

Isolation and characterization of irritant components of *Euphorbia pilulifera* L.

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Abstract: *Euphorbia pilulifera* is commonly found weed along road sides and loamy soils. This weed is commonly used as treatment of female disorders and respiratory problems. The latex of this weed causes irritation on hand on contact. To evaluate its irritant potentials, the dermatological investigation of irritant principles from locally occurring *Euphorbia pilulifera* was carried out. For this purpose, after collection and drying, a series of solvents with increasing polarity were used for the successive extraction of non-polar compounds (petroleum ether extract), constituents of intermediate polarities (chloroform extract) and polar constituents (methanol extract) from the whole herb of *Euphorbia pilulifera*. The chloroform extract of this weed was found most irritant to rabbit's skin. Chloroform extract was further subjected to column chromatography; four fractions Ep 1 to Ep 4 were isolated from active chloroform extract by column and thin layer chromatography. The irritant potentials of these isolated fractions were evaluated on rabbit's skin. Two fractions out of the four, Ep 1 and Ep 3 appeared to be the most irritant than others. A possible structure activity relationship of these active compounds was discussed in order to establish their activity.

Keywords: *Euphorbia pilulifera*; irritant; chromatography.

INTRODUCTION

Euphorbiaceae is a big family of angiosperms having 300 genera and around 7500 species. Most of the members of this family are herbs, some are shrubs and trees. *Euphorbia pilulifera* is common weed found throughout India on waste grounds and in loamy soils. It occurs mostly in summer and rainy seasons but found occasionally in winter season along road side and waste grounds. It is found in many tropical countries but it is exported mainly from India. The whole plant produces milky latex which is often irritating to the mucous membranes and skin (Kashyap and Joshi 1936; NIIR Board of consultants and engineers, 2005; Sivarajan and Bachanardran 2002; Henry 1999). *Euphorbia pilulifera* contains 0.4 % of a lycosidal substance, tannin, fatty acids, phorbic acid, sterols, eciphosterol, Jambulol melissic acid and sugars, 0.1 % alkaloids (Lanthers and Fleurentin 1987). Acids isolated are ellagic acid, gallic acid, maleic acid, tannic acid and tartaric acid. Flavonoids isolated include quercetin, quercitrin, quercitrol and derivatives. The latex contained inositol, taraxerol, friedelin, β -sitosterol, ellagic acid, kaempferol, quercitol and quercitrin (Chen 1991; Yoshida *et al.*, 1988; Elizabeth *et al.*, 2003; Blanc and De Saqui-Sannes 1972; Aqil and Khan 1999). The mineral contents of dried leaves sample were Ca 1.1% and P 0.3%, Fe 0.03%, Mg 0.5%, Mn 0.01%, Zn 0.01% and Cu 0.002% (Nguyen and Sosef 1999). According to the doctrine of signatures, *Euphorbia pilulifera* has reputation for increasing milk

flow in women, because of its milky latex and is used for other female diseases and diseases of respiratory tract. The plant has been used for female disorders but is now more important in treating respiratory ailments, especially cough, coryza, bronchitis and asthma. In India it is used to treat worm infections in children and for dysentery, gonorrhoea, jaundice, pimples, digestive problems and tumors. The fresh milky latex is applied to wounds and warts. Roots of the plant are used in sprains and inflammation, miscarriage, epilepsy, maggots in wounds and irregular growth of teeth (Chopra and Chopra 1958; Evans and Tylor 1983; Jha 1992). The main objectives of the research are to evaluate its irritant potential on animal skin and to identify irritant principles. This will possibly lead to the structure activity relationship of the irritant compounds present in this local species.

MATERIALS AND METHODS

Euphorbia pilulifera plants were collected from different parts of district Lahore, during June 2008. These were authenticated and the voucher specimen number GC-Herb-Bot-612 was deposited in the Dr Sultan Herbarium, GC University, Lahore for further reference. The whole herbs were dried under the shade at room temperature for about ten days. The dried plants were then pulverized to fine powder and stored in amber colored bottles.

Instruments

Instrument used in the present investigation include Soxhlet apparatus, Distillation apparatus (Quick fit, England), Rotary Vacuum Evaporator (Tokyo Rikakikai

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Co., Ltd.), Analytical Balance (Sartorius), Oven (Mettler, W. Germany), Refrigerator.

Chemicals

Following chemicals of B. D. H. analytical grade were used which were purchased from local market: Iodine, H₂SO₄ (Sulphuric acid), 2, 7-Dichloro Fluorocine, Sodium sulphate (anhydrous), Dichloromethane. Solvents of B. D. H. grade were used; all the solvents were re-distilled before use. Solvents used were Petroleum ether (40-60°C), Chloroform, Ethanol, Methanol, Ethyl acetate, Glacial acetic acid, Acetone, Distilled Water.

Chromatographic materials

The following chromatographic materials were used, Silica gel 60 (70-230 mesh ASTM) for column chromatography by E. Merck (Germany) Silica gel 60 GF₂₅₄ for thin layer chromatography by E. Merck (Germany). The isolated compounds were detected by different reagents. The Ultra violet Spectra were recorded on Shimadzu UV-160A Japan spectrophotometer using chloroform as a solvent. The Infrared Spectra were measured on Perk Elmer FT-IR SPECTRUM BX system by using direct method. 10 µl micro capillaries (Doumond Microcaps, USA) were used for topical application of the materials.

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 subspecies *Caprolagus hispidus* weighing 1.0-1.5kg were purchased from local market. These animals were acclimatized in the animal house for a period of three days and were provided with carrots, fresh green fodder (clover) and tap water *ad libitum*.

Extraction process

Successive extractions of the pulverized dried *Euphorbia pilulifera* plants were carried out by using 2.5 Liters, Petroleum ether (40-60°), Chloroform and methanol respectively. About 700grams of the powdered drug were used.

Chromatographic evaluation

Column chromatography of biological active chloroform extract was carried out. Glass column of 50X2.5 cm was used for column chromatography. 14 grams of chloroform extract of *Euphorbia pilulifera* were adsorbed on 20 grams of silica gel 60, using chloroform. Chloroform was completely evaporated and the dried silica gel adsorbed material after pulverization was added on the top of the column. The column was first run with petroleum ether, then the polarity of the system was changed by increasing the quantity of chloroform in petroleum ether. Thin layer chromatographic analysis of extracts was carried out using different solvent systems.

Bio-assay

The biological assay for irritancy was adopted from Evans and Schmidt's method (Schmidt and Moulton 1983; Evans and Schmidt 1979). 20, 40, 60 and 80µl solution from each dilution was applied to the inner surface of rabbit's one ear. This procedure was repeated using other rabbits and other dilutions. The untreated ear was used as control. The ears were examined for redness after 30 minutes of application and then after 30 minutes intervals, until two examinations indicated that further redness would not occur. Results were recorded according to Hacker's irritant grading given in table 1 (Hacker, 1971). Time for maximum erythma was noted. Four dilutions were chosen for the main assay to include one dilution that will give 100% positive response. The animals were also examined after 24 and 48 hours to ascertain the chronic inflammatory dose. The degree redness corresponding to "++" was noted giving result corresponding to "I. U" i.e. Irritant Units of Hacker (Hecker, 1971) and was also cited by Evans and Schmidt and Evans & Soper (Evans and Schmidt 1979; Saeed 1987; Evans and Soper 1978). If no redness was observed, the assay was repeated by using more concentrated solution of the extract on new rabbit's ear. For the main assay, a group of 6 rabbits for each dilution was used. 10µl of the most diluted solution of the chosen series was applied to one of the ears of rabbit in that group. The animals of the dilution group were also treated similarly, by increasing concentration of the irritant that is 20, 40 and 60 µl. Rabbits were examined after 30 minutes of application and then after 30 minutes intervals. Inflammation of the major blood vessels in rabbit ear was noted. ID₅₀ i.e. Irritant Dose in 50% individuals was taken as a dose corresponding to the 50% cumulative frequency. The dose causing an ear redness to the degree ++ is defined as irritant units (IU) and expressed in µg/ml per rabbit ear (Evans and Schmidt 1979; Hecker 1971; Saeed 1987; Evans and Soper 1978).

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.

RESULT

Phytochemical analysis

Petroleum ether extract, chloroform and methanolic extracts are obtained by successive extraction. Four major active compounds (namely Ep 1, Ep 2, Ep 3 and Ep 4) were isolated and purified from the active chloroform extract of *Euphorbia pilulifera*, by silica gel column and thin layer chromatography.

Spectral analysis

Isolated fraction Ep 1

The ultraviolet spectrum of this compound was determined in spectral grade chloroform and shown absorption maxima as $\lambda_{\max} = 231 \text{ nm}$ and 246 nm (fig. 1).

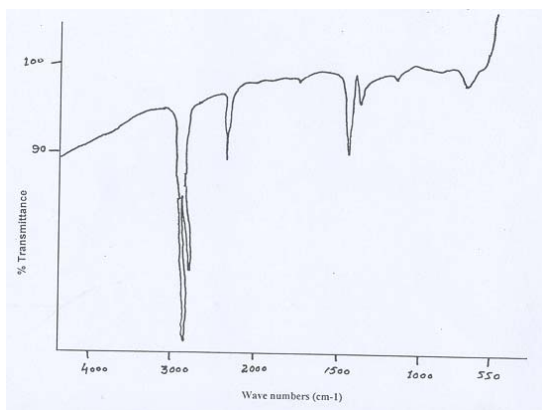


Fig. 1: Ultraviolet spectrum of compound Ep-1. Ultraviolet and Infrared spectrum of compounds isolated from chloroform extract of *Euphorbia pilulifera*

The infrared spectrum of this compound (fig. 2) exhibited absorption at 2850(strong), 2360 (weak), 1463 (medium), 1377 (weak) and 730 (weak) cm^{-1} .

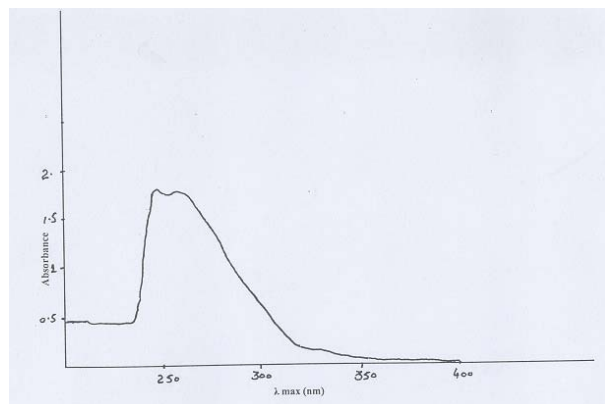


Fig. 2: Infrared spectrum of compound Ep-1.

Isolated fraction Ep 2

The ultraviolet spectrum of this compound was determined in spectral grade chloroform and shown absorption maxima as $\lambda_{\max} = 210 \text{ nm}$ and 256 nm .

The infrared spectrum of this compound exhibited absorption at 2854 (strong), 2360 (medium), 1458 (medium), 1377 (weak) cm^{-1} .

Isolated fraction Ep 3

The ultraviolet spectrum of this compound was determined in spectral grade chloroform and shown absorption maxima as $\lambda_{\max} = 234 \text{ nm}$ and 245 nm (fig. 3).

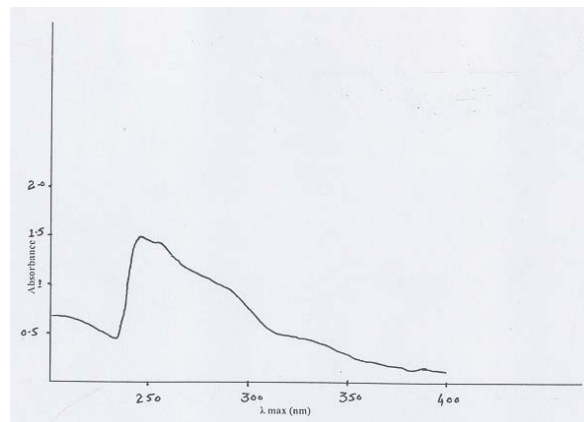


Fig. 3: Ultraviolet spectrum of compound Ep-3.

The infrared spectrum of this compound (fig. 4) exhibited absorption at 2926 (strong), 2854 (medium), 1710 (weak), 1604 (weak), 1460 (medium), 1377 (weak), 1260 (weak), 1188 (weak), 1081 (weak), 967 (medium), 895 (weak), 808 (weak) cm^{-1} .

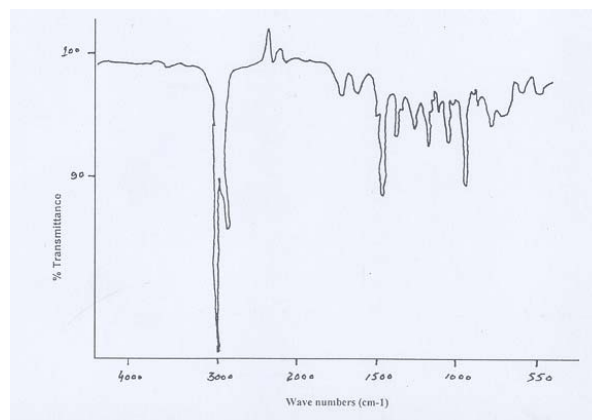


Fig. 4: Infrared spectrum of compound Ep-3

Isolated fraction Ep 4

The ultraviolet spectrum of this compound was determined in spectral grade chloroform and shown absorption maxima as $\lambda_{\max} = 236 \text{ nm}$ and 262 nm .

The infrared spectrum of this compound exhibited absorption at 2923 (medium), 2855 (medium), 1527 (strong), 1460 (weak), 1347 (strong), 1069 (weak), 851 (weak), 792 (weak), 681 (strong) cm^{-1} .

Irritancy assay

The results of the preliminary irritant responses of crude extract of *Euphorbia pilulifera* on rabbit's ear have been outlined in table 2. The results of irritant reactions of individual isolated compounds (Ep 1, Ep 2, Ep 3 and Ep 4) at four different doses have been outlined in table 3.

of locally occurring *Euphorbia pilulifera* and its isolated compounds had been carried out. Skin of albino rabbit's ears was used for this purpose. This aspect of dermatitis could easily be evaluated in animals by observing a quick response of animal against the different solvent extracts and the isolated compounds, with a suitable application dose (Saeed 1987; Mitchell and Rook 1979; Evans and Schmidt 1980; Marks and Samson 1977; Glickman 1979; Fregret 1982; Robert 1982; Anderson *et al.*, 1987).

DISCUSSION

One of the harmful dermatological potential i.e. Irritancy

Table 2: Preliminary Irritant Response of Solvent Extracts of *Euphorbia pilulifera* on rabbit's ear.

Dose 10mg/ml	Extracts	Acute response										Chronic response	
		15 min	30 min	45 min	1 h	1.5 h	2 h	2.5 h	3 h	3.5 h	4 h	24 h	48 h
20µl	Pet. Ether	-	-	-	-	-	-	-	-	-	-	-	-
	Chloroform	-	-	-	-	-	±	±	+	++	++	-	-
	Methanol	-	-	-	-	-	-	-	-	-	-	-	-
40µl	Pet. ether	-	-	-	-	±	-	-	-	-	-	-	-
	Chloroform	-	-	-	-	-	±	±	+	+	+	-	-
	Methanol	-	-	-	±	±	-	-	-	-	-	-	-
60µl	Pet. ether	-	-	-	-	±	-	-	-	-	-	-	-
	Chloroform	-	-	-	-	-	±	±	+	++	++	++	+
	Methanol	-	-	-	±	±	-	-	-	-	-	-	-
80µl	Pet. ether	-	-	-	-	-	-	-	-	-	-	-	-
	Chloroform	-	-	-	-	-	±	±	+	++	++	++	+
	Methanol	-	-	-	±	-	-	-	-	-	-	-	-

Table 3: Irritant Response of Isolated Compounds from Chloroform Extract of *Euphorbia pilulifera* on rabbit's ear at different doses.

Isolated Compounds	Dose [1mg/ml]	Acute response										Chronic response	
		15 min	30 min	45 min	1 h	1.5 h	2 h	2.5 h	3 h	3.5 h	4 h	24 h	48 h
Ep 1	10 µl	-	-	-	-	±	+	+	+	+	++	++	++
	20 µl	-	-	-	±	+	++	++	++	++	++	++	+
	40 µl	-	-	-	±	+	++	++	++	++	++	++	+
	60 µl	-	-	-	±	+	++	++	++	++	++	++	+
Ep 2	10 µl	-	-	-	-	-	-	-	±	+	+	-	-
	20 µl	-	-	-	-	-	-	±	±	+	+	-	-
	40 µl	-	-	-	-	-	-	±	±	+	+	-	-
	60 µl	-	-	-	-	-	-	±	±	+	+	-	-
Ep 3	10 µl	-	-	-	-	-	-	-	+	+	+	++	-
	20 µl	-	±	+	+	+	++	++	++	++	++	++	++
	40 µl	-	±	+	+	+	++	++	++	++	++	++	++
	60 µl	-	±	+	+	+	++	++	++	++	++	++	++
Ep 4	10 µl	-	-	-	-	-	-	-	±	±	±	-	-
	20 µl	-	-	-	-	-	-	-	±	±	+	-	-
	40 µl	-	-	-	-	-	-	-	±	±	+	-	-
	60 µl	-	-	-	-	-	-	-	±	±	+	-	-

Where
 - = No reaction
 ± = Doubtful reaction
 + = Slight redness
 ++ = Redness between two vessels
 +++ = Sever redness of blood vessels as well as surrounding areas

For the isolation and purification of phytochemical compounds from this species, successive solvent extraction was carried out. For this purpose, both non-polar and polar solvents i.e. petroleum ether (40-60°C), chloroform and methanol were used. The solvent extraction procedure was based on the assumption that petroleum ether (40-60°C) was a non-polar solvent; it probably extracted the least polar compounds. Methanol on the other hand was a polar solvent, extracted most of the polar components from the crude powder. Chloroform possessed an intermediate polarity and probably extracted the compounds with intermediate polarities. The dried powder of whole herb (700 g) of *Euphorbia pilulifera* was thus subjected to successive extraction in these three common solvents under the laboratory conditions. The results of the different solvent extraction in the form of percentage yield indicated that the production of both polar and non-polar components were not equal. Out of the three types of extracts, the polar components (22.71 %) were extracted in methanol and were in higher yield than others. The components with intermediate polarity (13.21%), which were extracted with chloroform, were next in the yield. On the other hand the non-polar components (7.28 %) which were extracted in petroleum ether were in the lowest yield. It could thus be concluded that the powdered material of *Euphorbia pilulifera* used in this investigation, contained a larger proportion of high polarity compounds than the non-polar components.

All three extracts were subjected to a comparative TLC analysis using different solvent systems. The main purpose of this analysis was to know the total number and chromatographic behavior of the compounds present in each extract. Out of different solvents systems used, the best solvent system which resolved the mixture of petroleum ether extract into six components was petroleum ether/chloroform (with ratios 80:20, 80:25 and 70:20). Chloroform extract was segregated maximum into eight components by the chloroform/ methanol (95:02, 95:03, 90:10 and 80:03 ratios) and petroleum ether/ chloroform (90:10 and 100% chloroform). On the other hand the mixture of polar components present in the methanol extract was best resolved into ten compounds by chloroform / methanol (in ratios of 70:25).

Preliminary irritancy assay was performed with the three solvent extracts and the isolated compounds of *Euphorbia pilulifera* on rabbit's ear. This method was originally innovated by Hecker in 1971 (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 (Evans and Schmidt 1979) and Evans and Soper (Evans and Soper 1978), for evaluating the irritancy of tigliane, daphnane and ingenane series of diterpene esters from various *Euphorbia* species (Saeed 1987; Evans and Soper 1978; Evans and Schmidt 1980). 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 for assessing the irritancy behavior. Although the erythema produced by these extracts/ isolated compounds were weak and diffused, but could easily be evaluated and could also be comparable to a similar reaction on other mammalian skins. Many authors used albino rabbits instead of mice for similar purpose (Anderson et al. 1987; Marzulli and Miabach 1975; Mitchell and Rook 1977; Sinigaglia et al., 1985).

The results further indicated that three solvent extracts except chloroform extract exhibited either no or doubtful irritant responses with various dose levels. Chloroform extract seemed to be more irritant than other two extracts at different dose levels and was more irritant at maximum dose up to 80 µg. Petroleum ether extract seemed to be nearly inert in its irritant reaction. It could thus be concluded that the constituents present in chloroform extract of *Euphorbia pilulifera* were responsible for such adverse reaction on the animal's skin.

The Chloroform extract of *Euphorbia pilulifera* which was found biologically active, was further subjected to column chromatographic analysis to isolate the active irritant compound/s, using an increasing quantity of chloroform in petroleum ether. The elution process was monitored by silica gel thin layer chromatography. Eleven pooled fractions were obtained. All the pooled column fractions did not give prominent irritant reactions.

Further, four compounds with more biological activity were isolated from the sixth, seventh, ninth and tenth pooled column fraction of the chloroform extract of *Euphorbia pilulifera*. They were named as Ep 1 to Ep 4.

Isolated fraction Ep 1

This was isolated and purified from the sixth column fraction. The spectral evidence showed that this fraction was probably a compound containing methyl, aryl, ketonic and carboxylic acid, amine or secondary amide group. Also phosphorous compound or traces of phosphorous were probably present (Williams and Fleming 1980).

Isolated fraction Ep 2

This was isolated and purified from the seventh column fraction. The spectral evidence indicated that this fraction probably contain methyl, aryl, ketonic and carboxylic acid, amine or secondary amide group. Bromide group showed the presence traces of halogens in the compound (Williams and Fleming 1980). This compound was very similar to Ep 1.

Isolated fraction Ep 3

Ep 3, was isolated from the ninth column fraction. The spectral evidence showed that the Ep 3 was probably a

compound containing methyl, aryl, ketonic and carboxylic acid, amine or secondary amide group. It also contains some alcohol and ether group in it. $-\text{SO}_2-\text{N}<$ group is also possibly present in it (Williams and Fleming 1980).

Isolated fraction Ep 4

This was isolated and purified from the tenth column fraction. The available spectral evidence showed that the Ep 4 was probably a compound containing methyl, aryl, ketonic and carboxylic acid, amine or secondary amide group. Also contain some alcohol and ether group in it (Williams and Fleming 1980).

CONCLUSION

Out of the four isolated fractions; only two fractions (namely Ep 1 and Ep 3) exhibited moderate to mild irritant responses on the rabbit's skin. Maximum response was demonstrated by Ep 3 when dose of 60 μg and 80 μg was applied on rabbit's ear. Erythema exhibited by this compound spread in an area of 2.5 cm^2 . The irritant response of the compound was started after 45 minutes and reached to a ++ intensity after two hours. The reaction lasted for about 48 hours, and then faded away. The remaining two isolated compounds with same concentration exhibited either no or doubtful irritant reactions on rabbit's ear.

These results were similar to the results found by other authors when they used weak irritant phytochemical compounds on animal skin, from natural plants sources (Schmidt and Moulton 1983; Evans and Schmidt 1979; Marzulli and Miabach 1975; Sinigaglia *et al.*, 1985; Saeed and Sabir 1994; Landsteiner and Chase 1942). It was further postulated from the results that the compound Ep 3 probably penetrated through the skin of rabbit's ear more easily as compared with other compounds. The presence of $-\text{OH}$, $-\text{COOH}$, or $\equiv\text{N}^+$, $=\text{NH}$ and $-\text{NH}_2$ groups or ketonic group were responsible to react with the cell membrane and cellular contents of both the superficial and deeper layers of epidermis. As a result, inflammation of both the superficial and the deeper layers occurred, which probably caused damage to the epidermis. The mechanism of action of the compound was probably similar to the results as that of moderate to strong irritant compounds previously investigated by other workers, on different plant sources (Schmidt and Moulton 1983; Evans and Soper 1978; Anderson *et al.*, 1987; Saeed and Sabir 1994). There might be two possible reasons for the moderate to weak reaction of ++ to + intensity, displayed by Ep 3. Firstly the compound itself penetrated to the skin easily. Secondly the nature of the molecule was not so drastic 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.

It could therefore be concluded that a detail chemical characterization and structural elucidation of these phytochemical compounds is necessary, to establish structure-activity relationship of such important molecules in terms of irritant activity.

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