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Quality control standardization and antioxidant activity of roots from *Eriosema chinense*

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ABSTRACT

Background: Roots of the plant *Eriosema chinense* Vogel are used as a vegetable by the people of Northern Australia and North East India. Traditionally, the roots of the plant are used for the treatment of diarrhea by the tribal people of North East India.
Aim: The present study was undertaken to perform quality control standardization and to evaluate antioxidant activity of roots from *Eriosema chinense*.
Methods: The roots were examined macroscopically, microscopically and various physicochemical parameters were evaluated using standard guidelines. Further, quantitative estimations of different phytoconstituents along with standardization of ethanol extract with HPTLC using lupinifolin as a marker compound was also done. The extract was also evaluated for *in vitro* antioxidant activity using different experimental models.
Results: The brown coloured bulb shaped, hairy roots showed the presence of cortical cells densely filled with tannins, lignified sclerenchymatous pericyclic fibers and central lignified xylem vessels with spiral thickening. Physicochemical parameters evaluated included ash values, extractive values, loss on drying, foaming index, swelling index, foreign matter, crude fiber content, pesticide residue and heavy metal analysis which were found to be in prescribed limits, Fluorescence powder drug analysis and total number of starch grains was also evaluated while total hemolytic activity was found to be moderate. Phytochemical screening of different extracts and quantitative estimations revealed the extract to be rich in carbohydrates, flavonoid, phenols and tannins, while lupinifolin quantified by HPTLC in ethanol extract was found to be 6.48% w/w. *In vitro* antioxidant studies depicted a potent antioxidant activity of extract that may be attributed to the presence of higher amount of flavonoids, phenols and tannins.
Conclusion: The quality control standards obtained in the present study will provide referential information to researchers for proper identification and authentication of plant and will help in maintaining its pharmaceutical, botanical and economical importance.

1. Introduction

Eriosema chinense Vogel (Leguminosae-Papilionoideae) is mainly distributed over the Eastern Himalayan region of India and China and is also found in countries like Thailand, Myanmar and Australia. The stem of plants is 30–50 cm in height, slender, erect, woody, little branched, and densely hairy. Leaflets are simple, linear-ligulate, and 2.5–5 cm in length. The flowers are yellow, 1–2, and borne in leaf-axils. The pods are oblong, about 2 cm in length, and densely hairy.[1] The roots of the plant are eaten raw and in the form of vegetable by the people of Northern Australia and North East India. It is used as a traditional medicine for the treatment of diarrhea by the tribal people of Meghalaya (India). The decoction of grains is used as astringent, diuretic, tonic, in cold sweats and is used during parturition to promote discharge of the lochia. A decoction of the grain with powdered pepper is given for diarrhea.[2] Recently, eight new prenylated flavonoids, khonklonginols A–H, together with six known compounds including five flavonoids, lupinifolinol, dehydrolupinifolinol, flemichin D, eriosemaone A, lupinifolin, and one lignan, yangambin, have been reported from this plant. Pharmacologically the plant has been evaluated for its cytotoxic and anti-tubercular activity.[3]

In developed as well as developing countries, medicinal plants have been used as a potential source of home remedies. It has also been observed that in recent years, there is great increase in

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demand of herbal medicines in pharmaceutical industries which covers a substantial proportion of the global drug market over the counter drug products. This has resulted into a huge turnaround in fields like botany, pharmacognosy and pharmacology and has become a major pillar of streams like pharmacy, medicine, natural product chemistry and many others. The above revolution has resulted in initiation of various active research programmes to produce effective standardized extracts and to isolate new lead compounds. Herbal drugs are prone to contamination and deterioration which may lead to variation in composition of constituents resulting in little or no therapeutic efficacy.[4] Therefore, care should be taken right from proper identification of plants, seasons and area of collection and their extraction and purification process in order to obtain quality oriented herbal formulations, To minimize the amount of adulteration and misinterpretation of herbal drugs or food materials, it is very essential to standardize it by evaluating various qualitative and quantitative parameters which may play a major role in developing referential standards of that particular drug or food materials.[5]

Even though the roots of the plant *E. chinense* is used as a vegetable food and is traditionally used for treating various ailments still, there is no any scientific data available describing its quality control profile. Therefore, the present study was carried out to evaluate different qualitative and quantitative parameters to facilitate the quality control standardization of *E. chinense*.

2. Material and methods

2.1. Authentication

The plant samples of *E. chinense* were obtained from different places in Shillong region (Khasi Hills district) and Jowai region (Jaintia Hills District) of Meghalaya (India) in May 2011 and after careful examination they were identified as *E. chinense* Vogel by Dr. B.K. Sinha (Scientist C, In charge), Botanical Survey of India, Shillong, India. For future reference, a voucher specimen (COG/EC/14) of the plant has been deposited in Department of Pharmaceutics, Indian Institute of Technology (Banaras Hindu University), Varanasi, India. The results represented in the present study provide a mean or common observations observed in different samples of *E. chinense*.

2.2. Macroscopic and microscopic evaluation

The macroscopical evaluation of the roots was done by observing them with reference to their color, shape, size, odor and taste etc. For microscopical examination, the roots of the plant were cut and were fixed in FAA (Formalin 5 mL + Acetic acid 5 mL + 70% Ethyl alcohol 90 mL) for about 24 h. Further, as per the schedule given by Sass,[6] the specimens were dehydrated with graded series of tertiary- butyl alcohol (TBA) followed by infiltration of the specimens by gradual addition of paraffin wax (melting point 58–60 °C) until TBA solution attained super saturation. The specimens were then casted into paraffin blocks which were further sectioned with the help of Rotary Microtome at a thickness of 10–12 μm followed by dewaxing as described by Johansen.[7] Staining of the sections was done using Toludine blue[8] and wherever required, fast-green, safranin and IKI (iodine in potassium iodide (for starch)) were also used as a staining agents. Photographs of different magnifications were taken with Nikon Trinocular Microscopic unit, Model E-200, Japan.

2.3. Physicochemical standardization

The roots of the plant were dried and grinded in a mixer grinder and were used for evaluation of various physicochemical parameters. Foreign matter present in the roots was determined visually using lenses, whereas loss on drying was determined by drying specified quantity of plant material in oven to a constant weight at 110 °C. Different ash values (total ash, acid insoluble ash and water soluble ash) were determined according to the standard procedure by incinerating the plant material at a temperature between 500 and 600 °C until it is white, indicating the absence of carbon. Extractive values of the roots in different solvents were evaluated by soaking it in respective solvents for about 18 h. Hemolytic activity of the roots was evaluated by determining its capacity to produce hemolysis in ox blood sample at a particular concentration compared to standard diosgenin. In addition, foaming index (by measuring the length of the foam) and swelling index (by measuring the volume occupied by the plant material in a measuring cylinder after 3 h) were also determined using the usual procedure described.[9] Lycopodium spore method, as described by Wallis[10] was used to determine the total number of starch grains in the roots. The amount of crude fiber present in the plant material was determined by boiling the plant material with 10% nitric acid followed by treating it with 2.5% NaOH.[11] The powdered roots were subjected to fluorescence powder drug analysis under day light, short UV and long UV according to the methods described by Chase and Pratt.[12]

2.4. Heavy metal analysis

The quantitative estimations of various heavy metals present in the roots of *E. chinense* was carried out using atomic absorption spectrophotometer by adopting the procedure mentioned in WHO guidelines.[9]

2.5. Pesticide content

The pesticide content of the roots was determined using the WHO guideline. A mixture of 350 mL of acetonitrile:water (65:35) was added to 50 g of grinded powdered roots which was blended at high speed for 5 min followed by filtration. 250 mL of filtrate was then transferred to a separating funnel to which further 100 mL light petroleum, 10 mL of sodium chloride (40%) and 600 mL of water were added with constant shaking up to 35–45 s. Aqueous layer was discarded from the solvent layer and the later was washed twice with 100 mL portions of water to which 15 g of anhydrous sodium sulfate was added with vigorous shaking. The extract was separated followed by reducing its volume to 5–10 mL which was allowed to pass through column packed with Florisil R grade 60/100 PR, activated at 650 °C at a rate of not more than 5 mL per minute. Three different elutes were obtained using three different ratios of ether: light petroleum mixture as mobile phase i.e. Elute 1 contained 6% of ether while elute 2 and 3 contained 15% and 50% of ether. The obtained elutes were transferred to a sample holder, and burned in a suitable combustion flask flushed with oxygen. The gases produced gets absorbed in a suitable solution in the combustion flask (water for chloride and H_2SO_4 in case of phosphate pesticides). In case of chloride pesticides 15 mL of the solution obtained after combustion was mixed with 1 mL of ferric ammonium sulfate (0.25 mol/L) and 3 mL of mercuric thiocyanate followed by swirling it where absorbance was measured at 460 nm. For determining phosphate pesticides, 7 mL of the solution obtained after combustion was mixed with 2.2 mL of sulfuric acid (300 g/L), 0.4 mL of ammonium molybdate (40 g/L) and 0.4 mL of aminonaphtholsulfonic acid followed by swirling it and heating it at 100 °C for 12 min which was then measured at 820 nm.[9]

2.6. Phytochemical standardization

Preliminary phytochemical screening of the different extracts of powdered roots obtained after cold maceration using different

solvents for the presence of various phytoconstituents was carried out.[13] The grinded roots of the plant (500 g) were extracted using ethanol (1.5 L) with Soxhlet apparatus until the whole drug was exhausted. Further, the extract was concentrated and evaporated (12.29% w/w) in a Rota evaporator and was kept in a desiccator until use. Total alkaloid content in the plant material was estimated using the usual gravimetric analysis[14] in which the plant material was first extracted with H_2SO_4 and was further given successive washes with chloroform and diethyl ether. The method described by Yemm and Willis[15] was used to determine total carbohydrates in ethanol extract of *E. chinense* (EEC) using anthrone reagent. Total phenols and tannin contents in EEC were estimated according to the method of Hagerman et al[16] using Folin ciocalteau reagent while total flavonoid and flavonol contents were determined following the methods of Kumaran and Karunakaran[17] using aluminum trichloride Total saponin content was estimated taking diosgenin as standard by implementing method of Baccou et al[18] using anisaldehyde-ethyl acetate reagent and H_2SO_4.

EEC was further standardized for the first time with lupinifolin after confirmation of its presence by thin layer chromatography using high performance thin layer chromatography (HPTLC). A stock solution of both EEC and standard lupinifolin in methanol was prepared in concentration of 5 mg/mL and 0.2 mg/mL respectively. The mobile phase for developing the chromatogram consisted of hexane and ethyl acetate mixture in the ratio 85:15 (v/v). The study was carried out using Camag- HPTLC instrumentation equipped with Linomat V sample applicator, Camag TLC scanner 3, Camag TLC visualizer and WINCATS 4 software for data interpretation. The R_f values were recorded and the developed plate was screened and photo-documented at ultra violet range with wavelength (λ_{max}) of 254 nm.

2.7. In vitro antioxidant studies

Since EEC showed the presence of flavonoids in very high quantity along with phenols and tannins therefore, it was further evaluated for its *in vitro* antioxidant activity by using various *in-vitro* methods. Phosphomolybdenum method was used to determine the total antioxidant capacity of EEC which is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at 695 nm in acidic pH. The assay of reducing power was performed by adopting potassium ferricynide method using ascorbic acid as standard. The free radical scavenging activity of EEC was determined by 1, 1-diphenyl-2-picryl-hydrazil (DPPH) method where ascorbic acid was used as standard and absorbance was measured at 517 nm. Nitric oxide scavenging assay was performed following the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions. EEC was also evaluated for H_2O_2 scavenging activity by using standard method and percent inhibition was calculated after taking the absorbance at 230 nm. Deoxyribose method was implemented for determining the hydroxyl radical scavenging activity of EEC using butylated hydroxy anisole (BHA) as a standard where absorbance was measured at 532 nm.[19,20]

3. Results

3.1. Macroscopical and microscopical evaluation

Macroscopically, the roots of the plant are bulb shaped, dark brown in color with smooth surface bearing root hairs. Their size ranges from 1 to 3 cm in length and 0.5–1.5 cm in width with a narrow pointed base. The roots bear, characteristic odor and bitter taste (Fig. 1).

The primary structure of the root in transverse section shows tetrarch condition, where secondary growth starts quite early. The formation of cork cambium is normal. The transverse section of roots before secondary growth shows the presence of cork cells, continuous lignified sclerenchymatous pericyclic fibers followed by continuous ring of phloem encircling the slightly ridged and furrowed solid zone of xylem (Fig. 2B).

In the mature root (Fig. 2A), the cork cells are compressed, thick walled, rectangular in shape with 7–10 layers and have their sizes ranging from 40 to 50 μm in length and 8–12 μm in width. This is followed by cork cambium showing 5–8 layers of thin walled rectangular to tabular shaped parenchymatous cells (35–55 μm in length and 10–15 μm in width). Next to the cork cambium appears, thick walled discontinuous lignified sclerenchymatous pericyclic fibers having size in the range of 150–300 μm in length and 10–25 μm in width. This is followed by secondary cortex constituting 8–10 layers of irregular shaped thin walled parenchymatous cells. In this zone, few parenchymatous cells are densely filled with tannin having size in the range of 25–45 μm in length and 15–35 μm in width which appear blue with ferric chloride solution (Fig. 2D). The entire secondary cortex region shows the presence of starch grains both simple and compound and few discrete xylem vessels with spiral thickening (150–300 μm in length and 15–45 μm in width). The central portion of the transverse section consists of wide zone of lignified xylem which consists of vessels, fibers and tracheids vessels showing spiral thickening and appears pink with pholoroglucinol (Fig. 2A, B and F). As growth proceeds, the number of furrows in the xylem become more deeper and are represented by radially elongated medullary rays 3–4 cells wide dissecting the xylem from 3 to 4 sides. Starch grains are present in large amount all throughout the section. Under the polarized light, the starch grains appears bright with dark background and are elliptical or circular in shape having size in the range of 8–14 μm in length and 7–12 μm in width (Fig. 2E).

Powder microscopy of roots shows the presence of sclerenchymatous pericyclic fibers (Fig. 2C) and parenchymatous cells filled with tannin appearing brown in color (Fig. 2D). The ground tissues are filled largely with starch grains which are more predominant (Fig. 2E). The grains are large, elliptical or ovate or circular with centric or eccentric hilum. The powder study also showed the presence of lignified xylem with spiral thickening (Fig. 2F).

3.2. Physicochemical standardization

The roots had 0.914% w/w of foreign matter, 3.853% w/w of moisture content and a swelling index of 7.25 mL/g, whereas length

Fig. 1. Roots of *E. chinense*.

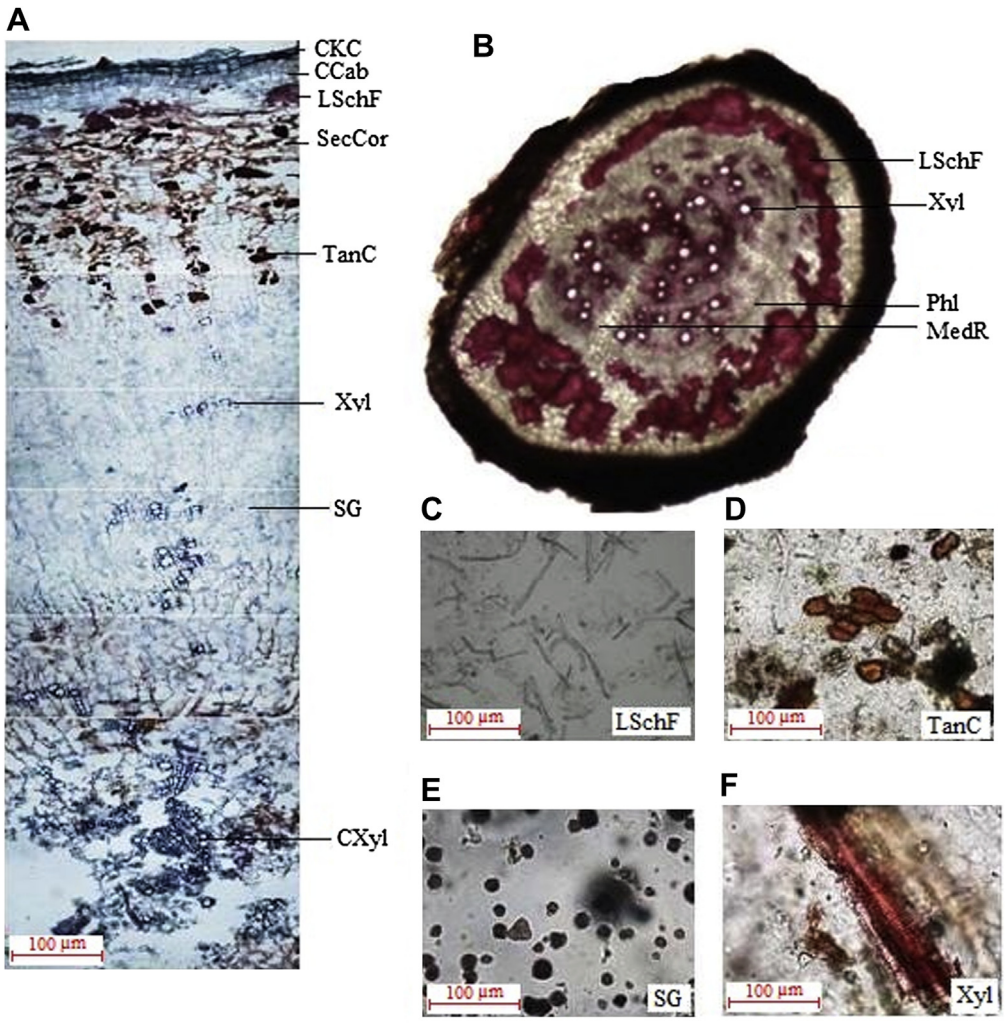


Fig. 2. Microscopy of *E. chinense*. Where A: Transverse section of mature root of *E. chinense*, B: Transverse section of young root of *E. chinense* and C–F represents Powder microscopy of *E. chinense* (C: Lignified sclerenchymatous fibers, D: Tanniferous cells, E: Starch grains and F: Xylem vessels). In figure, CCab: Cork cambium, CKC: Cork cells, LSchF: Lignified sclerenchymatous fiber, MedR: Medullary rays, Phl: Phloem, SecCor: Secondary cortex, SG: Starch grains, TanC: Tannin cells, Xyl: xylem vessels and CXyl: Central xylem vessels.

of the foam of powdered root was found to be less than 1 cm therefore, foaming index was reported to be less than 100. The roots showed the presence of total ash (5.612% w/w), acid insoluble ash (0.642% w/w) and water soluble ash (2.773% w/w), while extractive values in different solvents reported in % w/w were water: 5.150, methanol: 9.604, ethanol: 8.332, ethyl acetate: 4.975, chloroform: 5.778, hexane: 1.392, acetone: 5.441 and pet ether: 2.294 respectively. The total number of starch grains present in 1 mg of powder drug was found to be 162 325, while crude fiber content of roots was found to be 13.95% w/w of plant material. The hemolytic activity of roots of *E. chinense* was found to be 188.89 units per gram of powder. Fluorescence powder drug analysis of roots of *E. chinense* is represented in Table 1, whereas the results of heavy metal analysis of roots of *E. chinense* for the presence of various heavy metals are represented in Table 2.

3.3. Pesticide content

From the results, chlorinated pesticide present in the roots *of E. chinense* in first and the second elute was reported to be 0.479 and 0.315 mg/kg of plant material. The phosphated pesticide from the first and second elute of column was found to be 0.036 and

Table 1
Florescence analysis of *E. chinense*.

Test	Day light	Short UV	Long UV
Powder + 1 N NaOH in methanol	Corn silk	No fluorescence	Light green
Powder + 1 N NaOH in water	Orange red	Firebrick	Green yellow
Powder + 1 N HCL in methanol	Dark red	No fluorescence	Dark olive
Powder + 1 N HCL in water	Khaki	Dark golden red	Green yellow
Powder + 1 N HNO_3 in methanol	Corn silk	No fluorescence	Medium spring green
Powder + 1N HNO_3 in water	Golden red	No fluorescence	Spring green
Powder + 5% iodine	Corn silk	No fluorescence	Pale green
Powder + 5% $FeCl_3$	Maroon	No fluorescence	No fluorescence
Powder + 50% KOH	Dark olive green	No fluorescence	No fluorescence
Powder + 25% ammonia	Dark red	No fluorescence	Sea green
Powder + picric acid saturated	Dark golden red	Medium sea green	Green yellow
Powder + acetic acid	Dark red	Light green	Spring green

Table 2
Heavy metal analysis of *E. chinense*.

Heavy metals analyzed	Wavelength (nm)	Fuel (Litre/min)	Burner height (mm)	Conc in ppm
As	193.70	2.40	0.4	0.279
Cr	357.90	2.90	0.6	1.588
Cu	324.70	3.02	0.6	0.590
Fe	248.30	2.99	0.6	1.692
Hg	253.70	7.66	0.6	–
Ni	232.00	2.94	0.6	31.018
Pb	217.00	2.90	0.6	4.153

0.0641 mg/kg of plant material respectively while in third elute it was reported to be absent.

3.4. Phytochemical standardization

Preliminary phytochemical analysis of the EEC revealed the presence of phenols, flavonoids, tannins, alkaloids, steroids and carbohydrates as a major component (Table 3). Total phenolic content in EEC was reported to be 43.242 mg/g gallic acid equivalent while total tannin content was estimated to be 26.161 mg/g tannic acid equivalent. Total flavonoid and flavonol content were found to be 109.868 and 16.447 mg/g rutin equivalent. Total alkaloid and saponin estimated in the plant material were reported to be 0.552% w/w and 12.790 mg/g diosgenin equivalent, whereas total carbohydrate in EEC was found to be 146.807 mg/g D-fructose equivalent. The HPTLC analysis depicted well resolved peaks of EEC showing the presence of lupinifolin. The spots of the entire chromatogram were visualized under UV 254 nm and the percentage of lupinifolin (R_f 0.38) in EEC was reported to be 6.48% (w/w) (Fig. 3).

3.5. In-vitro antioxidant studies

Linear regression equation was used to determine the total antioxidant capacity of EEC and was expressed as the number of equivalent of ascorbic acid which was found to be 90.166 ± 1.641 μg/mL. Assay of reducing power is a concentration dependent reaction i.e. higher concentration indicates higher reducing power. The results demonstrated a potent reducing potential of *E. chinense* (0.263 ± 0.004 μg/mL) which was quite comparable with reducing power of standard ascorbic acid (0.419 ± 0.006 μg/mL). The capability of EEC to reduce DPPH by donating an electron or hydrogen to DPPH is indicative of free radical scavenging activity of the extract. The results depicted a IIC_{50} value of 146.357 ± 4.321 μg/mL of EEC as compared to ascorbic acid (IC_{50}: 79.120 ± 4.016 μg/mL). A considerably moderate scavenging potential of hydrogen peroxide by EEC was observed with an IC_{50} value of 221.048 ± 5.055 μg/mL compared to standard rutin IC_{50} 82.866 ± 6.396 μg/mL. Griess reagent was used to determine the nitric oxide scavenging activity which illustrated a moderate scavenging activity of EEC (IC_{50}: 232.945 ± 4.690 μg/mL) in comparison to rutin (IC_{50}: 76.436 ± 3.773 μg/mL). Fenton reaction was used to assess the potential of EEC in inhibiting the hydroxyl radical production through iron (II)-dependent deoxyribose damage assay. The results demonstrated a potent scavenging activity with an IC_{50} value of 170.234 ± 6.505 μg/mL compared to positive control BHA (IC_{50} 71.923 ± 4.934 μg/mL).

Table 3
Preliminary phytochemical screening of *E. chinense*.

Phytoconstituents	Extract							
	WE	ME	EE	EAE	CE	HE	AE	PEE
Alkaloids	−	+	+	+	+	+	+	+
Steroids	−	+	+	+	+	+	+	+
Anthraquinone glycoside	−	−	−	−	−	−	−	−
Cardiac glycoside	−	−	−	−	−	−	+	−
Cyanogenetic glycoside	−	−	−	−	−	−	−	−
Saponin	+	+	+	−	−	−	−	−
Phenolic compound	+	+	+	+	+	+	−	−
Carbohydrates	+	+	+	+	−	−	−	−
Proteins	+	+	+	+	−	−	−	−
Amino acid	+	+	+	+	−	−	−	−

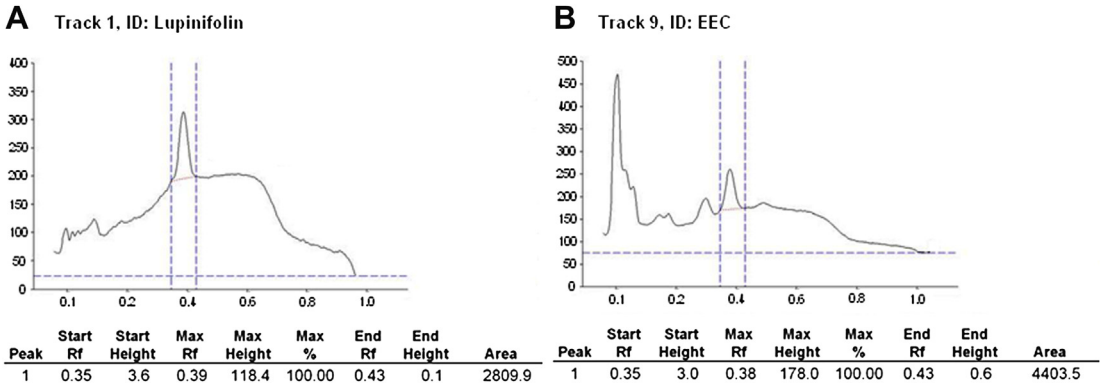
+ Indicates presence and − indicates absence. Where: WE: Water extract, ME: Methanolic extract, EE: Ethanolic extract, EAE: Ethyl acetate extract, CE: Chloroform extract, HE: Hexane extract, AE: Acetone extract, PEE: Petroleum ether extract.

4. Discussion

Quality control standardization of herbal drugs helps us in correct identification and authentication of the plant material. In recent years, there has been wide increase in therapeutic importance of herbal medicine thus, it is very essential to obtain a proper quality control profile for various medicinal plant used in traditional system of medicine. This may be helpful in minimizing the adulteration of these plants which occurs mainly due to improper knowledge regarding the varied geographical conditions, associated problems of different vernacular names, its morphology and microscopy. It is also said that correct identification and proper quality assurance of the starting materials is an essential prerequisite to ensure reproducible quality of herbal medicine which contributes to its safety and efficacy.[4,21]

Pharmacognostical evaluation of a plant/plant parts is considered to be the preliminary step in standardization of a plant that provides valuable information in terms of its morphological, microscopical and physical characteristics.[22] Macroscopical examination of a plant/plant parts represents detailed information regarding the qualitative assessment of plant based on its morphological and sensory characters such as size, shape color, taste, odor etc while microscopical evaluation provides us extensive knowledge about the cellular arrangement of tissues.[21] The specific microscopical characters of roots of *E. chinense* showed the presence of cortical cells densely filled with tannins, normal cork cambium, lignified sclerenchymatous pericyclic fibers, central lignified xylem vessels with spiral thickening and simple and compound starch gains spread all throughout the transverse section.

The results showed the presence of very low moisture content in the roots. It is very essential to control the moister content, since higher moisture content in plant material may lead to its deterioration and may therefore result in percentage variation of active constituents. The ash values represent inorganic salts naturally occurring, adhering or deliberately added to crude drug as a form of adulterant. Total ash in a plant material includes both physiological as well as nonphysiological ash while acid insoluble ash is a part of total ash and is an indicative of silica present, especially as sand and siliceous earth whereas, water soluble ash is the water soluble portion of the total ash.[4] From the results, it was found that the roots showed the presence of higher quantity of water soluble ash compared to acid insoluble ash. There was a consistent reduction in extractive values observed with a decreasing order of solvent polarity. The amount of active chemical constituents present in plant material depends on the extractive values extracted through different solvents for which as yet no suitable chemical or biological assay exist. Swelling index of a plant material is conclusive of the therapeutic or pharmaceutical value which may be attributed to the presence of gums, mucilage, pectin and hemicelluloses. The results demonstrated a lower swelling index of roots which may be due to low quantity of the above mentioned parameters. The foaming index of a plant material is the ability of an aqueous decoction of that plant materials and their extracts to



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area
1	0.35	3.6	0.39	118.4	100.00	0.43	0.1	2809.9

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area
1	0.35	3.0	0.38	178.0	100.00	0.43	0.6	4403.5

Fig. 3. HPTLC chromatogram of lupinifolin in ethanol extract from roots of *E. chinense* (EEC). In figure A: Standard peak of lupinifolin and B: Peak of lupinifolin present in EEC.

form persistent foam.[9] Fluorescence powder drug analysis is considered to be an important parameter in qualitative determination of crude drug since many compounds in plant material can be judged by their property of exhibiting florescence in day light or in ultra violet range (e.g. alkaloids like berberine).[4] The phenomenon of hemolysis is common in plants containing mainly saponins which cause hemoglobin to diffuse into the surrounding medium.[9] This principle is used for the determination of hemolytic activity of plant material which demonstrated a medium hemolytic activity of roots of *E. chinense*.

The heavy metals analysis performed in the present study showed the presence of As, Cr, Cu, Fe, Ni, Pb which were found to be in accordance with the prescribed limits of WHO. Heavy metals may accumulate in plants either through foliage or root systems and therefore, it is very essential to determine the level of toxic metals in the medicinal plants. The main contributing factor includes environmental pollutants, industrial and traffic emissions, agricultural expedients such as cadmium-containing dung, organic mercury fungicides and insecticide containing lead and arsenate. Heavy metal analysis also provides valuable information regarding metals that are natural essential components of coenzymes which play major role in growth, photosynthesis and respiration.[23]

Agricultural practice such as spraying and treatment of soils occurring throughout the processes of cultivation, and administration of fumigants during storage may result in contamination of medicinal plant/plant parts with pesticides. WHO has therefore, suggested that every nation dealing with production of medicinal plants/plant parts should have at least one central laboratory which will provide information regarding standard limits of pesticides.[9] The roots of *E. chinense* did show the presence of chlorinated and phosphated pesticides but were present in accordance with the standard limits of these pesticides.

The chemical nature of the active constituents present in the plant material can be identified by performing preliminary phytochemical screening of that plant material.[4] The results from the phytochemical analysis revealed the presence of flavonoids, phenols, carbohydrates, alkaloids, steroids, tannins and saponins. The quantitative estimations performed in the present study depicted the presence of carbohydrates, flavonoids, phenols and tannins in major quantities while saponins and alkaloids were present in quite considerable amount. Flavonoids have shown potential antioxidant and anti-inflammatory activities which are attributed to an increased capillary permeability and have been also associated in treatment of various cardiovascular diseases.[24,25] Phenols play beneficial role in active, quenching of oxygen-derived free radicals thus, neutralizing them by donating hydrogen atom or an electron to the free radicals. Therefore, they are considered as strong antioxidants and free radical scavengers with anticarcinogenic, antibacterial, anti-inflammatory activities and are also used in coronary heart disease, some types of tumors and coronary artery disease.[26,27] Tannins have a strong astringent action and are reported to have antibacterial, anti-inflammatory, anti-viral and antioxidant activities.[4,28,29] Alkaloids have been reported to posses wide range of therapeutic importance in the fields of cancer, malaria, pain, inflammation, parkinsonism, hypertension and number of central nervous system disorders.[30] The study also included HPTLC quantification of lupinifolin in EEC. Lupinifolin is a prenylated flavanone reported to have chemopreventive (anti-tumor promoters), antimycobacterial, anti-HSV-1, antibacterial and antioxidant activities.[3,31,32] Thus, lupinifolin can be used as a chemical marker for standardization of roots from *E. chinense*.

The results also depicted a potent *in vitro* antioxidant activity of EEC which was evident through total antioxidant capacity, reducing power assay and different free radical scavenging methods. Many studies have reported that, free radicals such as nitric oxide, hydroxyl radical and hydrogen peroxide in human body, get bound to DNA nucleotides and damages various biological systems thus, resulting in carcinogenesis, mutagenesis, and cytotoxicity. These impairments have been potentially improved by the use of plants rich in flavonoids, phenols and tannins as they neutralizes the free radicals by donation of hydrogen atom, quenching of oxygen and by chelation of metals which reduces the elevated oxidative stress.[19,33]

5. Conclusion

In conclusion, the present study provides various qualitative and quantitative standards of roots from *E. chinense* which will benefit interested researchers as a referential source of valuable information that will certify its identity and authenticity. The evaluated parameters may help in maintaining quality and purity of the vegetable root and will prevent its adulteration with drug of same or other genus having low potency.

Conflicts of interest

All authors have none to declare.

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Original article

Determination of ursolic acid in fractionated leaf extracts of *Ocimum gratissimum Linn* and in developed herbal hepatoprotective tablet by HPTLC

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ABSTRACT

Background: Ursolic acid was determined in dichloromethane and ethyl acetate fractions of methanolic extract of *Ocimum gratissimum* and in developed herbal hepatoprotective tablet by HPTLC method.
Methods: Hepatoprotective polyherbal formulation was prepared using five fractions of three plant extracts namely *O. gratissimum*, *Butea monosperma* and *Bauhinia variegata*. Among these three plants *O. gratissimum* contains ursolic acid. Chromatographic separation was performed on silica gel HPTLC plates with petroleum ether:ethyl acetate:acetone (8.2:1.8:0.1, v/v/v) as mobile phase. After drying, the plates were sprayed with 10% (v/v) ethanolic solution of sulfuric acid and heated to 120 °C for 3 min. Quantification was performed in absorbance/transmittance mode at a wavelength of 530 nm using a computer-controlled densitometer.
Results: The presented method was validated for linearity 400–1200 (ng/spot), intraday precision % C.V. (0.58–1.97), and interday precision % C.V. (1.46–2.22). Correlation coefficient (r^2 = 0.9960), detection limits as well as recovery values (97.5%–98.22%) were found to be satisfactory.
Conclusion: A good correlation was obtained among the standard, samples of polyherbal formulation and fractionated extract of *O. gratissimum* using HPTLC method.

1. Introduction

Ursolic acid is one of the biologically active compounds in *Ocimum gratissimum*. Ursolic acid has been isolated from the leaves.[1]

Ursolic acid is well known for its hepatoprotective effects in both acute chemically induced liver injury and chronic liver fibrosis and cirrhosis. They are still used alone or in combination with other hepatoprotective ingredients as oral medications. The beneficial effects of triterpenic acid on the liver could be due to its antioxidant and anti-inflammatory actions, and their effects on drug metabolizing enzymes. These triterpenoids are effective inducers of metallothionein, a small cysteine-rich protein acting like glutathione in the body's defense against toxic insults.[2] Oleanolic and ursolic acid are position isomers shown in Fig. 1.

Ursolic acid shows pharmacological properties like anti-inflammatory, hepatoprotective, antitumor, anti-HIV, antimicrobial, antifungal, antiulcer, gastroprotective, hypoglycemic and anti-hyperlipidemic activity.[3–6]

O. gratissimum (Lamiaceae) is a perennial, woody shrub that is a herbal medicine which has been practiced worldwide and distributed throughout India.[7] Traditionally, it is used in the treatment of diarrhea,[8,9] as a febrifuge and integral component of anti-malaria remedies,[10] mosquito/insect repellant, stomachic and general tonic, antiseptic in wound dressing, skin infections, conjunctivitis and bronchitis. '*Ocimum* tea', is dispensed as a remedy for fever and diaphoresis,[9] roots are used as sedative for children.[11]

Unfortunately, insufficient information is available concerning the distribution of ursolic acid in the *O. gratissimum* Linn (Lamiacea). The present study involves the determination of ursolic acid in both *O. gratissimum* leaves extracts and developed hepatoprotective tablet.

2. Material and methods

2.1. Apparatus

HPTLC system (Linomat 5, Camag, Switzerland) automatic sample applicator, TLC scanner IV (Camag), flat bottom and twin-

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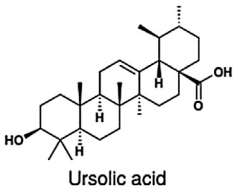

Fig. 1. Structure of ursolic acid.

trough developing chamber (15 × 10 cm), pre-coated silica gel aluminum plate (E. Merck, Darmstadt, Germany), electronic analytical balance, Shimadzu (AUX-220), micro syringe (100 μl) (Hamilton).

2.2. Reagents and standard

Ursolic acid was purchased from Yucca enterprises, Wadala, Mumbai and methanol AR grade from S.d. fine-Chem Ltd., Mumbai.

2.3. Plant materials

Polyherbal hepatoprotective tablet was prepared by using fractions obtained from alcoholic extracts of *Butea monosperma*, *Bauhinia variegata* stem bark and *O. gratissimum* leaves. All these ingredients were collected from Maliba Pharmacy College campus and were authenticated by Prof. Minoo H. Parabia, Department of Bioscience, Veer Narmad South Gujarat University, Surat. Voucher specimen (No: MPC/13032010/01, 02 and 03) has been deposited in the Department of Bioscience.

2.4. Extraction and fractionation

The powdered leaf of *O. gratissimum* was extracted with methanol at room temperature for seven days with shaking and stirring. Filtration was followed by evaporation of solvent using a rotary evaporator at low temperature and pressure. Crude methanolic extract was diluted with distilled water and the resultant mother solution was subjected to solvent–solvent partition using hexane, dichloromethane (DCM) and ethyl acetate (EtOAc).

2.5. Isolation of ursolic acid from polyherbal tablet

Weight accurately 500 mg equivalent of polyherbal tablet was transferred to the 10 ml volumetric flask and dissolved in 10 ml methanol. This solution was sonicated for 10 min and filtered through Whatman No. 1 paper to get solution containing 10 mg/mL. Concentrated solution obtained from polyherbal tablet was subjected to preparative TLC. Preparative TLC were performed on 20 cm × 10 cm TLC aluminum plate coated with 200 μm layer thickness of silica gel 60 F 254 using Petroleum ether:ethyl acetate:acetone (8.2:1.8:0.1, v/v/v) as mobile phase. After drying in a stream of warm air the plates were sprayed with 10% (v/v) sulfuric acid in ethanol, dried for 10 min and heated to 120 °C for 3 min. The quantification was carried out by densitometric scanning in absorbance mode at wavelength 530 nm. The silica in the respective marked area was scraped off and collected carefully in a test tube. The scraped silica, which contained the standard ursolic acid was extracted in methanol and filtered individually. The filtrates containing ursolic acid were evaporated under reduced pressure to obtain ursolic acid.

Melting point of ursolic acid was determined by open capillary method. Structural confirmation of the isolated ursolic acid was done by I.R. Spectroscopy and compared with that of standard.

2.6. Sample preparation

2.6.1. Preparation of standard solutions of ursolic acid

Stock solutions of ursolic acid were prepared by dissolving 20 mg ursolic acid in 100 ml of methanol (200 μg/mL). Standard solutions of concentration 400, 600, 800, 1000 and 1200 ng/ml were prepared by dilution of the stock solution with methanol.

2.6.2. Sample preparation from DCM and EtOAc fractions of O. gratissimum methanolic extract

Accurately weighed 100 mg of DCM and EtOAc fractions of *O. gratissimum* methanolic extract and polyherbal tablet were transferred to separate 10 ml volumetric flask and dissolved in 10 ml methanol. These solutions were sonicated for 10 min and filtered through Whatman No. 1 paper to get solution containing 10 mg/mL.

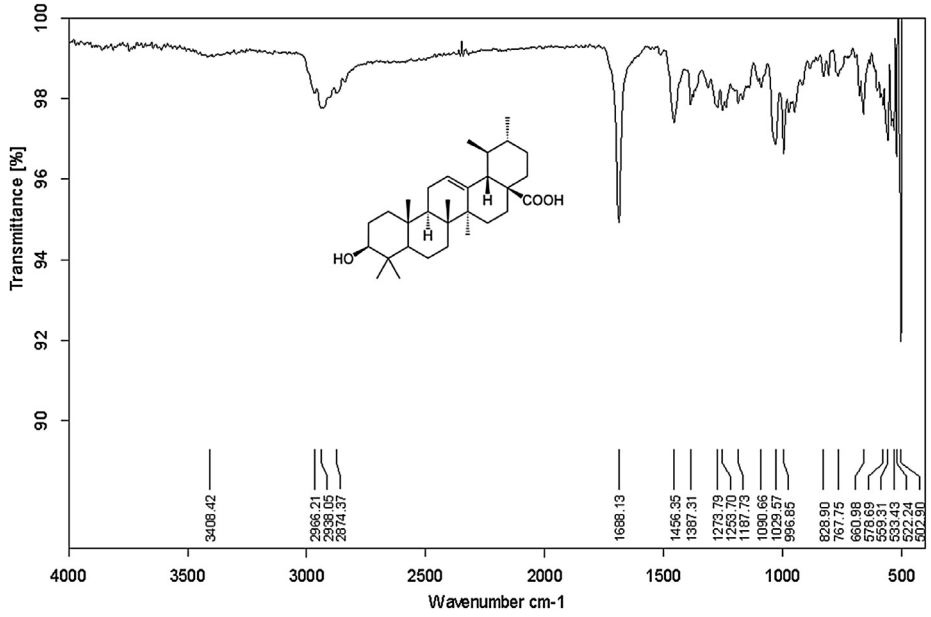

Fig. 2. IR spectrum of isolated compound (ursolic acid) from polyherbal tablet.

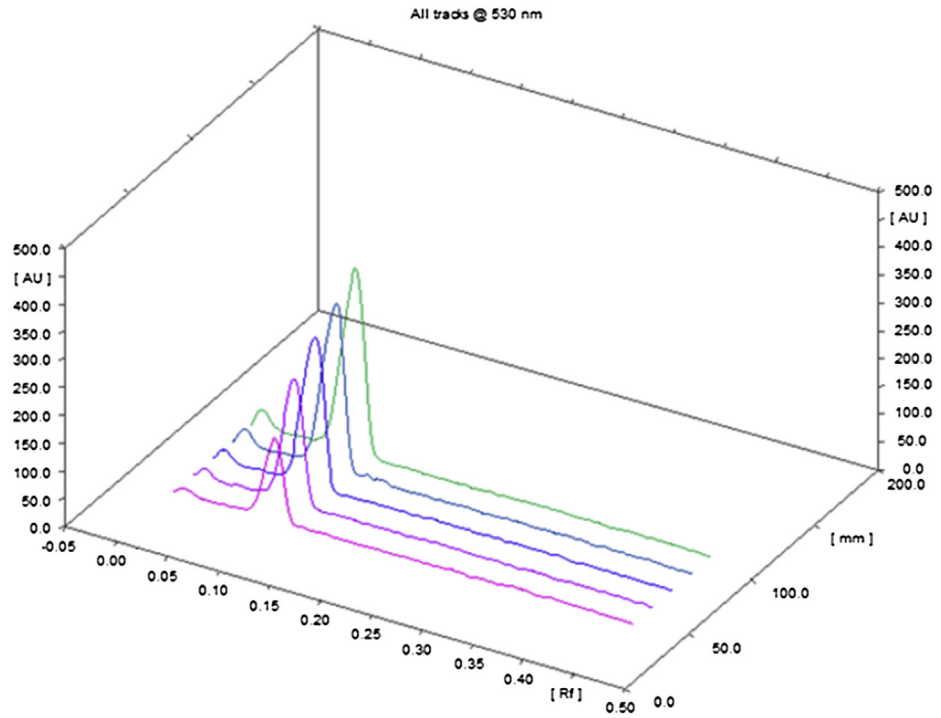

Fig. 3. 3D-Chromatogram of ursolic acid (200–1000 ng/spot).

2.6.3. Sample preparation from polyherbal tablet

Polyherbal tablet equivalent to about 100 mg of *O. gratissimum* extract was weighed and transferred to 50 ml volumetric flask containing 99.9% methanol. The resulting solution was centrifuged at 3000 rpm for 5 min and supernatant was analyzed for ursolic acid content.[12]

2.7. Chromatographic conditions for ursolic acid

Pre-coated HPTLC plates 20 cm × 10 cm were washed with methanol and dried in a stream of hot air before use. Two micro litres of standard solutions, 10 μL of sample solutions were spotted using an automatic applicator as 6 mm long streaks [track distance: 15.4 mm, distance from the left edge: 15 mm] and allowed to dry.

2.8. Calibration curve of ursolic acid

Different volumes of stock solution (200 μg/mL) were spotted on the TLC plate to obtain concentration 400, 600, 800, 1000, 1200 ng/spot of ursolic acid.

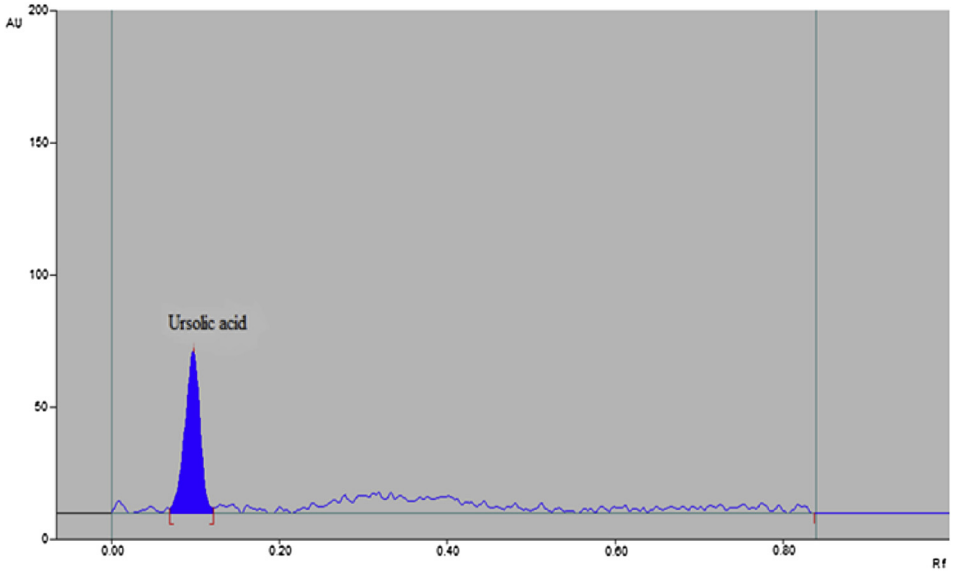

Fig. 4. Densitogram of ursolic acid (400 ng/spot) at 530 nm.

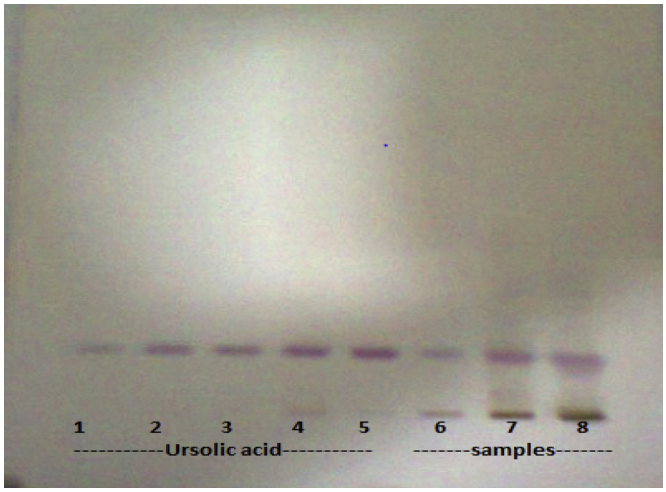

Fig. 5. Photograph of HPTLC plate: 1–5 (400–1200 ng/spot), calibration curve of ursolic acid, 6-polyherbal tablet, 7-dichloromethane, 8-ethyl acetate fractions of *Ocimum gratissimum* leaf.

2.9. Method validation

The proposed method was validated as per ICH guidelines.[13] Samples were prepared as per the earlier adopted procedure given in the experiment.

2.9.1. Linearity and range

Linearity is expressed in terms of correlation coefficient of linear regression analysis. The linearity response was determined by analyzing 5 independent levels of calibration curve in the range of 400–800 ng/spot of ursolic acid. The calibration curve of absorbance vs. concentration was plotted and correlation coefficient and regression line equations were determined.

2.9.2. Precision

Result of precision should be expressed as relative standard deviation (% R.S.D) or coefficient of variance (% C.V.).

2.9.2.1. Repeatability. Standard solutions were applied by Linomat 5 automatic sample applicator. Sample was spotted seven times for repeatability studies. The peak area obtained with each solution was measured and % C.V. was calculated.

2.9.2.2. Intraday precision. Mixed solution containing 600–1000 ng/spot of ursolic acid was analyzed three times on the same day and % C.V. was calculated.

2.9.2.3. Interday precision. Mixed solution containing 600–1000 ng/spot of ursolic acid was analyzed on three different days and % C.V. was calculated.

2.9.3. Accuracy

It was determined by calculating the recovery of ursolic acid by standard addition method.

Table 1
Linearity data for ursolic acid.

Ursolic acid (ng/spot)	Peak area
400	1205.47
600	2506.37
800	3904.28
1000	5126.22
1200	6122.73

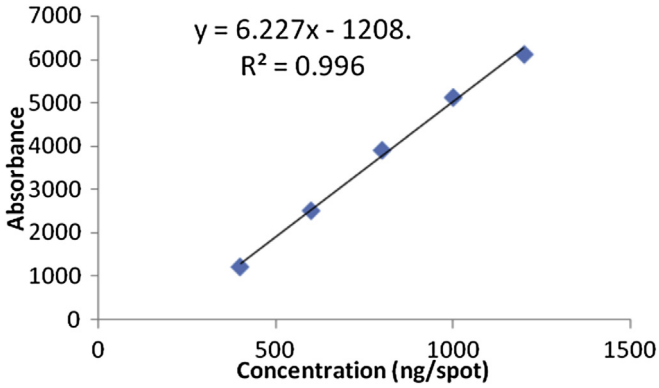

Fig. 6. Calibration curve for ursolic acid (400–1200 ng/spot).

Table 2
Repeatability data for estimation for ursolic acid.

Concentration (ng/spot)	Peak area
1000	5067.12
1000	5100.13
1000	5125.6
1000	5078.4
1000	5122.8
1000	5213.8
1000	5241.5
Average	5135.62 ± 66.84
% C.V.	1.30

2.9.4. Recovery studies

The accuracy of the method was established by performing recovery experiments at three different levels using the standard addition method. In 1 μL (1 μg/mL) of sample, known amounts of ursolic acid (400, 600, 800 ng per spot) standards were added by spiking. The values of percent recovery and average value of percent recovery for ursolic acid were calculated.

2.9.5. Limits of detection and limit of quantitation

The LOD and LOQ were estimated from the set of five calibration curves. The LOD and LOQ calculated as

$$LOD = 3.3 \times (SD/Slope)$$

$$LOQ = 10 \times (SD/Slope)$$

where,

SD = Standard deviation of the Y-intercepts of the five calibration curves.

Slope = Mean slope of the five calibration curves.

Table 3
Intraday precision data for estimation for ursolic acid.

Concentration of ursolic acid (ng/spot)	Peak area (±S.D.)	% C.V.
600	2455.59 ± 48.44	1.97
800	3884.37 ± 33.83	0.87
1000	5096.53 ± 29.82	0.58

Table 4
Interday precision data for estimation for ursolic acid.

Concentration of ursolic acid (ng/spot)	Peak area (±S.D.)	% C.V.
600	2551.84 ± 56.70	2.22
800	3904.55 ± 61.37	1.57
1000	5137.16 ± 75.38	1.46

Table 5
Recovery study for ursolic acid.

Concentration of ursolic acid in sample (ng/spot)	Amount of ursolic acid standard added (ng/spot)	Total concentration (ng/spot)	Mean concentration recovered (ng/spot)	% Recovery	% Recovery mean
200	200	400	198	99	97.50
200	200	400	195	97.5	
200	200	400	192	96	
200	400	600	387	96.75	98.16
200	400	600	396	99	
200	400	600	395	98.75	
200	600	800	582	97	98.22
200	600	800	596	99.33	
200	600	800	590	98.33	

Table 6
LOD and LOQ data for ursolic acid.

Mean slope (S)	6.227
S.D. of intercept (S)	−1208.
LOD (ng/spot)	5.99
LOQ (ng/spot)	18.17

Table 7
Estimation of ursolic acid in fractionated extracts of *Ocimum gratissimum* and tablet formulation.

Tracks	Rf values	Conc. (ng/10 mg)
Ursolic acid standards	0.10	–
Ursolic acid from EtOAc fraction of *Ocimum gratissimum*	0.10	399.55
Ursolic acid from DCM fraction of *Ocimum gratissimum*	0.10	372.66
Ursolic acid in polyherbal tablet (PTF)	0.11	255.14

3. Results and discussion

3.1. Identification of isolated compound from polyherbal tablet

Oleanolic and ursolic acids are position isomers.[14] The IR spectra of standard oleanolic acid and isolated compound from polyherbal tablet are displayed in Fig. 2 and were found to be comparable. Melting point of isolated compound was found to be 288 °C. IR spectra of isolated compound appears as a very intense absorption ribbon, around 3425 cm^{-1} due to OH group. A very intense absorption ribbon in between 2874 and 2966 cm^{-1}. At 1688 cm^{-1} appears a characteristic peak of carbonyl group (C═O). At 1456 cm^{-1} appears absorption ribbon from OH vibrations of planar distortion. At 1387 cm^{-1} appears a characteristic ribbon, which derives from C–H stretch and at 1108 cm^{-1} stretching vibrations of C–O group of carbonic acid. In this I.R. spectrum of isolated compound (ursolic acid) shown in Fig. 2, skeleton ring values are found to match with the reported values of oleanolic acid.[15]

3.2. Optimization of mobile phase

Various ratios of solvents were tried as a mobile phase and optimum mobile phase was selected was petroleum ether:ethyl acetate:acetone (8.2:1.8:0.1 v/v/v). This mobile phase allowed good resolution, dense, compact and well-separated spots at Rf value 0.10. Wavelength 530 nm for ursolic acid was used for quantification of the drug. Since there is only one peak seen, is shown in Fig. 3.

3.3. Quantification by HPTLC

3.3.1. Method development

In HPTLC chromatogram, all tracks for standard ursolic acid at wavelength 530 nm are shown in Figs 3 and 5. The Rf value of standard ursolic acid was found to be 0.10 and peak area was 2506.37 (Fig. 4).

3.3.2. Method validation

3.3.2.1. Linearity and range. Linearity was determined for ursolic acid at five concentration levels. The linearity was in the range 400–1200 ng/spot. Linearity data are depicted in Table 1 and Fig. 6. Correlation coefficient for calibration curve was 0.9960 and regression line equation for ursolic acid was $y = 6.227x - 1208$.

3.3.2.2. Precision. Precision considered at three level repeatability, interday and intraday precision.

3.3.2.2.1. Repeatability. The data for repeatability are shown in Table 2. The % C.V. for repeatability was found to be 1.30.

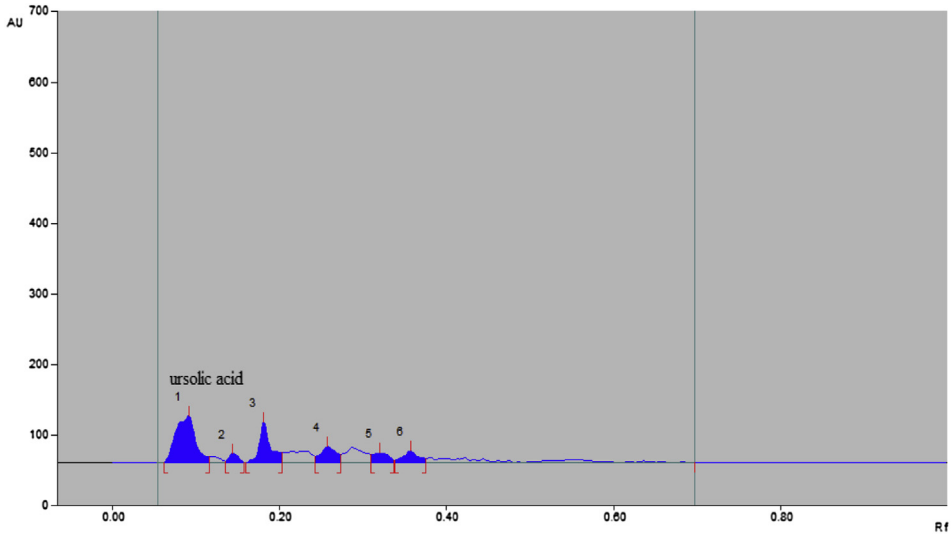

Fig. 7. Chromatogram of ethyl acetate fractions of *Ocimum gratissimum*.

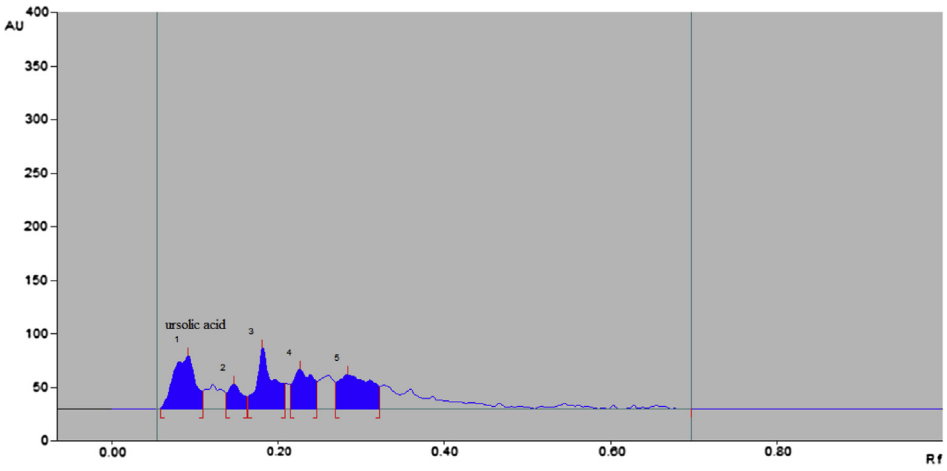

Fig. 8. Chromatogram of DCM fractions of *Ocimum gratissimum*.

3.3.2.2.2. Intraday precision. The data for intraday precision for ursolic acid are shown in Table 3. The % C.V. was found to be 0.58–1.97.

3.3.2.2.3. Interday precision. The data for interday precision for ursolic acid is shown in Table 4. The % C.V. for interday precision was found to be 1.46–2.22.

3.3.2.3. Accuracy. Accuracy of the method was confirmed by recovery at three level of standard addition. Percentage recovery for ursolic acid from DCM and ethyl acetate fractions of *O. gratissimum* and polyherbal hepatoprotective tablet was given in Table 5.

3.3.2.4. LOD and LOQ. Limit of detection and quantitation were determined by equation – LOD = 3.3 × (S/S) and LOQ = 10 × (S/S). LOD and LOQ results are shown in Table 6.

3.3.3. Estimation of ursolic acid in fractionated extracts of O. gratissimum and tablet formulation

The peak purity of ursolic acid from standard was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot. Good correlation (r^2 = 0.9960) was obtained between the standard and the samples of ursolic acid in the range 400–1200 ng/spot.

The identification of ursolic acid was done on the basis of Rf values. The concentrations of ursolic acid found using the presented method were found to be 399.55, 372.66 and 255.14 ng/10 mg in fractionated leaf extract of ethyl acetate fraction, dichloromethane fraction of *O. gratissimum* and polyherbal formulation respectively. The results are shown in Table 7.

Ethyl acetate fraction of *O. gratissimum* also showed six peaks, first peak of Ethyl acetate fraction Rf value 0.10 coincided with standard Rf value with peak area 1280 of standard (Fig. 7). The concentration of ursolic acid in Ethyl acetate fraction of *O. gratissimum* was found to be 399.55 (ng/10 mg).

Dichloromethane fraction also showed five peaks, the first peak with Rf value 0.10 coincided with standard Rf value with peak area was 1110 (Fig. 8). The concentration of ursolic acid in DCM fraction of *O. gratissimum* was found to be 372.66 (ng/10 mg).

Polyherbal tablet of PTF-2 formulation showed seven peaks. The Rf value (0.10) of third peak is coinciding with standard Rf value and its peak area was 935 (Fig. 9). The concentration of ursolic acid was found to be 344.53 (μg/10 mg).

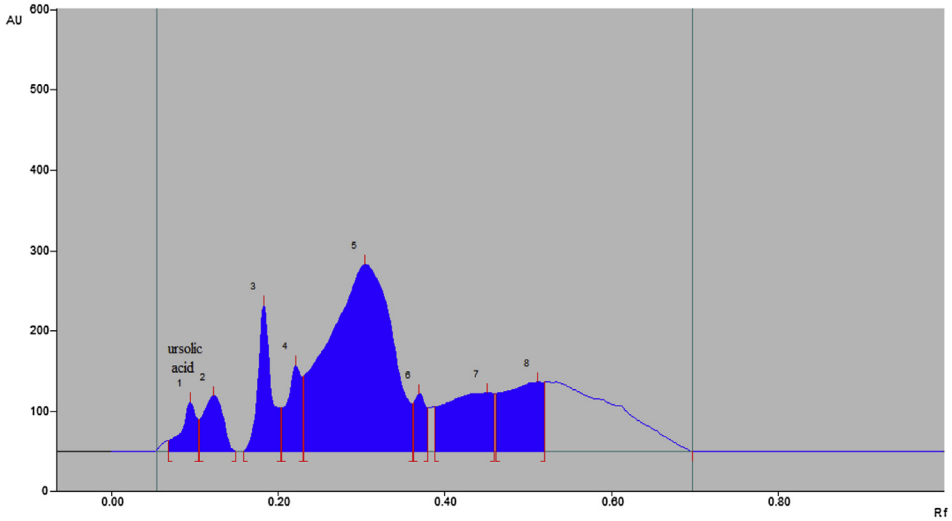

Fig. 9. HPTLC densitogram of polyherbal tablet showing ursolic acid.

Table 8
Summary of validation parameters.

Parameters	Result for ursolic acid
Linearity range	400–1200 (ng/spot)
Correlation coefficient	0.9960
Precision (% C.V.)	
Repeatability ($n = 7$)	1.30
Intraday precision ($n = 3$)	0.58–1.97
Interday precision ($n = 3$)	1.46–2.22
Accuracy (% recovery)	97.5–98.22
LOD (ng/spot)	5.99
LOQ (ng/spot)	18.17

3.3.4. Summary of validation parameters

The detailed summary of validation parameters is described in Table 8.

4. Conclusion

A good correlation was obtained among the standard, samples of polyherbal formulation and fractionated extract of *O. gratissimum*. An HPTLC method for quantitative estimation of ursolic acid present in fractionated leaf extract of *O. gratissimum* and polyherbal tablet has been developed and validated. The method can be used as an ursolic acid standard.

Conflicts of interest

All authors have none to declare.

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Original article

Investigating the pharmacological potential of *Iris kashmiriana* in limiting growth of epithelial tumors

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ABSTRACT

Background: *Iris kashmiriana*, a medicinal plant growing under Kashmir conditions, has been found very useful for pulmonary asthma, cancer, inflammation, liver and uterine diseases in traditional medicine. The medicinal importance of the plant prompted isolation of a variety of pharmacologically active compounds including quinones, triterpenoids, flavonoids, isoflavonoids and stilbene glycosides.
Objective: To evaluate the antiproliferative and antioxidant potential of methanolic extract of rhizomes of *I. kashmiriana*.
Material & methods: The effect of *I. kashmiriana* was evaluated against human epithelial cancer cell lines A549 and Caco-2 for their possible effect on cell proliferation. Free radical scavenging activity was tested by DPPH assay against known antioxidant Vitamin C. The extract was also analyzed for active components by RP-HPLC.
Results: The extract showed potent cytotoxic effect on both epithelial cell lines at all the tested concentrations with significant effect at 400 μg/ml ($IC_{50\ (A549)} = 128.7$ μg/ml; $IC_{50\ (Caco\text{-}2)} = 237.76$ μg/ml) as evaluated by MTT assay. The effect was, however, less pronounced on mouse fibroblast cell line NIH-3T3 ($IC_{50\ (NIH\text{-}3T3)} = 1182.92$ μg/ml), indicative of possible cell specific activity against epithelial cancers. Furthermore the free radical scavenging activity as verified by DPPH assay revealed that the methanolic extract of *I. kashmiriana* has strong antioxidant potential. HPLC analysis showed respectable amount of *Irigenin* and *Tectorigenin* present in the extract.
Conclusion: The results demonstrate pharmaceutical potential of *I. kashmiriana* for treatment of epithelial cancers and other inflammatory diseases.

1. Introduction

Plants are the main source of active principles for the treatment of diseases. The WHO reported that despite the availability of synthetic drugs, the big percentage of world's population relies on plant based therapies to cover the needs of primary health care.[1] Medicinal plants have got a priority position in the drug discovery and many modern day medicines have taken leads from the traditional use of medicinal plants. Despite major advances in molecular modeling, the medicinal plants remain an important source of new drugs and new drug leads.[2,3] Hence, for the treatment of disease states wherein drug therapy is a rational approach, plant materials represent legitimate starting materials for the discovery of new agents. In case of human cancers, many plant derived compounds have been approved for clinical use as anticancer drugs in the United States such as vinblastine, vincristine, vinorelbine, etoposide, teniposide, paclitaxel, docetaxel, topotecan, and irinotecan.[4–6]

Oxidative stress is defined as an imbalance between production of free radicals and reactive metabolites, so-called oxidants or reactive oxygen species (ROS), and their elimination by protective mechanisms, referred to as antioxidants. Free radicals, produced as a result of normal biochemical reactions in the body, play important role in the human body and become harmful only when they are produced in high amounts. Free radicals are implicated in wide spectrum of diseases such as cancer, atherosclerosis, aging,

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immunosuppression, inflammation, ischemic heart disease, diabetes, hair loss, and neurodegenerative disorders like Alzheimer's disease and Parkinson's disease.[7–9] Oxidative stress and cancer are highly interlinked. Cancer is a leading cause of death and may result from chronic injury to the epithelium by oxidants and other carcinogens. Under a sustained environmental stress, ROS over production may cause significant damage to cell structure and functions and may induce somatic mutations and neoplastic transformation.[10,11] Indeed, cancer initiation and progression has been linked to oxidative stress by increasing DNA mutations or inducing DNA damage, genome instability, and cell proliferation.[12] The human body possesses innate defense mechanisms to counter free radicals in the form of enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. However, these endogenous systems are often insufficient for complete scavenging of ROS. Vitamin C, vitamin E, selenium, β-carotene, lycopene, lutein and other carotenoids have been used as supplementary antioxidants. Plant secondary metabolites such as flavonoids and terpenoids also play an important role in the defense against free radicals.[13–15]

The *Iris* plant belonging to family *Iridaceae* is worldwide in distribution, known for their ornamental relevance and medicinal value. The species of the genus *Iris* are very useful for pulmonary asthma, cancer, inflammation, liver and uterus diseases.[15] The intensive phytochemical investigations of various iris species have resulted in the isolation of a variety of compounds including quinones, triterpenoids, flavonoids, isoflavonoids and stilbene glycosides.[16] Flavonoids and isoflavonoids are important plant secondary metabolites with structural diversity and are consumed by human as dietary constituents.[17] The isoflavone rich dietary consumption is reported to reduce risk of cancer particularly breast and prostate cancer.[18,19] The role of isoflavones in cancer[18,20] osteoporosis, cardiovascular diseases and menopausal symptoms in addition to their antioxidant[21] antimicrobial[22] anti-inflammatory and estrogenic activities[18,23] is well documented. The current study investigated the effect of methanolic extract of *Iris kashmiriana*, a local medicinal plant against human epithelial cell lines to evaluate its anticancer potential.

2. Materials & methods

2.1. Plant material

I. kashmiriana was collected in June 2011 from *Naranag* area of Jammu and Kashmir. The voucher specimen has been kept in the herbarium of the Indian Institute of Integrative Medicine (CSIR) Srinagar with voucher specimen (No. I001/2010-I005/2010).

2.2. Extraction

The whole plant was dried under shade. The air dried plant was chopped and cut into small pieces and then grinded to the powdered form. The powdered material of the plant was soaked in methanol for 48 h. The extract was separated by filtration and evaporated to dryness on rotary evaporator under reduced pressure, which afforded 98 g of MeoH extract. The methanol extract was kept at −20 °C until use.

2.3. Phytochemical screening

The phytochemical screening was done using various standardized chemical tests:

2.3.1. Test for flavonoids

5–10 drops of dilute HCl followed by a piece of Zn or Mg were added to the test tube containing 0.5 ml of methanolic plant extract. The solution was boiled for few minutes. Presence of flavonoids is confirmed from the change in color to pink or reddish pink.

2.3.2. Test for phenols (ferric chloride test)

Small quantity of alcohol or aqueous extract is dissolved in 2 ml of distilled water. Few drops of 10% aqueous ferric chloride solution are added to the extract. The blue or green color indicates the presence of phenols.

2.3.3. Test for saponins

The extract is mixed with some drops of sodium bicarbonate and shaken vigorously. Heavy comb-like froth formation confirms the presence of saponins.

2.3.4. Test for glycosides

Small quantity of extract was added to 1 ml NaOH solution; yellow color indicates the presence of glycosides.

2.3.5. Test for tannins (ferric chloride test)

To 1–2 ml of extract add few drops of 5% aqueous ferric chloride solution, a bluish black color is produced, which disappears on addition of dilute sulfuric acid solution and formation of yellowish brown precipitate indicates the presence of tannins.

2.4. Cell culture

Cell lines A549 (human lung adenocarcinoma), Caco-2 (human colon adenocarcinoma) and NIH-3T3 (mouse non-neoplastic fibroblast) were kindly provided by Hybridoma Lab., National Institute of Immunology, New Delhi. Cells were maintained in Dulbecco's minimal essential medium (DMEM) or RPMI supplemented with 10% fetal bovine serum, 100U/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO_2. The medium was changed every two days or until the cells became confluent and then used for the experimentation.

2.4.1. Proliferation assay

MTT assay was used as a standard test for determining the effect of methanolic extracts of *I. kashmiriana* on cell proliferation. MTT assay is simple and reliable technique, which measures cell viability and is widely used for the screening of anticancer agents. The methanolic crude extract of *I. kashmiriana* was dissolved in DMSO (dimethyl sulphoxide), filter sterilized and then further diluted to attain required concentrations. Cell suspension containing 2×10^4 cells per well was seeded into a 96 well microtiter plate. After 24 h of seeding, cells were treated either with extract (at different concentration) or DMSO alone. The DMSO served as solvent control. Each concentration was tested in triplicate. The cells were incubated at 37 °C in a humidified incubator with 5% Co_2 for 24 or 48 h. MTT solution was added to the cells at 0.1 mg/ml concentration followed by incubation for 4 h at 37 °C in dark. The supernatant was removed and an equal volume of DMSO was added to dissolve the formazan crystals. The absorbance was measured at 565 nm (EPOCH Microplate Reader, Bio-Tek Instruments, USA).

Percentage proliferation inhibition was calculated as:

$$\frac{(\text{O.D Control} - \text{O.D Test})}{(\text{O.D Control})} \times 100.$$

IC_{50} values were calculated after 48 h of treatment.

2.4.2. DPPH assay

In this assay, free radical scavenging activity of crude methanolic extract of *I. kashmiriana* was determined from the bleaching of

purple colored methanolic solution of DPPH.. The assay comprised of 1 mg/ml of DPPH in presence and absence of test material. The plate was then incubated at 37 °C for 1 h. After incubation, absorbance was read at 515 nm. From the absorbance, the free radical scavenging activity of each crude leaf extract relative to that of ascorbic was computed using the following:

$$\frac{(\text{O.D Control} - \text{O.D Test})}{(\text{O.D Control})} \times 100.$$

Discoloration of DPPH was taken as the indicator of antioxidant activity.

2.5. HPLC analysis

2.5.1. Sample preparation

The stock solution of extract and the standard were obtained by dissolving 5 mg of the extract in 1.0 ml MeOH and 1.0 mg of the standard in 1.0 ml MeOH respectively. The resulting solutions were filtered through 0.45 μm filter membrane. The appropriate concentration of working solutions was prepared by diluting stock solutions. The stability of stock as well as working solutions was monitored and found that the concentration of the solution does not vary.

2.5.2. Optimization of HPL C conditions

The HPLC conditions were optimized using the standard solution of marker compounds (*Irigenin* and *Tectorigenin*) and the solution of *I. kashmiriana* sample. Resolution of the peaks was tested and compared using different solvent systems. The separation of *Irigenin* and *Tectorigenin* was achieved on RP-18e column using methanol: water at a flow rate of 0.6 ml/min with run time of 55 min. The HPLC analysis was carried out by using photodiode array detector (DAD) and the spectra recorded at an optimum wavelength of 265 nm.

2.5.3. Calibration curves and recovery

The stock solutions of the reference compounds *Irigenin* and *Tectorigenin* was prepared and diluted to appropriate concentration for the construction of calibration curves. A minimum of four concentrations of *Irigenin* and *Tectorigenin* were injected in the concentration range of 0.08 μg/ml to 0.9608 μg/ml. The calibration curve was constructed by plotting the peak area versus the concentration of each analyte with detector wavelength set at 265 nm. Excellent calibration curve was obtained for the standard within the concentration range of 0.08 μg–0.96 μg/μl (r^2 = curve coefficients > 0.998). The recovery study was within the concentration range of the calibration curve. The recovery of the method was estimated by spiking *I. kashmiriana* sample with 2.0 μg/3.8 mg of the standard *Irigenin* and *Tectorigenin*. The data from the recovery studies revealed that the recovery of the method was in the range of 98.2–101.2% evaluating the accuracy of the method.

2.5.4. Quantification of Irigenin and Tectorigenin in different samples of I. kashmiriana

The developed RP-HPLC method coupled with UV-DAD separated *Irigenin* and *Tectorigenin* in all the tested samples, as depicted by chromatograms of the different extracts (IK-1 and IK-2) as well as the respective standard as shown below. *Irigenin* and *Tectorigenin* content were determined in the different extracts by comparing the retention time and UV spectra with those obtained by injecting standard in the same conditions.

2.5.5. Chromatographic conditions

HPLC analysis was performed on a Schimadzu Class VP HPLC system equipped with a binary pump (LC-10AT), an autosampler

Table 1
Phytochemical screening of methanolic extract of *Iris kashmiriana*.

Class of compounds	Present (+) or absent (−)
Flavonoids	+
Phenols	+
Saponins	−
Glycosides	+
Tannins	+

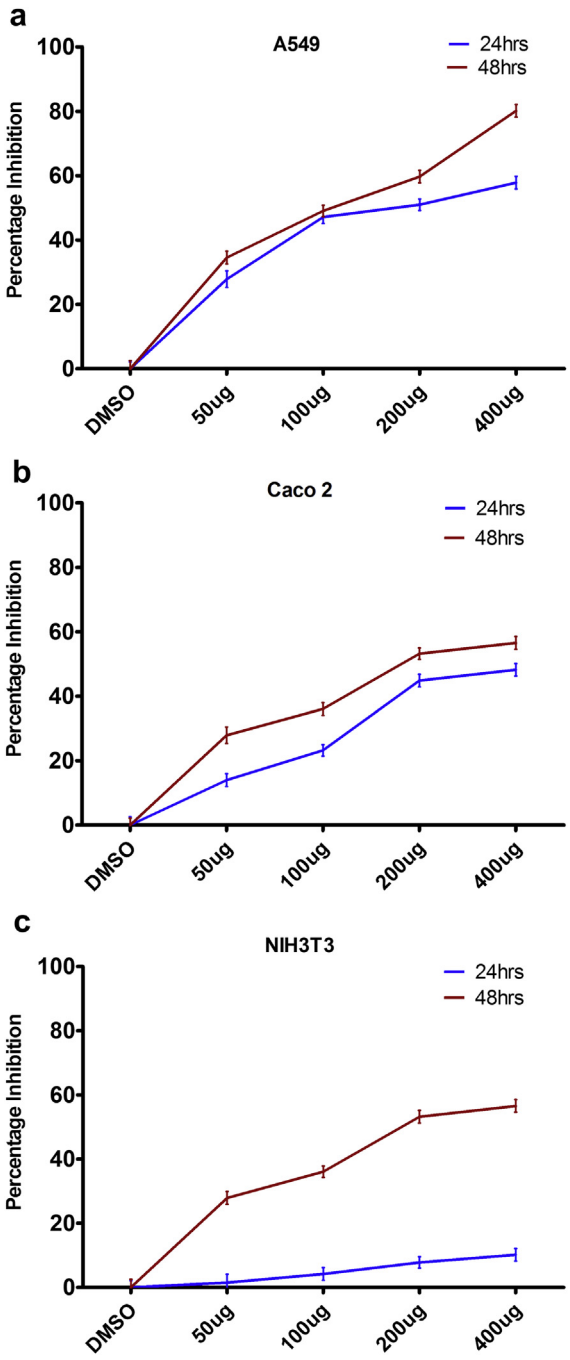

Fig. 1. Antiproliferative effect of methanolic extract of *Iris kashmiriana* on (a) A549 cell line, (b) Caco-2; (c) NIH-3T3. The values represent the mean percentage inhibition ± SD compared to the control ($N = 3$).

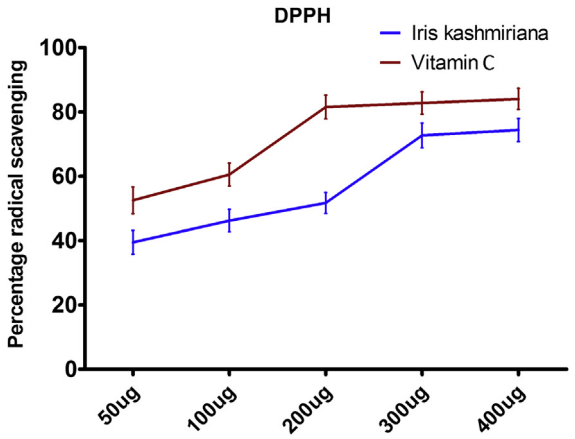

Fig. 2. Free radical scavenging activities relative to ascorbic acid of the crude methanolic extracts of rhizomes of *Iris kashmiriana* using the DPPH (1,1-diphenyl -2-picryl hydrazyl) assay. Each value represents the mean of three trials with three replicates per fraction per trial. Bars represent standard deviations.

(SIL-10AD), a column oven (CTO-10AS), vacuum membrane degasser (DGU-14A), a Diode array detector (SPD-M10A), and a system integrator (SCL-10A) controlled by a Class VP software which was used for data analysis and processing. Separation was carried out on a Chromolith RP-18e column (5 μm; 4.6 mm × 100 mm) with column oven temperature of 30 °C using an isocratic solvent system consisting of methanol and water (30:70; v/v). Elution was performed at a flow rate of 0.6 ml/min and the injection volume was 5.0 μl. The analytes were monitored at 265 nm.

3. Results

3.1. Phytochemical screening

The photochemical screening of the methanolic extract of *I. kashmiriana* showed the presence of flavonoids, phenols, glycosides and tannins (Table 1). The plant material tested negative for the alkaloids. These studies indicate that *I. kashmiriana* is the rich source of flavonoids and isoflavonoids.

3.2. Effect of methanolic extract of I. kashmiriana on cell proliferation

The antiproliferative activity of the extract were studied by MTT assay against human lung adenocarcinoma cell line (A549), human colon adenocarcinoma cell line (Caco-2) and mouse non-neoplastic fibroblast cell line (NIH-3T3). The methanolic extracts of *I. kashmiriana* showed significant activity (Fig. 1a, b) in inhibiting the growth of both adenocarcinoma cancer cell lines, A549 (IC_{50} = 128.7 μg/ml) and Caco-2 (IC_{50} = 237.76 μg/ml), in a dose dependent manner (50–400 μg/ml). The inhibition was, however, less pronounced on mouse fibroblast cell line NIH-3T3 (IC_{50} = 1182.92 μg/ml) with the percentage inhibition of only 20% (Fig. 1c) at maximal concentration (400 μg/ml). The differential effects of methanolic extract against various cell lines may be due to their varied origins, however, the possibility of selective action on cancerous cell lines cannot be ruled out. The results thus indicate that the extract contains promising antiproliferative agents that need to be tested as potential anticancer therapeutic drugs.

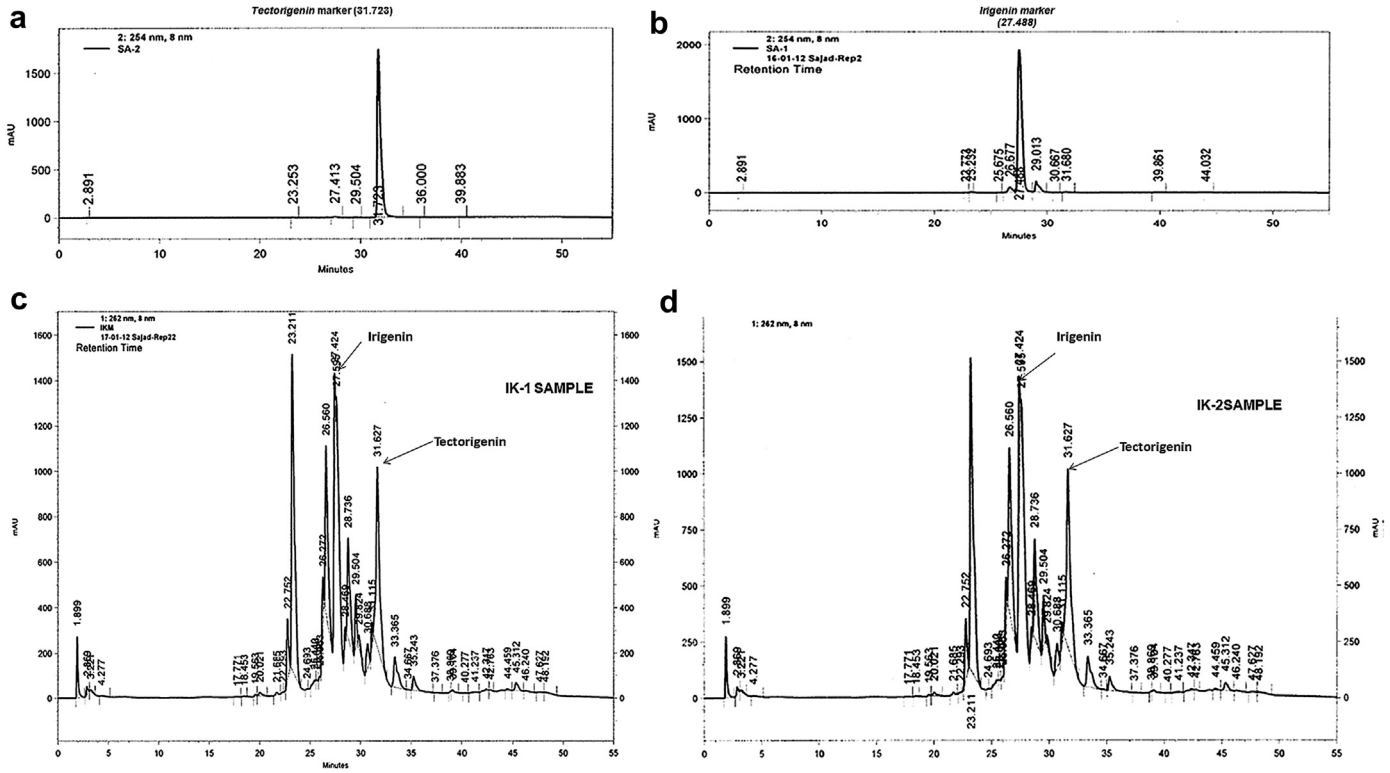

Fig. 3. HPLC profile of methanolic extracts of *Iris kashmiriana* against marker compounds *Tectorigenin* and *Irigenin*: (a) *Tectorigenin* showing peak at 31.723; (b) *Irigenin* showing peak at 27.488; (c) IK-1 sample showing peaks for *Tectorigenin* and *Irigenin*; (d) IK-2 sample showing peaks for *Tectorigenin* and *Irigenin*.

3.3. Antioxidant potential of methanolic extract of I. kashmiriana

Antioxidant properties, especially radical scavenging activities, are very important due to the deleterious role of free radicals in biological systems. The DPPH radical has been widely accepted as a tool for estimating free radical scavenging activities of various compounds and plant extracts. The extract showed a profound capacity to scavenge free radicals. The activity was comparable to known antioxidant, Vitamin C, indicating higher content of antioxidants present in the extracts. The oxidative potential was found to be dose dependent with the maximum effect at 400 μg (Fig. 2). The above activity can be attributed to redox properties of phenolic or flavonoid compounds present in the extracts which play an important role in absorbing and neutralizing free radicals and hence terminating the free radical chain reaction.

3.4. HPLC analysis

The extracts were also analyzed by HPLC for the presence of active components (Fig. 3). The extract showed good amount of *Irigenin* (4.80–6.13 mg/ml) and *Tectorigenin* 4.13–5.80 mg/ml) from two entirely different geographical locations (Table 2). The data depicted that the concentration of *Irigenin* and *Tectorigenin* was greatly variant among the same species collected from different eco-geographical regions. The concentration of *Irigenin* and *Tectorigenin* was highest in sample collected from *Naranag* (Forest area) than that collected from a local grave yard. The content of *Irigenin* was 6.13 mg/ml in IK-1 sample and 4.80 in IK-2 sample and that of *Tectorigenin* was 5.80 mg/ml in IK-1 sample and 4.13 mg/ml in IK-2 sample.

4. Discussion

Iris plant has been time tested for its remedial properties in cure of diseases wide range. The present study was carried out to evaluate the anticancer potential of the crude methanolic extract of *I. kashmiriana* on human cancer cell lines A549 (lung adenocarcinoma), Caco-2 (colon adenocarcinoma) and mouse non-neoplastic fibroblast cell line NIH-3T3 at different concentrations of the extract. Phytochemical screening of the methanolic extract showed presence of mostly phenolic compounds attributing strong antioxidant, anticancer and pharmacological properties to the extract. The extracts showed significant growth inhibition of adenocarcinoma cell line (A549) and colon adenocarcinoma (Caco-2) in a dose dependent manner. Furthermore, less pronounced effect on mouse fibroblast cell line (NIH-3T3) suggests cell-type specific effect of the extract.

The free radical scavenging activity is a crucial mechanism to protect cells against oxidative stress especially in case when the internal enzymes don't suffice. Several complementary and advanced assays are necessary in order to confirm the *in vitro* antioxidant potential of a particular extract or compound. In DPPH assay a dose dependent scavenging of the free radicals was observed, at par with the known antioxidant Vitamin C. The high radical scavenging activity of the crude extract indicates presence of potent antioxidants compounds. HPLC analysis revealed presence of two active compounds – *Irigenin* and *Tectorigenin*. This stresses upon the need for further purification for isolation and characterization of other constituents of the plant extract of pharmacological relevance.

Table 2
HPLC quantification of *Irigenin* and *Tectorigenin* in methanolic extract of *Iris kashmiriana* from two different eco-geographical locations.

	Sample	Content of *Irigenin* (μg/ml)	Content of *Tectorigenin* (μg/ml)
IK-1	*Iris kashmiriana* (from *Naranag* Kashmir)	**6.13**	**5.80**
IK-2	IK-1 (from local grave yard *Srinagar*)	**4.80**	**4.13**

Bold values indicate significant differences in the values of the compounds isolated from plants from two different geographical locations.

5. Conclusion

Our results demonstrate that methanolic extract of *I. kashmiriana* exhibits selective antiproliferative activity against epithelial cancer cell lines. The potent antioxidant nature of the extract adds to its anticarcinogenic potential suggesting a promising role in development of anticancer therapeutics in future. However, these findings warrant extensive studies to evaluate mechanistic action of antiproliferative and antioxidant principles of the plant.

6. Author contributions

AA, SHW, RAQ, QPH: concept design, **SHW**: literature search; **AA,SHW:** experimental studies; **AA, SHW:** data acquisition; **TAM,SB:** data analysis; **JIM,AHW:** statistical analysis; **AA,SHW,-TAM:** manuscript preparation; **RAQ,JIM,AHW**: manuscript editing; **RAQ, QPH**: manuscript review.

Conflicts of interest

All authors have none to declare.

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Review article

A review on phytochemical and pharmacological potential of genus *Chelidonium*

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Chelidonium

ABSTRACT

Many herbal remedies have so far been employed for the treatment of various ailments since the beginning of human civilization. *Chelidonium* is the smallest genus of family Papaveraceae, occurring in Europe and Asia. This review is intended to integrate traditional ethno-medical knowledge and modern scientific findings about *Chelidonium majus* in order to promote understanding of its therapeutic actions as well as its toxic potential. Through this review, the authors hope to attract the attention of natural product researchers throughout the world to focus on the unexplored potential of *Chelidonium* genus. An exhaustive literature survey revealed that alkaloids, flavonoids and phenolic acids constitute major classes of phytoconstituents of the genus. A few species of this genus have medicinal value, among these, *C. majus* Linn. (Papaveraceae) has been traditionally used in the treatment of skin diseases such as eczema, ringworm, oral infection, pains and nervous disorders. *C. majus* has also been included in homeopathic formulations which are in clinical use. Ukrain, a thiophosphate derivative of alkaloids from *C. majus*, exerts cytotoxic and cytostatic effects on tumor cells, simultaneously acting as an immune response modifier. *C. majus* seems to hold great potential for in-depth investigation for various biological activities, especially on central nervous system.

1. Introduction

The use of plants as medicines or tonics goes back to prehistoric times and has attracted the interest of scientists for centuries. Medicinal herbs are the local heritage with global importance. Owing to the global trend towards improved "quality of life", there is considerable evidence of an increase in demand for medicinal plants.[1] India has a rich source of a wide variety of plants having medicinal value. These plants are widely used by all sections of society whether directly as folk remedies or indirectly as pharmaceutical preparations of modern medicine.[2]

The present review emphasizes ethnopharmacology, morphology, phytoconstituents, pharmacological reports, clinical studies and toxicology of the prominent species of *Chelidonium*. Through this review, authors hope to attract the attention of natural product researchers throughout the world to focus on the unexplored potential of the *Chelidonium* species. This genus needs to be investigated systematically so that potential species can be exploited as therapeutic agents.

1.1. The genus Chelidonium

Chelidonium, the smallest genus of about 30 species of the family Papaveraceae, is distributed throughout Europe and Asia. The members of *Chelidonium* are herbaceous.[3,4] *Chelidonium majus* commonly known as Greater Celandine, is widely distributed in Europe and Western Asia where it grows on hedge banks.[5]

1.2. Ethnopharmacology

The use of *C. majus* Linn. in traditional medicine has been well known for many centuries. *C. majus* has been traditionally used as a herbal medicine for the treatment of gastric cancer, oral infection, liver disease and general pains.[6] The commercial drug (Herba *Chelidonii*) consists of the dried aerial parts harvested when blooming.[7,8] *Chelidonii* herba has been described in several European pharmacopoeias. In France and Germany, *C. majus* extracts are traditionally used for the treatment of various diseases. In the German market there are about a dozen OTC preparations with cholagogue and choleretic activities.[9] The extracts of *C. majus* are

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proven to be safe as components of veterinary and human phytopreparations.[10]

C. majus is used in various complementary and alternative medicine (CAM) systems, including homeopathy, mainly in combating diseases of the liver,[11] stomach and various skin disorders.[12]

1.3. Morphology

C. majus is a perennial herbaceous plant growing wild in both Europe and America, recognized readily by its pinnate leaves, small peduncled yellow flowers and yellow opaque juice. Roots several headed, branched, reddish brown; stem 50 cm long, light green, hairy; leaves 15 cm long, thin, petiolate; flowers in small, long peduncled umbels with two sepals and four yellow petals, each 1 cm long (Fig. 1); fruit linear, capsule two valved and many seeded; seeds small, black and possess an elaiosome which attracts ants to disperse the seeds.[3] The fresh plant contains a saffron colored milky juice, and has an unpleasant odor and acrid taste. The flowers appear from May to July.[3,13]

1.4. Microscopic characteristics

The transverse section of the stem is circular, covered by a thin cuticle. The epidermal cells have very thick walls. One or two layers of thin-walled chlorenchymatous hypodermis partly transformed into collenchyma. Cortex consists of polygonal, very thick-walled cells (sclerenchyma). Very few thin-walled 5–10 cells long, uniseriate covering trichomes, are present along with few anomocytic stomata.

The petiole has stem-like structure. The chlorenchyma is almost entirely transformed in collenchyma. There are multicellular, very long covering trichomes. Leaf epidermis has sinuous anticlinal walls; very few covering trichomes, uniseriate with 5–10 thin-walled striated cells. Stomata anomocytic, exclusively on the lower epidermis. Mesophyll is differentiated into a layer of palisade and two layers of spongy parenchyma made up of thin-walled chlorenchyma. Midrib consists of 1–2 layers of collenchyma and thin-walled parenchymatous ground tissue. Calcium oxalate crystals absent. The latex cells are present especially in the vascular region. Pollen grains spherical, with a finely pitted 3-furrowed exine, about 35 μm in diameter.[14–18]

1.5. General identity tests

Under ultraviolet light (365 nm) without treatment, the thin-layer chromatogram of Herba *Chelidonii* usually shows 2 or 3 blue and yellow-green fluorescent zones at R_f approximately 0.2; directly above this is the narrow yellow-green zone of chelerythrine, followed by the tailing yellow zone of sanguinarine. The pale white fluorescent zone of protopine is located in the same position as standard berberine.[19]

Fig. 1. Morphology of *Chelidonium majus*.

Table 1
Various physicochemical parameters.

Parameter	Value (%)
Foreign organic matter	NMT 1
Total ash	NMT 13
Acid-insoluble ash	NMT 2
Water soluble extractive	NLT 20
Loss on drying	NMT 10

NMT = Not more than; NLT = Not less than.

1.6. Physicochemical parameters

Table 1 shows the values of various physicochemical parameters.[14,16,20]

1.7. Phytoconstituents

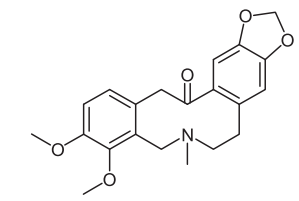
Exhaustive survey of literature revealed that only three species of *Chelidonium* have been investigated phytochemically. Table 2 summarizes the phytoconstituents of different species of *Chelidonium*. Structures of some of the constituents reported from various *Chelidonium* species are shown in Fig. 2.

1.8. Pharmacological reports

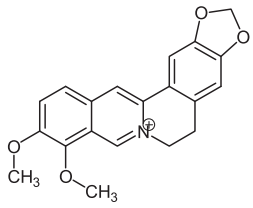
The available literature reveals that amongst 30 species of *Chelidonium*, only one species, i.e., *C. majus* has been evaluated for its pharmacological activity. The plant exhibits multiple biological actions such as antiviral,[44] antitumor,[45] antibacterial/antifungal[46] and anti-inflammatory effects.[47] In the following part these

Table 2
Phytoconstituents of different species of *Chelidonium*.

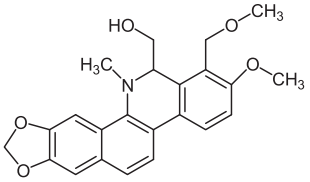
Species	Phytoconstituents
C. majus	**Alkaloids:** stylopine, chelidonine, 6-methoxydihydrochelerythrine, 6-methoxydihydrosanguinarine, dihydrosanguinarine, 8-oxocoptisine, canadine, protopine, allocryptopine, coptisine,[21] magnoflorine, oxysanguinarine, dihydrochelerythrine, dihydrochelilutine, dihydrochelirubine, *N*-methyl-9,10-dihydroxysanguinarine, stylopine β-metho-hydroxide, sanguinarine, berberine, corysamine,[22] norchelidonine,[23] isochelidonine,[24] homochelidonine, chelamine, chelamidine,[25] turkiyenine,[26] tetrahydrocoptisine,[27] scoulerine, *N*-methyl-stylopinium, sanguilutine, macarpine, sparteine, corytuberine[28]; **Non-alkaloidal constituents:** choline, histamine, tyramine, a saponin[29]; **Flavonoids:** rutin, quercetin, luteolin, hyperosid; **Phenyl carboxylic acids:** gallic acid, chlorogenic acid[30]; **Unsaturated fatty acids:** linoleic acid, oleic acid[31]; **Carotenoids/chlorophyll pigments:** neoxanthin, violaxanthin, zeaxanthin, lutein 342, α and β-cryptoxanthin, β-carotene 437, chlorophyll a 745 & chlorophyll b 481,[32] chelidoxanthin[33]; **Alcohol:** celidoniol[34]; **Mineral elements:** Al, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, S[35]; **Proteins**[36]; **Proteolytic enzymes**.[13,20,37–41]
C. japonicum	Sanguinarine derivatives, norsanguinarine, norchelerythine, protopine, bocconoline, sanguinarine, chelerythrine, choline, α-spinasterol.[42]
C. sinense/*C. majus* var. *asiaticum*	**Alkaloids:** stylopine, chelidonine, oxysanguinarine; **Triterpenoids:** 6α-hydroxy-A-neo-germacer-22(29)-en-30-oic acid, hop-22(29)-en-30-oic acid.[43]



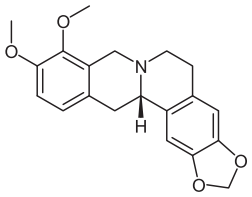
Allocryptopine



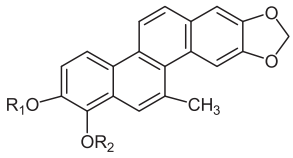
Berberine



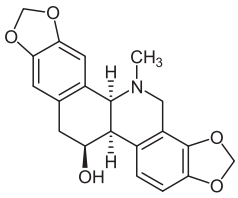
Bocconoline



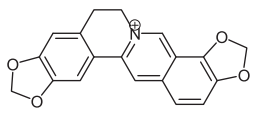
Canadine



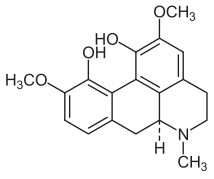
Chelerythrine R_1, $R_2 = CH_3$



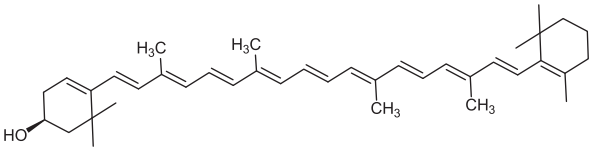
Chelidonine



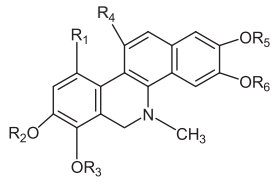
Coptisine



Corytuberine

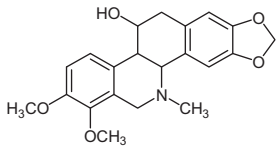


Cryptoxanthin

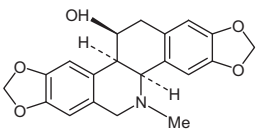


R_1=OMe, R_4=H, R_2=R_3=Me, R_5=R_6=CH_2 Dihydrochelilutine

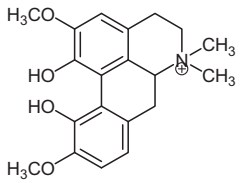
R_1=OMe, R_4=H, R_2R_3=R_5=R_6=CH_2 Dihydrochelirubine



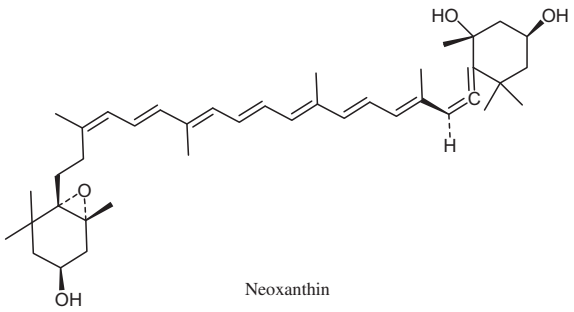
Homochelidonine



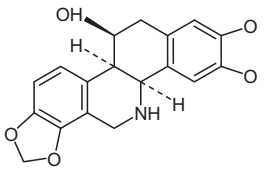
Isochelidonine



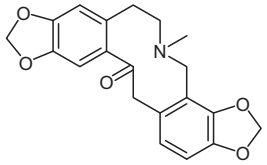
Magnoflorine



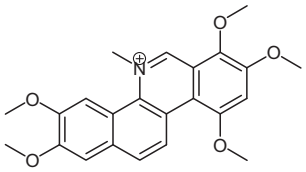
Neoxanthin



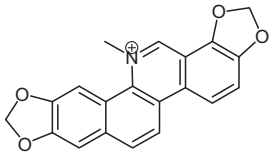
Norchelidonine



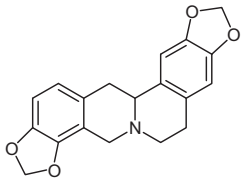
Protopine



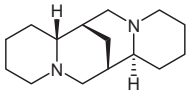
Sanguilutine



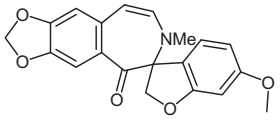
Sanguinarine



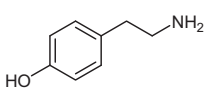
Stylopine



Sparteine



Turkiyenine



Tyramine

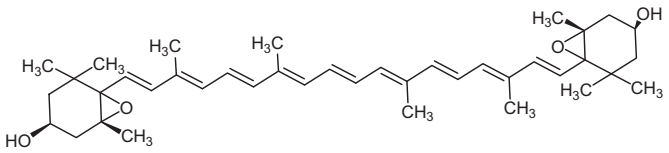
Fig. 2. Structures of some of the constituents reported from genus *Chelidonium*.

Fig. 2. (*continued*).

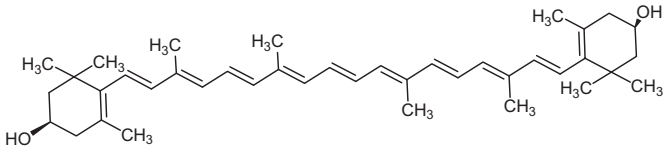
activities have been described with regard to the crude extract, the single alkaloids and the other non-alkaloid components of the plant.

1.9. Antiviral effects

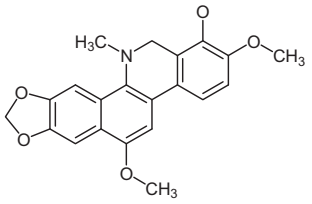
The effects of *C. majus* extracts against influenza virus have been studied *in vitro* and *in vivo*. Total alkaloids were active *in vitro*



Violaxanthin



Zeaxanthin



6-methoxydihydrochelerythrine

Fig. 2. (*continued*).

against influenza virus[48] and encephalomyocarditis.[49] In mice with influenza-virus-induced pneumonia, the total alkaloids were therapeutically effective when the injected dose of the virus was low.[48] Alkaloid extracts showed antiviral activity against human adenoviruses type 5 and 12, as well as against herpes simplex virus.[44,50] Antiviral properties of *C. majus* whole plant extracts were observed on the DNA herpes virus and the RNA poliovirus. The inhibitory effect of alkaline extract was higher than alcoholic extract, and the alkaline extract was more efficient against the herpes virus and the poliovirus.[51]

Alkaloids extracted in acidic pH show significant inhibitory effect on adenoviruses type 5 and 12 and herpes simplex virus type 1.[44] Protoberberine and benzophenanthridine alkaloids inhibited reverse transcriptase (RT) activity of RNA tumor viruses.[44,52–55]

1.10. Antitumor effects

Galenic preparations of *C. majus* aerial parts have been used in the treatment of malignant diseases for centuries, and the alkaloids are usually regarded as the tumor-inhibitory principles. The alkaloids chelidonine and protopine from *C. majus* extract were tested as potential tumor inhibitors in the treatment of sarcoma 180 and Ehrlich carcinoma. Chelidonine (50 μg/kg body weight of mouse) administered over 7 days exerted an insignificant tumor inhibition: 25% for sarcoma and 22% for Ehrlich carcinoma, with a mild cytotoxicity still present. Protopine (350 μg/kg) administered intraperitoneally in 7 days exerted only a mild tumor inhibition: 15% for sarcoma 180 and 26% for Ehrlich carcinoma. These findings indicated that chelidonine and protopine, although they exert certain antitumor activity, have no therapeutic value due to their high cytotoxicity at therapeutic doses.[56] Chelidonine *N*-oxide, a chelidonine derivative, had a higher anticancer activity than chelidonine.[57]

Sanguinarine and chelerythrine produce a dose dependent increase in DNA damage and cytotoxicity in both primary mouse spleen cells and L1210 cells.[58]

1.11. Antimicrobial activity

Pseudoalcoholates of sanguinarine and chelerythrine showed consistent antimicrobial activity.[59] These derivatives have a greater intercellular penetration and are active (MIC = 6.25 μg/ml) against: *Staphylococcus aureus*, *Escherichia coli*, *Salmonella gallinarum*, *Klebsiella pneumoniae*, *Mycobacterium smegmatis*, *Candida albicans*. Chelidonine, chelerythrine and homochelidonine inhibit the growth of Gram positive bacteria *in vitro*.[60]

Benzophenanthridine alkaloids are used for treating periodontal disease. Antibacterial products incorporated into toothpastes and mouth rinses containing an aqueous solution of sanguinarine chloride 0.3% showed an antiplaque effect.[61] Sanguinarine and chelerythrine were effective in controlling the production of volatile sulfur compounds responsible for bad breath.[62] Oriental drugs containing berberine and other compounds effective against dental caries have been added to dentifrices and are covered by a patent.[63]

Berberine in concentrations of 10–25 μg/ml inhibited the growth of different genera of fungi: Alternaria, *Aspergillus flavus*, *Aspergillus fumigatus*, *Candida albicans*, Curvularia, Drechslera, Fusarium, Mucor, Penicillium, *Rhizopus oryzae* and Scopulariopsis.[64] Chelerythrine and sanguinarine, have antifungal effect on some Trichophyton strains, *Microsporum canis*, *Epidermophyton floccosum* and *A. fumigates*.[65] Chelidonine N-oxide, a chelidonine derivative exhibits comparable antifungal activity against moulds and yeast.[57]

1.12. Anti-inflammatory activity

Sanguinarine, chelerythrine and quaternary benzophenanthridine fraction were screened for their anti-inflammatory activity in assays involving carrageenan-induced rat paw edema. Sanguinarine showed higher anti-inflammatory activity than chelerythrine.[47] Stylopine, isolated from the leaves of *C. majus*, in a dose of 0.001–500 mg/kg of body weight, is used to treat diseases caused by excessive inflammatory reactions.[66] Sanguinarine-HCl and chelerythrine-HCl exhibit a direct inactivating action on lytic activity of phages of 1017 and T2 types acting on *E. coli*.[67]

Ethanol extract of *C. majus* shows elastase activity inhibition effect, collagen synthesis promoting effect and skin wrinkle improving effect without causing any skin irritation.[68]

1.13. Anti-asthmatic activity

Total alkaloids from *C. majus* prolong the latent period of asthma induced by histamine and acetylcholine and lower the number of animals experiencing asthma convulsions. Total alkaloids from *C. majus* have marked anti-asthmatic action.[69]

1.14. Activity of Ukrain on central nervous system

The effects of the thiophosphoric acid alkaloid derivative Ukrain (UKSR-222) on the central nervous system of mice and rats were studied. Intraperitoneal administration of Ukrain in doses of 9.5 and 19 mg/kg to mice depressed spontaneous motor activity, decreased body temperature and potentiated the action of hexobarbital. Ukrain (19 mg/kg) also produced analgesic action in the hot plate test. It had no protective effect against electroshock. In rats, *i.p.* administration of Ukrain in dose of 14 and 28 mg/kg potentiated the action of amphetamine and apomorphine but had no effect on catalepsy induced by haloperidol. The central action of Ukrain seems to involve the stimulation of the dopaminergic system and the inhibition of the serotoninergic system.[64]

1.15. Effect of C. majus extracts on choleresis

The total ethanol extract, the phenol and the alkaloid fractions of *C. majus* herb were tested for their choleretic activity using the isolated perfused rat liver. The total extract induced choleresis: the bile flow was significantly increased and the amount of bile was more than doubled after 40 min as compared to the pretreatment value. The phenol and the alkaloid fraction caused a slight, but non-significant bile flow increase. The combination of both fractions caused an increase of about 20% over pretreatment value, however, not significant. Although the total extracts induced choleresis, it was not possible to assign this activity to either the alkaloid or the phenol fraction.[70]

1.16. Antihepatotoxic activity

An aqueous-ethanol extract of Herba *Chelidonii*, containing 41–45% of ethanol exerted significant hepatoprotective activity against carbon tetrachloride (CCl_4) toxicity in rats treated with varying doses of the extract. Intragastric administration of 12.5, 62.5 and 125 mg/kg body weight of the extract twice weekly over 3 weeks resulted in a reduction in CCl_4 induced hepatotoxicity. Increased plasma activities of the enzymes alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and lactate dehydrogenase, as well as the increased bilirubin level, induced by CCl_4, were significantly decreased by the extract.[71] A protection of the liver glycogen phosphorylase a and glucose 6-phosphatase activities against the toxic effects of CCl_4 is observed when the ethanol extract (0.01 ml/100 g, daily) is administered.[72]

1.17. Antispasmodic activity

Chelidonine exhibits spasmolytic activity with its main effect on inhibiting smooth muscle activity.[73] Protopine shows smooth muscle relaxant effects on isolated ilea of guinea-pigs, rabbits and albino rats and a marked relaxation of intestine *in situ* of anaesthetized dogs.[74] An aqueous-ethanol extract of the herb containing 0.81% total alkaloids, calculated as coptisine, as well as the individual compounds, caffeoylmalic acid and coptisine, were examined for their activity against acetylcholine-induced contractions in ileum isolated from rats. Acetylcholine-induced contractions were slightly reduced by the extract (12.7%; 2 mg/ml), and by coptisine (16.5%; 0.1 mg/ml).[75] In another study, acetylcholine-induced contractions in isolated guinea-pig ileum were antagonized by the addition of protopine and allocryptopine (IC_{50} 2.3 μM) to the bath media, whereas berberine potentiated the contractions.[76]

1.18. Clinical studies

In clinical trials, *C. majus* extracts showed a demonstrable reduction of the sensation of pain. The analgesia lasted for 4–48 h. The extracts produced an increase in the excretion of urine with retention of calcium and sodium chloride.[77] Ukrain, given *i.v.* in a dose of 10 mg every three days causes an increase in both T-cells and T-helper lymphocytes, a decrease in T-suppressor cells and normalization of the helper/suppressor (HIS) ratio. A significant improvement from the use of Ukrain as an immunostimulant in cancer patients can be achieved.[78]

A prospective observational study involving 608 patients treated orally with an aqueous-ethanol extract of the crude drug (5–7:1, mean daily dose 375–500 mg extract, corresponding to 9–12 mg of total alkaloids) has been reported. The outcomes were measured using the Physicians' Global Assessment of Efficacy (4-point scale). After an average of 22 days of treatment, symptoms (dyspepsia or cramps in the upper gastrointestinal tract) were reduced in most patients and the outcome was assessed as good or very good in 87.4% of the patients.[79]

1.19. Toxicology

On the isolated frog heart, chelidonine in a dose exceeding 0.05 mg produced arrhythmia, heart block and diastolic stoppage.[77] Oral ingestion of *C. majus* extract has been reported to produce hemolytic anemia. It coursed with intravascular hemolysis, renal failure, liver cytolysis and thrombocytopenia.[80] Acute hepatitis is also reported along with the intake of *C. majus*.[81–83] Intraperitoneal administration of 350 mg/kg body weight of methanol extract of the herb to mice for 7 days resulted in a 20% mortality rate. The median lethal intraperitoneal dose for chelidonine was 1300 mg/kg body weight in mice and 2000 mg/kg body weight in rats. Sub lethal doses of chelidonine induced sedation, tremor and decreased body temperature.[84]

1.20. Adverse reactions

Excessive ingestion of the decoction of *C. majus* may cause nausea and other gastrointestinal symptoms.[84] In rare cases, hepatic inflammation and an increase in liver enzyme activity and serum bilirubin have been reported, all of which are reversible following discontinuation of therapy.[85] A case of contact dermatitis was described after external use of the aerial parts of the plant.[86] A case of contact-derived allergic balanoposthitis and paraphimosis was observed after topical application of Herba *Chelidonii* juice.[87]

2. Conclusion

Survey of ethnopharmacologic records shows that such information is available only on *C. majus*. Three species of *Chelidonium* (Table 1) have been partially investigated for their phytoconstituents. A close scrutiny of literature on *Chelidonium* reveals that only one species (*C. majus*) has been investigated pharmacologically. Pharmacological studies infer that *C. majus* exhibits anti-inflammatory, hepatoprotective, antifungal, antiviral, cytotoxic, antibacterial and spasmolytic properties. *C. majus* has been included in a number of herbal and homeopathic formulations which are in clinical use for the treatment of various ailments. Mother tincture of the plant is available in Indian market, and is frequently used for the treatment of CNS disorders. Keeping in view the ethnopharmacology, phytochemical and pharmacological reports, low toxicity and frequency of use in homeopathic formulation, *C. majus* seems to hold great potential for in-depth investigation for various biological activities.

Few preliminary pharmacological reports support medicinal potential of *Chelidonium* species. These species need to be investigated systematically with a view to establish their pharmacological activities and mode of actions.

Conflicts of interest

All authors have none to declare.

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Short communication

In vitro hepatoprotective activity of *Corchorus depressus* L. against CCl_4 induced toxicity in HepG2 cell line

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ABSTRACT

Aim: To evaluate *in vitro* hepatoprotective activity of ethanolic extract from *Corchorus depressus* (CDEE) against CCl_4 induced toxicity in HepG2 cell line.
Methods: In vitro cytotoxicity and hepatoprotective potential of CDEE were evaluated using HepG2 cells. Based on the cytotoxicity assay, CDEE (50, 100, 200 μg/ml) was assessed for hepatoprotective potential against CCl_4 induced toxicity by monitoring cell viability, aspartate aminotransferase (AST), alanine aminotransaminase (ALT), lactate dehydrogenase (LDH) leakage, lipid peroxidation (LPO) and glutathione level (GSH).
Results: The results indicated that CCl_4 treatment caused a significant decrease in cell viability. In addition, the toxin treatment initiated lipid peroxidation (LPO), caused leakage of enzymes like transaminase (AST & ALT) and LDH with a significant decrease in GSH levels in HepG2 cells. It was observed that CDEE effectively alleviated the changes induced by CCl_4 in a concentration-dependent manner.
Conclusion: Our study revealed that CDEE has potent cytoprotective effect against CCl_4 induced toxicity in HepG2 cell line and which may be attributed to decrease in CCl_4 induced reactive oxygen species levels and resultant oxidative stress.

1. Introduction

Liver is a major organ of human that plays a crucial role in elimination and biotransformation of toxic substances. During the detoxification, reactive oxygen species (ROS) are generated within hepatocytes that result in oxidative damage, gross cellular changes and cell death causing hepatotoxicity or liver damage.[1] In absence of a reliable liver protective drug in the modern system of medicine, a number of medicinal preparations in Ayurveda, the Indian system of medicine, are recommended for the treatment of liver disorders. Natural remedies from medicinal plants are considered to be effective and safe alternative treatments for hepatotoxicity.[2] *Corchorus depressus* (Linn.) (Family: Tiliaceae) commonly known as 'bhaufali' is perennial herb, woody 6–9 inches in length and the plant is regarded as good sand binder in the desert.[3] It is distributed in arid and semi-arid regions of India and Pakistan to North and tropical Africa. Medicinal uses of this plant in general weakness, gonorrhea, diabetes, treachery troubles, improved sexual vigor, jaundice have been reported.[4–6] Antipyretic and analgesic activities of a new triterpenic acid isolated from *C. depressus* have been reported.[7,8] Ali and Ansari reported that application of the combined extract of *Emblica officinalis*, *Lawsonia inermis*, *Nardostachys jatamansi* and *C. depressus* prepared in the oil of *Sesamum indicum* diminished the falling of hair and gave them original color.[9] In a previous study, the methanol extract of *C. depressus* has already been investigated for its hepatoprotective and *in vivo* antioxidant effects against carbon tetrachloride induced hepatotoxicity in rats.[10] Chemical investigation of this plant has resulted in the isolation of various triterpenes,[11,12] phenolics and sterols.[13] However, to the best of our knowledge, the hepatoprotective effect of *C. depressus* in HepG2 cell line intoxicated with carbon tetrachloride (CCl_4) has not been demonstrated. Hence, this study was intended to investigate the *in vitro* hepatoprotective effect of ethanolic extract from *C. depressus*.

2. Materials and methods

2.1. Plant material

C. depressus (Linn.) was collected from Jodhpur district of Rajasthan, in month of August, 2011 and identified from Botanical

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Survey of India, Arid Zone Regional Centre, Jodhpur 342 008 and a voucher specimen no. LMC/AP/001 was deposited in the college for future reference.

2.2. Preparation of extract

C. depressus (whole plant) was dried under shade at room temperature. After drying, plant was subjected to size reduction to a coarse powder by using dry grinder. The powder was packed into Soxhlet apparatus and defatted with petroleum ether (60–80 °C). The marc was dried and extracted with ethanol at 80 °C for 24 h. *C. depressus* ethanol extract (CDEE) was concentrated to dryness under reduced pressure in a rotary evaporator and stored in airtight containers in refrigerator below 10 °C. The percentage yield of the ethanolic extract was found to 7.2% (w/w).

2.3. Cell culture

Human liver hepatoma cells (HepG2) (obtained from National Centre for Cell Sciences, Pune, India) were seeded (1×10^5 cells/T_{25} Flask) and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% Fetal Bovine serum (FBS) and penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (5 μg/ml) in a humidified atmosphere of 5% CO_2 at 37 °C until confluent. Cells were maintained in continuous passage by trypsinization of sub-confluent cultures using TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock culture was grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

2.4. In vitro cytotoxicity assay

The CTC_{50} (50% cytotoxic concentration) was determined by estimating mitochondrial synthesis using tetrazolium assay.[14]

2.5. CCl_4 induced toxicity in HepG2 cell line

The monolayer HepG2 cell culture was trypsinized and the cell count was adjusted to 1.0×10^5 cells/ml using DMEM medium containing 10% FBS. To each well of the 96-well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium. The cells were treated with 100 μl of toxicant (medium containing 1% (v/v) CCl_4), DMSO (medium containing 0.25% (v/v) DMSO) and medium only for various time intervals of 10 min, 30 min, 3 h, 6 h, 12 h and 24 h and the following assays were carried out to observe time-dependent changes.

2.5.1. Cell viability

Trypan blue exclusion test was carried out according to the method of Wu and Cederbaum with slight modification.[15]

2.5.2. AST, ALT and LDH leakage

The culture medium and cells were collected separately. The medium (0.2 ml) was used to measure concentration of AST, ALT and LDH as an indication of cell necrosis using Ecoline diagnostic kit (E–Merck Ltd., Mumbai, India).[16]

2.5.3. Lipid peroxidation

Malondialdehyde (MDA), the end product of lipid peroxidation, was measured using a thiobarbituric acid reactive substances (TBARS) assay.[16]

2.5.4. Glutathione levels

Total glutathione level was measured by DTNB-GSSG reductase recycling assay method. The total glutathione level was determined by the kinetic method from standard curve of GSH along with CDEE/Silymarin.[17]

2.6. Treatment with CDEE

HepG2 cells were plated in 96-well plates at a concentration of 1×10^5 cells/ml using DMEM medium containing 10% FBS incubated for 24 h at 37 °C under 5% CO_2 to attain confluency. Cells were treated with 100 μl each of serum free culture medium containing 0.25% (v/v) DMSO; 1% (v/v) CCl_4; silymarin (200 μg/ml); CDEE (200 μg/ml); 1% (v/v) CCl_4 + silymarin (50, 100, 200 μg/ml); 1% (v/v) CCl_4 + CDEE (50, 100, 200 μg/ml) for 24 h. Cell viability, AST, ALT, LDH leakage, lipid peroxidation and glutathione assays were performed using standard methods as described in previous section.

2.7. Statistical analysis

The results were expressed as mean ± S.E.M. The data were subjected to one-way analysis of variance (ANOVA) followed by Dunnett's test using GraphPad Prism 4.0 Software, San Diego, CA, USA to establish the statistical significance. Values of $P < 0.05$ were considered significant in all cases.

3. Results

3.1. In vitro cytotoxicity assay

CDEE evaluated for its cytotoxic activity by MTT assay. CDEE showed CTC_{50} value of 573.72 ± 11.83 μg/ml in HepG2 cell line.

3.2. CCl_4 induced toxicity in HepG2 cell line

Time-dependent toxic effects of CCl_4 in HepG2 cells were shown in Fig. 1. As compared to vehicle control, no significant change in cell viability, AST, ALT, LDH levels and lipid peroxidation as well as no significant change in GSH level was observed until an exposure period of 3 h, thus overruling any direct solvent mediated damage by CCl_4 in HepG2 cells. There after a time-dependent significant ($p < 0.01$) increase in leakage of AST, ALT, LDH and significant loss of cell viability was observed compared to vehicle control. Similarly, a significant ($p < 0.01$) increase in lipid peroxidation with concurrent significant decrease in glutathione was noted compared to vehicle control.

3.3. Cytoprotective effect of CDEE in HepG2 cells

Table 1 depicts the results of cell viability, leakage parameters-AST, ALT, LDH, MDA and GSH levels in all experimental groups. A significant ($p < 0.01$) decrease in viability of cells and a significant ($p < 0.01$) increase in the levels of AST, ALT, LDH was observed in the HepG2 cells exposed to CCl_4 as compared with Group 1 (normal control). These cells, when treated with CDEE (100 and 200 μg/ml) showed a significant restoration of the altered biochemical parameters towards the normal compared to CCl_4 treated group and is dose dependent.

4. Discussion

The present study reveals the hepatoprotective effect of CDEE against CCl_4 induced toxicity in HepG2 cells. In recent times *in vitro* cytotoxicity and hepatoprotective activity of plant extract and

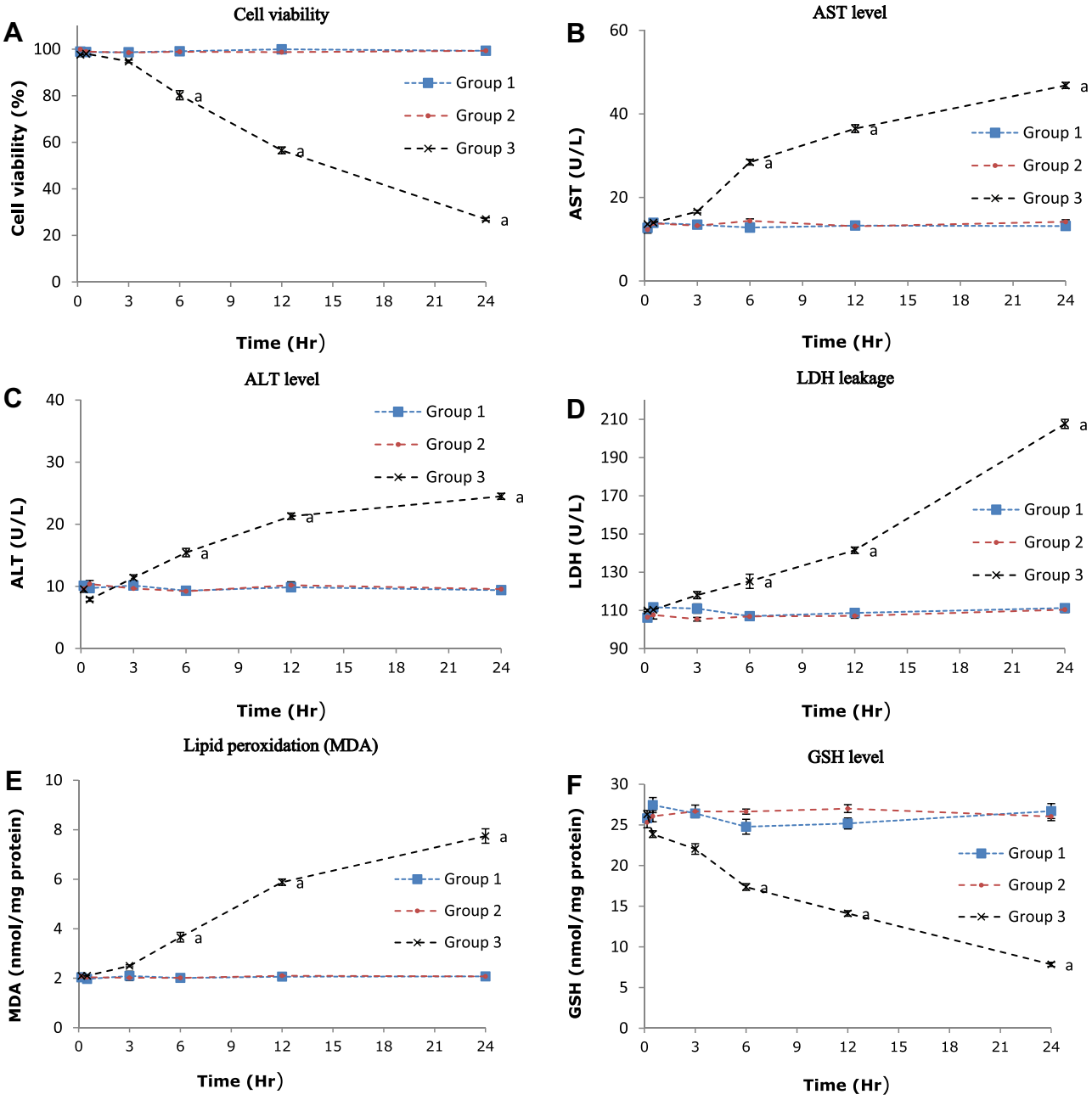

Fig. 1. Time-dependent changes observed in (A) Cell viability (B) AST levels (C) ALT levels (D) LDH leakage (E) lipid peroxidation (MDA) and (F) GSH level after exposure to 1.0% (v/v) of CCl_4 in HepG2 cells. Group 1 – Control; Group 2 – DMSO control 0.25% (v/v); Group 3 – 1.0% (v/v) CCl_4 in 0.25% DMSO. Results are expressed as mean ± SEM ($n = 6$). Level of significance $p < 0.05$. [a]Compared to vehicle control.

bioassay guided fractions has gained importance for primary level screening.[18] HepG2 cell line is a popular and an effective *in vitro* model for assessing hepatoprotective potential of phyto compounds and bioassay guided fractions due to its functional similarity to an intact liver.[1,19] CCl_4 is a hepatotoxic haloalkane whose mechanism has been studied intensively over the past years. It is one of the best studied solvent in regards to liver toxicity. It is well known that CCl_4 undergoes metabolic activation by a cytochrome P-450 dependent step to free radical products which can initiate lipid peroxidation.[20] The toxicity induced by CCl_4 *in vivo* and in cultured hepatocytes, involves the stimulation of lipid peroxidation, detected as an increase in malondialdehyde (MDA) formation.[21] Our results in conjunction with others reports,[14,22] proved that CCl_4 caused a time-dependent production of ROS and subsequent lipid peroxidation in HepG2 cells which was found to be maximum after an incubation period of 24 h. Therefore, HepG2 cells were incubated with CCl_4 for 24 h to study the protective effects of CDEE against CCl_4 induced toxicity. The loss of cell viability was measured as the end product of toxicity by means of trypan blue assay. The extent of cellular damage was measured in terms of release of leakage enzymes-AST, ALT and LDH. Increased release of these intracellular enzymes was observed at 24 h exposure to CCl_4. This indicated membrane damage and instability owing to oxidative injury created by the hepatotoxin. Likewise, toxin treatment caused significant increase in MDA levels, with a concurrent decrease in glutathione content in HepG2 cells. These cells, when

Table 1
Protective effect of ethanolic extract from *Corchorus depressus* on CCl_4 induced toxicity in HepG2 cell line.

Group no.	Experimental groups	Cell viability (%)	AST (U/L)	ALT (U/L)	LDH (U/L)	MDA (nmol/mg protein)	GSH (nmol/mg protein)
(I) Control							
1.	Normal control	99.31 ± 0.18	13.50 ± 0.40	9.56 ± 0.30	109.56 ± 1.25	2.07 ± 0.13	26.04 ± 0.57
2.	DMSO control (0.25%, v/v)	97.57 ± 0.49	12.52 ± 0.63	10.05 ± 0.56	108.88 ± 1.55	2.07 ± 0.13	24.05 ± 0.71
3.	Silymarin control (200 μg/ml)	98.90 ± 0.28	13.58 ± 0.21	9.87 ± 0.29	109.56 ± 1.25	2.11 ± 0.18	25.78 ± 0.71
4.	CDEE control (200 μg/ml)	98.62 ± 0.17	13.69 ± 0.33	9.78 ± 0.43	109.72 ± 0.63	2.05 ± 0.10	25.95 ± 0.47
(II) Toxin treatment							
5.	CCl_4 (1%, v/v)	26.95 ± 0.70[a]	46.82 ± 0.81[a]	21.29 ± 0.57[a]	207.60 ± 2.67[a]	6.75 ± 0.27[a]	9.85 ± 0.28[a]
(III) Silymarin treatment							
6.	CCl_4 (1%, v/v) + Silymarin (50 μg/ml)	57.55 ± 0.57[a]	34.52 ± 1.49[a]	18.48 ± 0.93[b]	173.56 ± 2.58[a]	5.85 ± 0.3[b]	13.29 ± 0.80[a]
7.	CCl_4 (1%, v/v) + Silymarin (100 μg/ml)	72.77 ± 0.84[a]	28.20 ± 0.98[a]	16.33 ± 0.77[a]	150.41 ± 3.11[a]	4.66 ± 0.23[a]	17.05 ± 0.60[a]
8.	CCl_4 (1%, v/v) + Silymarin (200 μg/ml)	81.62 ± 1.17[a]	19.48 ± 0.96[a]	12.91 ± 0.42[a]	127.01 ± 1.34[a]	3.64 ± 0.07[a]	21.35 ± 0.80[a]
(IV) CDEE treatment							
9.	CCl_4 (1%, v/v) + CDEE (50 μg/ml)	36.85 ± 1.13[a]	44.23 ± 1.16	19.06 ± 0.84	194.46 ± 3.32[a]	6.52 ± 0.31	8.83 ± 0.31
10.	CCl_4 (1%, v/v) + CDEE (100 μg/ml)	47.75 ± 2.29[a]	35.53 ± 0.88[a]	17.71 ± 0.46[a]	170.43 ± 1.65[a]	6.03 ± 0.14	12.89 ± 0.75[b]
11.	CCl_4 (1%, v/v) + CDEE (200 μg/ml)	54.70 ± 1.54[a]	26.52 ± 1.15[a]	15.29 ± 0.60[a]	150.05 ± 2.67[a]	5.10 ± 0.25[a]	16.17 ± 0.78[a]

Values are mean ± S.E.M., $n = 6$ in each group. [a]$p < 0.01$, [b]$p < 0.05$, Group II compared to Groups I and Group III–IV compared to Group II, No significant difference was noted between groups 1, 2, 3 and 4, (One-way ANOVA followed by Dunnett's test).
CDEE, ethanolic extract from *Corchorus depressus*; AST, aspartate aminotransferase; ALT, alanine aminotransaminase; LDH, lactate dehydrogenase; MDA, malondialdehyde; GSH, glutathione.

treated with different concentrations (50, 100 and 200/ml) of CDEE showed a significant restoration of the altered biochemical parameters towards the normal compared to CCl_4 treated group. The overall experimental strategy followed evaluated the plausible protective nature of CDEE against CCl_4 induced toxicity in HepG2 cell line. The mechanism involved was ascertained by selecting relevant parameters like leakage of enzymes, quantification of lipid peroxidation and measurement of intracellular glutathione levels. The possible underlying mechanism for the hepatoprotective effect of CDEE is because of its ability to inhibit lipid peroxidation and maintenance of glutathione in reduced state by virtue of its antioxidative powers. Several natural plants/plant products have been established as hepatoprotective against CCl_4 induced toxicity in HepG2 cells.[18,19,23,24] Moreover phytochemical analysis of CDEE has shown the presence of flavonoids and phenolic compounds[7–9] which have been known for their antioxidant and hepatoprotective activity.[24–26] Hence, the hepatoprotective effect observed in the present study mainly due to the presence of any of these compounds present in *C. depressus*. However, further studies on the active compounds and their biochemical mechanisms responsible for the hepatoprotective effect of *C. depressus* will be necessary.

5. Conclusion

Overall, it could be concluded that CDEE protects against oxidative injury induced by CCl_4 *in vitro* and it is also capable of enhancing the activities of hepatic enzymes implicated in combating ROS however further investigation should be carried out on the extract to identify the active constituents responsible for hepatoprotection.

Conflicts of interest

All authors have none to declare.

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