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Original article

Membrane stabilization – A possible mechanism of action for the anti-inflammatory activity of a Bangladeshi medicinal plant: *Erioglossum rubiginosum* (Bara Harina)

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ABSTRACT

Aims: The present study aimed at assessing the effect of methanol extract of *Erioglossum rubiginosum*, a widely used shrub like plant in folkloric medicine, in experimentally induced inflammation, using human red blood cell (HRBC) membrane stabilization as study method.
Methods: The methanol extract of leaves of *E. rubiginosum* and its pet-ether, carbon tetrachloride, chloroform and aqueous soluble partitionates were subjected to assays for lysis of erythrocytes and % of inhibition in hypotonic solution and heat induced lysis, using acetyl salicylic acid as standard drug, in an *in vitro* model.
Results: At the concentration of 1 mg/ml, methanol extract and its pet-ether, carbon tetrachloride, chloroform and aqueous soluble partitionates significantly inhibited hypotonic solution induced lysis of the human red blood cell membrane with values of 0.428 ± 0.005, 0.204 ± 0.003, 0.233 ± 0.002, 0.411 ± 0.003, 0.439 ± 0.003% respectively; which were comparable to the standard drug acetyl salicylic acid (0.1 mg/ml), 0.166 ± 0.003. In case of heat induced HRBC heamolysis, the plant extracts also showed significant activity where the values of inhibitory actions were 14.46 ± 0.344, 28.23 ± 0.315, 25.18 ± 0.303, 17.09 ± 0.365, 13.44 ± 0.470% for methanol extract, pet-ether, carbon tetrachloride, chloroform and aqueous soluble partitionates respectively.
Conclusion: It can be postulated from the observed results that the membrane stabilizing action and inhibition of erythrocyte lysis property of *E. rubiginosum* may be the possible mechanism of action of its anti-inflammatory activity. So, further studies are suggested to evaluate the anti-inflammatory and analgesic activities of the plant.

1. Introduction

Inflammation is a complex biological response of vascular tissues to harmful stimuli and also a part of non-specific immune response that occurs in reaction to any type of bodily injury. It is also an attempt of an organism to remove the injurious stimuli and initiate the healing process.[1] The inflammation process initiates with the activation and release of different types of mediators, such as: histamine, serotonin, slow reacting substances of anaphylaxis (SRS-A), prostaglandins, some plasma enzyme systems and the kinin system.[2,3] These mediators, working collectively, increase vasodilation and blood vessels permeability, which further lead to increased blood flow, exudation of plasma proteins and fluids, and migration of leukocytes, mainly neutrophils, outside the blood vessels into the injured tissues.[4] Some laboratory and pathological data also supported that inflammation has a role in both the initiation and the progression of atherosclerosis.[5–7] Again, the erythrocyte membrane resembles lysosomal membrane as such; the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane.[8–10] Therefore, as membrane stabilizes that interfere in the release and or action of mediators like histamine, serotonin, prostaglandins, leukotrienes etc.[11]

There are many agents or drugs namely anti-inflammatory agents or drugs, such as nonsteroidal anti-inflammatory drugs (NSAIDs) to treat the consequences of inflammation. The effect of these drugs including herbal preparation on the stabilization of erythrocyte membrane exposed to hypotonic and heat has been studied extensively.[9] But these studies showed that, these drugs are not free from adverse effects, as they are responsible for intestinal side effects and mucosal erosions that can progress into ulcers.[12] For these reasons many researchers have focused on medicinal plants for finding natural anti-inflammatory drugs.

Erioglossum rubiginosum (local name – *Bara Harina*) is a shrub or small tree, with a compact, bushy crown and up to 12 m tall. It

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belongs to the family *Sapindaceae* and is available in forests at low and medium altitudes throughout the Philippines, northern India to Indo-China, Thailand and also some tropical countries. This plant is extensively used as folkloric medicine; such as: roots are used as astringent, leaves and fruits are used for the treatment of fever and poulticing.[13,14] As a part of our continuing studies of medicinal plants of Bangladesh the methanol extract and the fractions of leaves of *E. rubiginosum* growing in Bangladesh were screened for membrane stabilizing activity for the first time and we, here in, report the results of our preliminary investigation.

2. Materials and methods

2.1. Collection and preparation of plant material

The leaves of *E. rubiginosum* were collected from Dhaka, Bangladesh, in July 2012. The plant was identified by the taxonomist of Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh and a voucher specimen was deposited in the herbarium unit. The sun dried powdered leaves (500 mg) of *E. rubiginosum* was macerated in 2.5 L of 99.8% methanol (Merck KGaA, Darmstadt, Germany). After 15 days the solution was filtered using filter cloth and Whatman® filter paper No. 1. The resulting filtrates were then evaporated in water bath maintained at 45 °c to dryness and thus a blackish–green semisolid mass of the extract was obtained. The concentrated methanolic extract was partitioned by modified Kupchan method[15] and the resultant partitionates i.e., pet-ether (PESF), carbon tetrachloride (CTCSF), chloroform (CSF), and aqueous (AQSF) soluble fractions were used for the experimental processes.

2.2. Collection of blood samples

Human RBCs were collected for the study. 7 ml of blood was collected from each of the healthy Bangladeshi male human volunteers (aged 20–23 years) without a history of oral contraceptive or anticoagulant therapy and free from diseases (using a protocol approved by Institutional Ethics Committee). The collected RBCs were kept in a test tube with an anticoagulant EDTA under standard conditions of temperature 23 ± 2 °C and relative humidity 55 ± 10%.

2.3. Reagents and chemicals

All the solutions, reagents used in this study were of analytical grades. They were procured from Sigma Chemical Co. Ltd. (St. Louis, MO, USA) and E. Merck (Germany). All the solutions, reagents and buffers were prepared with glass distilled water.

2.4. Assay of membrane stabilization

2.4.1. Erythrocyte suspension

The blood was washed three times using isotonic solution (0.9% saline). The volume of saline was measured and reconstituted as a 40% (v/v) suspension with isotonic buffer solution (pH 7.4) which contained in 1 L of distilled water: $NaH_2PO_4 \cdot 2H_2O$, 0.26 g; Na_2HPO_4, 1.15 g; NaCl, 9 g (10 mM sodium phosphate buffer). Thus the suspension finally collected was the stock erythrocyte (RBC) suspension.

2.4.2. Hypotonic solution induced hemolysis

The membrane stabilizing activity of the extract was evaluated by using hypotonic solution induced human erythrocyte hemolysis, designed by[16] with minor modification. To prepare the erythrocyte suspension whole blood (7 ml) was obtained using syringes (containing anticoagulant EDTA) from male volunteers through puncture of the anti-cubital vein. The blood was centrifuged, using centrifugal machine, for 10 min at 3000 g and blood cells were washed three times with solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4). The test sample, consisted of stock erythrocyte (RBC) suspension (0.50 ml), was mixed with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing either the extracts (1.0 mg/ml) or acetyl salicylic acid (0.1 mg/ml). The control sample, consisted of 0.5 ml of RBCs, was mixed with hypotonic-buffered saline alone. The mixture was incubated for 10 min at room temperature, centrifuged for 10 min at 3000 g and the absorbance of the supernatant was measured at 540 nm using UV spectrometer. The percentage inhibition of either hemolysis or membrane stabilization was calculated using the following equation:

$$\%\ \text{inhibition of hemolysis} = 100 \times (OD_1 - OD_2/OD_1)$$

Where,

OD_1 = Optical density of hypotonic-buffered saline solution alone (control) and,

OD_2 = Optical density of test sample in hypotonic solution.

2.4.3. Heat-induced hemolysis

Aliquots (5 ml) of the isotonic buffer, containing 1.0 mg/ml of different extracts of the plant were put into two duplicate sets of centrifuge tubes.[11] The vehicle, in the same amount, was added to another tube as control. Erythrocyte suspension (30 μL) was added to each tube and mixed gently by inversion. One pair of the tubes was incubated at 54 °C for 20 min in a water bath. The other pair was maintained at 0–5 °C in an ice bath. The reaction mixture was centrifuged for 3 min at 1300 g and the absorbance of the supernatant was measured at 540 nm using UV spectrometer. The percentage inhibition or acceleration of hemolysis in tests and was calculated using the following equation:

$$\%\ \text{inhibition of hemolysis} = 100 \times [1 - (OD_2 - OD_1/OD_3 - OD_1)]$$

Where,

OD_1 = test sample unheated,

OD_2 = test sample heated and,

OD_3 = control sample heated.

2.5. Statistical analysis

Data obtained were analyzed using SPSS version 16.0 (SPSS Inc. Chicago, IL. USA). All values are expressed as mean ± SD for three replicates. Data were analyzed by one-way ANOVA and the

Table 1
Effect of extracts of *Erioglossum rubiginosum* on hypotonic solution induced hemolysis of erythrocyte membrane.

Treatment	Concentration (mg/ml)	Optical density of samples in hypotonic solution (Mean ± SD)	Percentage inhibition of hemolysis
Control	1	0.59 ± 0.026	–
ME	1	0.428 ± 0.005***	27.46 ± 0.942***
PESF	1	0.204 ± 0.003**	65.36 ± 0.519**
CTSF	1	0.233 ± 0.002**	60.57 ± 0.428**
CSF	1	0.411 ± 0.003***	30.39 ± 0.592***
AQSF	1	0.439 ± 0.003***	25.65 ± 0.519***
Acetyl salicylic acid	0.1	0.166 ± 0.003**	71.92 ± 0.519

Level of Significance *** = $p < 0.001$, ** = $p < 0.01$, percent inhibition of migration was calculated relative to control.

Table 2
Effect of extracts of *Erioglossum rubiginosum* on heat induced hemolysis of erythrocyte membrane.

Treatment	Concentration (mg/ml)	Optical density of samples (Mean ± SD)		Percentage inhibition of hemolysis
		Heated solution	Unheated solution	
Control	1	0.620 ± 0.001	0.287 ± 0.011	–
ME	1	0.535 ± 0.002***	0.028 ± 0.002	14.46 ± 0.344***
PESF	1	0.455 ± 0.002**	0.037 ± 0.001	28.23 ± 0.315***
CTSF	1	0.475 ± 0.001**	0.044 ± 0.002	25.18 ± 0.303***
CSF	1	0.545 ± 0.003*	0.062 ± 0.003	17.09 ± 0.365***
AQSF	1	0.517 ± 0.002**	0.019 ± 0.001	13.44 ± 0.470***
Acetyl salicylic acid	0.1	0.503 ± 0.002**	0.250 ± 0.004	31.65 ± 0.504

Level of Significance *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$ percent inhibition of migration was calculated relative to control.

statistical significance differences were analyzed using paired t- test. $P < 0.05$ was considered statistically significant.

3. Result

The crude methanol extract of leaves of *E. rubiginosum*, as well as different partitionates derived from this extract, were subjected to assays for membrane stabilizing activities by following standard protocols and the obtained results were statistically represented in Tables 1 and 2. The results showed that the extracts (at concentration 1 mg/ml) were significantly potent on human erythrocyte, adequately protecting it against hypotonic solution and heat-induced lyses, when compared with the standard drug acetyl salicylic acid (0.10 mg/ml).

In hypotonic solution induced conditions, the samples were found to inhibit lysis of erythrocyte membrane within the range of 25.65 ± 0.519% to 65.36 ± 0.519%. Among the samples, the pet-ether soluble fraction (PESF) of leaves of *E. rubiginosum* displayed high inhibition (65.36 ± 0.519%) hemolysis of RBC as compared to 71.92 ± 0.519% demonstrated by acetyl salicylic acid, while the minimum inhibition capacity was observed for aqueous soluble fraction (AQSF) (Table 1). Besides, in heat-induced conditions, the samples were found to inhibit lysis of erythrocyte membrane within the range of 13.44 ± 0.470% to 28.23 ± 0.315%. Here, the maximum inhibitory capacity of RBC hemolysis was observed for pet-ether soluble fraction (PESF), 28.23 ± 0.315%, as compared to 30.55 ± 0.55% demonstrated by acetyl salicylic acid. In this case, the aqueous soluble fraction also revealed the minimal RBC hemolysis inhibition capacity (Table 2).

4. Discussion

It is relevant from the present study that the methanol extract and its different partitonates protected the human erythrocyte membrane against lysis induced by hypotonic solution and heat. During inflammation, lysosomal enzymes and hydrolytic components are released from the phagocytes to the extracellular space, which causes damages of the surrounding organelles and tissues and also assists a variety of disorders.[17] It was found that the NSAIDS act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membranes. Again, exposure of red blood cells (RBCs) to injurious substances such as hypotonic medium, heat etc. results in the lysis of the membranes, accompanied by oxidation and lysis of hemoglobin.[18] The inhibition of hypotonicity and heat induced red blood cell membrane lysis was taken as a measure of the mechanism of anti-inflammatory activity of the plant extract, because human red blood cell (HRBC) membranes are considered similar to lysosomal membrane components.[19]

It may be said that, the possible mode of action of the extract, its fractions and standard anti-inflammatory drugs could be connected with binding to the erythrocyte membranes with subsequent alteration of the surface charges of the cells. This might have prevented physical interaction with aggregating agents or promote dispersal by mutual repulsion of like charges which are involved in the hemolysis of red blood cells. In some research work, it has been reported that, some chemical constituents present in the extracts may exert the same mechanism, which are well known for their anti-inflammatory activity.[20] Both *in vitro* and *in vivo* study in experimental animals, demonstrated that flavonoids exert extensive stabilizing effects on lysosomes[21,22] while tannin and saponins have the ability to bind cations and other biomolecules, and are able to stabilize the erythrocyte membrane.[10,23]

In this research work it was found that all the extracts of the plant showed potent RBC membrane stabilization activity with good percentage protection against both hypotonic solution and heat-induced lysis. The pet-ether soluble fraction (PESF) of the extract of the plant was found be a better choice. Because, in both the test methods, that is hypotonic solution induced and heat induced heamolysis, the percentage (%) of heamolysis inhibitory action of pet-ether soluble fraction (PESF) was better than the other extracts. The heamolysis inhibitory action of the extracts may be due to synergistic effect produced by phyto-constituents present in these extracts.

5. Conclusion

On the basis of these results of the current study, it could be inferred that the extracts/fractions of *E. rubiginosum* contained principles that were capable of stabilizing human red blood cells membranes against hypotonic solution and heat induced lysis. The plant therefore could be regarded as a natural source of membrane stabilizers and could be used as an alternative remedy for the management and treatment of inflammatory related disorders and diseases. So, further studies are suggested to identify and isolate the chemical constituents responsible for the membrane stabilizing activity and also to evaluate the anti-inflammatory activity of the plant extract.

Conflicts of interest

All authors have none to declare.

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Original article

Phenolics content and antioxidant activity of some organic extracts of endemic medicinal plant *Anabasis aretioides* Coss. & Moq. from Algerian Sahara

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ABSTRACT

Aims: Anabasis aretioides Coss. & Moq. is one of the endemic medicinal plants from Algerian Sahara. It's used for many traditional therapies of various diseases. In this study, we investigate the content of different organic extracts on phenolics and flavonoids, and also their antioxidant activities.
Methods: Two conventional methods were used to carried out the antioxidant activity: the DPPH and hydrogen peroxide scavenging activities.
Conclusion: From the obtained results, we found that the ethanolic extract presented the high level of phenolic and flavonoid contents (231.85 ± 20.59 mg GAE/g and 132.8 ± 24.58 mg CEQ/g). The EC50 of different extracts were arranged between 47.71 and 86.73 μg/mL. A highest hydrogen peroxide activity was observed in ethyl acetate extract compared to antioxidants used as positive control (BHA and α-tocopherol).

1. Introduction

Because of their abundance in nature and their use by indigenous peoples for healing, some plants are placed in the medicinal world. For millennia, man draws in his environment knowledge necessary for its survival and its wellness. Before you even have the use of language, he could convey the experience of medicine and all types of natural remedies to finally develop what we call now traditional medicine or phytotherapy.

In recent years the use of traditional medicine is widespread throughout the world and has gained popularity not only people in developing countries have access to, but also those countries where biomedicine occupies an important place in their health systems.

The study of this ancient knowledge with modern science reveals some secrets of nature that enable man to continue its evolution.

Natural substances derived from plants have multiple uses in many industry, such as: cosmetology, dermopharmacy, and in the food industry. Among these compounds, secondary metabolites, which are mostly illustrated in therapy.

All forms of aerobic life are constantly subjected to oxidative pressure from reactive oxygen species (ROS), produced during the biochemical utilization of O_2.[1] ROS include hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot -}$), and hydroxyl radical ($HO^{\bullet}$), have the ability to react with a large variety of easily oxidizable cellular components such as lipids, proteins, and nucleic acids and it may trigger various diseases including cancer, cardiovascular diseases, cataracts, atherosclerosis, diabetes and aging.[2–5]

In industry, many synthetic antioxidants are used to prevent oxidative deterioration of fats and oily foods, especially BHT (butylated hydroxytoluene), BHA (butylated hydroxyanisol), and TBHQ (tertiary butylhydroquinone).[6] It is mentioned that natural and synthetic antioxidants have been shown to enhance product stability, quality and shelf. However, the use of synthetic antioxidant molecules currently being questioned because of potential toxicological risks.[6–9] Recently, intensive research has been carried out either to characterize antioxidant properties of extracts from several plant materials and/or to isolate and identify the compounds responsible for those activities.[10] Therefore, research into the determination of natural antioxidant sources is important. The number of reports about isolation and testing of natural antioxidants has increased immensely during the last decade.[11]

A great number of plants worldwide showed a strong antioxidant activity and a powerful scavenging activity against free radicals.

Nowadays, research has focused on medicinal plants to extract new natural antioxidants that can replace synthetic additives.

In addition, there is a conviction in public's mentality that phytochemicals (as additive) are inherently safer than synthetic chemicals used in foods.[12]

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Phenolic compounds are an important class of secondary metabolites of plant possessing various pharmacological activities. Actually, it is clear that phenolic compounds possess an excellent radical scavenging ability, which is mainly due to their redox properties, this one allow them to act as reducing agents, hydrogen donators, singlet oxygen quenchers agents, and they have also metal chelating potential.[13,14]

The valorization of local flora and especially endemic species is a priority of scientific research in countries who present a rich potential on medicinal plants.

Anabasis aretioides (Syn. *Fredolia aretioides* Coss. & Dur.) (Fig. 1) is one of the endemic medicinal plants of South–west Algeria and South–east Morocco. It's Common in the north–western Sahara from the Tafilalet, Tinghir in Morocco to Beni-Abbès in Algeria across the eastern Moroccan desert, Béni-Ounif, Ain Sefra and Béchar.[15] *A. aretioides* is used for various medicinal uses in Algerian folk medicine (Table 1). It grows on rocky and stony plateau. It's a vigorous cylindrical shrub, it looks like a huge cauliflower. The fruit is a small achene surrounded by transparent wings of the evergreen perianth.[16,17]

According to our knowledge, there are no data that characterize the potential antioxidant properties related to phenolic and flavonoid fractions of *A. aretioides*. With respect to this, the antioxidant activities of different extracts of leaves of this plant are presented in the study, together with the content of total phenolics and flavonoids in the investigated extracts.

2. Material and methods

2.1. Chemicals

Hydrogen peroxide, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), Butylated hydroxyanisol (BHA), Quercetin, α-Tocopherol, Folin–Ciocalteu's phenol reagent, Sodium carbonate, Sodium hydroxide and Methanol were from Sigma–Aldrich Chemical Company (Germany). All other chemicals were of analytical grade.

2.2. Plant materials

The whole plant of *A. aretioides* were collected in November 2010 from Beni-Abbès (Region of Bechar, south–west of Algeria) and dried away from direct sunlight. Dried plant material was then crushed into a mortar and stored at very low temperature until further use.

Fig. 1. *Anabasis aretioides* (desert cauliflower).[18]

Table 1
Popular use of *Anabasis aretioides* (Coss. & Moq.) in folk medicine.

Anabasis aretioides (Chenopodiaceae)	Synonymous: *Fredolia aretioides* Coss. & Dur. Ex Bunge Synonymous: *Noea aretioides*
Nomenclature	Arabic: Degâa French: Choux-fleur de Bouâmama
Part used	The leaves are prepared as an infusion or decoction[15]
Pharmacological action	Used as rheumatismal, as a diuretic, and as an antidote to poison[15] In Morocco, it's used in the treatment of diabetes[19]

2.3. Sample preparation

For this study we chose to take only the part used in traditional medicine. 10 g of aerial part of *A. aretioides* (leaves) was extracted by different organic solvents with increasing polarity, under reflux for 3 h. The extracts were then filtered and concentrated under reduced pressure at 60 °C using a rotary evaporator (Büchi Rotavapor R-200). The obtained residue was recovered in methanol and stored at +4 °C.

2.4. Total phenolic content

Total phenolic was estimated by the Folin–Ciocalteu method.[20] 0.1 mL of sample was mixed with 2 mL of sodium carbonate (2%) freshly prepared, the whole was vigorously mixed on a vortex. After 5 min, 100 μL of Folin–Ciocalteu reagent (1 N) were added to the mixture, all was left for 30 min at room temperature and the reading of absorbance (SPECORD 200 Plus) is performed against a blank at 750 nm. A calibration curve was performed in parallel under the same operating conditions using gallic acid as a positive control. The results are expressed as mg gallic acid equivalent per gram of dry extract (mg GAE/g).

2.5. Total flavonoid content

The total flavonoid content was determined by a colorimetric method as described in the literature.[21] Each sample (500 μL) was mixed with 2 mL of distilled water and subsequently with 150 μL of a $NaNO_2$ solution (15%). After 6 min, 150 μL of aluminum chloride ($AlCl_3$) solution (10%) was added and allowed to stand for 6 min. Then, 2 mL of NaOH solution (4%) was added to the mixture. Immediately, distilled water was added to bring the final volume to 5 mL and the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was then determined at 510 nm versus prepared water blank. Results were expressed as catechin equivalent per gram of dry extract (mg CEQ/g).

2.6. Determination of the scavenging effect on DPPH radicals

A methanolic solution (50 μL) of each sample or positive control at different concentrations was added to 1.95 mL of DPPH solution (6×10^{-5} M in methanol).[22] The studied compounds were tested with methanol as control, BHA, ascorbic acid, quercetin and α-tocopherol as antioxidant references. The absorbance at 515 nm was determined after 30 min of incubation at ambient temperature. The absorbance (A) of the control and samples was measured, and the DPPH scavenging activity (SA), in percentage, was determined as follow:

$$SA\,\% = \left[\left(A_{\text{control}} - A_{\text{sample}}\right) / A_{\text{control}}\right] \times 100$$

EC_{50} value (μg/mL) is the concentration at which that scavenging activity was 50%.

2.7. Determination of the scavenging effect on hydrogen peroxide

A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffered saline (PBS, 0.1 M, pH 7.4). 1 mL of sample or standards in methanol was added to 2 mL of hydrogen peroxide solution in PBS. The absorbance was measured at 230 nm, after 10 min against a blank solution that contained extracts in PBS without hydrogen peroxide.[23]

The percentage of H_2O_2 scavenging of examined extracts was calculated as:

$$\%\ \text{of scavenged}\ H_2O_2 = \left[\left(A_{\text{control}} - A_{\text{sample}}\right)/A_{\text{control}}\right] \times 100$$

where the control is the phosphate buffer with H_2O_2.

2.8. Statistical analysis

Data were reported as means ± standard deviation (SD) of three parallel measurements. Different statistical techniques such as analysis of variance (one-way ANOVA procedure), Duncan's multiple range method and regression analysis were carried out for analyzing the data. Sigma plot software was used to perform the statistical analysis. The values of $P < 0.05$ were considered to be statistically significant.

3. Results and discussion

3.1. Total phenolic and flavonoid contents

Total phenolic contents were estimated with Folin–Ciocalteu colorimetric method. This reagent is a mixture of phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic acids ($H_3PMo_{12}O_{40}$). It is reduced during the oxidation of phenols in a mixture of blue oxides of tungsten and molybdenum. The colour produced, whose absorption maximum is between 700 and 750 nm, is proportional to the amount of phenolic compound present in plant extracts. The total phenolic contents were reported as mg gallic acid equivalent per gram of dry extract.

In $AlCl_3$ colorimetric method, for determination of total flavonoid contents, aluminum chloride forms acid stable complex with the keto and/or the hydroxyl groups in the A or C ring of flavonoids.[21] The total flavonoid contents were reported as mg catechin equivalent per g dried extract.

On this plant and to the best of our knowledge, this study is the first to provide data on the quantification of phenolic and flavonoid contents. The results, as presented in Fig. 2, show that *A. aretioides* extracts contained high phenolic and flavonoid contents. The different organic solvents used in this study are chosen according to a gradient of increasing polarity to extract different groups of phenolic compounds. About the phenolic contents, we found a significant difference between the different solvents used (at $P < 0.05$), except for the two extracts: acetone and chloroform, which have close levels. This concept confirms the choice of organic solvents.

The order of total phenolic contents is: Methanol extract < Ethyl acetate extract < Acetone extract < chloroform extract < ethanol extract.

Concerning the flavonoid contents, we found a significant difference between the different solvents used (at $P < 0.05$), except for the two extracts: chloroform and ethyl acetate, which have close levels.

The order of total phenolic contents is: Methanol extract < Ethyl acetate extract < Acetone extract < chloroform extract < ethanol extract.

We can conclude, for this plant, that ethanol is an organic solvent recommended to extract phenolic compounds (phenolic and flavonoid). It's considered as an ecological solvent (green chemistry).

3.2. DPPH radical scavenging

The DPPH is a stable organic free radical with an absorption maximum band around 515–528 nm and it is a useful reagent for evaluation of antioxidant activity of different compounds.

The *A. aretioides* extracts were tested for their antioxidant scavenging effects on DPPH radical and their activity was compared to different positive controls: the synthetic antioxidant BHA, ascorbic acid and quercetin.

The results obtained at different concentrations of organic extracts of *A. aretioides* are given in Fig. 3. From these results, it is demonstrated that all the tested extracts showed a non-linear dose-dependant activity. The free radical scavenging activity is also expressed by the antioxidant concentration required for a 50% DPPH reduction (EC_{50}) (Table 2).

The model for scavenging stable DPPH free radicals can be used to evaluate the antioxidative activities in a relatively short time. The absorbance decreases as a result of a color change from purple to yellow as the radical is scavenged by antioxidants through donation

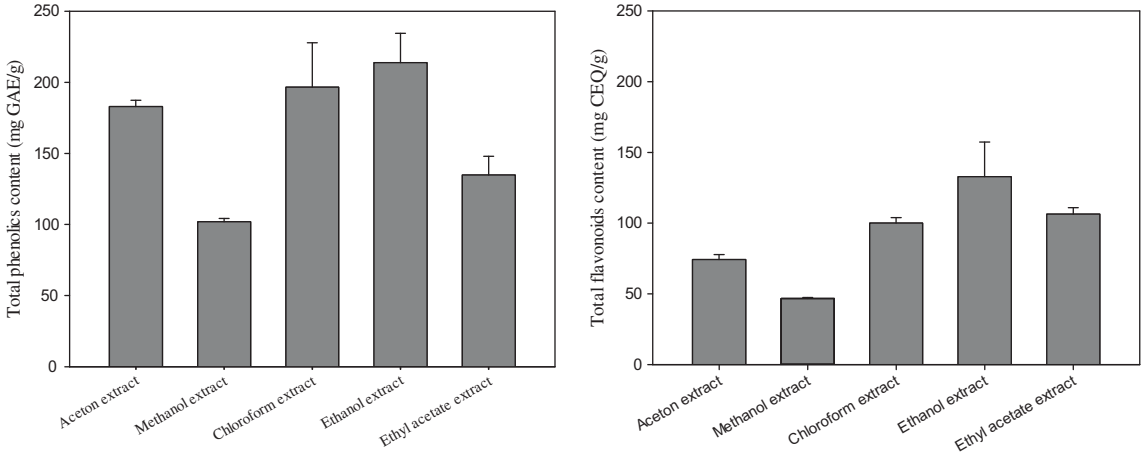

Fig. 2. Total phenolic and total flavonoid contents of different organic extracts of *A. aretioides*.

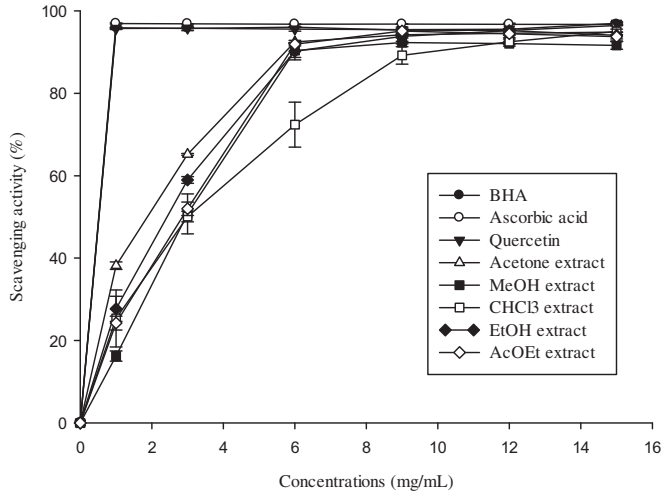

Fig. 3. Scavenging activity of *A. aretioides* extracts on the DPPH radical scavenging.

of hydrogen to form the stable DPPH-H molecule.[24] The effect of antioxidants on DPPH radical-scavenging was thought to be due to their hydrogen-donating ability.

The best free radical scavenging activities were exerted by the acetone extract. We remark that the methanol and ethyl acetate extracts were presented the same activity. Compared with positive controls, all extracts were presented a lower antioxidant activity (significant differences, $P < 0.05$).

3.3. Hydrogen peroxide radical scavenging

In this assay, since antioxidant compounds present in the extract are good electron donors, they may accelerate the conversion of H_2O_2 to H_2O.[25]

H_2O_2 is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (−SH) groups. Hydrogen peroxide can cross cell membrane rapidly. Once inside the cell, H_2O_2 can probably react with Fe^{2+} and possibly Cu^{2+} to form hydroxyl radical and this may be the origin of many of its toxic effects. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate.[23]

The *A. aretioides* extracts were tested for their antioxidant capacity by the hydrogen peroxide scavenging method. As shown in Table 3, all extracts, at the concentration of 100 μg/mL, showed a high scavenging capacity against H_2O_2 compared to positive control

Table 2
Total phenolic, total flavonoid contents and the EC_{50} values of *A. aretioides* extracts, BHA, ascorbic acid and quercetin.

	DPPH scavenging EC_{50} (μg/mL)	Total phenolic	Total flavonoid
Acetone extract	47.71 ± 1.13^{a}	183.01 ± 4.38^{a}	74.17 ± 3.52^{a}
Methanol extract	79.15 ± 4.23^{b}	101.85 ± 2.31^{b}	46.68 ± 0.74^{b}
Chloroform extract	86.73 ± 10.68^{c}	196.63 ± 31.2^{a}	100.01 ± 3.9^{c}
Ethanol extract	65.08 ± 1.98^{d}	231.85 ± 20.59^{c}	132.8 ± 24.58^{d}
Ethyl acetate extract	$72.15 \pm 1.04^{b,d}$	134.82 ± 13.27^{d}	106.38 ± 4.47^{c}
BHA	2.61 ± 0.13^{e}	-	-
Ascorbic acid	2.48 ± 0.09^{e}	-	-
Quercetin	2.59 ± 0.15^{e}	-	-

Each value represents the mean ± SD ($n = 3$). Total phenolic content was expressed as mg gallic acid equivalents/g dried extract. Total flavonoid content was expressed as mg catechin equivalents/g dried extract. EC_{50} values were expressed as final concentrations. Within the same column, means followed by different letters are significantly different at $P < 0.05$.

Table 3
Hydrogen peroxide scavenging activity of *A. aretioides* extracts.

	H_2O_2 scavenging activity (%)
Acetone extract	23.81 ± 2.13^{a}
Methanol extract	26.98 ± 2.99^{a}
Chloroform extract	29.28 ± 5.04^{a}
Ethanol extract	28.72 ± 3.03^{a}
Ethyl acetate extract	45.49 ± 3.84^{b}
BHA	24.13 ± 7.32^{a}
α-Tocopherol	32.44 ± 5.87^{a}

Each value represents the mean ± SD ($n = 3$). Within the column, means followed by different letters are significantly different at $P < 0.05$.

(BHA and α-tocopherol). There were no significant difference between organic extracts of *A. aretioides* and the two positive controls used ($P > 0.05$). However, we observed that the ethyl acetate extract of *A. aretioides* were presented a high activity significantly different to the positive control and two times more than BHA activity, this result suggest that this extract contain the necessary compounds for radical elimination. It is well established that hydrogen peroxide is not dangerous as it is, but may well be because of its ability to form the hydroxyl radical, thereby emphasizing on the importance of its elimination. Indeed, it has already been proven that dietary phenols protect mammalian and bacterial cells from cytotoxicity induced by H_2O_2,[26] indicating that the observed H_2O_2 scavenging activity of *A. aretioides* extracts could be due to the presence of phenols.

In conclusion, the use of different level of polarity in the choice of organic solvents allowed us to conclude that the solvent of extraction has a significant effect in phenolic extraction. For this plant the use of ethanol as extracting solvent is recommended. The results obtained in the present study shown that *A. aretioides* has the potent scavenging activity on DPPH radical and H_2O_2. This study is the first about this plant. The studies of organic fractions to isolate the compounds that have good antioxidant properties, and identify compounds from each fraction are the prospects for this work.

Conflicts of interest

All authors have none to declare.

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Original article

Psychopharmacological and antioxidant effects of hydroethanolic extract of *Alpinia zerumbet* leaves in mice

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ABSTRACT

Background: Alpinia zerumbet (Pers.) B.L. Burtt & R.M. Sm., Zingiberaceae, is traditionally used in Brazil to treat hypertension, inflammation, anxiety, and hysteria. However, investigations of antioxidant and central effects of *A. zerumbet* extract are lacking.
Objective: The aim of this study was to verify the effects of a hydroethanolic extract of *A. zerumbet* (HEA) on tail suspension and light/dark tests to screen for possible antidepressant- and anxiolytic-like activities, respectively. We also evaluated the *in vitro* antioxidant effects of HEA.
Materials and methods: Swiss male mice were orally treated with saline or HEA (200, 400 or 800 mg/kg) 60 min before testing. The *in vitro* antioxidant activity of HEA was determined using the ferric-reducing antioxidant property method and assays involving free radical and reactive oxygen species scavenging. Protections against glutathione oxidation and lipid peroxidation were also evaluated.
Results: HEA (200, 400 and 800 mg/kg) significantly reduced the period of immobility in the tail suspension test, similarly to imipramine. In the tail suspension test, HEA (400 and 800 mg/kg but not 200 mg/kg) and diazepam significantly increased time spent in the light side. The antioxidant activity of HEA was remarkable, as it showed significant ferric-reduction power, 1,1-diphenyl-2-picrylhydrazyl radical and hydrogen peroxide scavenging activity, and protection against lipid peroxidation.
Conclusion: This study showed the antioxidant, antidepressant- and anxiolytic-like effects of HEA in mice. More studies and the identification of active components of the extract are necessary to further assess the therapeutic potential of this species in the treatment of psychiatric diseases.

1. Introduction

Epidemiological studies show that millions of people suffer from different types of psychiatric disorders throughout the world, and this number is increasing gradually, mainly in developing countries.[1] Depression and anxiety are the major psychiatric disorders, which affect 10–15% of the population.[2] Despite heavy investments by the pharmaceutical industries in the research and development of new psychotropics, controlled trials have shown that there is little difference in the overall effectiveness of the drugs that are currently in use.[3,4] Therefore, bioprospecting studies are increasingly directed at the rational pursuit of value-added bioproducts, including new drugs. Thus, plants have emerged as potential alternatives for the treatment of psychiatry diseases.

Alpinia zerumbet (Pers.) B.L. Burtt & R.M. Sm., Zingiberaceae, which is known as colônia, falso-cardamomo and pacová, is a plant species found in northeast Brazil. The pharmacological properties attributed to *A. zerumbet* include diuretic and hypotensive effects as a result of its use in tea leaves.[5–7] Other medicinal properties include the anti-hysterical and vermifuge activities of the leaves, flowers, and rhizome.[8] *A. zerumbet* has also been used widely to treat hypertension, anxiety, inflammation, gastric distress, and

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rheumatism.[9] Recent studies have evaluated the effects of the volatile oil from this species in animal models of anxiety, depression and catalepsy. *A. zerumbet* essential oil potentiates apomorphine-induced catalepsy and increases the immobility time in forced swim and tail suspension tests, with a clear sedative effect.[10] The volatile oils of this species showed no activity in animal models used to evaluate anxiolytic-like activity.[10] In one study however, *A. zerumbet* essential oil showed anxiolytic activity in the elevated maze model.[11] An anxiolytic effect in rodents after inhalation of the essential oil in light–dark, elevated plus maze, and open field tests has also been reported.[12]

Therefore, the aim of the present study was to verify the effects of a hydroethanolic extract of *A. zerumbet* (HEA) in the tail suspension (TST) and light/dark (LD) tests, which are used widely for screening antidepressants and anxiolytics, respectively. Since free radicals and reactive oxygen species (ROS) production is involved in pathophysiology of several human disorders,[13,14] the *in vitro* antioxidant effects, as well as bioactive compounds concentration in HEA were also evaluated to provide a scientific basis for biological effects of *A. zerumbet*.

2. Material and methods

2.1. Animals

Experiments were performed using 127 two-month-old male Swiss mice (25–30 g), obtained from the Bioterism Center of Unochapecó. Seven mice were housed per cage (30 × 19 × 13 cm) and were maintained in our own animal facility under controlled environmental conditions 22 ± 1 °C, 12 h LD cycle, free access to food (Nuvilab CR1®) and water for at least two weeks before experiments. All procedures were performed in accordance with institutional policies related to the handling of experimental animals (approved by the URI Institutional Ethics Committee, process No. 091/PGA/11).

2.2. Plant material

The leaves of *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm., Zingiberaceae, were collected in Chapecó (SC) (26°58′36.06″S, 52°44′27.18″W) and were taxonomically identified by Osmar dos Santos Ribas (Curador do Herbário do Museu Botânico Municipal de Curitiba), with whom a voucher was deposited (MBM #306196).

2.3. Preparation of the extract

The leaves of *A. zerumbet* were dried at room temperature and powered (425 μm; 35 Tyler/Mesh). Powered leaves (500 g) were extracted using 70% (v/v) EtOH (17 L) by percolation, before evaporation under reduced pressure and further lyophilization (38.2% yield).

2.4. Drugs

Diazepam (UniãoQuímica, Brazil) was purchased from common commercial suppliers. Imipramine was obtained from Sigma–Aldrich (USA). All drugs were dissolved in saline (0.9% w/v NaCl). Drugs and vehicles were injected intraperitoneally (*i.p.*) or orally (*p.o.*) using a constant volume of 0.1 ml/10 g body weight.

2.5. Locomotion

Locomotion was measured using an open field apparatus that was divided into twelve equal quadrants (50 × 50 cm), (Insight, Ribeirão Preto, Brazil). The number of crossings was analyzed manually using a stopwatch. Groups ($n = 10$) of mice were treated orally with saline or HEA (200, 400 and 800 mg/kg). Mice were placed individually in the activity cages 60 min after treatments and locomotion was recorded for 6 min.

2.6. Tail suspension test (TST)

Mice were suspended 50 cm above the bench using a piece of adhesive tape that was placed approximately 1 cm from the tip of the tail. Mice were observed for 6 min, and the period of immobility was recorded using a stopwatch. Mice were considered to be immobile if they were hanging passive and motionless.[15] Mice ($n = 8–12$) were treated orally with the vehicle (0.9% w/v saline) or HEA (200, 400 or 800 mg/kg). An additional group was treated with imipramine (20 mg/kg, *i.p.*). Mice were submitted to TST at 30 or 60 min (for *i.p.* and *p.o.*-treated groups, respectively) after treatment.

2.7. Light/dark test

The LD apparatus consisted of a rectangular box (46 × 27 × 30 cm) that was divided into one small (18 × 27 cm) area and one large (27 × 27 cm) area with an opening door (7.5 × 7.5 cm) located in the centre of the partition at floor level. The small compartment was painted black, whereas the large compartment was painted white and illuminated brightly with a 60-W cold light source. Each animal was placed individually in the centre of the light compartment (facing away from the door) and the following parameters were recorded for 5 min: latency to enter in the dark compartment, time spent in the light zone, and the number of crossings between the light and dark compartments. The test was performed in a quiet darkened room.[16] The mice were kept in this room for at least 1 h before the test. Groups ($n = 9–11$) were treated orally with the vehicle (0.9% w/v saline), diazepam (1.0 mg/kg) or HEA (200, 400 or 800 mg/kg) 60 min before testing.

2.8. Determination of the total phenolic content

Quantification of the total phenolic content was performed using the Folin–Ciocalteu method.[17,18] Briefly, 100 μL of extract HEA solution was added to 1 ml of 10-fold-diluted Folin–Ciocalteu reagent. After agitation, the mixture was kept at room temperature for 5 min. Next, 1 ml of 10% (w/v) Na_2CO_3 was added, and the mixture was incubated in the dark for 90 min. The absorbance of the blue product, which indicated the capacity of phenolic compounds to reduce Folin–Ciocalteu in an alkaline medium, was measured at 725 nm against a blank sample. The total phenolic content was expressed as gallic acid equivalents, *i.e.* mg GAE/g dry weight (d.w.), and the values were calculated as the means of triplicate experiments.

2.9. Determination of the total flavonoid content

In spectrophotometric quantification of flavonoid compounds HEA (5 ml) was added to 2.5 ml of a mixture containing 5 mM $AlCl_3$ and 48 mM sodium acetate. After 30 min of incubation, the absorbance was measured at 425 nm and compared with a blank. A standard curve was prepared using quercetin and the results were expressed as quercetol equivalents, *i.e.* mg QE/g dry weight (d.w.), and calculated as the means of triplicate analyses.[19]

2.10. Determination of the ferric-reducing antioxidant power (FRAP)

The FRAP assay[20] was used with some modifications. This method is based on the reduction of Fe^{3+} by antioxidant molecules. Ferric-2,4,6-trypyridyl-s-triazine HEA in solution (TPTZ) was prepared by mixing 2.5 ml of 10 mM TPTZ solution with 40 mM HCl, 2.5 ml of 20 mM $FeCl_3.6H_2O$ and 25 ml of 0.3 M acetate buffer at pH 3.6. Thirty microlitres of different concentrations of HEA extract (5–250 μg/ml) or standard (ascorbic acid) were added to 990 μL of reaction mixture. After incubating at 37 °C for 15 min, the absorbance of the complex formed between Fe^{2+} and TPTZ was recorded at 593 nm. Controls containing the extract without TPTZ were also evaluated to determine the possible absorbance of natural compounds in the extract at the wavelength used to measure the absorbance of the Fe^{2+}-TPTZ complex. The absorbance of the extract at 593 nm in the absence of TPTZ was subtracted from that obtained using the extract plus the TPTZ mixture. The increase in the absorbance due to the Fe^{2+}-TPTZ complex formation induced by HEA was compared to that induced by ascorbic acid (standard), and the results were expressed as the mean absorbance of triplicate experiments ($n = 3$).

2.11. 1,1-Diphenyl-2-2picrylhydrazyl radical scavenging assay

A stable solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used to determine the total antioxidant capacity of HEA.[21] DPPH solution (0.24 mg/ml) was diluted with methanol until an absorbance of 1.10 ± 0.02 at 517 nm was obtained. The extract was diluted with methanol to produce different concentrations (5–250 μg/ml) and 50 μl of each dilution was mixed with 1.95 ml of methanolic DPPH solution. The antiradical power of the different concentrations of extract and the standard were determined by measuring the decrease in the DPPH absorbance after 24 h in the dark *versus* a blank. The addition of the antioxidant resulted in a relative decrease in the absorbance that was proportional to the antioxidant activity of the extract. The same procedure was followed for ascorbic acid to compare the antiradical capacity of HEA to that of ascorbic acid *in vitro*. This analysis was performed in triplicate ($n = 3$), and the results were expressed as mean % inhibition of the DPPH radical, which was calculated as follows: % inhibition $= [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$. The concentration of HEA that could scavenge half of the DPPH radical (IC_{50}) was calculated by nonlinear regression analysis using GraphPad Prism version 4.0 (La Jolla CA, USA).

2.12. Evaluation of the hydrogen peroxide scavenging capacity

The ability of HEA to scavenge hydrogen peroxide (H_2O_2) was assessed by measuring the decrease in the H_2O_2 absorbance at 240 nm in a medium used to assess catalase (CAT) antioxidant activity that contained 50 mM phosphate buffer (pH 7.0) and 17 mM H_2O_2.[22] The H_2O_2 scavenging ability of ascorbic acid was also evaluated to compare its H_2O_2 scavenging ability with that of HEA. The results were calculated as the mean of triplicate experiments ($n = 3$) and expressed as the CAT-like activity (k/mg d.w.).

2.13. Evaluation of the superoxide anion radical scavenging capacity

The ability of HEA to inhibit the auto-oxidation of epinephrine to adrenochrome, which is mediated by superoxide anions (O_2^-), was evaluated at 480 nm. The reaction assay contained 50 mM glycine buffer (pH 10.2) and 1 mM epinephrine at 30 °C.[23] This assay was performed in triplicate ($n = 3$) and the O_2^- scavenging ability of ascorbic acid solution (20 μM) was evaluated for comparison.

2.14. Protection against glutathione oxidation

The capacity of HEA to prevent GSH oxidation was assessed in the absence or presence of H_2O_2 by measuring the disappearance of the reduced sulfhydryl groups of GSH. The reduced sulfhydryl groups were quantified,[24] 30 min after GSH addition to a reaction mixture containing 200 mM potassium phosphate buffer (pH 6.4), HEA (5–250 μg/ml), and H_2O_2 (0 or 0.5 mM) at 39 °C. Controls containing extract lacking GSH were run to verify the possible absorbance of the extract at the wavelength used to assess GSH oxidation (412 nm). A low absorbance was detected in the extract, which was subtracted from that obtained with the extract plus GSH. The same procedure was adopted for ascorbic acid for comparison. This assay was assessed in triplicate ($n = 3$), and the results were expressed as the % of remaining sulfhydryl groups of GSH.

2.15. Protection against lipid peroxidation

Lipid peroxidation was performed using brain homogenates from mice. Animals were decapitated; the forebrain was immediately dissected and then homogenized in 50 mM Tris–HCl (pH 7.5; 1:9, w/v). The homogenate was centrifuged for 20 min at 2000 × *g* to yield a low-speed supernatant that was used for determination of lipid peroxidation. This supernatant (350 μL) was pre-incubated at 37 °C for 1 h in the presence or absence of 50 μM $FeCl_2$, 1 mM H_2O_2 and HEA (0–250 μg/dL) in a final volume of 500 μL, which was made up using 50 mM Tris–HCl. After pre-incubation, the level of thiobarbituric acid-reactive substances (TBARS) was determined.[25] A decrease in the formation of the pink chromogen in tubes pre-incubated with HEA was considered to be an indicator of the inhibition of lipid peroxidation, which was calculated as stated above (DPPH radical scavenging assay). The IC_{50} value, which represents the concentration of HEA that was necessary to inhibit 50% of lipid peroxidation, was determined by non-linear regression analysis using GraphPad Prism version 4.0 (La Jolla CA, USA).

2.16. Statistical analysis

The results (cumulative spontaneous locomotion counts, time(s) of immobility and *in vitro* antioxidant activity) were expressed as the mean ± S.E.M. Comparisons between groups were performed using ANOVA followed by Tukey's test, using SPSS 20.0. Differences between data were analyzed using the Student's *t*-test to determine the *in vitro* antioxidant activity (different concentrations of HEA × different concentrations of ascorbic acid). For the lipid peroxidation assay, comparisons using different concentrations of HEA were analyzed by one-way ANOVA followed by Tukey's test using SPSS 20.0. $P < 0.05$ was considered significant.

3. Results

Fig. 1 shows the effects of HEA on the tail suspension test. HEA significantly reduced immobility time in the TST ($F_{(4,47)} = 15.0$, $p < 0.0001$). Imipramine was not more active than HEA in this test. Spontaneous locomotion of groups treated with HEA did not differ from controls ($F_{(4,\ 24)} = 0.54$, data not shown).

Fig. 2 shows the effects of HEA on light/dark test. HEA (200, 400 and 800 mg/kg) and diazepam (1.0 mg/kg) significantly increased the latency for the first crossing from the light to the dark compartment ($F_{(4,45)} = 7.3$, $p < 0.0001$; Fig. 2A). The number of crossings between compartments was significantly increased by

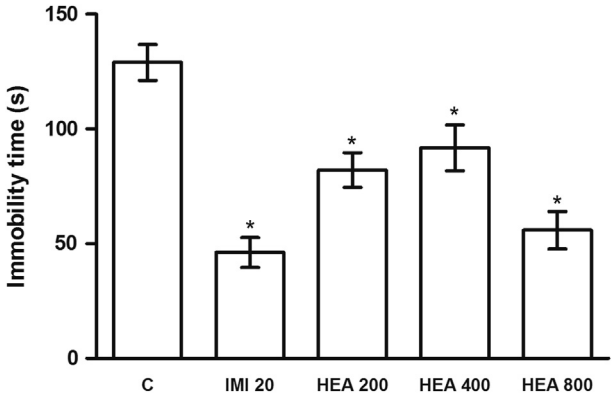

Fig. 1. Effects of HEA (200, 400 and 800 mg/kg, *p.o.*) and imipramine (20 mg/kg, *i.p.*) in the TST. Each column represents the mean ± S.E.M. $N = 8–12$. * = $p < 0.0001$ *versus* saline. ANOVA/Tukey.

HEA (400 and 800 mg/kg) and diazepam (1.0 mg/kg) in the light/dark test ($F_{(4,45)} = 8.7$, $p < 0.0001$, Fig. 2B). Like diazepam (1.0 mg/kg), HEA (400 and 800 mg/kg) significantly increased the time spent in the light side of the apparatus ($F_{(4,45)} = 6.8$, $p < 0.0002$, Fig. 2C).

The total phenolic content expressed as gallic equivalent (GAE) was found to be around 80.29 ± 1.38 mg GAE/g d.w., while flavonoid content was recorded in quercetol equivalents (QE) and was around 4.85 ± 0.05 mg QE/g d.w.

Fig. 3 shows the results of the FRAP assay. One-way ANOVA showed that HEA and ascorbic acid had significant reducing power at a concentration of ≥5 μg/ml. However, the ferric-reducing power of ascorbic acid was significantly higher than that of HEA for all concentrations evaluated, according to the Student's *t*-test.

Fig. 4 shows DPPH radical scavenging antioxidant activity. The DPPH radical scavenging ability of HEA was lower than that of ascorbic acid, but it was high in the evaluated concentration range. For 5 and 10 μg/ml, the inhibition of DPPH by HEA was 27% and 46%, respectively, and it produced >80% of inhibition for all concentrations between 20 and 250 μg/ml. The IC_{50} value for DPPH radical scavenging activity of HEA was 9.86 μg/ml (confidence interval = 5.71–17.02), whereas for ascorbic acid it was 4.94 μg/ml (confidence interval = 3.87–6.32).

HEA also had H_2O_2 scavenging activity (Fig. 5). However, this property was less evident compared with its reducing potential and DPPH radical scavenging activity (Figs. 3 and 4, respectively), *i.e.* it was evident at concentrations of 50–150 μg/ml HEA (Fig. 5) but not at higher concentrations (data not shown). HEA failed to prevent the H_2O_2-induced oxidation of the thiol groups of GSH and it also had no superoxide radical (O_2^-) scavenging activity (data not shown). Ascorbic acid is not shown in Fig. 5 because it also did not present protective effect against GSH oxidation.

Fig. 6 shows the protective effect of HEA during the Fenton reaction-induced lipid peroxidation of homogenates of mice brains. The extract inhibited lipid peroxidation at all concentrations, where the inhibition rate was 46–79% at concentrations of 5–250 μg/ml, while the IC_{50} value for HEA was 15.7 μg/ml (confidence interval = 9.3–26.4).

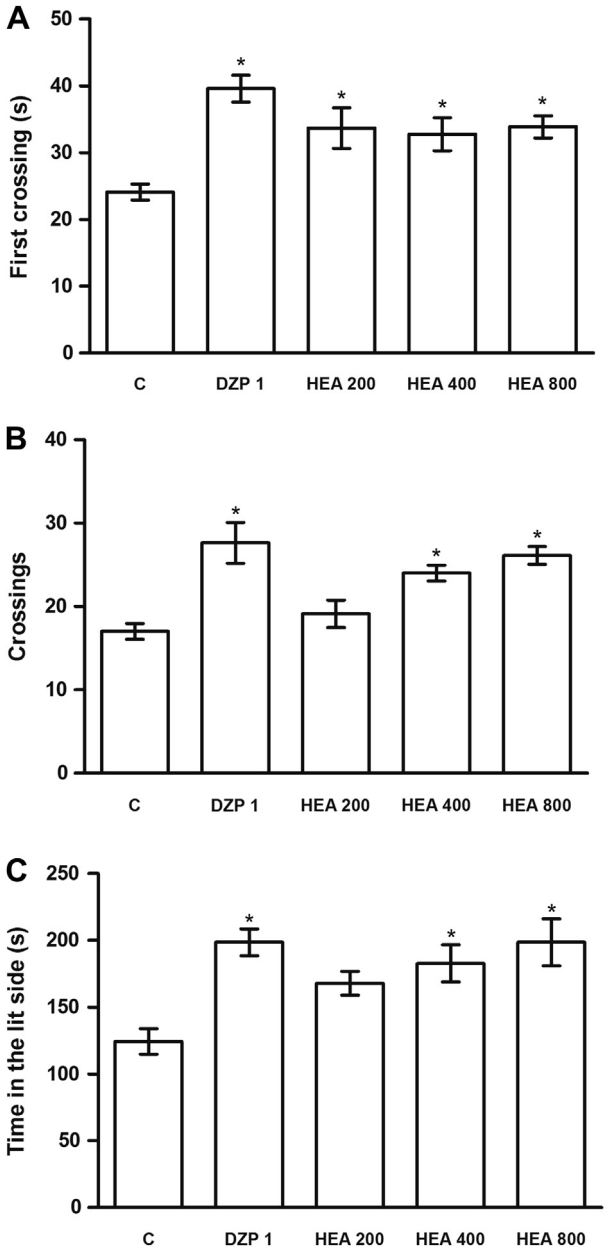

Fig. 2. Effects of HEA (200, 400 and 800 mg/kg, *p.o.*) and diazepam (1 mg/kg, *p.o.*) on latency for first crossing (A), number of crossings (B) and time in the lit side (C) in the light/dark test. Each column represents the mean ± S.E.M. $N = 9–11$. * = $p < 0.0001$ *versus* saline. ANOVA/Tukey.

4. Discussion

Preclinical evaluation is essential for studies involving new potential drugs. In this context, this study evaluated the effects of *A. zerumbet* on the central nervous system in animal models. Our results showed that HEA (200, 400 and 800 mg/kg) had an antidepressant-like activity in TST. Furthermore, this effect was comparable to the tricyclic antidepressant imipramine. In addition to this antidepressant-like activity, HEA (400 and 800 mg/kg) had an anxiolytic-like profile in the LD test that was comparable to diazepam.

Phenolic compounds are found mainly in fruits, herbs, and vegetables, and their antioxidant properties are recognized in addition to their many other biological effects.[26,27] Flavonoids, such as flavonols and flavanes, are a major class of phenolic compounds found in plants.[27] In this study, the total phenolic and flavonoid contents were quantified in HEA, which could be related to the antioxidant properties of this extract.

The antioxidant activity of a substance is correlated to its reducing properties, so FRAP is a reliable method for assessing the

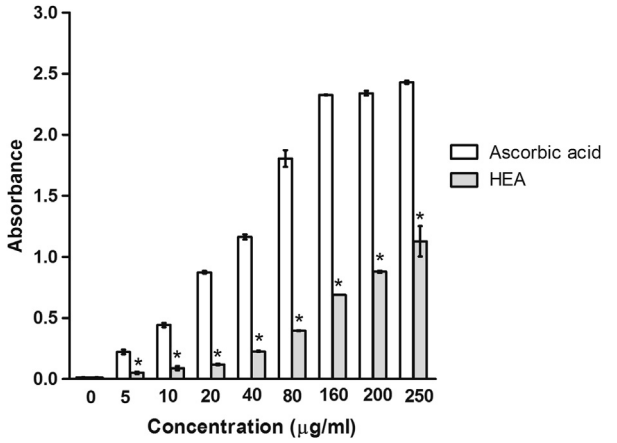

Fig. 3. Ferric-reducing antioxidant power (FRAP) of HEA. Results are expressed as mean ± S.E.M. ($n = 3$). *Different from ascorbic acid solution at the same concentration. $p < 0.001$, *t*-Student's test.

antioxidant activity of several compounds, including polyphenols.[20] The FRAP assay measures the ability of an antioxidant substance to reduce the 2,4,6-trypiridyl-s-triazine-Fe(III) complex to 2,4,6-trypiridyl-s-triazine-Fe(II) complex by donating an electron.[20] HEA had a remarkable reducing activity with the 2,4,6-trypiridyl-s-triazine-Fe(III) complex, which indicated the presence of compounds that could donate electrons. The reducing activity could be also attributed to compounds other than phenolics in the present study, however the activity of leaf and seed extracts of *Abelmoschus moschatus* Medik., was strongly suggested to be attributable to polyphenol compounds.[28] Indeed, phenolic compounds are widely recognized as important reducing agents.[29]

The reducing power of HEA was supported by the DPPH radical scavenging assay, which also evaluated the ability of antioxidants to transfer a single electron. DPPH scavenging was detected at all concentrations (5–250 μg/ml) evaluated. These results strongly suggest that the DPPH radical scavenging capacity of HEA was related to its reducing properties in the FRAP assay. The DPPH scavenging capacity of the extract was lower than that of ascorbic acid at all concentrations evaluated. However, the antioxidant potential of HEA was similarly high because ascorbic acid inhibited 90–92% of DPPH oxidation at 10 μg/ml, whereas HEA inhibited 81–87% at concentrations of 20–250 μg/ml.

HEA was also capable of removing H_2O_2, which is an ROS that is produced continuously during cellular oxidative metabolism.[30] H_2O_2 scavenging occurred at 50–150 μg/ml of HEA and it was lower than its reducing power and DPPH scavenging activity, which

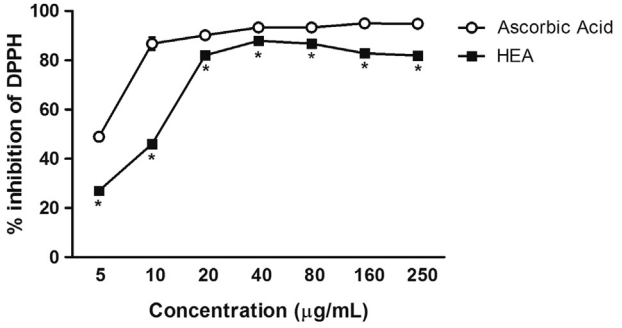

Fig. 4. DPPH radical scavenger activity of HEA. Results are expressed as means ± S.E.M. ($n = 3$). *Different from DPPH radical scavenger activity of ascorbic acid solution at the same concentration. $p < 0.001$, *t*-student`s test.

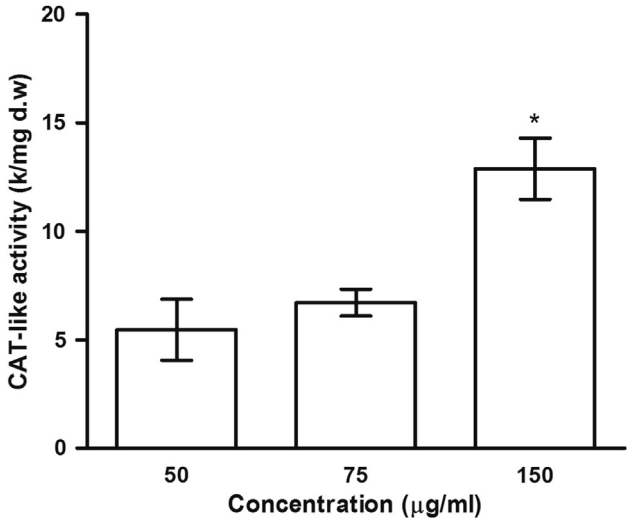

Fig. 5. H_2O_2 scavenger activity of HEA. Results are expressed as means ± S.E.M. ($n = 3$). *Different from H_2O_2 scavenging activity of lower concentrations of HEA (50 and 75 μg/mL).

were observed from 5 μg/ml. The H_2O_2 scavenging capacity indicates indirect protection against −OH radical formation, which is produced by Fenton-type reactions involving H_2O_2 and transition metals.[30]

Extracts of other plants have H_2O_2 scavenging capacity that may be attributed to phenolic compounds, which can transfer electrons to H_2O_2 and reduce it to water.[28,31,32] However, the weak H_2O_2 scavenging ability of HEA appeared to be insufficient to inhibit the H_2O_2-induced oxidation of GSH, because none of the extract concentrations tested produced this effect. The lack of H2O2 scavenging with ascorbic acid may be explained by H_2O_2-induced ascorbic acid oxidation in the reaction mixture used in these assays, as previously demonstrated.[33]

The inhibition of lipid peroxidation is considered to be an important index of antioxidant activity because membrane lipid peroxidation is an endpoint of biological damage that occurs in several diseases, including neurodegenerative disorders[34] However, antioxidants may protect from lipid peroxidation by scavenging

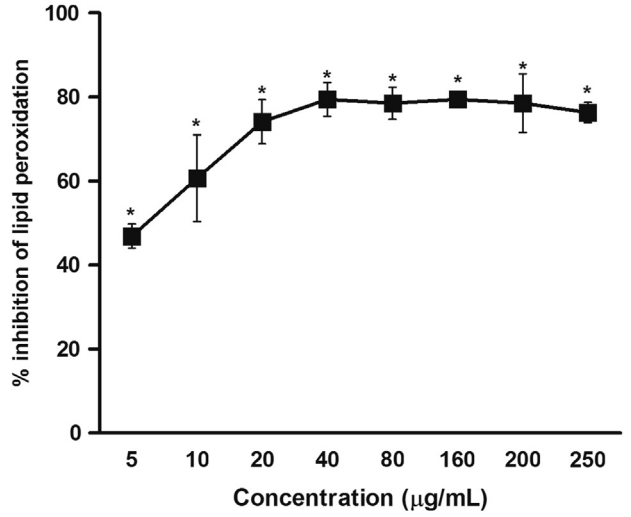

Fig. 6. Inhibition of Fenton reaction-induced lipid peroxidation of HEA. Results are expressed as means ± S.E.M ($n = 5$). *Different from control (0 μM of HEA) at the same conditions.

free radicals, thereby preventing diseases.[28,35] Therefore, the inhibition of lipid peroxidation is recognized as an important index of antioxidant activity. Interestingly, the present study showed that HEA inhibited lipid peroxidation and provided a good degree of protection against oxidative damage to lipids. This protective effect may be related to its capacity for scavenging the −OH radical, which is produced by the Fenton reaction between iron and H_2O_2.[36] When present in excess, the −OH radical may initiate a lipid peroxidation chain that terminates in brain injury.[37]

Oxidative damage to lipids has recently been detected in anxiety- and depression-like rat models[38] and in patients with major depressive disorders.[39] The inhibition of lipid peroxidation by HEA and its ability to scavenge free radicals might be a link between antioxidant activity and its central effects in the present study using mice. Further *in vivo* studies should be performed to corroborate this hypothesis.

In summary, this study demonstrated the *in vitro* antioxidant, antidepressive, and anxiolytic effects in mice of HEA. The *in vitro* antioxidant activity may be attributable to its radical scavenging ability and protection against lipid peroxidation. The central nervous system activities of HEA might be in part due to antioxidants and flavonoids detected in the plant.

Conflicts of interest

All authors have none to declare.

Acknowledgement

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Original article

Triterpenoid saponins from *Lysimachia candida* Lindl.

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ABSTRACT

Background: Identification of chemical constituents is very important for ensuring the function of traditional herbal medicine. The objectives of this study were to determine the activity chemical constituents of *Lysimachia candida* Lindl.
Methods: Solvent fractionation and chromatographic separation techniques were used to isolate the chemical constituents of *Lysimachia candida* Lindl.
Results: Three triterpenoid saponins were isolated from the antifungal activity fraction of *Lysimachia candida* Lindl. Their structures were elucidated on the basis of spectroscopic data. They were established as a new compound 3-*O*-[β-D-Xylopyranosyl-(1→2)-β-D-Glucopyranosyl-(1→4)-[β-D-Glucopyranosy-(1→2)]-α-L-arabinopyranoside]-13,28-Epoxy-3,16,22-oleananetriol, named Lysimanoside **(1),** along with two known compounds Lysikokianoside I **(2)** and Anagallisin C **(3)**.
Conclusion: Anagallisin C showed to be the main antifungal compound against *Aspergillus flavu.* in *Lysimchia candida* Lindl.

1. Introduction

Lysimachia candida Lindl. is a folklore medicinal plant that grows in South China. The whole plant is used for treating fever, swelling, dermatitis and bone fracture.[1] In the course of our investigation on antifungal natural products from plants, we found the crude extract of *L. candida* Lindl. showed activity against *Aspergillus flavus* with a dosage of about 500 μg on filter diffusion assay. The crude extract was suspended in H_2O, and partitioned with $CHCl_3$, EtOAc and *n*-BuOH successively. The *n*-BuOH extract showed activity against *A. flavus* with a dosage of about 200 μg on filter diffusion assay. In further investigation on the antifungal constituents of this plant, a new compound, 3-*O*-[β-D-xylopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 4)-[β-D-glucopyranosyl-(1 → 2)]-α-L-arabinopyranoside]-13,28-Epoxy-3,16,22-oleananetriol, named Lysimanoside (**1**), along with two known compounds Lysikokianoside I (**2**) and Anagallisin C (**3**) were isolated from the stems of *L. candida* Lindl. Anagallisin C showed to be the main antifungal compound against *A. flavus* in *L. candida* Lindl.

2. Results and discussion

The MeOH extract of the stems of *L. candida* Lindl. was suspended in H_2O, and partitioned with $CHCl_3$, EtOAc and *n*-BuOH successively. Separation of *n*-BuOH extract by a combination of silica gel and ODS column chromatography and preparative HPLC afforded Lysimanoside (**1**), Lysikokianoside I (**2**) and Anagallisin C (**3**) (Fig. 1). Their chemical structures were determined on the basis of spectroscopic analysis and comparison to the reported values in literature.

Compound **1** was obtained as an amorphous white powder. $[\alpha]^{20}_D$ − 13.9° (*c* = 1.0, MeOH). The molecular formula was determined as $C_{52}H_{86}O_{22}$ from ESIMS ions at *m/z* 1085 $[M + Na]^+$, 1101 $[M + K]^+$ in positive and 1061 $[M - H]^-$, 1097.5 $[M + Cl]^-$ in negative as well as an HRESIMS ion at *m/z* 1063.56616 $[M + H]^+$ (Calcd for $C_{52}H_{86}O_{22}$, 1063.56890). The negative-ion ESIMS showed fragment ion peaks at *m/z* 929 $[M-C_5H_8O_4-H]^-$ due to the loss of a pentose unit, *m/z* 767 $[M-C_5H_8O_4-C_6H_{10}O_5-H]^-$ due to the loss of a pentose unit and a hexose unit and *m/z* 605 $[M-C_5H_8O_4-C_6H_{10}O_5-C_6H_{10}O_5-H]^-$ due to the loss of a pentose unit and two hexose units. Compound 1 displayed 52 carbon signals in ^{13}C NMR spectrum (Table 1), of which 30 carbon signals could be assigned to the signals of the aglycon. ^{13}C NMR spectrum exhibited seven sp3 carbons at δ 16.69, 16.69, 18.79, 20.11, 26.02, 28.34 and 33.86 and signals for an oxygenated methylene at δ 77.62 and a quaternary carbon at δ 88.31. These data coupled with information from the ^1H NMR spectrum of seven methyl singlets at δ 1.25, 1.14, 1.05, 0.98, 0.94, 0.89 and 0.84 and a pair of oxygenated methylene protons at δ 3.37(d, *J* = 8.0 Hz) and 3.66 (d, *J* = 8.0 Hz) indicated that the aglycon of **1** is based on a 13,28-epoxyoleanane skeleton.[2] The ^1H NMR and ^{13}C NMR spectrum indicated the presence of one

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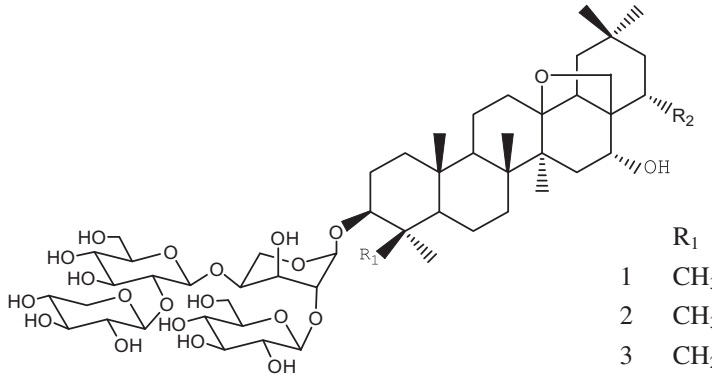

	R_1	R_2
1	CH_3	OH
2	CH_3	H
3	CH_2OH	H

Fig. 1. Structures of **1**, **2** and **3**.

α-arabinopyranosyl unit [δ_H 4.69 (d, J = 4.0 Hz); δ_C 104.32] two β-glucopyranosyl units [δ_H 4.97 (d, J = 8.0 Hz); δ_C 104.82; δ_H 4.91 (d, J = 8.0 Hz); δ_C 105.59], and one β-xylopyranosyl unit [δ_H 4.51 (d, J = 4.0 Hz); δ_C 107.34]. The above analysis revealed that compound **1** was a triterpenoid saponin with four monosaccharide units. ^{1}H–^{1}H COSY, ^{13}C–^{1}H COSY, NOESY and HMBC experiments enabled us to identify the aglycone of 1 as priverogenin B.[3] A ^{13}C NMR spectral comparison of **1** with a known compound anagallosaponin VII showed that compound **1** varies structurally from anagallosaponin VII only in its saccharide moieties, these sugar units are also affixed to the C-3 position.[4] The sugar sequence of compound **1** was determined by the HMBC spectrum (Fig. 2). Long-range correlations were seen between C-3 (δ 91.4) of the aglycone and H-1 (δ 4.69) of the arabinose, C-2 (δ 80.3) of the arabinose and H-1 (δ 4.91) of the glucose, C-4 (δ 77.9) of the arabinose and H-1 (δ 4.97) of the glucose, and C-2 (δ 85.2) of the glucose and H-1 (δ 4.51) of the xylose. Thus, the tetrasaccharide unit in **1** was determined as β-D-xylopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 4)-[β-D-glucopyranosyl-(1 → 2)]-α-L-arabinopyl-. The values of ^{13}C NMR spectral of the sugar units are similar to that of known compound anagallosaponin IV and desgluconagalloside B.[3] So the structure of compound **1** was elucidated as 3-*O*-[β-D-xylopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 4)-[β-D-glucopyranosyl-(1 → 2)]-α-L-arabinopyranoside]-13, 28-Epoxy-3,16,22-oleananetriol, which is a new compound, named Lysimanoside.

Compound **2** was obtained as an amorphous white powder. $[\alpha]^{20}_D$ − 4.3° (c = 1.0, MeOH). The molecular formula was determined as $C_{52}H_{86}O_{21}$ from ESIMS ions at m/z 1069 $[M + Na]^+$, 1085 $[M + K]^+$ in positive and 1045 $[M - H]^-$, 1081.5 $[M + Cl]^-$ in negative. The negative-ion ESIMS showed fragment ion peaks at m/z 913 $[M-C_5H_8O_4-H]^-$ due to the loss of a pentose unit, m/z 751 $[M-C_5H_8O_4-C_6H_{10}O_5-H]^-$ due to the loss of a pentose unit and a hexose unit and m/z 589 $[M-C_5H_8O_4-C_6H_{10}O_5-C_6H_{10}O_5-H]^-$ due to the loss of a pentose unit and two hexose units. Compound **2** displayed 52 carbon signals in ^{13}C NMR spectrum (Table 1), of which 30 carbon signals could be assigned to the signals of the aglycon. ^{13}C NMR spectrum exhibited seven sp3 carbons at δ 16.73, 16.77, 18.83, 19.93, 24.95, 28.38 and 33.92 and signals for an oxygenated methylene at δ 77.61 and a quaternary carbon at δ 88.36. These data coupled with information from the ^{1}H NMR spectrum of seven methyl singlets at δ 1.22, 1.15, 1.05, 0.95, 0.90, 0.90 and 0.84 and a pair of oxygenated methylene protons at δ 3.35 (d, J = 8.0 Hz) and 3.67 (d, J = 8.0 Hz) indicated that the aglycon of **2** is based on a 13,28-epoxyoleanane skeleton. In the ^{1}H NMR spectrum, two carbinylic proton signals assignable to H-3 and H-16 of the aglycon were observed at δ 3.26 (d, J = 8.0 Hz) and 3.70 (d, J = 8.0 Hz). Thus, the aglycon was identified as 13,28-epoxy-3β,16α-dihydroxyoleanane. ^{1}H NMR spectrum showed four anomeric proton signals at δ 4.99 (d, J = 8.0 Hz), 4.90 (d, J = 8.0 Hz), 4.68 (d, J = 4.0 Hz), 4.51 (d, J = 4.0 Hz). The above analysis revealed that compound **2** was a triterpenoid saponin with four monosaccharide units. The sequence of the sugar chain was assigned with the same structure as that of compound **1** by comparing their ^{1}H NMR and ^{13}C NMR data. The ^{1}H NMR and ^{13}C NMR data agreed with the known compound lysikokianoside I.[5] Thus, compound **2** was identified as lysikokianoside I.

Compound **3** was obtained as an amorphous white powder. $[\alpha]^{20}_D$ − 18.2° (c = 0.5, MeOH). The molecular formula was determined as $C_{52}H_{86}O_{22}$ from ESIMS ions at m/z 1085 $[M + Na]^+$, 1101 $[M + K]^+$, 1063 $[M + H]^+$ in positive and 1061 $[M - H]^-$, 1097.5 $[M + Cl]^-$ in negative. The negative-ion ESIMS showed fragment ion peaks at m/z 929 $[M-C_5H_8O_4-H]^-$ due to the loss of a pentose unit, m/z 767 $[M-C_5H_8O_4-C_6H_{10}O_5-H]^-$ due to the loss of a pentose unit and a hexose unit and m/z 605 $[M-C_5H_8O_4-C_6H_{10}O_5-C_6H_{10}O_5-H]^-$ due to the loss of a pentose unit and two hexose units. Compound 3 displayed 52 carbon signals in ^{13}C NMR spectrum (Table 1), of which 30 carbon signals could be assigned to the signals of the aglycon. ^{13}C NMR spectrum exhibited six sp3 carbons at δ 13.13, 17.19, 18.83, 19.91, 24.94 and 33.90 and signals for an oxygenated methylene at δ 77.62 and a quaternary carbon at δ 88.40. These data coupled with information from the ^{1}H NMR spectrum of six methyl singlets at δ 1.24, 1.14, 0.94, 0.92, 0.90 and 0.70 and a pair of oxygenated methylene protons at δ 3.34 (d, J = 8.0 Hz) and 3.70 (d, J = 8.0 Hz) indicated that he aglycon of **3** is based on a 13,28-epoxyoleanane skeleton. The ^{13}C NMR data of the aglycon were similar to that of compound 2, but the methyl group signal of C-23 was replaced by a hydroxymethyl at δ 64.62. This assignment was confirmed by the HMBC correlation between δ_H 0.70 (H-24) and δ_C 64.62. ^{1}H NMR spectrum showed four anomeric proton signals at δ 4.95 (d, J = 8.0 Hz), 4.91 (d, J = 8.0 Hz), 4.68 (d, J = 4.0 Hz), 4.58 (d, J = 4.0 Hz). The above analysis revealed that compound **3** was a triterpenoid saponin with four monosaccharide units. The sequence of the sugar chain was assigned with the same structure as that of compound 1 by comparing their ^{1}H NMR and ^{13}C NMR data. The ^{1}H NMR and ^{13}C NMR data agreed with the known compound anagallisin C.[6] Thus, compound **3** was identified as anagallisin C.

Compounds (**1**–**3**) were evaluated for their activities against *A. flavus* by filter diffusion assay. Compound **3** showed growth inhibitory activity against *A. flavus* with IC_{50} value 28.03 μg/ml.

3. Experimental

3.1. General experimental procedures

Melting points were determined on an MD-S2 micro-melting point apparatus (Yanagimoto Seisakusho Co. Ltd., Japan) and are

Table 1
^{1}H NMR and ^{13}C NMR Data for compounds **1**–**3** in CD_3OD.

Carbon no.	**1**		**2**		**3**	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
C-1		40.18		40.23		39.95
C-2		27.22		27.23		26.59
C-3	3.16 (m)	91.37	3.26 (d, J = 8.0 Hz)	91.36	3.59 (m)	83.88
C-4		40.6		40.6		44.3
C-5	0.72 (d, J = 10.5 Hz)	56.78	0.72 (d, J = 10.5 Hz)	56.82	1.16	48.13
C-6		18.71		18.73		18.25
C-7		35.05		35.15		34.58
C-8		43.44		43.25		43.21
C-9	1.28 (m)	51.28	1.28 (m)	51.36	1.28 (m)	51.32
C-10		37.8		37.82		37.6
C-11		19.86		19.85		19.88
C-12		33.45		33.3		33.29
C-13		88.31		88.36		88.4
C-14		45.92		45.36		45.37
C-15		36.87		37.03		37.03
C-16	3.71 (d, J = 8.0 Hz)	75.63	3.70 (d, J = 8.0 Hz)	78.73	3.72 (d, J = 8.0 Hz)	78.74
C-17		50.43		45.27		45.37
C-18	1.45 (m)	52.07	1.44 (m)	52.33	1.48 (m)	52.35
C-19		38.95		39.79		39.78
C-20		33.67		32.4		32.4
C-21		46.04		37.34		37.35
C-22	4.3 (m)	71.13		32.14		32.16
C-23	1.05 (s)	28.34	1.05 (s)	28.38	3.75, 4.22 (d, J = 10.0 Hz) Hz)	64.62
C-24	0.84 (s)	16.69	0.84 (s)	16.77	0.7 (s)	13.13
C-25	0.89 (s)	16.69	0.9 (s)	16.73	0.9 (s)	17.19
C-26	1.14 (s)	18.79	1.15 (s)	18.83	1.14 (s)	18.83
C-27	1.25 (s)	20.11	1.22 (s)	19.93	1.24 (s)	19.91
C-28	3.37 (d, J = 8.0 Hz), 3.66 (d, J = 8.0 Hz)	77.62	3.35 (d, J = 8.0 Hz), 3.67 (d, J = 8.0 Hz)	77.61	3.34 (d, J = 8.0 Hz), 3.70 (d, J = 8.0 Hz)	77.62
C-29	0.94 (s)	33.86	0.95 (s)	33.92	0.94 (s)	33.9
C-30	0.98 (s)	26.02	0.9 (s)	24.95	0.9 (s)	24.94
Ara						
1	4.69 (d, J = 4.0 Hz)	104.32	4.68 (d, J = 4.0 Hz)	104.34	4.68 (d, J = 4.0 Hz)	104.34
2		80.25		80.23		80.14
3		74.35		74.31		74.43
4		77.9		77.87		77.9
5		65.76		65.77		65.83
Glu (Inner)						
1	4.97 (d, J = 8.0 Hz)	104.82	4.99 (d, J = 8.0 Hz)	104.81	4.95 (d, J = 8.0 Hz)	104.67
2		85.15		85.1		85.14
3		77.57		77.56		77.58
4		71.08		71.08		71.08
5		79.32		79.4		79.67
6		62.53		62.54		62.54
Glu (Terminal)						
1	4.91 (d, J = 8.0 Hz)	105.59	4.90 (d, J = 8.0 Hz)	105.6	4.91 (d, J = 8.0 Hz)	104.75
2		75.9		76.01		75.95
3		78.12		78.03		78.12
4		71.98		71.96		71.81
5		78.07		78.03		78.07
6		63.29		63.29		63.14
Xyl						
1	4.51 (d, J = 4.0 Hz)	107.34	4.51 (d, J = 4.0 Hz)	107.3	4.58 (d, J = 4.0 Hz)	107.33
2		76.01		75.88		75.9
3		77.81		77.8		77.79
4		70.96		70.94		70.94
5		67.42		67.39		67.39

uncorrected. Optical rotations were obtained on a Perkin–Elmer 341 polarimeter (Perkin–Elmer, Inc., Waltham, MA). HRESIMS data were obtained on a Bruker Bio TOF IIIQ mass spectrometer (Bruker Biospin Gmbh, Rheistetten, Germany). ESIMS data were collected on an MDS SCIEX API 2000 LC/MS/MS instrument (Applied Biosystems, Inc., Forster, CA). The ^{1}H (400 MHz), ^{13}C (100 MHz), and 2D NMR spectra were recorded in CD_3OD on a Bruker DRX-400 instrument using TMS as an internal reference (Bruker Biospin Gmbh, Rheistetten, Germany). Preparative HPLC was conducted with a Shimazu LC-6A pump and a Shimazu RID-10A refractive index detector (Shimazu Corp., Japan) using an XTerra prep MS C-18 column (10 μm, 300 × 19 mm, Waters Corp., Milford, MA). For column chromato graphy, silica gel 60 (100–200 mesh, Qingdao Marine Chemical Ltd., Qingdao, China), Develosil ODS (75 μm, Nomura Chemical Co. Ltd., Japan) and Sephadex LH-20 (GE healthcare, Uppsala, Sweden) were used. TLC was performed on precoated plates (Kieselgel 60GF254, Merck, Darmstdt, Germany).

3.2. Plant material

The plant of *L. candida* Lindl. was collected from Dinghu mountain conservation and identified by Professor Huagu Ye, South

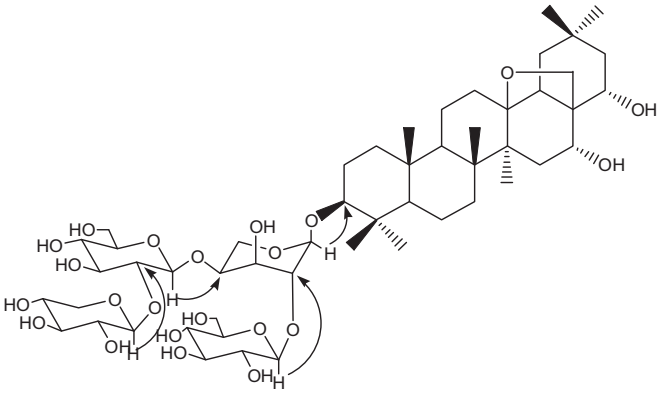

Fig. 2. The Main HMBC correlation for **1**.

China Botanical Garden, Chinese Academy of Sciences, China. A voucher specimen (No. 612966) has been deposited at the Herbarium of South China Botanical Garden, Chinese Academy of Sciences.

3.3. Extraction and isolation

The crude extraction (84 g) was suspended in H_2O and sequentially extracted with $CHCl_3$, EtOAc and *n*-BuOH. The extracts were evaporated under vacuum to yield a $CHCl_3$ fraction (0.9 g), an EtOAc fraction (0.35 g), an *n*-BuOH fraction (9.3 g), and a H_2O fraction. Each fraction was tested *in vitro* for antifungal activity against *A. flavus*. The *n*-BuOH fraction, exhibited antifungal activity against *A. flavus*, was chromatographed over silica gel column, eluted with $CHCl_3$:MeOH (7:3, 6:4, 5:5, and 4:6, each 2000 ml) to give six fractions (Fr.1: 0.69 g, Fr.2: 0.28 g, Fr.3: 0.26 g, Fr.4: 1.66 g, Fr.5: 0.36 g, and Fr.6: 0.74 g). The six fractions were tested *in vitro* for antifungal activity against *A. flavus*. Fr.5 (90 mg) that exhibited antifungal activity against *A. flavus* was separated on an ODS C-18 column, eluted with 76% MeOH and then purified by prepared HPLC using 72% MeOH to afford compound 1 (19 mg), compound 2 (46 mg) and compound 3 (25 mg).

3.4. Antifungal assay

The microorganisms used in the antifungal assay *A. flavus* (GIM3.18) were maintained at the Guangdong Institute of Microbiology. *A. flavus* were cultivated on PDA medium. Compounds (1–3) were evaluated for their activities against *A. flavus* by using filter diffusion assay.[7] The sterile paper disk of 6 mm diameter impregnated with the compound. The mycelia actively grown on PDA plates were dissolved in sterile water to be used to inoculate to the agar plate (concentration was 0.5×10^3–2.8×10^3 CFU/ml). Ketoconazole was used as positive control. The impregnated disks were placed on the medium suitably spaced apart and the plates were incubated at 28 °C for 48 h. The diameter (mm) of growth inhibition halos caused by the compound was examined.

Conflicts of interest

All authors have none to declare.

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Review article

Morin Hydrate: Botanical origin, pharmacological activity and its applications: A mini-review

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ABSTRACT

Flavonoids are one of the secondary metabolites belongs to a polyphenolic group, and are commonly found from different parts of the plant sources like fruit, vegetables, nuts, stems, seeds, flowers, tea, wine, propolis and honey. Flavonoids have been used in different ailments like anti-inflammatory, antibacterial, and antiviral activity etc. In the past decade, Morin Hydrate (3,5,7,2′,4′-pentahydroxyflavone) a poly phenol compound has been extensively studied for different pharmacological activities in various human disorders, with slight side effects. This mini-review summarises the origin, nomenclature and highlights of its pharmacological activities.

1. Introduction

Plants produce different types of low molecular weight products. Most of them are helpful in plant defence mechanisms. Among them, flavonoids are a group of secondary metabolites related to a polyphenolic group distributed widely in plant kingdom having 3000 types of flavonoids.[1] Flavonoids are having the different pharmacological activities like antihepatotoxic, anti inflammatory, anti-ulcer activity.[2] In spite of the various bioflavonoids, Morin Hydrate was one of the bioflavonoid in nature has a marked attention in nature.

Morin Hydrate (3,5,7,2′,4′ pentahydroxyflavone), was a yellow crystalline polyphenolic compound coming from branches of *Morus alba* L (white mulberry) and red Wine. It is ubiquitisoly distributed in the family of Moraceae [white mulberry (*M. alba*)] and in almond (*Prunus dulcis*, family Rosaceae), in sweet chestnut (*Castanea sativa*, family Fagaceae) and other fruits also.[3,4] From long back onwards Morin Hydrate produces different types of pharmacological benefits such as free radical scavenging activity,[5] xanthine–oxidase inhibitor property,[6] anti-inflammatory activity,[7] Protective effect of DNA from damage caused by free radical,[8] prevention of low density lipoprotein oxidation,[9] and anticancer activity.[10] Morin Hydrate was used in food and traditional herbal medicines. Further studies indicate that its health benefits in vitro and in vivo.[3]

In spite of the vast research progress made on Morin Hydrate pharmacological/biological activities, there is no such article which comprisely explains its botanical origin, pharmacological activities. So, in this present Review paper we tried to compile the few pharmacological activities of Morin Hydrate.

2. Chemistry of Morin Hydrate

Synonym: 2′, 3, 4′, 5, 7-pentahydroxyflavone.

Linear formula: $C_{15}H_{10}O_7 \cdot XH_2O$

The Morin Hydrate structure [Fig. 1] represents an isomeric form of quercetin, both having OH in position 3, a resorcinol moiety, and a carbonyl group in position 4; the only difference between them is the hydroxylation pattern on B-ring, which is meta in Morin Hydrate but Ortho in quercetin. Even though quercetin is regarded as one of the flavonoid with the highest antioxidant potential because it has all those groups, as well as an ortho hydroxylation pattern on B-ring, it has been demonstrated a higher effectiveness of Morin Hydrate facing certain oxidative processes.[11]

3. Pharmacological activities

An increasing number of studies showed that Morin Hydrate having the different pharmacological activities, including cardiovascular disease, diabetic mellitus, neurodegenerative disease, cancer and anti-inflammatory activities. As inflammation leads to the various oxidative stress related disorders, thus anti-oxidant and anti-inflammatory activities of Morin Hydrate play a critical role in

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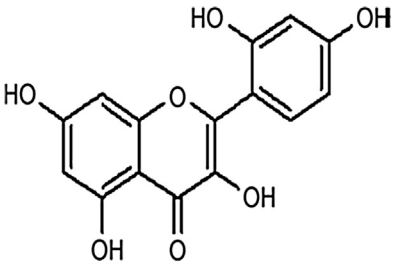

Fig. 1. Morin Hydrate.

the therapeutic processing, even though the cellular mechanisms of action need to be processed.

3.1. Anti-oxidant and anti-inflammatory effects of Morin Hydrate in cardiovascular diseases

Cardiovascular Diseases (CVD) are the chief mortality causes worldwide. Myocardial infarction (MI) is one of the CVD disorder. MI is the condition in which there is imbalance between the coronary supply and its myocardial demand and causes necrosis of myocardial tissue.[12] As Morin Hydrate is mostly ubiquitous in white mulberry and in almond shows cardiovascular benefit qualities in isoproterenol-induced myocardial infarction in rats due to its free-radical scavenging activity attributed by the polyphenolic group.[13] Morin Hydrate showed the significant beneficial effect on lipid profiles, blood pressure, serum glucose levels from the high fat diet induced hypertensive rats.[14] And also, Subash and Subramanian showed that by the administration of Morin Hydrate (30 mg/kg) by oral administration offered the protection against hyperammonemia by acting through reducing of blood pressure, oxidative stress and by increasing the antioxidant system in ammonium-chloride induced hyperammonemic rats induced by at dose of 100 mg/kg/b.w i.p.[15]

Isoproterenol [1-(3, 4-dihydroxyphenyl)-2-isopropylaminoethanol hydrochloride] was one of the synthetic catecholamine and β-adrenergic agonist, which has been documented to produce severe stress in the myocardium, resulting in MI, if administered in supramaximal doses.[16] Isoproterenol (ISO)-induced cardiac necrosis include increased oxygen consumption, increased calcium overload and accumulation, increased myocardial cAMP levels, deranged electrolyte milieu, changes in the myo-cardial cell metabolism, alterations of membrane permeability, intracellular acidosis and increase in lipid peroxides.[12] Al-Numair et al[13] showed that pre treatment of Morin Hydrate (20, 40 and 80 mg/kg, respectively) daily for a period of 30 days, decreases the activities of cardiac marker enzymes in serum and increased activities in the heart, and decreased activities of calcium-dependent adenosine triphosphatase and magnesium-dependent adenosine triphosphatase in the heart, and activity of the sodium potassium-dependent adenosine triphosphatase increased in the heart. And also, showed a significant decrease in glycoprotein levels in serum and heart. ISO produces the myocardial infarction via free radicals mediated β-adrenoreceptor mechanism[17] In particular, Morin Hydrate (40 mg/kg) significantly ($p < 0.05$) decreases the isoproterenol induced myocardial infarction in rats. The observed effects may be due to its anti-oxidant,[5] anti-inflammatory and free-radical scavenging activity[7] of Morin Hydrate. Up to now, the molecular mechanism of actions are not elucidated, further studies are required to prove the molecular mechanisms of Morin Hydrate.

3.2. Xanthine oxidase and uricosuric activity

Gout is a general systemic joint disorder that affects the 1% of western population.[18] Hyperuricemiasis was a hallmark of gout.[19] A serum uric acid level of more than 9 mg/dl was considered as gouty arthritis and incidence was 4.9%.[20] Pathologically production of gouty arthritis occurs due to over production or decreased excretion of purine metabolic end product i.e. uric acid.[21] Recently, a urate-anion transporter (URAT1) was identified in the brush-border membrane of the proximal tubule in human kidney, mainly involved in the serum uric acid level through reabsorption of urate from the lumen to the cytosol in kidney tubules.[22] Different mechanism of drugs are used in the treatment of gout. These include Xanthine oxidase inhibitors (e.g. allopurinol), inhibitors of urate reabsorption at proximal renal tubule like probenecid and benzbromarone are employed as hypouricemic agents. Moreover these agents along with hypouricemic activity posses some undesirable side effects like hepatotoxicity associated with the benzobromarone.[23] Some natural herbs were exhibited the xanthine oxidase inhibitor activity,[24,25] along with other types of mechanisms which were helpful in the treatment of gouty arthritis and hyperuricemia related disorders. Morin Hydrate, which is a natural Chinese herb having the xanthine oxidase inhibitor activity along with other type of mechanism involved to reduce the rheumatic disorders. Because, mainly it inhibits the urate reabsorption at the brush border of proximal renal tubule membrane vesicles,[6] explains this acts on the kidney to inhibit the urate reabsorption. By Lineweaver–Burk plot mechanism Morin Hydrate explained the inhibition of the urate reabsorption through competition mechanism, with a k_i value of 17.4 μM. In addition, to this Morin Hydrate also had the mixed type of xanthine oxidase inhibitor activity i.e. K_i and K_{ies} values were being 7.9 and 35.1 μM, respectively. By using oxonate-induced hyperuricemic rat model, Morin Hydrate showed the uricosuric activity4. Based on this above information Morin Hydrate has been used in the treatment of rheumatic disorders, along with further investigation also.

3.3. Anti-inflammatory activity

Free radicals, nitric oxide, leuckotrienes were the main inflammatory parameters in the intestinal inflammation. They are involved in the production of inflammatory mediators in the intestinal inflammatory conditions.[7]Morin Hydrate shown to inhibit the leuckotriene-β_4 synthesis and inhibition of nitric oxide synthase activity.[26] Moreover, it also inhibits the myeloperoxidase activity, which was evidently increased in the intestinal inflammation marker of neutrophil infiltration.[27] Another anti-inflammatory activity attributed to the Morin Hydrate, due to its inhibition of interleukine-β which is a one of the pro-inflammatory cytokine responsible for the induction of inducible nitric oxide Synthase (iNOS) activity in enterocytes.[28]

In intestine, inflammatory bowel disease (IBD) is a chronic phase of inflammatory disorder associated with two closely related conditions, namely Crohn's disease, and Ulcerative colitis. The main aetiological events occurred in the development of IBD was synthesis and up-regulation of pro-inflammatory mediators, such as reactive oxygen species, cytokines and platelet-activating factors.[29] Now-a-days, the drugs used for the management of IBD are 5-amino salicylic acid and local or systemic gluco-corticosteroids, to exert their benefit through various mechanisms.[30] Rats were impart colitis by single injection of colonic instillation of 30 mg of the hapten trinitrobenzenesulphonic acid dissolved in 0.25 mL of 50% ethanol. And the colitic rats were treated with Morin Hydrate 25 mg/kg orally for 4 weeks. Morin Hydrate showed the beneficial effect on 4th week following colitis insult, both macroscopically and microscopically.[7] The anti-inflammatory activity associated with it by decreasing the colonic myeloperoxidase, which is previously attributed its action in experimental colitis. Different biochemical mediators are involved in the colonic inflammation are

myeloperoxidase, leukotriene B4, interleukin-1β synthesis, glutathione and malondialdehyde levels and nitric oxide Synthase activity. The anti-inflammatory activity of Morin Hydrate was mainly due to its inhibition of synthesis of most important cytokine interleukine 1β and decreased in the nitric oxide Synthase, free radicals involved in the inflammatory cascade. The anti-inflammatory activity of Morin Hydrate against experimental colitis in rats, due to its ameliorative effect of such as free radicals, leukotriene B4, nitric oxide and interleukin-1.[27]

3.4. Anti-cancer activity

For decades, increasing studies focussed the potential anticancer activity of Morin Hydrate, in various kinds of cancers. For example, Morin hydrate, showed the anti-inflammatory activity in the acute phase of trinitrobenzoic acid induced colitis in rats,[31] exhibited the chemoprotective effect of chemically produced rat tongue carcinogenesis,[32] and inhibit the phorbol-ester induced transformation of rat hepatocytes.[33] Moreover, Morin Hydrate inhibits the peroxisome-proliferated activator receptor induced keratinocyte differentiation by inhibiting the lipoxygenase pathway.[34] It also showed the inhibitory activity in release of inflammatory cytokines such as IL-8, interleukin (IL)-6, and tumour necrosis factor (TNF) from mast cells.[35]

Morin Hydrate has shown its anticancer activity in cancer models like inhibit the growth of COLO205 cells in nude mice.[36] The role of transcription factor nuclear transcription-ǩB (NF-ǩB) is involved in various kinds of cell proliferation, cell survival, tumorigenesis, and inflammation. By using the DNA binding assay study that induction of NF-ǩB activation pathway induced by tumour necrosis factor (TNF), phorbol 12-myristate 13-acetate, ceramide, lipopolysaccharide, interleukin-1, and H_2 O_2 was suppressed by Morin Hydrate, through inhibition of lǩB (inhibitory subunit of NF-ǩB) kinase, leads to suppression of phosphorylation and degradation of lǩBa and consequent p65 nuclear translocation. Morin Hydrate also inhibited the NF-ǩB dependent reporter gene expression activated by TNF receptor, TNF, TNFR-associated factor 2, TNFR1-associated death domain, NFǩB inducing kinase, IǩB kinase, and the p65 subunit of NF-ǩB. Morin Hydrate also inhibits the NF-ǩB related products that are involved in the cell survival i.e. inhibitor of apoptosis protein 1 & 2, survivin, X-chromosome linked IAP, and BcL-xL, invasion (matrixmetalloproteinase-9) and proliferation (cyclin D1and cyclooxygenase-2) were down-regulated by Morin Hydrate.[10]

3.5. In nephrotoxicty

During the metabolic process we require the various metals for the enzymatic and non-enzymatic process in organic and inorganic form. But, the requirement of metals is in a limited range. However, various heavy metals are available in earth, mercury is a one among them. Mercury is a wide-spread environmental pollutant causes severe alterations in humans and animals.[37] Human beings are mostly exposed to heavy metals through the diet and inhalation.[38] Morin Hydrate at 200 mg/kg, p.o. for 10days simultaneously, showed the protection from mercuric chloride induced nephrotoxicity at dose of 5 mg/kg, i.p. for 5 days. Morin Hydrate significantly ($p < 0.01$).[20] Moreover, it showed the protection from serum markers like LDH, AST, ACP levels in significantly ($p < 0.05$),which are increased in renal nephritis and renal infarction.[39]

4. Further studies

From the different studies reviewed in this article, Morin Hydrate has been useful in the management of different human disorders. Despite the in vivo and in vitro studies trying to elucidate the mechanisms of Morin Hydrate, and more studies are required to elucidate molecular mechanism of it. Moreover, clinical experimentations are required urgently to provide a basis for prospective usefulness of Morin Hydrate in the treatment and mitigation of human diseases.

Conflicts of interest

The author has none to declare.

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Short communication

Cytotoxic effect of *Anthocephalus cadamba* Miq. leaves on human cancer cell lines

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ABSTRACT

Introduction: *Anthocephalus cadamba* Miq. (Rubiaceae) is used as folk medicine in the treatment of fever, anemia, leprosy, dysentery, blood and skin diseases.
Methods: Powdered leaves of *A. cadamba* were extracted sequentially with hexane, chloroform, ethanol, ethanol:water (50:50) and water by maceration, and evaluated for cytotoxic potential using SRB assay against four human cancer cell lines lung (A-549), ovary (IGR-OV-1), prostate (PC-3) and CNS (SF-295).
Results: Chloroform extract exerts potent cytotoxic effect against human lung (A-549), ovary (IGR-OV-1), prostate (PC-3) and CNS (SF-295) cancer cell lines IC_{50} of 8, 57, 49 and 39 μg/ml respectively. Ethanolic extract was found to be active only against one cell line CNS (SF-295).
Conclusion: The present study demonstrates the cytotoxic potential of *A. cadamba* leaves extract (chloroform) against different human cancer cell lines.

1. Introduction

Anthocephalus cadamba Miq. (Rubiaceae) commonly known as kadam, is a large deciduous tree found all over the India on the slopes of evergreen forests up to 500 m. It has been used in Indian traditional medicine for treatment of different ailments and conditions such as fever, anemia, uterine complaints, snake bite, menorrhigia, blood and skin diseases, diarrhea, colitis, dysentery and in improvement of semen quality.[1,2] The root extract was found beneficial in different urinary problems such as dysuria, calculi and glycosuria[3] and also reported to possess antimicrobial, anthelmintic, hypolipidemic and antioxidant activities.[4,5] *A. cadamba* was screened for hepatoprotective activity against carbon tetrachloride induced liver injury.[6] The stem bark has been reported to have anti-inflammatory, antipyretic, diuretic and laxative activities.[7] To the best of our knowledge, no previous cytotoxic study of this plant has been reported. In this communication, we report the in vitro cytotoxic potential of *A. cadamba* leaves extracts on four different human cancer cell lines A-549 (lung), IGR-OV-1 (ovary), PC-3 (Prostate) and SF-295 (CNS).

2. Material and methods

2.1. Plant material

The leaves were collected from botanical garden of Guru Nanak Dev University, Amritsar and authenticated by Dr. Adarsh Pal Vig, Department of Botanical and Environmental Sciences, GNDU. A specimen has been deposited at the herbarium of same department (voucher no, 0480).

2.2. Preparation of leaf extract

The air-dried powdered leaves of *A. cadamba* (550 g) were extracted by maceration using hexane, chloroform, ethanol, ethanol:water (50:50) and water. The crude extracts were obtained by evaporating the solvent using vacuum rotary evaporator (Fig. 1).

2.3. Cell lines

The human cancer cell lines procured from National Cancer Institute, Frederick, U. S. A. were used in present study. Cells were grown in tissue culture flasks in complete growth medium (RPMI-1640 medium with 2 mM glutamine, pH 7.4 supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 units/ml penicillin) in a carbon dioxide incubator (37 °C, 5% CO_2, 90% RH). The cells at subconfluent stage were harvested from the flask by

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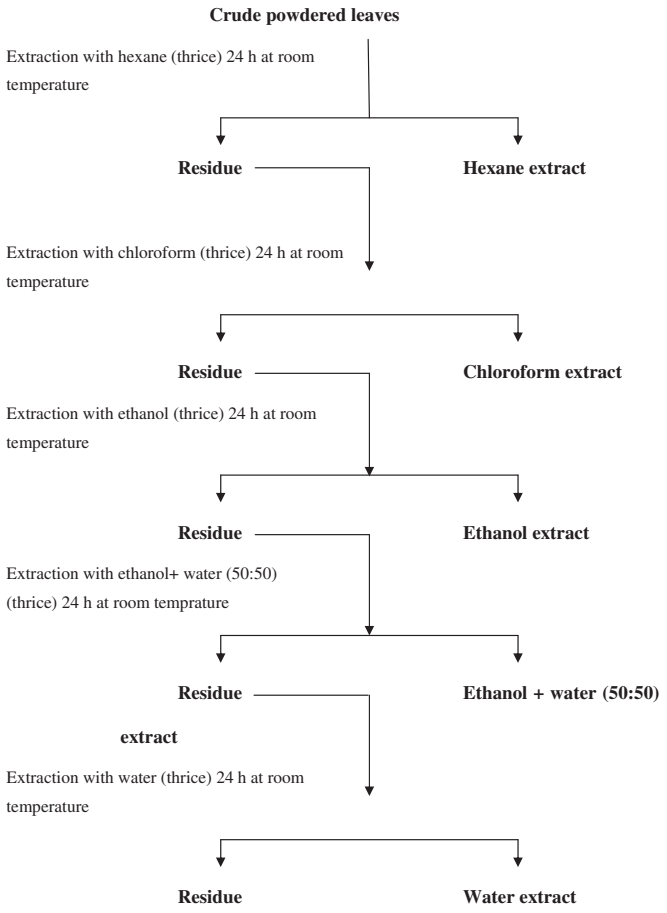

Fig. 1. Extraction of *Anthocephalus cadamba* leaves in increasing order of solvent polarity.

treatment with trypsin (0.05% in PBS (pH 7.4) containing 0.02% EDTA) and single cell suspension made in above mentioned medium.

2.4. Cytotoxicity assay

In vitro cytotoxicity against various human cancer cell lines was determined using 96-well cell culture plates. The 100 µl of cell suspension was added to each well of the 96-well cell culture plates. The cells were allowed to grow in CO_2 incubator (37 °C, 5% CO_2, 90% RH) for 24 h. Different concentrations of crude extract in complete growth medium (100 µl) were added after 24 h incubation to the wells containing cell suspension. The plates were further incubated for 48 h (37 °C in an atmosphere of 5% CO_2 and 90% relative humidity) in a carbon dioxide incubator and then the cell growth was stopped by gently layering trichloroacetic acid (50% TCA, 50 µl) on top of the medium in all the wells. The plates were incubated at 4 °C for 1 h to fix the cells attached to the bottom of the wells. The liquid of all the wells was gently pipetted out and discarded. The plates were washed five times with distilled water to remove TCA, growth medium low molecular weight metabolites, serum proteins etc. and air-dried. Cell growth was measured by staining with sulforhodamine B dye. The adsorbed dye was dissolved in Tris–HCl Buffer (100 µl/well, 0.01 M, pH 10.4) and plates were gently stirred for 10 min on a mechanical stirrer. The optical density (OD) was recorded on ELISA reader at 540 nm.[8]

3. Results

The different extracts (ACH, ACC, ACE, ACAA and ACA) of *A. cadamba* leaves were evaluated for their cytotoxic potential against four human cancer cell lines viz. lung (A-549), ovary (IGR-OV-1), Prostate (PC-3) and CNS (SF-295) using the SRB assay. The results of cytotoxic studies of the different extracts against human cancer cell lines were presented in Table 1. Paclitaxel has been used as standard in case of lung (A-549), ovary (IGR-OV-1), Mitomycin-C in case of Prostate (PC-3) while Adriamycin in case of CNS (SF-295). Among the different extracts of *A. cadamba* leaves, only the chloroform extract was able to be inhibit the growth of different cancer cells as revealed by the value of IC_{50} (Table 1) and especially against Lung (A-549) cancer cell line 8 µg/ml which is comparable to standard drug Paclitaxel (IC_{50} 4.1 µg/ml) used. The ethanolic extract bears moderate cytotoxicity only against the CNS (SF-295) cancer cell line, the other tested cell line were found resistant. The hexane (ACH), alc: aq (50:50) (ACAA) and aqueous (ACA) extracts were not found to be active against human cancer cell lines (Table 1).

4. Discussion

It is well established that plants have been a useful source of clinically important antitumor compounds and still there have been worldwide efforts to discover new anticancer agents from plants. The examples of anticancer agents developed from plants includes antileukemic alkaloids vinblastine and vincristine, isolated from the Madagascar periwinkle (*Vinca rosea*), taxane derivative paclitaxel (isolated from *Taxus brevifolia*), later known as taxol and the alkaloid camptothecin (from the Chinese tree *Camptotheca acuminata*).[9] Of all available anticancer drugs between 1940 and 2002, 40% were natural products or natural product-derived with another 8% natural product mimics.[10]

A. cadamba is a member of cinchonoideae, a subfamily of Rubiaceae, the largest flowering plant family which comprises more than 13,000 species. Some Rubiaceae plants are found to have antitumor activity, *Morinda citrifolia* fruit juice has been reported to possess antitumor activity in the Lewis lung peritoneal carcinoma model.[11] The dichloromethane fraction of *Gardenia jasminoides* was found to be capable of inducing apoptotic cell death by DNA topoisomerase 1 inhibition in KB cells.[12] The role of indole based alkaloids vinblastin and vinblastin as antineoplastic drugs has been well established. The phytochemical studies of leaves and stem bark of A. Cadamba have revealed the presence of cadamine, isocadamine,[13] cadambine, 3α-dihydrocadambine,[14] 3 β-isodihydrocadambine, 3 β-dihydrocadambine,[15] cadambagenic acid,[16] triterpenoids saponins and glycoside[17,18]. The widespread use of *A. cadamba* in traditional medicine and presence of indole based phytoconstituents

Table 1
Cytotoxic activity (IC_{50} µg/ml) of different extracts of *A. cadamba* leaves.

Extract[a]/standard	Cell line type			
	Lung	Ovary	Prostate	CNS
	A-549	IGR-OV-1	PC-3	SF-295
(ACH)	>100	>100	>100	>100
(ACC)	8	57	49	38
(ACE)	>100	>100	>100	67
(ACAA)	90	>100	>100	>100
(ACA)	>100	>100	>100	>100
Paclitaxel	4.1	4.5	–	–
Mitomycin-C	–	–	1.5	–
Adriamycin	–	–	–	0.05

[a] ACH (hexane), ACC (chloroform), ACE (alcoholic), ACAA (alc:aq (50:50)) and ACA (aqueous).

encouraged us to explore the cytotoxic potential. The compounds which have been previously isolated from the *A. cadamba* contains indole nucleus in the present study, cytotoxic action against cancer cells may be attributed to these active ingredients of *A. cadamba* extract, which can induce anti-proliferation pathway leading to cancer cell death. The result of the present study is the first report of cytotoxic activity of *A. cadamba* and for the cytotoxic activity, each cell line responded differently to the treatment with the tested extracts. Chloroform extract exerts potent cytotoxic effect against all tested human cancer cell lines particularly Lung (A-549) and CNS (SF-295). Further analysis and purification of the active principles of *A. cadamba* appears worthwhile in order to clarify the chemical nature and mode of action of the bioactive components responsible for cytotoxic potential.

Conflicts of interest

All authors have none to declare.

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Short communication

In vitro biological standardization, formulation and evaluation of directly compressed polyherbal anthelmintic tablets

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ABSTRACT

Introduction: Herbal medicines are free from side effects, adverse effects and they are economical and easily available will be beneficial for the mankind over the centuries. Helminth infections are the common infections affecting the large portion of the world's population and can be easily treated by using medicinal plants.
Methods: The present study was aimed to formulate the polyherbal anthelmintic tablets having composition of *Uraria lagopoides*, *Punica granatum*, and *Saraca asoca* by direct compression method using Super Tab 11SD, Primojel, talc and magnesium stearate as excipients and subjected to evaluation of pre compression and post compression parameters. Finally the unit dosage form was evaluated for its anthelmintic activity using Indian adult earthworm *Pheretima posthuma*.
Results and conclusion: Time taken for paralysis, death of the worms was recorded. It was found to be statistically significant.

1. Introduction

Herbal medicines are major sources of health care for the mankind over the centuries.[1] WHO report 80% of the world population relies on the drugs which are from natural origin.[2–4] Helminthic infections are now identified as cause of acute as well as chronic illness among the human beings as well as cattle's. More than half of the world's population suffers from infection of one or the other and majority of cattle's suffers from worm infections.[5] Many reports claims the efficacy of several natural plants in eliminating worms,[6] keeping this in mind the present work was designed to formulate and evaluate the anthelmintic activity of polyherbal formulation.

2. Material and methods

2.1. Materials

The whole plants of *Uraria lagopoides*, *Punica granatum* (including fruits), and *Saraca asoca* (including flowers and seeds) were collected from botanical gardens of Sri Venkateswara University, Tirupathi and authenticated by Dr. Madha Chetty, taxonomist and HOD of Botany, SVU, Tirupathi. Super Tab 11SD (Spray dried lactose), Primojel (Sodium starch glycolate), talc and magnesium stearate, carboxy methyl cellulose (CMC) of Pharmacopeial grade were gift samples from DFE Pharma, Banglore, India. Albendazole pure drug was provided by Bell Bulk drug Industry, Hyderabad, A.P., India. All other solvents and chemicals used were of analytical grade. Adult earthworm of the genus and species, *Pheretima posthuma*

2.2. Preparation of extract

The whole plant in case of *U. lagopoides*, individual whole plant parts in case *of P. granatum* including matured fruits and *S. asoca* including flowers and seeds after collection were shade dried, powdered (40 mesh size) to get a coarse powder and subjected to Soxhlet extraction using methanol as a solvent. The extract was filtered and concentrated at reduced temperature on a rotary evaporator. The percentage (%) yield was found to be 32.15, 47.12 and 42.31 respectively and then subjected to preliminary phytochemical,[7–11] organoleptic, physiochemical, heavy metal, microbiological and pathogen analysis[12] shown in Table 1.

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Table 1
Preliminary phytochemical, organoleptic, physiochemical, heavy metal, microbiological and pathogen analysis.

S. no.	Parameter	*Uraria lagopoides*	*Punica granatum*	*Saraca asoca*
1.	**Phytochemical analysis**			
	Carbohydrates	+	+	+
	Proteins & amino acids	+	+	−
	Steroids	+	+	+
	Glycosides	+	+	+
	Flavonoids	+	+	+
	Alkaloids	−	+	+
	Tannins	+	+	+
	Saponins	−	+	+
2.	**Organoleptic characters**			
	Colour	Greenish-yellow	Dark brown	Reddish brown
	Odour	Characteristic	Characteristic	Characteristic
	Taste	bitter	Characteristic	Characteristic
	Physical appearance	Free flowing powder	Free flowing powder	Free flowing powder
3.	**Physiochemical characters**			
	WSE	52.12%	87.61%	77.38%
	ASE 50	76.28%	81.35%	81.27%
	PH 1% w/v solution	4.22	4.24	5.72
	LOD	5.0%	5.17%	4.10%
	Ash content	8.12%	6.97%	4.02%
	Acid insoluble ash	1.72%	1.25%	0.68%
	Moisture content by K.F	52.12%	2.68%	3.21%
4.	**Heavy metal analysis**			
	Lead	5 PPM	7 PPM	8 PPM
	Arsenic	1 PPM	1 PPM	1 PPM
	Cadmium	0.2 PPM	0.2 PPM	0.2 PPM
	Mercury	1 PPM	1 PPM	1 PPM
5.	**Microbiological analysis**			
	Total aerobic count	260 CFU/g	420 CFU/g	250 CFU/g
	Yeast & mould	30 CFU/g	40 CFU/g	20 CFU/g
6.	**Pathogen analysis**			
	E. coli	−	−	−
	Salmonella	−	−	−
	Pseudomonas aeruginosa	−	−	−
	Staphylococcus aureus	−	−	−

'+'Present, '−' absent.

2.3. Anthelmintic investigation

P. posthuma, Indian adult earth worms were used to screen the anthelmintic activity. Earthworms were obtained from moist soil, and washed out in to normal saline water to remove all the fecal matter and waste surrounding their body, having 3–5 cm in length, 0.1–0.2 cm in width, weighing 0.8–3.04 g are selected. The earthworms resembled the intestinal roundworm parasites of human beings both anatomically and physiologically.[13,14]

Indian adult earth worms were used for the in vitro anthelmintic bio assay[15] of individual plant methanolic extract and polyherbal extract. The worms were divided into the respective groups containing six-earth worms in each group. All the prototypes were suspended in minimum quantity of 0.5% v/v CMC and the volume was adjusted to 10 ml with normal saline for making the concentration of 25, 50, 100 mg/ml. All the prototypes and the standard drug solution were freshly prepared before commencement of the screening. All the earthworms were washed in normal saline solution before they were released into 10 ml of respective formulation as follows, vehicle (0.5% v/v CMC in normal saline), and Albendazole (25 mg/ml) and prototypes (25, 50 and 100 mg/ml) the anthelmintic activity was determined.

Paralysis was said to occur when the worms do not revive even in normal saline. Death was concluded when the worms lost their motility followed with fading away of their body colour. Six worms of about the same size per petridish were used. They were observed for their spontaneous motility and evoked responses. Observations were made for the time taken to paralysis and death of individual worms.

Statistical evaluation of the data by Analysis of variance (ANOVA) test was performed and the results were expressed as mean ± SEM using graph pad prism V 5.0 ($n = 6$).all the results were shown in Table 2 and Figs. 1–7.

Table 2
Effect of Albendazole and methanolic extract (mg/ml) of *3* Indian medicinal plants (*Saraca asoca, Uraria lagopoides, Punica granatum*) on Indian earth worm *Pheretima posthuma*.

Group	Treatment	Concentration	Time taken by earthworm for	
			Paralysis (min.)	Death (min.)
I	Control	–	–	–
II	Albendazole	25 mg/ml	$15.83 \pm 0.48^{***}$	$45.94 \pm 0.04^{***}$
III	SAME[a]	25 mg/ml	$50.80 \pm 2.23^{*}$	$60.25 \pm 2.12^{*}$
		50 mg/ml	$38.58 \pm 2.14^{*}$	$48.25 \pm 2.11^{*}$
		100 mg/ml	$13.26 \pm 2.22^{**}$	$37.56 \pm 2.25^{**}$
IV	ULME[b]	25 mg/ml	$55.35 \pm 3.23^{*}$	$72.28 \pm 2.82^{*}$
		50 mg/ml	$40.85 \pm 1.16^{**}$	$61.10 \pm 1.21^{**}$
		100 mg/ml	$14.56 \pm 1.32^{**}$	$40.56 \pm 1.46^{**}$
V	PGME[c]	25 mg/ml	$40.35 \pm 2.23^{*}$	$62.28 \pm 2.32^{*}$
		50 mg/ml	$28.74 \pm 2.13^{**}$	$51.10 \pm 1.71^{**}$
		100 mg/ml	$13.86 \pm 1.11^{*}$	$42.15 \pm 1.21^{*}$
VI	PHME[d]	25 mg/ml	$45.61 \pm 6.13^{**}$	$62.78 \pm 2.52^{**}$
		50 mg/ml	$32.11 \pm 0.12^{***}$	$47.25 \pm 0.12^{***}$
		100 mg/ml	$10.04 \pm 0.87^{***}$	$30.29 \pm 2.56^{***}$
VII	PHT[e]	25 mg/ml	$47.61 \pm 4.11^{*}$	$59.78 \pm 2.51^{*}$
		50 mg/ml	$34.13 \pm 0.22^{***}$	$49.81 \pm 0.11^{**}$
		100 mg/ml	$11.14 \pm 1.17^{**}$	$31.21 \pm 1.04^{**}$

Values are expressed in mean ± SEM, $n = 6$, $^{*}P < 0.001$, $^{**}P < 0.01$, $^{***}P < 0.05$.
[a] SAME: *Saraca asoca* methanolic extract.
[b] ULME: *Uraria lagopoides* methanolic extract.
[c] PGME: *Punica granatum* methanolic extract.
[d] PHME: *Polyherbal methanolic* extract.
[e] PHT: Polyherbal tablet.

2.4. Formulation of polyherbal anthelmintic tablets

For formulation of polyherbal tablets, direct compression method[16] is selected because, direct compression (DC) is by far the simplest means of production of a pharmaceutical tablet and high dose formulations.[17] It requires only that the active ingredient is properly blended with appropriate excipients before compression. Apart from simplicity of formulation and manufacture, the key advantages of direct compression include reduced capital, labour and energy costs for manufacture and the avoidance of water for granulation for water sensitive drug substances.[18] Three key factors for successful tableting are flow and compactability of the

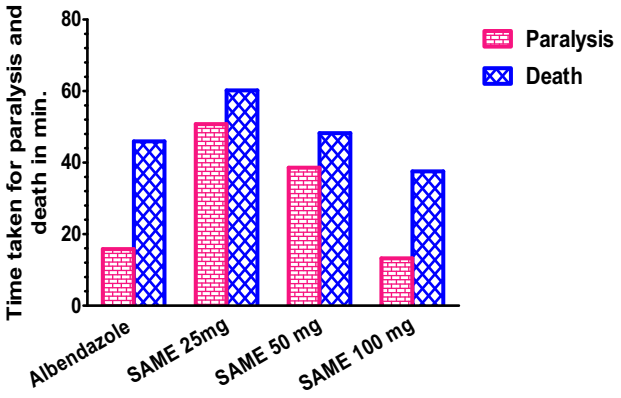

Fig. 1. Effect of Albendazole and methanolic extract (mg/ml) of *Saraca asoca* on Indian earth worm *Pheretima posthuma*.

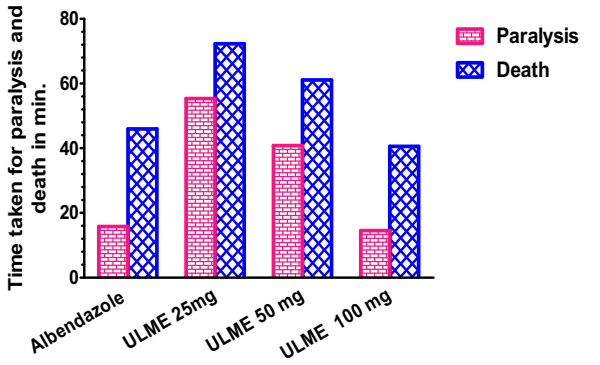

Fig. 2. Effect of Albendazole and methanolic extract (mg/ml) of *Uraria lagopoides* on Indian earth worm *Pheretima posthuma*.

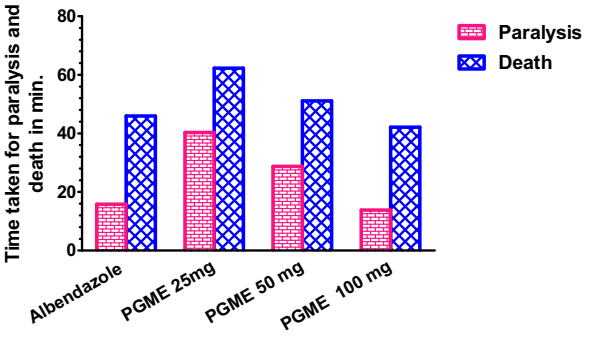

Fig. 3. Effect of Albendazole and methanolic extract (mg/ml) of *Punica granatum* on Indian earth worm *Pheretima posthuma*.

compression mix, and drug content uniformity in the mix and the final tablets.[19]

The biologically potent methanol extract was used for developing of herbal tablet formulation. The plant extracts were mixed with super tab 11 SD, Primojel, magnesium stearate and talc as excipients and compressed into tablets. Table 3 and Fig. 8.

2.5. Evaluation of powder blend for pre compression parameters

The granules obtained for the trial batches PHF-I, II & III were satisfactory. The pre compression parameters are like physical properties i.e., bulk density, true density Hausner's ratio, Carr's compressibility and angle of repose were calculated. All the parameters are within the limits and shown good flow properties[20–23] and the data was shown in Table 4.

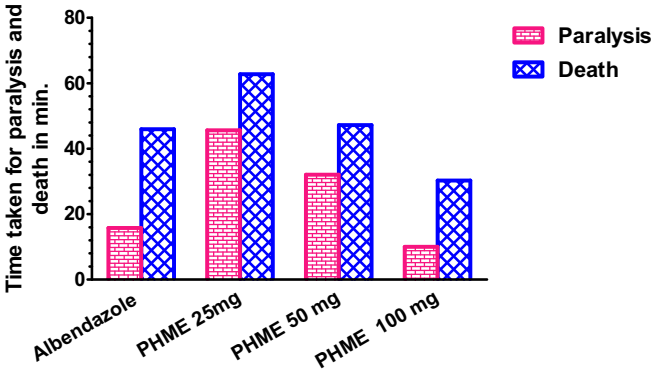

Fig. 4. Effect of Albendazole and polyherbal methanol ic extract (mg/ml) on Indian earth worm *Pheretima posthuma*.

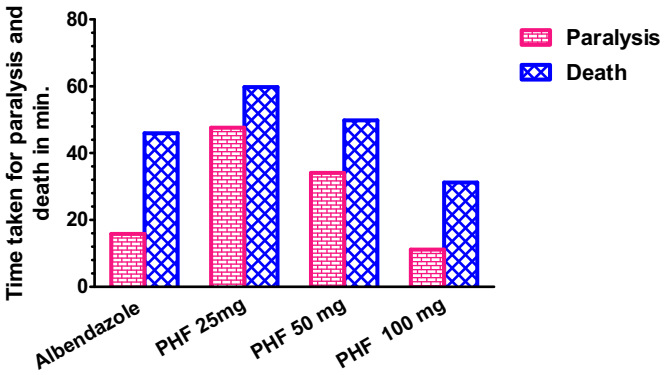

Fig. 5. Effect of Albendazole and polyherbal formulation (PHF-III) on Indian earth worm *Pheretima posthuma*.

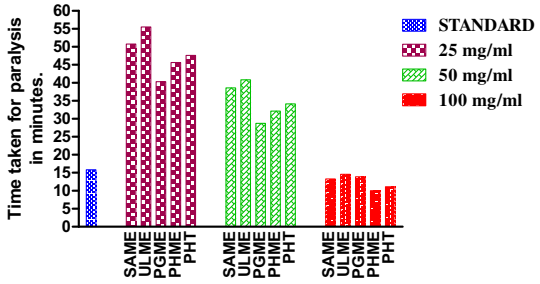

Fig. 6. Effect of methanolic extract (mg/ml) of 3 Indian medicinal plants (*Saraca asoca, Uraria lagopoides, Punica granatum*) on Indian earth worm *Pheretima posthuma* – time taken for paralysis (min.).

2.6. Evaluation of polyherbal tablets for post compression parameters

The polyherbal tablets were prepared using the methanolic extracts of *U. lagopoides, P. granatum* and *S. asoca* are subjected to evaluation of different post compression parameters like hardness, friability, uniformity of weight, uniformity of content, thickness, disintegration, stability and in vitro dissolution studies. The data within the range of Pharmacopeial specifications was shown in Table 5.

2.7. In vitro drug release

The in vitro drug release from the developed polyherbal anthelmintic was carried out by using a USP type-II dissolution test

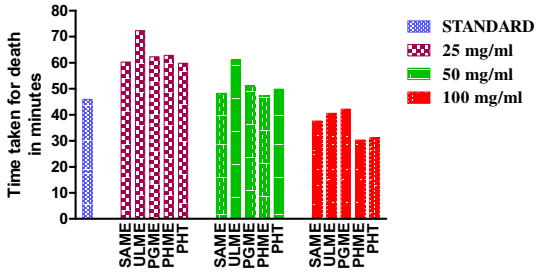

Fig. 7. Effect of methanolic extract (mg/ml) of 3 Indian medicinal plants (*Saraca asoca, Uraria lagopoides, Punica granatum*) on Indian earth worm Pheretima posthuma – time taken for death (min.).

Table 3
Formulation of tablets.

S. no.	Ingredients (mg)	PHF-I	PHF-II	PHF-III
1.	PHE (API)	300	300	300
2.	Super tab 11 SD (diluent-binder)	164	154	144
3.	Primojel (super disintegrant)	30	40	50
4.	Magnesium stearate (lubricant)	3	3	3
5.	Talc (glidant)	3	3	3
Total weight		**500**	**500**	**500**

Fig. 8. Developed polyherbal tablets by direct compression method.

apparatus with 0.1 N HCl (pH 1.2) as a medium. The tablet containing 300 mg of API were placed in a rotating basket (50 rpm) filled with 900 ml of the dissolution medium, maintained at 37 ± 0.5 °C. At scheduled time intervals, the samples (5 ml) were withdrawn by using syringes and replaced immediately with fresh dissolution medium. The samples were assayed spectrophotometrically at 309 nm for the dissolved drug, where samples were automatically filtered before measuring the absorbance against 0.1 N HCl as blank. The drug release was calculated from graph drawn in between time vs % cumulative drug release and shown in Table 6 and Fig. 9.

2.8. UV-Vis spectrophotometric method development for estimation of drug content in developed polyherbal anthelmintic tablet

In order to find out the wavelength (λ_{max}) of maximum absorption of the extract, different concentrations of the extract (10 μg/ml, 20 μg/ml and upto 200 μg/ml) in 0.1 N HCl were scanned using spectrophotometer within the wavelength range of 200–400 nm against 0.1 N HCl as blank and the wavelength corresponding to maximum absorbance was noted.

Table 4
Pre compression parameters for polyherbal granules.

S. no.	Parameter	PHF-I	PHF-III	PHF-II
1.	Bulk density (g/cc)	0.49	0.51	0.52
2.	True density (g/cc)	2.36	2. 41	2.41
3.	Hausner's ratio	1.16	1.19	1.21
4.	Carr's compressibility (%)	19.62	20.52	14.31
5.	Angle of repose (Ø)	32.51	28.22	25.6

Table 5
Post compression parameters for polyherbal tablets.

S. no.	Parameter	PHF-I	PHF-II	PHF-III
1.	Hardness (kg/cm^2)	5.6 ± 0.21	5.4 ± 0.31	5.1 ± 0.12
2.	Friability (%)	0.87 ± 0.51	0.82 ± 0.1	0.75 ± 0.43
3.	Uniformity of weight (mg)	497 ± 0.97	501 ± 0.18	499 ± 0.54
4.	Uniformity of content	302 ± 0.34	303 ± 0.65	299 ± 0.26
5.	Thickness (mm)	4.22 ± 0.4	4.20 ± 0.14	4.12 ± 0.21
6.	Disintegration time (min.)	13.41 ± 0.11	12.35 ± 0.21	9.24 ± 0.41

All values are expressed as mean ± SD, $n = 6$.

Table 6
In vitro dissolution profile (cumulative drug release) of polyherbal anthelmintic tablet.

S. no.	Time (min.)	PHF-I	PHF-II	PHF-III
1.	0	00 ± 00	00 ± 00	00 ± 00
2.	10	8.68 ± 6.34	12.12 ± 2.45	16.32 ± 1.21
3.	15	17.45 ± 2.45	19.08 ± 0.34	25.25 ± 1.42
4.	20	24.67 ± 1.76	26.56 ± 2.46	37.76 ± 4.56
5.	25	29.13 ± 3.67	32.12 ± 4.66	49.89 ± 2.12
6.	30	32.57 ± 3.64	41.23 ± 1.87	57.12 ± 5.34
7.	35	40.46 ± 2.56	49.21 ± 1.45	62.34 ± 4.56
8.	40	48.36 ± 3.89	57.13 ± 0.34	69.33 ± 1.23
9.	45	54.47 ± 1.46	64.11 ± 1.45	73.23 ± 2.89
10.	50	59.46 ± 1.56	72.24 ± 1.21	81.54 ± 1.97
11.	55	67.45 ± 2.21	81.88 ± 0.12	90.32 ± 7.56
12.	60	76.12 ± 0.13	88.23 ± 1.12	99.35 ± 2.32

2.8.1. Preparation of standard stock solution

Accurately weighed 100 mg of extract was dissolved in 5 ml of methanol in 100 ml volumetric flask and volume was made up to the mark with 0.1 N HCl to give a clear solution of 1000 μg/ml concentration.

2.8.2. Preparation of working standard solutions and construction of calibration curve

A series of different concentrations of polyherbal methanolic extract were prepared from working stock solution. From 0.1 ml and upto 2.0 ml solutions were pipetted out from the working stock solution and were transferred in to 10 ml volumetric flasks. 10,20,30,40 up to 200 μg/ml solutions were obtained respectively on making up the solution to 10 ml with 0.1 N HCl. The absorbance of all these solutions were measured against a blank at respective λ_{max} using a UV double beam spectrophotometer (UV/VIS-1800, Shimadzu, Japan). A standard plot of absorbance v/s concentration of extract gives the standard calibration curve of the extract. This curve was used to determine in vitro drug release and drug content of polyherbal tablets.

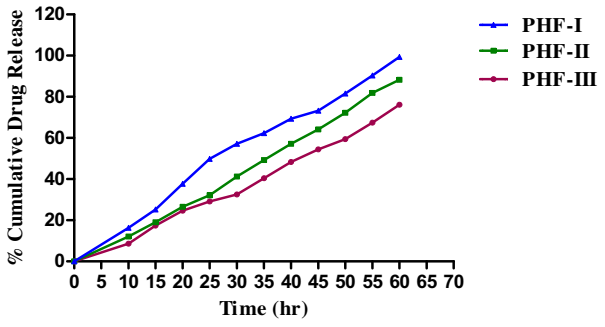

Fig. 9. In vitro dissolution profile of developed poly herbal formulations (PHF-I, II & III).

Table 7
Stability data of the optimized formulation PHF-III.

Time	Description, average weight and % drug content at different storage conditions		
	Zone-II, 25 °C/60% RH	Zone-III, 30 °C/65% RH	Zone-IV, 40 °C/75% RH
I Month	Dark brown colour, characteristic odour, 500 mg and 99.35%	Dark brown colour, characteristic odour, 500 mg and 99.31%	Dark brown colour, characteristic odour, 500 and 98.94%
II Month	Dark brown colour, characteristic odour, 500 mg and 99.30%	Dark brown colour, characteristic odour, 501 mg and 98.52%	Dark brown colour, characteristic odour, 501 mg and 98.12%
III Month	Dark brown colour, characteristic odour, 501 mg and 99.31%	Dark brown colour, characteristic odour, 501 mg and 97.62%	Dark brown colour, characteristic odour, 501 mg and 96.21%

3. Results and discussion

The methanolic extract of *U. lagopoides*, *P. granatum*, *S. asoca* and polyherbal extract at the doses of 25, 50 mg/ml were not able to produce significant activity, whereas at the dose of 100 mg/ml ($P < 0.05$) produce significant activity comparable that of standard drug Albendazole. The earth worms are more sensitive at 100 mg/ml concentration shows paralysis ranging from loss of motility to loss of response to external stimuli, which eventually progressed to death. The results were compared with the standard drug Albendazole and it was found that instead of the individual methanolic extract, polyherbal methanolic extract containing equal portions of above said plants at 100 mg/ml concentration was more effective than the selected standard drug. The Mean ± SEM values were calculated for each extract and polyherbal extract and shown in Table 2 and Figs. 1–7. Preliminary Phytochemical analysis show the presence of flavonoids, terpenoids, glycosides, alkaloids and tannins among other chemical constituents contained within them. Tannins were shown to produce anthelmintic activities and chemically polyphenolic in nature.[24] It is possible that tannins bind to free proteins in the gastrointestinal tract of host animal or glycoprotein on the cuticle of the parasite and may cause death. In addition, tannins or their metabolites have a direct effect on the viability of the pre-parasitic stages of helminthes and other phytochemicals may responsible for have an anthelmintic effect include terpenoids, flavonoids and alkaloids.[25]

The various composition of developed polyherbal anthelmintic tablet formulations (PHF-I, II & III) are shown in Table 3. The pre compression parameters were determined for developed polyherbal methanolic extract. The results (Table 4) of bulk density, true density, angle of repose, Carr's Index and Hausner's ratio indicated that the polyherbal powder mixture possess good flow properties and good packing ability. After a formulation by a direct compression method using Remek 10 station automated punching machine, developed polyherbal tablets were subjected to measuring of post compression parameters like uniformity of weight, uniformity of content, hardness, friability, thickness, and disintegration time of the tablets are given in tables. All the parameters of the test products are complied with the Pharmacopeial requirements. The polyherbal tablets were also tested for their stability[26] and the results (Table 7) are reproducible, even on tablets that had been stored for 3 months at different storage conditions like 25 °C/60% RH, 30 °C/65% RH, and 40 °C,/75% RH. The absorption curve of polyherbal methanolic extract showed characteristic absorption maximum at 309 nm in 0.1 N HCl. The drug obeyed Beer's law in the concentration range of 10 μg/ml to 200 μg/ml, and it was found to be linear with $R^2 = 0.999$, regression equation $Y = 0.014x + 0.004$. In vitro dissolution studies were conducted on polyherbal tablets of each of the formulations such as PHF-I, PHF-II, and PHF-III. The mean cumulative percent of drug released at different time intervals for each formulation is shown in Table 6 and Fig. 9. It was found that the release rate of drug increases from 76.12 ± 0.13 (PHF-I) to 99.21 ± 0.11 (PHF-III) as the concentration of Primojel was increased from 30 mg to 50 mg in 60 min. These results indicate the optimized formulation is PHF-III. The formulation also shows significant anthelmintic activity.

Conflicts of interest

All authors have none to declare.

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Letter to the Editor

In vitro antimicrobial activity of *Muntingia calabura* fruit extracts against food borne pathogens

Food products are prone to spoilage and are excellent sources for many pathogens to colonize in a new host. Foodborne outbreaks from microbial contamination, chemicals and toxins are common in many countries.[1] Trading of contaminated food between countries increases the potential for outbreaks and health risks.[2] The burden of foodborne disease remains substantial and safety of food is an important health, social and economical issue which has become the global topic of increasing research efforts. An increasing number of customers prefer foods with mild processing and without chemical preservatives. Food borne illnesses caused by microbial contamination is due to the growth of pathogenic bacteria and their toxins in food. Concerns also arise from emergence or recognition of the importance of certain microbial food pathogens or spoilage organisms.[3] Natural products which inhibit the growth of pathogenic bacteria in food have been developed and used since ancient times.

Muntingia calabura (Elaeocarpaceae) is native to American continent and is widely grown in warm regions of Asia. The plant has been reported to possess antinociceptive, antiproliferative, antioxidant and antipyretic effects.[4–7] Various parts (bark, roots and leaves) contain flavonones, flavones, flavans and biflavans which exhibited cytotoxic effects.[8–10] Recently, gastroprotective activity of the leaf extracts was also reported.[11] Antimicrobial activity of *M. calabura* was documented by previous studies.[12] In our previous study, we have found out the significant antibacterial activities of various parts of *M. calabura* against human and plant pathogens.[13] Those findings encouraged us to evaluate the antimicrobial activity of *M. calabura* fruit extracts against food borne pathogens.

Ripened fruits of *M. calabura* were collected, homogenized and extracted at room temperature with petroleum ether, chloroform, ethyl acetate, acetone, methanol and distilled water in the ratio of 1:25 (w/v). Each solvent was collected and filtered using Whatman No.1 filter paper to obtain the respective solvent extracts. The filtrates were concentrated under reduced pressure and dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions. Varying polar solvent extracts of *M. calabura* fruits were screened for their phytoconstituents[14,15] and antibacterial activities.

Preliminary in vitro antimicrobial activity was performed against *Bacillus cereus* ATCC 10876, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Salmonella typhi* MTCC 3224, *Shigella flexneri* ATCC 12022 and *Candida albicans* ATCC 10231 using well diffusion assay. In brief, 100 μl of the appropriate bacterial suspension was inoculated on Mueller Hinton agar (bacteria) and Sabouraud's dextrose agar (yeast) using sterile swabs. 20 μl of the extract was added into the 5 mm wells and the plates were allowed for pre-diffusion of the extract before incubation. The diameter of zone of inhibition mean of two replicates ± SD as indicated by clear area which was devoid of growth of microbes was measured to determine antibacterial activity. The experiment was replicated twice to confirm the reproducible results.

The minimum inhibitory concentration (MIC) assay was performed in both well diffusion and broth dilution method. Briefly, different concentrations (160, 80, 40, 20 and 10 μg ml^{-1}) were prepared in DMSO and the zone diameter of inhibition was determined for the well diffusion assay. Broth dilution assay was performed by dissolving the extracts in DMSO and added into Luria–Bertani (LB) broth to obtain a concentration of 320 μg ml^{-1} and serially diluted to achieve concentrations of 160, 80, 40, 20 and 10 μg ml^{-1}. A 10 μl standardized suspension of each tested organism (10^7 CFU/ml) was transferred to each tube. The control tubes containing only bacterial suspension were incubated at 37 °C for 24 h. The lowest concentration of the extract which did not show any growth of tested organism was determined as the MIC.

The data obtained were statistically analyzed and the results were expressed as means along with standard deviation of three parallel measurements.

Phytochemical analysis of various solvent extracts of *M. calabura* fruits revealed the presence of alkaloids, flavonoids, glycosides, phenols, saponins, tannins, steroids and terpenoids. Results obtained from preliminary antimicrobial screening of *M. calabura* fruit extracts showed its effectiveness against all the Gram positive organisms tested with zone diameter of inhibition in the range of 5–21 mm. S. flexneri ATCC 12022 was found sensitive to acetone and methanol extracts whereas, *E. coli* ATCC 8739 and *S. typhi* MTCC 3224 were found to be resistant against all the extracts tested. The Gram negative bacterium, *S. flexneri* ATCC 12022 was inhibited by the acetone and methanolic fruit extracts of the plant and highest inhibition was observed in *C. albicans* ATCC 10231 (21 mm) against petroleum ether extract.

Minimum inhibitory concentration (MIC) values (Table 1) in the range of 20–40 μg ml^{-1} were recorded against *B. cereus* ATCC 10876 with zone diameter of 7.4–12.0 mm, whereas it was 10–40 μg ml^{-1} in case of *B. subtilis* ATCC 6633 (5.5–10.1 mm). For *S. aureus* ATCC 6538, the range was between 20 and 160 μg ml^{-1} (6.7–16.1 mm) and a moderate inhibitory action was recorded with *S. flexneri* ATCC 12022 with the zone diameter of 5.9–9.3 mm. Highest inhibitory action was recorded with *C. albicans* ATCC 1023 (19.6 mm) with the MIC value of $\leq$40 μg ml^{-1}. Various degrees of inhibitory action were observed with varying solvents. In general, more inhibition was seen with increasing concentrations. None of the extracts were able to inhibit the growth of *E. coli* ATCC 8739 and *S. typhi* MTCC 3224 throughout the study. Both petroleum ether and chloroform were ineffective against most of the pathogens tested and

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Table 1
MIC of *M. calabura* fruit extracts against food borne pathogens (μg ml^{-1}).

Organism	Pet	Chl	Eta	Ace	Met	Aqu
B. cereus ATCC 10876	–	–	≤20	≤40	40	–
B. subtilis ATCC 6633	–	–	40	≤10	20	≤10
S. aureus ATCC 6538	≥160	–	20	≤10	40	≤20
E. coli ATCC 8739	–	–	–	–	–	–
S. typhi MTCC 3224	–	–	–	–	–	–
S. flexneri ATCC 12022	–	–	–	20	≥160	–
C. albicans ATCC 10231	≤40	–	–	–	–	–

Pet – petroleum ether, Chl – chloroform, Eta – ethyl acetate, Ace – acetone, Met – methanol, Aqu – aqueous.

the acetone extract of *M. calabura* fruits seemed to be the most promising with the MIC values of 10–40 μg ml^{-1} which exerted strong inhibition against *B. cereus* ATCC 10876, *B. subtilis* ATCC 6633, *S. aureus* ATCC 6538 and *S. flexneri* ATCC 12022. Significant inhibitory activity was observed with aqueous extracts against *B. subtilis* ATCC 6633and *S. aureus* ATCC 6538 with a considerably low MIC.

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Letter to the Editor

Pharmacognosy of plant *Acalypha fruticosa* Forssk.

Acalypha fruticosa. Forssk. is said to be attenuant, alternative, stomachic and alexipharmic.[1] Root and leaf paste is prepared in water and applied externally to treat skin diseases.[2] This plant extract is reported to be anticancer, antioxidant and anti-inflammatory.[3,4]

1. Taxonomy of *Acalypha fruticosa* Forssk.

Domain: *Eukaryota* Whittaker & Margulis, 1978 – eukaryotes
Kingdom: *Plantae* Haeckel, 1866
Subkingdom: *Viridaeplantae* Cavalier-Smith, 1981 – Green Plants
Phylum: *Tracheophyta* Sinnott, 1935 Ex Cavalier-Smith, 1998 – Vascular Plants
Subphylum: *Euphyllophytina*
Infraphylum: *Radiatopses* Kenrick & Crane, 1997
Class: *Magnoliopsida* Brongniart, 1843 – Dicotyledons
Subclass: *Dilleniidae* Takhtajan, 1967
Superorder: *Euphorbianae* Takhtajan Ex Reveal, 1992
Order: *Euphorbiales* Lindley, 1833
Family: *Euphorbiaceae* (yoo-for-bee-AY-see-ee) J.F. Gmelin, 1777, Nom. Cons.
Subfamily: *Acalyphoideae*
Tribe: *Acalypheae*
Sub tribe: *Acalyphinae*
Genus: *Acalypha* (ak-uh-LY-fuh) C. Linnaeus, 1753
Species: *Fruticosa* Forssk.

An aromatic shrub up to 4 m tall (Fig. 1a). Stems pubescent and greenish at first, later glabrescent and reddish-brown. Petioles 0.5–3 cm longleaf blades 2–7 × 1–4.5 cm, ovate to rhombic-ovate, shortly caudate-acuminate at the apex, crenate-serrate to dentate on the margin, rounded to wide-cuneate or subtruncate at the base, membranous to thinly chartaceous, sparingly or evenly yellowish-pellucid gland-dotted beneath, sparingly to evenly pubescent on both surfaces, and usually more densely so along the midrib and main nerves beneath, 5 (7)-nerved from the base; lateral nerves in 2–4 pairs.

Stipules 3–4 mm, narrowly lanceolate, puberulous, chestnut-brown. Plants usually monoecious. Inflorescences rarely exceeding 2 cm in length, spicate, axillary, usually androgynous with a densely congested terminal male portion and with 1–4 bracteate female flowers at or near the base; male bracts 1 mm long, ovate, densely white-pubescent; female bracts foliaceous, accrescent to c. 8–10 × 10–15 mm, broadly ovate to reniform, crenate or repand-dentate, sparingly yellow gland-dotted and often fairly prominently ribbed on the lower surface, sparingly pubescent, 1-flowered. Male flowers subsessile; buds tetragonous-subglobose, densely pubescent or white-tomentose. Female flowers sessile; sepals 3, 1 mm long, ovate-lanceolate, ciliate; ovary 0.7 mm in diameter, 3-lobed to subglobose, ±smooth, yellow-glandular in the grooves, densely pubescent; styles 4 mm long, ±free, laciniate, pink or red. Fruits 2 × 3 mm, 3-lobed, yellow gland-dotted, evenly pubescent-pilose. Seeds 1.5–2 × 1–1.3 mm, ellipsoid-ovoid, smooth, brown, with an elliptic vulviform caruncle. *A. fruticosa* occurs in coastal and deciduous bush land and thickets, wooded grassland, riverine grassland, on rocky shores or outcrops, and in humid localities, from sea-level up to 1400 m altitude. It is common in overgrazed areas. This species is globally distributed in Tropical Africa to India, Myanmar and Sri Lanka. Within India, it is said to be found from Orissa to Tamil Nadu, Karnataka and Kerala. *A. fruticosa* occurs in coastal and deciduous bush land and thickets, wooded grassland, riverine grassland, on rocky shores or outcrops, and in humid localities, from sea-level up to 1400 m altitude.

2. Pharmocognocy – Sirucinni- (*Acalypha fruticosa*. Forssk.)

Fresh plants were collected from Vandavasi, Tamil Nadu, India and identified by Botanist, Siddha Central Research Institute, Chennai – 106.

Root, stem, petiole and leaf were fixed in FAA solution (70% ethyl alcohol, formalin and acetic acid in the ratio of 90 ml:5 ml:5 ml). The materials were left in the fluid for three days, after which they were washed in water and dehydrated with tertiary butyl alcohol, paraffin wax was infiltrated and the specimens were embedded in wax for sectioning. Alcoholic safranin (0.5%) counter stained with 0.25% fast green. All slides, after staining in safranin were dehydrated by employing graded series of ethyl alcohol (30%, 50%, 70%, 90% and absolute alcohol) and stained fast green in clove oil and xylol–alcohol (50–50) and passed through xylol and mounted in DPX mountant.

Clearing of leaves for studying stomatal number and stomatal index was done by using 5% sodium hydroxide along with chlorinated soda solution supplemented with gentle heat. Photomicrographs were taken with the help of Nikon Eclipse E200 Microscope.

Root: Long, unbranched tap root with lateral roots, brown outside and cut surface yellowish, fracture short; no characteristic odour and taste.

Stem: Green, erect, branched, pubescent, odour aromatic.

Leaf: Leaves ovate–oblong, 25–76 mm long, glandular beneath, pubescent, odour aromatic.

3. Microscopic

3.1. T.S. of root

Transverse section of root is circular in outline (Fig. 1b). Epiblema crushed. Cork is made up of rectangular 10 to 12 layers of

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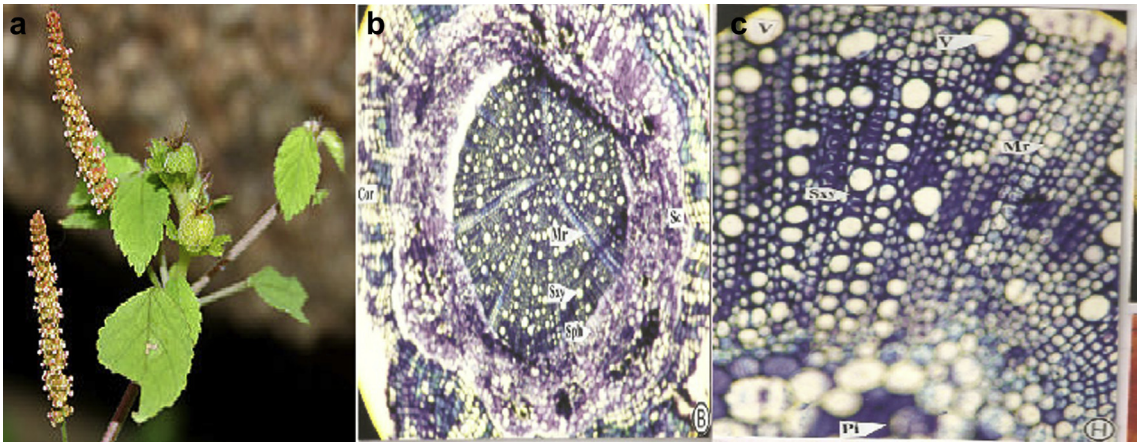

Fig. 1. a. *Acalypha fruticosa* Forssk. b. T.S. Root. c. T.S of stem with xylem and paranchyma cells.

parenchyma cells. Secondary cortical cells show the presence of laticifers and large druses; stone cell patches present; phloem narrow, phloem parenchyma occasionally having druses. Vessels circular, mostly solitary, sometimes in radial groups of 2–4, widely spaced in a large zone of xylem parenchyma. Rays biseriate–triseriate, which start from the centre of the wood. The ray cells are radially elongated and thin walled. Pith absent.

3.2. T.S. of stem

Transverse section of stem is circular in outline (Fig. 1c). The cork which forms the outermost tissue is a narrow zone formed of 4–6 rows of small thin walled, cubical to rectangular cells. The secondary cortex is made up of spherical to oval thick walled parenchyma cells. There is no clear demarcation between the cortex and bast. The phloem is composed of sieve tubes, companion cells and small, thin walled parenchyma cells. It is followed by secondary xylem. The xylem vessels are solitary and arranged as a row and are surrounded by mechanical cells. There are 2–4 rows of medullary rays. The ray cells are radially elongated and thin walled. The central pith region composed of thick walled spherical to polyhedral parenchyma cells.

3.3. T.S. of petiole

Transverse section of petiole is nearly oval in outline. Epidermis is single layered made up of small rectangular cells. A few epidermal cells contain druses of calcium.oxalate crystals. The subepidermal region is made up of 2 or 3 layers of collenchyma and inner region consists of 3 or 4 layers of rounded parenchyma cells.

Vasculature is represented by a deep crescent widely spaced five collateral bundles. The parenchyma cells in the central region are made up of closely arranged penta–octagonal cells. Solitary or groups of pericyclic fibres are seen above the vascular bundle. Huge clustered crystals and a few prisms of calcium oxalate crystals are seen in the parenchyma cells.

3.4. T.S. of leaf

Leaf is dorsiventral in nature.

3.5. T.S. of lamina

Epidermis is single layered made up of barrel shaped cells and covered by a thick cuticle. The palisade tissue is made up of single layer of closely arranged columnar cells. Idioblasts occur in this region. Spongy tissue is made up of loosely arranged rounded parenchyma cells. Vascular bundles of the veins are accompanied by sclerenchyma. On adaxial side a small vascular bundle is present opposite to the large vascular bundle.

3.6. T.S. of Midrib

Transverse section of midrib shows a small projection on the dorsal side and convexity on the abaxial side. The adaxial and abaxial subepidermal region contains 1 or 2 layers of collenchyma cells. Palisade tissue runs along the midrib region, below the collenchyma cells.

A large collateral vascular bundle is seen in the centre. A few sclerenchyma fibres are seen below this bundle. On adaxial side, a small vascular bundle is seen opposite to the larger bundle. The rest of the region is made up of parenchyma cells. Some cells are filled with druses of calcium oxalate crystals.

4. Epidermis in surface view

Adaxial foliar epidermis is made up of undulate anticlinal walls, stomata are absent. Abaxial foliar epidermis is made up of wavy contour. It is perforated by numerous paracytic (rubiaceous) stomata. Disc shaped multicellular glands are seen.

4.1. Trichomes

Sessile, disc shaped multicellular glandular trichomes occur.

4.2. Powder

Green shows cork cells, parenchyma, vessels, collenchyma, druses, leaf fragments with paracytic stomata palisade cells, spongy cells and glandular trichomes.

5. Quantitative microscopy

Stomatal number: Abaxial surface – 40–50/mm^2
Stomatal index: Abaxial surface – 26–31/mm^2
Vein islet number – 8–12/mm^2
Palisade ratio – 5–7

The above study of *A. fruticosa* Forssk., will give a further insight in understanding this plant and its medicinal values and this information will be useful to botanists as well as medicinal plant analysts.

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Letter to the Editor

Evaluation of antioxidant potential of *Celosia argentea* extracts

Sir,

Free radicals are generated naturally required for body's basic metabolism or chemically due to malfunctioning in the antioxidant enzymes systems leading to cellular damage by the excess unstable reactive oxygen species (ROS) or reactive nitrogen species (RNS).[1]

These ROS are free radicals with unpaired electrons (such as O_2^- and H_2O_2) that are highly reactive and can react with biologic macromolecules, modify the structure and function of proteins, and cause oxidative damage to DNA through oxidative stress-induced destruction of pyrimidine and purine bases and oxidation of protein thiols and lipids.[2,3] The most destructive ROS are superoxide radical ($\cdot O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$) generated due to one, two or three electron transfers to dioxygen (O_2).[4] They are responsible for the disorders like arthritis, hemorrhagic shock and coronary diseases, cataract, cancer and AIDS as well as neuro-degeneration.[2]

An antioxidant may be defined as 'any substance which when present at low concentrations, compared with those of the oxidizable substrate significantly delays or inhibits oxidation of that substrate'.[5] They help prevent the above disorders by either quenching the free radicals, chelating metal ions, peroxide reduction or by stimulating the antioxidant defense enzymes.[6]

Phenols contain one or more hydroxyl/carboxyl substituents which help them to chelate metal ions and thus block the superoxide-driven Fenton reaction, believed to be the most important pathway for generation of ROS. They can also suppress the lipid per-oxidation by trapping the lipid alkoxyl radical.[7,8]

Hence in present work, *Celosia argentea* reported to contain high amount of plant phenolics was evaluated for its ability to scavenge the harmful radicals generated by H_2O_2, DPPH, ABTS and FRAP.

Fresh whole plant of *C. argentea* Linn. was collected from Bhor, Pune, Maharashtra, authenticated by Botanical Survey of India, (BSI/WC/Tech/2011), powdered and extracted successively with n-hexane, ethyl ether followed by methanol (80%).

Total Phenolic Content (TPC) was determined by mixing 100 µl of extract with 0.5 ml Folin–Ciocalteu (FC) reagent (diluted 10 times with distilled water). 7 ml of distilled water was added and allowed to stand at room temperature for 5 min. Then, 1.5 ml sodium bicarbonate (60 mg/ml) solution was added to the mixture and left in dark place for 2 h. Absorbance was read at 725 nm against blank using UV–Visible spectrophotometer (Perkin Elmer Lambda 35, USA). A calibration curve was prepared, using a standard solution of gallic acid. Results were expressed as gallic acid equivalents mg (GAE)/100 g.[9]

In the Hydrogen Peroxide Antioxidant Assay, Hydrogen peroxide solution (H_2O_2) (2 mM/L) was prepared in standard phosphate buffer (pH 7.4). Different concentration of the extracts (0.2, 0.4, 0.6, 0.8 mg/ml) in distilled water were added to 0.6 ml of H_2O_2 solution. Absorbance was determined at 230 nm after 10 min against a blank solution containing phosphate buffer compared with the standard ascorbic acid (0.2, 0.4, 0.6, 0.8 mg/ml).[10]

In the DPPH Assay *a* solution of 3.3 mg DPPH in 100 ml methanol was prepared and 1.0 ml of this solution was mixed with 1.0 ml of extract (25, 50, 75, 100 µg/ml) in methanol containing 0.02–0.1 mg of the extract. The reaction mixture was vortexed thoroughly and left in the dark at 25 °C for 30 min. The absorbance of the mixture was measured at 517 nm. BHT (25, 50, 75, 100 µg/ml) was used as reference.[10]

$$\%\text{Radical scavanging activity} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

In the ABTS scavenging activity the stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml $ABTS^+$ solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. Plant extracts (1 ml) (25, 50, 75, 100 µg/ml) were allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min using the spectrophotometer compared with that of BHT (25, 50, 75, 100 µg/ml).[10]

In the FRAP (Ferric Reducing Antioxidant Potential) Assay the stock solutions included 300 mM acetate buffer (3.1 g sodium acetate and 16 ml acetic acid), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-*s*-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3$ solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml $FeCl_3$. The temperature of the solution was raised to 37 °C before using. Plant extracts (150 µl) (50 µg/ml) were allowed to react with 2850 µl of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve was linear between 200 and 1000 µM Trolox. The FRAP scavenging capacity of the extract was compared with that of BHT (50 µg/ml) and the percentage inhibition was calculated.[10]

The seed extract was found to be the richest in Total Phenolic content (23.39 µg/ml) of all the three extracts at the concentration of 0.1 mg/ml.

The % Inhibition of H_2O_2 radical was maximum for the seed extract at the concentration of 0.8 mg/ml followed by aerial parts extract. Root extract showed insignificant results when compared with the standard-ascorbic acid (97.63%).

The % inhibition of DPPH radical was highest for the seed extract at the concentration of 100 µg/ml followed by aerial parts extract.

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Table 1
% Inhibition of H_2O_2, DPPH and ABTS radical by various parts extract of *C. argentea*.

Test extracts	% Inhibition		
	H_2O_2 assay (0.8 mg/ml)	DPPH assay (100 μg/ml)	ABTS assay (100 μg/ml)
Aerial part	67.13	70.81	62.25
Seed	79.12	88.18	86.05
Root	51.14	36.16	30.80
Standard	97.63	99.51	87.76

Std for H_2O_2 Assay: Ascorbic acid.
Std for DPPH & ABTS Assay: BHT.

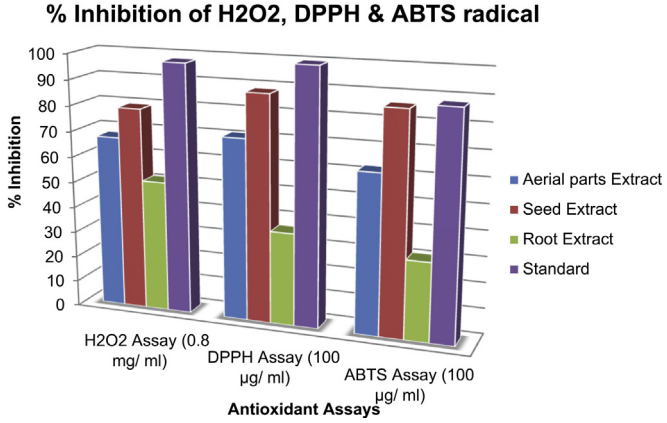

Fig. 1. % Inhibition of various extracts at the highest concentration compared to standard.

Root extract compared showed insignificant results compared with the standard-BHT showing % Inhibition of 99.51% at the same concentration. Thus indicating highest antioxidant activity for the seed extract compared with the standard-BHT.

The % Inhibition of ABTS radical was highest for the seed extract at the concentration of 100 μg/ml followed by aerial parts extract. Root extract, compared with the standard-BHT showed insignificant results. Thus indicating highest antioxidant activity for the seed extract compared with the standard- BHT (Table 1)(Fig. 1).

In the FRAP assay, the lower the TEAC value more is the antioxidant potential, the TEAC was found to be least for the seed extract (1.92), followed by the aerial parts extract (2.14), at the concentration of 50 μg/ml. Thus proving the seed extract to be the most potent antioxidant.

Since the plant is reported to be rich in phenols, the total phenolic for the seed extract was significant compared for all the three extracts. As per the *in vitro* antioxidant assays the ability to scavenge the generated harmful radicals was more for the seed extract followed by the aerial parts extract. However the antioxidant potential for the root extract was found to be negligible.

Hence, it could be stated that seed extract of *C. argentea* could help protect the damage due to harmful free radicals by scavenging and suppressing them possibly be due to its abundant polyphenols.

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