Fig. 2(D)) indicated that the compound with R_f of 0.80 in the hexane extract was a false-positive, having the white spot on the yellow background (Fig. 2(D)).

4. Discussion

Polarity of extracting solvent plays an important role on the quality and quantity of the extract. According to this extraction procedure, dichloromethane fraction yielded the highest contents of didehydrostemofoline and stemofoline (Table 1).

Thin-layer chromatograms of each extract were shown in Fig. 2(A) and (B). Major alkaloids constituents, didehydrostemofoline and stemofoline were found with R_f values of 0.55 and 0.45, respectively. Active AChE inhibitory activity was found in hexane and dichloromethane extracts while methanol extract possessed very low activity (>1000 μg/ml). It was found that didehydrostemofoline (R_f value = 0.55) and stemofoline (R_f value = 0.45) contributed mainly to the activity (Fig. 2(A)–(C)). In order to confirm true enzyme inhibition, a false-positive test was carried out to determine the chemical inhibition of thiocholine and DTNB. After comparing with the false-positive assay, the compound with R_f value of 0.80 in the hexane extract was proved to be a false-positive (Fig. 2(D)) whereas didehydrostemofoline and stemofoline spots did not appear on the false-positive test. Comparing the results from microplate assay with those from TLC assay, the highest AChE inhibitory activity (73.78 μg/ml) was found in the dichloromethane extract which contained the highest contents of both major alkaloids. It could be deduced that didehydrostemofoline and stemofoline were responsible for AChE inhibitory activity of *S. collinsiae* roots (Fig. 2 and Table 1).

5. Conclusion

AChE inhibitory activity of stemofoline, didehydrostemofoline and the dichloromethane extract from the roots of *S. collinsiae* were 102.1, 131.3, and 1.30 μM, respectively (Table 1). These scientific findings of AChE inhibitory activity could support the traditional claims of this plant as a natural insecticide.

Conflicts of interest

All authors have none to declare.

Acknowledgments

This study is a part of a Ph.D. thesis of Mahidol University financially supported by Thailand Research Fund and Mahidol University (Royal Golden Jubilee Ph.D. Program Grant No. PHD/0139/2550). The project is also supported by the Office of the Higher Education Commission and Mahidol University under the National Research Universities Initiative. We thank Assoc. Prof. Dr. Srunya Vajrodaya for identification of the plant sample and Mr. Panupon Khumsupan for his kind help in proofreading the manuscript.

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Pharmacognosy Journal 5 (2013) 60–65

Contents lists available at SciVerse ScienceDirect

Pharmacognosy Journal

journal homepage: www.elsevier.com/locate/phcgj

Original article

Studies on nitrogen fixing bacteria and their application on the growth of seedling of *Ocimum sanctum*

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ARTICLE INFO

Article history:
Received 19 January 2013
Accepted 9 March 2013
Available online 19 March 2013

Keywords:
Indole acetic acid
Bio-fertilizer
Azotobacter
Azospirillum

ABSTRACT

Introduction: Representatives of the *Azotobacteriaceae* family are regular inhabitants of soils of roots (rhizosphere) and on the root surface of free-living nitrogen fixing bacteria of the genera *Azotobacter* and *Azospirillum*. They are the regular associates of the roots of many tropical grasses and cereals. Their associations with medicinal plants are of paramount importance. The medicinal plants such as *Aloe vera* and *Datura alba* were selected to access the nature of association in the rhizosphere and on the roots by the above bacteria.

Methods: The roots of *A. vera* had the dominance of nitrogen fixing bacteria (132×10^2/g). The characteristics of the selected bacteria revealed the presence of *Azotobacter chroococcum*, *Azotobacter beijerinckii*, *Azotobacter vinelandii* and *Azospirillum lipoferum*. The carbon sources such as sucrose, lactose, glucose, maltose, rhamnose, xylose and mannitol either individually or in combination induced the growth of selected nitrogen fixing bacteria *(A. chroococcum*, *A. beijerinckii*, and *A. vinelandii)*.

Results: The maximum growth was recovered with maltose and glucose (1.36S0.D). The mixed carbon sources such as mannitol, maltose and xylose showed elevated growth of the bacterium *(A. chroococcum* and *A. beijerinckii)*. The production of IAA, a growth promoting hormone by *A. chroococcum*, *A. beijerinckii* and *A. vinelandii* was studied at varying pH ranges from 6 to 9 in the growing medium. The increase in the pH stimulated the growth as well as the synthesis of IAA. Both the growth and the growth hormones are similar in the growing liquid broth. The above observations paved the way for accessing the growth of the seedling of *Ocimum sanctum*. The application of *Azotobacter* and *Azospirillum* species as biofertilizers is a testimony to the effect that IAA on seedlings growth.

Conclusion: With this we conclude that IAA with its growth promoting capacity dominates the Microbial and Agricultural Biotechnology in the years to come.

1. Introduction

Bio-fertilizers are the formulation of living microorganisms which are able to fix atmospheric nitrogen in the available form to plants either by living freely in the soil or being associated symbiotically with plants. They are capable of mobilizing nutritive elements from non-usable form to usable form through biological process.[1] The primary object of this study is on *Azotobacter*, a member of the family *Azotobacteriaceae* and it has been isolated from both soil and rhizosphere environment. *Azotobacter* spp. are mesophilic bacteria whose growth and nitrogen fixing ability are highly dependent on the temperature. The application of free-living N_2 fixing *Azotobacter* and *Azospirillum* as bio-fertilizers is known to result in increased productivity of a variety of field ground crops. *Azospirillum* is a Gram negative soil bacterium living in close association with roots of plants.[2]

Medicinal plants are the most important source of life saving drugs for the majority of the world's population. Medicinal plants constitute an important component of flora and are widely distributed in India. The pharmacological evaluation of substances from plants is an established method for the identification of lead compounds which can lead to the development of novel and safe medicinal agents. Medicinal plants are the vital source of medication in developing countries. According to the World Health Organization (WHO) in 1977 "a medicinal plant" can be of any plant, which is one or more of its part contains substances that can be used for the therapeutic purposes or which are precursors for the synthesis of useful drugs. Among 250,000 higher plant species on

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0975-3575/$ – see front matter
http://dx.doi.org/10.1016/j.phcgj.2013.03.003

earth, more than 80,000 species are reported to have both at least some medicinal value and therapeutic value. They are classified according to the part used, habit, habitat, therapeutic value, etc.

Azotobacter synthesizes some biologically active substances, including some phyto-hormones such as auxin, thereby stimulating plant growth. They also facilitate the mobility of heavy metals in soil and enhance bioremediation of soil from heavy metals such as mercury, lead, etc. Many workers have reported on the production of plant hormones by *Azotobacter*[3,4] and by *Azospirillum*. It's ability to fix molecules of nitrogen, increase soil fertility and stimulate plant growth.[5–7]

The present study deals with the distribution of nitrogen fixing *Azospirillum* spp. in rhizosphere and soil sediments and also the potentiality of indole acetic acid production.

2. Materials and methods

The following medicinal plants were collected for the isolation of N_2 fixing bacteria such as *Aleo vera* (family; Asphodelaceae), and *Datura* family; (Solanoideae) from SASTRA University campus and the plants were authenticated and herbarium was made for further studies.

2.1. Enumeration and isolation of bacteria from rhizosphere and on the root associated soil samples

For measuring bacterial load, serial dilution and pour plate techniques were followed as per Pramer and Schmidt.[8] Bacteria were counted by plate method in which 1 ml of a suitable dilution (10^{-1}) of the original sub- sample of rhizosphere and root associated soil sample was pipetted out into a sterile Petri dish. About 20 ml of the (*Azotobacter* medium) at (40 °C) the ear bearing temperature was added to the sample in the Petri dish and then mixed by rotating it in clockwise and anti-clockwise directions. With regard to the aerobic (*Azotobacte*r spp., and *Azospirillum* spp.) in rhizosphere and root associated soil sample and their enumeration the pour plate method was followed[9,10] and showed that the temperature of the molten agar at the time of pouring was important in relation to the number of the colonies developing on pour plates. The above procedure was done in the laminar flow chamber to prevent contamination from externals. The inoculated petri dishes on solidification of the medium were inverted, to prevent the condensed water from falling on the medium and then kept in an incubator for 7 days at room temperature (28 °C). The bacterial colonies were counted after 7 days of incubation period. For obtaining pure cultures the colonies were streaked on agar plates and then subcultured on the same medium slants. On satisfactory growth of the bacteria on the slants, these were stored in a refrigerator (4 °C) till further use. *Azotobacter* medium was used for the isolation of *Azotobacter* with pH 8.5.

2.2. Preparation of soil extract (for 100 ml)

250 g of soil sample was weighed out and transported to a flask containing 100 ml of sterile water. To it 0.5 g of calcium chloride was added and the flask was then autoclaved. After this, the mud was filtered and the clear solution was again sterilized and stored for further use.

2.3. Plant sources

Isolation and enumeration of N_2 fixers were done in the rhizosphere and root associated soils. Here, 1 g each of rhizosphere soil sample and root were taken from the medicinal plants selected and treated with distilled water for 10 min to isolate *Azotobacter* and *Azospirillum*. The roots and soils were crushed using sterile 9 ml blank (10^{-1} dilution). This was aseptically transferred to the dilution level of 10^{-2}. 1 ml from that dilution was aseptically transferred to each sterile Petri dish. About 15–20 ml of sterilized media (*Azotobacter* medium) were aseptically transferred to each Petri dish respectively. The plates were rotated clockwise and anti-clockwise directions to get uniform mixing of the samples and the medium. They were incubated at room temperature till the appearance of microbial colony forming units for 7–10 days.

2.4. Enumeration and isolation of Azospirillum

Azospirillum was isolated in the same way as *Azotobacter*. For isolation an N/b basal medium was employed. Total number of colonies of *Azotobacter* and *Azospirillum* in each petri dish was counted and recorded. The dry weight of the sample (rhizosphere and root associated soil samples) were noted for both the medicinal plants selected in the present investigation.

2.5. Identification of Azotobacter and Azospirillum by staining techniques (Beijerinck, 1901a ;1925)

Cell morphology of the above bacteria was studied from Gram staining and also the biochemical studies. In this Gram staining technique a thin heat fixed smear of *Azotobacter* was made on a clean glass slide. To this, a few drops of crystal violet (primary stain) staining reagent was added and left for 1 min. The slide was washed in tap water. The smear was flooded with a few drops of gram iodine (mordent) and left for 1 min. The smear was washed gently in tap water and then decolorized with 95% of ethanol. The slide was washed with tap water and counter stained with saffron in "O", washed in tap water and blot dried. The slide was observed under microscope in oil immersion (100×). Gram negative rods were observed for the identification of both *Azotobacter* and *Azospirillum* species.

2.6. Effect of carbon sources

Mannitol, glucose, lactose, rhamnose, xylose, maltose and sucrose were utilized for the growth of *Azotobacter* and *Azospirillum*. For the effect of carbon sources on their growth the *Azotobacter* broth medium was prepared with the ingredients as before without agar. At different ways, the carbon sources were weighed at 1% level to assess the effect on their growth either with individual carbon source or in combinations and added into the 100 ml broth in a conical flask. The flasks were then steamed for 15 min. These were disbursed with 10 ml each in a tube and inoculated with *Azotobacter* isolated from the different samples and incubated for five days at room temperature. The turbidimetric reading of the bacterial culture was observed using UV–Vis Spectrophotometer at 600 nm (Unicam).

2.7. Indole acetic acid estimation

Tryptophan is an essential amino acid that can undergo oxidation by way of the enzymatic activities of some bacteria. Conversion of tryptophan into metabolic products mediated by the ability to tryptophanase. The ability to hydrolyze tryptophan with the production of indole is not the characteristic of all microorganisms and therefore it serves as a biochemical marker. *Azotobacter* is known to have the ability to hydrolyze tryptophan and produce indole acetic acid (IAA). The determination of IAA production was followed according to Tien et al[1] The isolates of *Azotobacter* were grown in 100 ml of the respective medium mentioned with various pH levels (6, 7, 8 and 9) supplemented with tryptophan (100ug/L) along with

100 mg/l of yeast extract for 7 days in dark. The broth was then centrifuged at 3000xG for 30 min. The supernatant was brought to pH 2.8 with 1 N HCl and extracted twice with equal volume of diethyl ether at 4 °C. The ether phase was dried and dissolved in 20 ml methanol. IAA present in the methanol extract was estimated by using Salper's reagent (1.0 ml of 0.5 M ferric chloride in 50 ml of 35% perchloric acid) according to Gorden and Paleg.[11] The color intensity was read at 535 nm with appropriate blank (2 ml methanol + 4 ml Salper's reagent) in a UV–Vis Spectrophotometer (Unicam).

2.8. Testing of the biofertilizers for the growth of seedling, Ocimum sanctum

In this experiment, polythene bags were disinfected with some common laboratory disinfectant and filled in with uniform amount of garden soil already sterilized. Efficient strains of *Azotobacter chroococcum*, *Azotobacter vinelandii*, *Azotobacter beijerinckii* and *Azospirillum lipoferum* were multiplied in a pure forming their respective medium. Both *Azotobacter* and *Azospirillum* broths were employed. Mixed cultures of phosphobacteria and *Streptomyces* along with the above bacteria were utilized for inoculation to the seedling. Before inoculation of the above microbial cultures, the plant height, number of leaves; number of branched roots were counted. About 5 ml broth of the inoculants each was poured into the respective plant (*O. sanctum*) .Suitable control plant was kept for comparison. Plants are watered as and when required and allowed to growth for about 10 days. Differences between inoculated plant and control plants in respect of height, number of leaves and number of branched roots were noted.

3. Results

The rhizosphere and soil samples were collected from Thanjavur city during the period of 2 months. Two plants were collected from Thanjavur city. The plants were *Aloe vera* and *Datura alba* (Fig. 1). Quantitative studies of *Azotobacter* and *Azospirillum* in all samples were carried out. Using seven carbon sources namely lactose, mannitol, glucose, xylose, rhamnose, sucrose, maltose as individually or in combination at one concentration (1%), the growth of *Azotobacter* and *Azospirillum* was studied. Production of IAA at different pH by *Azotobacter* and *Azospirillum* isolated from rhizosphere and soil samples was also estimated.

Of the rhizosphere and soil sample collections from two different medicinal plants, the maximum population of *Azotobacter* and *Azospirillum* recorded was (132×10^2/g) and no growth of fungal and actinomycete populations were found respectively. The roots of *Aloe vera* had the dominance of nitrogen fixing bacteria, *Azotobacter chroococcum* (3rd collection) (Table 1). The second highest population recorded was (124×10^2/g) (Ist collection). The minimum bacterial population obtained from rhizosphere sample was (82×10^2/g) (IInd collection) (Table 1).

For the present study, root associated soil sample of *A. vera* and *D. alba* were selected and strains were isolated from the root associated soil sample. Of these, maximum bacterial population of (77×10^2/g) was obtained from *A. vera* (Table 1). The second highest population recorded was (34×10^2/g) (Table 1). The minimum bacterial population recorded was (27×10^2/g) (Table 1).

Along with the estimation of microbial population of *Azotobacter*, enumeration of *Azospirillum* (Table 2) was also carried out. It was noticed in the rhizosphere and root associated soil samples of medicinal plants. Maximum population of *A. lipoferum* (21×10^2/g) was obtained from *A. vera* rhizosphere sample (Table 2). The minimum population of *A. lipoferum* (9×10^2/g) was obtained from root associated soil sample of *D. alba* (Table 2).

It was noted that no fungal and actinomycetes population were obtained from any of the above samples (Table 2). Since the bacterial strains were isolated, it is necessary to characterize the strains of *Azotobacter* spp. and *Azospirillum* spp. based on the morphological, physiological and biochemical characteristics according to Bergey's Manual of Systematic Bacteriology (Table 3). The strains of *Azotobacter* and *Azospirillum* were characterized on the basis of pigment variations, pair formation, carbon sources, pH, acid production. The strains of *A. chroococcoccum*, *A. beijerinckii*, *A. vinelandii* and *A. lipoferum* were identified based on the "Gram staining" methodology (Table 3). The strains of *Azotobacter* and *Azospirillum* were Gram–ve rods. *A. chroococcum*, *A. vinelandii*, *A. beijerinckii* identified as Gram–ve rods (pink). *A. lipoferum* are *flagella* type. The above strains were identified through light microscope under oil immersion (100×).

(Fig. 2) The effect of carbon sources from rhizosphere and root associated soil sample, the maximum growth of *A. chroococcum* was recovered with maltose (1.105) and glucose (1.365 O.D.) (Table 4). The second highest population of *A. chroococcum* was recovered with lactose (0.886 O.D.), xylose (0.884) and rhamnose (0.858) respectively (Table 4). The minimum population of *A. vinelandii* was recovered with mannitol (0.593), sucrose (0.566) respectively (Table 4).

The *Azospirillum lipoferum* show the highest population was recovered with sucrose (0.42) and lactose (0.34) respectively. The strains of *Azotobacter chroococcum*, *A. vinelandii*, *A. beijerinckii* recovered the maximum population (0.719) with combination of carbon sources such as glucose, sucrose and lactose respectively. The strain of *Azospirillum lipoferum* showed the highest population

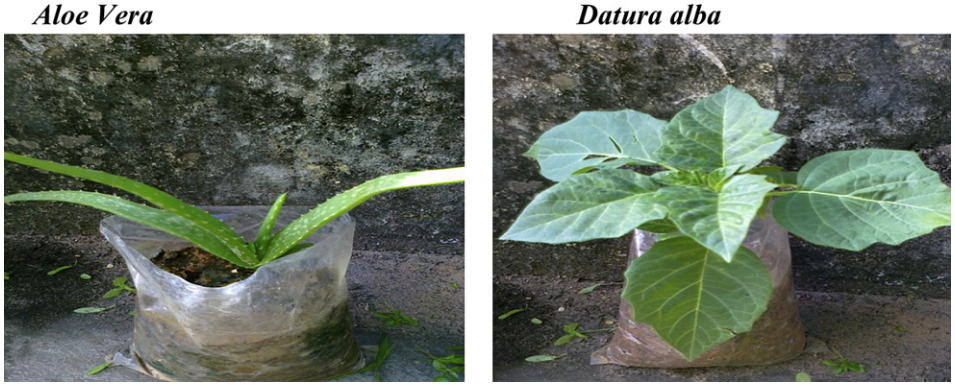

Fig. 1. Medicinal plants used in study.

Table 1
Enumeration of microbial population from the medicinal plants (*Aloe vera* and *Datura alba*).

S. no	Medicinal plant	Date of collection	Media	No of colonies CFU × 10^2/g		Dry weight (g)
				Bacteria	Fungi	
1.	*Aloe vera* (Root)	05.01.2011	AA[a]	124	1	1.12
				77	–	
2.	*Datura alba* (Root)	05.01.2011	AA[a]	–	–	0.94
				–	–	
3.	*Aloe vera* (Root)	10.01.2011	AA[a]	82	–	1.13
				21	–	
	Aloe vera (Soil)	10.01.2011	AA[a]	–	–	1.23
				27	–	
4.	*Datura alba* (Root)	10.01.2011	AA[a]	–	–	0.96
				–	3	
	Datura alba (Soil)	10.01.2011	AA[a]	9	–	1.17
				–	–	
5.	*Aloe vera* (Root)	18.01.2011	AA[a]	132	–	1.51
				–	–	
	Aloe vera (Soil)	18.01.2011	AA[a]	34	–	1.15
				–	–	
6.	*Datura alba* (Root)	18.01.2011	AA[a]	–	–	0.98
				–	–	
	Datura alba (Soil)	18.01.2011	AA[a]	–	–	1.28
				–	1	

[a] AA – *Azotobacter* agar medium.

(0.489) with combination of mannitol, maltose and xylose respectively (Table 4). The production of indole acetic acid (IAA), a growth promoting hormone, by *Azotobacter* was studied at varying pH from 6 to 9 in the growing medium for rhizosphere and root associated soil samples. At pH 9, *Azotobacter chroococcum* showed the maximum IAA production (0.168) and growth (2.001) respectively (Table 5). At pH 7 the second highest growth of *Azotobacter beijerinckii* was recovered as 1.976 and IAA production recovered as 0.147 at pH 7. The third highest growth of *Azotobacter vinelandii* was recovered as 1.889 at pH 6 and IAA production recovered as 0.132 at pH 6. The minimum growth of *A. lipoferum* was recovered as 1.503 at pH 8 and IAA production recovered as 0.101 at pH 8 respectively (Table 5 and Fig. 3) The above observation enhances the growth of the seedlings of *O. sanctum* and the testing of bio-fertilizers were carried out through the application of the *Azotobacter* spp. and *Azospirillum* spp. respectively. The number of leaves, roots and height of the *O. sanctum* were noted (Table 5). The strains of *A. chroococcum* ,*A. beijerinckii*, *A. vinelandii* and *A. lipoferum* were inoculated into the seedlings separately and the one seedling placed as control (Table 5). The height, number of root, leaves after the growth period of 10 days were recovered and noted (Table 5). The nitrogen fixers isolated from medicinal plants such as *A. vera* and *D. alba* have specific influence over the other medicinal plant such as *O. sanctum*. There were not many variations over the growth of the seedling by the selected independent bacteria. However, the microbial proliferation of the above biofertilizers is because of the root exudates of the seedlings selected on the selected microorganisms. Moreover, many of the environmental factors such as temperature, light, carbon dioxide, etc. are also known to influence the microbes in the rhizosphere It is not yet known to what extend seedlings can select a constant rhizosphere microbial community from the high contrasting reservoirs of the soil population. The synergetic effect between these bacteria in the rhizosphere had been well documented.[12] Hence, individual or combined bacterial inoculation will reduce 40–50% use of fertilizers in agricultural crops.

Table 2
Identification of bacterial strains from medicinal plants (*Aloe vera* and *Datura alba*).

S. no	Date	Samples	Strains	Appearance
1.	02.02.2011	*Aloe vera* (Root)	Gram negative	Rod
			Gram negative	Rod cyst
		Datura alba (Root)	Gram negative	Rod slightly curved
			Gram negative	Rod cyst
		Aloe vera (Soil)	Gram positive; Gram negative	Rod; cocci
			Gram positive; Gram negative	Rod; cocci
		Datura alba (Soil)	Gram negative	Rod; cocci
			Gram negative	Rod; cocci
2.	04.02.2011	*Aloe vera* (Root)	Gram negative	Rod cyst
			Gram negative	Rod cyst
		Aloe vera (Soil)	Gram negative	Rod
		Datura alba (Root)	Gram negative	Rod
		Datura alba (Soil)	Gram negative; Gram positive	Rod cyst
3.	05.02.2011	*Aloe vera* (Root)	Gram negative	Rod slightly curved
			Gram negative	Rod slightly curved
		Aloe vera (Soil)	Gram positive; Gram negative	Rod cyst
		Datura alba (Root)	Gram negative	Rod cyst
		Datura alba (Soil)	Gram negative	Rod; cocci cyst

4. Discussion

In the present investigation, *Azotobacter* spp and *Azospirillum* spp were isolated from rhizosphere and root associated soil sample of two medicinal plants (*A. vera* and *D. alba*). The significance of a microbial community in facilitating a high rate of nitrogen fixation was recognized. Tables 1 and 2 summarized the bacterial population obtained from *A. vera* and *D. alba* and their rhizosphere and root associated soil samples. Though *Azotobacter* spp. were not obtained highly in all collections of *D. alba* (rhizosphere and root associated soil sample), the results showed a higher count in *A. vera* collection samples. The fungal and actinomycete population recorded from *A. vera* and *D. alba* were comparatively low. The high growth of *Azotobacter* also led to a high nitrogen fixing activity. It was found that distribution of nitrifiers depend on nutrient concentration. This could led to the increase in *Azotobacter* colonies obtained from *A. vera* (rhizosphere and root associated soil sample). Among the two plants *Aloe vera* showed and recorded a maximum bacterial population.

Azospirillum spp. are soil microorganisms occurring in the rhizosphere of higher plants. *A. lipoferum* was isolated with an N/b medium,[13] and isolated this organism from several sources and

Table 3
Effect of carbon sources at 1% on the growth of *Azotobacter species* from medicinal plants.

S. no	Date	Medicinal plants	Bacterial strain	Carbon sources (1%)	*O.D @ 535 nm
1.	23.02.2011	*Aloe vera* (Root)	*Azotobacter chroococcum*	Glucose *(H)	1.365
		Aloe vera (Soil)	*Azotobacter chroococcum*	Maltose *(L)	1.105
		Aloe vera (Root)	*Azotobacter beijerinckii*	Sucrose	0.566
		Aloe vera (Soil)	*Azotobacter beijerinckii*	Starch	0.34
		Aloe vera (Root)	*Azotobacter vinelandii*	Mannitol	0.886
		Aloe vera (Soil)	*Azotobacter vinelandii*	Rhamnose	0.858
2.	04.03.2011	*Datura alba* (Root)	*Azotobacter chroococcum*	Xylose	0.884
		Datura alba (Soil)	*Azotobacter chroococcum*	Glucose	0.554
		Datura alba (Root)	*Azotobacter beijerinckii*	Sucrose	0.523
		Datura alba (Soil)	*Azotobacter beijerinckii*	Lactose	0.42
		Datura alba (Root)	*Azotobacter vinelandii*	Rhamnose	0.827
		Datura alba (Soil)	*Azotobacter vinelandii*	Mannitol	0.31
3.	04.03.2011	*Aloe vera* and	*Azotobacter chroococcum*	Glucose	0.719
		Datura alba (Root)	*Azotobacter beijerinckii*	Sucrose	
			Azotobacter vinelandii	Lactose	
		Aloe vera and	*Azotobacter chroococcum*	Mannitol	0.489
		Datura alba (Soil)	*Azotobacter beijerinckii*	Maltose	
			Azotobacter vinelandii	Xylose	

*O.D – optical density; *H – high; *L – low.

produced enlarged forms, very reminiscent to the behaviors of *Azotobacter* under similar conditions. Growth of *Azotobacter* spp. subjected to 7 different carbon sources at 1% concentration was studied. In case of maltose and glucose maximum growth of (1.105) and (1.365) was recovered. The considerable quantities of nitrogen are reported to be fixed by *Azotobacter* under natural condition. Out of all carbon sources, sucrose was the least preferred carbon source by *Azotobacter* and *Azospirillum* isolated from rhizosphere of *D. alba*. The individual effect of glucose and maltose showed the highest preference than in combination of carbon sources. All the bacterial strains showed an affinity to glucose, maltose rather than sucrose. The fixed nitrogen sources such as nitrate and ammonia are potent repressors of the synthesis of nitrogenase in bacteria while nitrogen itself is not probably required for enzyme synthesis.

Several factors control the rate of bacterial nitrogen fixation including the provision of carbon and energy sources, the oxygen tension and the concentration of fixed nitrogen sources. The present investigations revealed that *Azotobacter* spp. can grow in all 7 carbon sources. The successfully conduction of a similar study using mannitol, sucrose and glucose as carbon sources. Another study showed with *Azotobacter* spp., the requirement for carbon source and the oxygen tension are closely linked. Since for nitrogen fixation to occur there must be an abundance of carbon sources to satisfy requirements of respiratory protection.

Purushothaman et al, suggested the synthesis of a growth promoting hormone like Indole acetic Acid (IAA) by *Azotobacter* from agricultural soil.[6] Though studies reported the synthesis of IAA by bacteria (*Azotobacter*) associated with seaweeds, no report of IAA production from estuarine fish and marine sediments are known.

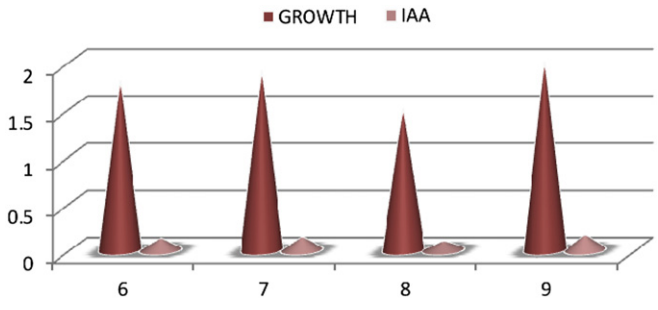

Fig. 2. Effect of pH on growth and indole acetic acid production from *Azotobacter* spp.

Table 5 clearly showed the amount of IAA produced by Azotobacter from rhizosphere and root associated soil sample. It was noted that the *A. lipoferum* produced less IAA than *Azotobacter* spp. Studies of IAA production from the *Azospirillum* spp were mainly conducted in plants. Fnlchieri et al studied the effect of IAA produced by *Azospirillum lipoferum* in maize.[14] Evidence that growth hormones produced by bacteria can in some instance increase growth rates and improve yields of host plants have been produced by Barea and Brown.[4]

Application of *Azospirillum* and *Azotobacter* as biofertilizers in the fields of agriculture began decades ago. It has been attempted on IAA with its application of *Azotobacte*r and *Azospirillum* spp. as biofertilizers for the growth of seedlings of *O. sanctum*. In the present study most of the promoters such as root height, number of roots, leaf area showed the increased value in the seedlings. It is quite obvious that *Azospirillum. amazonense* combines well with *Streptomyces* than *Azotobacter* in growth promoting activity as observed by Rajitha[15] and by Nisha.[16] This may be due to the fact that the biosynthesis of antibacterial substances by *Azotobacter* significantly decreased by supplementing their cultures with metabolic products from *Azospirillum* but were stimulated by metabolic products from *Streptomyces* cultures as observed by Elshanshoury[12] on wheat development.

It is not yet known to what extend seedlings can select a constant rhizosphere microbial community from the high contrasting reservoirs of the soil population. The synergetic effect between these bacteria in the rhizosphere had been well documented by Elshanshonry.[12] Hence, individual or combined bacterial inoculation will reduce 40–50% uses of chemical fertilizers in agricultural crops. It is also presumed that microorganisms act on seedlings both through IAA production and through nutrient absorptions. Release of IAA and free enzymes and their subsequent participation in the promotion of growth and nutrient regeneration would assess

Table 4
Effect of pH on the growth and indole acetic acid production in *Azotobacter* spp.

S. no	Date	pH	Growth (*O.D 600 nm)	*IAA (*O.D 535 nm)
1.	28.03.2011	6	1.889	0.132
2.	28.03.2011	7	1.976	0.147
3.	28.03.2011	8	1.503	0.101
4.	28.03.2011	9	2.001	0.168

*O.D – optical density; *IAA – indole acetic acid production.

Table 5
Testing of the biofertilizer for the growth of seedling (*Ocimum sanctum*).

S. no	Date	Treatments	Height of plant (cm)		Number of			
			Initial (g)[a]	Final (g)[a]	Roots		Leaves	
1.	04.04.2011	Control	10	15	2	6	12	21
2.	04.04.2011	*Azotobacter chroococcum*	11.4	18	3	8	14	36
3.	04.04.2011	*Azotobacter beijerinckii*	10.7	16.3	5	7	18	23
4.	04.04.2011	*Azotobacter vinelandii*	10.2	12.4	2	6	19	21
5.	04.04.2011	*Azospirillum lipoferum*	10.5	16	5	9	17	29
6.	04.04.2011	*Azotobacter chroococcum*+*Streptomyces*+Phosphobacteria	10.9	21.2	4	12	15	41

[a] G – growth.

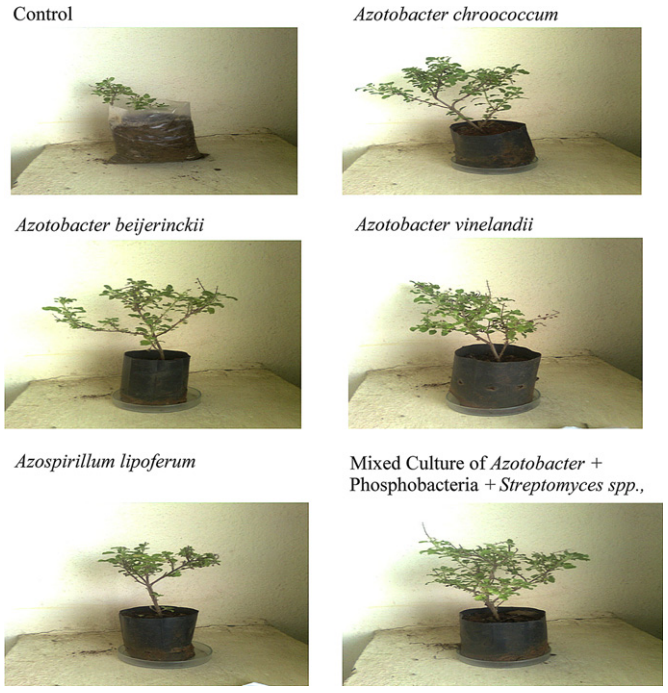

Fig. 3. Comparative testing of the bio-fertilizer for the growth of seedling *(Ocimum sanctum).*

the potential fertility of the environment and the growth of the seedling, *Avicennia officinalis* by preventing soil pollution. It should be remembered that following the release of IAA and nutrients, their subsequent availability to seedling under natural conditions.

Conflicts of interest

All authors have none to declare.

Acknowledgment

We thank the Management of Sastra University – Thanjavur for providing facilities to carry out this research work.

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Pharmacognosy Journal 5 (2013) 66–71

Contents lists available at SciVerse ScienceDirect

Pharmacognosy Journal

journal homepage: www.elsevier.com/locate/phcgj

Original article

Evaluation of antioxidant and anti-fatigue properties of *Trigonella foenum-graecum* L. in rats subjected to weight loaded forced swim test

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ARTICLE INFO

Article history:
Received 31 January 2013
Accepted 21 March 2013
Available online 28 March 2013

Keywords:
Trigonella foenum-graecum
Anti-fatigue activity
Antioxidant activity
Fenugreek
Physical performance

ABSTRACT

Background: *Trigonella foenum-graecum* L. (Fabaceae) is commonly known as fenugreek. Aim of the present study was to assess anti fatigue potential of fenugreek hydro-alcoholic extract (FHE).
Methods: The anti fatigue activity of FHE was investigated in *in vivo* rat model subjected to weight loaded forced swim test (WFST) at a dosage of 10 mg/kg body weight. *In vitro* antioxidant activities were carried out by spectrophotometric methods.
Results: Exhaustive swimming time in WFST was increased by more than 2-fold in FHE supplemented group to that of control group on day 13. The FHE treatment lowered malondialdehyde and lactic acid levels in liver and muscle tissues compared with control exercised group ($p < 0.05$). FHE also reduced serum lactic acid, blood urea nitrogen and creatine phosphokinase activities significantly to that of control. Administration of FHE significantly protected the depletion of serum glucose, liver and muscle glycogen, and activities of antioxidant enzymes i.e. SOD, CAT and GPx (6.05 ± 0.5; 22.0 ± 1.1 and 2084 ± 158 U/mg protein, respectively).
Conclusion: In the present study we report that FHE ameliorates various impairments associated with physical fatigue.

1. Introduction

Fatigue is a physiological phenomenon that appears with physical stress or exhaustive exercises, which reduces the physical endurance capacity.[1] It has been demonstrated that exercise leads to increase in free radical formations thus causing oxidative damage to membranes. Chronic fatigue sometimes causes various neurological, psychiatric and systemic diseases and is often associated with aging, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis and depression.[2,3] The supplementation of antioxidants has a major role in reducing the degree of fatigue by the oxidation of inter or intra cellular oxidizable substrates.[4]

Fenugreek (*Trigonella foenum-graecum* L.) is an annual crop belonging to the legume family. This crop is native to an area extending from Iran to Asia, but is now widely cultivated in China, north and east Africa, Ukraine and Greece.[5] In Asia, the fenugreek leaves and seeds have been used extensively to prepare extracts and powders for medicinal uses. Fenugreek seed is reported to have antidiabetic,[6] hypocholesterolaemic,[7] anticancer,[8] antifertility, antimicrobial, antiparasitic, and anti viral.[9] The seeds of fenugreek contain several beneficial phytochemicals including polyphenols, steroidal sapogenins, fiber galactomannans, antioxidants, rare amino acids such as 4-hydroxyisoleucine and proteins.[10]

In the preset study, we have evaluated the antioxidant and anti-fatigue activity of fenugreek seed extract in *in vitro* and *in vivo* rat model by weight loaded forced swim test. Biochemical changes on serum, liver and muscle tissues were observed to prove endurance capacity of fenugreek seed hydro-alcoholic extract (FHE). Thus, the evaluation of anti-fatigue properties of drugs or herbal preparations requires understanding of its potential to scavenge reactive oxygen species (ROS) and enhance antioxidant defense in the body.

2. Materials and methods

2.1. Seed material and extraction

Fenugreek seed samples were collected from the local market of Mysore, India. Dry seeds (100 g) were cleaned and imbibed in distilled water for 12 h and macerated with 90% ethanol to get all the bioactive components. Both water and 90% ethanol fractions were combined together, evaporated the solvent and lyophilized for further use.

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0975-3575/$ – see front matter
http://dx.doi.org/10.1016/j.phcgj.2013.03.005

2.2. Quantification of total polyphenols, flavonoids and amino acids

Total polyphenols were determined by the Folin–Ciocalteu procedure.[11] The amount of total polyphenols was calculated from the calibration curve of gallic acid standard solutions, concentration of total phenols was expressed as mg/g of dry extract. Total flavonoid contents were determined by the method of Ordon et al,.[11] Flavonoid concentration of the extract was expressed as mg/g equivalent of quercetin. Amino acid content was estimated by ninhydrin reagent to produce purple color and measured absorbance at 570 nm. A calibration graph of glycine was plotted and amino acid concentration of the extract was expressed as mg/g equivalent of glycine.[12]

2.3. Determination of in vitro antioxidant activities of FHE

2.3.1. DPPH radical scavenging activity

The free radical scavenging activity of the fenugreek extract was determined *in vitro* by DPPH (1, 1 diphenyl 2, picryl hydrazyl) assay.[13] DPPH in methanol (0.1 mM) was prepared and 3.0 ml of this solution was added to 40 μl of extract solution in water at different concentrations. The mixture was incubated at room temperature for 30 min and the absorbance was measured at 515 nm against corresponding blank solution. Percentage inhibition of DPPH free radical was calculated based on the control reading. The antioxidant activity of the extract was expressed as IC_{50}, which the concentration (μg/ml) of extract inhibits formation of DPPH radicals by 50%.

2.3.2. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging capacity was measured using modified method as described previously by Halliwell and Gutteridge.[14] The assay was performed by adding EDTA (0.1 ml; 1 Mm), $FeCl_3$ (0.01 ml; 10 mM), H_2O_2 (0.1 ml; 10 mM), deoxyribose (0.36 ml; 10 mM), phosphate buffer (0.33 ml; 50 mM, pH 7.4), ascorbic acid (0.1 ml) and 1.0 ml of extract (50–300 μg/ml). The mixture was then incubated at 37 °C for 1 h and followed by addition of equal amounts of trichloroacetic acid (10%) and thiobarbituric acid (0.05%) to develop the pink chromogen, which was measured at 532 nm and the activity of the extract was reported as the percentage of inhibition of deoxyribose degradation.

2.3.3. ABTS radical scavenging activity

The ABTS radical scavenging activity [2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical] assay was followed by the method of Re et al.[15] The stock solutions included 7 mM ABTS solution and 2.4 mM potassium per sulfate solution. The working reagent was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in dark. Sample extract was allowed to react with 1 ml of the working reagent and the absorbance was taken at 734 nm after 7 min using the spectrophotometer against corresponding blank solution.

2.3.4. Metal chelating activity

Metal chelating activity was observed with the percentage inhibition of ferrozine-Fe^{2+} complex formation.[16] Briefly, the extract (25–100 μg/ml) was added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml), the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine – Fe^{2+} complex formation was calculated with the absorbance of the extract or Na_2EDTA (positive control).

2.4. In vivo physical endurance capacity of FHE

2.4.1. Animal experiment

Animal studies were conducted according to the institute animal ethical committee regulations approved by the committee for the purpose of the control and supervision of experiments on animals (CPCSEA). Eighteen male albino rats of Wistar strain weighing 120–140 g (3–4 week old) were selected from the stock colony of Defence Food Research Laboratory, Mysore, India, housed in an acryl fiber cage in a temperature controlled room (25 ± 2 °C) and was maintained in 12 h light/dark cycle. The rats were randomly divided into the following 3 groups: sedentary, control and treatment groups (FHE). The treatment group rats were administered orally with FHE (10 mg/kg of body weight/per day) for a period of two weeks. Sedentary and control rats were orally administered with equal amount of distilled water. The animals were fed with a commercial pellet diet (Sri Venkateswara Enterprises, Bangalore, India) and water *ad libitum*.

2.4.2. Weight loaded forced swim test (WFST)

The weight loaded forced swim test (WFST) was performed as described previously with some minor modifications.[17] The rats of FHE administered group and control group were allowed to swim with constant loads (tagged to the tail base) corresponding to 5% of their body weight. The swimming exercise was carried out in small tank with 30 cm deep with water maintained at 25 ± 2 °C. Exhaustion was determined by observing loss of coordinated movements and failure to return to the surface within 10 s.[18] This experiment was repeated every alternate day for a period of three weeks. Animals were sacrificed under mild anesthesia immediately after the last exercise. Blood was collected from the heart using a heparinized syringe into centrifuge tubes. Separated plasma and tissue samples of brain, liver and muscle were stored at −80 °C until further analysis.

2.4.3. Measuring blood biochemical parameters

Serum BUN, CK, lactic acid and plasma glucose were determined using commercially available kits from Agappe Company, India.

2.4.4. Determination of glycogen and lactic acid

Liver and muscle tissues were digested with 2 ml of KOH (30%) and boiled in water bath for 30 min with occasional shaking and then allowed to cool at room temperature. Saturated Na_2SO_4 solution was added to the mixture and stirred well. Glycogen was precipitated by adding 5 ml of ice cold ethanol to the sample mixture and centrifuged at 10,000 rpm for 10 min. One ml of HCl (1.2 N) was added to the supernatant (1: 1 v/v) and incubated at 90 °C for 2 h, and then allowed to cool. DNS method was followed to determine hydrolyzed product of tissue glycogen.[19] Lactic acid content was measured by the method of Sawhney and Singh.[20] Tissue samples were homogenized in phosphate buffer (100 mM, pH 7.2), and deproteinized with TCA (10%). These deproteinized samples were centrifuged at 5000 rpm for 15 min. To the supernatant, 1 ml of copper sulfate solution (20%) and 6 ml of H_2SO_4 (conc.) were added and kept in boiling water bath for 5 min and allowed to cool. One hundred micro liters of p-hydroxydiphenyl reagent was added to the above sample mixture and incubated at 37 °C for 30 min. The absorbance was measured at 560 nm.

2.4.5. Determination of SOD, CAT and GPx activities

Liver tissues were homogenized in 50 mM phosphate buffer saline (pH 7.4). SOD and GPx activities were determined with the commercially available kits (Randox, Canada; Cat no. SD 125 and RS 504, respectively). CAT was determined by measuring the decay of

6 mM H_2O_2 solution at 240 nm by spectrophotometric degradation method.[17] Briefly, Liver tissue was homogenized in phosphate buffer (50 mM; pH 7.0). Centrifuged at 8000 rpm for 20 min and supernatant was diluted in phosphate buffer (1:100). To 100 µl of supernatant, 200 µl of phosphate buffer and 700 µl of H_2O_2 (660 mM) was added and absorbance was read at 240 nm for 3min at 15 s interval.

2.4.6. Determination of thiobarbituric acid-reactive substances (TBARS)

TBARS as malondialdehyde (MDA µmol/cm/g) was analyzed by Buege and Aust.[21] Brain, liver and muscle tissues (100 mg) were homogenized in 2 ml of phosphate buffer (pH 7.0). TCA (10%), 0.5 ml and 2 ml of TBA mixture were added to tissue homogenate (0.5 ml). The TBA mixture contains TBA (0.35%), SDS (0.2%), $FeCl_3$ (0.05 mM) and BHT in glycine-HCl buffer (100 mM, pH 3.6). The above reaction mixture was boiled at 100 °C for 30 min and then allowed to cool. The mixture was centrifuged at 8000 rpm for 10 min and the absorbance was measured at 532 nm.

2.5. Statistical analysis

The data are expressed as mean ± standard deviation of the mean (S.D). Data was analyzed using one-way ANOVA. Differences at $p < 0.05$ were considered to be significant.

3. Results and discussion

3.1. In vitro antioxidant activities of FHE

Fenugreek (*T. foenum-graecum L.*) is a multi-purpose medicinal plant. It is being used as nutraceutical, functional food, as well as forage crop for livestock feed.[22] Phytochemistry of the fenugreek seeds reveal the presence of tannic acid, fixed and volatile oils, steroidal saponin diosgenin, alkaloids trigonelline, trigocoumarin, trigomethyl coumarin, and steroidal saponins such as gitogenin, trigogenin and vitamin A.[23] Phenolic compounds are a class of antioxidant agents which act as free radical scavengers and are considered as a major group of compounds that contribute to the antioxidant activities of plant materials because of their neutralizing ability on free radicals due to their hydroxyl groups.[24] Flavonoids are a group of polyphenolic compounds with known properties of free radical scavenging, antibacterial and anti-inflammatory action.[25] Different extraction protocols of fenugreek were reported in a vast range of polyphenols and flavonoids by Bukhari et al,[26] and the range was 1.35–6.85 mg/g GAE and 208–653 µg/g QE, respectively. However, the present extraction methodology of fenugreek had given more polyphenol (22 ± 1.5 µg/mg GAE) and flavonoid (16.6 ± 1.2 µg/mg QE) contents when compared to other extraction process by earlier researchers.[26] It is well-known that phenolic compounds contribute to quality and nutritional value in terms of modifying color, taste, aroma, flavor and also in providing health beneficial effects. The present results showed that the FHE extract is rich in amino acids contents. Moreover, fenugreek seed is rich with several amino acids like lysine, trypthophan[6] and 4-hydroxyisoleucine, which is reported as a major bioactive compound for hypoglycemic activity.[27,28] In the present study, FHE also showed high quantity of amino acid i.e. 50.0 ± 2.8 µg/mg glycine equivalents. This implied that the amino acids in the FHE can enhance exercise ability.

Oxidative stress plays an important role in the pathophysiology of exhaustive exercises with fatigue condition.[29] Free radicals cause oxidative damage to nucleic acids, proteins, carbohydrates and lipids. The antioxidant property of an extract neutralizes the free radicals and controls the oxidative damage of tissues in the treatment of fatigue syndrome.[30,31] The antioxidant activity of the FHE increased with an increasing amount of extract. FHE was able to reduce the stable free radical DPPH to yellow colored diphenylpicrylhydrazine with an IC_{50} of 395 µg/ml ($R^2 = 0.9712$). In the hydroxyl radical mediated 2-deoxy-D-ribose degradation assay, IC_{50} value were calculated using linear regression equation ($R^2 = 0.9889$) and showed $IC_{50} = 188.6$ µg/ml. The result of ABTS radical scavenging activity and metal chelating activity of FHE are also in dose depended manner with IC_{50} values of 96.7 µg/ml ($R^2 = 0.9929$) and 69.7 µg/ml, respectively ($R^2 = 0.9978$). Moreover, in the present study, FHE is observed with excellent antioxidant activity and assume that these scavenging activities could help in delaying the fatigue or in the formation of free radicals in exercised stress conditions.

3.2. In vivo endurance capacity of FHE

3.2.1. Effect of FHE on prolonged swimming time

In the present study, 10 mg/kg bwt./day of FHE was orally fed to the rats for a period of two weeks to know the swimming endurance capacity. Present results showed that there was no significant change in weight gain by the rats in sedentary group, control group and FHE supplemented group (Results not shown). Muralidhara et al,[32] showed that the fenugreek powder, administered intra gastrically to mice and rats of both sexes failed to induce any signs of toxicity or mortality up to a maximum dosage of 2 and 5 g/kg body weight, respectively. Khalki et al,[33] reported with no mortality or treatment-related signs of abnormal behavioral changes at the dosage of 500 and 1000 mg/kg bwt. in the female rodents. However, Araee et al[34] has considered that in view of the presence of the steroidal saponin diosgenin in fenugreek seeds, in high doses adversely influences bone marrow cell proliferation. Hence, the dosage of FHE used in the study (10 mg/kg bwt./day) has been proved to be non toxic.

The weight loaded forced swim test (WFST) was carried out with 5% tail load for a period of three weeks (first two weeks with FHE treatment and then, third week without FHE treatment). The forced swim test is perhaps one of the most commonly used animal models for evaluating the physical endurance capacity.[35] On the first and second day of WFST, no significant difference of swimming time was recorded between control and FHE treated groups ($p > 0.05$). Third day onwards, the swimming time was gradually increased with FHE treatment when compared to that of control

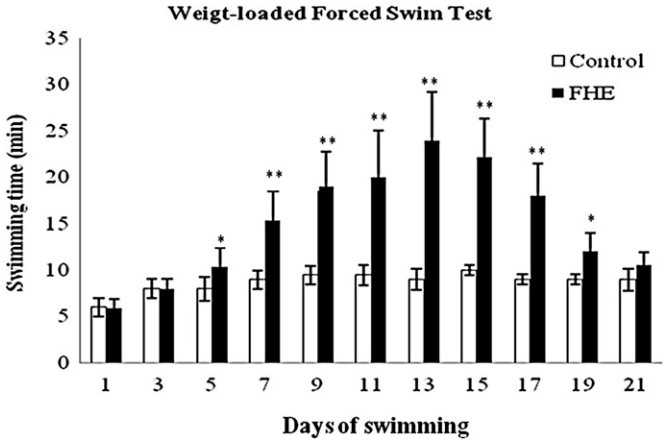

Fig. 1. Effect of FHE on physical endurance capacity by weight loaded forced swim test. Data express the mean ± S.D. for 6 rats. Different symbols above the bar indicates statistically significant differences for the same day, $^*p < 0.05$, $^{**}p < 0.01$ *vs* control; FHE = fenugreek hydro-alcoholic extract.

group ($p < 0.05$). Maximum swimming time was recorded on day 13 of the FHE treatment with 24 ± 4.2 min (Fig. 1). The day 15 onwards it was observed in decreased swimming performance due to withdrawal of the extract. However, the swimming time of the FHE treated group was more when compared with control group. In the present study, the swimming time data showed that the administration of FHE could evidently extend swimming time for exhaustion, indicating FHE has anti-fatigue activity and could elevate the exercise tolerance.

3.2.2. Effect of FHE on blood biochemical parameters

The blood biochemical levels of BUN, CK, glucose and lactic acid of all studied groups are shown in Table 1. The homeostasis of blood glucose plays an important role in prolonging endurance exercise. Continuous exercises often lead to hypoglycemia and can suppress the active functioning of the brain. Therefore, the amount of blood glucose can illustrate the speed and degree of fatigue development.[18] Simple exercise begins with an increase in aerobic muscular activity. However, intensive exercises switch over to anaerobic metabolism, which converts LDH to lactic acid.[36] In the present experiment the exposure to the WFST led to decrease in plasma glucose and increase in serum lactic acid levels in control group comparison with the sedentary group ($p < 0.05$). However, the FHE treatment reversed these levels (Table 1).

Blood urea nitrogen represents normally kidney function; however, there are many factors like protein breakdown, dehydration, stress, fatigue, etc., that can cause an alteration in BUN levels. This protein and amino acids are metabolized to meet the energy requirement when the body cannot derive energy from carbohydrate and fat. There may be a positive correlation between the urea nitrogen and exercise tolerance.[18] The present results indicate that the levels of BUN in FHE group were lower than that of control group ($p < 0.01$), suggesting that FHE may reduce protein catabolism for energy. One of the functions of creatine kinase in cells is to add a phosphate group to creatine, to synthesize high-energy phosphocreatine molecule, utilized as a rapid source of energy by the cells. During the process of extreme muscle stress, the muscle cells degenerate and their contents find in the blood stream. Because most of the CK in the body normally exists in the muscle, an increase in CK in the blood indicates that muscle damage has occurred or is occurring.[37] The most intriguing finding of the study was the significant decreases in serum CK (a biomarker of muscle fiber damage) by FHE group, when compared to the control group ($p < 0.05$). The homeostasis of blood glucose plays an important role in performance enhancement.[38] As shown in Table 1, the blood GLU levels of FHE were higher than that of the control group. Intensive exercise leads to the accumulation of blood lactic acid,[18,37] the present results confirms that the LA levels were significantly lowered by FHE treatment when compared with control group ($p < 0.05$).

Table 1
Effect of FHE on blood biochemical parameters after the weight loaded forced swim test.

Group	BUN (mg/dL)	CK (U/L)	GLU (mmol/L)	LA (mg/dL)
Sedentary	17.7 ± 1.5	172 ± 30.7	7.15 ± 0.8	59 ± 8
Control	21.1 ± 2.0**	199 ± 25.2**	6.62 ± 0.5*	75 ± 11**
FHE	18.3 ± 1.6$^{\#\#}$	184 ± 19$^{\#}$	6.74 ± 0.3	68 ± 9$^{\#}$

Values are expressed as mean ± S.D; $n = 6$ in each group, statistically significant differences, $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* sedentary; and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs* control. BUN = blood urea nitrogen, CK = creatine phosphokinase, GLU = glucose, LA = lactic acid; sedentary = rat unexposed to the WFST and treated with distilled water, control = rat exposed to the WFST and treated with distilled water. FHE = rat exposed to the WFST and treated with fenugreek extract.

Table 2
Effect of FHE on antioxidant enzymes of liver tissues after the weight loaded forced swim test.

Group	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)
Sedentary	6.31 ± 0.9	29.0 ± 2.1	2202 ± 181
Control	4.91 ± 1.0**	20.8 ± 3.3**	1689 ± 175**
FHE	6.05 ± 0.5$^{\#\#}$	22.0 ± 1.1$^{\#}$	2084 ± 158$^{\#\#}$

Values are expressed as mean ± S.D; $n = 6$ in each group, statistically significant differences, $^{**}p < 0.01$ *vs* sedentary; and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs* control. SOD = Superoxide dismutase, CAT = Catalase, GPx = Glutathione peroxidase. Sedentary = rat unexposed to the WFST and treated with distilled water, control = rat exposed to the WFST and treated with distilled water. FHE = rat exposed to the WFST and treated with fenugreek extract.

3.2.3. Effect of FHE on antioxidant enzymes activity

Activity of liver SOD, CAT and GPx were decreased by swimming exercise when compared with sedentary group rats ($p < 0.01$). However, these antioxidant activities were up regulated with supplementation of FHE by 23.2%, 5.7% and 23.3% respectively, when compared with control group (Table 2). Recent studies show that oxidative stress plays a very important role in the etiology of chronic fatigue syndrome and that antioxidants might be useful in its treatment.[29] The primary antioxidant enzymes include SOD, GPx and CAT.[37] SOD dismutates superoxide radicals to form H_2O_2 and O_2. GPx is an enzyme responsible for reducing H_2O_2 to water and alcohol, respectively. CAT catalyzes the breakdown of H_2O_2 to form water and O_2. These antioxidant defense mechanisms become weaker during chronic fatigue and other disease conditions.[37] So, the improvement in the activities of these defense mechanisms can help to fight against fatigue. In the present results, the exogenous antioxidants from FHE may act directly or interact with endogenous antioxidants to form a cooperative network of cellular antioxidants to defend fatigue and strengthen the endurance capacity.

3.2.4. Effect of FHE on lipid peroxidation, glycogen and lactic acid

Lipid peroxidation is known to play an important role in the pathophysiology of fatigue syndrome.[30] Lipid peroxidation in liver and muscle tissues represented as malondialdehyde (MDA μmol/cm/g) and results are shown in Fig. 2. Swimming exercise significantly increased MDA concentration in liver and muscle when compared with sedentary group ($p < 0.05$). However, FHE supplemented group decreased the MDA levels in liver and muscle tissues by 4.6% and 25.1% respectively, compared with control group ($p < 0.01$). Glycogen is an important source of energy during physical exercises, hence glycogen contents are sensitive

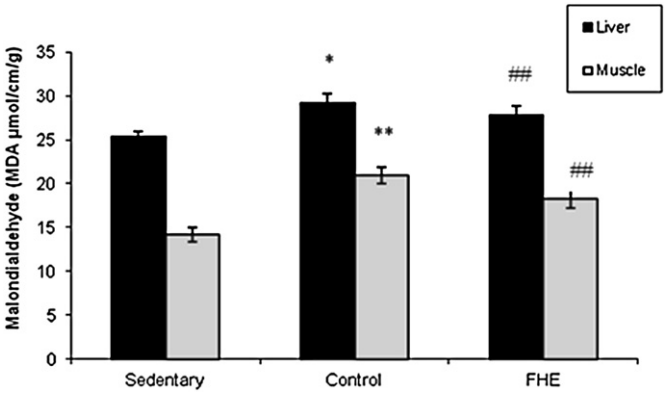

Fig. 2. Effect of FHE on thiobarbituric acid-reactive substances (TBARS). Data express the mean ± S.D. for 6 rats. Different letters above the bar indicates statistically significant differences, $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* sedentary; and $^{\#\#}p < 0.01$ *vs* control, FHE = fenugreek hydro-alcoholic extract.

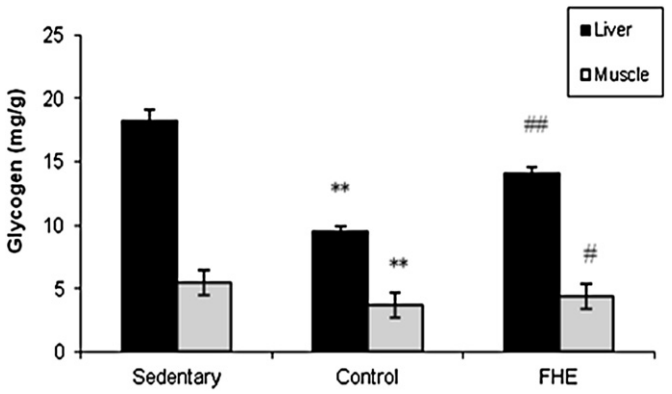

Fig. 3. Effect of FHE on glycogen content in liver and muscle. Data express the mean ± S.D. for 6 rats. Different symbols indicates statistically significant differences, $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* sedentary; and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs* control, FHE = fenugreek hydro-alcoholic extract.

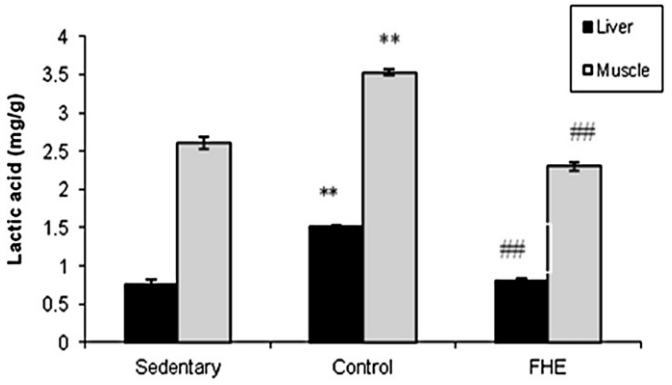

Fig. 4. Effect of FHE on lactic acid content in liver and muscle. Data express the mean ± S.D. for 6 rats. Different symbols above the bar indicates statistically significant differences, $^{**}p < 0.01$ *vs* sedentary; and $^{\#\#}p < 0.01$ *vs* control, FHE = fenugreek hydro-alcoholic extract.

biomarkers related to fatigue. As per the previous reports, the depletion of liver and muscle glycogen is common phenomena in physical exercises.[35] Depletion of liver glycogen may lead to hypoglycaemia impairing nervous function in over exercise fatigue condition.[39] In the study, swimming exercise reduced the levels of liver and muscle glycogen when compared to that of sedentary group ($p < 0.01$). As shown in Fig. 3, the glycogen content of liver and muscle were also significantly increased by the FHE supplementation ($p < 0.01$ and $p < 0.05$, respectively). Lactic acid is one of biomarkers judging the degree of fatigue.[18] In the present study, the lactic acid levels were significantly reduced by FHE treatment in both liver ($p < 0.01$) and muscle ($p < 0.05$) (Fig. 4).

4. Conclusion

The results show that hydro-alcoholic extract of *T. foenum-graecum* is rich in polyphenol, flavonoid and amino acid contents. The results have also concluded that FHE is rich with *in vitro* antioxidant activities along with physical endurance capacity to delay fatigue in rat model. The present study has demonstrated the potential of FHE in reducing physical fatigue by prolonged the exhaustion swimming time and modulating several biochemical markers. Therefore, FHE can elevate the endurance capacity and facilitate recovery from fatigue. However, further research is warranted to elucidate its mechanism.

Conflicts of interest

All authors have none to declare.

Acknowledgments

The authors are grateful to the Director, Defence Food Research Laboratory (DFRL), Mysore, India for providing technical support and valuable suggestions.

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Pharmacognosy Journal 5 (2013) 72–76

Contents lists available at SciVerse ScienceDirect

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Original article

Formulation and standardization of Medhya Rasayana – A novel Ayurvedic compound nootropic drug

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ARTICLE INFO

Article history:
Received 10 January 2013
Accepted 15 March 2013
Available online 28 March 2013

Keywords:
Medhya Rasayana
Ayurvedic nootropic
Poly herbal formulation
HPTLC

ABSTRACT

Introduction: Medhya Rasayana is a poly herbal formulation widely used in Ayurvedic clinical practice with multi fold benefits, specifically to improve memory and intellect by their Prabhava (specific action) namely Medhya (Nootropic). There is no work on record on formulation and standardization aspect of a compound formulation containing nootropic herbs. This study highlights physico-chemical characterization, TLC and HPTLC densitogram profiling of Medhya Rasayana which can be applied for authentication of this poly herbal formulation.
Methods: Four Medhya Rasayana herbs namely, Mandukaparni (*Centella asiatica* Linn.), Yastimadhu (*Glycyrrhiza glabra* Linn.), Guduchi (*Tinospora cordifolia* (Wild) Miers.) and Shankhapushpi (*Evolvulus alsinoids* L.) were authenticated botanically. Tablets were prepared by combining all these four drugs and subjected for detailed physico-chemical and HPTLC analyses.
Results: Set of standardization parameters were derived for the compounded tablet containing four Medhya Rasayana herbs by physico-chemical characterisation. The tests proposed would serve as diagnostic parameters for the identity of this poly herbal formulation. HPTLC fingerprint profile which can serve as a fingerprint for the identification of the formulation has been obtained.
Conclusion: The proposed method of making tablet from four Medhya Rasayana herbs will aid in yielding concentrated medicament with the same efficacy as per the classically proposed drug dosage at lower dose. Standards for the poly herbal formulation has been developed for the quality check of the formulation.

1. Introduction

Ayurveda, the Indian system of medicine is the first recorded medical science widely practiced in India since ancient times. In recent years there is global revolution worldwide towards acceptance of this holistic science owing to its effectiveness and safety. The increasing demand at the global level has created great need to standardize herbal medicines. The earliest references of drug standardizations are mentioned in Ayurveda classics under the speciality of Bhaishajya Kalpana and Rasa Shastra which exclusively deal with drug formulation and manufacturing. Most of the tests described in ancient literature appear to be based on observations and seems to be subjective without valid scientific backing. Hence standardization and development of reliable quality protocols are important.[1]

Medhya Rasayana are group of drugs widely used in Ayurveda since antiquity with manifold benefits specifically to improve memory and intellect by their Prabhava (specific action) namely Medhya (Nootropic). Medhya Rasayana comprises group of 4 drugs namely Mandukaparni (*Centella asiatica* Linn.), Yastimadhu (*Glycyrrhiza glabra* Linn.), Guduchi (*Tinospora cordifolia* (Wild) Miers.) and Shankhapushpi (*Convolvulus pleuricaulis* Chois or *Evolvulus alsinoides*),[2] that can be used singly or in combinations. They are specially mentioned for children with wide range of applications on different systems. Considering the overall effect of the individual Medhya Rasayana, a thought was given to develop a combination of all these and to standardize the same for wide spectrum use. As the majority of the drugs used in the preparation are predominant of Tikta rasa (bitter), obviously the prepared drug will also be the same. Hence, classical dosage forms of these drugs would be difficult to be given to children owing to unacceptable palatability. Storage of drugs over a period in the classical described form is also a difficult task. Fresh preparation and administration thereon of drug to large population is also not easy. Maintaining

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0975-3575/$ – see front matter
http://dx.doi.org/10.1016/j.phcgj.2013.03.004

uniform dosage also will be a challenge. Considering these facts, it was thought to develop tablets from the combination drug which would be most suitable mode of administration. More over the proposed method of making it into Rasakriya tablet will also aid in yielding concentrated medicament with the same efficacy as for the classically proposed drug dosage at lower dose. The drug thus prepared will also be benefited with enhanced shelf life of 1 year.[3] Development of a composite standardization protocol for Medhya Rasayana (MR) in tablet form is aimed in the current study.

2. Materials and methods

2.1. Plant materials

Required plant medicines such as whole plant of Mandookaparni, stem of Guduchi and root of Yastimadhu were collected from authorized raw drugs suppliers of SDM Ayurveda Pharmacy, Kuthpady, Udupi. Shankhapushpi was collected from M/s Dhanwantari Vana, the Garden for Medicinal plants in Bangalore, Government of Karnataka undertaking. The raw materials were first identified and authenticated by experts at SDM Ayurveda Pharmacy, Kuthpady, Udupi.

2.2. Preparation of Medhya Rasayana tablet

As per the classical description Guduchi Kashaya (decoction),[4] Shankhapushpi Kalka (macerated paste of fresh plant)[5] and Mandukaparni Swarasa (fresh juice)[6] prepared separately and were later mixed thoroughly and heated on Mandagni (mild fire). When decoction became thick, Yastimadhu Churna (powder) was added and heated till it became semi solid in consistency (method of preparation of Rasakriya).[7] Tablets of 250 mg (drug weight) were punched and packed in air tight containers.

2.3. Instrumentation and techniques

Physico-chemical determination of total ash, acid insoluble ash and water soluble ash, loss on drying at 110 °C, water soluble extractive, and alcohol soluble extractive tests were done at Bangalore Test house as per API[8] standards. Assay for bitter substances and glycyrrhizin contents were done as per the protocol in standardization of botanicals.[9] Disintegration time of the tablets was assessed as per IP.[10] HPTLC studies were done at SDM Centre for Research in Ayurveda and Allied Sciences, Kuthpady, Udupi as per standard procedure.[11,12] To develop HPTLC fingerprinting of chloroform and alcohol (successive) extract of MR, 5 g of the powdered tablets were extracted successively with chloroform and ethanol using a Soxhlet extractor. The extracts were separately distilled off and dissolved in 30 ml of respective solvents. 10, 20 and 30 μl of the above extract is applied on precoated silica gel F_{254} on aluminium plates to a band width of 7 mm at 3 concentration levels of 10, 20 and 30 μl using Linomat 5 TLC applicator. The chloroform extract was developed in toluene:ethyl acetate (6.5:2.5) and the alcohol extract in toluene:ethyl acetate:acetic acid:methanol (3:5.5: 0.8: 0.1).

HPTLC fingerprint profile MR tablet and its ingredients were obtained by extracting 3 g each of the samples in 10 ml ethanol by cold percolation for 48 hours. The filtrates were made up to 30 ml and 20 μl of the above extracts were applied on a precoated silica gel F_{254} on aluminium plates to a bandwidth of 8 mm using Linomat 5 TLC applicator. The plate was developed in toluene–ethyl acetate (7:2.5) and the developed plates were visualized and scanned under UV 254, 366, and after derivatisation in vanillin-sulphuric acid spray reagent at 620 nm. R_f, colour of the spots, densitogram were recorded.

3. Results

Physico-chemical standards for poly herbal formulation MR is presented in Table 1. R_f values of the spots and their colour by TLC photo-documentation of chloroform and alcohol extracts of MR have been developed. Chloroform extract of MR at 254 nm showed 8 spots (0.11 Dark green, 0.22 Green, 0.31 Dark green, 0.36 Green, 0.43 Green, 0.65 Green, 0.69 Green, 0.80 Dark green) whereas under 366 nm it showed 15 spots (0.02 Fluorescent (F) brown, 0.04 F blue, 0.07 F pink, 0.09 F blue, 0.11 F blue, 0.18 F blue, 0.22 F blue, 0.33 F blue, 0.43 F violet, 0.51 F light blue, 0.57 F violet, 0.65 F blue, 0.71 F pink, 0.76 F pink, 0.84 F pink) and 9 spots (0.22 Yellow, 0.33 Dark pink, 0.43 Yellow, 0.54 Green, 0.57 Green, 0.65 Pink, 0.74 Pink, 0.84 Light green, 0.92 Dark green) after derivatisation using toluene:ethyl acetate (6.5:2.5) as solvent system. Alcohol extract of MR at 254 nm showed only 3 spots (0.18 Light green, 0.35 Light green, 0.45 Green) whereas under 366 nm it showed 7 spots (0.18 F green, 0.43 F blue, 0.52 F green, 0.61 F violet, 0.66 F blue, 0.70 F yellow, 0.75 F pink) and 5 spots (0.06 Brown, 0.09 Yellow, 0.56 Orange, 0.64 Pink, 0.70 Blue) after derivatisation using toluene:ethyl acetate:acetic acid:methanol (3:5.5:0.8:0.1) as solvent system.

The product MR tablet was analysed for its fingerprint in comparison with its four ingredients by TLC photo documentation using toluene:ethyl acetate (7:2.5) as solvent system. At 366 nm, 4 spots of the MR showed same R_f value (0.03 F pink, 0.52 F pink, 0.62 F green and 0.78 F pink) as that of *E. alsinoides*. Spots with R_f 0.62 (F green) and 0.78 (F pink) being of same colour indicated the presence of *E. alsinoides* in the formulation. Out of 12 spots of MR at 366 nm, 5 spots showed same R_f values (0.03 F pink, 0.52 F pink, 0.62 F green, 0.71 F pink and 0.78 F pink) in *C. asiatica*. Spots with R_f 0.62 (F green), 0.71 (F pink) and 0.78 (F pink) being of same colour indicating the presence of *C. asiatica* in MR as one of the ingredient. 5 spots of MR showed same R_f value (0.03 F blue, 0.06 F blue, 0.14 F blue, 0.34 F blue and 0.62 F green) in *G. glabra*. Spots with R_f 0.03 (F blue), 0.06 (F blue) and 0.34 (F blue) were being of same colour indicating the occurrence of *G. glabra* in MR. None of the spots detected in MR corresponded to that in *T. cordifolia* at 366 nm which may be due to the presence of high polar of compounds which were not resolved in the solvent system used. HPTLC fingerprint profile and densitometric scan of chloroform and alcohol extract as well as MR in comparison to its ingredients at UV 254 nm, 366 nm, and post derivatisation at 620 nm were developed to help in the standardization of the phytochemicals in the formulation (Figs. 1 and 2).

4. Discussion

Despite the advent of modern technology in standardization of compound formulations, only a few Ayurvedic poly herbal medicines are standardized so far. The novel concentrated compound formulation Medhya Rasayana vati thus developed yield better

Table 1
Physico-chemical characters of Medhya Rasayana tablet.

Parameters	Results
Loss on drying at 110 °C (% w/w)	6.065
Total ash (% w/w)	10.80
Water soluble ash (% w/w)	8.13
Alcohol soluble extractive (% w/w)	15.3
Water soluble extractive (% w/w)	25.01
Average weight (g)	0.3614
Hardness (kg/cm^2)	2
Disintegration time (min)	2
Bitter content (% w/w)	4.81
Glycyrrhizin content (% w/w)	2.82

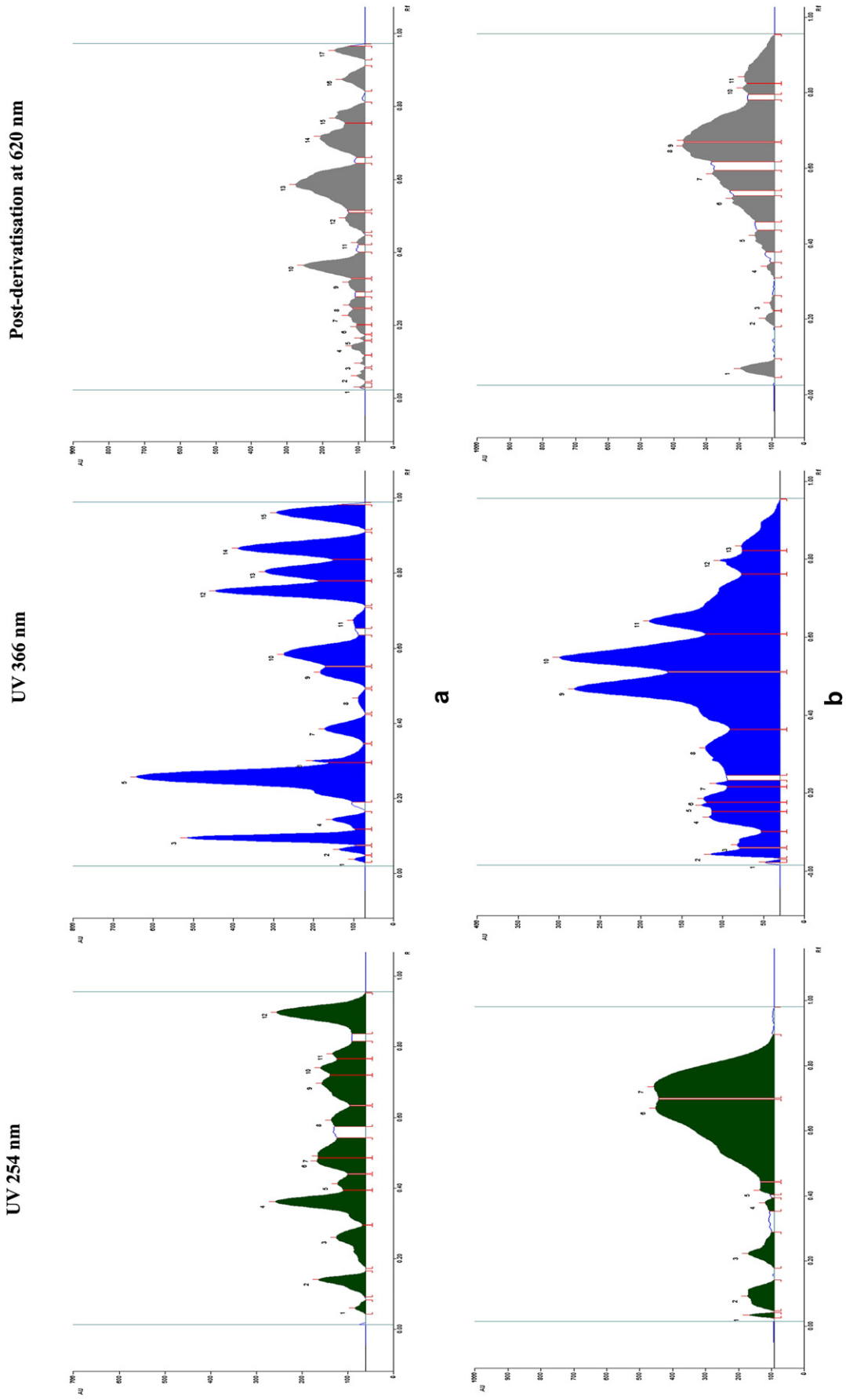

Fig. 1. HPTLC densitometric scan of chloroform and alcohol extract of Medhya Rasayana. (a): Chloroform extract using toluene:ethyl acetate (6.5:2.5), (b): Alcohol extract using toluene:ethyl acetate:acetic acid:methanol (3:5.5:0.8:0.1).

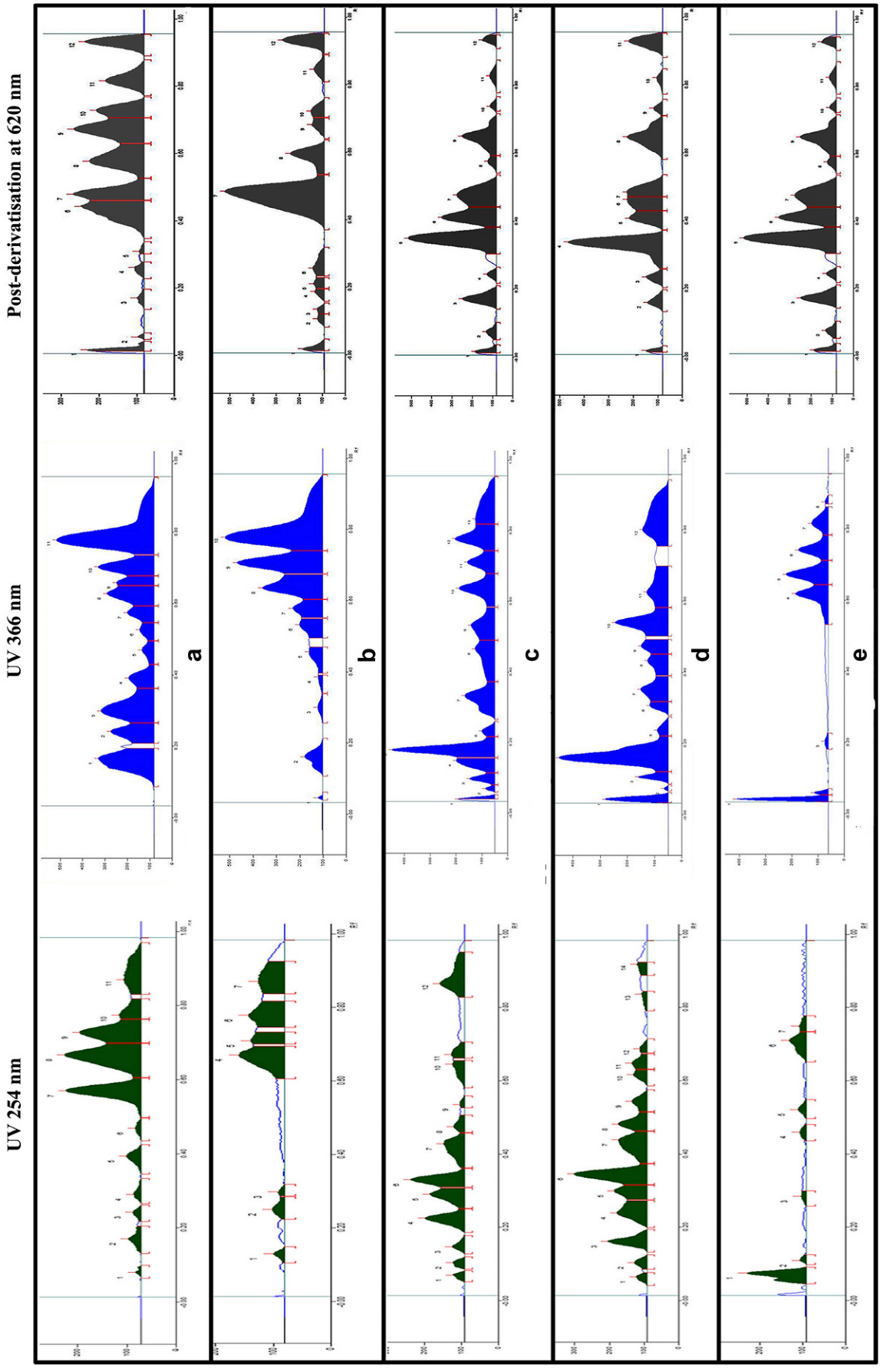

Fig. 2. HPTLC densitometric scan of Medhya Rasayana with its ingredients. (a): *E. alsinoides*, (b): *C. aciatica*, (c): Medhya Rasayana, (d): *G. glabra*, (e): *T. cordifolia*.

acceptability in terms of palatability, increased shelf life and as compared to prescribed forms in a lesser dose. The physico-chemical standards would serve as preliminary test for the standardization of the formulation. The unique R_f values, densitometric scan and densitogram obtained at different wavelengths pre- and post-derivatisation can be used as fingerprint to identify the poly herbal combination Medhya Rasayana. The current investigation can be used as standardization test for the newly developed compound formulation for Nootropic action. Further, detailed macro-microscopic examination of the raw drug as whole and powder form in comparison with the formulation would add to the standardization test of the Medhya Rasayana vati.

Conflicts of interest

All authors have none to declare.

Acknowledgements

Authors are highly grateful to our revered President, Dr. D. Veerendra Heggade and Dr. B. Yashoverma, Secretary, SDM Educational Society for the encouragement. Authors highly regard the constant support of Dr. Prasanna N Rao, Principal, Dr. Shailaja U, Head, Department of Kaumarabhritya and Dr. Girish K J, Research co-ordinator SDM College of Ayurveda, Hassan. Authors thank Dr. B. Ravishankar, Director, SDM Centre for Research in Ayurveda and Allied Sciences for providing the facilities and Miss Rajalekshmi and Miss Sangeetha for help in carrying out the studies.

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Pharmacognosy Journal 5 (2013) 77–79

Contents lists available at SciVerse ScienceDirect

Pharmacognosy Journal

journal homepage: www.elsevier.com/locate/phcgj

Short communication

Volatile oil composition and antimicrobial activity of two *Thymus* species

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ARTICLE INFO

Article history:
Received 10 May 2012
Accepted 7 September 2012
Available online 19 March 2013

Keywords:
Medicinal plants
Essential oil
Antimicrobial and antifungal activity

ABSTRACT

Background: Medicinal plants have potential for using as antimicrobial agents against pathogens. In genus *Thymus* (Lamiaceae), phenolic compounds with terpene origin are responsible of these effects.
Objective: Evaluated essential oil constituents and antimicrobial activity of *Thymus daenensis* compared with *Thymus vulgaris*.
Materials and methods: Staphylococcus aureus, *Bacillus subtilis*, *Escherichia coli*, *Candida albicans* and *Aspergillus niger* were used for antimicrobial activity assay with using disc diffusion technique. Volatiles oils compositions were analyzed by GC/MS.
Results: Escherichia coli was resistant to essential oils. Essential oil obtained from *T. daenensis* showed better antimicrobial activity than with *T. vulgaris*. Thymol (84.45%) and carvacrol (46.62%) were the main components of *T. daenensis* and *T. vulgaris* respectively.
Conclusion: The results showed that *T. daenensis* was more effective and could be an alternative for *T. vulgaris*. In addition, these essential oils could be used as natural antimicrobial agents.

1. Introduction

Antimicrobial effects especially against infectious pathogens are one of potentials of medicinal plants. *Thymus* genus (Lamiaceae) has with about 215 species.[1] In genus *Thymus*, phenolic compounds with terpene source are responsible for these effects. Most of their properties are due to essential oils.[2] Volatile oils possess antimicrobial, antitussive, digestive, expectorant and carminative activities.[3] *Thymus daenensis* which is endemic of Iran[4] has antimicrobial properties[5] and its beneficiary for asthma and bronchitis has been proved.[6] *Thymus vulgaris* is a well-known species of *Thymus* genus. The oil extracted of *T. vulgaris* is used as food preservative, antiworm,[7,8] antiseptic and antispasmodic.[9] In traditional medicine, juices of flowers have been generally used for relieve headache, dysmenorrhea, and digestive disorders in some countries.[10] Aerial parts of plants containing glands which are rich in essential oil.[11,12] Antimicrobial and antiseptic effects of genus *Thymus* have been studied by many researchers, although fewer studies carried out on *T. daenensis*. Present study indicated comparison of antimicrobial activity and essential oil compounds of *T. daenensis* and *T. vulgaris*.

2. Materials and methods

2.1. Plant materials

T. daenensis and *T. vulgaris* were collected from Daran (East of Esfahan province, Iran) and Abadeh (Northwest of Fars province, Iran) respectively in early of June 2011 in flowering stage. *T. daenensis* identity was confirmed and voucher specimen was deposited at the Forest and Rangel research center of Iran with No. 13353 and voucher specimen of *T. vulgaris* deposited in Shiraz Faculty of Pharmacy herbarium with No. pm153.

2.2. Essential oil extraction

Essential oils were obtained from aerial, air shad dried parts, after powdered and subjected to hydro-distillation using Clevenger system during 5 h, according to the method recommended in British Pharmacopoeia. The oils were dehydrated by sodium sulfate and stored in refrigerator in dark vials.[13]

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0975-3575/$ – see front matter
http://dx.doi.org/10.1016/j.phcgj.2012.09.002

2.3. Gas chromatography/mass spectrometry conditions

A Hewlett–Packard GC/MS 6890 system with an HP-5M capillary column (phenyl methyl siloxan, 25 m × 0.25 mm i.d., Hewlett Packard part No.190915.433, USA) was used for the identification of volatile compounds. GC oven temperature was kept constant at the rate of 3 °C/min. Mass spectra (Hewlett Packard 5973,USA) were taken at 70 eV. The injector temperature was 250 C; mass range was between 30 and 600 *m/z*. Compounds identify was accordance on comparison of their RT and mass spectra with Willey (275) and Adams libraries spectra.[14]

2.4. Standard bacteria and fungi

Standard germs (ATCC) were used for investigation of antimicrobial activities. The germs were prepared from center of microbial collection of Iran and stock cultures were stored in refrigerator. *Staphylococcus aureus* (1112), *Bacillus subtilis* (1023), *Escherichia coli* (1397), *Candida albicans* (5027) and *Aspergillus niger* (5162) were the microorganisms.

2.5. Culture media

Muller Hinton agar media (Merck, Darmstadt, Germany) was used for antibacterial assay and Sabouraud dextrose agar (Merck, Germany) was used as a medium for antifungal assay. Agar diffusion and Agar dilution technique were used for this study.[15]

Table 1
Chemical constitutes of essential oils.

Compound	KI	*T. daenensis*	Compound	KI	*T. vulgaris*
α-Pipene	941	0.676	α-Thujene	932	1.164
p-Cymene	1027	2.662	α-Pipene	941	0.851
1,8-Cineole	1034	3.739	Camphene	958	0.537
γ-Terpinene	1065	2.851	β-Pinen	983	0.805
Thymol	1291	84.41	Myrcene	996	1.209
β-Caryophyllene	1427	4.482	α-Phellandrene	1006	0.459
			α-Terpinen	1020	2.116
			β-Phellandrene	1032	0.616
			1,8-Cineole	1033	1.198
			γ-Terpinene	1064	9.699
			Linalool	1098	2.408
			Borneol L	1172	0.952
			α-Terpineol	1193	0.851
			1-Isopropyl-2-methoxy-4-methyl Benzene	1199	1.814
			Carvacrol, methyl ether	1248	1.142
			Carvacrol	1303	46.62
			β-Caryophyllene	1429	1.859
			Δ-Cadinene	1528	0.313
			Caryophyllen oxide	1584	0.19
			γ-Selinene	1586	0.089
			9-Octadecenoic acid	1589	1.5
			Docosane	2205	1.299
			Tricosane	2306	0.806
			Tetracosane	2405	3.595
			Pentacosane	2504	6.361
Monoterpene hydrocarbons		6.189			17.45
Oxygen containing monoterpenes		88.149			52.02
Sesquiterpene hydrocarbons		–			12.463
Oxygen containing sesquiterpenes		4.482			4.691
Other		–			1.814
Total		98.82			88.453

Table 2
Antimicrobial activity of essential oil by disc diffusion method.

Plant	*Thymus daenensis*				*Thymus vulgaris*				
Microbe	2 μL	3 μL	4 μL	5 μL	2 μL	3 μL	4 μL	5 μL	Control
Staphylococcus aureus	31.7	33.7	35	38.4	25	29.7	33	35.7	23_{Am}
Bacillus subtilis	35.7	38.4	40.4	43	30.4	32.4	33	39.7	17_{Am}
Escherichia coli	15	18.4	23	27.7	12.4	13.4	15	19.7	19.7_{Ge}
Candida albicans	26.4	31	35	37.7	25.7	28.4	29.7	33	$—_{Pm}$
Aspergillus niger	41.7	49.7	49	53.7	36.4	41.7	40.4	45.7	$—_{Pm}$

Am: ampicilin, Ge: gentamycin, Pm: polymixin.

2.6. Antimicrobial assay

Paper disk diffusion method was used for investigating inhibition zones of bacterial and fungal growth.[16] 50 ml of culture media was applied for each plate and autoclaved at 121 °C for 15 min. Solutions containing 1.5×10^8 of each microbes per ml equivalent were prepared to 0.6 and 0.5 standard of McFarland's tube from germs strain respectively and were read by spectrophotometer. Sterile media inoculated, and then loaded disk with essential oil was placed in the center of the plates by sterile forceps (one disk in each plate). Ampicilin and gentamicin were used for Gram-positive and Gram-negative as a positive control. For fungi, polymixin was applied (300 unites per disc). Concentrations used were 5, 4, 3 and 2 μl of essential oils. After that plates were incubated at 37 °C during 18–24 h. Diameters of non-growth zones were measured using a caliper.

3. Results and discussion

Analyzing the spectra of GC/MS, retention indices and mass spectra of objects detected and compared with standard compounds and references; six components identified in *T. daenensis* and 25 components identified in *T. vulgaris* which are accounts for 98.82% and 88.45% of total essential oil respectively (Table 1). Thymol (84.41%) and β-caryophyllene (4.48%) were the main compounds of *T. daenensis* and oxygen containing monoterpenes were major group of terpene compounds (Table 1). There is just one sesquiterpen component which is β-caryophyllene (4.482%). Some other reports show that identified compounds were 24 which main components were thymol (74.61%), p-cymene (4.6%), γ-terpinene (4.48%), carvacrol methyl ether (4.27%)[6] and 26 compounds which thymol (74.7%), p-cymene (6.5%), β-carvacrol (3.8%)[17] or 38 compounds which thymol (16.4%), carvacrol (52.3%)[18] were the main components. Inhibitory effects of *T. daenensis* are shown in Table 2 and indicated significant activity against Gram-positive and Gram-negative bacteria and also fungal. *E. coli* had most resistance among the selected microbes and the best result was observed in treatment with *A. niger*. Main compounds of *T. vulgaris* were carvacrol (46.62%), γ-terpinene (9.69%), pentacosane (6.36%) and oxygen containing monoterpenes were major groups of terpene compounds (Table 1). Essential oil of *T. vulgaris* was containing 0.64% unknown and 1.81% non-terpene components. Main compounds in other reports were thymol (79.15%), carvacrol (4.63%), p-cymene (3.27%)[9] or thymol (39.9%), p-cymene (19.5%), γ-terpinene (15.4%) and borneol (5.3%).[19] Inhibitory effects of *T. vulgaris* are shown in Table 2. Result indicated antimicrobial activity against all of microorganisms but effects in case of *E. coli* was less than positive control. Both oils had the highest effect on *B. subtilis* among the selected bacteria.

4. Conclusion

Both of these essential oils have antimicrobial activity against all of cases but *T. daenensis* has stronger effects in compare with *T. vulgaris* especially in the case of *E. coli*. Most effects were on *A. niger*

while positive control (polymixin, 300 unit per disc) indicated no activity against fungal. Considering high amount of thymol and according to our results, *T. daenensis* is a good alternative in antimicrobial cases for *T. vulgaris* and has potential for future works in this field.

Conflicts of interest

All authors have none to declare.

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Pharmacognosy Journal 5 (2013) 80–82

Contents lists available at SciVerse ScienceDirect

Pharmacognosy Journal

journal homepage: www.elsevier.com/locate/phcgj

Short communication

Chemical constituents of *Artocarpus camansi*

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ARTICLE INFO

Article history:
Received 24 April 2012
Accepted 12 June 2012
Available online 14 March 2013

Keywords:
Artocarpus camansi
Moraceae
Friedelinol
Cycloartenol acetate
Cycloartenol

ABSTRACT

The dichloromethane extract of the leaves of *Artocarpus camansi* afforded friedelinol (**1**), squalene (**2**), β-sitosterol (**3**), stigmasterol (**4**) and phytol (**5**), while the stems yielded polyprenol (**6**), cycloartenol (**7**) and cycloartenol acetate (**8**). The structures of **1**, **7** and **8** were elucidated by extensive 1D and 2D NMR spectroscopy. The cytotoxicity of these triterpenes were tested using the sulforhodamine B assay against the human cancer cell lines: lung adenocarcinoma A549 cells, stomach adenocarcinoma AGS cells, colon adenocarcinoma HT29 cells and prostate cancer PC3 cells. Results of the study indicated that **1**, **7** and **8** are non-cytotoxic against these cell lines.

1. Introduction

Artocarpus camansi (Moreaceae family), commonly known as breadnut and kamansi grows in tropical region and occasional in the Quezon province in the Philippines.[1] The fruit of *A. camansi* (breadnut) either mature or young is boiled and cooked as a vegetable, while the seeds are very starchy and are eaten boiled and roasted. A decoction of the bark is used as a vulnerary, emollient and in the treatment of dysentery.[2]

Previous studies reported the nutritional composition of *A. camansi* seeds[3] and the isolation and partial characterization of a lectin from the seeds of *A. camansi*.[4] A number of *Artocarpus* species have been used as food and traditional folk medicines in South-East Asia. An extract of *Artocarpus altilis* (breadfruit) leaves was shown to contain compounds with significant anti-inflammatory activities.[5] Isoprenylated flavonoids, namely morusin, artonin E, cycloartobiloxanthone, and artonol B from the bark of *A. altilis* have shown high toxicities against brine shrimp *Artemia salina* lethality test with LC_{50} of 55.90, 5.69, 33.79, and >1000.90 μg/mL, respectively.[6]

We report herein the isolation and identification of five compounds from the leaves of *A. camansi*: friedelinol (**1**), squalene (**2**), β-sitosterol (**3**), stigmasterol (**4**) and phytol (**5**), and three compounds from the stem of *A. camansi*: polyprenol (**6**), cycloartenol (**7**) and cycloartenol acetate (**8**) (Fig. 1). To the best of our knowledge, this is the first report on the isolation of these compounds from *A. camansi*.

2. Materials and methods

2.1. General experimental procedures

^{1}H and ^{13}C NMR spectra were recorded on Varian Unity Inova 500 MHz or VNMRS 600 MHz spectrometer and chemical shifts in ppm were referenced to the residual solvent. Column chromatography was performed with silica gel (230–400 mesh). TLC was performed on aluminum plates coated by silica gel 60 F_{254}. The plates were visualized by spraying with vanillin–sulfuric acid, followed by warming.

2.2. Plant material

The leaves and stems of *A. camansi* were collected from Bureau of Plant Industry (BPI), Department of Agriculture, Manila, Philippines in April, 2011. The sample was authenticated by Josephine T. Garcia from the BPI. A voucher specimen # 004 of *A. camansi* was deposited at School of Chemical Engineering and Chemistry, Mapúa Institute of Technology – Manila.

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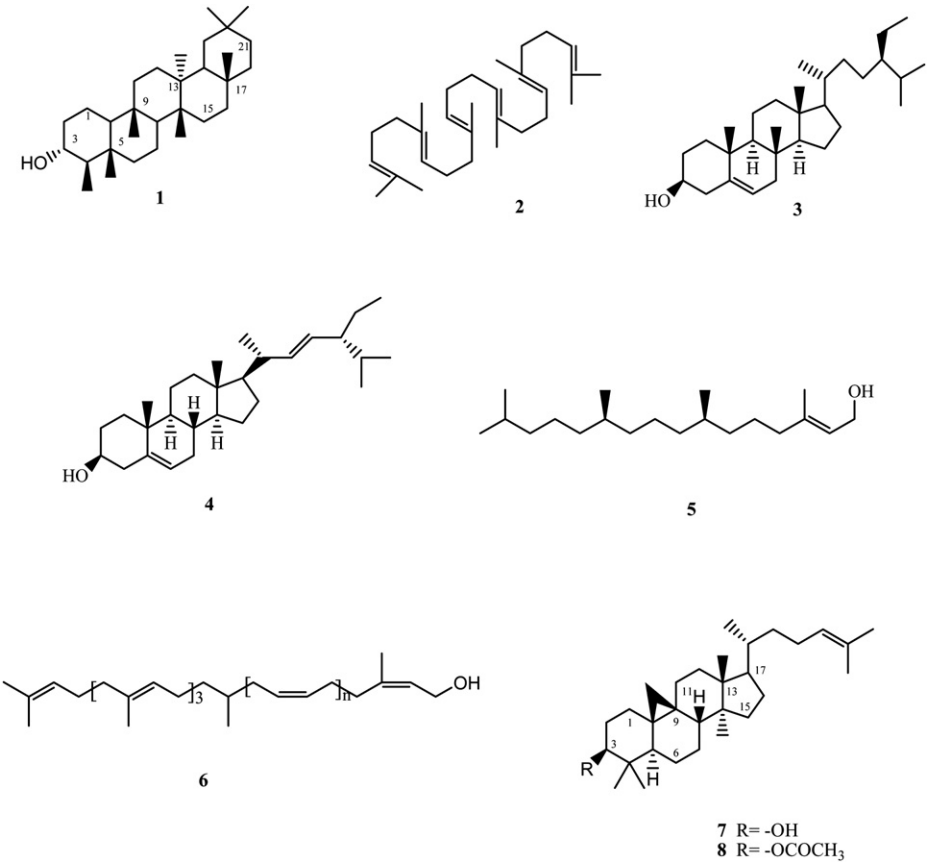

Fig. 1. Chemical constituents of *A. camansi*: friedelinol (**1**), squalene (**2**), β-sitosterol (**3**), stigmasterol (**4**), phytol (**5**), polyprenol (**6**), cycloartenol (**7**) and cycloartenol acetate (**8**).

2.3. Extraction and isolation of the chemical constituents from the leaves of A. camansi

The air-dried leaves (592 g) of *A. camansi* were ground in an osterizer, soaked in dichloromethane (DCM) for three days, and then filtered. The filtrate was concentrated by evaporation under vacuum to afford a crude extract (13.6 g).

The extract was chromatographed in increasing proportions of acetone in dichloromethane (DCM) at 10% increment. The DCM fraction was rechromatographed (3×) in hexane to afford **2** (12 mg). The 20% acetone in DCM fraction was rechromatographed (4×) in 10% EtOAc in hexane to afford **5** (10 mg). The 60% acetone in DCM fraction was rechromatographed (2×) in 15% EtOAc in hexane to afford **1** (8 mg). The 100% acetone fraction was rechromatographed (3×) in DCM to afford a mixture of **3** and **4** (15 mg).

2.4. Extraction and isolation of the chemical constituents from the stems of A. camansi

The air-dried stems (105 g) of *A. camansi* were ground in an osterizer, soaked in methanol for seven days, and then filtered. The crude extract (4 g) was concentrated by evaporation under vacuum.

The methanol extract was dissolved in H_2O (150 mL) and then partitioned with ethyl acetate. The ethyl acetate-soluble fraction (2 g) was rechromatographed (3×) in 10% EtOAc in hexane to afford **6** (5 mg) and **8** (3 mg). The more polar fractions were rechromatographed with DCM to afford **7** (10 mg) after washing with hexane.

*Friedelinol (**1**)*

^{13}C NMR (150 MHz, $CDCl_3$): δ 15.8 (C-1), 36.1 (C-2), 72.7 (C-3), 49.1 (C-4), 39.3 (C-5), 41.7 (C-6), 17.5 (C-7), 53.2 (C-8), 37.1 (C-9), 61.3 (C-10), 35.3 (C-11), 30.6 (C-12), 37.8 (C-13), 38.3 (C-14), 32.3 (C-15), 35.5 (C-16), 30.0 (C-17), 42.8 (C-18), 35.2 (C-19), 28.2 (C-20), 32.8 (C-21), 39.7 (C-22), 11.6 (C-23), 16.4 (C-24), 18.2 (C-25), 18.6 (C-26), 20.1 (C-27), 32.1 (C-28), 31.8 (C-29), 35.0 (C-30).

*Cycloartenol (**7**)*

^{13}C NMR (150 MHz, $CDCl_3$): δ 32.0 (C-1), 30.4 (C-2), 78.8 (C-3), 40.5 (C-4), 47.1 (C-5), 21.1 (C-6), 28.1 (C-7), 48.0 (C-8), 20.0 (C-9), 26.1 (C-10), 26.0 (C-11), 26.9 (C-12), 45.3 (C-13), 48.8 (C-14), 32.9 (C-15), 26.6 (C-16), 52.3 (C-17), 18.0 (C-18), 29.9 (C-19), 35.9 (C-20), 18.3 (C-21), 36.3 (C-22), 24.9 (C-23), 125.2 (C-24), 130.9 (C-25), 17.6 (C-26), 25.7 (C-27), 19.3 (C-28), 14.1 (C-29), 25.4 (C-30).

*Cycloartenol acetate (**8**)*

^{13}C NMR (150 MHz, $CDCl_3$): δ 31.6 (C-1), 26.8 (C-2), 80.7 (C-3), 39.4 (C-4), 47.2 (C-5), 20.9 (C-6), 28.1 (C-7), 47.8 (C-8), 20.1 (C-9), 25.9 (C-10), 25.8 (C-11), 35.5 (C-12), 45.3 (C-13), 48.8 (C-14), 32.8 (C-15), 26.5 (C-16), 52.2 (C-17), 18.0 (C-18), 29.8 (C-19), 35.9 (C-20), 18.2 (C-21), 36.3 (C-22), 25.4 (C-23), 125.2 (C-24), 130.9 (C-25), 17.6 (C-26), 25.7 (C-27), 19.3 (C-28), 15.1 (C-29), 25.4 (C-30), 171.0, 21.3 (OAc).

2.5. Sulforhodamine B assay

Cells suspended in RPMI-1640 medium containing 10% FBS were dispended into 96-well microtiter plates and maintained in a humidified incubator at 37° in 5% CO_2/95% air. After 24 h, 4 wells of each cell line were fixed *in situ* with trichloroacetic acid to represent a measurement at the time of drug addition (T0). Additionally, vehicle or the test compound was added to the cells and incubated

for additional 72 h. The assay was terminated by the addition of cold trichloroacetic acid. After rinsing the plates 3 times with tap water, sulforhodamine B solution 0.4% (weight per volume in 1% acetic acid) was added to each well and the plates were incubated for 10 min at room temperature. Unbound dye was removed by rising 3 times with 1% acetic acid and the plates were air-dried. Bound sulforhodamine B was subsequently solubilized with 10 mM Tris base (Sigma Chemical Co.) and the absorbance was read at a wavelength of 515 nm. From absorbance measurement of cells at time zero (T0), cells with vehicle treatment (C) and cells in the presence of the test compound (Tx), the percent of growth was calculated at each test compound concentration. Percentage of growth inhibition was calculated using the formula, $100\% - \{[(Tx-T0)/(C-T0)] \times 100\%\}$. The anti-proliferation activity of tested compound is presented as GI_{50} that indicates the concentration of 50% of growth inhibition of tested compound.

3. Results and discussion

The structures of **1**, **7** and **8** were elucidated by extensive 1D and 2D NMR spectroscopy and confirmed by comparison of their ^{13}C NMR data with those of friedelinol,[7] cycloartenol and cycloartenol acetate[8] reported in the literature. The structures of **2**–**6** were identified by comparison of their ^{1}H NMR data with those reported in the literature for squalene,[9] β-sitosterol,[10] stigmasterol,[10] phytol,[11] and polyprenol,[12] respectively. It is noted that the leaves and stems of *A. camansi* contained different chemical constituents. It is also noted that the stems contain cycloartane-type triterpene, while the leaves contain friedelane-type triterpene.

Cytotoxicity tests on **1**, **7** and **8** were conducted against the human cancer cell lines A549, AGS, HT29 and PC3 at the concentration of 100 μM. Triterpenes **1**, **7** and **8** were non-cytotoxic against A549 with growth inhibition of 14.17%, 0.15% and 6.68%, respectively. They were also non-cytotoxic against AGS with growth inhibition of 46.24%, 10.77% and 11.77%, respectively. Furthermore, these triterpenes were non-cytotoxic against HT29 with growth inhibition of 26.82%, 17.23% and −2.81%, respectively. In addition, they were non-cytotoxic against PC3 with growth inhibition of 39.56%, 29.98% and 26.90%, respectively.

Literature search revealed some of the biological activities of the isolated compounds as follows. Squalene has chemopreventive activity against colon carcinogenesis and antioxidant properties.[13,14] β-Sitosterol has been shown to induce apoptosis in human tumors for colon and breast cancers.[15] Stigmasterol has been shown to decrease plasma cholesterol, inhibit intestinal cholesterol and plant sterol absorption.[16] The polyprenols from *Ginkgo biloba* L. leaves exhibited hepatoprotective effects on CCl_4-induced hepatotoxicity in rats.[17] Friedelinol showed only antibacterial activity (MIC = 12.5–100 μg/ml) and no antifungal activity[18] (Tamokou et al, 2009). Cycloartenol exhibited moderate and selected antimicrobial activity against *Escherichia coli* and *Pseudomonas aeruginosa* with an MIC value of 512 μg/ml[19] (Momo et al, 2011).

Conflicts of interest

All authors have none to declare.

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Pharmacognosy Journal 5 (2013) 83–86

Contents lists available at SciVerse ScienceDirect

Pharmacognosy Journal

journal homepage: www.elsevier.com/locate/phcgj

Short communication

Preliminary pharmacognostic evaluation and volatile constituent analysis of spathe of *Phoenix dactylifera* L. (Tarooneh)

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ARTICLE INFO

Article history:
Received 11 January 2013
Accepted 28 February 2013
Available online 6 March 2013

Keywords:
Date palm
GC–MS
Phytochemical screening
Spathe
Volatile oil

ABSTRACT

Background: Spathe of date palm (which is called Tarooneh by Persian folk) and its aroma water is widely used in Persian traditional medicine but according to best of our knowledge lack is done on its chemical composition or standardization of the herb for its quality control in herbal market.
Methods and material: In this study microscopical and preliminary pharmacognostic properties of fractions obtained from date palm spathe (Tarooneh) were screened by thin layer chromatography. In addition, essential oils of the spathe were analyzed by GC–MS spectroscopy.
Results: Different fractions of the spathe contain steroids, triterpene steroids, oils and flavonoids in different amount. Fourteen compounds accounting for 93% of the oil were identified by GC–MS analysis. Oxygen containing monoterpenes were the main class of components (73%) with carvacrol (37%), linalool (24%) and thymol (10%) as major constituents of the oil.
Conclusion: Volatile constituents and preliminary pharmacognostic evaluation of Tarooneh can give some useful data for further phytochemical analysis, quality control and standardization of Tarooneh.

1. Introduction

Phoenix dactylifera L. (Arecaceae) is widely cultivated in Middle East and America for nutritional values of its fruits which are rich in carbohydrates and tannins.[1] In Persian traditional medicine the fruits are used as detersive, vermicide, astringent, febrifuge and aphrodisiac.[2] Also, the spathe which is called Tarooneh and its aroma water are used as sedative, tranquilizer, nerve tonic and for rheumatoid arthritis.[3] Palm spathe is a large bract that forms a sheath to enclose the flower cluster of the palm. According to the best of our knowledge there is not any report on pharmacognostic properties or chemical constituents of the spathe or its aroma water.

2. Materials and methods

Spathe of the *P. dactylifera* L. (Tarooneh) was purchased from Shiraz herbal market and authenticated by Miss Sedigheh Khademyan, Taxonomist, Department of Pharmacognosy, Shiraz School of Pharmacy and the voucher specimen was preserved with the code PM-172 in the department for further references. The plant material was powdered, passed through sieve number 100 and kept in dark closed container.

2.1. Microscopieal studies

One drop of chloral hydrate solution was added to a small amount of root powder on a slide and mixed well. The cover slip was added and heated on a flame. For detection of mucilage and starch, water was applied instead of chloral hydrate and the slides were not heated.[4]

2.2. Evaluation of extractive values

One hundred gram of root powder was extracted with n-hexane applying soxhlet apparatus for 6 h (n-hexane extract). The residuum was dried and macerated with dichloromethane, followed by ethanol in a dark and closed glass container, two days for each. Hexane, dichloromethane and ethanolic extracts were concentrated with a rotary evaporator and dried in a speed vacuum apparatus at 40 °C. The dried extracts were weighed out and kept in teflon caped tubes at −20 °C.

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0975-3575/$ – see front matter
http://dx.doi.org/10.1016/j.phcgj.2013.02.005

2.3. Thin layer chromatography

Solutions of 5 mg/ml of different fractions were prepared and 10 µl of them were applied with HPTLC (CAMAG) to the silica gel plate $60F_{254}$ (10 × 20 cm) from Merck. The plates were run in non-polar (toluene: acetone, 80:20), semi-polar (toluene: chloroform: acetone, 40:25:35) and polar (n-butanol: glacial acetic acid: water, 50:10:40) mobile phases. Chromatographic spots were visualized first using ultraviolet lamps emitting at 254 and 365 nm and then using different spray reagents.[5] For detection of essential oils and fatty acids phosphomolybdic acid reagent (vis.) and for alkaloids, Dragendorff reagent was applied. Also, 5% potassium hydroxide for coumarins (UV_{365} nm) and anthraquinones (vis. & UV_{365} nm), orcinol for glycosides, NP (Ethanolamine diphenylborate)/PEG for Flavonoids (UV_{365} nm), Liebermann–Burchard for steroids and triterpenes, 3% $FeCl_3$ for tannins and other phenolic compounds (vis.), vanillin-sulfuric acid and anisaldehyde-sulfuric acid as general reagents were sprayed on thin layer chromatography (TLC) plates.[6,7] All chemicals and solvents were of analytical grade purchased from Merck or Sigma Aldrich.

2.4. Analysis of the volatile constituents

The volatile oil was extracted for 3 h by hydrodistillation Clevenger-type apparatus. One milliliter of n-hexane was added to collect the volatile oil. The oil was dried over anhydrous sodium sulfate and n-hexane was vaporized under a stream of N_2. The gas chromatography–mass spectroscopy (GC/MS) analysis was carried out using a Hewlett–Packard 6890. The gas chromatograph was equipped with an HP-5MS capillary column (phenylmethylsiloxane, 25 m, 0.25 mm i.d.). The oven temperature was programmed from 60 °C (4 min) to 250 °C at a rate of 3 °C/min and increased at a rate of 5 °C/min to 280 and held for 10 min. The carrier gas was helium with a flow rate of 1.2 ml/min. The mass spectrometer was operating in the EI mode at 70 eV. The interface temperature was 250 °C; mass range was 30–600 *m/z*. The Kovats index (KI) of components was calculated for all compounds using a homologous series of n-alkanes under the same operational conditions of analysis. Identification of components was based on a comparison of their KI and mass spectra with Wiley (275), Adams libraries spectra and Pherobase Kovats Index Database.[8,9]

3. Results and discussion

Microscopical features of the spathe, including thickened inner parenchyma (A), sclerenchyma (B) and a piece of spathe tissue containing elongated cells (C) is shown in Fig. 1. The extractive values of herbal drugs can be considered as a representative of different class of their chemical constituents. The extractive values of n-hexane, dichloromethane and ethanolic fractions were determined 0.76%, 0.20% and 0.70% respectively.

The classes of these secondary metabolites were screened by thin layer chromatography and a variety of chemical regents. Some chemical of the spathe like essential oils could be detected under UV lamps without chemical treatments. The spry reagent used made a great knowledge of secondary metabolites of the plant spathe. The n-hexane fraction chromatographed in non-polar mobile phase revealed several blue spots with phosphomolybdic acid reagent which shows that the root contains several essential oils. With Liebermann-Burchard reagent saponin and steroidal components in n-hexane and dichloromethane fractions of the spathe which were chromatographed in non-polar and semi-polar mobile phases appeared as brown zone in visible light and green florescent under UV_{365} nm (Fig. 2). Tannins gave faded red-brown zones. Alkaloids in the petroleum ether fraction which was run in non-polar solvent gave red-orange color with Dragendorff regent. Polyphenols gave blue spots with $FeCl_3$ reagent in ethanol fraction. According to best of our knowledge there is not any report on essential oils, polyphenols, saponin or steroidal constituents of spathe of *P. dactylifera*. Ardekani et al reported the presence of phenolic components in 14 different varieties of Iranian date palm (*P. dactylifera* L., Arecaceae) seed extract. Different amount of polyphenols,[10,11] several tannins,[12,13] flavonoids and procyanidin oligomers such as luteolin, quercetin, and apigenin have been identified in extracts of date fruits.[14] In 2005, Mansouri et al studied phenolic profile of different varieties of ripe date palm fruit and detected *p*-coumaric, ferulic, sinapic acids, cinnamic acid derivatives and isomers of 5-*o*-caffeoylshikimic acid. They also identified different types of flavonoids, mainly flavones, flavanones and flavonol glycosides.[15] The presence of estradiol, esteriol, estrone and some novel flavonoids was reported from date palm pollen by Abbas and Ateya in 2011.[16] Estrone and cholesterol have been detected also by Reymond et al from date palm seeds and pollen.[17] Photochemistry and secondary metabolites of date palm fruits,

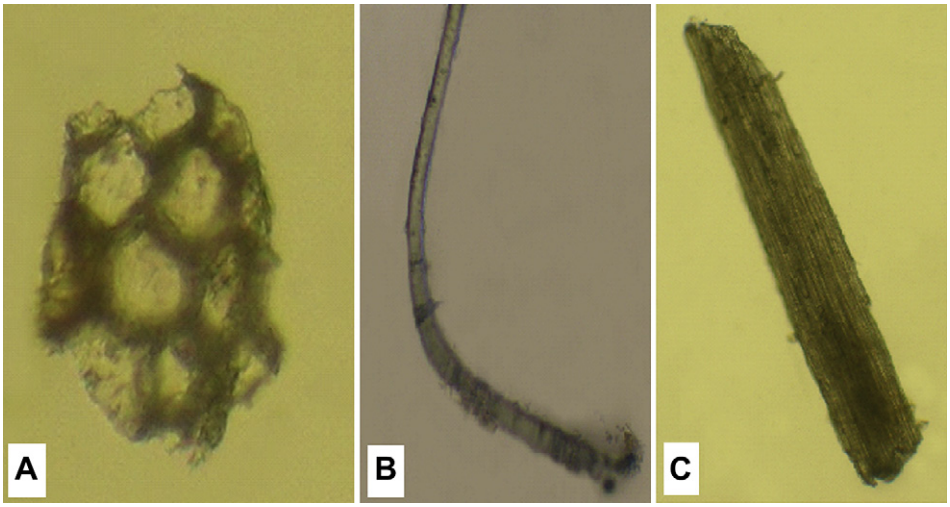

Fig. 1. Microscopical features of spathe of *P. dactylifera* L., A: thickened inner parenchyma (×10), B: sclerenchyma (×10) and C: a piece of spathe tissue containing elongated cells (×10).

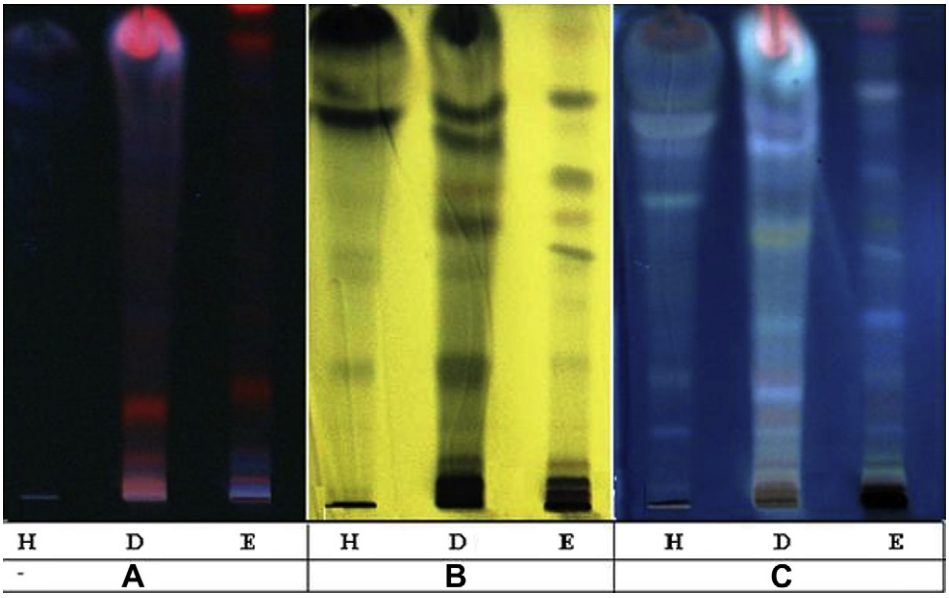

Fig. 2. TLC fingerprints of n-hexane (H), dichloromethane (D) and ethanolic (E) fractions of spathe of *P. dactylifera* run in semi-polar mobile phase. A: Under UV_{365} lamp and without reagent treatment, B: treated with phosphomolybdic acid reagent and C: under UV_{365} lamp after treatment with Liebermann–Burchard reagent.

seeds and pollen have been reviewed in several articles[18,19] and books.[20,21]

The volatile oils of the spathe, mostly miscible in water were trapped in hexane and gave a colorless solution with characteristic odor. With GC–MS analysis of the oil, 12 compounds were identified which represented about 92.86% of the total detected constituents. The identified compounds, their percentage and KI are summarized in Table 1. The main class of the compounds was found to be monoterpene hydrocarbons (0.52%), oxygen containing monoterpenes (73.76%), sesquiterpene hydrocarbons (6.07%) and oxygen containing sesquiterpenes (7.55%).

Carvacerol (35.45%) and linalool (24.10%) were the major components. Other compounds (up 2%) were thymol (10.93%), spathulenol (7.55%), beta-caryophyllene (4.33%), 3, 4 dimethoxytoluene (4.33%) and carvacrol methyl ether (2.44%). In 2011, Amira et al reported a low percentage of linalool (0.80 and 0.40%) in Degla cultivar of date fruit.[22] Also the percentage of 4- terpineol in Horra and Gosbi cultivars of date fruit was almost similar to this work (0.10–1.20%). In another report the principal volatile oils of the fruits were identified as ethyl acetate, acetaldehyde, isopropyl acetate, δ-valerolactone, octanal, furfuryl alcohol, 5-methyl furfural, linalool, δ-valerolactone and γ-undecalactone.[23]

Seeds which are rich in oleic acid, lauric acid, phenolics and tocopherol[24,25] have been reported for antioxidant activity.[26,27] Also, extracts of leaves and pits of *P. dactylifera* exhibited antifungal and antiviral properties.[28,29] Azmat et al analyzed hexane extract of seeds and leaves of *P. dactylifera* and reported that the leaves extract contains fatty acid ethyl ester but fatty acid methyl esters along with steroids and terpenoids were mostly identified in seeds extract.[30]

Table 1
Volatile constituent of Tarooneh (spathe of *P. dactylifera* L.).

Compound	Retention time (min.)	Kovats index	Percentage
p-Methyl anisole	8.21	1024	0.63
Epoxy linalool	10.22	1073	0.34
Linalool	12.06	1116	24.10
Terpineol-4	14.87	1179	0.52
Carvacrol methyl ether	17.84	1246	2.44
3,4 Dimethoxytoluene	18.09	1252	4.33
Unknown	18.42	1259	2.01
Thymol	20.81	1314	10.93
Carvacrol	21.57	1332	35.45
Unknown	21.88	1339	1.73
Carvacrol acetate	23.64	1380	0.50
Beta-caryophyllene	25.18	1417	4.33
Aromadendrene	25.89	1434	0.87
Alpha-humulene	26.49	1449	0.33
Ledene	28.20	1491	0.54
Spathulenol	31.71	1581	7.55
Unknown	33.91	1640	0.89
Unknown	43.80	1926	0.73

4. Conclusion

Different fractions of date palm spathe contain steroids, triterpene steroids, oils and flavonoids in different amount. Oxygen containing monoterpenes were the main class of components with carvacrol, linalool and thymol as major constituents of the oil. In this study pharmacognostic characteristic and volatile constituents of Tarooneh were determined successfully which can give some guideline for further phytochemechical analysis in future and also useful data for quality control and standardization of Tarooneh aroma water which is sold commonly in herbal markets.

Conflicts of interest

All authors have none to declare.

Acknowledgments

The present article was extracted from Pharm. D. thesis written by Samaneh Rivaz and was financially supported by Meymand Medicinal Plant Research Center and Shiraz University of Medical Sciences grants no. 90-5768.

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Contents lists available at SciVerse ScienceDirect

Pharmacognosy Journal

journal homepage: www.elsevier.com/locate/phcgj

Short communication

Comparative *in vitro* antibacterial evaluation of different extracts of *Camellia sinensis* leaves form different geographical locations in India

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ARTICLE INFO

Article history:
Received 9 December 2012
Accepted 11 February 2013
Available online 21 February 2013

Keywords:
Camellia sinensis
Antibacterial
Polyphenols
Leaf

ABSTRACT

Introduction: The present study was conducted to assess and compare the *in vitro* antibacterial property of four different solvent extracts from *Camellia sinensis* (tea) leaves collected form five different geographical regions of India.
Methods: All the extracts at the concentration of 4 mg/ml was tested for their *in vitro* antibacterial potential by cup plate method against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Staphylococcus epidermidis*.
Results: All the test tea extracts were found to exhibit remarkable antibacterial effect except against *S. aureus*. The tea sample form Assam was found to be the most active.
Conclusion: The marked and differential antibacterial effect of *C. sinensis* leaf from different Indian locations was plausibly due to geographical variations in chemical constituents especially polyphenol contents of *C. sinensis* leaf.

1. Introduction

The use of higher plants and their preparations to treat infectious diseases is an age-old practice and in the past possibly the only method available. However, the systematic study of higher plants for detecting antimicrobial activity is of comparatively recent origin.[1] These investigations have been triggered by the emergence and spread of antibiotic resistant microorganisms causing the effective life-span of existing antibiotics limited.[2] Hence, the plant kingdom is being screened for newer and effective chemotherapeutic agents. Higher plants can serve both as potential antimicrobial crude drugs as well as a source of newer anti-infective agents.[3]

Camellia sinensis L. (Theaceae), commonly known as tea is a large evergreen shrub indigenous to Eastern Asia where it is cultivated extensively. Tea is actually a product made from leaf and bud of the plant, *C. sinensis*, is the second most consumed beverage in the world.[4] The dried cured leaves of *C. sinensis* have been used to prepare beverages for more than 4000 years. The method of curing determines the nature of the tea to be used for infusion. Green tea is a type of cured tea that is 'non fermented' and produced by drying and steaming the fresh leaves; whereas black tea leaves are withered, rolled, fermented and then dried.[5] *C. sinensis* leaf has been used medicinally for centuries in the Traditional Chinese Medicine (TCM). Recently there has been renewed interest on green tea in prevention of several disease risks ands other important health benefits.[6] Previous researchers have reported several pharmacological and toxicological properties of *C. sinensis* leaf on animals and humans.[7–20] The present study was conducted to assess and compare the *in vitro* antibacterial property of different extracts from tea leaves collected form different geographical regions of India.

2. Materials and methods

2.1. Plant material

The mature green leaves of *C. sinensis* L. (Theaceae) were collected during the month of August 2008 from five different locations of five states in India. The sources of the tea leaves were from the Darjeeling (West Bengal), Guwahati (Assam), Coonoor (Tamil Nadu), Coorg (Karnataka) and Munnar (Kerala) regions of India. Just after collection, the tea leaves were shade dried at room temperature (24–26 °C) and ground mechanically into a coarse powder.

2.2. Chemicals

All the chemicals used were of analytical grade, obtained from Merck. The culture media were obtained from Himedia.

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0975-3575/$ – see front matter
http://dx.doi.org/10.1016/j.phcgj.2013.02.002

Table 1
Different solvent extractive values of *C. sinensis* leaves from different locations.

Solvents	% Extractives				
	Assam	Darjeeling	Tamil Nadu	Karnataka	Kerala
Ethyl acetate	16.44	19.23	14.66	12.98	17.00
Methanol	22.09	34.12	24.86	25.94	26.52
Ethanol	30.41	49.37	25.22	28.37	30.99
Aqueous methanol	33.47	32.71	26.93	35.22	31.48

2.3. Extraction

The powdered leaves of green tea (40 g) were extracted separately with different solvents namely ethyl acetate, ethanol, methanol and aqueous methanol (60:40) by boiling under reflux for 8 h. All the extracts were concentrated by distilling off the solvents at reduced temperature using vacuum. Then the dry extracts were weighed and percentage of different extractive values was calculated with respect to the air dried powdered plant material. These results are presented in Table 1.

2.4. Test microorganisms

Standard bacterial cultures of *Bacillus subtilis* (NCIM-2708), *Staphylococcus aureus* (NCIM-2079), *Escherichia coli* (NCIM-2685) and *Staphylococcus epidermidis* (NCIM-2478) were obtained from Al-Ameen Biotechnology and Research center, Bangalore 560027, India. The microorganisms were maintained in usual laboratory conditions by sub-culturing at regular intervals.

2.5. Preparation of inoculums

A 24 h old culture was used for the preparation of bacterial suspension. The suspension of bacteria was made in sterile isotonic saline (0.9% w/v) solution. The turbidity of bacterial cultures was adjusted with sterile saline according to 0.5 McFarland turbidity standard (1.5×10^6 cells/ml), for preparation of the inoculums.

2.6. Test samples

Test samples for *in vitro* antibacterial bioassay were prepared freshly from the dry extracts. All the five extracts obtained from different samples *C. sinensis* were dissolved in 10% w/w of dimethyl sulfoxide (DMSO) to get a concentration of 4 mg/ml. Ampicillin was used as reference and its solution was prepared at a concentration of 2 mg/ml in sterile distilled water.

2.7. Evaluation of antibacterial activity

The antibacterial activities of the test extracts were evaluated by cup plate method as reported by previous workers,[21] with minor modifications. Nutrient agar medium previously prepared and sterilized was cooled down to approximately 45–50 °C. 20–25 ml of this media were poured into 9 cm sterile glass Petri dishes previously marked suitably at the bottom surface, to a depth of approximately 4 mm. The inoculum (1% of medium) was added to the molten agar media at 45 ± 0.5 °C in the Petri dishes and the plates were swirled gently to disperse the microorganisms homogeneously. The plates were then allowed to solidify. Then 4–5 bores were made on the medium by using sterile borer under aseptic conditions. 0.1 ml of the different extract samples was added to the respective bores. Similarly, 0.1 ml of ampicillin solution was employed as reference. 10% DMSO was used as control. The Petri dishes were kept in the refrigerator at 4 °C for 1 h for diffusion. After diffusion the Petri dishes were incubated at 37 ± 1 °C for 24 h and the zones of inhibition were observed and measured using a zone recorder in mm. Antibacterial activity of all the extracts was carried out against all four organisms in similar manner. All the experiments were performed in triplicate and results averaged.

3. Results and discussion

The antibacterial activity of four different solvent extracts from five *C. sinensis* leaf samples against four bacterial strains was assessed by cup plate method. This method is based on diffusion of antimicrobial component from reservoir hole to the surrounding inoculated nutrient agar medium, so that the growth of microorganisms is inhibited as circular zone around the hole.[21] The results are presented in Tables 2–6.

Based on the data obtained from the present study, except *S. aureus* all the three test bacteria were found to be sensitive against all the test tea extracts at the applied concentration of 4 mg/ml. No zone of inhibition was observed against *S. aureus* for all test extracts. However, the reference antibacterial agent ampicillin was effective against *S. aureus* at a concentration of 2 mg/ml. *S. epidermidis* was found to be the most sensitive against all test extracts whereas *S. aureus* was least sensitive (practically insensitive) against all the test tea extracts.

The tea sample form Assam was found to be the most active. The semi polar solvent extracts namely ethanol and aqueous methanol (60:40) extracts of all five samples were found to be more effective in all cases. This indicated ethanol, water and methanol extracted maximum antibacterial principles (presumably polyphenols) form *C. sinensis* leaves. Different solvents have the capacity to extract different antimicrobial constituents from plants. Since nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds, they are most often obtained through initial ethanol, hydroalcohol or methanol extraction.[2,22]

Tea leaves contain varying amounts of polyphenols particularly flavonoids. Polyphenols are well known natural products known to possess several notable biological properties including excellent antimicrobial activity.[23] The main flavonoids present in green tea leaves include catechins (flavan-3-ols) and most importantly epigallocatechin-3-gallate (EGCG).[24] Tea leaf also contains some phenolic acids such as chlorogenic acid, gallic acid, caffeic acid, etc which also have antioxidant effect.[24,25] The observed differences in antioxidant activity of tea leaf extracts could be attributed to the

Table 2
Antibacterial activity of *C. sinensis* extracts from Assam.

Bacteria	Zone of inhibition of (mm)				
	Ethyl acetate extract	Methanol extract	Ethanol extract	Aqueous methanol extract	Ampicillin
S. aureus	NI	NI	NI	NI	22.2 ± 0.23
S. epidermidis	10.03 ± 0.08	13.7 ± 0.26	17.9 ± 0.15	18.2 ± 0.42	24.1 ± 0.10
E. coli	13.7 ± 0.26	15.8 ± 0.31	14.8 ± 0.27	17.9 ± 0.13	21.03 ± 0.10
B. subtilis	12.2 ± 0.23	14.7 ± 0.23	16.5 ± 0.14	16.7 ± 0.26	25.4 ± 0.35

Data are presented as Mean ± Standard Error Mean (SEM). NI = No Inhibition.

Table 3
Antibacterial activity of *C. sinensis* extracts from Tamil Nadu.

Bacteria	Zone of inhibition (mm)				
	Ethyl acetate extract	Methanol extract	Ethanol extract	Aqueous methanol extract	Ampicillin
S. aureus	NI	NI	NI	NI	21.03 ± 0.10
S. epidermidis	11.27 ± 0.12	10.2 ± 0.08	11.43 ± 0.08	9.46 ± 0.14	22.2 ± 0.23
E. coli	10.05 ± 0.18	10.63 ± 0.03	12.7 ± 0.20	10.36 ± 0.23	25.4 ± 0.35
B. subtilis	11.6 ± 09	11.03 ± 0.08	12.13 ± 0.08	11.43 ± 0.17	24.1 ± 0.1

Data are presented as Mean ± Standard Error Mean (SEM). NI = No Inhibition.

Table 4
Antibacterial activity of *C. sinensis* extracts from Karnataka.

Bacteria	Zone of inhibition (mm)				
	Ethyl acetate extract	Methanol extract	Ethanol extract	Aqueous methanol extract	Ampicillin
S. aureus	NI	NI	NI	NI	21.03 ± 0.10
S. epidermidis	11.1 ± 0.05	12.7 ± 0.28	9.9 ± 0.17	10.16 ± 0.9	22.2 ± 0.23
E. coli	10.7 ± 0.26	10.03 ± 0.03	11.2 ± 0.9	12.1 ± 0.9	25.4 ± 0.35
B. subtilis	10.43 ± 0.23	11.3 ± 0.14	11.43 ± 0.24	11.4 ± 0.11	24.1 ± 0.1

Data are presented as Mean ± Standard Error Mean (SEM). NI = No Inhibition.

Table 5
Antibacterial activity of *C. sinensis* extracts from West Bengal (Darjeeling).

Bacteria	Zone of inhibition (mm)				
	Ethyl acetate extract	Methanol extract	Ethanol extract	Aqueous methanol extract	Ampicillin
S. aureus	NI	NI	NI	NI	21.03 ± 0.10
S. epidermidis	14.29 ± 0.06	14.1 ± 0.45	16.4 ± 0.65	17.4 ± 0.44	22.2 ± 0.23
E. coli	12.91 ± 0.14	12.0 ± 0.08	14.2 ± 0.55	15.5 ± 0.17	25.4 ± 0.35
B. subtilis	13.21 ± 0.17	14.1 ± 0.05	13.4 ± 0.14	14.1 ± 0.12	24.1 ± 0.1

Data are presented as Mean ± Standard Error Mean (SEM). NI = No Inhibition.

Table 6
Antibacterial activity of *C. sinensis* extracts from Kerala.

Bacteria	Zone of inhibition (mm)				
	Ethyl acetate extract	Methanol extract	Ethanol extract	Aqueous methanol extract	Ampicillin
S. aureus	NI	NI	NI	NI	21.03 ± 0.10
S. epidermidis	10.9 ± 0.23	11.4 ± 0.15	13.4 ± 0.15	14.3 ± 0.20	22.2 ± 0.23
E. coli	10.4 ± 0.08	13.3 ± 0.14	16.56 ± 0.14	16.6 ± 0.15	25.4 ± 0.35
B. subtilis	11.21 ± 0.31	9.43 ± 0.08	15.43 ± 0.23	13.46 ± 0.12	24.1 ± 0.1

Data are presented as Mean ± Standard Error Mean (SEM). NI = No Inhibition.

variations of composition in the polyphenol contents of *C. sinensis* leaves grown in different geographical locations due to diverse environmental or climatic factors.

The present preliminary study confirms remarkable *in vitro* antibacterial activity of four different solvent extracts of *C. sinensis* leaf collected from five different geographical locations against four bacterial strains. All the leaf extracts demonstrated marked antibacterial property against *Bacillus subtilis*, *E. coli* and *S. epidermidis*. *C. sinensis* leaf collected form Assam was found to be the most active. The differential antibacterial effect of *C. sinensis* leaf from different locations of five Indian states was plausibly due to geographical variations in chemical constituents especially polyphenol contents of *C. sinensis* leaf.

Conflicts of interest

All authors have none to declare.

Acknowledgment

The authors are thankful to the authority of Al-Ameen College of Pharmacy, Bangalore 560027, India for providing necessary facilities for the present study.

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Letter to the Editor

In vivo anti-inflammatory activity and flavonoid identification of medicinal *Eupatorium* species

In the last years, it is becoming clear that inflammation plays a key role in the development and progression of various chronic diseases, including cancer, type-2 diabetes, Alzheimer's disease, cardiovascular and renal diseases.[1] The limitation of current anti-inflammatory therapies is acknowledged, and continuous efforts are made to improve the available treatments and to find new drugs with less secondary effects. In this search, plants that have long been used in traditional medicine for inflammation, pain and wound healing, represent a rich source of new anti-inflammatory compounds.

One of the most popular and extensively used species from the genus *Eupatorium*, *Eupatorium perfoliatum*, has been widely used by native Indians in North America and nowadays it is used there and in Europe for inflammation-associated diseases like arthritis and rheumatism.[2]

In a previous work we have found four flavonoids with significant anti-inflammatory activity in the TPA-induced ear edema in mice and in the NF-κB expression assays, isolated through bioassay-guided fractionation of an active extract of *Eupatorium arnottianum*.[3] These results led us to evaluate the anti-inflammatory activity of other five native medicinal species of *Eupatorium*, with traditional uses for inflammation and pain by indigenous and rural cultures, and to investigate the presence of some of those anti-inflammatory flavonoids in the active extracts of the selected species.

Eupatorium subhastatum, *Eupatorium laevigatum*, *Eupatorium macrocephalum*, *Eupatorium hecatanthum* and *Eupatorium candolleanum* are described as used in folk medicine for the treatment of different inflammation-related ailments. These herbs are consumed either as teas, gargarisms, by chewing different plant parts or used topically in different forms. Stems, leaves and fresh flowers of *E. hecatanthum* are chewed against teeth pain and stomachaches[4,5]; decoctions or infusions of its aerial parts are used in washes for gangrene and ulcerations.[6] *E. laevigatum* leaves are used for wound healing and as analgesic.[7,8] *E. macrocephalum* flowers are chewed for sore throat.[4] *E. subhastatum* is used to cure pimples and insect bites, in gargarisms for sores, and as descongestive.[8] Although these species have been widely used in folk medicine, no pharmacological data related to inflammation has been reported previously.

Dichloromethane and aqueous extracts of *E. subhastatum*, *E. laevigatum*, *E. macrocephalum*, *E. hecatanthum* and *E. candolleanum* were assayed in the topical and systemic models of inflammation: TPA-induced ear edema and carrageenan-induced edema assays. Furthermore, HPLC profiling and flavonoid identification of the active dichloromethane (DCM) extracts of the five species was carried out. Acute toxicity of the aqueous extracts was also evaluated.

Plants were collected in the surroundings of Paraná, Entre Ríos province (Argentina) in February 2008. *E. subhastatum* was collected in Castelar, Buenos Aires province (Argentina) in December 2008. Specimens were identified by Ing. Juan de Dios Muñoz, and Ing. Gustavo Giberti. Voucher specimens were deposited at Instituto de Tecnología Agropecuaria de Entre Ríos Herbarium and at the Herbarium of the Museo de Farmacobotánica Juan A. Domínguez, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires.

Aqueous extracts (5%) were prepared according to Argentine Pharmacopoeia VI edn.[9] Briefly, 200 ml of boiling distilled water were added to 10 g of air-dried aerial parts in a stopper flask and left to settle for 20 min. The aqueous extracts were filtered and lyophilized. Dichloromethane extracts (DCM) were prepared by maceration of 25 g of the dried and ground aerial parts in a stopper flask in contact with 250 ml of dichloromethane during 24 h. Extracts were then filtered and re-extracted twice. The filtered extracts were mixed and dried under vacuo. The yields of all dried extracts were calculated referring them to 100 g of dried plant material.

The *in vivo* assays were performed according to international guiding principles and local regulations concerning the care and use of laboratory animals for biomedical research.[10] Animals were housed in standard environmental conditions (22 ± 1 °C, with a 12 h light/dark cycle) with free access to a standard commercial diet and water *ad libitum*.

The ear edema assay was carried out according to Carlson et al.[11] Groups of 10 animals (male Swiss mice, 25–30 g) were used. The right ear of each mouse received a topical application of 2.5 μg of 12-O-tetradecanoylphorbol-13 acetate (TPA) as 0.125 μg/μl acetone solution (10 μl to each side of the ear). Extracts (0.5, 1 and 2 mg/ear/20 μl, dissolved in acetone), were applied topically immediately after TPA. The left ear, used as control, received the vehicle only. Indomethacin was used as reference drug (0.5 mg/ear/20 μl). After 4 h, the animals were sacrificed. Disks of 6 mm diameter were removed from each ear and their weight was determined. The swelling was measured as the difference in weight between the punches obtained from right and left ears.

Carrageenan-induced edema assay was performed as described by Winter et al.[12] Groups of 5 female Sprague–Dawley rats (150–200 g) were used. Paw swelling was elicited with 0.1 ml 1% carrageenan in 0.9% (w/v) saline injected in the right hind foot. Extracts (100 and 200 mg/kg) and the reference drug indomethacin (10 mg/kg) dissolved in EtOH–Tween 80–water 5:5:90 were administered orally 1 h before carrageenan injection. A control group received the vehicle only (5 ml/kg). The inflammation was quantified by measuring the volume displaced by the paw, using a

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plethysmometer (Ugo Basile) at 0 and 1, 3, and 5 h after the carrageenan injection. The difference between the left and the right paw volumes (indicating the degree of inflammation) was determined and the percent inhibition of edema was calculated in comparison to control animals.

For acute toxicity assays, groups of 10 CF-1 mice (6 weeks old), 5 male and 5 female, were used. The control group received only vehicle (water) and the remaining groups received increasing doses up to 2 g/kg (0.5 ml/25 g body weight) of the different aqueous extracts of *Eupatorium* spp., orally, by means of a gastric catheter. The number of deaths, weight loss, abdominal constrictions, palpebral ptosis, movement, lethargy, stereotypy, ataxia, tremors, convulsions, diarrhea and presence of secretions were recorded. Animals were observed twice a day, for up to 15 consecutive days.

Statistical analysis was performed using a one-way analysis of variance (ANOVA), followed by Dunnett's test and Bonferroni test. A p value <0.05 was considered significant. Results were expressed as mean ± SEM.

DAD–HPLC analysis of the active DCM extracts was performed in a Waters 600/DAD Waters 2996 device. Empower Pro data processor, UV range: 200–400 nm, was used. 0.5 g of each dried DCM extract was resuspended in 10 ml of a mixture of hexane–diethyl ether 1:1 and 30 ml 70% methanol were added. After shaking, the organic phase was discarded. The methanolic–aqueous phase was taken to dryness. The dried methanol residues were dissolved in 2 ml methanol HPLC-grade and filtered by 0.45 μm filters. 20 μl of this solution were injected. Jaceosidin, nepetin, hispidulin and eupatilin (reference compounds previously isolated from *E. arnottianum*) were used. These compounds were dissolved in methanol at the concentration of 0.5 mg/ml and 20 μl of each solution were injected. Extracts and reference compounds were run in two chromatographic systems as follows:

System 1. Column: Phenomenex Luna C18, 5 μm, 250 × 4.6 mm. Solvent A: water–formic acid 97:2.5. Solvent B: methanol–formic acid 97:2.5. Gradient 0–5 min: 50% B; 30 min: 100% B. Flow: 1 ml/min. System 2. Column: Agilent Zorbax Eclipse XDB-C8, 5 μm, 150 × 4.6 mm. Solvent A: water–formic acid 97:2.5. Solvent B: methanol–formic acid 97:2.5. Gradient 0–10 min: 45% B. 15 min: 60% B. 20–25 min: 100% B. Flow: 1 ml/min. Peak purity was determined by comparison of the UV spectra at upslope and downslope inflexion points. Identification of the peaks in the active extracts was done by comparing the retention times (Rt) and UV spectral data with those of the reference compounds.

The yields of DCM extracts (w/w) were: *E. subhastatum* 15.1%, *E. laevigatum* 20.0%, *E. macrocephalum* 9.0%, *E. hecatanthum* 7.3%, *E. candolleanum* 6.2%. Aqueous extracts yields (w/w) were: *E. subhastatum* 15.0%, *E. laevigatum* 19.0%, *E. macrocephalum* 14.0%, *E. hecatanthum* 19.6% and *E. candolleanum* 15.8%.

The five tested species were effective in reducing the ear thickness induced by TPA (Table 1). *E. subhastatum*, *E. laevigatum*, and *E. hecatanthum* DCM extracts were the most active ones, with maximum inhibition of 86.4, 89.7, 94.4% and ED_{50} of 0.35, 0.51, 0.92 mg/ear, respectively. *E. candolleanum* and *E. macrocephalum* also reduced the ear edema (ED_{50} 1.0 and 0.79 mg/ear), but produced a lower maximum inhibition. The aqueous extracts could not be tested due to their low solubility either in acetone or absolute ethanol, solvents used to dissolve the dried extracts in the topical TPA assay.

Only DCM and aqueous extracts of *E. subhastatum*, at a dose of 200 mg/kg, were active in the carrageenan-induced paw edema assay, both producing a moderate edema inhibition (37% in the 5th hour). None of the other species presented activity in this assay (100 and 200 mg/kg).

Table 1
Topical anti-inflammatory activity of dichloromethane extracts of *E. subhastatum*, *E. laevigatum*, *E. hecatanthum*, *E. macrocephalum* and *E. candolleanum* in the TPA-induced mouse ear oedema.

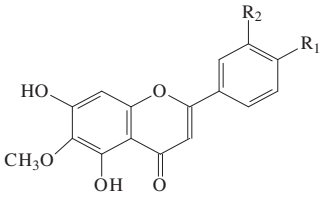
Treatment	Weight (mg)	Oedema inhibition (%)
Control	21.4 ± 0.9	0
Indomethacin	5.1 ± 0.8*	76.2
E. subhastatum DCM		
0.5 mg	9.2 ± 1.4*	57.0
1.0 mg	3.6 ± 0.5**	83.2
2.0 mg	2.9 ± 0.8**	**86.4**
E. laevigatum DCM		
0.5 mg	13.0 ± 1.3*	39.2
1.0 mg	2.2 ± 0.2**	**89.7**
2.0 mg	3.3 ± 0.8**	84.6
E. hecatanthum DCM		
0.5 mg	16.4 ± 0.4	23.4
1.0 mg	10.7 ± 0.8*	50.0
2.0 mg	1.2 ± 0.1**	**94.4**
E. macrocephalum DCM		
0.5 mg	13.5 ± 2*	36.9
1.0 mg	9.1 ± 0.8**	57.5
2.0 mg	8.9 ± 1.0**	58.4
E. candolleanum DCM		
0.5 mg	13.6 ± 0.6*	36.4
1.0 mg	9.3 ± 0.9**	56.5
2.0 mg	10.7 ± 1.7*	50.0

DCM: dichloromethane extract. Results were obtained by topical administration of different doses (0.5, 1 and 2 mg/ear) of each extract and 0.5 mg/ear of indomethacin. Statistical differences compared to controls ($n = 10$) were determined by Dunnett's test $^{*}p < 0.05$, $^{**}p < 0.01$. Bold numbers mean higher maximum inhibitions obtained.

The administration of aqueous extracts of the tested species was not lethal even up to an oral dose of 2000 mg/kg p.o. Therefore, their oral LD_{50} was higher than 2 g/kg in mice. No significant difference in body weight gain was observed between the control and any of the treated groups at any period time. Besides, the extracts did not produce any sign of toxicity during the observation period and at necropsy no macroscopic changes in organs could be detected in the treated groups, demonstrating that these extracts did not produce acute toxicity.

Jaceosidin, nepetin, eupatilin and hispidulin (Fig. 1) were identified by HPLC–DAD in the active dichloromethane extracts of the investigated species.

Jaceosidin was identified in all the tested species with the exception of *E. laevigatum*. This compound proved to be the major one in *E. subhastatum*, *E. macrocephalum* and *E. candolleanum*.



	R_1	R_2
Nepetin	**OH**	**OH**
Jaceosidin	**OH**	**OCH_3**
Hispidulin	**OH**	**H**
Eupatilin	**OCH_3**	**OCH_3**

Fig. 1. Structure of the flavonoids identified in *Eupatorium* DCM extracts.

Nepetin was identified in *E. macrocephalum* and *E. hecatanthum*, while eupatilin was present in *E. macrocephalum* and *E. candolleanum*. Hispidulin was only identified in *E. laevigatum*.

Jaceosidin, nepetin and hispidulin have demonstrated anti-inflammatory effect in the same experimental model used in this investigation[3,13]; besides, eupatilin inhibited croton oil induced edema.[14]

Other flavonoid compounds were detected in the DAD–HPLC profiles of the extracts, so further investigations should involve isolation and identification of these compounds and the determination of their participation in the anti-inflammatory activity of *Eupatorium* extracts included in this study.

Since there are no previous references about the toxicity of these species, the safety of their aqueous extracts was evaluated. The oral LD_{50} of the aqueous extracts were greater than 2 g/kg in mice. The lack of acute toxic effects of the aqueous extracts of these plants via the oral route in mice could support their safety as traditional medicines.

In conclusion, this is the first report on the anti-inflammatory activity and acute toxicity of these *Eupatorium* species. The topical anti-inflammatory effect found for these species could support their medicinal uses for localized inflammations. The presence of the active compounds eupatilin, jaceosidin, nepetin and/or hispidulin in these DCM extracts is reported here for the first time and could justify, at least in part, the observed anti-inflammatory effects of these extracts in the TPA assay.

The finding of anti-inflammatory effect and absence of acute toxicity of the studied species, together with the chromato graphic flavonoid profiling and the identification of active principles in their extracts, can contribute to the quality control and serve as a support for the efficacy and security of these traditional medicines.

Acknowledgments

This work was supported by grant 20020110200267 from Secretaría de Ciencia y Técnica de la Universidad de Buenos Aires (UBACYT). Authors are grateful to Ing. J. de D. Muñoz (Instituto de Tecnología Agropecuaria de Entre Ríos), Ing. G. Giberti (Museo de Farmacobotánica Juan A. Domínguez, Facultad de Farmacia y Bioquímica de la Universidad de Buenos Aires) for collection and botanical identification of plant material and to María Teresa Argerich (CONICET) for her technical support.

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28 December 2012
Available online 19 March 2013

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Pharmacognosy Journal 5 (2013) 94–96

Contents lists available at SciVerse ScienceDirect

Pharmacognosy Journal

journal homepage: www.elsevier.com/locate/phcgj

Letter to the Editor

Elemental analysis, physicochemical characterization and lithontriptic properties of *Lapis judaicus*

Jews stone is the larger spines of regular echinoids, especially cidaroids such as *Balanocidaris glandifera*, called *Lapis judaicus* (English: Jews' stones; Persian: Sang-e-Jahudan; Arabic: Hajarul Yahud). It is commonly ornamented by a series of longitudinal, finely tuberculated striae. It was formed in the Late Jurassic (Oxfordian to Tithonian) in Europe, North Africa and the Middle East. The spines have a short neck and a globular head ornamented with beaded ribs. The head tapers rapidly to a point distally. In terms of provenance, spines of *B. glandifera* are most likely to have come from the Upper Oxfordian 'Glandarienkalk' limestones of the Mount Hennon district of what is now southern.[1]

Lapis judaicus has a long history of use in both eastern and western traditional medicines for urinary diseases. Moreover, its beneficiaries in the treatment of bowel bleeding, wounds, stings, and snakebites were also noted.[2] Dioscorides Pedanius (first century AD) recommended the use of *Lapis judaicus* for dissolving urinary calculi. It should be bruised to make a pulverized powder, suspended in water and drunk.[3] Later medieval European physicians were also recommended this drug for similar indications.[4] Ibn Sina, the mediaeval Persian scientist believed that it was one of the most useful drugs for urinary calculi healing.[5] A review on historical text of medieval Al-Sham indicated that it was extensively used for internal and skin diseases.[6] Specimens of Jews' stones are still sold in certain bazaars of Iran, Iraq, Afghanistan,[7] Jordan,[8] India, and Pakistan.[9]

Beside extensive use of *Lapis judaicus*, information about its chemical constitutes and pharmacological activity is very limited. An *in vivo* study showed that it has an inhibitory effect on the crystallization of calcium oxalate.[10]

We have recently done a double blind randomized clinical trial for evaluation lithotriptic effect of *Lapis judaicus* on patients which have calcium oxalate stone(s) in lower pole of their kidney (clinicaltrials.gov/ct2/show/NCT01443702). In this regards, we will introduce chemical constitutes and some physiochemical parameters of this drug. Moreover we will demonstrate *Lapis judaicus* potentials in size reduction of calcium oxalate stones *in vitro*.

Lapis judaicus was purchased from different cities in Iran. Fossils were identified by Department of Paleontology, Shiraz University and voucher specimens deposited in Shiraz School of Pharmacy collection.

Various stone shapes were present in the bulk of Jews' stone. We categorized them depending on their size and morphologic shape. Randomized sampling was done for calculating the percentage of each form. Organoleptic features of the powder were evaluated by observing color, odor and taste.[11]

Water, ethanol and acid soluble extractives, pH, total and acid insoluble ash and loss on drying were measured according to methods described in United States pharmacopeia.[11] Each study was repeated 6 times.

Samples were analyzed for their carbon, hydrogen and nitrogen (CHN) concentration on a Costech ECS 4010 Elemental combustion system (Valencia, CA) with pneumatic auto sampler. It was set up for CHN analysis. Reactor 1 consisted of chromium (III) oxide/silvered cobaltous–cobaltic oxide catalysts at 980 °C. Reactor 2 consisted of reduced high purity copper wires at 650 °C. Helium was used as the carrier gas at a flow rate of 100 ml min^{-1}. This was filtered for hydrocarbons upstream of the instrument. A packed (Porous polymer, HayeSep Q) 3 m GC column (SS 6 × 5 mm–2m-HayeSep q 60/80) was used for separation of the gases. A thermal conductivity detector (TCD-L-3) was used to calculate the signal of each sample.

Three 10% *Lapis judaicus*/n-hexane powder (w/w) extracts were prepared by maceration method for 24 h. The resulted extracts were filtered and kept in −20 °C for injection to Gas chromatography–mass spectrometry (GC/MS). The GC/MS analyzes were carried out using a Hewlett–Packard 6890. The gas chromatograph was equipped with an HP-5M capillary column (phenyl methyl siloxan, 25 m × 0.25 mm i.d., Hewlett–Packard Part No. 190915.433, USA). The oven temperature was programmed from 50 °C (3 min) to 250 °C at the rate of 3 °C/min and finally held for 10 min at 250 °C. The carrier gas was He with the flow rate of 1.2 ml/min. The mass spectrometer (Hewlett–Packard 5973, USA) was operating in EI mode at 70 eV. The interface temperature was 250 °C; mass range was 30–600 *m*/*z*. Identification of components was based on a comparison of their RI and mass spectra with Willey (275).[12]

A wavelength-dispersive sequential X-ray spectrometer (PW 25040, Philips, The Netherlands) equipped with super-Q software for quantitative analysis was used for measuring the Kα line of all elements.

Crystal identifications were carried out on a X-ray powder diffraction (XRD) analyzer (Xpert–MPD, Philips, The Netherlands) system operating at the Co Kα wavelength of 1.7889 Å, 30 mA, and 40 kV. Step size was 0.02°/s.

The samples were detected for presence of heavy metals such as lead, arsenic and mercury. Determination of heavy metals was done

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by A Perkin Elmer AAnalyst 700 (Norwalk, CT, USA) atomic absorption spectrometer apparatus.[11]

We added 1 g of *Lapis judaicus* powder to a 250 ml beaker, which contain 100 ml of phosphate buffer (pH = 6.0). For negative control, nothing was added to the buffer. Calcium oxalate calculi expelled by surgery from patients weighed and put in each the buffer. For each group (*Lapis judaicus* and control) we used four samples. Beaker shacked and after 48 and 120 h the weight changes were measured. As there is not any standard drug for size reduction in calcium oxalate stone, we didn't have positive control.

Jews' stones have a variety of morphological presentations (Fig. 1). Genser (1565) and Mercati (1791) were also reported these variations.[1,3] Olive shape (77.13% w/total weight) and bone shape (15.21% w/total weight) are major morphologies (Table 1). Olive, bone and spindle shapes are Echinoid fossils and shell shapes are Goniorhynchia fossils. The powder is Khaki in color and without noticeable taste or odor. Loss of weight on drying was found to be 3.13 ± 1.55 w/w, which is not too high, hence could limit bacterial, fungal, or yeast growth. Extractive value was low in water (4.33% ± 0.32 w/w) and ethanol (5.00% ± 0.01 w/w). However, its solubility in acid was high (94.87% ± 0.99 w/w) which may indicate high solubility in gastric syrup. The pH of 1% and 10% w/w solution was nearly 9.52 ± 0.01 and 9.25 ± 0.04 respectively, which indicates basic behavior of Jew's stone in solution. Total ash value was 38.35% ± 9.47 w/w which 22.24% ± 5.13 w/w was soluble in acid.

CHN elemental analysis indicated that *Lapis judaicus* powder conation 26.55 ± 1.24% carbon, 0.97 ± 1.24% hydrogen and almost no nitrogen. In average, carbon, hydrogen and nitrogen are 27.68 ± 1.43% w/w of total powder. In the other hand, GC/MS apparatus did not find any significant compound in the prepared extracts. Based on CHN and GC/MS results we didn't find any organic material in *Lapis judaicus*.

X-ray fluorescence semi quantitative results that almost 43.76% of materials were lost on ignition. Major components of the powder after ignition were CaO (52.44 W/W), MgO (1.75% W/W), SiO_2 (1.07% W/W), Fe_2O_3 (0.50% W/W), Al_2O_3 (0.33% W/W) and Sr (0.08% W/W). Moreover, trace amounts (less than 0.001%) of Phosphorus, Chlorine, Nickel, Palladium, Sodium, Sulfur, Titanium, Chromium, Copper, Potassium, Manganese, Palladium, Gadolinium, Bismuth, Ruthenium, Indium and Cerium were founded. Elemental analysis of different morphologies presented in *Lapis judaicus* showed that chemical constitutes in different morphologies were almost similar although the percentage of some constitutes are different (Table 1). X-ray powder diffraction qualitative identifications lead to identifying five different mineral crystals in *Lapis judaicus* powder. These minerals were Calcite ($CaCO_3$), Dolomite ($CaMg(CO_3)_2$), Quartz (SiO_2), Boehmite ($Al_2O_3(H_20)$) and Muscovite ($KAl_2(Si_3AlO_{10})(OH)_2$). Moreover, no heavy metals were identified by atomic absorption spectrometer method (<1 ppb).

In vitro studies indicated that *Lapis judaicus* powder can reduce the size of calcium oxalate stones 35.1 ± 7.9% after 2 days and 58.2 ± 1.64% in 5 days ($p < 0.05$).

Inorganic materials have been in the inventory of medicinal substances of various cultures since ancient times. Most of them and their usage did not fade away when modern medicine took over; they exist to the present day in the traditional systems of medicine around the world. Traditional healers have used them alone or combined with a wide array of other geological and botanical ingredients, to treat a diversity of ailments. Unlike medicinal plants for which WHO has issued official standardization methods, there is no official standardization reference for inorganic substances used in traditional medicines.[13]

This study, not only investigated physicochemical properties of *Lapis judaicus*, but also chemical constitutes and *in vitro* pharmacological activities. *Lapis judaicus* contain magnesium, which is a protective agent in calcium oxalate crystal growth. Moreover its basic pH nature could be another inhibitor for stone production.[14] Furthermore SiO_2 can change calcium oxalate monohydrate to calcium oxalate dihydrate, which is more soluble.[15] In the other hand

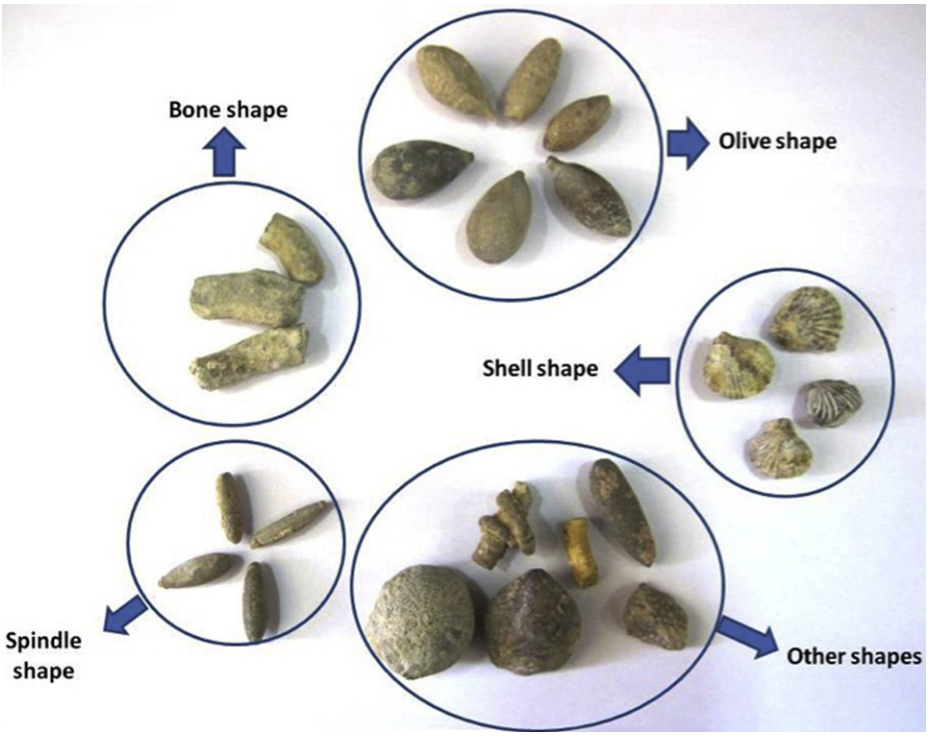

Fig. 1. Different fossils present in the bulk of *Lapis judaicus*.

Table 1
Lapis judaicus various shapes and its XRF elemental analysis.

Morphological shape	Shape %	Loss on ignition %	CaO %	MgO %	SiO_2 %	SO_3 %	Al_2O_3 %	Fe_2O_3 %	Sr%
Olive shape	78.13 ± 2.43	44.27	49.77	4.28	0.43	0.20	0.38	0.60	0.06
Bone shape	15.21 ± 1.42	43.70	53.28	1.06	1.28	trace	0.42	0.26	0.05
Shell shape	3.23 ± 1.49	40.90	46.90	1.73	6.77	trace	2.41	0.81	0.05
Spindle shape	1.49 ± 1.18	43.20	53.20	1.80	0.87	0.42	0.41	trace	0.09
Total powder	–	43.76	52.44	1.75	1.07	0.17	0.33	0.50	0.08

high amount of calcium make a controversy because it is a promoter for calcium oxalate crystallization.[14] Results of *in vitro* study indicated that *Lapis judaicus* could be a candidate for future studies in the field of renal stone dissolving agents.

The results obtained in this study is not only useful for researchers in the filed of urinary stones but also considered as a showcase for determining authenticity and purity of a non-organic traditional drugs for quantitative standardization.

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22 February 2013
Available online 14 March 2013