REPORT

Assessment of *Fumaria indica, Dicliptera bupleuroides* and *Curcuma zedoaria* for their antimicrobial and hemolytic effects

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Abstract: The present investigation was undertaken to evaluate the antibacterial, antifungal and hemolytic activities of organic and aqueous fractions of *Fumaria indica*, *Dicliptera bupleuroides* and *Curcuma zedoaria*. The methanolic extracts of the plants were dissolved in the water (distilled) separately and then partitioned with the *n*-hexane, $CHCl_3$, EtOAc and *n*-BuOH sequentially. Antibacterial activity was checked against *Escherichia coli*, *Pasturella multocida*, *Bacillus subtilis* and *Staphylococcus aureus* by the disc diffusion method using streptomycin sulphate, a standard antibiotic, as positive control. Antifungal activity was studied against four fungi i.e. *Aspergillus niger, Aspergillus flavus, Ganoderma lucidum* and *Alternaria alternata* by the disc diffusion method using fluconazole, a standard antifungal drug, as positive control. It was revealed that aqueous fraction of *F. indica* showed very good antibacterial activity against *P. multocida* with zone of inhibition 26mm and MIC of 98µg/mL. Its $CHCl_3$ and *n*-BuOH fractions also displayed good results. Its $CHCl_3$ fraction showed good antifungal activity against *G. lucidum* with zone of inhibition 24mm and MIC of 115µg/mL. Other polar fractions of *F. indica* showed good activity against somefungal strains. The $CHCl_3$ and EtOAc fractions of *D. bupleuroides* displayed good antibacterial activity against some bacterial strains. Its EtOAc fraction showed good antifungal activity only against *G. lucidum*. The $CHCl_3$ fraction of *C. zedoaria* showed good activity against all studied bacterial strains, while its EtOAc and *n*-BuOH fractions displayed good results against some bacterial strains. None of the fractions of *C. zedoaria* displayed antifungal activity against the under test strains. All the studied fractions of three plants showed very less toxicity except *n*-hexane fraction of *D. bupleuroides* which showed 79% toxicity.

Keywords: *Fumaria indica*, *Dicliptera bupleuroides*, *Curcuma zedoaria*, antibacterial potential, antifungal, disc diffusion method, hemolytic effects.

INTRODUCTION

Medicinal plants are being used for treatment of diseases from ancient times. Due to the efficacy and therapeutic properties of medicinal plants, these are prescribed frequently even if we don't know about their chemical constituents completely (Maciel *et. al*., 2002). Thus, phytotherapists introduced cheaper and shorter production of drugs in the market because unlike other drugs the plant based drugs do not require strict quality control regarding their efficacy and safety (Silva and Fernandes, 2010). In developing countries, infectious diseases are the source of mortality and morbidity among general population. So, in recent years, pharmaceutical companies are developing new antimicrobial drugs because microorganisms are becoming resistant to the conventional antimicrobial drugs. Therefore, search for the plant based new antimicrobial drugs must be emphasized (Nascimento *et. al*., 2000; Sakagami *et. al*., 2002).

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Exploration of plant based antimicrobials is required because these represent an unexploitable source for medicines. The plant based antimicrobials possess a massive therapeutic potential against bacterial, fungal and viral infections. Over past two decades, great interest has been developed to investigate natural materials for the advent of novel antimicrobial drugs. Researchers have tested and evaluated various extracts of traditional medicinal plants and herbs which showed their great effectiveness against microorganisms. So, such traditional ethno medicinal plants are the foundations for modern medicine and may lead to advent of new therapeutic agents, neutraceuticals, pharmaceuticals, food supplements and cosmetics (Girish and Satish, 2008). In addition, the synthetic drugs for treatment of various diseases in developing countries are inadequate and expensive as well adulterated, having many side effects. So, search for new infection-fighting strategies is required to cope with microbial infections (Sieradzki *et. al*., 1999).

So keeping in view the affluent potentiality of medicinal plants' species as vast sources of antibacterial and

antifungal agents, an investigation was undertaken for screening the antifungal, antibacterial and hemolytic activities of *Fumaria indica* Pugsley, *Dicliptera bupleuroides* Nees. and *Curcuma zedoaria* Rosc. which are important medicinal plants. *F. indica* belongs to family Fumariaceae. The plant is used as antidyspeptic, blood purifier, anthelmintic, cholagogue, diaphoretic, diuretic, stomachic, sedative, laxative and tonic. It is used for treatment of jaundice, abdominal cramps, diarrhoea, fever, leprosy, syphilis and constipation. It possess anthelmintic, hepatoprotective, antipyretic and hypoglycemic properties (Riaz *et. al*., 2012a). *D. bupleuroides* belongs to family Acanthaceae, used for tonic debility. It is also used for the treatment of eye diseases. Its fresh crushed leaves are used to apply gently on effected portion of body three times in a day for a week in eczema (Riaz *et. al*., 2012b). *C. zedoaria*, commonly known as white turmeric, belongs to family Zingiberaceae. Its various parts are commonly used in Ayurveda and also in other tribal and folk system of medicines. Its rhizome is used in the cure of stomach diseases, toothache, blood stagnation, tuberculosis, enlargement of spleen and leucoderma. It also exhibits anti-inflammatory, antimicrobial, hepatoprotective, antiulcer, anti-allergic, antiamoebic, antitumor, antipyretic, anti metastasic, analgesic and anti-inflammatory effects. Its rhizome is extensively used for treatment of various cervical and ovarian cancers. Its rhizome is used to aid digestion, for purification of blood, for treating colds and infections and for providing relief for colic (Riaz *et. al*., 2011).

In this study, we have characterized various organic and aqueous fractions of these plants to estimate their antibacterial, antifungal and hemolytic activities, as such type of detailed work has not been performed yet on these plants. Antibacterial activity was checked against two types of Gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus* and two types of Gram-negative bacteria such as *Escherichia coli* and *Pasturella multocida* by the disc diffusion routine using streptomycin sulphate, a standard antibiotic, as positive control. Antifungal activity of crude fractions of plant was studied against four fungi i.e. *Aspergillus niger, Aspergillus flavus, Ganoderma lucidum* and *Alternaria alternata* by the disc diffusion method using fluconazole, a standard antifungal drug, as positive control. The fractions were also checked for their toxicity by their hemolytic effects.

MATERIALS AND METHODS

Plant material

The plants, *F. indica* and *D. bupleuroides* and were collected from district Kotli, Azad Kashmir, while *Curcuma zedoaria* was collected from Chhanga Manga forest, Pakistan, in 2010. These were identified by a taxonomist, Mr. Muhammad Ajaib, Dept. of Botany, GC University, Lahore. The Voucher numbers of the specimens, (*Fumaria indica*: (GC. Herb. Bot. 969), *Dicliptera bupleuroides*: (GC. Herb. Bot. 968), *Curcuma zedoaria*: (GC. Herb. Bot. 967)], have been placed in GC university's herbarium.

Extraction and fractionation

Each of the shade-dried and ground whole plants (2kg) was extracted exhaustively with the methanol (5L×4) at the room temperature. Methanolic extracts of each plant were evaporated on rotavapour separately to yield the residues, that were dissolved in the distilled water (1L) and after this partitioned with *n*-hexane (1L×3), $CHCl_3$ (1L×3), EtOAc (1L×3) and *n*-BuOH (1L×3) respectively (Riaz *et. al*., 2012c). All these organic fractions as well as remaining water fraction were concentrated separately on rotavapour. The residues obtained were further used to assess their *in vitro* antibacterial and antifungal potential as well as for hemolytic activities.

Antibacterial and antifungal assay

Microbial strains

The samples both irradiated and un-irrdiated were tested separately against two strains of Gram-positive bacteria: *Bacillus subtilis* JS 2004 and *Staphylococcus aureus*, API Staph TAC 6736152, and two strains of Gram-negative bacteria: *Pasteurella multocida* (local isolate) and *Escherichia coli* ATCC 25922 and four fungal strains *Aspergillus niger, Aspergillus flavus, Ganoderma lucidum* and *Alternaria alternata*. Pure bacterial and fungal strains were collected from the Dept. of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan. Verification of the identity and purity of the strains was done by the Institute of Microbiology of the same university. In the Nutrient agar (NA, Oxoid), the bacterial strains were cultivated at 37ºC overnight.

Disc diffusion method

Disc diffusion method was used to check antibacterial and antifungal activities of plant extracts. Suspension of the tested microorganisms (100µL), contained 10^7 CFU/ml (colony-forming units) of bacteria cells on the Nutrient agar medium. The 9mm diameter filter discs were impregnated separately with extracts' solution and placed on agar plates that were already inoculated with tested microorganisms. Streptomycin sulphate (Oxoid, UK) (30 µg/ml/dish) was taken as positive reference for bacterial strains to compare the sensitivity of isolate/strain in the analyzed microbial species. The discs with no samples were taken as negative control. Plates were kept at 4ºC for 2 hours and then incubated for 18 hours at 37ºC for bacterial strains. Evaluation of the antibacterial and antifungal activity for the organisms was done by measurement of diameter of the growth inhibition zones in millimeters and by comparing to that of positive and negative controls (CLSI, 2010).

Measurement of MIC

The MIC (minimum inhibitory concentration) was stated as the lowest concentration of sample that has capability to inhibit complete growth of bacterial strain being tested (Andrews, 2001). It was calculated graphically as an extraplotation of linear relationship to zero value.

Hemolytic activity

Hemolytic activity of the plant extracts was performed according to standard method (Powell *et al*., 2000; Sharma and Sharma, 2001). Blood was collected from various volunteers after their counseling and consent. Heparinized human blood (3mL), freshly drawn, was taken and centrifuged at 1000x*g* for 5 minutes. After discarding plasma, cells were washed with 5mL of cooled (4°C) sterile isotonic PBS (Phosphate-buffered saline) having pH 7.4, three times. For each assay the concentration of erythrocytes were maintained at 10^8 cells per mL. Then 100µL of each plant extract was mixed separately with human blood (10^8cells/mL). Incubation was done at 37°C for 35min and agitation was started after 10 minutes. The samples were placed for 5 minutes on ice immediately after the incubation, then centrifugation was done at 1000x*g* for 5 minutes. From each tube, 100µL of supernatant was taken, and diluted with cooled (4°C) PBS 10 fold. PBS was used as negative control while triton X-100 (0.1% v/v) was used as positive control and passed through the same process. Absorbance of each sample was measured, using µ Quant (Bioteck, USA), at 576 nm. Calculation of % RBCs lysis for each sample was done by following formula.

$$\text{Percentage hemolysis} = \frac{Abs.of\ sample - Abs.of\ blank}{Abs.of\ positive\ Control} \times 100$$

All readings were taken in triplicate and mean values were calculated.

The study protocol was approved by the institutional Ethical Committee (Approval No.DGS/8786-89 dated 09-03-2015), University of Agriculture, Faisalabad, Pakistan and was conducted in accordance with 1964 Declaration of Helsinki and its subsequent amendments (Abbasi *et al*., 2016).

RESULTS

Antibacterial activities of all the studied fractions of *F. indica*, *D. bupleuroides* and *C. zedoaria* were checked against two Gram-positive bacteria i.e. *S. aureus* and *B. subtilis* and two Gram-negitive bacteria i.e. *P. multocida* and *E. coli* by the disc diffusion method using streptomycin sulphate, a standard antibiotic, as positive control. Zones of growth inhibition were measured in mm and MIC was also calculated. The results have been summarized in table 1. Antifungal activity of all the studied fractions was checked against *A. niger*, *A. flavus*, *G. lucidum* and *A. alternata* and the results of zones of growth inhibition as well MIC have been given in table 2. All the studied fractions were checked for their toxicity by their hemolytic effects and the results are given in table 3.

DISCUSSION

Antibacterial activity

In recent years, more attention is being paid on plant extracts as well as on the biologically active compounds that are being isolated from important medicinal plants. Medicinal plants have a vital role in developing countries to meet basic health needs and such plants offer substantial activities against the infective micro-organisms and can be new sources of antifungal and antibacterial agents (Mun˜oz-Mingarro *et al*., 2003; Coelho de Souza *et al*., 2004). Secondary metabolites e.g. phenolics, flavones, flavonoids, terpenes, alkaloids, phenolic glycosides, tannins, sugars, organic acids, quinones etc. have anticeptic action. These disintegrate cytoplasmic membrane and destabilize electron flow system. Terpenes disturb cell structure of micro-organisms. Flavonoids and terpenes inactivate enzymes which synthesize structural components of bacteria (Silva and Fernandes, 2010). The phytochemical tests have been performed on these plants by Riaz *et al*., (2012a; b; 2011) which showed that these plants are rich in such types of active compounds e.g. phenolics, flavonoids and terpenoids etc.

Table 3: Hemolytic activities of various fractions of *Fumaria indica*, *Dicliptera bupleuroides* and *Curcuma zedoaria*

Plant	Sample	Toxicity % ± S.E.M[a)]
Fumaria indica	*n*-Hexane fr.	1.7±0.2
	Chloroform fr.	2.5±0.3
	EtOAc fr.	1.8±0.3
	n-Butanol fr.	1.5±0.1
	Aqueous fr.	1.6±0.1
Dicliptera bupleuroides	*n*-Hexane fr.	79.3±1.1
	Chloroform fr.	1.0±0.3
	EtOAc fr.	1.1±0.7
	n-Butanol fr.	3.1±0.1
	Aqueous fr.	1.1±0.1
Curcuma zedoaria	*n*-Hexane fr.	8.6±0.1
	Chloroform fr.	2.7±0.1
	EtOAc fr.	3.6±0.1
	n-Butanol fr.	2.6±0.1
	Aqueous fr.	6.2±0.1
Triton[b]		100.0±0.1
Phosphate Buffer Saline[c]		0.00

a) Standard mean error of three assays.
b) Positive control having 100% toxicity.
c) Negative control having 0% toxicity.

Antibacterial activity of all the studied fractions of the three plants was checked against *S. aureus*, *B. subtilis*, *P. multocida* and *E. coli* (table 1). Zones of inhibition were measured in mm. For *Fumaria indica*, it was observed that $CHCl_3$ fraction showed good activity against all the

Table 1: Zones of inhibition (mm) and MIC values of various fractions of *Fumaria indica*, *Dicliptera bupleuroides* and *Curcuma zedoaria* against Gram-positive and Gram-negative bacteria

Plant	Sample	Zones of inhibition (mm)				MIC (µg/mL)			
		S. aureus	*B. subtilis*	*P. multocida*	*E. coli*	*S. aureus*	*B. subtilis*	*P. multocida*	*E. coli*
Fumaria indica	*n*-Hexane fr.	9	-	7	-	312	-	357	-
	$CHCl_3$ fr.	20‡	22‡	22‡	20‡	136	128	119	132
	EtOAc fr.	20‡	24‡	14	18	112	106	252	202
	n-BuOH fr.	16	12	16	14	226	276	227	251
	Aqueous fr.	14	16	26‡	16	249	224	98	225
Dicliptera bupleuroides	*n*-Hexane fr.	-	-	14	12	-	-	251	276
	$CHCl_3$ fr.	14	20‡	14	16	250	126	251	226
	EtOAc fr.	16	18	20‡	22‡	225	199	125	118
	n-BuOH fr.	12	14	12	14	278	251	278	251
	Aqueous fr.	16	14	18	14	225	226	202	252
Curcuma zedoaria	*n*-Hexane fr.	-	6	-	5	-	362	-	398
	$CHCl_3$ fr.	20‡	20‡	22‡	22‡	174	176	116	148
	EtOAc fr.	16	22‡	20‡	18	223	112	201	200
	n-BuOH fr.	10	20‡	12	12	304	174	276	275
	Aqueous fr.	10	14	12	14	304	252	278	254
Streptomycin sulfate		32	36	30	32	40	19	49	39

Table 2: Zones of inhibition (mm) and MIC values of various fractions of *Fumaria indica*, *Dicliptera bupleuroides* and *Curcuma zedoaria* against Fungi

Plant	Sample	Zones of inhibition (mm)				MIC (mg/mL)			
		A. niger	*A. flavus*	*G. lucidum*	*A. alternata*	*A. niger*	*A. flavus*	*G. lucidum*	*A. alternata*
Fumaria indica	*n*-Hexane fr.	-	4	-	6	-	387	-	356
	$CHCl_3$ fr.	14	20‡	24‡	18	251	155	115	199
	EtOAc fr.	12	14	16	22‡	275	251	224	122
	n-BuOH fr.	-	12	-	20‡	-	276	-	158
	Aqueous fr.	12	14	22‡	16	275	251	120	224
Dicliptera bupleuroides	*n*-Hexane fr.	-	-	-	-	-	-	-	-
	$CHCl_3$ fr.	14	12	16	-	251	278	224	-
	EtOAc fr.	12	12	20‡	14	276	274	126	251
	n-BuOH fr.	14	12	16	-	252	276	224	-
	Aqueous fr.	12	12	12	14	275	276	273	251
Curcuma zedoaria	*n*-Hexane fr.	-	-	-	-	-	-	-	-
	$CHCl_3$ fr.	-	12	-	14	-	274	-	250
	EtOAc fr.	12	12	10	14	275	274	298	251
	n-BuOH fr.	14	12	12	-	252	278	276	-
	Aqueous fr.	-	12	-	10	-	275	-	298
Fluconazole		30	30	32	34	51	49	40	30

‡$p < 0.05$ comparative to blank i.e. negative control ($p < 0.05$ was considered as significant).

studied strains of bacteria i.e. 20mm, 22mm, 22mm and 20mm respectively. EtOAc fraction showed good activity against *S. aureus* (20mm) and *B. subtilis* (24mm). Aqueous fraction showed good results only against *P. multocida* (26mm). All the other fractions showed no significant results. MIC values of all fractions were also calculated (table 2). Aqueous fraction showed good MIC value i.e. 98µg/mL against *P. multocida*. EtOAc fraction showed good MIC values against *S. aureus* (112µg/mL) and *B. subtilis* (106µg/mL).

The results for antibacterial activity of the studied fractions of *Dicliptera bupleuroides* showed that $CHCl_3$ fraction demonstrated good activity against *B. subtilis* i.e. 20mm. Its MIC value was found as 126µg/mL. EtOAc fraction demonstrated good activity against *P. multocida* and *E. coli* i.e. 20mm and 22mm respectively. Their MIC values were observed as 125µg/mL and 118µg/mL. All the other fractions showed no significant results. For *Curcuma zedoaria*, it was observed that $CHCl_3$ fraction showed good activity against all the studied strains i.e.

20mm, 22mm, 22mm and 20mm respectively. EtOAc fraction showed good activity against *B. subtilis* and *P. multocida* 22mm and 20mm respectively. *n*-BuOH fraction showed good result against *B. subtilis* (20mm). All the other results were found non-significant. $CHCl_3$ fraction demonstrated good MIC value against *P. multocida* i.e. 116µg/mL. MIC value of EtOAc fraction was found as 112µg/mL against *B. subtilis*.

The results mentioned as good were found significant ($p< 0.05$) while all the other results were found non-significant ($p>0.05$) when compared with the blank. These observations have been made on the basis of measurements of zones of inhibition. Statistical analysis of variance (ANOVA) and the Duncan *t*-test supported the experimental results obtained. Streptomycin Sulfate, a reference antibacterial drug was taken as positive control. Phytochemical screening tests done by Riaz *et al*. (2012a; b; 2011) showed that the fractions which showed good acitivities are rich in active compounds such as phenolics, flavonoids and terpenoids while the fractions which showed less or no activity contain very less or no such active compounds.

Antifungal activity

The bioactive molecules, present in medicinal plants, inhibit the fungal growth. Some of the fungitoxic compounds from the plants are: Luteone (in *Lupinus albus*), Sakuranetin (in *Ribus nigrum*), and Nobiletin (in *Citrus* spp.) (Walton and Brown, 1999). Antifungal activity of all the studied fractions of three plants was checked against *A. niger, A. flavus, G. lucidum* and *A. alternata* and results have been summarized in table 2. Results for *F. indica* showed that $CHCl_3$ fraction demonstrated good activity against *G. lucidum* and *A. flavus* i.e. 20mm and 24mm respectively. Their MIC values were found as 155 and 115µg/mL respectively. *n*-BuOH and EtOAc fraction showed good activity only against *A. alternata* i.e. 22mm and 20mm respectively. Their MIC values were calculated as 122 and 158µg/mL respectively Aqueous fraction showed good activity against *G. lucidum* (22mm) having MIC 120µg/mL. All the other fractions showed very less or no activity.

In *D. bupleuroides*, EtOAc fraction showed good activity only against *G. lucidum* having zone of inhibition 20 mm and MIC 126µg/mL. All the other fractions showed moderate activity. *n*-Hexane fraction showed no activity at all. In *C. zedoaria* that *n*-hexane fraction showed no activity at all. All the other fractions showed moderate activity. Fluconazole was taken as a reference antifungal drug. The results mentioned as good were found significant ($p<0.05$) while all the other results were found non-significant ($p>0.05$) when compared with the blank. These observations have been made on the basis of measurements of zones of inhibition in mm. Polar fractions of these plants As indicated by phytochemical screening tests done by Riaz *et al*., (2012a; b; 2011), the fractions which showed good activities found to be rich in active compounds such as phenolics, flavonoids and terpenoids while the fractions which showed less or no activity contain very less or no such active compounds.

Hemolytic activity

Plant derived natural compounds have been gained much attention due to their potential to act as chemopreventive and cytotoxic activity. To perform hemolytic assay is very important to determine whether the specific drug that possesses antioxidant, antimicrobial and other bioactivities, can be used in the pharmacological applications. The *in vitro* hemolytic activities are now-a-days becoming new area of research in the drug lead discoveries (Mukherjee and Rajasekaran, 2010). In the exploration of the action of the plant extracts on the human blood, it is essential to determine hemolytic activity because this is the indicator of cytotoxicity and bioactivity. *In vitro* hemolysis tests have been employed by many researchers for the toxicological evaluation of the various plants (Oliveira *et al*., 2009).

So, all the studied fractions of three plants were checked for their toxicity by their hemolytic effects and the results are given in table 3. The hemolytic activities of the plants' extracts were compared with the triton, taken as +ve control, having 100% toxicity and phosphate buffered saline (PBS), taken as -ve control, having 0% toxicity. *F. indica* showed very less toxicity (1-2%). The EtOAc, chloroform and *n*-butanol fraction of *D. bupleuroides* were also found very less toxic only 1-3%. These fractions have been found good antioxidant and antimicrobial agents, so these might be useful in drug formulations. The *n*-hexane fraction of *D. bupleuroides* showed 79% toxicity. All the studied fractions of *C. zedoaria* showed very less toxicity (1-8%).

CONCLUSIONS

This study concluded that aqueous fraction of *F. indica* showed highest activity against *P. multocida* (26mm) having MIC 98µg/mL as compared to its other fractions. Its $CHCl_3$ and *n*-BuOH fractions also displayed good results. Its $CHCl_3$, *n*-BuOH and aqueous fractions showed good antifungal activity against some strains. The $CHCl_3$ and EtOAc fractions of *D. bupleuroides* displayed good antibacterial activity against some bacterial strains. Its EtOAc fraction showed good activity only against *G. lucidum*. The $CHCl_3$ fraction of *C. zedoaria* showed good activity against all studied bacterial strains, while its EtOAc and *n*-BuOH fractions displayed good results against some bacterial strains. None of the fractions of *C. zedoaria* displayed antifungal activity against studied strains. All the studied fractions of three plants showed very less toxicity except *n*-hexane fraction of *D. bupleuroides* which showed 79% toxicity. All the studied

fractions of these plants which showed very less toxicity and displayed good antimicrobial and antioxidant potentials, might be used as neutraceuticals as well as in pharmacological preparations to produce economic, safe and nontoxic drugs.

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