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Original article

Antiepileptic activities of the extract and fractions of *Mondia whitei* (Hook f.) Skeel leaves

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ABSTRACT

Objectives: To investigate the antiepileptic potentials of *Mondia whitei* (Hook f.) Skeel leaves as identified in the folklore recipes for the management of paralysis, epilepsy. *M. whitei* is also used in antimalarial, antihelminthic recipes as well as in the treatment of impotence and sexual dysfunction.
Methodology: The antiepileptic study of the methanol extract, hexane and ethyl acetate fractions were carried out using the pilocarpine induced seizure model while the Phytochemical and fluorescence analysis were carried out using standard procedures.
Result: The presence of saponins, flavonoids, tannins and resins were detected in the leaves of *M. whitei*. The extract and fractions exhibited a dose-dependent effect in the delay of the onset of seizures and reduction in the duration of seizure.
Conclusion: This study justifies the use of *M. whitei* in folklore medicine as a recipe in the management of epilepsy.

1. Introduction

Epilepsy a neurological condition characterized by recurrent seizures and convulsions is third most common neurological disorder which affects about 50 million people[1] most especially in the developing countries. The inbalance between excitatory and inhibitory decrease in GABAergic and glutamatergic transmission is involved in the generation of epilepsy.[2]

Several antiepileptic medications are employed in the management of epilepsy however, the side effects exhibited by these medications are of great concerns In addition, the cost of these drugs has made the management of epilepsy a challenging one particular in developing countries. Epilepsy has been a main cause of mortality hence an increased interest in the use of herbal remedies for its management.

Mondia whitei (Hook f.) Skeels (Apocynaceae) a climbing shrub of about 8–20 m, is one of the two species of the genus *Mondia*.[3] It is characterized by latex and woody roots, which are aromatic when old. It is commonly known as white's ginger or tonic root[4] and Isirigun (Yoruba, SW Nigeria).

M. whitei is endemic to South, East and West Africa, widely distributed from Guinea to East Africa. In traditional medicine, the various parts of *M. whitei* are used for the management of several diseases and ailments such as impotence and sexual dysfunction,[5–8] constipation, abdominal pain, appetite stimulant,[9] urinary infections, gonorrhoea,[10] as analgesic pains, inducement of labour. In addition, the roots are chewed to treat stress, tension[9,11] in the management of paralysis, epileptic attacks and fits in children,[11,12] for milk production in lactating mothers.[13] In Central African Republic, the seeds are used in preparing arrow poisons,[9,11] as antimalarials[14,15] and anthelminthics.[16]

Previous biological studies have reported the androgenic effect, aphrodisial properties[5,17] analgesic, antimicrobial properties,[10] anti-inflammatory, antioxidant effect. From the roots, β-sitosterol and propane, an unusual chloropropane have been isolated.[4] The aim of this study is to investigate the antiepileptic activities of the extract and fractions of *M. whitei*.

2. Plant collection

The fresh leaves of *M. whitei* were collected in September, 2012 from Ilaro Ogun State and authenticated at Forestry Research Institute of Nigeria and the Department of Pharmacognosy, Olabisi Onabnjo University where voucher specimens with number FHI 109513 have been deposited.

3. Preparation of extract and fractions

The fresh leaves of *M. whitei* were cleaned, air dried and ground into powder. The powdered leaf sample was macerated in 80%

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Table 1
Phytochemical analysis of *Mondia whitei*.

Saponins	Flavonoids	Tannins	Cyanogenetic glycosides	Anthraquinone		Alkaloids	Resins	Cardiac glycosides	Steroids
				Free	Combined				
++	+	++	−	−	−	−	+	−	−

++ = Highly present; + = moderately Present; − = Absent.

methanol for 72 h. The filtrate was concentrated under reduced pressure. The dried extract was made aqueous and partitioned successively with hexane and ethyl acetate to obtain the hexane and ethyl acetate fractions.

3.1. Test animals

Wistar albino rats (200–220 g) were used for this study. The rats were obtained from the animal house of the University of Ibadan, Oyo State. The rats were allowed to acclimatize and kept in accordance with the International Guidelines on animal care and kept in cages at 25 ± 2 °C (12/12 h light/dark cycle) They were fed with rat pellets and water *ad libitum*.

4. Phytochemical and fluorescence screening

The Phytochemical and fluorescence screening were carried out using standard procedures.[18–20]

5. Evaluation of antiepileptic activity of *M. whitei* leaves

The antiepileptic activity of *M. whitei* was investigated by adopting the method of Turski et al, 1983[21] by using the Pilocarpine induced seizure model (400 mg/kg of Pilocarpine).

Group 1: Rats received 1 ml/kg of normal Saline
Group 2: Rats received 100 mg/kg extract/fraction
Group 3: Rats received 50 mg/kg extract/fraction
Group 4: Rats received 25 mg/kg extract/fraction
Group 5: Rats received 5 mg/kg Diazepam

The rats ($n = 5$) were administered the test sample/reference drug and thirty (30) min after, they received 400 mg/kg body weight of pilocarpine intraperitoneally.

6. Statistical analysis

The results are presented as mean ± SEM. The results were analysed by the one-way analysis of variance (ANOVA).

Table 2
Fluorescence Analysis of the leaves of *Mondia whitei*.

Treatment	Normal daylight	UV	
		254 nm	366 nm
5% NaOH	Army green	Ash	Ash
5% KOH	Brown	Green	Coffee brown
5% FeCl	Deep green	Black	Black
Na_2CO_3	Green	Green	Grey
5% H_2SO_4	Light green	Black	Black
$AgNO_3$	Dark green	Grey	Brown
Dil. NH_3	Dark green	Deep green	Black
Conc. H_2SO_4	Foaming red	Black	Black
5% HCl	Brown	Lemon	Orange
Dil. HNO_3	Light green	Light green	Brown
Conc. HCl	Dark green	Green	Dark brown

7. Result

M. whitei (Apocynaceae) has been reported to possess several biological activities such as antifertility, androgenic activity, antiinflammatory, antibacterial and antisickling properties. Previous phytochemical studies reported the presence of reducing sugars and triterpenes[22] while Quasie et al,[23] reported presence of alkaloids, flavonoids, saponins and phenolics.[24] In this study, the leaves of *M. whitei* revealed the presence of saponins, flavonoids, tannins, resins and absence of cyanogenetic glycosides, Anthraquinone, alkaloids and cardiac glycosides (Table 1). This is similar to the reports of Quasie et al,.[23] The variation reported in *M. whitei* from various areas could be due to certain environmental factors, seasonal variations or differences in varieties of the species.

In the fluorescence analysis, the leaves of *M. whitei* reacted with various chemical reagents showing various colour reactions in normal daylight and under the UV light at both 254 nm and 366 nm. These reactions exhibited will enable the characterization and identification of the purity of the leaf of *M. whitei* (Table 2).

The use of pilocarpine in inducing epilepsy in this study activated the muscarinic receptors in the brain of the rats. This led to cholinomimetic syndromes characterized by salivation, oro-facial movement, brightening of the eyes and lacrimation, raised body furs, increased gastric motility and urinary incontinence as well as tonic clonic seizures of the hind limbs which mimicked the features of temporal lobe epilepsy in human beings.[25]

The methanol extract, ethyl acetate and hexane fractions of *M. whitei* at 100 mg/kg 50 mg/kg, 25 mg/kg exhibited a dose-dependent effect in delaying the onset and reducing the duration of seizures. The hexane fraction at 100 mg/kg appeared to delay onset of seizures (25.3 min) while the ethyl acetate fraction shortened the duration of seizure at the same dose (27 min). Though the extract and fractions did not prevent seizures, the methanolic extract at all doses tested protected the rats from mortality while the fractions exhibited partial recovery (Fig. 1).

Several studies have attributed the presence of monoterpenes, triterpenoid glycoside[26,27] alkaloids, flavonoids, saponins[28] to antiepileptic activities. This study therefore indicates that the presence of the various constituents (Saponins, tannins, flavonoids)

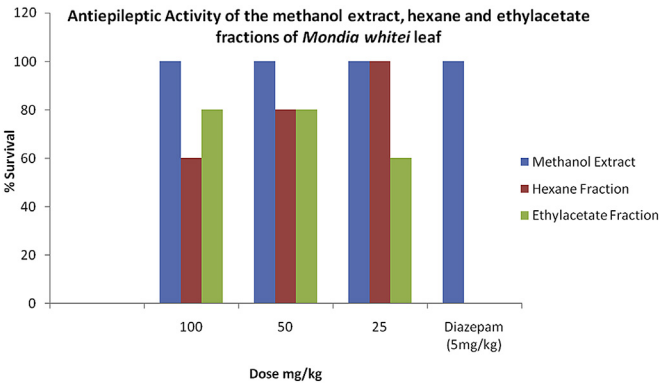

Fig. 1. Antiepileptic activity of the methanol extract, hexane and ethyl acetate fractions of *Mondia whitei* leaf.

may be responsible for different mode of actions in the antiepileptic activities of the extract and fractions of *M. whitei* leaves. This study highlights the need for further studies.

Conflicts of interest

All authors have none to declare.

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Original article

Development of HPTLC method for estimation of piperine, guggulsterone E and Z in polyherbal formulation

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ABSTRACT

Aim: Triphala guggul a polyherbal tablet formulation is used for sinusitis, allergies, boils, constipation, piles, high cholesterol, rheumatism, mal-absorption, purgative and as blood purifier. In the present study an attempt has been made to develop a simple, precise, rapid and cost-effective high performance thin-layer chromatographic (HPTLC) method for quantitative estimation of piperine, guggulsterone E and Z in Triphala guggul formulation.

Method: The different batches of formulation were prepared in laboratory by using authenticated raw material and were subjected to various physical and chemical evaluations. Then the prepared formulation and three commercial formulations were investigated for the qualitative and quantitative estimation of mentioned constituents. The methanolic extract of all formulations were quantified by using HPTLC studies. Linear regression data for the calibration curves of standards viz. piperine, guggulsterone E and Z showed a good linear relationship over a concentration range of 0.06–0.14 μg/spot, 2.5–17.5 μg/spot, 5–30 μg/spot respectively with the correlation coefficient of 0.99085, 0.99847, 0.9990 respectively and thus exhibits good linearity between concentration and area. The content of guggulsterone E (14.68 %w/w, 13.05 %w/w, 6.36 %w/w, 14.36 %w/w); guggulsterone Z (31.81 %w/w, 26.95 %w/w, 11.62 %w/w, 23.86 %w/w); and piperine (0.068 %w/w, 0.0150 %w/w, 0.321 %w/w, 0.0375 %w/w) were found in TF, TN, TM, TP prepared and three marketed formulation respectively.

Conclusion: The proposed HPTLC method was found to be rapid, simple and linear for quantitative estimation of piperine, guggulsterone E & Z in different formulations and extracts.

1. Introduction

Herbal medicine has been enjoying renaissance among the customers throughout the world. However, one of the impediments in the acceptance of the Ayurvedic formulation is the lack of standard quality control profile. Although, considerable work is being done to evaluate herbals for their quality, safety and efficacy, there is a need of a well-defined specific method for routine analysis of herbal raw materials and formulations with regard to constituents responsible for its efficacy. Development of methods for analysis of plant products poses difficulty, due to their unknown chemical profile, complex nature and inherent variability of the chemical constituents. It is therefore difficult to establish quality control parameter for plant based drugs due to which unregulated sales of adulterated and spurious drugs are observed.[1]

'Triphala guggul' is a traditional Ayurvedic herbal formulation consisting of the dried fruits of three medicinal plants, *Terminalia chebula* (Combretaceae), *Terminalia belerica* (Combretaceae) and *Emblica officinalis* (Euphorbiaceae), these are combined with *Commiphora mukul* (Burseraceae) and *Piper longum* (Piperaceae) for the treatment of sinusitis, allergies, boils, constipation, piles, high cholesterol, mal-absorption and as a purgative, blood purifier, anti-inflammatory and anti-rheumatic.[2–5] The pharmacopoeia standards in Ayurvedic or herbal pharmacopoeia appear inadequate to ensure the quality of plant drugs or their formulations.

In the present study, an attempt has been made to develop a simple, rapid and accurate HPTLC method for estimation of piperine as well as guggulsterone E and Z in marketed formulations of Triphala guggul tablet. These constituents are considered to be

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the active components and can be considered as marker compounds.[3–5] The method developed was also used for chemical fingerprint analysis.

2. Materials and methods

2.1. Equipment and chromatographic condition

A CAMAG HPTLC system equipped with a sample applicator Linomat IV using 100 μl syringe and connected to a nitrogen tank; twin trough plate development chamber; CAMAG TLC scanner-3 with winCATS software. Each HPTLC plate precoated, silica gel G 60 F_{254} size 20 × 10 cm accommodated twenty tracks of standards and samples, applied according to following settings: bandwidth 4 mm; distance between bands 5 mm; The plates were developed to 8 cm in a twin trough glass chamber, saturation time 30 min, scanning mode Absorbance/Reflectance; temperature 20 ± 5 °C and separation technique ascending.

2.2. Chemicals

Standard piperine (Sigma Aldrich), guggulsterone E and Z (Yucca Enterprises, Mumbai), precoated silica gel G 60 F_{254} TLC aluminium plates (20 × 20 cm, 0.2 mm thick) (Merck Ltd. Germany) and AR grade chemicals were used. The marketed samples were purchased from local market of Nagpur, Maharashtra.

2.3. Drugs

T. chebula (Hirda), *T. belerica* (Baheda), *E. officinalis* (Amla), *P. longum* (Pimpli) and *C. mukul* (Guggul) authenticated crude drugs were procured from Natural Remedies, Bangalore. The raw material was subjected to physical and chemical evaluation.[1,6,7] The formulation was prepared in laboratory in different batches as per the formula given in the Bhaishajya Ratnavali.[8] The three batches of marketed formulations from three different manufacturers in India namely, Formulation 1 Triphala guggul by Unza Pharmaceutical (batch C-21 [TM1], C-26 [TM2], D-17 [TM3]), Formulation 2 Triphala guggul by Baidyanath (batch 329 [TN1], 356 [TN2], 357 [TN3]), Formulation 3 Triphala guggul, by Ayurvedic Rasashala (batch 030035 [TP1], 051052 [TP2], 050302 [TP3]) were procured from local market of Nagpur, Maharashtra. These formulations were subjected to the pharmacopoeial evaluation of tablets.[9,10] Batches which pass the evaluation test were used for qualitative and quantitative study using HPTLC.

2.4. HPTLC method for estimation of phytoconstituents[11–14]

2.4.1. Preparation of standard guggulsterone E solution

A stock solution of 0.5 mg/ml was prepared by dissolving 5 mg of guggulsterone E in methanol and volume was made to 10 ml and different amounts 5, 10, 15, 20, 25, 30, 35 μL were applied in triplicate on TLC plates, using a Camag Linomat IV sample applicator.

2.4.2. Preparation of standard guggulsterone Z solution

A stock solution of 1 mg/ml was prepared by dissolving 10 mg of guggulsterone Z in methanol and volume was made to 10 ml and different amounts 5, 10, 15, 20, 25, 30 μL were applied in triplicate on TLC plates.

2.4.3. Preparation of standard piperine solution

A stock solution of 0.1 mg/ml was prepared by dissolving 1 mg of piperine in methanol and volume was made to 10 ml and 6, 8, 10, 12, 14 μL were applied in triplicate on TLC plates.

2.4.4. Sample preparation

The sample was weighed (250 mg); sonicated with methanol for 25 min, filtered through Whatmann filter paper and volume was made to 10 ml in volumetric flask. 5 μL (for estimation of guggulsterone E & Z) and 10 μL (for estimation of piperine) of prepared and marketed formulations samples were applied on plate in 4 mm band with the help of Linomat IV applicator and developed under the same conditions as described for the standards.

2.5. Calibration curve

Calibration curve was constructed according to requirement of ICH guidelines.[15] Each concentration was applied to a plate (20 × 10 cm) in triplicates of 4 mm band length with a distance of 5 mm between each two bands. The distance from the plate side edge was 10 mm and from the bottom of the plate was also 10 mm. The application rate was 15 μL/s, standard zones were quantified by linear densitometric scanning using Camag TLC scanner. Deuterium lamp was utilized as a source of radiation. Evaluation was done using linear regression analysis via peak areas and calibration curve was prepared by plotting peak area vs. concentration applied.

2.6. Method validation

2.6.1. Linearity

The linearity of the HPTLC method was evaluated by analysing a series of different concentrations of the standards (guggulsterone E, Z and piperine), where each concentration was applied in triplicate. Linear regression data for the calibration curves of standards guggulsterone E and Z, piperine showed a good linear relationship.

2.6.2. Specificity

The specificity of the method was determined by analysing the drug standard and test samples. The peak for test sample was confirmed by comparing its Rf and spectrum with those of the standard.

2.6.3. System precision

The system precision was assessed by determination of six different concentrations of standards each applied in triplicate.

2.6.4. Method precision (repeatability)

Repeatability (precision) was determined by repeated analysis of standard samples using the same equipment, same analytical procedures and same laboratory and on the same plate. Repeatability of measurement was determined by spotting 10 μL of standard drug solution on TLC plate, after development spot was scanned six times without changing position. The % RSD was determined.

2.6.5. Limits of detection and quantitation

Limit of detection and limit of quantitation were validated based on signal to noise ratio where the minimum concentration at which the standard solutions can be reliably detected was recorded. Limits of detection (LODs) and limits of quantization (LOQs) were calculated using the expressions $3.3\sigma/s$ and $10\sigma/s$, respectively, in which σ is intercept standard deviation and s is the slope of calibration curve.

2.6.6. Recovery study

The accuracy of proposed method was evaluated by addition of standard drug solution to pre-analysed tablet sample solution at three different concentration levels at 50, 100, and 150% of linearity. This parameter shows the proximity between the experimental values and the real ones.

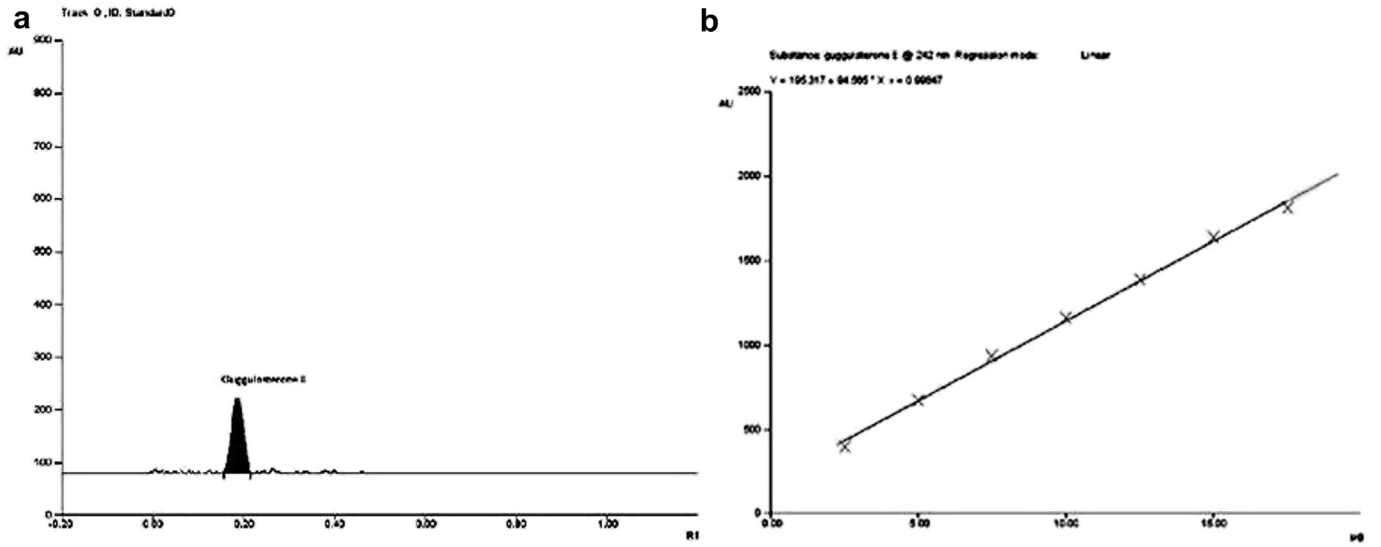

Fig. 1. (a) HPTLC scan densitogram (b) Calibration curve of standard guggulsterone E.

2.6.7. Robustness

By introducing small changes in the mobile phase composition, mobile phase volume and duration of mobile phase saturation, the effects on the results were examined. Robustness of the method was done in triplicate at a concentration level of 10 µg/spot for guggulsterone E, Z and 0.10 µg/spot for piperine.

2.6.8. Sample analysis

The developed method can be applied in determination of guggulsterone E & Z as well as piperine in polyherbal formulations. Three batches of laboratory formulation were prepared by using authentic raw material and three batches of three different marketed companies were selected and analysed by the proposed method.

3. Results

Standard guggulsterone E (Rf: 0.19, Fig. 1), guggulsterone Z (Rf: 0.27, Fig. 2) and piperine (Rf: 0.62, Fig. 3) showed single peak in HPTLC chromatogram. Calibration curve was prepared by plotting peak area vs. concentration applied. The linear regression data for the calibration curves of standards guggulsterone E, Z and piperine showed a good linear relationship over a concentration range of 2.5–17.5 µg/spot, 5–30 µg/spot, 0.06–0.14 µg/spot respectively with the correlation coefficient of 0.99847, 0.9990 and 0.99085 respectively and linear regression equation was found to be: $y = 195.317 + 94.585x$, $y = 332.423 + 101.518x$, $y = 5417.084 + 38873x$ respectively, where y is the spot area and x is the concentration of the analyte. It was observed that other constituents present in the formulations did not interfere either with the peak of guggulsterone E, Z and piperine therefore the method was specific. The spectrum of respective standards and respective spots present in the samples was found to be similar or overlapped (Fig. 4). The proposed HPTLC methods were validated for intra and interday variations. The values of percent relative standard deviations (RSDs) were found to be 0.80, 1.17, 0.69 (interday) 1.22, 2.25, 0.22 (intraday) for guggulsterone E, Z and piperine respectively which indicate that the method was precise. Limit of detection and limit of quantitation where the minimum concentration at which the standards solution can be reliably

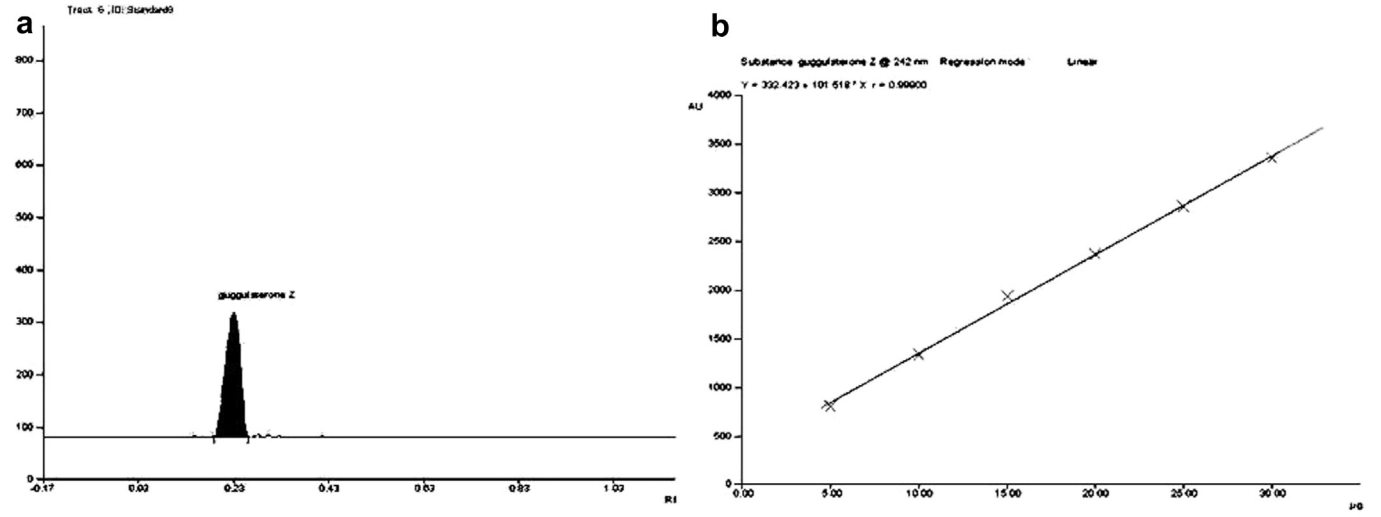

Fig. 2. (a) HPTLC scan densitogram (b) Calibration curve of standard guggulsterone Z.

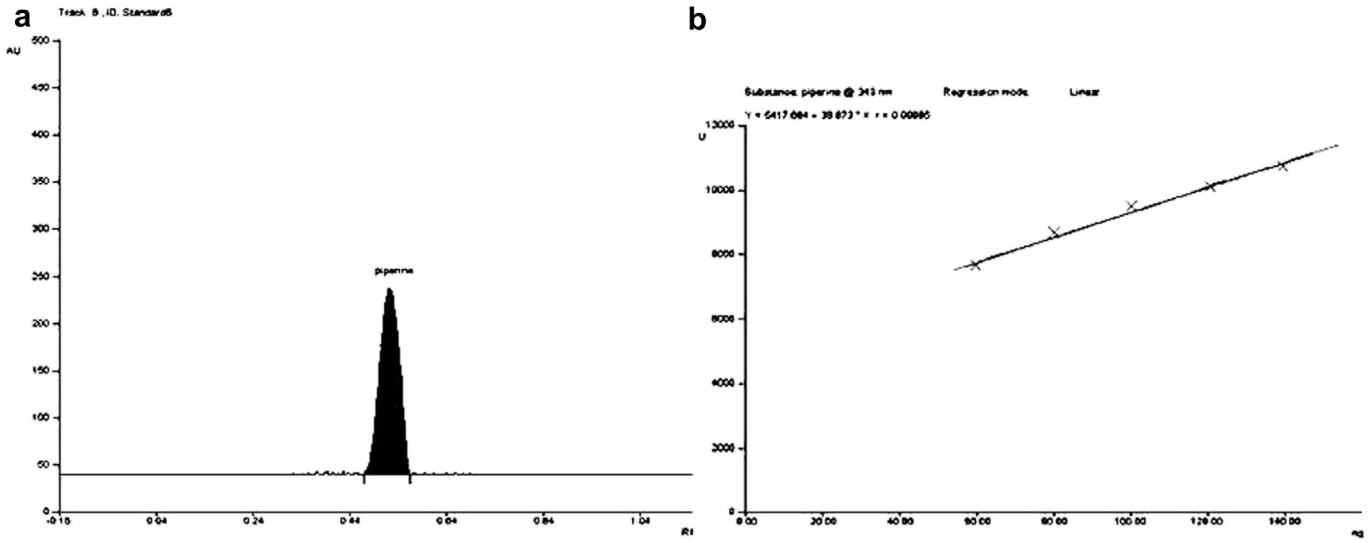

Fig. 3. (a) HPTLC scan densitogram (b) Calibration curve of standard piperine.

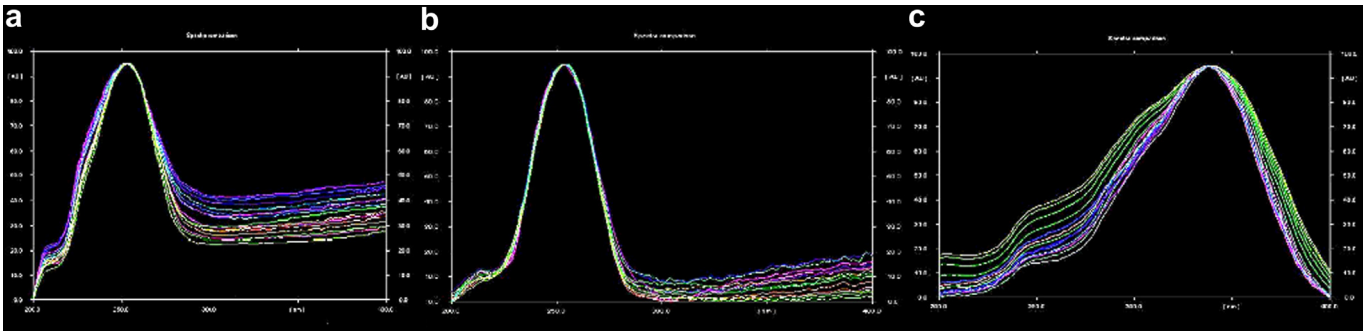

Fig. 4. Overlapping spectra (a) guggulsterone E (b) guggulsterone Z (c) piperine.

detected was recorded as 1.07 μg/spot, 1.54 μg/spot, 0.12 μg/spot and minimum concentration at which the analyte can be reliably quantified was found to be 3.28 μg/spot, 4.68 μg/spot, 0.36 μg/spot for guggulsterone E, guggulsterone Z and piperine respectively (Table 1). To study the accuracy of the method, recovery studies were performed at three different concentration levels at 50, 100, and 150% of linearity. The recovery ranged from 98.80 to 101.05%, 98.69 to 101.81%, 97.75 to 102.33% for guggulsterone E, Z and piperine respectively (Table 2) indicating that the proposed HPTLC method was accurate. All the batches of prepared and marketed formulation were subjected to analysis by optimized HPTLC method. Table 3 shows the percentage of guggulsterone E, Z and piperine present in the all formulations. The above study reflects that the percentage of guggulsterone E and Z is much less in formulation TM as compared to other formulations and percentage of piperine is much less in formulation TN, even it shows variation in percentage of above mentioned constituents in different batches.

Table 1
Validation parameters of the HPTLC method for estimation of standards.

Parameter	Guggulsterone E	Guggulsterone Z	Piperine
Linearity range (μg/spot)	2.5–17.5 μg/spot	5–30 μg/spot	0.06–0.14 μg/spot
Detection wavelength (λ_{max})	242 nm	242 nm	343 nm
Mobile phase	Petroleum ether:Ethyl acetate (3:1)	Petroleum ether:Ethyl acetate (3:1)	Benzene:Ethyl acetate:Diethyl ether (6:3:1)
Rf value	0.19	0.27	0.62
Regression equation	$y = 195.317 + 94.585x$	$y = 332.423 + 101.518x$	$y = 5417.084 + 38873x$
Slope (b)	94.585	101.518	38873
Intercept (a)	195.317	332.423	5417.084
Correlation coefficient (R^2)	0.99847	0.999	0.99085
Limit of detection (μg/spot)	1.07	1.54	0.12
Limit of quantitation (μg/spot)**	3.28	4.68	0.36
Intraday precision (RSD, %)**	1.22%	2.25%	0.22%
Interday precision (RSD, %)**	0.80%	1.17%	0.69%

**Average of six determinations.

Table 2
Results of recovery study.

Marker	Initial amount (μg/spot)	Amount added (μg/spot)	Amount of marker added in formulation (μg/spot)	Amount recovered*(%)	Recovery ± SD*(%)	% RSD
Guggulsterone E	18.4	50%	9.2	98.80	0.10	0.37
	18.4	100%	18.4	101.05	0.14	0.39
	18.4	150%	27.6	99.24	0.15	0.34
Guggulsterone Z	46.4	50%	23.2	99.088	0.57	0.82
	46.4	100%	46.4	98.69	0.59	0.65
	46.4	150%	69.6	101.81	1.01	0.86
Piperine	0.02	50%	0.01	102.33	0.0052	1.70
	0.02	100%	0.02	97.75	0.0081	2.08
	0.02	150%	0.03	99.58	0.013	2.66

*Average of three determinations.

Table 3
Percentage of piperine, guggulsterone E and Z in Triphala guggul formulation.

Types of sample	Guggulsterone E		Guggulsterone Z		Piperine	
	Total area included in peak	%w/w	Total area included in peak	%w/w	Total area included in peak	%w/w
TF1	1889.27	14.32	4790.8	35.14	6288.96	0.0896
TF2	1967.45	14.92	5206.8	38.416	6174.44	0.0778
TF3	1944.76	14.8	5149.7	37.985	5774.74	0.0367
TM1	1439.07	1.05	1838.7	11.87	5759.28	0.0351
TM2	1299.38	9.35	2028.9	13.37	8956.22	0.364
TM3	1224.6	8.7	1553.9	9.62	9770.51	0.55
TN1	2537.74	15.11	4565.9	33.36	5543.15	0.0128
TN2	1930.35	14.68	3716.6	26.67	5487	0.0071
TN3	1304.48	9.38	2976.5	20.84	3663.92	0.0253
TP1	1808.27	13.68	3134.8	22	5538.92	0.0124
TP2	2070.34	15.02	3592.1	25.7	5827.43	0.0422
TP3	1964.74	14.4	3362	23.88	5981.93	0.058

4. Discussion

The HPTLC method optimized in this work for the quantification of guggulsterone E, Z and piperine is simple, rapid, accurate, reproducible and sensitive. It can be useful to analyze a wide variety of guggul and piperine containing products. The method was established taking requirements of high precision and economy into consideration. The validation parameters for the developed method were the specificity, calibration curve, precision (repeatability), recovery and accuracy. The method resulted in a sharp, symmetrical, and well resolved peak. The spectrum of standard and sample shown end to tail well overlapping. Linear regression data for the plot confirmed the good linear relationship and the resulting equation was operational in the concentration range of 2.5–17.5 μg/spot for guggulsterone E, 5–30 μg/spot for guggulsterone Z, 0.06–0.14 μg/spot for piperine. The optimized method was found precise and accurate.

The wide variations in the labelled content in the marketed formulations were observed. This shows that Ayurvedic formulations are not standardized and thus obviously lead to marked differences in the therapeutic efficacy of the formulations, when administered. Hence, the newly developed HPTLC method could be proposed for routine quality control process for the estimation of guggulsterone E, Z and piperine in formulations. It can be adopted to standardize the product inprocess and the content of guggulsterone E, Z and piperine can be altered during its formulation stage, thus ensuring desired therapeutic efficacy of the herbal product. This would also minimize or avoid the batch-to-batch variation. This content of research will also be useful in reckoning the precise shelf life and stability of the respective formulations having these phytoconstituents. These types of analytical protocols following sophisticated modern techniques are important for worldwide acceptance and to bring the herbal formulations at par with allopathic formulations. It also serves as a rapid and specific tool in the herbal research, thereby, allowing the manufacturers to set quality standards and specifications so as to seek marketing approval from regulatory authorities for therapeutic efficacy, safety and shelf life of herbal drugs.

Conflicts of interest

All authors have none to declare.

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Original article

Phytochemical and GC–MS analysis of bioactive compounds of *Sphaeranthus amaranthoides* Burm

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ABSTRACT

Objective: To isolate and analyze the phytochemical constituents of *Sphaeranthus amaranthoides* using GC–MS.
Method: Preliminary phytochemical screening of the extract was carried out according to the standard method described by Brindha et al. GC–MS analysis was performed on the methanolic extract of *S. amaranthoides* to find out the chemical constituents.
Results: Phytochemical screening revealed the presence of steroids, alkaloids, sugars, phenolics, flavonoids, saponins, tannins and amino acids with mottled degree. GC–MS results revealed the presence of 23 different phytocompounds viz., 2-Propenoic acid, 2-methyl-, 2-[[2,3,3a,4,7,7a(or 3a,4,5,6,7,7a)-hexahydro-4,7-methano-1H-indenyl]oxy] ethyl ester (32.73%), Methanone, (1-hydroxycyclohexyl)phenyl – (13.71%), Methyl 2-bromomethyl-10-tetrahydropyranyloxy-2-decenoate – (7.84%),4,7-Methano-1H-indene,3a,4,5,6,7,7a-hexahydro-5-(2-propenyloxy) – (6.27%), Primidone – (4.50%), 2-Propenoic acid, 1,7,7-trimethylbicyclo[2.2.1]hept-2-yl ester, exo – (3.78%) and normorphine, bis(o-trimethylsilyl) – (3.65%) etc.
Conclusion: The presence of various bioactive compounds confirms the application of *S. amaranthoides* for various diseases by traditional practitioners. However, isolation of individual phytochemical constituents may proceed to find a novel drug.

1. Introduction

Medicinal plants have been used for centuries as remedies for human disease.[1–3] In India plenty of plants are being used as drug due to their medicinal properties. The plant kingdom still holds many species of plant contain substance of medicinal values which are yet to be discovered.[4] Extensive studies of the adverse effects of these herbal medicines and establishment of a good correlation between biomarkers and plants are essential for ensuring the efficiency and quality of the herbal medicines. Recently, there has been growing interest in exploiting the biological activities of flora and fauna owing to their natural origin, cost effectiveness and lesser side effects.[5,6] Plant based natural constitutions can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seed, etc.[7] The medicinal actions of the plants unique to particular plant species or groups are consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct.[8] The spectrometric and chromatographic screening method could provide the needed preliminary observations to select crude plant extracts with potentially useful properties for further chemical and pharmacological investigations.

The determination of phytoconstituents is largely performed by the relatively expensive and often laborious techniques such as gas (GC) and liquid chromatography (LC) combined with specific detection schemes.[9] In the last few years, GC–MS has become firmly established as a key technological metabolic profiling in both plant and non-plant species.[10–12] One of them is *Sphaeranthus amaranthoides*. *S. amaranthoides* Burm.f. is a small procumbent herb, with steam rooting and pubescent with appressed hair leaves palmately 3-foliolate. Features of the herb: low annuals with spreading branches, stem – erect, glabrous, sometimes as thick as the little finger, but short, branches – not winged and 8–12 inches, leaves – 2–4 inches, linear, oblong narrowed at the base. This plant is well known for its medicinal value for the treatment of eczema, blood disorder, stomach worms, filarial, fever and as a remover of kapha, vata, and piles. It is also known to cure skin diseases.[13]

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Table 1
Preliminary phytochemical screening of methanolic extract of *Sphaeranthus amaranthoides*.

Compounds	Methanol extract
Steroids	+
Alkaloids	+
Sugar	+
Phenolics	+
Flavonoids	+
Saponins	+
Tannins	+
Amino acids	+

S. amaranthoides belongs to plant kingdom, Dicotyledon class, Gamopetalae sub class, Inferae series, Asterales order, and Asteraceae (Compositae) family. It is weed of paddy field of southern India particularly in Thoothukudi Dist., Tamil Nadu, India (Dec. 2012). Crude extracts and medicines manufactured of the principles of natural compounds even by pharmaceutical companies may lead to large scale exposure of humans to natural products. In order to promote the use of medicinal plants, it should be thoroughly investigated with their composition, activity and thus validate their use.[14] The literature search reveals that still no work have been done on this plant. And nobody has isolated this crude extract from methanolic solvent and analyzed the crude extract by GC–MS. For this reason, the aim of this work was to isolate, investigate and characterize the bioactive chemical constitution in this organic crude extract by using photochemical test and GC–MS analysis.

2. Material and methods

2.1. Collection of the plant material

The plant *S. amaranthoides* was collected from the Thoothukudi Dist., Tamil Nadu, India and all the primary works done (washing, drying…etc.). The plant materials were identified and authenticated by Dr. V. Chelladurai, Retired Research Officer – Botany, Central Council for Research in Ayurveda and Siddha (C.C.R.A.S). Govt. of India, Tirunelveli. The collected plant material was free from disease and also free from contamination of other plants.

2.2. Preparation of plant extract

100 g of *S. amaranthoides* air-dried and coarsely powdered plant material was extracted with 500 ml methanolic solvent by using Soxhlet extractor. After extraction the sample was kept in dark for 72 h with intermittent shaking. Then the solvent was evaporated under reduced pressure using Rota-vapor and to obtain viscous semi solid masses.

2.3. Phytochemical screening

The methanolic extract was tested for steroids, alkaloids, sugar, phenolic compounds, flavonoids, saponins, tannins, anthraquinone and amino acids. Phytochemical screening of the extract was carried out according to the standard method.[15]

2.4. GC–MS analysis

The GC–MS analysis of methanolic crude extract of *S. amaranthoides* was performed using a GC–MS equipment Thermo GC-TRACE ultra ver: 5.0, Thermo MS DSQ II. Experimental conditions of GC–MS system were as follows: TR 5-MS capillary standard non-polar column, dimension: 30 m, ID: 0.25 mm, Film: 0.25 μm was used and flow rate of mobile phase (carrier gas: He) was set at 1.0 ml/min. In the gas chromatography part, temperature program (oven temperature) was 40 °C raised to 250 °C at 5 °C/min and injection volume was 1 μL. Samples which dissolved in chloroform were run fully at a range of 50–650 *m/z* and the results were compared by using Wiley Spectral library search program. The mass spectra detected in 36 min.

3. Result & discussion

The phytochemical screenings of *S. amaranthoides* extract revealed that the methanolic extract contains steroids, alkaloids,

Table 2
Compounds present in the methanolic extract of *Sphaeranthus amaranthoides* using GC–MS analysis.

No.	RT	Name of the compound	MF	MW	Peak area %	Compound nature
1	3.10	(2RS,3aRS,7aSR)-2-(3-Hydroxy-1-methoxypropyl)perhydroindan-4-one	C14H24O4	256	1.08	Ketone
2	8.07	Dimethyl derivative of vitamin D3 – triol	C28H48O3	432	0.92	Sterol
3	9.33	7,8 Bi(trimethylsilyl)benzo(5,6-g)-1H,3H-quinazoline-2,4-dione	C18H24N2O2Si2	356	1.89	Alkaloid
4	12.61	1-Propanone, 2-bromo-1-phenyl – (CAS)	C9H9BrO	212	1.44	Ketone
5	13.23	1,3-Bis(4-chlorobenzyl)-5,6-dihydrobenzo[f]quinazoline	C26H20Cl2N2	430	0.72	Alkaloid
6	14.34	2-Propenoic acid, 1,7,7-trimethylbicyclo[2.2.1]hept-2-yl ester, exo-	C13H20O2	208	3.78	Ester
7	17.12	2-tert-Butyl-4-isopropyl-5-methylphenol	C14H22O	206	0.49	Phenol
8	17.71	4,7-Methano-1H-indene, 3a,4,5,6,7,7a-hexahydro-5-(2-propenyloxy)-	C13H18O	190	6.27	Ester
9	20.57	Methanone, (1-hydroxycyclohexyl)phenyl-	C13H16O2	204	13.71	Ketone
10	22.99	2-Propenoic acid, 2-methyl-, 2[[2,3,3a,4,7,7a(or 3a,4,5,6,7,7a)-hexahydro-4,7-methano-1H-indenyl]oxy]ethyl ester	C16H22O3	262	32.73	Ester
11	25.71	Hexadecanoic acid, methyl ester	C17H34O2	270	0.75	Ester of fatty acid
12	29.01	Primidone	C12H14N2O2	218	4.50	Alkaloid
13	32.72	1,2,4-Trioxolane-2-octanoic acid, 5-octyl-, methyl ester (CAS)	C19H36O5	344	1.80	Ester of fatty acid
14	33.40	4,4′-isopropylidene-bis-(2-cyclohexylphenol)	C27H36O2	392	0.83	Phenol
15	33.81	4,5-Bis(p-bromophenoxy)-1,2-dicyanobenzene	C20H10Br2N2O2	468	0.66	Aromatic
16	34.53	Epoxygedunin	C28H34O8	498	0.63	Saponin/steroids
17	36.00	6-(t-Butylimino)-8-(3′-trifluoromethylphenyl)-3,4-dihydro-2H,6H-pyrimido[2,1-b][1,3]thiazine-7-carbonitrile	C19H19F3N4S	392	0.87	Alkaloid
18	36.54	7a,9c-(Iminoethano)phenanthro[4,5-bcd]furan,4aà,5-dihydro-3-methoxy-12-methyl- 9(CAS)	C18H19NO2	281	0.67	Alkaloid
19	36.85	Diethyl2-(2-furyl)-4-hydroxy-4-methyl-6-oxo-1,3-cyclohexanedicarboxylate tbdms	C23H36O7Si	452	1.00	Ester
20	37.14	2,9-bis(2′,6′-dimethoxyphenyl)-1,10-phenanthroline	C28H24N2O4	452	1.36	Alkaloids
21	37.61	6,7-Dihydro-6,6-dimethyl-2,3-diphenylindazol-4(2H,5H)-one	C21H20N2O	316	1.03	Alkaloids
22	37.98	Di-(2-ethylhexyl)phthalate	C24H38O4	390	2.54	Ester
23	38.58	Normorphine,bis(o-trimethylsilyl)	C22H33NO3Si2	415	3.65	Alkaloid

sugars, phenolics, flavonoids, saponins, tannins and amino acids compounds except anthraquinone (Table 1).

The results pertaining to GC–MS analysis lead to the identification of number of compounds from GC fractions of the methanolic extracts of *S. amaranthoides*. They were identified through mass spectrometry attached with GC. GC–MS analysis of methanolic extract of *S. amaranthoides* was put into a (Table 2). The result revealed the presence of 23 different phytocompounds viz., (2RS,3aRS,7aSR)-2-(3-Hydroxy-1-methoxypropyl)perhydroindan-4-one – (1.08%), Dimethyl derivative of vitamin D3 – triol – (0.92%), 7,8-Bis(trimethylsilyl)benzo(5,6-g)-1H,3H-quinazoline-2,4-dione – (1.89%), 1-Propanone, 2-bromo-1-phenyl – (1.44%), 1,3-Bis(4-chlorobenzyl)-5,6-dihydrobenzo[f]quinazoline – (0.72%), 2-Propenoic acid, 1,7,7-trimethylbicyclo[2.2.1]hept-2-yl ester, exo – (3.78%), 2-tert-Butyl-4-isopropyl-5-methylphenol – (0.49%), 4,7-Methano-1H-indene,3a,4,5,6,7,7a-hexahydro-5-(2-propenyloxy) – (6.27%), Naphthalene – (0.72%), Methanone, (1-hydroxycyclohexyl) phenyl – (13.71%), 2-Propenoic acid, 2-methyl-, 2-[[2,3,3a,4,7,7a(or 3a,4,5,6,7,7a)-hexahydro-4,7-methano-1H-indenyl]oxy] ethyl ester – (32.73%), Hexadecanoic acid, methyl ester – (0.75%), Primidone – (4.50%), 1,2,4-Trioxolane-2-octanoic acid, 5-octyl-, methyl ester – (1.80%), 4,4′-isopropylidene-bis-(2-cyclohexylphenol) – (0.83%), 4,5-Bis(p-bromophenoxy)-1,2-dicyanobenzene – (0.66%), epoxygedunin – (0.63%), 6-(t-Butylimino)-8-(3′-trifluoromethylphenyl)-3,4-dihydro-2H, 6H-pyrimido[2,1-b][1,3]thiazine-7-carbonitrile – (0.87%), 7a,9c-(Iminoethano)phenanthro[4,5-bcd]furan, 4aà,5-dihydro-3-methoxy-12-methyl- (CAS) – (0.67%), Diethyl 2-(2-furyl)-4-hydroxy-4-methyl-6-oxo-1,3-cyclohexanedicarboxylate tbdms – (1.00%), 2,9-bis(2′,6′-dimethoxyphenyl)-1,10-phenanthroline – (1.36%), 6,7-Dihydro-6,6-dimethyl-2,3-diphenylindazol-4(2H,5H)-one – (1.03%), Di-(2-ethylhexyl)phthalate – (1.03%), normorphine, bis(o-trimethylsilyl) – (3.65%). The GC–MS spectrum confirmed the presence of 23 components with the retention time 3.10, 8.07, 9.33, 12.61, 13.23, 14.34, 17.12, 17.71, 19.72, 20.57, 22.99, 25.71, 29.01, 32.72, 33.40, 33.81, 34.53, 36.00, 36.54, 37.14, 37.61, 37.98 and 38.58, respectively (Fig. 1).

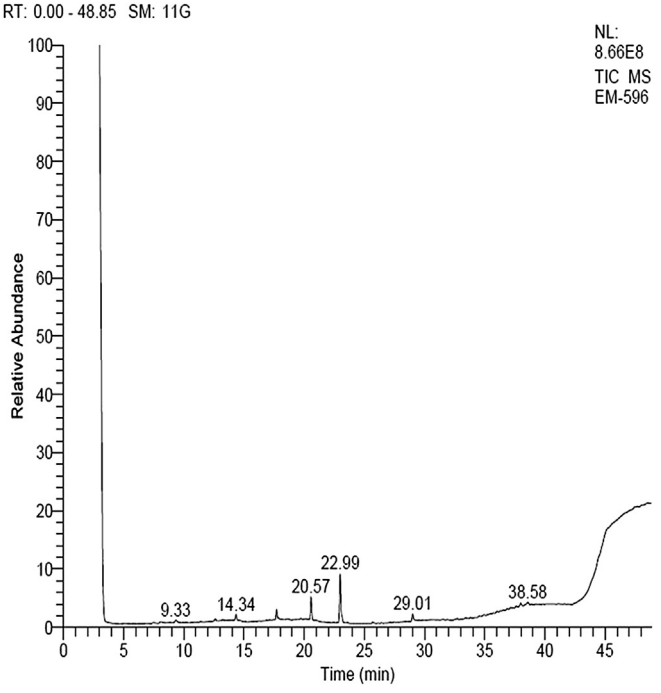

Fig. 1. GC–MS chromatogram of methanolic extract of *Sphaeranthus amaranthoides*.

In the present study, methanolic extract of the plant of *S. amaranthoides* is analyzed through GC–MS. Till date no reports exist on the GC–MS analysis of *S. amaranthoides*. In terms of percentage amounts epoxygedunin, hexadecanoic acid, Di-(2-ethylhexyl) phthalate and primidone predominant in the extract. These four major compounds have some important medicinal activity in future drug discovery system. Such as epoxygedunin having anticancer activity, on the other hand hexadecanoic acid had antimicrobial, nematicide, hemolytic 5-alpha reductase inhibitor and antioxidant activity. Hemophiliacs, kidney dialysis activity were present in Di-(2-ethylhexyl) phthalate compound. Primidone shows anti-convulsant activity. The individual fragmentation for few of the components is illustrated in Fig. 2A–D. The name, molecular weight, molecular formula and structure of the component of test material were determined. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. GC–MS analysis of methanolic extract of *S. amaranthoides* was tabulated in Table 2.

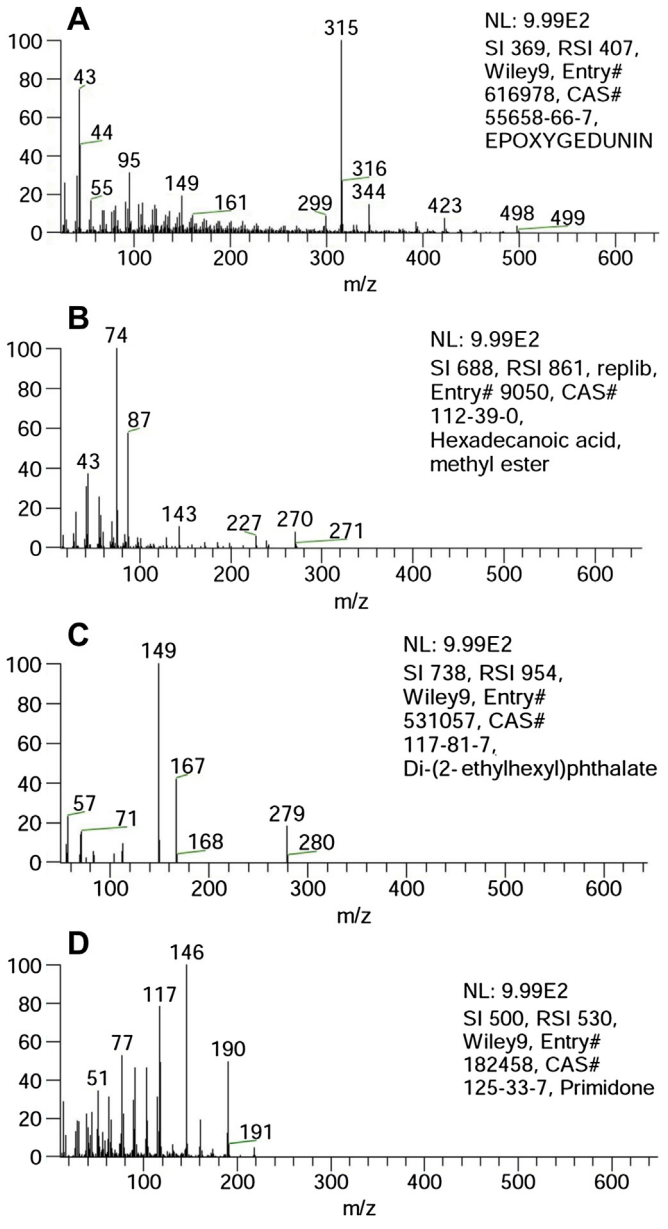

Fig. 2. A) Epoxygedunin. B) Hexadecanoic. C) Di-(2-ethylhexyl) phthalate. D) Primidone.

4. Conclusion

The present study results confirmed the presence of phenolics, alkaloids, steroids, saponins, tannins and flavonoids with varied degree. In addition to this, GC–MS profile can be used as biochemical markers in the pharmaceutical industries to identify the authentic mother plants and differentiate from its adulterants.

Conflicts of interest

All authors have none to declare

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Original article

Evaluation of antioxidant and antimicrobial activities of the phenolic composition of Algerian *Arbutus unedo* L. roots

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ABSTRACT

Introduction: The composition of phenols and flavonoids present in ethyl acetate and methanolic extracts of Algerian *Arbutus unedo* L. roots was in this work investigated using a combination of analytical techniques.
Methods: Firstly, the total phenolic and flavonoid content was established by an analysis of both extracts using capillary GC-MS and ^{1}H, ^{13}C NMR spectroscopy after fractionation via column chromatography. Secondly, a screening for the antioxidant and antibacterial properties of the compounds identified was performed via 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, as well as ferric-ion reducing power (FRAP) assays. Subsequently, the antibacterial activity of the compounds extracted was determined via disc-diffusion against both Gram-positive and Gram-negative bacteria.
Results: It was found that the ethyl acetate extract of Algerian *A. unedo* L. roots was characterized by higher concentrations of phenolic acids, which also has about 2-3 times better radical scavenger properties than the reference antioxidant used, ascorbic acid. In addition, this extract showed the best antibacterial activity obtained in this study against both Gram-positive bacteria as well as Gram negative bacteria, with minimal inhibition dosage values as low as 25 Ag/disc.
Conclusion: To conclude both the antioxidant and the antibacterial properties of the extracts of Algerian *A. unedo* L. roots could hence be of practical value.

1. Introduction

Flavonoids are a major class of polyphenols present in plants, and several thousand derivatives sharing a common phenylchromane scaffold have been documents. Flavonoids are widely distributed in the plant kingdom, being present in a broad range of commonly consumed fruits and vegetables and plant-derived products such as cocoa, tea or wine. The interest in dietary flavonoids has grown in the last fifteen years, among other reasons due to the publication of several epidemiological studies showing an inverse correlation between dietary consumption of flavonoid-rich products and the incidence of and mortality from cardiovascular disease and cancer.[1–3]

Indeed, polyphenols, and among those particularly the group of flavonoids, possess a wide spectrum of chemical and biological activities, including antioxidant properties.[4] Prominent members of this class are quercetin and (epi)catechin, as they are major flavonoids present in the human diet and which are hence of practical interest. These compounds behave as powerful antioxidants and free radical scavengers[5,6] as well as being able to interact with several key enzymes.[7] The numerous beneficial effects attributed to phenolic products sparked new interest in finding vegetal species with high phenolic content and associated relevant biological activity. In this vein, we in this work examined the roots of *Arbutus unedo*, which constitutes a rich dietary source of phenolic compounds with antioxidant and bioactive properties which has been traditionally harnessed in folk medicine in the Mediterranean

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region as well as North Africa.[8–12] In Algerian traditional medicine, a decoction of the root has been shown to exhibit antihypertensive properties and its dried leaves are used also for other indications.[13,14] The use of *A. unedo* in the prevention and treatment of platelet aggregation in conjunction with arterial hypertension could be supported by previous research.[15,16] On the analytical level, several compounds, including polyphenols, have been isolated from *A. unedo* extracts[9,12] and an antioxidant activity was demonstrated in several extracts.[17–20] The present study expands on those results by examining the antioxidative properties of the catechin and solvent extracts (namely ethyl acetate and methanolic extracts) of *A. unedo* roots, *via* employing 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric-ion reducing power (FRAP) assays. Results are compared with an antioxidant standard, namely ascorbic acid. In addition, the flavonoid and total phenol contents were determined by colorimetric methods and their antimicrobial effects were evaluated *via* the disc-diffusion method. Finally, nine phenolic compounds in ethyl acetate extract were characterized using GC–MS and NMR spectroscopy.

2. Materials and methods

2.1. Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, gallic acid, Folin–Ciocalteu, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine reagents were purchased from Sigma (Sigma, France). All other chemicals were of analytical grade and obtained from the same vendor.

2.2. Plant material

Fresh roots of *A. Unedo* were collected in the Terni forest (at about 20 km south of Tlemcen). Voucher specimens were deposited in the herbarium of Abou Bekr Belkaid University, Tlemcen, Algeria.

2.3. Analytical protocols (NMR, CC and GC–MS)

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were obtained on a Bruker AMX 300 instrument using acetone-d_6 as an internal reference. Chemical shift (δ) values were reported in ppm and coupling constants (J) in Hz. Each ^{1}H NMR spectrum consisted of 128 scans requiring 10 min and 26 s acquisition time. A presaturation sequence was used to suppress the residual H_2O signal with low power selective irradiation at the H_2O frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz. Gas chromatographic analysis was performed using an Agilent 6890 Series GS System gas chromatograph fitted with a splitless injector for a low background HP-5MS fused silica capillary column (60 m × 0.25 mm i.d. ×0.25 μm film thickness) supplied by Agilent. A silanized injector liner split/splitless (2 mm i.d.) was used. Detection was carried out with a 5973 mass-selective single quadrupole detector (Agilent Technologies). The GC–MS operation control and the data process were carried out by Chem-Station software package (Agilent Technologies). The injector temperature was 250 °C. The oven temperature was held at 90 °C for 1 min, then increased to 220 °C at a heating rate of 6 °C min^{-1}, then to 290 °C at 10 °C min^{-1} and held for 1.23 min and finally to 310 °C at a rate of 40 °C min^{-1} and the temperature was held for 7.5 min. The total run time was 38.5 min. The detector temperature was 280 °C. The carrier gas used was helium at a flow rate of 1.0 ml min^{-1}. The samples were injected in the splitless mode and the splitter was opened after 5 min (delay time). The sample volume in the direct injection mode was 1 μl. The conditions for electron impact ionization (EI) were an ion energy of 70 eV and the mass range scanned was 140–465 *m/z*. The identification of chemical compounds was assigned on the basis of comparison of their retention indices and mass spectra with to the NIST Library and those given in the literature.[21,22] Column chromatography (CC) was performed using silica gel (200–400) and TLC analyses were carried out on silica gel GF254 plates. Given that silylation is a suitable procedure for the gas chromatographic (GC) analysis of non-volatile and thermolabile compounds this method has been applied to the phenolic extract and standards.[23] For the silylation procedure, a mixture of 200 μl BSTFA, 200 μl ethyl acetate extract of roots of *A. unedo* in acetonitrile, and 50 μl pyridine were mixed and mechanically shacked for 2 min at room-temperature and consecutively placed in a water bath at 80 °C for 60 min. At this point the sample was ready to be injected into the gas chromatography–mass spectrometer.

2.4. Preparation of extracts

Boiling water extracts (100 ml) of plant samples (100 g) obtained under reflux conditions (hydrodistillation process) were extracted three times (20 ml in each run) with organic solvents (ethyl acetate and methanol). The ethyl acetate and methanolic extracts were filtered and concentrated under vacuum to obtain two extracts in yields of 0.35% and 0.56% (w/w), respectively. The organic solvent extracts were dried over anhydrous sodium sulfate and then stored in sealed glass vials at 4 °C prior to analysis. Each extraction was performed in triplicate.

2.5. Extraction and isolation of catechin

The roots of *A. unedo* (1.5 kg) were extracted with methanol (MeOH) three times at room temperature. The methanol extracts were combined and concentrated under reduced pressure, removing all solvent. The extractions (68 g in total) were suspended in H_2O and extracted three times with EtOAc to give the EtOAc-soluble fraction (10.2 g). The EtOAc-soluble fraction was further separated using silica gel column chromatography (CC) and eluted with ether/EtOAc/MeOH (3:1:1) to yield four fractions, A (3.26 g), B (3.04 g), C (1.76 g) and D (2.14 g). Fraction A was subjected to CC on silica gel, eluted with ether/acetone (7/3) containing 0.1% acetic acid to give two sub-fractions (**A1**: 1.12 g and **A2**: 2.14 g). Sub-fraction **A1** was purified on silica gel CC, eluting with petroleum ether/acetone (7:3), and followed by recrystallization using acetone to obtain **a-1** (0.84 g; catechin; see Fig. 1).

2.6. Total phenolic contents

Total phenolic contents of the extracts were determined using the Folin–Ciocalteu reagent according to the method previously reported by Slinkard and Singleton (1977)[24] using gallic acid as a standard, and as modified recently by Li et al (2008)[25] 200 μl of the diluted extract solution containing 40 μg of the extract were added to 1 ml of Folin–Ciocalteu reagent (10 times diluted in distillated water). After 4 min, 800 μl of Na_2CO_3 (75 mg/ml) solution were added and the mixture was allowed to stand for 45 min at room temperature. At the end of the incubation, the absorbance was measured at 760 nm. The same procedure was also applied to the standard solutions of gallic acid to obtain a standard curve as a control. The concentrations of phenolic compound expressed as mg gallic acid equivalent per g (mg GAE/g) of extract were calculated according to the standard gallic acid graph. All experiments were carried out in triplicate, and gallic acid equivalent values were reported as X ± SD of triplicates.

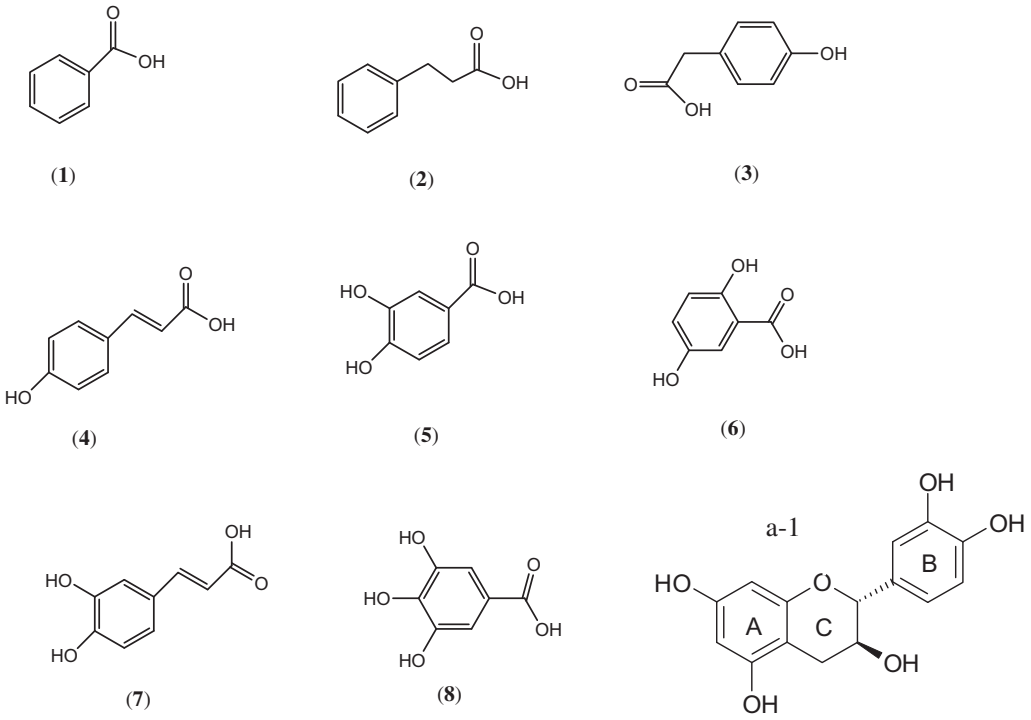

Fig. 1. Structures of compounds **1**–**8** and **a-1** identified from an ethyl acetate extract of *A. unedo* roots.

2.7. Total flavonoid contents

Total flavonoid contents of the extracts were determined using the Dowd method as adapted by Querttier et al (2000)[26] 1 ml of 2% methanolic aluminum trichloride ($AlCl_3$) solution was mixed with the same volume of extracts. Absorption at 430 nm was measured after 10 min against a sample consisting of 1 ml methanol without $AlCl_3$. The concentrations of flavonoid compounds expressed as mg quercetin equivalent per g (mg QE/g) of extract were calculated according to the standard quercetin graph. All experiments were carried out in triplicate, and quercetin equivalent values were reported as X ± SD of triplicates.

2.8. Antioxidant activity

2.8.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity

The antioxidant activity of the samples was determined by applying the 2,2-diphenyl 1-picrylhydrazyl (DPPH) radical scavenging method.[27,28] The scavenging activity on DPPH radicals assay is extensively used as a basic screening method for testing the antiradical activity of different plant materials. DPPH is a stable free radical that possesses a characteristic absorption maximum between 515 and 517 nm, which is diminished in the presence of a compound (*i.e.* antioxidants) capable of reducing it to its hydrazine form by hydrogen/electron donation.[29,30] In test tubes, 0.25 ml of 0.8 mM DPPH in MeOH were added to aliquots of the extracts dissolved in 3.75 ml of MeOH, corresponding to concentration ranges of extract between 1 and 10 μg/ml. After mixing the samples were maintained in the dark at room temperature for 30 min. Absorbance at 517 nm was measured using a UV/Vis Spectrophotometer (Optizen POP) and compared to a control without extract. A control was prepared for each sample by using methanol instead of the DPPH solution and ascorbic acid was used as reference compound. Antioxidant activity was expressed as a percent inhibition of DPPH radical, and calculated as follows:

$$\text{Scavenging activity}(\%) = 100 \cdot \text{Abs control} - \text{Abs sample}/\text{Abs control}$$

IC_{50} values were determined from the graphs of scavenging activity against the concentration of the extracts. These values are defined as inhibitory concentration of the extract necessary to decrease the initial DPPH radical concentration by 50% and are expressed in μg/ml. Triplicate measurements were carried out.

2.8.2. Ferric-reducing power (FRAP) assay

The total antioxidant capacity of sample was determined using a FRAP assay of Oyaizu (1986).[31] This approach evaluates the ability of a substance to reduce Fe^{3+} to Fe^{2+}, which is measured by the formation of a colored complex with potassium ferricyanide that can be read spectrophotometrically at 700 nm. Since the antioxidant activity of a substance is usually correlated to its reducing capacity, this assay provides a reliable method to evaluate the antioxidant activity.[32] For this assay different concentrations of extracts (100 and 150 μg/ml) in distilled water were mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferricyanide ($K_3Fe(CN)_6$) (1%). The mixture was incubated at 50 °C for 20 min after which 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (5 ml) was mixed with 5 ml of distilled water, 1 ml of $FeCl_3$ (0.1%) was added and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as the standard compound in this step and phosphate buffer (pH 6.6) was used as blank solution. The data presented are the average of three measurements given as mean ± standard deviation.

Table 1
Total phenol and flavonoid contents of *A. unedo* roots solvent (ethyl acetate and methanol) extracts employed in the current study.

Extracts	Total polyphenol content[a]	Total flavonoid content[b]
Ethyl acetate	555.19 ± 18	28.50 ± 2
Methanolic	335.16 ± 7	26.30 ± 2

Values expressed are means ± SD of three parallel measurements.
[a] mg gallic acid equivalent per g of extract (mg GA/g).
[b] mg quercetin equivalent per g of extract (mg QE/g).

Table 3
Antioxidant properties of catechin and solvent extracts from *A. unedo* roots as determined ferric-reducing antioxidant power (FRAP) assays.

Extracts	FRAP (A° = 700 nm)	
	100 (μg/ml)	150 (μg/ml)
Ethyl acetate	1.89 ± 0.14	2.37 ± 0.06
Methanolic	0.25 ± 0.05	0.45 ± 0.03
Catechin	1.01 ± 0.07	1.72 ± 0.04
Ascorbic acid	0.66 ± 0.06	1.2 ± 0.01

Values expressed are means ± SD of three parallel measurements.

2.9. Antimicrobial activity

2.9.1. Bacterial and yeast strains

The bacterial strains used in this study, *i.e. Staphylococcus aureus* and *Listeria monocytogenes* (Gram positive), and *Escherichia coli* and *Pseudomonas aeruginosa* (Gram negative) were isolated at the Medical Reanimation Department of the Hospital University Center of Tlemcen in Algeria. The yeast *Candida albicans* was isolated at the Dermatology Department of the same hospital.

2.9.2. Preparation of inocula

Bacterial strains, preserved in nutrient agar at 4 °C, were revivified in nutrient solution and incubated at 37 ± 1 °C for 24 h. 0.1 ml of each culture was added to 10 ml BHIB (Brain Heart Infusion Broth, Pronadisa Hispanalab). *C. albicans*, preserved at 4 °C in Sabouraud agar supplemented with chloramphenicol, was revivified in nutrient solution and incubated at 30 ± 1 °C for 48 h. 0.1 ml of each culture was added to 10 ml sterile physiological water (0.85 g NaCl/l H_2O). For the antimicrobial assay bacterial strains were grown on Mueller–Hinton Agar (MHA, Pronadisa Hispanalab) while *C. albicans* was grown on Sabouraud Dextrose Agar with added chloramphenicol (SDA, Merck).

2.9.3. Disc-diffusion method

Bacterial and yeast inocula reached microbial densities in the range 10^6–10^7 cfu/ml. Antibacterial activities of samples of the roots were assessed using the paper disk agar diffusion method according to Rios et al (1987).[33] Absorbent disks (Whatman disk of 6-mm diameter) were impregnated with 25 μg of extracts and then placed on the surface of inoculated plates (90 mm) and incubated at 37 °C for 24 h. Negative controls were prepared using a disk impregnated with the same solvent as that used to dissolve the samples, respectively. Gentamicin and amphotericin B were used as the positive control antibiotics for bacteria and fungi, respectively. Antimicrobial activity was assessed by measuring the inhibition zone. All the tests were performed in triplicate.

3. Results and discussion

3.1. Total phenolic and flavonoid contents of solvent extracts

The amount of total phenolics in the extracts was determined spectrophotometrically according to the Folin–Ciocalteu procedure and calculated as gallic acid equivalent. The amounts of total phenols found in *A. unedo* extracts are shown in Table 1. The total phenolics and contents of the ethyl acetate and methanolic extracts of *A. unedo* roots were 555.19 and 335.16 mg/g, respectively. The results showed that the ethyl acetate extract has a higher total phenol and flavonoid content than the methanolic extract. Similarly, the ethyl acetate extract was found to be richer in flavonoids (28.50 mg/g) than the methanolic extract (26.30 mg/g). Overall roots of *A. unedo* possess high total phenolic sand it is of interest to compare our values with previous studies. For example, the total phenolic values were comparable to those reported in *A. unedo* bark and leaves from Portugal[18] which fall in the range 254–328 mg GAE/g. However, the values were much higher than that in *A. unedo* leaves and fruits from Northeast of Portugal[12] which falls in the range 17–170 mg GAE/g.

3.2. Antioxidant activity

The antioxidant activity of catechin and solvent extracts was assessed using DPPH and FRAP which provide information on different aspects of the antioxidative properties of a particular compound. Table 2 presents the results of the antioxidant activity obtained for the studied extracts, expressed in terms of the amount of extract needed to decrease the DPPH concentration by 50% (IC_{50}). The IC_{50} value for ascorbic acid was also obtained and reported in Table 3 for comparative purposes. It was found that the ethyl acetate extract of *A. unedo* roots had the highest radical scavenging activity, corresponding to the lowest IC_{50} value of 1.2 μg/ml. This activity was higher than that of the catechin alone, with an IC_{50} value of 2.1 μg/ml, as well as the methanolic extract with an IC_{50}

Table 2
Antioxidant properties of catechin and solvent extracts from *A. unedo* roots as determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging.

Source	Antiradical activity (%)						
Ethyl acetate	Extract concentration (μg/ml)	0.5	1.0	1.5	2.0	2.5	
	Scavenging effect on DPPH (%)	33.35	42.35	52.36	82.50	98.36	
	DPPH IC_{50} (μg/ml)						1.2 ± 06
Methanolic	Extract concentration (μg/ml)	4.0	6.0	7.0	8.0	10	
	Scavenging effect on DPPH (%)	22.35	42.35	50.36	68.36	79.36	
	DPPH IC_{50} (μg/ml)						6.9 ± 0.8
Catechin	Extract concentration (μg/ml)	1.5	2	2.5	3	3.5	
	Scavenging effect on DPPH (%)	36.25	49.36	59.35	75.35	88.35	
	DPPH IC_{50} (μg/ml)						2.1 ± 0.2
Ascorbic acid	Extract concentration (μg/ml)	2	2.5	3	3.5	4	
	Scavenging effect on DPPH (%)	33.25	42.53	51.36	61.25	82.36	
	DPPH IC_{50} (μg/ml)						2.8 ± 0.5

Values expressed are means ± SD of three parallel measurements.

Table 4
Antibacterial activity of extracts and catechin of *A. unedo* roots.

Microorganisms	Zone of inhibition in mm (% inhibition)				
	Methanolic extract	Ethyl acetate extract	Catechin	Gen[a]	AmB[b]
Gram-positive bacterium					
L. monocytogenes	8 (40)	10 (50)	12 (60)	20	nd
S. aureus	11 (48)	16 (80)	20 (86)	23	nd
Gram-negative bacterium					
P. aeruginosa	–	14 (100)	12 (85)	14	nd
E. coli	–	–	–	22	nd
Yeast					
C. albicans	–	–	–	nd	22

Mean diameter of zone of inhibition in millimeters.
Figures in parentheses are inhibition percentages.
nd: not determined.
–: no activity.
[a] GEN: Gentamicin.
[b] AmB: Amphotericin B.

value of 6.9 μg/ml. In addition, the DPPH scavenging abilities of both catechin and the ethyl acetate extract were higher than that of the standard, ascorbic acid, with an IC_{50} value of 2.8 μg/ml. The activities obtained in the FRAP assay for the ethyl acetate extract as well as catechin at a concentration of 100 μg/ml were 1.89 and 1.01, respectively, which again were stronger than the corresponding value for ascorbic acid of 0.66. The FRAP value obtained for the methanolic extract was significantly lower on the other hand with a value of 0.45. At higher concentrations (150 Ag/ml), the FRAP of ethyl acetate (2.37), methanolic (0.45) extract and catechin (1.72) also increased (as summarized in Table 3). The ethyl acetate extract is hence most potent, with an activity of more than twice than that of ascorbic acid. According to the results just presented we find a correlation between the total phenol content and antioxidant activity of the extract which is important to note.

3.3. Antimicrobial activity

The antibacterial activities of ethyl acetate and methanolic extracts and catechin obtained *in vitro* against the employed bacteria were qualitatively and quantitatively assessed by the presence or absence of inhibition zones, the results of which are summarized in Table 4. It can be seen that the ethyl acetate extract showed the highest inhibition activity (*P. aeruginosa*: 100%, *S. aureus*: 80%) and catechin showed the second highest inhibition activity (*S. aureus* 86%, *P. aeruginosa*: 85%, *L. monocytogenes*: 60%). On the other hand, the inhibition activity for methanolic extract was the lowest with inhibition of 48% and 40% against *S. aureus* and *L. monocytogenes*, respectively. No inhibition was obtained for *E. coli* and *C. albicans* strains. Hence also the antibacterial activity of extracts correlates well with the total phenolic contents of *A. unedo* root extracts. It can hence be hypothesized that the phenolic compounds present in the extracts are also causally responsible for their antibacterial activity, which is in line with previous studies.[34] We can conclude that *A. unedo* roots show significant antibacterial activity and could be considered as a source of natural antibiotics for medicinal use.

Table 6
Main phenolic acids compounds identified in ethyl acetate extract from *A. unedo* roots.

N°	Rt (min)	Compounds	Characteristic fragments
1	21.96	Benzoic acid	194. 179. 135. 105.77
2	27.54	Hydrocinnamic acid	310. 293. 196. 179.73
3	40.86	p-Hydroxyphenylacetic acid	296. 281. 252. 179. 164. 147.73
4	50.24	p-Coumaric acid	308. 293.279. 219.73
5	53.16	Protocatechuic acid	370. 355. 311. 281. 193. 73
6	59.62	Gentisic acid	355. 281. 267. 223. 147.73
7	69.97	Caffeic acid	219. 396. 381. 191.73
8	77.62	Gallic acid	458. 443. 355. 281. 179. 174.73

3.4. Structure elucidation

Compound **a-1** (catechin) was obtained as a red powder. The ESI-MS gave a quasi-molecular ion peak at *m/z* 289 $[M - H]^-$, corresponding to the molecular formula $C_{15}H_{14}O_6$. ^{13}C NMR showed a total of 12 sp^2 carbons, seven of which are quaternary, the six others being tertiary carbons (see Table 5 for details). The deshielding of 3 out of the seven quaternary carbons indicated that these are all bound to heteroatoms. Moreover, proton NMR indicated the presence of a broad peak around 6.75 ppm. The *trans* stereochemistry was deduced from the ^1H spectrum which showed two rather large coupling constants ($J = 8.1$ and $J = 5.4$ Hz). The ^1H NMR spectral features of **a-1** were similar to those of catechin.[34]

3.5. Ethyl acetate composition

The identification of each compound was based on a combination of retention time of standard silylated compounds, their molecular weights (MW) and characteristic fragments. The structures identified in the ethyl acetate extract are reported in Table 6 and Fig. 1 and comprise benzoic (1), hydrocinnamic (2), p-hydroxyphenylacetic

Table 5
^1H NMR. ^{13}C chemical shifts and coupling constants (Hz) of catechin (**a-1**).

Ring	Position	δ ^1H (ppm); m; J (Hz)	δ ^{13}C (ppm)	δ ^1H (ppm); m; J (Hz)[a]
C	2	4.58 (d. $J = 7.5$)	82.84	4.55 (d. $J = 7.5$)
	3	3.98 (m)	68.80	4.04 (m)
	4	2.52 (dd. $J = 16.0.\ 8.1$). 2.85 (dd. $J = 16.0.\ 5.4$)	28.50	2.49 (dd. $J = 16.1.\ 8.2$). 2.83 (dd. $J = 16.0.\ 5.4$).
A	4[a]		100.82	
	5		157.55	
	6	5.87 (d. $J = 2.2$)	96.30	5.91 (d. $J = 2.2$)
	7		157.82	
	8	5.94 (d. 2.27)	95.51	/
	8[a]		156.90	
B	1′		132.22	
	2′	6.75 (d. $J = 1.7$)	115.25	/
	3′	6.72 (d. $J = 8.1$)	116.07	6.75 (d. $J = 8.0$)
	4′		146.23	
	5′		146.20	
	6′	6.85 (dd. $J = 8.1.\ 1.8$)	120.02	/

d: doublet; dd: double doublets; m: multiplet.
[a] Data of literature.[39]

(3), p-coumaric (4), protocatechuic (5), gentisic (6), caffeic (7) and gallic (8) acids. Gallic acid has been associated with a wide range of biological actions, including antioxidant, antitumoral, antimicrobial, between many others.[35–37] The antioxidant and antimicrobial activities of phenolic compounds from plants are well known.[5,6,38] Given that the ethyl acetate extract of *A. unedo* roots exhibited strong antioxidant activity and a significant antimicrobial activity against the *P. aeruginosa* and *S. aureus* strains these biological activities can possibly be attributed to the phenolic compounds present in the extracts, which is supported by the correlations between phenolic contents and antibacterial activity described above. In addition, up to 6 TMS-derivative compounds were unknown and are the subject of future purification and characterization efforts (data not shown).

4. Conclusions

Our results demonstrate that *A. unedo* roots extracts exhibit potent free radical scavenging activities, which are multiple times higher than that of the positive control, ascorbic acid, as determined in both DPPH and FRAP assays. In addition, in particular the ethyl acetate extract of *A. unedo* roots also possesses significant antimicrobial activity against a variety of strains employed in this study. Hence, the results of this study suggest that extracts of *A. unedo* roots may have potential uses as natural antimicrobials and antioxidants, which can hence be proposed as new sources of natural additives for the food and/or pharmaceutical industries. Our findings from chemical analyses indicate that the roots of *A. unedo* are a promising source of bioactive compounds such as catechin and phenolic acids, the properties of which will be followed up also in future studies.

Conflicts of interest

All authors have none to declare.

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Original article

Induction of tetraploid plants of *Pogostemon cablin* (Blanco) and its quality evaluation

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ABSTRACT

Background: To establish and optimize the rapid propagation technology of *Pogostemon cablin* (Blanco) and to induce polyploidy plants of *P. cablin* (Blanco).
Methods: Shoot tips from *in vitro*-grown plants were treated with four different concentrations of colchicine solution (0.01%, 0.04%, 0.08%, 0.1%), and shaken (100 rpm) at 25 °C for 2, 4, 8 h, respectively. The induced buds were identified by stem-tip chromosome determination and stomatal apparatus observation. Six selected tetraploid lines were transferred to the field, and the leaf characteristics, contents of volatile oil were respectively evaluated to provide evidence of high-yield and good qualities of tetraploid *P. cablin* (Blanco).
Results: Six lines of tetraploid plants were obtained. The optimum concentration of colchicine treatment for tetraploid induction was 0.04% for 2 h. The chromosome number of the tetraploid plantlet was $2n = 4 \times = 56$. All tetraploid plants showed typical polyploidy characteristics. Three elite tetraploid lines possessed higher productivity of volatile oil than those of the control.
Conclusion: Obtained tetraploid lines will be of important genetic and breeding value and can be used for further selection and plant breeding.

1. Introduction

In plant breeding programs, polyploidy plants have been used to develop superior varieties and to restore the fertility of interspecific or intergeneric hybrids. Furthermore, the leaves, stems, roots, and flowers in polyploidy plants like *Echinacea purpurea* L[1] and *Cucumis melon*[2] are usually bigger than those of the diploid plants to have an increased biomass and yield. In recent years, the technique of *in vitro* polyploidy induction with colchocine has been employed in many medicinal plants, such as *Morinda officinalis*,[3] *Sophora flavescens* Aiton[4] and *Zingiber officinale* Rosc.[5] The desired active compounds of medicinal plants store in the leaves, stems, flowers or roots. Researches proved that some polyploid plants with giant characteristics have more active compounds than the diploids. It was reported that tetraploid plants of *Z. officinale* Rosc[5] possesses higher rhizome yield and overall productivity of volatile oil and gingerol than the diploids.

Pogostemon cablin (Blanco) belongs to Lamiaceae family. It is widespread in the south of China, Korea and Japan.[6] *P. cablin* (Blanco) is also a typical traditional Chinese medicine which has been widely used for the treatment of removing dampness, relieving summer-heat, exterior syndrome, stopping vomiting and stimulating the appetite. The one of the main medicinal components in *P. cablin* (Blanco) is volatile oil distributed mainly on the leaves.[7] Up to now, there has been no report on the *in vitro* tetraploid induction of *P. cablin* (Blanco). In this paper, the studies on the induction of tetraploid plant of *P. cablin* would be investigated.

2. Materials and methods

2.1. Plant material

P. cablin (Blanco) ($2 \times = 28$) were obtained from the medicinal planting garden of Guangdong Pharmaceutical University in the city of Guangzhou of Guangdong Province in China. The original plant was identified by the Department of traditional Chinese medicine at Guangdong Pharmaceutical University.

2.2. Material disinfection and shoots regeneration

Leaf explants and stem cuttings were collected and washed in running tap water for about 20 min and surface-sterilized by using 70% alcohol. Then the explants were washed three times with sterilized distilled water to remove the traces of alcohol thoroughly.

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Finally, the explants were sterilized with 0.1% mercuric chloride solution[8] for 8 min under aseptic condition. The explants were rinsed with sterile distilled water three to five times and transferred to a Petri dish containing sterile filter papers to remove excess surface water. The surface-sterilized explants were transferred to Murashige and Skoog (MS)[9] medium containing 3% (w/v) sucrose and 0.6% (w/v) agar powder supplemented with 0.2 mg L^{-1} 6-benzylaminopurine (BAP) and 0.5 mg L^{-1} α-naphthaleneacetic acid (NAA) to induce callus. The inoculated explants were kept at a temperature of 26 °C and provided with 1500 L × light intensity from fluorescence cool tube light for a 16 h photoperiod.[10] The induced callus were transferred to MS medium supplemented with 0.1 mg L^{-1} BAP to generate shoots.

2.3. Induction of polyploid plantlets

Shoot tips from *in vitro*-grown shoots were treated with five different concentrations of colchicine solution (0.01%, 0.04%, 0.08% and 0.1% (w/v)), and shaken (100 rpm) at 25 °C for 8 h. Treated buds were then transferred to the MS solid medium supplemented with 0.1 mg L^{-1} BAP and cultured in an illuminated incubator for a 16 h photoperiod of 1500 L × light intensity at 26 °C for 20 days for bud proliferation. Alternatively, the buds were submerged into 0.04% (w/v) colchicine solution for 1,2, 4 and 8 h, then transferred to the MS solid medium supplemented with 0.1 mg L^{-1} BAP and cultured in an illuminated incubator for a 16 h photoperiod of 1500 L × light intensity at 26 °C for 20 days for bud proliferation. The buds (approximately 3 cm in length) of the subculture materials were excised the tips of stems for subsequent chromosome determination.

2.4. Chromosome determination

Stem tips approximately 0.5 cm in length were excised at about 10 o'clock in the morning and pretreated in the 0.2% w/v colchicine solution for 2 h. After pretreatment, the stem tips were transferred to 0.075 mg L^{-1} KCL for 30 min at 26 °C. Then the stem tips were transferred to Carnoy's fixative (containing 3:1 ethanol and glacial acetic acid) and stored at 4 °C for 2–24 h, rinsed with distilled water for three times, each time for 10 min, respectively. Then it was macerated for 1.5 h with enzyme (containing 2.0% pectinase and 2.5% cellulase) at 37 °C. After soaking in Carnoy's fixative for 30 min, the fixed stem tips were stained with improved Carbol fuchsin (1.8 g sorbitol dissolved in 10 ml Carbol fuchsin, and then mixed with 45% v/v acetic acid, 90 ml). A photomicroscope (ML11, China) was used for chromosome determination. The chromosome count of each tetraploid (4 × = 56) line was repeated for at least three generations.

The buds (approximately 3 cm in length) of each tetraploid line were excised and transferred to the rooting medium consisting of the solid MS medium at half the macronutrient concentration and supplemented with 0.2 mg L^{-1} IBA to induce roots. The rooted plants were transplanted into a seedling bed for leaf characteristics' evaluation.

2.5. Stomatal measurements

Leaf characteristics were obtained from 6-month-old fully established glasshouse plants. For stomatal apparatus measurements, an area about 0.1 cm^2 on the upper epidermis of the unifoliate leaf was peeled off and spread onto a glass microscope slide. A photomicroscope (ML11, China) was used to measure the stomatal apparatus length and width. Four unifoliate leaves were chosen from the same parts of each of five diploid control plants and each of five tetraploid plants. Twenty stomatal apparatus were measured for each leaf.

2.6. The contents of chlorophyll of polyploidy plants

The determination of chlorophyll contents in leaves of all obtained tetraploid plants were carried out according to the method established by Xia Lan.[11] 0.05 g accurately weighted fresh leaves was placed in a test tube, submerged with 6 ml 95% ethanol for 24 h, and then it was used as sample solution. The absorbance (A) of sample solution was measured by UV-spectrophotometry (UV-1800, China) at 645 nm and 663 nm. Ethanol was used as blank control. The content of chlorophyll was calculated by the following equation and each sample was repeated for three times:

$$\text{Content of chlorophyll a (mg/g)} = (12.7 \times A_{663} - 2.69 \times A_{645}) \times V/1000w$$

$$\text{Content of chlorophyll b (mg/g)} = (22.7 \times A_{645} - 4.68 \times A_{663}) \times V/1000w$$

$$\text{Total content of chlorophyll (mg/g)} = \text{Content of chlorophyll a} + \text{Content of chlorophyll b}.$$

Here, ***V*** was the volume of test solution used at measuring, ml; w was the wealth of leaves at measuring, g.

2.7. The contents of volatile oil of polyploidy plants

Tetraploid plants (grown half a year old) were harvested in the field and were used to determine the contents of volatile oil. The measurement of volatile oil contents in leaves of all obtained tetraploid plants was conducted according to the guideline of China Pharmacopoeia (edition 2010). Three leaves samples (each was 3.0 g accurately weighted fine-grinded powder (W, g)) from each tetraploid line were used to measure the volatile oil content.

3. Results

3.1. Effect of colchicine treatment on tetraploid buds induction

To evaluate the effect of colchicine concentration on the tetraploid induction, the buds were submerged in colchicine solution for 8 h. The effect of colchicine concentration on the tetraploid

Table 1
The influence of different colchicine concentration on polyploid induction in *Pogostemon cablin*.

Colchicine concentration (%)	Treatment number	Survival number	Survival percentage (%)	Variation number	Variation rate (%)
0	39	38	97.44	0	0
0.01	30	20	66.67	2	6.67
0.04	40	24	60.0	10	25.0
0.08	30	13	43.33	1	3.33
0.1	21	2	9.52	0	0

Buds were submerged in different concentrations of colchicine solution for 8 h and cultured for 20 days on MS solid medium supplemented with 0.1 mg L^{-1} BAP.

induction is shown in Table 1. The results showed that after treatment with increasing colchicine concentration for 8 h, the percentages of buds that survived decreased significantly with the colchicine concentration, while almost all of the untreated buds survived. When colchicine concentration was 0.01% (w/v), the survival rate of buds was 66.67%, and as colchicine concentration increased to 0.1% (w/v), the survival rate of buds decreased to 9.52%. Chromosome determination was used to identify the tetraploids from the regenerated plants. According to the chromosome counts, the tetraploid induction rates were also influenced by the colchicine concentration, the induction rate increased in the first three concentrations, but decreased when the colchicine concentration reached 0.08% (w/v). The highest induction rate was found at the concentration of 0.04% (w/v), reached to 25%. These results indicated that the optimum concentration of colchicine treatment for tetraploid induction was 0.04% (w/v).

To assess the effect of treated time on the tetraploid induction, the buds were submerged in 0.04% (w/v) colchicine solution for different time (Table 2). When the buds were immersed in distilled water for 12 h, the survival number was decreased to less than 50%. It showed that the buds should not be immersed in solution for more than 12 h. The results showed that with the treated time increasing, the number of survival buds decreased. The results in Table 2 also showed that the tetraploid induction rate was affected by the treated time in 0.04% (w/v) colchicine solution. According to the chromosome count, immersing buds in the 0.04% (w/v) colchicine solution for 1, 2, 4 and 8 h were effective in induction of polyploidy buds, and the induction rate increased to the highest when the treated time reached 2 h. The percentage of polyploidy buds was 63.33% when immersing in 0.04% (w/v) colchicines for 2 h. This is by far the highest induction ratio in our experiments. Chromosome count revealed that the tetraploid plantlets had 56 chromosomes (4x = 56) [Figs. 1 and 2], and 55 tetraploid plantlets were obtained in our experiments.

3.2. Morphological differences between diploid and tetraploid

The morphological features of tetraploid plants were evaluated and compared with diploid control plants to determine whether they could be used to identify putative tetraploids. Tetraploid plants with thicker stems were taller and stronger than diploid plant. The leaves of tetraploid plants appeared normal in shape. The length, width, and area of the leaves for 6-month-old glasshouse-grown plants showed obvious difference. The length and width of glasshouse-grown diploid leaves were about 3.41 cm and 3.26 cm, respectively, while the same dimensions in glasshouse-grown tetraploid leaves were about 7.63 cm and 5.97 cm, respectively. The length and width of tetraploid leaves increased about 2.2 and 1.8 times than the diploids, respectively. The surface area of glasshouse-grown tetraploid leaves was therefore about 2.3 times greater than that of leaves from diploid plants. The sizes of stomatal apparatus of glasshouse-grown diploid and tetraploid leaves were measured and found to be significantly different. In general, tetraploids possessed longer and wider stomatal apparatus. The length and width of the stomatal apparatus of glasshouse-grown diploid leaves were about 0.52 μm and 0.29 μm, respectively, while the same dimensions in glasshouse-grown tetraploid leaves were about 0.60 μm and 0.44 μm, respectively (Table 3, Figs. 3 and 4).

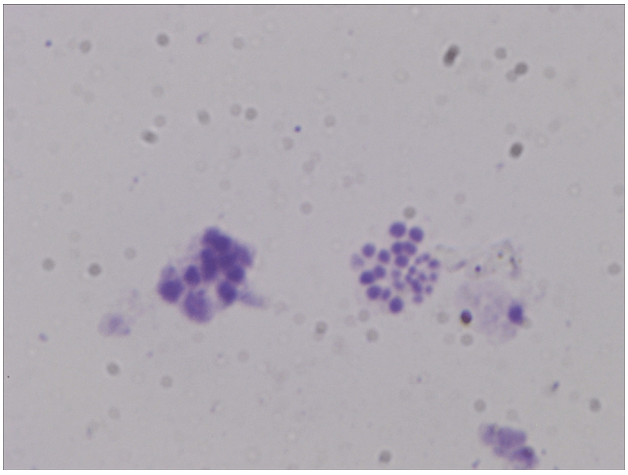

Fig. 1. The chromosome of diploid plant, 2n = 2 × = 28.

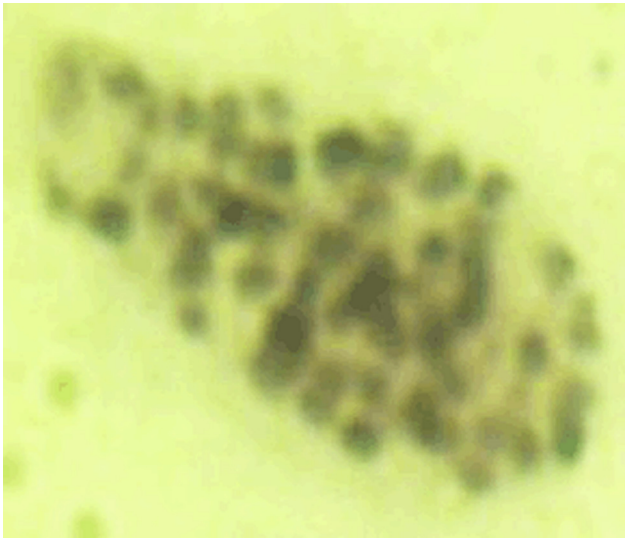

Fig. 2. The chromosome of tetraploid plant, 2n = 4 × = 56.

3.3. Determination of the leaves content of chlorophyll

The content of chlorophyll in each tetraploid line is shown in Table 4. The content of both chlorophyll a and chlorophyll b in M-1 line was about twice more than that of the diploid, respectively. The results indicated that the tetraploid lines showed higher chlorophyll content than the control.

Table 2
The effect of different treated time of 0.04%(w/v) colchicine solution on polyploid induction in *Pogostemon cablin* (Blanco).

Treatment time (hours)	Treatment number	Survival number	Survival percentage (%)	Variation number	Variation rate (%)
1	30	28	93.33	6	20.0
2	30	21	70.0	19	63.33
4	33	21	63.64	9	27.27
8	40	24	60.0	10	25.0

Table 3
Comparisons of plant characteristics of diploid and tetraploid in *Pogostemon cablin*.

Ploidy	Plant height/cm	Stem diameter/cm	Leaf length/cm	Leaf width/cm	Leaf area/cm^2	Stomatal apparatus length/μm	Stomatal apparatus width/μm
Diploids	18.50 ± 0.436a	0.93 ± 0.03a	3.41 ± 0.12a	3.26 ± 0.08a	13.00 ± 0.08a	0.52 ± 0.03a	0.29 ± 0.01a
Tetraploids	38.80 ± 0.361b	2.23 ± 0.08b	7.63 ± 0.21b	5.97 ± 0.18b	30.08 ± 0.37b	0.60 ± 0.03b	0.44 ± 0.02b

Values represent the mean ± standard error. Within each row, means followed by the same letter are not significantly different at $P = 0.05$ level by Duncan's multiple range test, the minuscule number indicates 0.05 level. The same as the following tables.

3.4. Determination of the content of volatile oil

One of the important purposes of *P. cablin* (Blanco) breeding is to improve the yield of medicinal compound of volatile oil, so the volatile oil yields of all diploid and tetraploid plants were evaluated. The volatile oil yield in each tetraploid line is shown in Table 5. The results indicate that all of the tetraploid lines showed higher volatile oil yield than that of control significantly. The highest content of volatile oil in tetraploid lines was found in line M-1, and the volatile oil content was 0.11 ml per 3 g, about 57% higher than that of the control.

4. Discussion

Polyploid plants are good-quality, high-yielding plants resistant to environment stress, pest and diseases. Medicinal polyploid plants can not only own more harvests, but also have more effective constituent. It is meaningful to induce polyploid *P. cablin* for the desired active compounds of *P. cablin* stored in the leaves and stems.[12–16]

There are different treatments to induce polyploid plant, including physic method, biologic method and chemical method. Compared with traditional methods, tissue culture is more effective and convenient to obtain a large number of materials for induction. Tissue culture could be used to speed up the polyploid breeding process, and improve the success rate of induction.[3,16–18] *P. cablin* (Blanco) is propagated mainly by vegetative propagation or cutting seedling for its seldom flowering. In this study, we established a successful the regeneration system of *P. cablin* (Blanco), and obtained lots of buds of *P. cablin* for the induction of polyploidy *P. cablin*.

Colchicine plays a significant role in inducing tetraploid. More and more reports showed that colchicine is effective to induce polyploidy plants.[19] The effect of colchicine concentration and treatment time is different for different plants. In our research, buds treated with low concentration of colchicine, the variation rate increased with the treating time. However, with the increasing of time and concentration of colchicines treatment, the death rate was increasing and the variation rate was decreasing for the toxicity of the colchicine. In our research, we found that the optimum concentration of colchicine treatment for the induction of tetraploid was 0.04%, and the optimum treated time was 2 h.

Morphological characteristics observation is an effective method for ploidy analysis. In our research, the tetraploid plants of

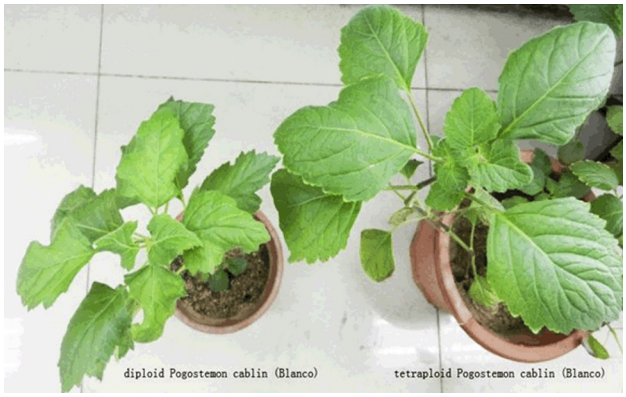

Fig. 3. The diploid and tetraploid *Pogostemon cablin*.

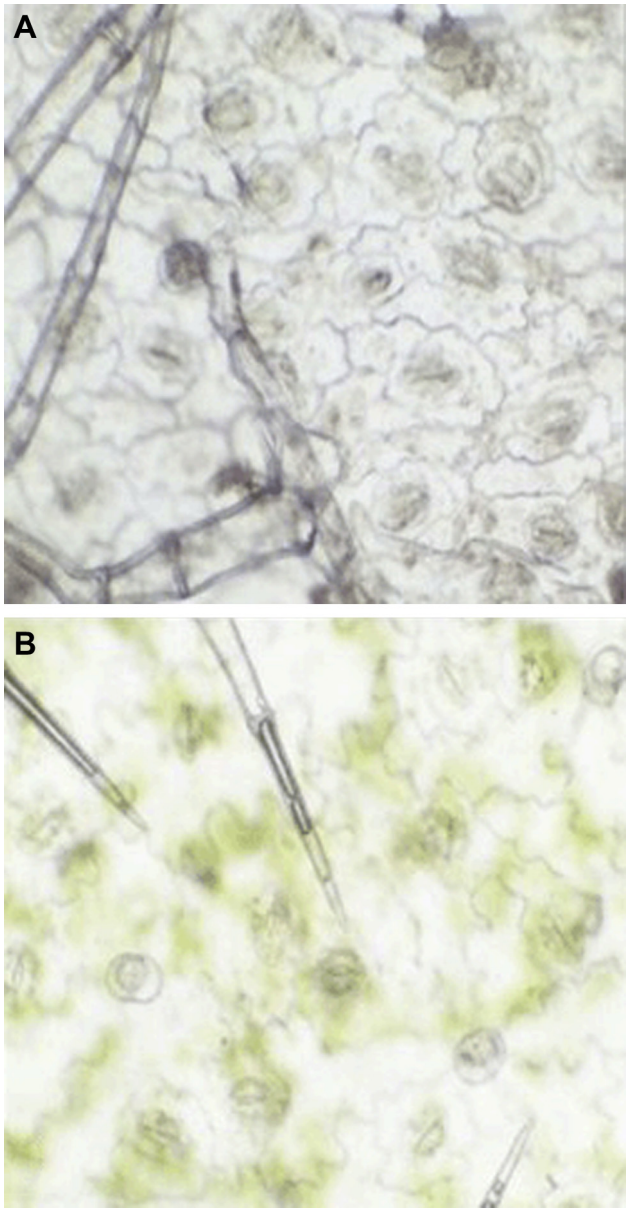

Fig. 4. The stomata of the diploid and tetraploid in *Pogostemon cablin* (Blanco). A: diploid, B: tetraploid.

Table 4
Comparisons of the content of chlorophyll in the leaves of diploid and tetraploid *Pogostemon cablin*.

Lines	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Content of chlorophyll (mg/g)
CK	0.679 ± 0.055a	0.377 ± 0.005a	1.055 ± 0.053a
M-1	1.418 ± 0.004b	0.676 ± 0.059b	2.094 ± 0.060b
M-2	0.834 ± 0.028a	0.378 ± 0.031c	1.211 ± 0.027c
M-4	1.110 ± 0.036c	0.423 ± 0.007d	1.533 ± 0.040d

Ck: diploid plant. M-1, M-2, M-3: tetraploid lines.

Table 5
Comparison of the content of volatile oil of diploid and tetraploid *Pogostemon cablin* (Blanco).

Lines	Volatile oil (mL)
Ck	0.07
M-1	0.11
M-2	0.10
M-4	0.10

P. cablin (Blanco) were shown significant larger characteristics than the diploids, such as plant height, stem diameter, leaf area, stomatal length and stomatal width. Before chromosome determination, the utility of the stomatal size in distinguishing plants with different ploidy levels has been used in other plant types which could be an auxiliary method for ploidy screen.[20] The stomata frequency decreases and stomata length and width increase in ploidy plants. Our report showed that induced tetraploids lines of *P. cablin* shown longer and wider stomata than the diploids.

In addition, our research also showed the content of chlorophyll of tetraploid was higher than those of diploids. Marisa Miller, etc also found the increased chlorophyll content in polyploidy *Arabidopsis thaliana* hybrids.[21]

In medicinal plants, the leaves, stems, flowers, and roots usually are the source of the desired active compounds, so the increased biomass associated with polyploidy plants is a very attractive characteristic. The higher yield or higher active compound content of these plants is important for the extraction of natural products and their clinical use in many countries such as China and India. In this research, the tetraploid plants of *P. cablin* were successfully induced. It can be used for further selection and plant breeding of *P. cablin*.

In summary, three elite traploid lines of *P. cablin* (Blanco) were generated from buds by submerging in 0.04% (w/v) colchicine solution for 2 h. They were selected to be used in further breeding programs to obtain superior new varieties for commercial production.

Conflicts of interest

All authors have none to declare.

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Original article

Hepatoprotective effects of *Adenanthera pavonina* (Linn.) against anti-tubercular drugs-induced hepatotoxicity in rats

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ABSTRACT

Objective: The aim of the current study was to evaluate the hepatoprotective action of the leaves of *Adenanthera pavonina* against isoniazid (INH) and rifampicin (RIF)-induced liver damage in experimental animals.

Methods: Five groups of six rats each were selected for the study. A methanolic (50%) extract of *A. pavonina* at a dose of 100 and 200 mg/kg as well as silymarin 100 mg/kg were administered orally once daily for 28 days in INH + RIF treated groups. The serum levels of glutamic oxaloacetic transaminase (SGOT), glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), bilirubin, total protein, albumin and lactate dehydrogenase (LDH) were estimated along with activities of superoxide dismutase (SOD), catalase, glutathione, thiobarbituric acid reactive substances (TBARS). Histopathological analysis was carried out to assess injury to the liver tissue.

Result: The methanolic extract of *A. pavonina* was safe up to a dose of 2000 mg/kg. The significantly elevated serum enzymatic activities of SGOT, SGPT, ALP, bilirubin and LDH due to INH + RIF treatment were restored to near normal in a dose dependent manner after the treatment with methanolic extract of leaves of *A. pavonina*. Also the increased level of total protein and albumin towards normal by extract of *A. pavonina* leaves. In the anti-oxidant studies a significant increase in the levels of glutathione, catalase and superoxide dismutase was observed. In addition, methanolic extract also significantly prevented the elevation of hepatic malondialdehyde formation in the liver of INH + RIF intoxicated rats in a dose dependent manner. The biochemical observations were supplemented with histopathological examination of rat liver sections.

Conclusions: These findings suggested that the methanolic extract of leaves of *A. pavonina* exhibited hepatoprotective effects against INH + RIF induced hepatic damage in rats as compared to standard drug silymarin.

1. Introduction

Adenanthera pavonina Linn. (Family: Fabaceae) commonly known as red wood and red-bread tree, is a deciduous tree, 18–24 m tall, erect and 60 cm in diameter.[1] Many species of *Adenanthera*, including *A. pavonina*, have been used as traditional herbal medicine against a variety of diseases including diabetes, lipid disorders, diarrhoea, haemorrhage from the stomach, haematuria and as anti-inflammatory agent in gout. Traditionally, the ground seed of *A. pavonina* is widely used for the treatment of various human ailments such as treatment of boils, inflammation, blood disorders, arthritis, rheumatism, cholera, paralysis, epilepsy, convulsion, spasm and indigestion.[2,3] Phytochemically, the seeds contain an anti-inflammatory active principle, O-acetylethanolamine. The leaves possess octacosanol, dulcitol, glucosides of β-sitosterol and stigmasterol. The bark furnishes stigmasterol glucoside,[4] and pods contain glycosides, saponins and steroids.[5,6] A new five-membered lactone ring with an exo-cyclic double bond compound, pavonin was isolated from the methanol soluble part of *A. pavonina*.[7] The methanol seed extract has also been reported to demonstrate anti-inflammatory and analgesic activities.[8] The crude extract of *A. pavonina* showed blood pressure lowering effect[9] antifungal, anti-oxidant and cytotoxic,[8] anti-diabetic[10] and anti-hyperlipidemic activities.[11]

Liver is the most important organ concern with the biochemical activities in the human body. It regulates many important metabolic functions and hepatic injury is associated with alteration of these metabolic functions.[12,13] The disorder associated with the liver is numerous and varied[12] as it is the frequent target of number of toxicants. Although viral infection is one of the main causes of

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hepatic injury, xenobiotics, hepatotoxins, excessive therapy, environmental pollutants, and chronic alcohol ingestions can also cause severe liver injury.

Many traditional remedies employ herbal drugs for the treatment of liver ailments.[14] The current study was undertaken to evaluate the hepatoprotective effects of methanolic extract of *A. pavonina* leaves against isoniazid and rifampicin induced liver damage in Sprague-Dawley rats. Isoniazid and rifampicin (INH + RIF), being the first line drugs used as antituberculous chemotherapy, are known to be associated with hepatotoxicity.[15,16]

2. Materials and methods

2.1. Preparation of plant extract

The fresh leaves of *A. pavonina* were collected from the field area of Pallavaram, Chennai, India, in the month of June 2010. The plant specimen was authenticated by National Institute of Herbal Science, Plant Anatomy Research Center, Chennai, Tamil Nadu (Voucher specimen no. PARC/2011/954 & 955). 500 g of the coarsely powdered material was packed in muslin cloth and subjected to a Soxhlet extractor for continuous hot extraction with methanol (50%) for 72 h at 30 °C. Thereafter methanolic extracts of *A. pavonina* were filtered through Whatman paper no. 42 and the resultant filtrates were concentrated under reduced pressure and finally vacuum dried. The yield of the methanolic extract was 11.2% w/w.

2.2. Materials and animals

All solvents, chemicals, solutions and reagents used in the study were of analytical grade procured from SD Fine Chemicals Pvt. Ltd., Mumbai, India; Fischer Inorganics and Aromatics Pvt. Ltd., Lucknow. Isoniazid and rifampicin were obtained as a gift sample from Lupin Drug Laboratory Limited, India. Silymarin was obtained from Ranbaxy Laboratories Limited, India. All biochemical estimation kits were obtained from Robonik diagnostic, Lifechem diagnostic and Span diagnostics kit (India) Ltd. Major instruments used for the study were Autoanalyser (Merck Microlab 200, M/s Vital Scientific, The Netherlands) and Spectrophotometer (160A UV–Vis, Shimadzu, Japan).

Male Sprague-Dawley (SD) rats weighing 150–200 g and Swiss albino mice (25–30 g) were kept in the departmental animal house of Faculty of Pharmacy, Integral University, Lucknow, Uttar Pradesh (India) at a temperature of 25 ± 2 °C and 12 h light/dark cycle, respectively, for one week before and during the experiments. Animals were allowed to access standard rodent pellet diet (Dayal animal feed, Lucknow, India) and drinking water. Food was withdrawn for overnight before the experiment though water was allowed *ad libitum* and allocated to different experimental groups. The study was performed in accordance with the guide for the care and use of laboratory animals, as adopted and promulgated by the Institutional Animal Ethics committee, CPCSEA, India (Reg. No. 1213/ac/2008/CPCSEA/IU).

2.3. Toxicity studies

Acute toxicity study was performed for the methanolic extract of leaves of *A. pavonina* according to the Organisation for Economic Co-operation and Development guidelines-No. 423 (2001) for acute toxic classic method.[17,18] Swiss albino mice of either sex were used for each step in this study. The animals were fasted for overnight with only water available, after which the extracts were administered intragastrically at different doses of 50 and 300 mg/kg. Food and water were withheld for a further 1–2 h after drug administration. Mice were closely observed for the initial 4 h after administration, and then once daily for 14 days to observe mortality. If mortality occurred in two of the three animals at any dose, then this dose was assigned as a toxic dose. If mortality occurred in one animal, then this same dose was repeated to confirm the toxic dose. If mortality did not occur, the procedure was repeated for further higher doses, i.e. 2000 mg/kg. One-tenth and one-twentieth of the maximum tolerated dose of the extract tested (2000 mg/kg) for acute toxicity did not show mortality and were selected for evaluation of the effect of *A. pavonina* 100 and 200 mg/kg for hepatoprotective effects.

2.4. Isoniazid and rifampicin induced hepatotoxicity

Isoniazid and rifampicin (50 mg/kg body wt. each, p.o) suspension were prepared separately in carboxy methyl cellulose (CMC). Rats were treated with isoniazid (INH), co-administered with rifampicin (RIF) for 28 days orally to produce hepatotoxicity.[19]

2.5. Preparation of doses

A known quantity of methanol extracts was suspended in 1% (w/v) carboxy methyl cellulose with distilled water to make the respective stock solutions. Water was used to dissolve silymarin (100 mg/kg) with carboxy methyl cellulose. From these stock solutions, the doses (100 mg/kg and 200 mg/kg) of the extracts were prepared. The doses were prepared fresh each day.

2.6. Experiment design

Male Sprague-Dawley rats (150–200 g) were divided into five groups comprising six animals in each group.

Group I (NC): Normal control (1% CMC)
Group II (HC): INH + RIF (50 + 50 mg/kg, p.o. 28 days)
Group III (MEAP1): INH + RIF (50 + 50 mg/kg) + *A. pavonina* (100 mg/kg, b. wt, p.o. 28 days)
Group IV (MEAP2): INH + RIF (50 + 50 mg/kg) + (200 mg/kg) *A. pavonina* + (200 mg/kg, b. wt, p.o. 28 days)
Group V (HCSD): INH + RIF (50 + 50 mg/kg) + Silymarin (100 mg/kg, b. wt, p.o. 28 days)

2.7. Assessment of liver function

2.7.1. Biochemical estimations

Twenty-four hours after administration of the last dose of the treatment schedule with drugs and extracts, the animals were euthanized by an overdose of diethyl ether. Whole blood was withdrawn from the rats by sino-orbital puncture after an overnight fast. The blood was allowed to coagulate at room temperature for 30 min and then centrifuged at 2000 rpm for 15 min for separation of serum. The serum was used for estimating the biochemical parameters viz., glutamic oxaloacetic transaminase (SGOT), glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), bilirubin, total protein, albumin and lactate dehydrogenase (LDH), albumin, total protein (TP), total bilirubin. The supernatant of the liver homogenate was used for the estimation of enzyme anti-oxidants like catalase (CAT),[20] tissue glutathione by Ellmann method, superoxide dismutase (SOD) by a colorimetric method[21] and thiobarbituric acid reactive substances (TBARS). The contents of malondialdehyde (MDA) were determined by the method of Chaurasia.[22]

Table 1
Effect of methanolic extracts of *A. pavonina* and different biochemical parameters in INH + RIF induced hepatotoxic rats.

Drug treatment	SBL (mg/dl)	SGPT (IU/L)	SGOT (IU/L)	ALP (IU/L)	LDH (U/L)	Total protein (g/L)	Albumin (g/L)
Normal control	0.628 ± 0.008	51.48 ± 0.834	79.172 ± 1.407	151.59 ± 3.389	340.87 ± 2.652	6.72 ± 0.072	2.94 ± 0.049
Hepatotoxic (INH + RIF)	1.79 ± 0.127$^{\#}$	162.83 ± 2.476$^{\#}$	146.41 ± 1.991$^{\#}$	290.36 ± 3.190$^{\#}$	577.79 ± 2.638$^{\#}$	3.72 ± 0.170$^{\#}$	1.420 ± 0.121$^{\#}$
MEAP 100 mg	0.88 ± 0.013*	93.95 ± 1.267*	90.131 ± 2.407*	175.10 ± 2.926*	358.12 ± 2.718*	4.77 ± 0.134*	2.09 ± 0.027*
MEAP 200 mg	0.83 ± 0.007**	74.07 ± 3.801**	84.143 ± 1.991**	164.68 ± 1.863**	352.45 ± 1.441**	5.50 ± 0.155**	2.25 ± 0.112**
Silymarin 100 mg	0.76 ± 0.026$^{@}$	66.54 ± 3.590$^{@}$	81.177 ± 1.407$^{@}$	156.31 ± 2.028$^{@}$	348.12 ± 2.718$^{@}$	5.66 ± 0.219$^{@}$	2.27 ± 0.054$^{@}$

Values are expressed as mean ± S.E.M. $n = 6$.
$^{\#}P < 0.001$, Hepatotoxic vs normal control.
$^{@}P < 0.001$, Standard treatment vs hepatotoxic.
$^{**}P < 0.01$, Treatment vs hepatotoxic.
$^{*}P < 0.05$, Treatment vs hepatotoxic.

2.7.2. Histopathological studies

Slices of liver from each of the five animals in all groups were preserved in 10% buffered neutral formalin (pH 7.4). The tissues were mounted in the laboratory by embedding paraffin sections of 5–10 μ size.[23] These sections were then stained with haemotoxylin–eosin dye. The degree of liver damage was examined by a pathologist of R. S. Diagnostic Centre, Lucknow, for observation under a low power microscope for any pathological changes. Centrilobular necrosis, fatty infiltration, fibrosis, lymphocyte infiltration, etc. was noted.

2.8. Statistical analysis

A result of biochemical estimation has been expressed as Mean ± Standard Error of Mean (S.E.M). The values were subjected to One Way Analysis of Variance (ANOVA) using SPSS-16 (Statistical Package for the Social Sciences) software. The variance in a set of data has been estimated by Dunnett's *t*-test.

3. Results

3.1. Acute toxicity study

Mice administered with methanolic extract of *A. pavonina* up to 2000 mg/kg did not show any abnormal behaviour, during initial 4 h after drug administration. No mortality was observed during 14 days after treatment with methanolic extract of *A. pavonina* in either sex.

3.2. Effect of methanolic extract on biochemical parameters

The results of hepatoprotective effects of methanolic extract of *A. pavonina* on INH + RIF intoxicated rats are shown in Table 1. Administration of INH + RIF at a dose of 50 mg/kg b. wt, p.o. each significantly ($P < 0.001$) elevated SGPT, SGOT, ALP, LDH and, Serum Bilirubin (SBL) activities when compared to the normal control. Treatment of methanolic extract of *A. pavonina* at a dose of 100 mg/kg and 200 mg/kg b. wt, 1 h prior to INH + RIF administration significantly protected the elevation of transaminases and ALP activities towards normal. Serum Bilirubin (SBL) and LDH were significantly ($P < 0.01$) reduced by administration of *A. pavonina* at a dose of 100 mg/kg and 200 mg/kg as compared to hepatotoxic controlled rats. The protection was better on dose 200 mg/kg and a significant increase ($P < 0.01$) was observed in the levels, TP and albumin in the serum, against the hepatotoxic control group (Table 1).

3.3. Effect of methanolic extract on anti-oxidant parameters

Activities of hepatic SOD, CAT, glutathione and TBARS are presented given in Table 2. SOD and glutathione activities were significantly ($P < 0.01$) enhanced after the treatment of *A. pavonina* + INH + RIF treated group. However, the hepatic CAT activity was improved significantly ($P < 0.01$) when compared to the hepatotoxic control. Further the activity of GSH was enhanced and normalized in the *A. pavonina* + INH + RIF treated. Hepatic MDA level was significantly ($P < 0.05$) elevated in INH + RIF control group than the normal group. It was significantly reduced by administration of *A. pavonina* at a dose of 100 mg/kg and 200 mg when compare to hepatotoxic rats.

3.4. Effect of methanolic extract on histopathology of liver

Histopathological examinations of liver tissues were performed by the method of Belur et al, 1990.[23] For histological examinations on the 28th day, the liver was isolated and preserved in 10% neutral buffered formalin. Histopathological observation of tissues was carried out in a Pathology laboratory, at R.S. Diagnostic Centre, Lucknow, India. After fixation, the tissues were embedded in paraffin, clear in xylene and dehydrated in descending series of ethanol. At least four cross-sections were taken from each tissue of 5 μm thickness and stained with haematoxylin and eosin (H&E).

Table 2
Effect of methanolic extract of *A. pavonina* in different parameters in INH + RIF induced hepatotoxic rats.

Drug treatments	GSH (μg/mg)	CAT (μmol of H_2O_2 consumed min/mg of tissue protein)	SOD (units/mg tissue protein)	MDA (μmol/mg protein)
Normal control	4.14 ± 0.009	16.13 ± 0.654	4.68 ± 0.274	25.42 ± 0.915
Hepatotoxic (INH + RIF)	1.61 ± 0.007$^{\#}$	8.66 ± 0.377$^{\#}$	1.48 ± 0.064$^{\#}$	72.92 ± 0.713$^{\#}$
MEAP 100 mg	2.00 ± 0.009*	11.04 ± 0.395*	2.33 ± 0.097*	62.47 ± 1.282*
MEAP 200 mg	2.07 ± 0.006**	12.63 ± 0.616**	3.72 ± 0.105**	39.57 ± 0.614**
Silymarin 100 mg$^{@}$	3.22 ± 0.008$^{@}$	14.32 ± 0.544$^{@}$	4.24 ± 0.163$^{@}$	31.86 ± 1.190$^{@}$

Values are expressed as mean ± S.E.M.; $n = 6$.
$^{\#}P < 0.001$, Hepatotoxic vs normal control.
$^{@}P < 0.001$, Standard treatment vs hepatotoxic.
$^{**}P < 0.01$, Treatment vs hepatotoxic.
$^{*}P < 0.05$, Treatment vs hepatotoxic.

Following two changes xylene washes of 2 min each, tissue sections were mounted with DPX mount. The slides were evaluated for histopathological changes and photomicrographs were taken using microscope system. H&E staining was used to visualize and differentiate between tissue components in normal and in hepatotoxic control model. Histological evaluation of the liver tissues in the hepatotoxic model rats at the magnification of 40× showed marked changes at the periphery. The degeneration and necrosis of liver cells, presence of pycnotic nuclei, granular cytoplasm and increase in intercellular spaces (Fig. 1B). Similar focal changes are also seen in the central areas. Microscopic examination on normal liver section shows intact mucosal lining of flattened epithelial cells (Fig. 1A). Mucosal glands are seen compactly arranged, consisting of cells with vesicular nuclei with nucleoli and abundant eosinophilic cytoplasm. These glands are separated by thin strands of fibro connective tissue. Basement membrane is thick and intact. Glands near basement membrane have more basophilic cytoplasm and few bundles of fibrous tissue and occasional blood vessels are also seen (Fig. 1A). Hepatotoxic group treated with silymarin 100 mg/kg, body weight as reference drug (Fig. 1C) shows intact mucosal lining of flattened epithelial cells. Mucosal glands are seen compactly arranged without any abnormality or any degenerative changes of hepatocytes. In rats group treated with AP extract in two different doses (Fig. 1D and E), shows marked changes at the periphery, granular cytoplasm and decrease in intercellular spaces as compared to hepatotoxic control rats. Liver sections with minimal degenerative changes of hepatocytes with minimal swelling and necrosed area. The treatment with above extracts showed that there is a significant reduction in tissue damage along with minimal evidence of inflammation. Histological examination of liver tissues in rats supplemented with *A. pavonina* extract at the dose of 200 mg/kg body weight showing nearly normal tissue architecture, absence of inflammatory cells in the central areas showing significant.

4. Discussion

In our study, the combinatorial effect of anti-tubercular drugs (INH + RIF) was used to induce hepatotoxicity in the experimental animals as an already established model.[24] The model has been reported in literature to produce various grades of liver damage, including centrilobular necrosis, liver cell proliferation and suppression of anti-oxidant system.[27] Many researchers have suggested that part of hepatocellular injury induced by combination of anti-tubercular drugs has been mediated through cytochrome P_{450}.[28] Reduction in hepatic anti-oxidant function has also been suggested as one of the other mechanisms for hepatotoxicity caused by anti-tubercular drugs.[25] The combination of these two anti-tubercular drugs-induced hepatotoxicity, manifested mainly as hepatocellular steatosis and centrilobular necrosis, possibly associated with cholestasis, and it has been suggested that toxic isoniazid metabolites bind covalently to cell macromolecules in both animal and human case studies.[26] During the metabolism of INH, hydrazine was produced directly (from INH) or indirectly (from acetyl hydrazine). From earlier study it was evident that hydrazine plays a crucial role in INH-induced liver damage in rats. The combination of INH and RIF was reported as higher rate of inhibition of biliary secretion, an increase in liver cell lipid peroxidation and cytochrome P_{450} was thought to be involved the synergistic effects of RIF on INH.

The serum levels of a number of studied hepatic enzymes behave as diagnostic indicators for hepatic injury.[27] Increased levels of SGPT, SGOT, LDH and ALP in serum of the INH + RIF induced animals certainly indicate liver damage. An increase in the levels of these marker enzymes in serum was due to the leakage of the enzymes from liver as a result of tissue damage. On concurrent treatment with methanolic extract of *A. pavonina* at dose of 100 and 200 mg/kg respectively, the serum marker enzyme levels were near to normal indicating protection against liver damage (Table 1 & Fig. 1D, E). This protective effect could be possibly due to the reduction in the tissue damage brought by the methanolic extract of *A. pavonina*. The results were compared with the standard silymarin. It is a general perception that, the serum bilirubin levels are elevated in hepatic injury. A marked elevation was observed in serum bilirubin levels of INH + RIF induced rats, whereas total protein (TP) and albumin levels in the serum were markedly decreased. A reduction in synthesizing proteins was seen following

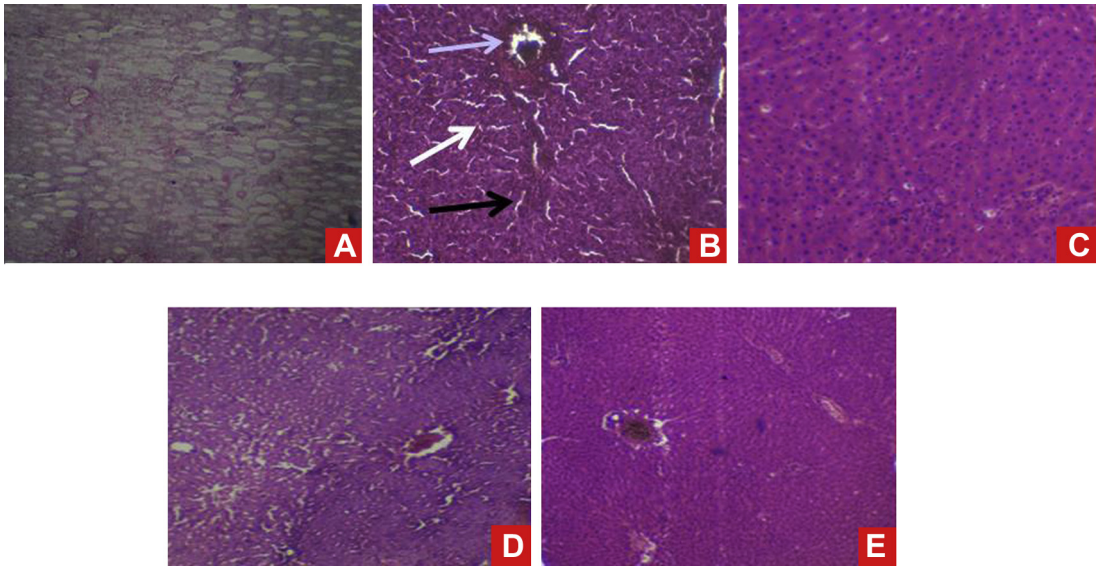

Fig. 1. (A–E): Effect of methanolic extracts of *A. pavonina* against anti-tubercular drugs (INH + RIF) induced histopathological changes in normal rat liver. (A) Normal rats showed normal hepatocytes with well preserved cytoplasm with normal lobular structural design of the liver, (B) anti-tubercular drugs (INH + RIF) induced rat liver, where white arrow indicates necrosis and black arrow indicates inflammation, (C) silymarin (100 mg/kg), (D) methanolic extract of *A. pavonina* 100 mg/kg (E) methanolic extract of *A. pavonina* 200 mg/kg (H&E 40×).

intoxication of the liver with hepatotoxicants. As seen in the silymarin treated group and methanolic extract of *A. pavonina*, all studied parameters were restored to normal condition from the abnormal ones.

Suppression of the anti-oxidant system in anti-tubercular drugs intoxicated rats has been reported earlier.[28] The decreased activities of SOD and CAT, the primary anti-oxidant enzymes, are observed in the anti-tubercular drugs (INH + RIF) induced rats which may be due to the interaction of accumulated free radicals with the associated metal ions or with the active amino acids of these enzymes.[29] In our study, the groups treated with methanolic extract of *A. pavonina* and silymarin, were found to restore the levels of anti-oxidant enzymes which could be due to the ability of the constituents in the administered compounds to scavenge reactive oxygen species. Hepatocellular disintegrate and the inflammation in the liver was observed in the centrilobular region by histopathological examination in INH + RIF treated groups. This could be only possible by the overall protective character of the extract.[30–32] A plethora of reports has been published that flavonoids, alkaloids and saponins played a major role in protecting the liver from injuries. *A. pavonina* was found to be richly containing flavonoids and saponins. Thus the hepatoprotective effect of the methanolic extract of *A. pavonina* could be possibly due to the presence of flavonoids, alkaloids, glycosides and saponins.

5. Conclusion

The results of this study suggest that the methanolic extract of *A. pavonina* has protective effects against INH + RIF induced hepatic damage in experimental rats. Administration of the extract attenuates the hepatotoxic effects by decreasing MDA production and through an increase of anti-oxidant defences. Our study demonstrates the health benefits traditionally claimed to this medicinal plant in liver disease.

Conflicts of interest

All authors have none to declare.

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