Identification and evaluation of counter-irritant potential of crude extract of *Malva parviflora* L. by WHO recommended methods

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Abstract: Plenty of medicinal plants are available in Pakistan and are in human use as herbal medicines from ancient time. Present work is based on the evaluation of the use of *Malva parviflora* in skin irritation problems. For this purpose, powdered plant material (The aerial part and roots separately) was extracted by using successive solvent extraction method using petroleum ether, chloroform and methanol. Resulting three crude fractions were subjected to counter-irritant investigations on rabbit's ear. Petroleum ether fraction exhibited prominent counter- irritant potential. Five compounds named, as MP-1, MP-2, MP-3, MP-4 and MP-5 were isolated from petroleum ether extract by column and thin layer chromatography. These compounds were subjected to UV spectrophotometer for detection of absorption of light, then FTIR for specific functional group identification and counter-irritant potentials was evaluated on rabbit's ear skin. The MP-1 and MP-2 exhibited excellent counter-irritant activity in different dilutions than others. However, dilution 100µg/ml showed maximum activity.

Keywords: *Malva parviflora*: counter-irritant, chromatography.

INTRODUCTION

Malva parviflora is an annual herb, which is native to Europe and may found in waste places, cultivated fields, disturbed sites, rocky slopes and grasslands (Fernald *et.al*., 1950). In Pakistan, found in N.W Himalayas, Sind, Punjab (Nandkarni, 1976 and vizgirdas, 2006). Tea of this plant is given due to its soothing effect on bronchial mucosa for treating dry, irritative cough and bronchitis (Kane, 2006). Carbohydrate portion mainly consists of polysaccharides which is innate immunity stimulant and can be used synergistically with other immune stimulating drugs. The drug constituents are mainly excreted in urine therefore used in cystitis, urethritis and lithiasis (Moore, 1997). Seeds and leaves used alone or in combination with *Artemisia douglasiana* or *A. filifolia* (kane, 2006; Bucay, 2007) in stress and poor diet related gastritis (Bucay and Martinzen, 2007). Whole herb show healing properties (Lans, 2007) and externally used in resolving boils, abscesses and removing splinter tissue (Moerman, 1998). Anti inflammatory, antimicrobial, antioxidant and wound healing abilities have been reported by (Lanes *et al.,* 2007).

MATERIALS AND METHODS

Plants of *Malva parviflora* were collected during 2008 from the Jallo Park, Lahore and were authenticated from Herbarium of Government College University Lahore, Pakistan. The plant material was brought to laboratory and aerial parts and roots were separated, dried under shade and then pulverized. The pulverized aerial plant parts and roots were stored separately in amber colored bottles.

Dried pulverized aerial parts (1.5kg) and roots (220g) of *M. parviflora* were subjected to successive extraction in pet. ether, chloroform and methanol respectively under the laboratory conditions. The dried aerial parts yielded least polar component (5.02%), polar components (4.84%), the components with intermediate polarity (2.05%).The extracted root material contained, the polar components (17.73%), intermediate polarity components (9.24%) and non-polar components (5.89 %) . This could be concluded that the powdered material of aerial parts of *M. parviflora* in these investigations, contained a larger proportion of non-polar components. Whereas the powdered material of roots of *M. parviflora* contained a larger proportion of polar components. Roots yield higher amount of pet. ether extract therefore it was subjected to open column chromatographic analysis to isolate the active compound/s and biological assay.

Sixteen pooled fraction were obtained based on thin layer chromatographic analysis and only five fractions were subjected to biological assay.

Biological assay for counter- irritancy

For biological assay healthy adult male/female albino rabbits (*Oryctolagus cuniculus)* weighing 1.0-1.5kg were purchased from local market. These animals were acclimatized in the animal house university college of pharmacy, university of the Punjab Lahore, for a period of three days and were provided with carrots, fresh green fodder (clover) and tap water *ad libitum.* Assay for counter irritant effect of solvent extracts/column fractions/isolated compounds was performed with some modifications in main assay (Evans and Schmidt's 2003). Sand paper with fine particles was used to irritate inner

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skin surface of rabbit's ear skin in clockwise direction for about 10 mints. Irritation, Redness, erythema was produced in area of 2.0cm^2 in diameter. 100µl solution from each solvent extracts/column fractions/isolated compounds were applied to the irritated area. Untreated area 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.

RESULTS

Total sixteen pooled fractions obtained from chromatographic separation and tested dermatologically for counter- irritant properties. Out of these sixteen column fractions, only eleven fractions were dermatologically active. They produced counter-irritant effect almost after 1 hour and in most instances, this effect lasts for 72 hours (Table I and II). Among eleven tested fractions, fraction No.2, 3, 5, 9 and 15 seemed to contain the active compounds that were responsible for dermatological effect. Well-marked counter-irritant effect within upper skin layer observed. Compounds showing activity named as Mp-1, Mp-2, Mp-3, Mp-4 and MP-5. These five most active compounds subjected to UV (figs. 3, 5, 7, 9 and 11) and FTIR (figs. 4, 6, 8, 10 and 12) spectral analysis respectively. MP-1 and MP-2 were showed the maximum counter-irritant activity. All dermatologically active five compounds are discussed.

DISCUSSION

Compound MP-1 was isolated and purified from the second column fraction. The UV spectral analysis indicate that compound Mp-1 had strong or absorption at λ max = 265 nm. This strong absorption was probably due to n → π* and π → π* transition, which suggested the presence of carbonyl group either of a ketone or an aldehyde or carboxylic acid along with some conjugated diene chromophoric system in its molecule(Anonymous,1996). The infrared spectrum of Mp-1, showed a strong peak at 2915cm^{-1} which was possibly due to some methylene group (CH_3, = CH_2, ≡CH). The strong peak along with a weak peak at 2954 cm^{-1} and 2858 cm^{-1} showed the C-H stretching vibrations in aliphatic alkyl compounds. Often such strong bands are shown by the methyl; methylene and alkylene groups resulted from symmetrical and asymmetrical stretching of C-H modes. A strong methylene/methyl peak at 1454 cm^{-1} and a weak methyl peak at 1377cm^{-1} plus a peak at 723cm^{-1} (methylene rocking vibration was indicative of a long chain linear aliphatic compounds. The presence of medium peak at 723cm^{-1} along with weak peaks at 792 cm^{-1} and weak peaks at 777 cm^{-1} indicated the aliphatic chloro compounds(C-Cl) with stretching vibration. Weak peak at 2362 cm^{-1} and 2331 cm^{-1} indicated the presence of nitrite (C≡N) compound. A medium peak at 684.59cm^{-1} indicated the presence of thio or thiol ether compounds (CH_2-S-) along with C-S stretching (William and Flemming *et al.,* 1980).

The available spectral evidence showed that the compound Mp-1 was probably a conjugated diene containing methyl, aliphatic chloro compounds, nitrile or thiol or thio ether compounds(William and Flemming *et al.,*1980).

Compound MP-2 was isolated and purified from the third column fraction. The compound Mp-2 had strong UV absorption at λ max = 245 nm. This strong absorption was probably due to n → π* and π → π* and transition, which suggested the presence of carbonyl group either of a ketone or an aldehyde or a carboxylic acid (Anonymous,1996) Infrared spectrum of Mp-2 also exhibited a number of strong and weak intensity bands. A strong peak at 2923 cm^{-1} which was possibly due to some methylene group (> CH_2). The strong peak along with medium peaks at 2946 cm^{-1} and 2846 cm^{-1} showed the C-H stretching vibrations in aliphatic (alkanes/alkyl) compounds. Often such strong bonds are shown by the methyl, methylene and alkyl groups resulted from symmetrical and asymmetrical stretching of C-H modes. A medium peak at 1438cm^{-1} and weak peak at 1354 cm^{-1} plus peaks at 723 cm^{-1} and 746 cm^{-1} is indicative of a long chain aliphatic linear structure. A medium peak at 2358 cm^{-1} along with weak peak at 2335 cm^{-1} indicated nitrile C≡N group. Weak peak at 1700 cm^{-1} indicate the presence carbonyl group such as ketone, an aldehyde, an ester. and a carboxylic acid. A weak peak at 1600cm^{-1} indicated the presence of conjugation with double bond which might be due to an aromatic ring. A weak peak at 1354 cm^{-1} indicated the presence of aromatic tertiary amine (C-N stretching). A weak peak at 1238 cm^{-1} indicated the presence of aromatic phosphate possibly due to P-O-C stretching. A weak peak at 1023 cm^{-1} indicated the presence of organic siloxane or silicone (Si-O-S)A medium peak at 569 cm^{-1} indicated the presence of aliphatic iodo compound (C-I) with stretching. Further, a strong peak at 723 cm^{-1} indicated the aromatic absorption in finger print region of the molecule. This was possibly due to aromatic stretching of π bonds in the molecule (William and Flemming *et al.,* 1980).

The available spectral evidence showed that the compound Mp-2 was probably a conjugated diene containing methyl, ketone, aldehyde, carboxylic acid, aliphatic iodo compounds, ester, nitrile, tertiary amines, aromatic phosphate, siloxane or silicone and iodo compounds (William and Flemming *et al.,*1980).

Compound MP-3 was isolated and purified from the 5th pooled column fraction.. The compound Mp-3 had strong UV absorption at λ max = 263 nm. This strong absorption was probably due to n → π* and π → π* and transition, which suggested the presence of carbonyl group either of a Ketone or an aldehyde or a carboxylic acid (Anonymous, 1996). Infrared spectrum of Mp-3 also showed two peaks one strong at 2919 cm^{-1} and weak at 2953 cm^{-1} indicated alkyl/methyl/alkyne groups stretching vibration possible due to C-H. Another medium peak at 2862 cm^{-1}, confirmed C-C stretching vibration. Such bonds were resulted from symmetrical and asymmetrical stretching vibration of C-H modes. The presence and the number of $–CH_3$, $=CH_2$ and ≡ CH groups in the molecule were further indicated by the peaks in the finger print region at 1462 cm^{-1}, 1377 cm^{-1} and 1065 cm^{-1}.Two weak peaks at 754 cm^{-1} and 715 cm^{-1} indicated a long chain aliphatic linear structure. A medium peak at 2385 cm^{-1} and a weak peak at 2362 cm^{-1} was due to presence of nitrile compounds. Peaks at 1739 cm^{-1} and 1639 cm^{-1} indicated the presence of ester or amide compounds respectively. Peaks at 839 cm^{-1} and 800 cm^{-1} indicated the presence of P- disubstituted aromatic ring. A band at 684.59cm^{-1} was possibly due to some thio or thio ether CH_2-S-(C-S stretching) compounds (William and Flemming *et al.,* 1980).

The available spectral evidence showed that compound Mp-3 was probably a methyl, a aryl, nitrile, amide, ester, P-disubstitued aromatic and some thio, thio-ether compounds (William and Flemming *et al.,* 1980).

Compound Mp-4 was isolated and purified from the 9th pooled column fraction. The compound Mp-4 had strong UV absorption at λ max = 263 nm. This strong absorption was probably due to n → π* and π → π* and transition, which suggested the presence of carbonyl group either of a ketone or an aldehyde or a carboxylic acid (Anonymous, 1996).

The infrared spectrum of Mp-4 also exhibited a strong band at 2916 cm^{-1} was possibly due to some alkyl group absorption possibly due to $–CH_3$, $=CH_2$ and ≡ CH. Another strong peak at 2946 cm^{-1}along with a weak band at 2854 cm^{-1} also indicated C-H stretching vibrations present in aliphatic compounds. Such strong bands resulted due to symmetrical and asymmetrical stretching vibration of C-H modes. The presence of $–CH_3$, $=CH_2$ and =CH groups in the molecule were further indicated by the peaks at 1446 cm^{-1} and 731 cm^{-1} which indicated the bending and rocking vibration of methylene groups along with aromatic absorption. Weak peaks at 2346 cm^{-1} and 2315 cm^{-1} represented the presence of nitrile C≡N group. Weak band at 1362 cm^{-1} indicated the presence of the sulphonated compounds. Weak peak at 1023 cm^{-1} represented the presence of silicon oxy compounds or organic silioxane or silicone (Si-O-Si) compounds. Such peaks at 808 cm^{-1} showed aromatic ring with 1, 4 disubstitution at the para position and further absorption at 777 cm^{-1} confirm the chloro group substitution in aromatic ring. Weak peak at 685 cm^{-1} showed the presence of thio, thio - ether group presence (CH_2-S-) with C- S stretching in the molecule. Three peak appeared at 808, 777, 731 cm^{-1} in the finger print region further showed a typical aromatic absorption, possibly due to deformation of C-C and C-H bands (William and Flemming *et al.,* 1980).

The available spectral evidence showed that compound Mp-4 was probably a methyl, a aryl, nitrile, sulphonated, silicone oxy or silioxane or silicone, chloro and some thio, thio-ether compounds (William and Flemming *et al.,* 1980).

Compound Mp-5 was isolated and purified from the 15th pooled column fraction. The compound Mp-5 had strong UV absorption at λ max = 254nm. This strong absorption was probably due to n → π* and π → π* and transition, which suggested the presence of carbonyl group either of a ketone or an aldehyde or a carboxylic acid along some conjugated diene chromatographic system present in its molecule(Anonymous,1996).

The infrared spectrum of Mp-5 showed a number of strong, medium and weak intensity band figure. A strong peak at 2920 cm^{-1} was possibly due to some alkyl group (>CH_2). Strong peak at 2954 cm^{-1} showed the C-H stretching vibrations in aliphatic compounds such as CH_3, = CH_2 and ≡CH. Such strong bands shown methylene and alkyl groups resulted due to symmetrical and asymmetrical stretching of C-H modes. The presence of – CH_3 = CH_2 and ≡CH groups in the molecule were further indicated peak at 1450 cm^{-1}, 1362 cm^{-1}, 731 cm^{-1}. Medium peak at 2827 cm^{-1} indicated the presence of the methoxy, methyl ether (O-CH_3) groups along C-H stretching vibrations. Weak peak at 2369 cm^{-1} showed the presence of C≡N group. Weak peak at 1727 cm^{-1} indicated the presence of aldehyde group. Another weak peak at 1031 cm^{-1} indicated the presence of aliphatic fluoro compound with C-F stretching vibration. A medium peak at 769 cm^{-1} indicated 1,2-disubstituted aromatic ring at ortho positions. A medium peak at 685 cm^{-1} indicated the presence of thio or thio ether CH_2-S-compounds along with C-S stretching vibrations (William and Flemming *et al.,* 1980).

The available spectral evidence showed that compound Mp-5 was probably a methyl, a aryl or a nitrile or methoxy or methyl ether, fluoro and some 1,2-disubstitued and thio, thio-ether compounds (William and Flemming *et al.,*1980).

Table 1: Counter-irritant response of the crude solvent extract of roots of *Malva parviflora* L. on rabbit's ear

Dose levels µg/ml	Counter-irritant response (healed areas in cm) after *												
	Extracts	1hr	2hr	3hr	4hr	5hr	6hr	7hr	8hr	9hr	10hr	24hr	48hr	72hr
100 µg/ml	Pet.eher	-	0.52	0.54	0.59	0.62	0.9	0.9	0.8	0.8	0.8	0.7	0.7	0.7
	Chloroform	-	-	-	-	-	-	-	-	-	-	-	-	-
	Methanol	-	0.31	0.35	0.39	0.41	0.47	0.50	0.5	0.5	0.5	0.5	0.5	0.5
50 µg/ ml	Pet.eher	-	-	0.42	0.47	0.50	0.55	0.57	0.6	0.6	0.6	-	-	-
	Chloroform	-	-	-	-	-	-	-	-	-	-	-	-	-
	Methanol	-	-	-	0.26	0.30	.35	0.4	0.4	0.4	0.4	-	-	-
40 µg/ ml	Pet.eher	-	-	-	0.35	0.35	0.35	-	-	-	-	-	-	-
	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

Table 2: Counter-irritant response of the isolated compound mp-1-5 from petroleum ether extract of roots of *Malva parviflora* on rabbit's ear

	Dose levels µg/ml	Counter-irritant response (healed areas in cm) after*														
		1hr	2hr	3hr	4hr	5hr	6hr	7hr	8hr	9hr	10hr	11hr	12hr	24hr	48hr	72hr
MP-1	100 µg/ml	-	0.53 ± 0.01	0.55 ± 0.01	0.6 ± 0.02	0.7 ± 0.03	0.9 ± 0.02	0.9 ± 0.11	0.8 ± 0.02	0.8 ± 0.03	0.8 ± 0.04	0.8 ± 0.01	0.8 ± 0.02	0.7 ± 0.02	0.7 ± 0.04	0.7 ± 0.03
MP-2	100 µg/ml	-	0.53 ± 0.02	0.55 ± 0.01	0.6 ± 0.02	0.7 ± 0.03	0.9 ± 0.02	0.9 ± 0.03	0.8 ± 0.02	0.8 ± 0.03	0.8 ± 0.04	0.8 ± 0.05	0.8 ± 0.01	0.7 ± 0.02	0.7 ± 0.03	0.7 ± 0.01
MP-3	100 µg/ml	-	0.5 ± 0.02	0.5 ± 0.03	0.6 ± 0.02	0.7 ± 0.03	0.8 ± 0.01	0.8 ± 0.09	0.6 ± 0.02	0.6 ± 0.01	0.6 ± 0.03	0.6 ± 0.04	0.6 ± 0.05	0.5 ± 0.02	-	-
MP-4	100 µg/ml	-	-	0.3 ± 0.02	0.3 ± 0.03	0.5 ± 0.05	0.5 ± 0.02	0.5 ± 0.03	0.5 ± 0.05	-	-	-	-	-	-	-
MP-5	100 µg/ml	-	0.3 ± 0.05	0.3 ± 0.06	0.5 ± 0.02	0.5 ± 0.03	0.5 ± 0.06	0.5 ± 0.01	0.5 ± 0.14	-	-	-	-	-	-	-
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 replicates. hr= Hours of healing

All the five isolated compounds, exhibited a strong to moderate counter-irritant responses on rabbit's ear skin. Maximum response observed by Mp-1 and Mp-2 by 100µg/ml dose of these compounds. The counter-irritant response observed after 30 minutes of samples application, which continued to increase with time and gained peak levels in about 6 hour. This counter-irritant effect lasted for about 72 hour. The effect shown by the other three compounds Mp-3, Mp-4, Mp-5 seemed to be of lesser in frequencies as compared to Mp-1 and Mp-2. These results were similar to the results shown by other authors when they used counter- irritant phytochemical compounds on animal's skin, from the same natural plant sources. Based upon the results it was observed that compound MP-1 and MP-2 probably easily penetrated through the irritated skin of rabbit's ear, reach to the dermis layer easily as compared to other separated compounds, and heal the erythmatous eruption. The presence of the C≡N group and the conjugated diene systems with chloro or nitile group were responsible for their counter- irritant effect. Further previous studies indicated that compounds having ion-channel modulating and mast cell degranulating properties are effective counter-irritant agents. Mostly counter-irritant activity of the plant compounds was due to their free radical scavenging activity and antioxidant activity because wound healing process involves a series of cellular and cytokine mediated events resulting in the contraction and closure of wound and restoration of a functional group. These events include inflammation, tissue formation and skin remodeling. Inflammation results in the generation of reactive oxygen species which have been found to play both beneficial and deleterious effect in the wound healing process (Vigrag, 2002). The harmful effect of overproduction of free radicals on cells and tissues consequently leads to diseases such as skin cancer, psoriasis and impaired skin wound healing. Since *M. parviflora* has high percentage of flavonoids and phenolic compounds particularly flavones and hesperidin which acts as a free radical scavengers due to which on one side

they exert counter- irritant effect (Afolayan, 2008) by converting free radicals such as superoxide (O_2^-) singlet oxygen and hydroxyl ion (O^-,OH^-) back to their original state in order to prevent production of reactive oxygen moieties and on other side themselves are converted to a free radical in a form that does not usually react with oxygen and thus exerting counter-irritant effect. It was concluded that the compound Mp-1 and Mp-2 exhibited well marked counter-irritant activities possibly due to the presence of flavonoid and phenolic compounds.

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