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Full Length Article

Phytochemical Investigation of Irritant Constituents of *Cuscuta reflexa*

Syed Saeed ul Hassan[2], Shahid Rasool[1*], Muhammad Khalil-ur-Rehman [2], Saiqa Ishtiaq[2], Shahid ul Hassan[2], Imran Waheed[2] and M. Asif Saeed[2]
[1]*Faculty of Pharmacy, University of Sargodha, Sargodha-40100, Pakistan*
[2]*University College of Pharmacy, University of the Punjab, Lahore-54000, Pakistan*
*For correspondence: shahid_rph@hotmail.com

Abstract

Dodder (*Cuscuta reflexa* Roxb.) is found locally as parasitic weed on host plants. This plant often causes irritation on the hands. The main objective of the present investigation was to evaluate its irritation potential on animal skin. Skins irritating chemical constituents were separated in the form of various fractions. To do this, solvents with various polarities were used to extract least-polar compounds (petroleum ether extract), constituents of intermediate polarities [Chloroform ($CHCl_3$) extract] and polar constituents [methanol (MeOH) extract] from pulverized biomass of *Cuscuta reflexa.* Ten fractions were collected from methanol extract by Liquid Column Chromatography and purified by Thin Layer Chromatography. The irritation potential of these purified fractions was evaluated on rabbit's skin. Fractions eluted by $CHCI_3$ / MeOH (90:10), $CHCI_3$ / MeOH (40:60) and $CHCI_3$ / MeOH (20:80) showed more irritant potential. These biologically active purified fractions were characterized by Ultra Violet (UV) and Fourier Transform Infrared (FTIR) spectroscopy. The presence of –OH, –COOH, or ketonic group and a double bond in these fractions were liable to be reacted with the cell membrane and cellular contents of both superficial and deeper layers of epidermis causing irritancy. It was concluded that *Cuscuta reflexa* contained skin irritant compounds. © 2014 Friends Science Publishers

Keywords: *Cuscuta reflexa;* Solvent extraction; Liquid Column Chromatography; Irritating compounds; UV and FTIR spectroscopy

Introduction

Dermatitis caused by plants is commonly encountered in the practice of dermatology. Irritant properties of the plants have long been discovered by Indians, Chinese and Arab physicians (Bah *et al.,* 2012). Different plants produce different reactions on coming in contact with the skin, depending upon nature of plant, the type of skin and other varying factors (Behl *et al.,* 2004)

Cuscuta reflexa commonly known as Dodder or Akash, belongs to family Convolvulaceae. It is tropical and subtropical herb found as parasite weed on host plants. There are about 15 species of genus *Cuscuta* (Kirtikar, 1985; Battacharjee, 2001). It contains coumarin, flavonoids, α-amarin, β- amarin, oleonolic acid, stigmasterol and β-sitisterol which were detected from roots of the plant (Ramachandran *et al.,* 1992; Anis *et al.,* 1999). The presence of high molecular weight carboxymethylcellulose was also found in this plant (Chatterjee *et al.,* 1997). Soluble phenolic constituents mainly caffeic acid were extracted from *C. Reflexa* (Leoffler *et al.,* 1995). Seedlings of this parasitic plant synthesize ascorbic acid (Tommasi and Gara, 1990).

Cuscuta has ability to take up and accumulate alkaloids of host plant (Czygan *et al.,* 1988). The major glycoside, Isohamentin 3-O-neohesperidoside was isolated along with flavonol glycoside (Dandapani *et al.,* 1989). In addition to these compounds, aromandendrin and taxi-folin were also isolated from this plant which has anti-HIV activity (Mahmood and khan, 1989). *C. reflexa* is purgative, expectorant, tonic, diaphoretic, diuretic and aphrodisiac (Kirtikar, 1985). This and other related plants have antiviral activity against HIV (Mahmood and khan, 1989; Salehan *et al.,* 2013). This plant shows inhibitory effect towards *Bacillus subtilus*, *Pseudomonas aeuroginosa* and *Shigella dysenteriae* (Anjum and Khan, 2003). It also has antifungal activity against *Colletotrichum capsici* (Sinha *et al.,* 2004). Methanolic extract of *C. reflexa* delayed maturation in mice due to suppressed ovarian steroidogenesis (Mazumdera and Bhattacharya, 2003).

It was observed in practice that *C. reflexa* often cause irritation on the hands of those who collect it. As our efforts to explore the flora of Pakistan with respect to irritancy (Hassan *et al.,* 2012; Hassan *et al.,* 2013), the main objective of the present investigation was to evaluate its irritant potential on animal's skin.

Materials and Methods

Plant Material

Fresh wines of *Cuscuta reflexa* were collected from the villages of tehsil Phalia district Mandi Bahawal Din,

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Punjab, Pakistan. Plant was authenticated by Dr. Zaheer ud Din, Department of Botany, Government College University Lahore (Voucher specimen No. 651). Plant was spread on the laboratory tables and dried under the shade at room temperature for 7 days. Dried plant material was pulverized by using an electric mill.

Chemicals

Petroleum ether (40-60˚C), Chloroform, Methanol, Distilled water and Acetone were of analytical grade (BDH Company, England). Silica gel 60 (70-230 mesh ASTM) was used for Column Chromatography (E. Merck, Germany). Prepared TLC plates (20 x 20 cm) coated with silica gel on aluminium foils were used (E. Merck, Germany). A boro-silicate glass column (Pyrex) of 50 x 2.5 cm size was used for column chromatography.

Instruments

Rotary vacuum evaporator (Tokyo Rikakikai Co., Ltd, Japan), UV spectrophotometer (Hitachi-270-30), FTIR (Pye-Unicam SP-8-400).

Animals

Healthy adult male/female albino rabbits were purchased from the local market and identified as *Caprolagus hispidus*. These animals (1.0-1.5 kg body weight) were acclimatized in the animal house, University College of Pharmacy, University of the Punjab Lahore, for three days and were *ad libitum* fed carrots, fresh green fodder (clover) and tap water.

Solvent Extraction

Dried pulverized plant material (1 kg) was extracted successively in petroleum ether (40-60^{o}C) chloroform and methanol, using 2.5 L of each solvent for soaking. Maceration was carried out in each solvent for four days at room temperature (25±2.5^{o}C). Solvent of each extracted material was removed with rotary evaporator under reduced pressure and the extracts were weighed as shown in Fig. 1 (Brain and Turner, 1975).

Column Chromatography

Column was packed uniformly with 250 g of silica gel, (which was already activated by heating at 120˚C in an oven for 3 h) by slurry method. Chloroform was used for packing the column. A 20 g of methanol extract was adsorbed on 20 g of silica gel, using chloroform. Chloroform was completely evaporated and the dried silica gel adsorbed material after pulverization was put on top of the column. The column was first run with a mixture of chloroform and methanol then the polarity of the system was changed, by increasing the quantity of methanol in chloroform.

Table 1: Grading of irritant reactions

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 area in between.
+ + +	Intense reddening of the entire ear often accompanied with macroscopic visible hyperplasia.
+ + + +	Visible exudative lesion with marked epidermal damage.

Where, – = 0 = No reaction; ± = 1 = Doubtful reaction; + = 2 = Slight reddening of main vessels; ++ = 3 = Marked reddening of main vessels; +++ = 4 =Intense reddening of the entire ear

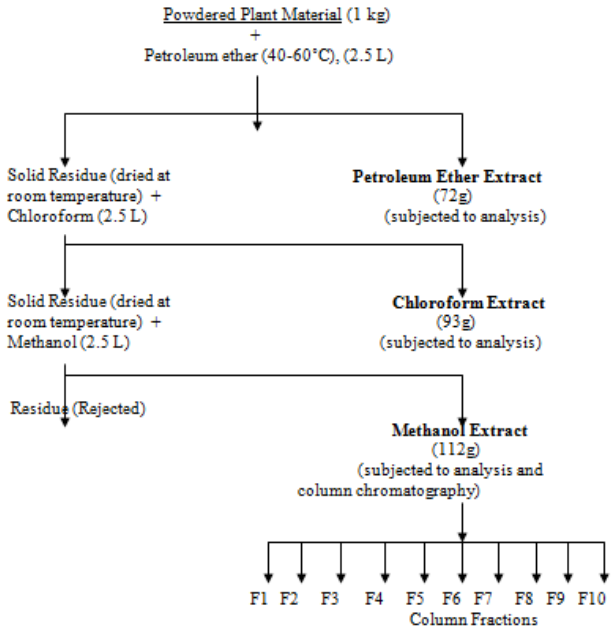

Fig. 1: Flow diagram showing solvent extraction and column fractions procedure

Ten fractions were collected in glass test tubes. These fractions were purified by PTLC using different solvent systems.

Irritancy Test

The biological assay for irritancy was adopted from Evans and Schmidt method (Evans and Schmidt 1980; Schmidt and Moult, 1983). A 20–50 µL solution from different dilutions was applied to the inner surface of rabbit's ear. Acetone was used as solvent as well as control. The ears were examined for redness after 15 min. of application and then after 30 min. intervals, until two examinations indicated that further redness would not occur. Time for maximum erythema was noted. The animals were also examined after 24 and 48 h to ascertain the chronic

inflammatory dose. Evaluation of irritant response of the extracts / fractions was done as given in Table 1. The dose causing an ear redness to the degree ++ was defined as irritant unit (IU) and expressed in µg/mL per ear (Hecker, 1971; Evans and Schmidt, 1980; Schmidt and Moult, 1983).

Characterization of Active Purified Fractions

Biologically active purified fractions were characterized by UV and FTIR spectroscopy. Ultraviolet Spectra were recorded on Hitachi-270-30 spectrophotometer using ethanol as a solvent. Infrared Spectra were measured on Pye-Unicam SP-8-400 spectrophotometer using thin film on sodium chloride disc.

Results

Solvent Extraction and %age Yield of Extracts

Out of the three types of extracted materials, the polar components (11.20%) which were extracted in methanol were yielded grater than other extracts. The components with intermediate polarity (9.33%), which were extracted by chloroform, were next in the yield. Conversely, non-polar fractions (7.24%) which were extracted in petroleum ether were the lowest in yield. Thus the powdered plant of *C. reflexa* contained greater fraction of polar compounds and those with intermediate polarities as compared to non-polar components.

Separation of Fractions

The methanol extract of *C. reflexa* which was present in sufficient amount, was used for column chromatography to obtain the fractions. Ten pooled fractions were collected and purified by PTLC using different mobile phases (Table 2).

Irritancy Assay

Results of preliminary irritation responses of crude extracts on rabbit's ear are given in Figs. 2–4. Methanol extract causes more irritancy as compared to chloroform and petroleum ether extracts. Results of irritant reactions of purified column fractions of methanol extract on rabbit's ear have been shown in Fig. 5. The result indicated that fractions eluted by $CHCl_3$/MeOH (90:10), $CHCl_3$/MeOH (40:60) and $CHCl_3$/MeOH (20:80) showed more irritant potential.

Characterization of Active Purified Fractions

Purified fractions 4, 6, 7, 9 and 10 showed more irritant potential. These biologically active purified fractions were characterized by UV and FTIR spectroscopy as shown in Table 3 and Fig. 6 and 7. UV spectra of these purified fractions showed absorption at λ_{max} (nm) 225, 245, 270, 280 and 255, respectively which was probably due to $n \rightarrow \pi^*$ transition, which suggested the presence of some double bonds in their molecules.

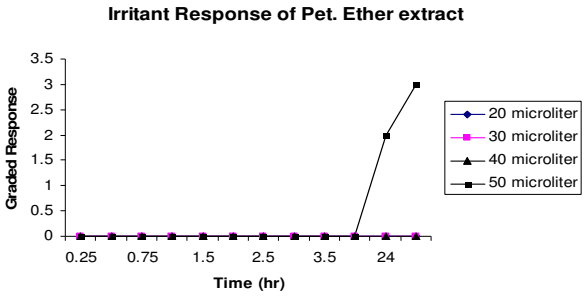

Fig. 2: Irritant response of Petroleum Ether extract

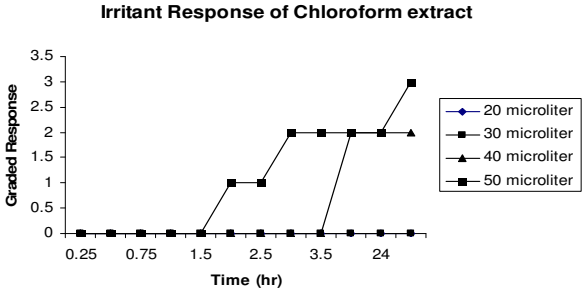

Fig. 3: Irritant response of Chloroform extract

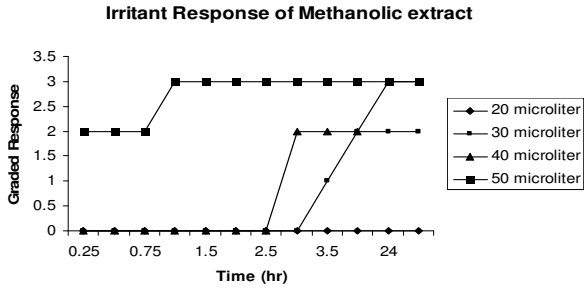

Fig. 4: Irritant response of Methanolic extract

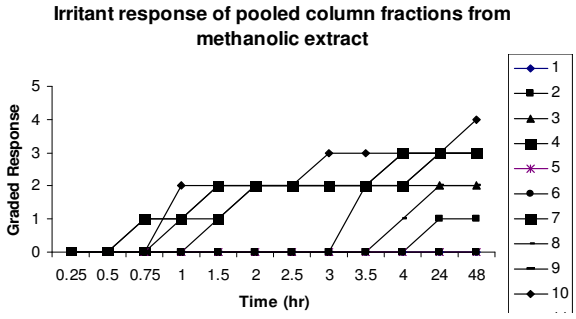

Fig. 5: Irritant response of column fractions from Methanolic extract

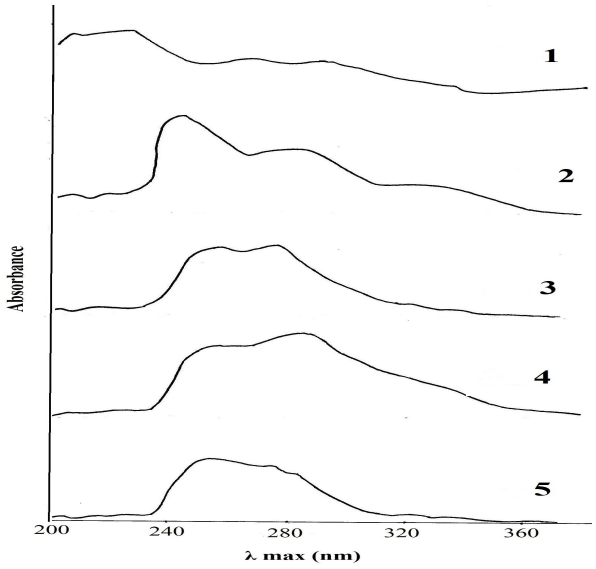

Fig. 6: UV spectra of isolated compounds

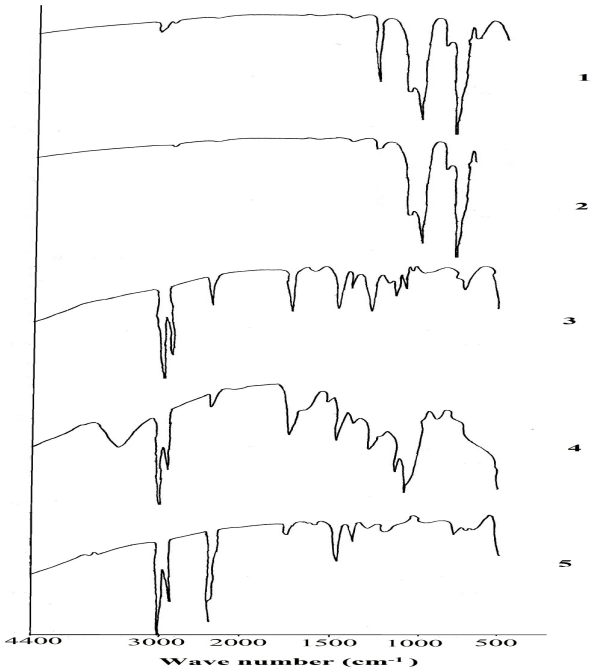

Fig. 7: FTIR spectra of isolated compounds

The available FTIR spectral evidence showed that the Fractions 4, 9 and 10 probably contained methyl/aryl/ketonic/carboxylic acid/acid anhydride/amine/nitrile or some secondary amide group along with some –OH groups due to some alcohol or phenol. Fractions 6 and 7 contained methyl/aryl/ketonic/carboxylic acid/acid anhydride/amine/ nitrile or some secondary amide group without –OH group.

Table 2: PTLC Analysis of pooled column fractions of methanolic extract of *C. reflexa*

Pooled Fraction	Eluting Solvent	PTLC solvent systems	No. of Spots	hRf values	Detecting Agents		
					UV light	Iodine	Liebr Bur Reagent
1	$CHCl_3$(100%)	$CHCl_3$ / MeOH (40:60)	1	89	l-pink	l – yel	l- bro
2	$CHCl_3$ / MeOH (95:5)	$CHCl_3$ / MeOH (30:70)	1	76	d-pink	d – yel	d- bro
3	$CHCl_3$ / MeOH(90:10)	$CHCl_3$ / MeOH (20:80)	1	85	l – blu	Yel	Bro
4	$CHCl_3$ / MeOH(90:10)	$CHCl_3$ / MeOH (10:90)	1	83	l – blu	l – yel	l- bro
5	$CHCl_3$ / MeOH(80:20)	$CHCl_3$/MeOH/Ace.A (90:10:1)	1	45	d– pur	d- yel	l- bro
6	$CHCl_3$ / MeOH(70:30)	$CHCl_3$/MeOH/Ace.A (80:20:2)	1	41	d – pink	d – yel	l- bro
7	$CHCl_3$ / MeOH(60:40)	$CHCl_3$/MeOH/Ace.A (90:10:5)	1	50	l - blu	l- yel	d- bro
8	$CHCl_3$ / MeOH(50:50)	$CHCl_3$/MeOH/Ace.A (80:20:5)	1	62	l – pur	Yel	l- bro
9	$CHCl_3$ / MeOH(40:60)	$CHCl_3$ / MeOH/HCl (80:20:2)	1	89	l – pink	l – yel	d- bro
10	$CHCl_3$ / MeOH(20:80)	$CHCl_3$ / MeOH/HCl (80:20:5)	1	33	d-pink	d- yel	l- bro

$CHCl_3$ = chloroform, MeOH = methanol, Ace.A = acetic acid, Liebr.Bur. = libermann burchad, l-pin = light pink, d-pin = dark pink, l-pur = light purple, l-blu = light blue, yel =yellow color, l-yel = light yellow, d-yel = dark yellow, bro = brown color, l-bro = light brown, d-bro = dark brown

Table 3: UV and FTIR spectra characterization of active fractions

Active fractions	*UV* $\lambda_{max\ (nm)}$	*FTIR (cm^{-1})* –OH	C=O	$–CH_3$	$–NH_2$
4	225	3358	1622	1399, 2410	2930
6	245	-	700, 864	1012, 1413	2962
7	270	-	1730	1463, 1272	2950
9	280	3388	1500, 1462	1462, 1074	2920
10	255	3600	1733	1378, 1174	2914

Discussion

The results indicated that two solvent extracts except methanol extract exhibited either no or doubtful irritant responses when the low doses of 20, 30 and 40 µL were used but at 50 µL dose level redness of +, ++ and +++ intensity was observed on rabbit's ears (Table 2). Methanol extract seemed to be more irritant than other two extracts at this dose level. Chloroform extract showed little irritation response. Petroleum ether extract seemed to be nearly inert in its irritation reaction with all the four doses used. Thus to conclude polar constituents of *C. reflexa* were responsible for such adverse reaction on the animal's skin.

The fractions No. 4, 6, 7, 9 and 10 seemed to be most active fractions (Fig. 5). They exhibited a strong to moderate irritant responses on rabbit's skin. Maximum irritant response was demonstrated by fractions 4, 9 and 10 when the dose of 20 µL was applied on rabbit's ears. The irritant responses of ++ intensity by these fractions were observed after 2 h of their application, which continued to increase with time and gained +++ intensity

levels in about 4 h. These reactions lasted for about 48 h then faded away. On the other hand, two other fractions 6 and 7 demonstrated moderate irritant response (Fig. 5). It was further postulated from UV and FTIR spectra (Table 5; Fig. 6 and 7) that the fractions 4, 9 and 10 probably penetrated through the skin of rabbit's ear with much ease. The presence of –OH, –COOH, or ketonic group and a double bond in these compounds were liable to be reacted with the cell membrane and cellular content of both the superficial and deeper layers of epidermis. As a result, inflammation of superficial as well as the deeper layers occurred, which probably causes damage to epidermis. The mechanism of action of these compounds was probably like the other strong to moderately irritating previously (Mitchell and Dupuis, 1971; Evens and Schmidt, 1980; Fregret, 1981; Benazra *et al.,* 1985). Fractions 6 and 7 displayed a moderate to weak reactions (++ to + intensity), which might be due to two possible reasons. 1) The compounds themselves entered the skin with some difficulty and were not completely accessible for the skin and 2) the nature of their molecules was not much strong to severely damage to epidermis of skin.

In conclusion, phytochemical investigation indicated that *C. reflexa* contains several *c*onstituents that can be isolated by column chromatography. Five of the fractions were biologically active and caused skin irritation. The level of activity varies from slight to severe irritation. In our best of knowledge, irritant activity of crude extracts and fractions of *C. reflexa* is first time reported. Furthermore, the mechanism of irritancy can be investigated and different drugs can be formulated for the treatment of this irritancy.

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