

Full Length Research Paper

Irritant effects of *Euphorbia prostrata*

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Irritant dermatological investigations of locally occurring *Euphorbia prostrata* were carried out to discover the irritant principle present in it. For this purpose, successive solvent extraction was carried out by using polar, non polar as well as intermediate polarity solvents for the extraction of active irritant compounds from the whole herb. Irritant potentials of these extracts were evaluated on rabbit's skin. Biological assay showed that only the chloroform extract was active because it produced irritancy at a certain dose level. Five fractions Ep-1 to Ep-5 were isolated from the active chloroform extract of the plant by column and thin layer chromatography. Irritant dermatological prospective of these fractions were also evaluated on rabbit's ear skin. Out of the five isolated fractions, Ep-2 and Ep-4 appeared to be the strong irritant fractions, while Ep-1, Ep-3 and Ep-5 proved to be moderately irritant fractions at the same dose level. A possible structure-activity relationship of these active fractions was also discussed based on the ultraviolet (UV) and infrared (IR) spectrum.

Key words: Irritancy, Euphorbiaceae, chromatography.

INTRODUCTION

Euphorbia prostrata is a weed; it grows in the cultivated fields of wheat, rice or other crops and also in the gardens along with grass. It is commonly observed that during the removal of this weed, irritation on the hands of workers often occur. The character and uniqueness of such irritant compounds present in this *Euphorbia* species and its mechanism of action has not been previously investigated. Taking into account all these observations, the current study of irritant contact dermatological studies of *E. prostrata* was conducted. *E. prostrata* belongs to the family Euphorbiaceae; a large family of Dicotyledonous Angiosperms (Charles et al., 2007). Family Euphorbiaceae is widely distributed throughout both hemispheres and ranges in morphological form from largest dessert succulents to trees and even small herbaceous types (Cutler et al., 1987).

E. prostrata is native to West Indies, but is now widely distributed throughout the tropics and subtropics (Turner,

1995). It occurs throughout tropical Africa and the Indian Ocean Islands (Singh, 1994). *E. prostrata* grows in gardens, on distributed grounds, in cultivated land and roadside, especially in sandy soils, from sea-level to high altitude (Carter and Leach, 2001). It is also found throughout India as a weed in the plains and at lower elevations (Nguyen and Sosef, 1999). The major constituents include anthraquinone glycosides, flavanoids, phenols, phlobotannins, polysaccharides, saponins, tannins and terpenoids. Alkaloids were not present in very high amounts (Singla and Pathak, 1991). A range of hydrolysable ellagitannins were isolated, including prostratin A, B, and C, euphorbins G and H, tellimagradin I and II, rugosin A, D, E and G from different fractions of extracts of the dried leaves. Flavanoids isolated from the aerial parts include: kaempferol, cosmosin (apigenin-7-glucoside), rhamnatin-3-galactoside, quercitin and quercitin-3-rhamnoside. Other constituents of the aerial parts include the sterols β -amyryne acetate,

β -sitosterol, campesterol, stigmasterol and cholesterol. Aerial parts also contain the terpene alcohol β -terpenol, gallic acid, corilagin, 1, 2, 3-tri-O-galloyl-D-glucose, geraniin, and various amino acids, including n-valeramide and N, N-dimethyl-4-benzoxybutylamine. Roots contain a myricyclic alcohol and two triterpenes, taraxerol and tirucalol (El-Mahy, 2004; Yoshida et al., 1990).

All parts of *E. prostrata* are used as traditional medicine around the globe. Its leaves are used as antidote for stings especially of wasp and scorpion sting. This weed is also used as anti abortive agent and for painful menstruation. In Uganda, pregnant women eat the boiled shoots, mixed with sesame to reduce the risk of miscarriage (Neuwinger, 2000; Kamatenesi-Mugisha and Oryem-Origa, 2007; Kokwaro, 1993; Ogwal, 1996). Leaf powder mixed with palm oil is rubbed on the head to treat headache. Crushed whole plant was eaten with bread against kidney stones. Around the Indian Ocean Islands, an infusion of the leaves or aerial parts was taken either alone or combined with other plants to treat diarrhea, dysentery and stomachache. It showed activity against *Shigella dysenteriae* type I induced diarrhea in rats (Watt and Breyer-Brandwijk, 1962; Kamgang et al., 2007). *E. prostrata* showed antibacterial activities as well as inhibitory effects against HIV-1 protease and hepatitis C virus protease (Hussein et al., 1999, 2000). It possessed anti-fungal activity against certain dermatophytes in experimentally infected animals (Pal and Gupta, 1979). In India, the latex was used to treat diabetes, as it was considered to have hypoglycaemic activities (Akhtar et al., 1984). Many species of family Euphorbiaceae are commonly used as sources of medicines by various tribal and ethnic communities in Pakistan.

MATERIALS AND METHODS

Plants

E. prostrata plants were collected from the Botanical Garden, Government College University, Lahore and from different areas around and within Lahore. These were authenticated by Dr. Sultan, Herbarium, Department of Botany, Government College University, Lahore against specimen number G. C. Herb. Bot. 605. The herbaceous plants were dried under the shade at room temperature for about ten days. The dried plants were then pulverized to fine powder and stored in black polythene bags.

Instruments

Instrument used in this research were distillation apparatus (Quick fit, England), electric balance (Sartorius), oven (Mettler, W. Germany), water bath, Soxhlet apparatus.

Chemicals

All the chemicals used were of BDH analytical grade. The following chemicals were used, which were purchased from local market, H₂SO₄, acetic anhydride, aluminium chloride, anisaldehyde, vanillin, iodine, sodium sulphate (anhydrous).

Solvents

All the solvents used were of BDH analytical grade. The following solvents were commonly used, which are purchased from local market. All the solvents were re-distilled before use: petroleum ether (40 to 60°C), chloroform, ethanol, methanol, glacial acetic acid, acetone, dichloromethane, hydrochloric acid, acetic acid, distilled water.

Chromatographic material

Chromatographic materials used were, silica gel 60 (70-230 mesh ASTM) for column Chromatography – by E. Merck (Germany), Silica gel G₆₀ (HF 254) for thin layer chromatography by – E. Merck (Germany).

Spectral analysis

The ultra violet spectra were recorded on UV-2450 SHIMADZU spectrophotometer by using chloroform as solvent. The infrared spectra were measured on IRPRESTIGE-21 SHIMADZU spectrophotometer using thin film on NaCl disc by mull method.

Micro capillaries

10 μ l microcapillaries (Doumond Microcaps, U.S.A) were used for topical application of the materials on the skin of the animals.

Animals

The study was carried out as per approved protocol by the Animal Ethics Committee, University College of Pharmacy, University of the Punjab, Lahore, Pakistan. Healthy adult male/female rabbits of albino strain of species *Oryctolagus cuniculus* and subsp. 18 rabbits weighing 1.0 to 1.5 kg were purchased from local market. All the rabbits were acclimatized in the animal house of University College of Pharmacy, University of the Punjab, Lahore for a period of three days and were provided with carrots, fresh green fodder and tap water *ad libitum*.

Vehicle

Unless otherwise stated, redistilled acetone was used as a vehicle for bioassay.

Solvent extraction

The pulverized dried *E. prostrata* plants (600 g) were extracted successively in petroleum ether (40 to 60°C), chloroform and methanol by using 2.0 L of each solvent for soaking. Maceration was carried in each solvent for 4 days at room temperature (25 \pm 2.5°C). The solvent of each extracted material was removed under reduced pressure and the residues were weighed.

Thin layer chromatography (TLC)

20 \times 5 cm glass plates were used for this purpose. 30 g silica gel G₆₀ was made into slurry by mixing with 90 ml of distilled water and spread uniformly on 21 plates of 20 \times 5 cm size with the help of moving spreader Dosga applicator, which was already adjusted at 0.25 mm. The plates were dried at room temperature and activated

Table 1. Grading of irritant reactions. Adopted from Hacker (1971).

Reaction grades	Explanation
-	No reaction
±	Doubtful reaction, diffused inflammation with no clear visible symptoms
+	Slight reddening of the main vessels without reddening the area in between
++	Marked reddening of the main vessels with reddening of the areas in between
+++	Intense reddening of the entire ear often accompanied with macroscopic visible hyperplasia
++++	Visible exudative lesion with marked epidermal damage

in an oven at 100°C.

Spotting on plates

Small quantity of liquid extract was taken and spotted on layers at 1.5 cm above the baseline of the plate with the help of microcapillary tube. The distance between the two spots was kept at 1.5 cm.

Development of plates

The spots on thin layer plates were dried by air-dryer and developed in chromatographic jars. Inner side of the tank was made saturated with the solvent, after attaching the solvent soaked filter paper in it. 30 to 35 ml of solvent was poured into the tank, so that it rises 1 to 2 cm above from bottom of the tank. The plates were placed inside the tank and the lid was closed. The plates were allowed to develop. The solvent was run up to 1.5 cm from the upper edge of the plates. Solvent front was marked and after drying the plates with air-dryer, the spots were detected under UV light and with iodine. Different solvent systems were used for TLC of three solvent extracts that is, petroleum ether extract, chloroform extract and methanol extract.

Column chromatography

The active extract was further subjected to column chromatography. Different fractions were obtained from the column by monitoring with thin layer chromatographic procedure. Similar fractions were pooled and five isolated fractions were obtained at the end, which were subjected to main assay for irritancy.

Biological assay for irritancy

Dilution series

10 mg of the dried extract was accurately weighed by using electric balance and made up to 10 mg/10 ml (w/v) solution with acetone. The dilution series was prepared according to the equation, as was given by Evans and Schmidt (Schmidt and Moul, 1983).

$$C_m = C_o \times a^{-m}$$

Where: C_o = Initial concentration; C_m = concentration after m dilution; a = dilution factor. The dilution factor in all the cases was kept at 2, and six or seven dilutions were prepared.

Pilot assay

The biological assay for irritancy was adopted from Evans and Schmidt's method (Evans and Schmidt, 1979). 20, 40, 80 and 120

µl solutions from each dilution were applied to the inner surface of rabbit's ear and the untreated ear was used as control. The ears were examined for redness after 30 min of application and according to time mentioned in Table 2, until two examinations indicated that further redness would not occur. Time for maximum erythema was noted. Four dilutions were chosen for the main assay to include one dilution that will give maximum positive response. The animals were also examined after 24 and 48 h to ascertain the chronic inflammatory dose. The degree of redness corresponding to "++" was noted giving result corresponding to "IU" that is, irritant units of Hacker (1971) and cited by Evans and Schmidt (Evans and Schmidt, 1979). If no redness was observed, the assay was repeated by using more concentrated solution of the extract on new rabbit's ear.

Main assay

For the main assay, a group of 6 rabbits for each dilution was used. 20 µl of the most diluted solution of the chosen series was applied to one of the ears of rabbit in that group by using 10 µl microcapillaries. The animals of other dilution group were also treated similarly by increasing concentration of irritants. Rabbits were examined after 30 min of application and then after 30 min intervals. The number of ears showing marked inflammation of the major blood vessels was recorded. ID_{50} that is, irritant dose in 50% individuals was taken as a dose corresponding to the 50% cumulative frequency. The evaluation of irritant response has been described in Table 1. The dose causing an ear redness to the degree ++ is defined as IU (Hacker, 1971) and expressed in µg/ml per ear.

RESULTS AND DISCUSSION

Solvent extraction

Successive extraction with petroleum ether, chloroform and methanol showed that the percentage yields of extracts of petroleum ether, chloroform and methanol were 2.95, 4.07 and 13.38%, respectively.

Thin layer chromatography

TLC results of petroleum ether, chloroform and methanol extracts of whole herbs of *E. prostrata* showed that following were the solvent systems which resolved the extracts into maximum components. Petroleum ether extract was resolved into five components by solvent system; petroleum ether/chloroform with ratios 95:5, 95:7

Table 2. Irritant response of the crude solvent extracts of *Euphorbia prostrata* on rabbit's ear.

Dose ($\mu\text{g}/\mu\text{l}$)	Extracts	Response after – acute time (h)								Chronic time (h)				
		1	2	3	4	5	6	7	8	9	10	24	48	72
20/20	Pet. ether	-	-	-	-	-	-	-	-	-	-	-	-	-
	Chloroform	-	-	-	-	-	-	-	-	-	-	-	-	-
	Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-
40/40	Pet. ether	-	-	-	-	-	-	-	-	-	-	-	-	-
	Chloroform	-	-	-	-	-	-	-	-	-	-	-	-	-
	Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-
80/80	Pet. ether	-	-	-	-	-	-	-	-	-	-	-	-	-
	Chloroform	-	-	-	±	+	++	++	+	+	+	±	±	-
	Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-
120/120	Pet. ether	-	-	-	-	-	-	-	-	-	-	-	-	-
	Chloroform	-	-	+	+	++	+++	+++	+++	+++	++	++	+	+
	Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-

and 95:10. Chloroform extract was resolved into six components by solvent systems; petroleum ether/chloroform/methanol with ratios 20:80:3 and 20:80:5. Methanol extract was best resolved into four components by solvent system; chloroform/methanol with a ratio of 90:10.

Isolated compounds

Chloroform extract of the plant was subjected to column chromatography, and five different compounds were isolated from the extract namely: Ep-1, Ep-2, Ep-3, Ep-4 and Ep-5.

Spectral analysis

Compounds Ep-1

The ultraviolet and infrared spectrums of the isolated compounds were obtained and are shown in Figures 1 to 10.

Irritancy assay

The results of preliminary irritant responses of crude extracts of *E. prostrata* on rabbit's ear have been outlined in Table 2. The results of irritant reactions of isolated compounds of chloroform extract of *E. prostrata* on rabbit's ear have been outlined in Table 3, while the irritant results of individual isolated compounds (Ep-1, Ep-2, Ep-3, Ep-4 and Ep-5) at four different doses have been outlined in Tables 4 to 8.

DISCUSSION

For isolation of phytochemical fractions from this species, successive solvent extraction was carried out. For this purpose, both non-polar and polar solvents that is, petroleum ether (40 to 60°C), chloroform and methanol were used (Brain and Turner, 1975). The solvent extraction procedure was based on the assumption that petroleum ether (40 to 60°C) was a non-polar solvent; it probably extracted the least polar compounds. Methanol on the other hand was a polar solvent, and extracted most of the polar components from the crude powder. Chloroform possessed an intermediate polarity and probably extracted the compounds with intermediate polarities (Singla and Pathak, 1989). The dried powder of whole herb (600 g) of *E. prostrata* was thus subjected to successive extraction in these three common solvents under the laboratory conditions. Results of the broad solvent extraction in the form of percentage yield indicated that the production of both the polar and non-polar components were not equal. Out of the three types of extracted materials, the polar components (13.37%) were extracted in methanol and were in higher yield than others. The components with intermediate polarity (4.07%), which were extracted with chloroform, were next in the yield. On the other hand, the non-polar components (2.95%) which were extracted in petroleum ether were in the lowest yield. It could thus be concluded that the powdered *E. prostrata* contained high proportion of polar compounds than others.

All of the three extracts were subjected to a comparative TLC analysis using different solvent systems in each case. The main purpose of this analysis was to have an idea of the total number and chromatographic behavior of the compounds present in each extract. The

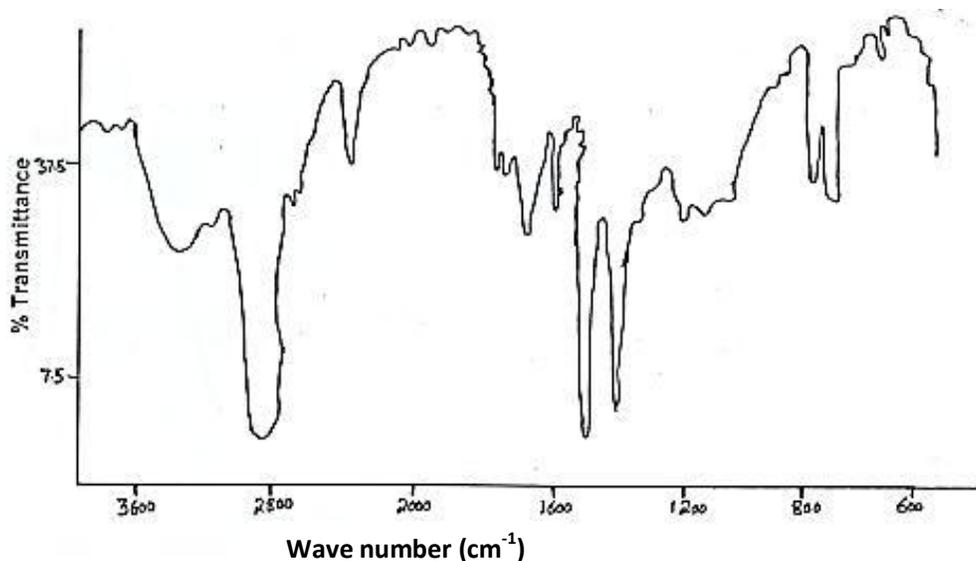


Figure 6. Infrared spectrum of Ep 3.

Table 7. Irritant response of the isolated compound Ep-4 from chloroform extract of *Euphorbia prostrata* on rabbit's ear.

Dose levels ($\mu\text{g}/\mu\text{l}$)	Response after – Acute time (h)												Chronic time (h)		
	1	2	3	4	5	6	7	8	9	10	11	12	24	48	72
120/120	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++
80/80	+	++	++	++	++	+++	+++	+++	+++	++	++	++	++	++	++
40/40	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-
20/20	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-

Table 8. Irritant response of the isolated compound Ep-5 from chloroform extract of *Euphorbia prostrata* on rabbit's ear.

Dose levels ($\mu\text{g}/\mu\text{l}$)	Response after – Acute time (h)												Chronic time (h)		
	1	2	3	4	5	6	7	8	9	10	11	12	24	48	72
120/120	++	++	++	++	++	+++	+++	+++	+++	++	++	++	++	++	+
80/80	+	+	+	+	++	++	++	++	++	++	++	++	++	++	++
40/40	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-
20/20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

results of this analysis showed that the best solvent system which resolved the mixture of petroleum ether extract into five components seemed to be petroleum ether/chloroform (with ratios 95:5, 95:7 and 95:10). Chloroform extract was segregated maximum into six components by the petroleum ether/chloroform/methanol (20:80:3 and 20:80:5). On the other hand, the mixture of polar components present in the methanol extract was best resolved into four components by chloroform/methanol (90:10).

Preliminary irritancy assay was performed with all three types of solvent extracts and the isolated compounds of *E. prostrata* on rabbit's ear. This method was originally

innovated by Hecker (1971) for evaluating the irritant principles from croton oil on mice's ears, which was later on followed with minor modifications by Evans and Schmidt (1980) and Evans and Soper (1978), for evaluating the irritancy of tigliane, daphnane and ingenane series of diterpene esters from various *Euphorbia* species. These authors used albino mice as an animal model for their investigations. In the present work, the same method was used, but instead of mice, rabbits were used as animal model to assess the irritancy activities. Although the erythema produced by these extracts/isolated compounds was of weak and diffused type, but could easily be evaluated. Many other authors

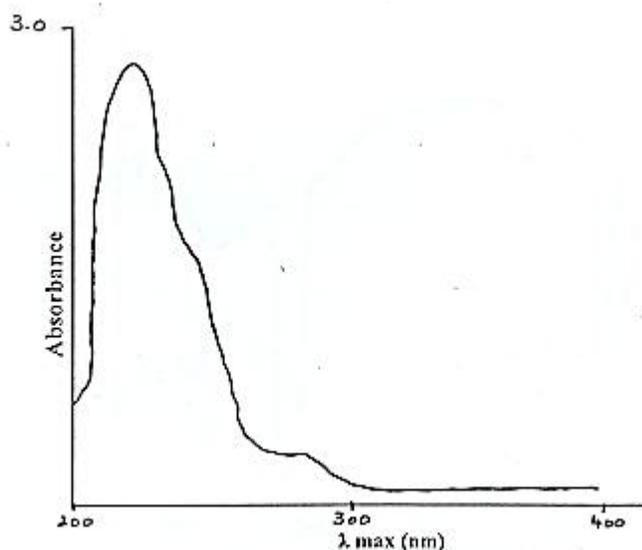


Figure 7. Ultra violet spectrum of Ep 4.

used albino rabbits instead of mice for similar purpose (Anderson et al., 1987; Benazra et al., 1985).

Results indicated that all three solvent extracts exhibited no irritant responses when the low doses of 20 and 40 μg were used but at higher doses of 80 and 120 μg , only chloroform extract produces redness of +, ++ and +++ intensity on rabbit's ears. Hence from all the three extracts, chloroform extract seemed to be irritant than other two extracts at this dose level. Methanol and petroleum ether extract seemed to be inert in its irritant reaction with all the four doses used. It could thus be concluded that intermediate polarity constituents of *E. prostrata* were responsible for such adverse reaction on the animal's skin (Table 2).

The chloroform extract of *E. prostrata* was further subjected to column chromatographic analysis to isolate the active irritant compounds, using an increasing quantity of methanol in chloroform. The elution process was monitored by silica gel thin layer chromatography. Thirteen pooled fractions were obtained. All the pooled column fractions gave irritant reactions, but level of irritation intensity is variable with reference to time.

Further, five fractions were isolated from the first, third, sixth, eleventh and thirteenth pooled column fractions of the chloroform extract of *E. prostrata* on the basis of their superior irritation activity of +++ (intense reddening of the entire area). They were named as Ep-1 to Ep-5.

Isolated fraction Ep-1 was isolated from the first column fraction and was brown in color. The thin layer chromatography of this fraction indicated only one major spot, when solvent system (petroleum ether/chloroform in ratio 40:60) was used. It gave light yellow colour with iodine. Under UV, the spot appeared light pink fluorescence. The compound, Ep-1, had strong absorption at $\lambda_{\text{max}} = 252 \text{ nm}$ (Figure 1) which was probably due

to $n \rightarrow \pi^*$ transition. This value is assigned to aldehydes. It also indicated the presence of conjugated polyenes Me ($\text{C}\equiv\text{C}$)Me.

The infrared spectrum of Ep-1 (Figure 2) showed strong and broad absorption band between 3600 to 3000 cm^{-1} due to $-\text{OH}$ absorption which possibly indicated some alcoholic or phenolic group. Absorption in this region was possibly due to the presence of some water incorporated during the recording of spectrum. The stronger band at 2900 cm^{-1} showed the $\text{C}-\text{H}$ stretching present in alkane/alkene and alkynes. Often, such bands are shown by the methyl, methylene or aryl groups resulted from symmetrical or asymmetrical stretching $\text{C}-\text{H}$ modes. The weak absorption at 2700 cm^{-1} was possibly due to presence of some bands $-\text{NH}_2$, $=\text{NH}$ or $\equiv\text{N}$. This indicated the presence of amyl/amines or ammonium. Bands in this region were due to $\text{N}-\text{H}$ stretching. The band at 2350 cm^{-1} was a weak absorption and was possibly due to the presence of carboxylic group present in compound. The weak absorption at 1950 cm^{-1} possibly indicated the presence of some cyanates. The medium band at 1740 cm^{-1} indicated carboxyl absorption possibly due to $\text{C}=\text{O}$ stretching vibration. Such vibration was present in keto esters, six ring or lactones. Absorption at 1660 cm^{-1} was probably due to β -keto esters in H-bonding in enol form. Strong peak at 1460 cm^{-1} also indicated the $-\text{C}-\text{N}=\text{C}$ stretching vibrations possibly due to $-\text{NH}_2$, $=\text{NH}$ or $\equiv\text{N}$ absorption. The absorption at 1390 cm^{-1} was probably due to the presence of phosphates in the compound. The medium absorption at 720 cm^{-1} indicated the presence of 5 adjacent H and monosubstituted benzene ring (Williams and Fleming, 1980). The available spectral evidence showed that Ep-1 was probably a compound containing aldehyde, amide, alcoholic, phenolic, cyanate and lactone ring. It also indicated the presence of phosphates in the compound.

Isolated fraction Ep-2 was isolated from the third column fraction. It was a brown fraction. Thin layer chromatography of this compound indicated only one major spot, when solvent system (petroleum ether/chloroform in ratio 40:60) was used. It gave yellow colour with iodine. Under UV, the spot appeared light pink fluorescence.

The fraction, Ep-2 had strong absorption at $\lambda_{\text{max}} = 250 \text{ nm}$ (Figure 3) which was probably due to $n \rightarrow \pi^*$ transition. This value is assigned to unsaturated acids/esters, nitriles or amides. The infrared spectrum of Ep-2 (Figure 4) showed broad and medium absorption band between 3600 to 2930 cm^{-1} due to $-\text{OH}$ absorption which possibly indicated some alcoholic or phenolic group. Absorption in this region was possibly due to the presence of some water incorporated during the recording of spectrum. Stronger band at 2900 cm^{-1} showed the $\text{C}-\text{H}$ stretching vibration present in alkane/alkene or alkynes. Often, such bands are shown by the methyl/methylene or aryl groups resulted from symmetrical or asymmetrical stretching $\text{C}-\text{H}$ modes. The

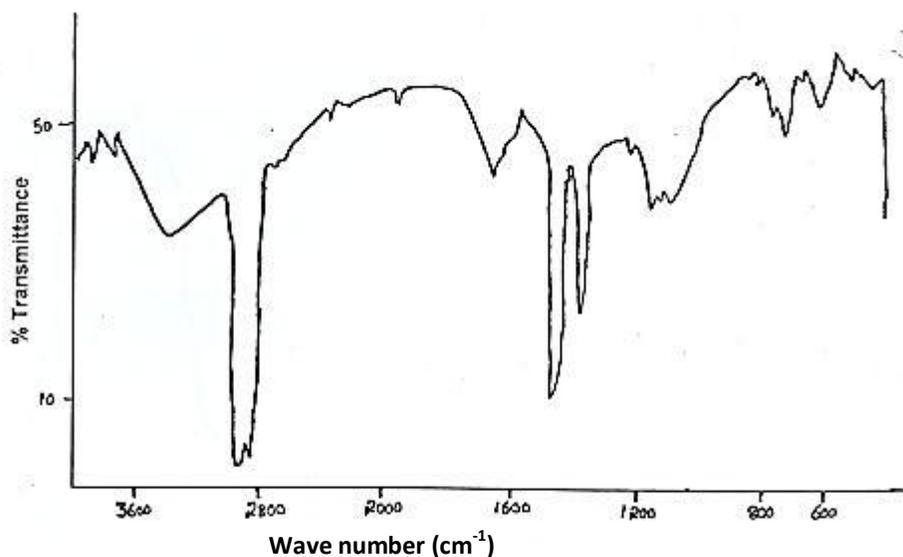


Figure 8. Infrared spectrum of Ep 4.

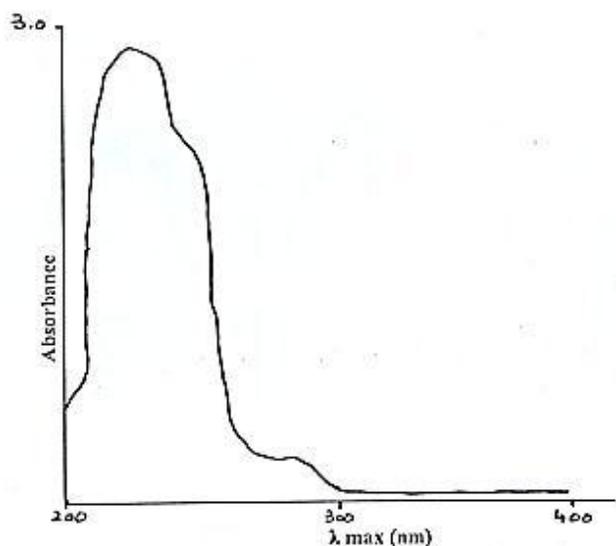


Figure 9. Ultra violet spectrum of Ep 5.

strong absorption at 2840 cm^{-1} was possibly due to the C-H stretching present in aryl esters ($\text{C}-\text{O}-\text{CH}_3$). Weak absorption at 1950 cm^{-1} possibly indicated the presence of some cyanates. Strong absorption at 1680 cm^{-1} was probably due to presence of $> \text{C}=\text{N}$ - present in imines. Absorption at 1470 cm^{-1} was a medium band and possibly indicated the presence $\text{CH}_2=\text{CH}_3$ - groups which was present in the alkanes or alkenes. Medium absorption at 1350 cm^{-1} was probably due to presence of nitrates in the compound. Absorption at 1080 cm^{-1} was a weak band and indicated presence of $\equiv\text{C}-\text{O}-\text{C}\equiv$ present in ether (Williams and Fleming, 1980). The available spectral

evidence showed that Ep-2 was probably a compound containing alcoholic/phenolic/carbonyl/alkane/alkene/alkyne/cyanate/s/nitrites or ether.

Isolated fraction Ep-3 was isolated from the sixth column fraction. It was light yellow fraction. The thin layer chromatography of this compound indicated only one major spot, when solvent system (petroleum ether/chloroform in ratio 40:60) was used. It indicated light yellow colour with Iodine. Under UV, the spot appeared light pink fluorescence. The fraction, Ep-3 had strong absorption at $\lambda_{\text{max}} = 242\text{ nm}$ (Figure 5) which was probably due to $n \rightarrow \pi$ and $\pi \rightarrow \pi$ transition. This absorption perhaps indicated some double bonds in the molecule. The infrared spectrum of Ep-3 (Figure 6) showed broad and medium absorption band between 3300 to 3050 cm^{-1} due to $-\text{OH}$ absorption which possibly indicated some alcoholic or phenolic group. Absorption in this region was possibly due to the presence of some water molecule incorporated during the recording of spectrum. Broad and strong band at 2920 to 2820 cm^{-1} indicated stretching vibration in plane possibly due to $\equiv\text{CH}=\text{CH}_2$ or $-\text{CH}_3$ groups present in the molecule. Weak absorption at 2710 cm^{-1} was possibly due to presence of overtone bands $-\text{NH}_2$, $=\text{NH}$ or $\equiv\text{N}$ stretching vibrations possibly due to presence of some amyl/amines, or ammonium. Medium absorption at 1730 cm^{-1} possibly indicated the presence of keto esters/six ring or larger lactones. Medium absorption at 1450 cm^{-1} possibly indicated presence of $\text{CH}_2=$, CH_3- groups which was present in the alkanes or alkenes. The strong absorption at 1380 cm^{-1} was probably due to presence of nitro ($\text{C}-\text{NO}_2$) and methyl ($-\text{CH}_3$) in the compound. Medium absorption at 1140 cm^{-1} indicated presence of sulphur group ($=\text{SO}_2$). Medium absorption at 690 cm^{-1}

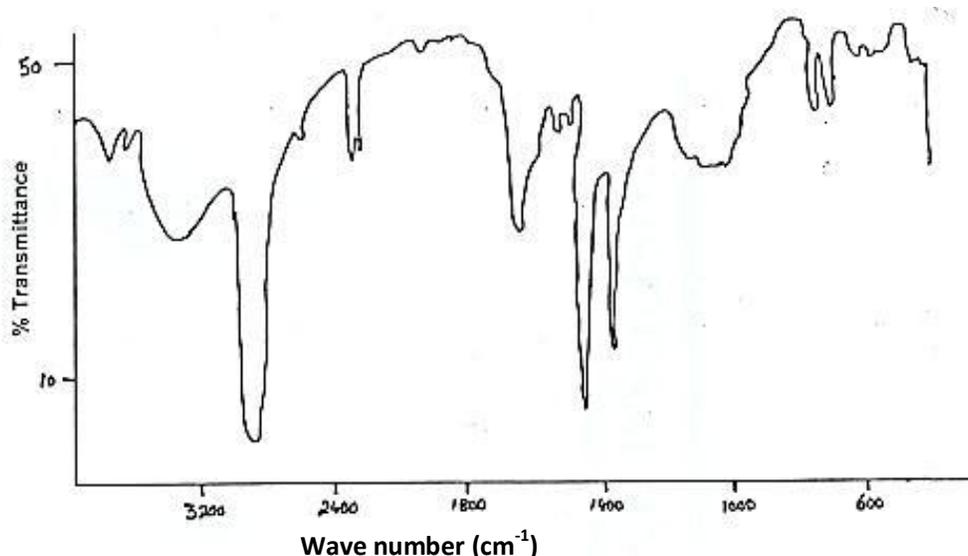


Figure 10. Infrared spectrum of Ep 5.

possibly indicated the presence of halogen groups (Williams and Fleming, 1980). The available spectral evidence showed that Ep-3 was probably a compound containing alcoholic/phenolic/carbonyl/methyl/alkane/alkene/aryl/amines/nitro/ammonium/keto esters/halogen groups and 6 ring lactone. It also indicated the presence of sulphur in the compound.

Isolated fraction Ep-4 was isolated from the eleventh column fraction. It was a dark brown compound. The thin layer chromatography of this compound indicated only one major spot, when solvent system (petroleum ether/chloroform in ratio 95:3) was used. It gave light yellow colour with Iodine. Under UV, the spot appeared as a light pink fluorescence. The fraction, Ep-4, had strong absorption at $\lambda_{\max} = 230$ nm (Figure 7) which was probably due to $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transition. This absorption perhaps indicated some double bonds in the molecule. The infrared spectrum of Ep-4 (Figure 8) showed strong and broad absorption band at 3500 to 3060 cm^{-1} due to $-\text{OH}$ absorption which possibly indicated some alcoholic or phenolic group. Absorption in this region was possibly due to the presence of some water incorporated during the recording of spectrum. The strong and broad band at 2970 to 2840 cm^{-1} indicated alkane/alkene/alkynes or oximes. Weak absorption at 1670 cm^{-1} was probably due to stretching vibration of carboxylic acid/ketones or aldehyde. The strong absorption at 1450 cm^{-1} possibly indicated the stretching vibrations of $-\text{NH}_2/\text{=NH}/\equiv\text{N}$ or $-\text{NH}_2\text{CO}/\text{=NHCO}/-\text{CN}$. Medium absorption at 1360 cm^{-1} was probably due to presence of nitrates in the compound. Weak absorption at 1080 cm^{-1} probably indicated the deformation of $\equiv\text{CH}$, $=\text{CH}_2$ or CH_3 (Williams and Fleming, 1980). The available spectral evidence showed that Ep-4 was probably a

compound containing alcoholic, phenolic, carbonyl, alkane, alkyne, oximes, esters, amide or some nitrates.

Isolated fraction Ep-5 was isolated from the thirteenth column fraction. It was a dark brown compound. The thin layer chromatography of this compound indicated only one major spot, when solvent system (petroleum ether/chloroform in ratio 95:3) was used. It gave yellow colour with Iodine. Under UV, the spot appeared yellow. The fraction, Ep-5 had strong absorption at $\lambda_{\max} = 240$ nm and a weak absorption at 284 nm (Figure 9). Both the strong and weak absorptions are probably due to $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transition. The infrared spectrum of Ep-5 (Figure 10) showed strong and broad absorption band between 3500 to 3200 cm^{-1} due to $-\text{O}-\text{H}$ stretching vibration possibly due to some alcoholic or phenolic group. Absorption in this region was possibly due to the presence of some water incorporated during the recording of spectrum. The strong and broad band at 2980 to 2820 cm^{-1} showed the C—H stretching present in alkane/alkene or alkynes. Often, such bands are shown by the methyl/methylene or aryl groups resulted from symmetrical or asymmetrical stretching C-H modes. Weak absorption at 2700 cm^{-1} was possibly due to presence of overtone bands $-\text{NH}_2/\text{=NH}/\equiv\text{N}$. This indicated the presence of aryl/amines or ammonium. Bands in this region were due to N—H stretching. The weak band at 2320 cm^{-1} was possibly due to the presence of carboxylic group present in compound. Weak absorption at 1670 cm^{-1} was probably due to H-bonding in enol form. The strong absorption at 1450 cm^{-1} possibly indicated presence of alkane/alkene or alkyne ($-\text{CH}_3$, $=\text{CH}_2$, $\equiv\text{CH}$). A medium absorption at 1375 cm^{-1} was probably due to the presence of sulphur in the compound. The absorption at 700 cm^{-1} was a weak band and indicated the presence of some halogen groups (Williams and Fleming, 1980).

The available spectral evidence showed that Ep-5 was probably a compound containing amide/alcoholic/phenolic/alkane/alkene/alkyne/amine/ammonium/carboxylic group or halogen. All five isolated compounds (namely Ep-1 to Ep-5) exhibited mild to moderate irritant responses on the rabbit's skin. Maximum response was demonstrated by Ep-2 and Ep-4 when a dose of 120 µl of 1 mg/ml was applied on rabbit's ear. Reddening produced by these two compounds spread in an area of 3.0 cm. The irritant response of these compounds started after 1 h and reached to a +++ intensity after three hours in case of Ep-2 and four hours in case of Ep-4. The intensity of the reaction starts declining after about 8 h. The reaction lasted for about 72 h, and then clears. The compound Ep-1, Ep-3 and Ep-5, demonstrated a mild response, the reddening produced by these compound was spread in an area of 1.5 cm. It was further postulated from the results that the compounds Ep-2 and Ep-4 penetrated through the skin of rabbit's ear more simply as compared to other compounds. The presence of -OH, -COOH, or ketonic group and a double bond with conjugated diene system was accountable to react with the cell membrane and cellular ingredients of both the superficial and deeper layers of epidermis. As a result, inflammation of both the superficial and the deeper layers occurred, which probably hurt the epidermis. The mechanism of action of these compounds was possibly same as that of moderate to strong irritant compounds previously investigated by other scientists (Anderson et al., 1987; Benazra et al., 1985). There might be two possible reasons for the mild to moderate irritancy of +++ to + intensity displayed by Ep-1 to Ep-5. Initially, the compound itself penetrated to the skin. Secondly, the nature of the molecule was not so strong or severe enough to cause any terrible damage to the epidermal tissues of the skin but was strong enough to produce little dilatation of blood vessels and inflammation of the superficial layers.

Conclusion

Based on the data generated in this study, it could be concluded that a detailed chemical characterization of these phytochemical compounds is necessary, so that a structure-activity relationship of such important molecules in terms of irritant activity could be established.

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