Counter irritant activity of *Carthamus oxycantha*

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Abstract: Many locally occurring species of Asteraceae are used as medicinal plants by various tribal and ethnic communities in Pakistan. *Carthamus oxycantha* is often occurs as weed in cultivated fields. Folk medicines indicated its use as an anti inflammatory and wound healing plant. It is used for wound healing by the local population in the form of powder paste. No scientific report, about the behavior of this plant has so far been published. The counter irritant studies of locally occurring *Carthamus oxycantha* was carried out. The main objectives of the project were to evaluate its wound healing effects on animal skin and the identity and characterization of chromatographically isolated fractions. For this purpose, different solvents with a broad range of polarity were successively used to extract non-polar compounds (petroleum ether extract), constituents intermediate polarities (chloroform extract) and polar constituents (methanol extract) from the whole herb of *Carthamus oxycantha*. The counter irritant activity of the crude extracts and isolated fractions was evaluated on rabbit's skin. Five fractions Co-1 to Co-5 were isolated from the active chloroform extract by column and thin layer chromatography. Co-1, Co-3 and Co-5 appeared to be the most potent counter irritant than others. A possible structure-activity relationship of these active compounds was investigated by using spectroscopy (UV and FTIR analysis).

Keywords: Oxycantha, Counter irritant, chromatography, rabbit's skin, spectroscopy.

INTRODUCTION

Carthamus oxycantha is found as weed in the cultivated fields. It belongs to family Asteraceae which is the largest family of dicotyledon angiosperm. It includes more than 1000 genera and about 10000 species distributed all over the world. Mostly it contain herbs or under shrubs, rarely trees or climbers (Ahmad e*t al*; 2007-2010). *Carthamus oxycantha is* found in India on dry parts of Punjab and Uttar Pradesh extending to Pakistan in the west. It has great potential as an oil seed crop. It is a good source of edible and drying oil. The oil is known as Poli oil; the chief ingredient in Afridi wax and is used as glass cement (http://www.floracafe.com). *Carthamus oxycantha* seeds yields two types of oils, oleic oil and linoleic oil (Kashyap and Joshi, 1936; Nadkarni, 1976; Chopra *et al*., 1982; Fernandez-Martinez *et al*., 1993). Fatty acid oil composition of oleic oil is palmitic acid 5-6 %, stearic acid 1.5 -2 %, oleic acid 74-80 %, linoleic acid 13-18 % and traces of linoleic acid and longer chain fatty acids (Singh and Kumar, 1947). *Carthamus oxycantha* fruit also contains proteins 20-25 %, hull 60 %, residual fat 2-15 %. Flowers of *Carthamus oxycantha* contain two major pigments, the water soluble, yellow carthamidin and the formally important dye carthamin, flavonone which is orange red. Flowers also contain 0.3-0.6 % carthamin. Flavonoids, glycosides, sterols and serotonin derivatives have been identified from flowers and seeds (Firestone, 1999). By mass spectrometric and extensive spectroscopic analysis two new glycosides, 2-O-methylglucopyranosyl-carthamoside and beta-D-fructofuranosyl carthamoside, along with the known compound 3',4',5,7-tetrahydroxy-flavanone have been isolated from Carthamus oxyacantha, using recycling preparative HPLC. (Hassan *et al*, 2010).

Young leaves of *Carthamus oxycantha* were used as a vegetable, whereas seeds were used in cooking. The fruit was used as bird feed. *Carthamus oxycantha* herbage is valuable as green fodder in many countries. The straw was also used as fodder. Flowers were used to treat cerebral thrombosis, male infertility, rheumatism and bronchitis (Fernandez-Martinez *et al*., 1993; Singh, Kumar, 1947). *Carthamus oxycantha* based medicines also showed beneficial effect on pain and swelling associated with trauma. Flowers of this species were also used to treat jaundice, while the seeds were considered as laxative. The sap was believed to reduce salivation. The oil was applied to treat scabies. The edible oil extracted from the seed was now the main product of *Carthamus oxycantha*. Although the oil was suitable for paint production, it was used mainly in cooking and for making salad dressings and margarine. *Carthamus oxycantha* had been used traditionally to make roghan wax used in the batik industry (Firestone, 1999). *Carthamus oxycantha* had long been grown for the dye extracted from the flowers. Depending on the dyeing procedure and the addition of other colourants and mordants, it imparts a yellow, red, brown or purple colour to cloth. However, dyes were still produced on a small scale for traditional and religious purposes. *Carthamus oxycantha* was a substitute or adulterant for true saffron as a natural food colourant. Flowers of this species were commonly mixed

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with rice, bread, pickles and other food to give them an attractive orange colour. The seed cake was used as animal feed. The cake from undecorticated seeds containing matairesinol-glucoside was only suitable for ruminants. *Carthamus oxycantha* meal and flour from decorticate seed were used in the production of high-protein human diet supplements (Fernandez-Martinez *et al*., 1993). The crude extract of *Carthamus oxycantha* and its fractions *in vitro* caused an atropine sensitive spasmogenic effect in guinea-pig ileum (Gilani *et al;* 2005). *Carthamus oxycantha* processes significant antihyperlipidemic activity (Ahmad *et al.,* 2009). It has shown significant reduction during harvesting the cereal crops, local people suffer irritant problems by different kind of weeds. The folk medicine indicated the counter irritant potentials of some species of weed, including, *Carthamus. oxycantha*. Such common observation during the harvesting period of wheat in the month of April/May inspired to investigate the biological activity of this species.

MATERIAL AND METHODS

Carthamus oxycantha was collected from different wheat fields of Lahore and was authenticated from Dr Sultan Harbarium of GC university Lahore-Pakistan vide specimen no GC-Herb-Bot.611. The plant material was dried under shade and whole plant was pulverized. The pulverized plant was stored in amber colour bottles.

Instruments

Instrument used in the present investigation include Soxhlet apparatus, Rotary Vacuum Evaporator (Tokyo Rikakikai Co., Ltd.), Analytical Balance (Sartorius), Oven (Memmert, W. Germany), Spectrophotometer (Shimadzu UV-160A Japan), FTIR SPECTRUM BX system (Perk Elmer), Refrigerator.

Chemicals

Following chemicals of B. D. H. analytical grade were used which were purchased from local market: Iodine, H_2SO_4 (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 (40-60ºC), 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 fractions 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 ìl micro capillaries (Doummond 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 solvent extractions of the pulverized dried *Carthamus oxycantha* plants were carried out by using 2.5 Liters of Petroleum ether (40-60º), Chloroform and methanol respectively. About 1000grams 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. 20 grams of chloroform extract of *Carthamus oxycantha* were adsorbed on 25 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, 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.

Biological assay for counter- irritancy

Assay for counter irritant effect of solvent extracts/ column fractions/isolated fractions, was performed with some modifications in assay described by Evans and Schmidt (1979). Sand paper with fine particles was used to irritate inner skin surface of rabbit's ear skin in clockwise direction for about 10 minutes. Irritation, Redness, erythema was produced in area of 2.0 cm^2 in diameter. 100µl solution from each solvent extracts/ column fractions/isolated fractions was applied to the irritated area. Untreated ear was used as control. Ears were examined for intensity of erythema after every 30 minutes up to seven observations. A group of 6 rabbits for each dilution was used while performing main assay. The most diluted solution of the chosen series applied to one of the ears of rabbit in that group. The animals of the other groups for other dilutions were also treated similarly, by increasing concentration of irritants. Rabbits were examined after every 30 minutes. The numbers of

ear showing decreased irritancy, redness and erythema were recorded. The method was originally presented by Hecker (Hecker, 1971; Ishtiaq *et al.,* 2012). The authors used albino mice as experimental animal for their investigations. In the present work, same method was used, but instead of mice, rabbits were used as animal model for assessing the counter irritancy behavior. The counter irritancy procedure 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 this purpose (Anderson, 1987; Marzulli and Miabach, 1975; Lampe and Fagerstorm, 1968; Benazra C, Sigman CC, Perry, Helmes and Maibach, 1985).

STATISTICAL ANALYSIS

The results obtained were analyzed by calculating mean and standard error of mean by using statistical package SPSS version 20. This helped to understand the effect of the crude extract and isolated fractions of the plant.

RESULTS

The results of the successive solvent extraction indicated that the polar components extracted in methanol (4.11%), were in higher yield than others. The components with intermediate polarity extracted with chloroform (2.66%) were next in yield. On the other hand, the non- polar components extracted in petroleum ether (1.95%) were in lowest yield. It could thus be concluded that the powdered *Carthamus oxycantha* used in this investigation, contained a larger proportion of polar compounds or compounds with intermediate polarity than the non-polar components. All three solvent extracted materials were subjected to a comparative TLC analysis using different solvent systems (Stahl, 1969). The main purpose of these analyses was to have an idea of the total number and chromatographic behavior of the different compounds present in each extract. Out of the different solvent systems used, the best solvent system which resolved the mixture of petroleum ether extract into six components was petroleum ether/$CH2Cl_2$ ratios (90:10, 85:5 and 70:25). Chloroform extract was segregated maximum into five components by $CHCl_3$/MeOH (90:5 and 90:10). On the other hand the mixture of polar components present in the methanol extract was best resolved into major six compounds by MeOH/$CHCl_3$ (90:15). The Results of primary counter irritancy assay with all the three solvent extracts at different doses are shown in table 1. Main counter irritancy assay was performed with the isolated fraction (Co-1 to Co-5) of biologically active chloroform extract and results are presented in the table 2. The spectroscopic (VU and Infrared) finding of all the isolated fractions (Co-1 to Co-5) are highlighted in the figs. 1-10.

DISCUSSION

Preliminary counter irritant assay was performed with all three types of solvent extracts of *Carthamus oxycantha* on irritated rabbit's ear. Three extracts were applied on irritated rabbit's ears to examine their counter irritant activities. The counter irritant intensity of these extracts could be evaluated by healed area. The result indicated that petroleum ether; chloroform and methanol extracts exhibited counter irritant responses, when the doses of 100ug/10ml of each extract were applied. Petroleum ether extract showed nearly inert in its counter irritant response. Chloroform extract showed well marked counter irritant property when a dose of 100 µg/10ml was used on irritated rabbit's ear. Methanol extract on the other hand, showed weak counter –irritant response even at high dose. It could thus be concluded that the highly polar and intermediate polar constituents of *Carthamus oxycantha* were responsible for its counter irritant and anti-inflammatory property on irritated animal skin. Since the chloroform extract of *Carthamus oxycantha* was active and obtained in sufficient amount, it was further subjected to column chromatographic analysis to isolate the active compounds using an increasing quantity of chloroform in petroleum ether. The elution process was monitored by thin layers. Twelve pooled fractions were obtained. Five pooled column fractions out of twelve were dermatologically active. They produced well marked counterirritant responses on irritated rabbit's ear after 30 minutes of application and in most instances this reaction lasted for about 72 hours. Their counterirritant responses started after nearly 2 hours and lasted for even more than 72 hours. Well marked counter irritant response healing intensity of irritated skin was observed. Five fractions were isolated from the second, third, fifth, seventh and eighth active pooled column fractions of the chloroform extract of *Carthamus oxycantha*. They were named as Co-1, Co-2, Co-3, Co-4 and Co-5.

Isolated fraction Co-1

This fraction was isolated from the second column fraction. It was yellowish orange. Thin layer chromatography of this fraction indicated only one major spot, when a number of solvent systems. It gave a yellow colour with iodine vapours possibly due to some unsaturation. Under UV light, the spot appeared with blue fluorescence. It gave pink coloured spot with Liberman Burchad reagent. It probably indicates some steroid/ diterpenes or triterpenes.

The fraction Co-1 had strong UV absorption at max = 285 nm. This strong absorption was probably due to $n \rightarrow \pi^*$ transition, which suggested the presence of carbonyl group either of a ketone or an aldehyde or carboxylic acid (Williams and Fleming, 1980). In infrared system of Co-1, showed a strong band at 2987 cm^{-1} due to the -CO-CH_3 group which was often very weak bonding. Second peak

Table 1: Counter-Irritant response of crude solvent extracts of *Carthamus oxycantha* on rabbit's ear

Dose levels µg/10ml	Counter irritant Response (Healed Area – cm^2) after *												
	Extract	1hr	2hr	3hr	4hr	5hr	6hr	7hr	8hr	9hr	10hr	24hr	48hr	72hr
100	Pet. ether	–	–	–	–	–	–	–	–	–	–	–	–	–
	Chloroform	–	0.41	0.43	0.48	0.52	0.79	0.79	0.79	0.79	0.79	0.99	0.99	1.1
	Methanol	–	0.21	0.24	0.29	0.31	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
50	Pet. ether	–	–	–	–	–	–	–	–	–	–	–	–	–
	chloroform	–	–	–	0.39	0.41	0.41	0.41	0.45	0.45	0.65	0.70	–	–
	Methanol	–	–	–	0.16	0.20	0.25	0.30	0.30	0.30	0.30	–	–	–
40	Pet. ether	–	–	–	–	–	–	–	–	–	–	–	–	–
	Chloroform	–	–	–	0.24	0.30	0.34	0.40	–	–	–	–	–	–
	Methanol	–	–	–	–	–	–	–	–	–	–	–	–	–
25	Pet. ether	–	–	–	–	–	–	–	–	–	–	–	–	–
	chloroform	–	–	–	–	–	–	–	–	–	–	–	–	–
	Methanol	–	–	–	–	–	–	–	–	–	–	–	–	–
Control		2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0

Where*=Mean reading of healing area of treated rabbits ear group. Hr= hours from application of drug.

Table 2: Counter-Irritant response of isolated fractions of chloroform extracts of *Carthamus oxycantha* on rabbit's ear

Isolated fraction	Dose levels µg/10ml	Counter irritant Response (Healed Area – cm^2) after *														
		1hr	2hr	3hr	4hr	5hr	6hr	7hr	8hr	9hr	10hr	11hr	12hr	24hr	48hr	72hr
Co-1	100	-	.53±.01	.55±.01	.6±.02	.7±.03	.9±.02	.9±.01	.8±.02	.8±.03	.8±.04	.8±.01	.8±.02	.9±.02	1.1	1.1
	50	-	-	-	.5±.01	.6±.03	.6±.03	.6±.05	.7±.05	.7±.05	.7±.05	.8±.04	.8±.04	-	±.04	±.03
	40	-	-	-	.3±.04	.3±.03	.3±.01	.3±.02	-	-	-	-	-	-	-	-
	25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
															-	-
Co-2	100	-	.53±.02	.55±.01	.6±.02	.7±.03	.7±.03	.8±.02	.8±.02	.8±.03	.8±.04	.8±.05	.8±.01	.9±.02	.9±.03	.9±.01
	50	-	-	-	.2±.01	.3±.03	.3±.02	.4±.07	.4±.04	.4±.03	.4±.06	.4±.05	.4±.02	-	-	-
	40	-	-	-	.35±.02	.35±.03	.35±.04	-	-	-	-	-	-	-	-	-
	25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Co-3	100	-	.5±.02	.5±.03	.6±.02	.7±.03	.8±.01	.8±.09	.8±.02	.8±.01	.9±.03	.9±.04	.9±.05	.9±.02	1.2	1.2
	50	-	-	.4±.03	.4±.02	.4±.01	.5±.03	.5±.05	.5±.04	.5±.02	.6±.01	-	-	-	±.02	±.02
	40	-	-	-	.2±.02	.2±.01	.3±.01	.3±.02	-	-	-	-	-	-	-	-
	25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
															-	-
Co-4	100	-	.3±.02	.3±.02	.3±.03	.5±.05	.5±.02	.5±.03	.6±.05	.8±.02	.9±.02	.9±.02	.9±.02	-	-	-
	50	-	-	-	.3±.02	.3±.01	.5±.03	.5±.05	.5±.04	.5±.02	.6±.01	-	-	-	-	-
	40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Co-5	100	-	.3±.02	.3±.02	.3±.03	.5±.05	.5±.02	.5±.03	.6±.05	.8±.02	.9±.02	.9±.02	.9±.02	-	-	-
	50	-	-	-	.3±.02	.3±.01	.3±.04	.3±.03	.3±.05	.3±.01	.3±.08	.3±.01	.3±.02	-	-	-
	40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Control		2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0

Where * = Mean reading of healing area of treated rabbits ear group. Hr= hours from application of drug

at 2852 cm^{-1} indicated the stretching vibration due to CH_3,=CH_2 and ≡CH and due to=NH,–NH_2,–NHCO or =NH_2CO. Another peak at 2360 cm^{-1} was also possibly due to N-H stretching, vibration shown by the presence of amine, ammonium and amide absorption. The presence and number of CH_3=CH_2 and ≡CH groups in the molecule were further indicated by peaks in the finger print region at 1469 and 1272 cm^{-1}. Two weak peaks at 947 and 719 cm^{-1} also revealed the presence of adjacent hydrogen atoms on the benzene ring, possibly due to the C–H stretching vibration in aromatic compound.The available spectral analysis indicated that fraction Co-1 was probably contained methyl, aryl, ketone, carboxylic acid or some secondary amides (Williams and Fleming, 1980).

Isolated fraction Co-2

This fraction was isolated from the third column fraction. It was yellowish orange. Thin layer chromatography of this fraction indicated only one major spot, when a number of solvent systems. It gave yellow colour with iodine possibly due to some unsaturation. Under UV, the spot appeared as blue fluorescent. It gave pink coloured spot by using Liberman Burchad reagent. It probably indicated some steroid/diterpenes or triterpenes nature of the molecule.

The fraction Co-2 had strong UV absorption at max = 260 nm. This strong absorption was probably due to n → π* transition, which suggested the presence of carbonyl group either of a ketone or an aldehyde or carboxylic acid

(Williams and Fleming, 1980). The infrared system of Co-2, showed a strong band at 2925 cm^{-1} possibly due to the C-CH_3 group which was often very weak bonding. Second peak at 2852 cm^{-1} indicated the presence of -CHO group. Another weak peak at 2360 cm^{-1} possibly due to N-

H group stretching, showed the presence of amine, ammonium and amide group. IR region also showed a band at 1737 cm^{-1} which was due to C=O group indicates the carbonyl group absorption possibly due to some aldehyde/ketone/ carboxylic acid/ acid anhydride. Another possibility of the band at 1458 cm-1 was due to presence of $-CH_3$ and $=CH_2$ groups in the molecule. Further peaks in finger print region at 1378 cm^{-1} and a weak intensity peak at 1171 cm^{-1} also indicated the presence of carbonyl group (C=O) absorption of aldehyde, ketone or some carboxylic acid. The presence of carboxylic group also confirmed by a weak intensity peaks at 721 cm^{1}. The available spectral evidence showed that the fraction Co-2 was similar to Co-1 which also contained methyl, aryl, ketonic, carboxylic acid and some secondary amide group.

Isolated fraction Co-3

This fraction was isolated from the fifth column fraction. It was of light yellow colour. Thin layer chromatographic analysis revealed that this fraction had one major spot, when a number of solvent systems. It showed yellow colour with iodine possibly due to some unsaturation. Under UV light, the spot appeared with blue fluorescence. It gave pink spot with Liberman Burchad reagent. It probably indicated some steroid/ diterpenes or triterpenes.

The isolated fraction Co-3 had strong UV absorption at max = 280 nm. This strong absorption was probably due to $n \rightarrow \pi^*$ transition, which suggested the presence of carbonyl group either of a ketone or an aldehyde or carboxylic acid. Infrared spectrum of Co-3 exhibited band at 3411 cm^{-1} which was possibly due to the OH group which indicated the presence of alcohol and phenol, it also indicated free OH absorption thus was possibly incorporated with the analytical sample. Second peak at 2924cm^{-1} was possibly due to some $–CO-CH_3$ stretching vibration. Third strong peak at 2852cm^{-}indicated presence of vibration of the -CHO group. Another weak peak at 2361cm-1 was due to N-H stretching, showed the presence of amine, ammonium and amide group. A strong peak 1737 cm^{-1} was possibly due to C=O group which indicated carbonyl absorption possibly due to some acidic anhydride/ ketone/ aldehyde/ some acid chloride. A medium peak at 1518 cm^{-1} was due to -NH bending. Medium peak at 1456 cm^{-1} indicated CH_2 and CH_3 groups. Medium peak at 1347 cm^{-1} was in the finger print region. A strong peak at 1259 cm^{-1} was further confirmed the presence of stretching of carbonyl group. One medium peak at 1168 cm^{-1} and a strong at 1123 cm^{-1} also indicated some organosulphur compound. Peaks at 986 cm^{-1}, 849 cm^{-1}, 812 cm^{1}, and 706 cm^{-1} further confirmed the alkene/alkyne absorption.

The available spectral evidence showed that the fraction Co-3 was similar to some extent with Co-1 and Co-2 isolated fractions. It was also a fraction with methyl, aryl, ketonic, carboxylic acid/sulphur/alkene, amine or secondary amide group with some -OH groups due to some alcohol or phenol (Williams and Fleming, 1980).

Isolated fraction Co-4

This fraction was isolated from the seventh column fraction. It was a light yellowish sticky oily fraction. Thin layer chromatography of this fraction indicated only one major spot, when a number of solvent systems. It gave yellow colour with iodine possibly due to unsaturation. Under UV light, the spot appeared as blue fluorescent. It gave light purple colour with Liberman Burchad reagent, which indicated some steroid/diterpenes or triterpenes nature of the molecule.

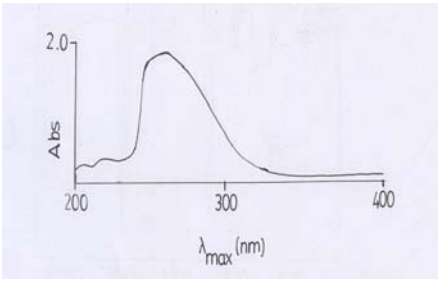
The fraction Co-4 had strong UV absorption at max=255 nm. This strong absorption was probably due to $n \rightarrow \pi^*$ transition, which also suggested the presence of carbonyl group either of a ketone or an aldehyde or carboxylic acid (Williams and Fleming, 1980). The infrared spectrum of Co-4 also exhibited a number of strong and weak intensity bands. Two strong intensity bands at 2939 cm^{-1} and 2853 cm^{-1} showed saturated C-H stretching vibrations possibly due to some alkane/alkene or alkyne. This band resulted from symmetrical and asymmetrical stretching mode in which two -CH bands of methyl groups were extending while third one was contracting. Another band at 2360 cm^{-1} indicated the presence of N--H symmetrical and asymmetrical stretching vibration possibly due to the $=NH_2$, ≡NH, ≡N or -CONH group in the molecule. A medium intensity peak at 1462 cm^{-1} indicated the presence of $-CH_3$, $=CH_2$, ≡CH groups in the molecule which was further shown by peak in finger print region at 1378 cm^{-1}. Two weak peaks at 876 cm^{-1} and at 720 cm^{-1} further showed the presence of alkene or aromatic groups (Williams and Fleming, 1980).

Isolated fraction Co-5

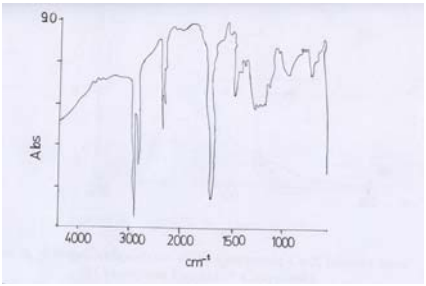
This fraction was isolated from the eighth column fraction. It was yellowish orange oily fraction. Thin layer chromatographic analysis of this fraction indicated only one major spot, when a number of solvent systems. It gave yellow colour with iodine possibly due to some unsaturation. Under UV light, the spot appeared with pink fluorescence. It exhibited light purple colour with Liberman Burchad reagent. It probably indicated some steroid/ diterpenes or triterpenes nature of the molecule.

The fraction Co-5 had strong UV absorption at max = 275 nm. This strong absorption was probably due to $n \rightarrow \pi^*$ transition, which suggested the presence of carbonyl group either of a ketone or an aldehyde or a carboxylic acid. Infrared spectrum of Co-5 showed band at 3331 cm^{-1} due to -OH absorption which was possibly due to some carboxylic, alcoholic or phenolic groups. The broadening of this band was due to the stretching vibration of -OH

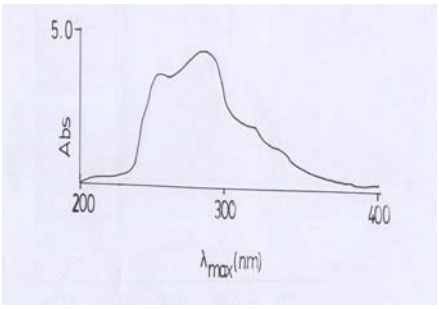
Spectral analysis of the fractions isolated by column chromatography



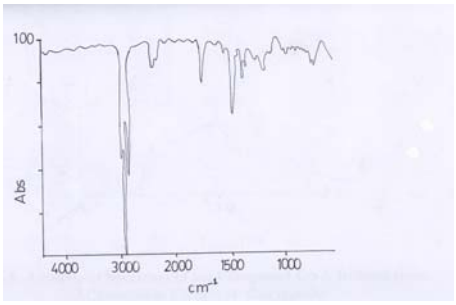
Ultraviolet spectrum of Co – 1



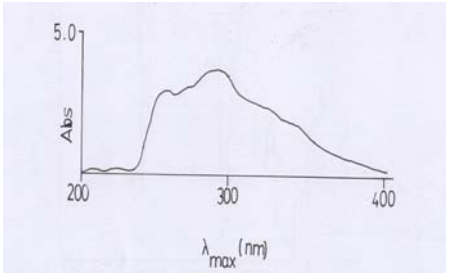
Infrared spectrum of Co – 1



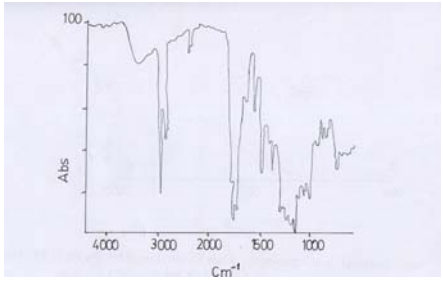
Ultraviolet spectrum of Co – 2



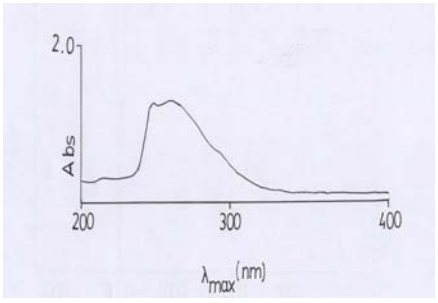
Infrared spectrum of Co – 2



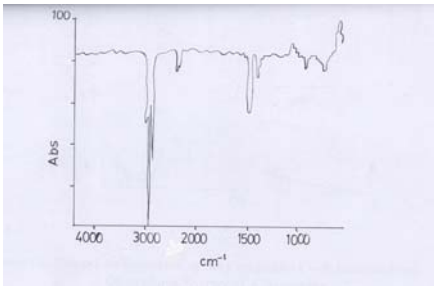
Ultraviolet spectrum of Co – 3



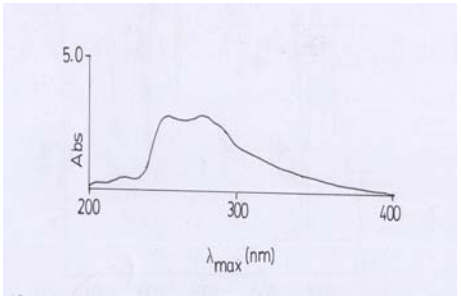
Infrared spectrum of Co – 3



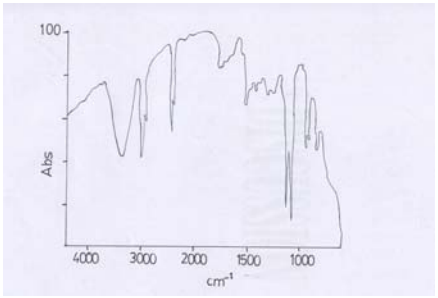
Ultraviolet spectrum of Co – 4



Infrared spectrum of Co – 4



Ultraviolet spectrum of Co – 5



Infrared spectrum of Co – 5

with intra molecular H-bonded at OH. A band at 2930 cm^{1} showed the C-H stretching vibration present in alkane or alkene. Such band shown by the methyl and methylene or aryl groups were possibly resulted due to the symmetrical and asymmetrical stretching of C-H modes. A medium intensity peak at 2342 cm^{-1} indicated the presence of amine, ammonium or amide possibly due to N-H stretching. A medium peak at 2360 cm^{-1} indicated the presence of N-H symmetrical and asymmetrical stretching vibration of $=NH_2$, ≡NH, ≡N or -CONH group in the molecule (Williams and Fleming, 1980). Weak peak at 1722cm^{-1} showed carbonyl group (C=O) absorption. A medium peak at 1462 cm^{-1} showed N=O which indicated the nitro group. Three peaks one weak at 1210 cm^{-1} and two strong at 1084 cm^{-1} and 1039 cm^{-1} indicated esters. Another peak at 883 cm^{-1} showed isolated -H group substitution pattern of benzene ring. Last medium intensity peak 783 cm^{1} indicated the alkene or aromatic group (Williams and Fleming, 1980).

The available spectral evidence revealed that the fraction Co-5 was not very similar to first four isolated fractions as it contained esters, carboxylic acid or some secondary amide group along with some -OH groups due to some alcohol or phenol.

CONCLUSION

All these five isolated fractions exhibited strong to moderate counter irritant response on the rabbit skin. Maximum irritant healing response was demonstrated by Co-1, Co-3 and Co-5, under the influence of 100µg/10ml dose. The healing intensity exhibited by these isolated fractions was similar. On the other hand, two other isolated fractions Co-2 and Co-4 demonstrated moderate healing response. All these isolated fractions started their action after two hours and continued up to 72 hrs.

During this period, nearly all the irritated area was healed and scars of the same size were observed, as compared to the controlled. Their effects seemed to be intensified not only with time but also with the increase in dose (from 50 µg to 100µg). Three isolated fractions (Co-1, Co-3 and Co-5) appeared to be stronger counter irritant than other two. They showed comprehensive counter irritant properties within 48 to 72 hours under the influence of a dose of 100µg/10ml. It was further postulated from the results that the isolated fractions Co-1, Co-3 and Co-5 probably penetrated through the skin damage of rabbit's ear with much ease when compared with the other isolated fractions. The presence of –OH, -COOH, or ketonic group in this fraction, was liable to be reacted with the inflamed cell membrane and cellular content of both the superficial and deeper layers of epidermis. As a result the inflamed and the damaged superficial and deeper layers probably healed. These results were similar to the results found by other authors and the mechanism of action of these isolated fractions was probably like the other counter irritant/ anti-inflammatory compounds previously investigated. There might be two possible reasons for weak counter irritant reactions exhibited by other two isolated fractions. Firstly the compound itself penetrated the skin barriers with a little difficulty, thus was not fully available to the damaged skin. Secondly the nature of its molecule was not so drastic or severe enough to cause any quick healing response to the epidermal tissues of the skin, but strong enough to enhance the repairing mechanism of the damaged superficial layers of skin over a longer time. It could be concluded that a detail chemical characterization of these phytochemical compounds is necessary, so that a structure-activity relationship of such important molecules in terms of counter irritant activity could be established.

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