

## PHARMACOLOGICAL EVALUATION OF *FUMARIA INDICA* (HAUSSKN.) PUGSLEY; A TRADITIONALLY IMPORTANT MEDICINAL PLANT

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### Abstract

Plants represent an illimitable bank of molecules for new drug discovery and development. The current study was aimed to scientifically validate and test the traditionally important medicinal plant '*Fumaria indica*' using multimode standardized assays. The plant was screened for antioxidant, antimicrobial, cytotoxic, antidiabetic and protein kinase inhibitory potential employing a range of solvents in the extraction process. Highest amount of gallic acid equivalent total phenolic ( $25.59 \pm 0.18$   $\mu\text{g}$  GAE/mg dry weight (DW) and quercetin equivalent total flavonoid ( $9.38 \pm 1$   $\mu\text{g}$  QE/mg DW) contents were quantified in methanol+distilled water extract (M+Dw). RP-HPLC profiling revealed the presence of appreciable quantity of apigenin, myricetin, quercetin and rutin ranging from 0.030 to 0.215  $\mu\text{g}$ /mg DW. Highest DPPH free radical scavenging efficiency ( $\text{IC}_{50}$  81.03  $\mu\text{g}$ /ml), total antioxidant capacity ( $16.43 \pm 0.14$  ascorbic acid equivalent (AAE)/mg DW) and total reducing power ( $41.17 \pm 2.22$  AAE/mg DW) were quantified in the M+Dw extract. Significant antibacterial activity was observed against *Micrococcus luteus* by ethanolic extract (MIC < 3.70  $\mu\text{g}$ /ml) while acetone+distilled water and ethanol extracts showed substantial activity against *Salmonella typhimurium* (MIC 33.33  $\mu\text{g}$ /ml). Significant cytotoxicity against brine shrimps ( $\text{LC}_{50}$  349.55  $\mu\text{g}$ /ml) and Hep G2 cell line ( $\text{IC}_{50}$   $8.45 \pm 2.11$   $\mu\text{g}$ /ml) was manifested by n-hexane and n-hexane+ethyl acetate extracts respectively while a conspicuous protein kinase inhibitory activity against *Streptomyces* 85E strain was exhibited by the n-hexane+ethanol extract ( $18 \pm 0.23$  mm bald phenotype at 100  $\mu\text{g}$ /disc). A moderate *in vitro* antidiabetic activity by virtue of alpha amylase inhibition was manifested by ethyl acetate extracts. Our study highlighted the importance of using a multi-solvent extraction process in bioprospecting of medicinal plants. It also validates the traditional medicinal uses of *F. indica* and emphasizes the need of further screening, isolation and characterization of its biologically active principles.

**Key words:** *Fumaria indica*, Medicinal plant, *Salmonella typhimurium*, multimode standardized assays

### Introduction

Plants have provided a source of inspiration for novel drug compounds throughout the history of mankind, as medicine obtained from plants have made enormous contributions to human health and well-being (Shinwari & Kaiser, 2011). According to world health organization (WHO), about three-fourths of world population depends upon traditional remedies for its healthcare needs, which include herbs mainly (Khan & Shinwari, 2016). Herbal medicine also known as traditional or natural medicine has existed in one way or another in various civilizations such as Chinese, Western, Japanese, Egyptians and Unani or Tibb (south Asia) (Tareen *et al.* 2016; Gilani, 2005a). A few prominent examples of drugs obtained from medicinal plants are: Aspirin, artemisinin, atropine, colchicine, digoxin, ephedrine, morphine, physostigmine, pilocarpine, quinine, reserpine, taxol, tubocurarine, vincristine and vinblastine. Traditional cures and folklore use of various herbs are the main source of natural product drug discovery (Gilani, 2005b). Approximately 10,000 to 53,000 herbs/plant species are used traditionally worldwide, however the number of these traditional remedies screened biologically is quite low and appropriate screening is an obligatory prerequisite to validate and explore the true pharmacological potential of these plants (Saslis-Lagoudakis *et al.*, 2012; Sarwat *et al.* 2012).

The genus *Fumaria* (Family: Fumariaceae) comprises of 46 species in the world and are generally known as 'fumittery, fumitory, fumus, earth smoke, vapor or wax dolls' in English (Orhan *et al.*, 2012). *Fumaria indica* (Hausskn.) Pugsley; Commonly known as pitpara, is a small

(up to 61 cm in height), sub-erect, scandent, annual herb growing wild in the plains and lower hills of India, Pakistan, Afghanistan and Mangolia (Baquar, 1989). The flowering and fruiting period is between March-June (Khan & Qureshi, 2013). *F. indica* has ethnopharmacological importance and is considered to be an anthelmintic, antidyspeptic, blood purifier, diaphoretic, diuretic and is also known to be useful in alleviating liver obstruction. It is also reported to be used as a laxative, stomachic, sedative, and tonic in the indigenous system of medicine (Gupta *et al.*, 2012a). The plant has also been evaluated pharmacologically and evidence obtained from various studies suggest that *F. indica* possess antidiarrheal (Gilani *et al.*, 2005b), anti-inflammatory and antinociceptive (Rao *et al.*, 2007), central nervous system depressant (Singh & Kumar, 2010), chemopreventive (Hussain *et al.*, 2012) and hepatoprotective properties (Rathi *et al.*, 2008). The plant is reported to be safe and do not possess any toxic effects (Gupta *et al.*, 2012a).

The major chemical constituents of the plant include several alkaloids i.e. cryptopine, fumaramine, fumariline, fumarophycine, fuyuziphine, narlumicine, narlumidine, papracine, paprafumicine, papraline, paprarine, protopine etc; steroids i.e. campesterol, sitosterol, stigmasterol; organic acids i.e. caffeic acid and fumaric acid (Rao *et al.*, 2007; Pandey *et al.*, 2008). These plants also contain certain kinds of fatty acids with antioxidant effects, phospholipids are a part of these lipids (Gupta & Rao, 2012b).

Plants contain a wide range of structurally and chemically diverse constituents with varied physicochemical characteristics (Shaheen & Shinwari, 2012). Extraction solvents used commonly might not cover potentially active

constituents due to their variable affinity and solubility profile (Sarwat *et al.*, 2012). It results in unreliable results of different plant screening protocols. Review of literature revealed a gap on part of studying the pharmacological spectrum of *F. indica* using multiple solvent systems covering a broader polarity range and application of a cascade of bioassays in an organized way to determine the true pharmacological potential of subject plant. Our study was aimed to apply various polarity based solvents for the extraction, to detect, identify and quantify the extracted phytoconstituents and to screen all the extracts for possible antioxidant, antimicrobial, cytotoxic and antidiabetic potential of *F. indica* by employing various *in vitro* bioassays and elucidation of solvent effect on the scale of observed activity.

## Materials and methods

**Collection and identification:** Fresh plant was collected in the month of June, 2014 during its flowering season from village Toru; district Mardan, Khyber Pakhtoonkhwa, Pakistan. Prof. Dr. Rizwana A. Qureshi, Department of Plant sciences, Quaid-i-Azam University, Islamabad identified the plant and a voucher specimen was deposited in the herbarium of medicinal plants, Quaid-i-Azam University, Islamabad, Pakistan with a herbarium number PHM 486.

**Reagents and solvents:** Analytical grade reagents and solvents were used in the study. Solvents i.e. n-Hexane, Chloroform, Acetone, Ethyl acetate, Methanol and Ethanol employed in the extraction process, were purchased from Sigma (Sigma-Aldrich, Germany); Dimethylsulfoxide (DMSO) was acquired from Sigma (Sigma Aldrich, USA). Standard antibacterial drugs (Cefixime, Ciprofloxacin), Standard antifungal drugs (Clotrimazole), Trichloroacetic acid (TCA), Trypton soya broth (TSB) and RPMI-1640 medium were purchased from Sigma (Sigma-Aldrich Germany). All other chemicals and reagents used in the study i.e. Aluminium chloride ( $\text{AlCl}_3$ ), 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) reagent, Ferric chloride ( $\text{FeCl}_3$ ), Monosodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4$ ), Folin–Ciocalteu reagent (FC), ascorbic acid, caffeic acid, (+) catechin, gallic acid, kaempferol, myricetin and quercetin were purchased from Merck (Darmstadt, Germany) unless otherwise specified in the text.

**Drying and extraction:** The collected plant material was sorted for any unwanted herbs and decayed or rotten plant parts. It was then followed by rinsing the plant material with tap water. The plant was then cut into small pieces and was shade dried for up to three weeks in a place having sufficient air circulation. Appropriately dried plant material was then pulverized to get a coarsely grounded powder. The powdered plant was then used in the extraction process. Powdered plant (40 g) was soaked in respective solvent in 1000 ml Erlenmeyer flask; separate flasks were used for each extract. The solvent systems and their combinations (1:1) employed in current study include the following with increasing order of polarity: n-hexane (nH), n-hexane+ethyl acetate (nH+Ea), n-hexane+ethanol (nH+E), chloroform (Ch), ethyl acetate (Ea), methanol+chloroform (M+Ch), ethyl acetate+methanol (Ea+M), acetone+methanol (Ac+M), methanol (M), acetone

(Ac), ethanol (E), acetone+distilled water (Ac+Dw), methanol+distilled water (M+Dw) and distilled water (Dw). Soaking of powdered material was done for three days with occasional shaking using an ultrasonic bath with an operational frequency and temperature of 25 KHz and 25°C respectively. Extraction process was performed thrice with each solvent. Filtration of the extracted plant material was performed using Whatmann No.1 filter paper and the extracts were concentrated by vacuum evaporation in rotary evaporator (Buchi, Switzerland) and dried at 45°C in vacuum oven (Yamato, Japan) under reduced pressure. The crude extracts were then kept at 20°C until further analysis.

**Percent extract recovery:** Plant extracts after been dried were weighed to get the percent recovery of crude extracts by using the following formula:

$$\text{Percent extract recovery} = (a / b) \times 100$$

a = Total weight of the crude extract

b = Total dry weight of the powdered plant material in each extraction (40 g)

## Phytochemical analysis

**Total phenolic content estimation:** Standard procedure described by Haq *et al.*, (2012) was followed for the determination of phenolic content. The reagent used was Folin–Ciocalteu reagent (FC), while gallic acid served as a standard (positive control). An aliquot of 20  $\mu\text{l}$  from each crude extract (4mg/ml DMSO) was transferred via micropipette to the wells of a 96 well microtiter plate followed by addition of 90  $\mu\text{l}$  of FC reagent. The plate with the mixture was incubated at room temperature for a period of 5 min, 90  $\mu\text{l}$  of sodium carbonate (6% w/v) was then added to the wells carrying the mixture. The absorbance was measured at 630 nm with the help of a microplate reader (Biotek, USA, microplate reader ELX 800). The experiment was run in triplicate, and a calibration curve ( $y = 0.016x - 0.017$ ,  $R^2 = 0.969$ ) was drawn employing the same experimental conditions with gallic acid (2.5, 5, 10, 20, 40  $\mu\text{g/ml}$ ) being used as a positive control. The total phenolic content (TPC) was expressed as  $\mu\text{g}$  gallic acid equivalent (GAE)/ mg of dry weight (DW).

**Total flavonoid content estimation:** The total flavonoid content (TFC) of the crude extracts of *F.indica* was estimated using aluminium chloride based colorimetric method (Fatima *et al.*, 2015). Quercetin was used as a positive control. The assay was performed in 96 well plate, and each well used in the test contained a mixture having 20  $\mu\text{l}$  of test sample (4mg/ml DMSO), 10  $\mu\text{l}$  of 1 M potassium acetate, 10  $\mu\text{l}$  of 10% (w/v) aluminum chloride and 160  $\mu\text{l}$  distilled water. After incubation at room temperature for 30 min, the absorbance was measured at 415 nm using microplate reader. Quercetin at final concentrations of 2.5–40  $\mu\text{g/ml}$  was used in the study in order to draw a calibration curve ( $y = 0.032x - 0.220$ ,  $R^2 = 0.974$ ) and the resultant TFC was expressed as  $\mu\text{g}$  quercetin equivalent (QE) per mg DW. The experiment was run in triplicate.

**HPLC-DAD analysis:** A previously reported protocol by Fatima *et al.* (2015) was adopted for the determination and

quantification of polyphenols in various solvent extracts of *F.indica* using high performance liquid chromatography. The HPLC system (Agilent Chem station Rev. B.02-01-SR1[260]) had a Zorbex-C8 analytical column (4.6 x 250 mm, 5µm particle size, Agilent, USA) in conjunction with a diode array detector (DAD; Agilent technologies, Germany). Reverse phase chromatographic analysis was used and two mobile phases named A and B of different composition were prepared for polyphenols analysis. Mobile phase A had acetonitrile:methanol:water:acetic acid in a ratio of 5:10:85:1, while mobile phase B contained acetonitrile:methanol:acetic acid in a ratio of 40:60:1 and their flow rate being maintained at 1ml/min. Samples for HPLC analysis were dissolved in methanol at 10 mg/ml concentration. Sample volume of 20 µl was injected through an injection port into the column. Next analysis was preceded by a 9 min column reconditioning phase. The gradient volume of mobile phase B in the first 0-20 min was 0-50%, from 20-25 min it was 50-100% and from 25-30 min it was 100%. Apigenin, caffeic acid, catechin, gallic acid, kaempferol, myricetin, quercetin and rutin were used as reference standards and their stock solutions were prepared and further diluted using methanol as a solvent to get a final concentration of 50 µg/ml. The solutions were freshly prepared just before analysis and all were filtered through a 0.2 µm sartolon polyamide membrane filter and degassed.

The analysis was performed at ambient temperature, absorbance was measured at four different wavelengths for respective compounds i.e. 257 nm, 279 nm, 325 nm and 368 nm (rutin and gallic acid: 257 nm, catechin: 279 nm, caffeic acid: 325 nm, myricetin, quercetin and kaempferol: 368 nm). Results were expressed as µg/mg DW. Retention time and UV absorption spectra of samples were compared with those of standards for the identification and quantification of different polyphenols.

## Biological evaluation

### Antioxidant assays

**Free radical scavenging assay (DPPH assay):** A previously reported protocol by Bibi *et al.* (2011) was followed with slight modifications. The free radicals scavenging activity was indicated by the discoloration of the purple colored DPPH solution (9.2 mg/100 ml methanol). Initially, 20 µl of the sample (4mg/ml DMSO) was taken from their stock solution and was transferred to the corresponding wells of a 96 well microplate, it was then followed by the addition of 180 µl DPPH reagent to each well containing the sample. The final concentration of the sample was 400 µg/ml. The mixture was then incubated at 37°C for 60 min period in dark. Ascorbic acid (1 mg/ml) was used as a standard (positive control) while DMSO was used as a negative control. Absorbance of the mixture was measured at 517 nm with a microplate reader. The drop in absorbance of the mixture having DPPH reagent in it shows the presence of radical scavenging properties in the sample. Samples which showed greater than 70% scavenging activity at initial concentration were further tested by using three fold serial dilution to calculate IC<sub>50</sub> values.

Percent radical scavenging activity was calculated using the following formula;

$$\% \text{ scavenging activity} = (1 - \text{Ab}_s / \text{Ab}_c) \times 100$$

Ab<sub>s</sub> and Ab<sub>c</sub> = Absorbance of sample and negative control respectively

**Total antioxidant capacity (TAC):** Phosphomolybdenum based analysis technique as reported previously by Bibi *et al.* (2011) was used to evaluate the total antioxidant capacity of *F.indica*. A reagent solution comprising of 4 mM ammonium molybdate, 28 mM sodium phosphate and 0.6 M sulphuric acid was used and 100 µl of all the plant extracts (4 mg/ml DMSO) were mixed with 1 ml of the reagent solution. Incubation was done at 90°C for 90 min and it was followed by cooling at room temperature. Absorbance was measured at 645 nm using a microplate reader. Ascorbic acid (1 mg/ml DMSO) at final concentration of 6.25-100 µg/ml was used as a standard (positive control) and calibration curve was drawn ( $y = 0.032x - 0.25$ ), DMSO was used as blank (negative control). The test was performed in triplicate and the resultant TAC was expressed as µg ascorbic acid equivalent (AAE) per mg DW.

**Total reducing power (TRP):** The reducing power of crude extracts of *F.indica* was estimated by following the previously reported protocol (Jafri *et al.*, 2014) with slight modification. The test was performed by taking 200 µl of each sample (4 mg/ml DMSO stock solution) and 400 µl each of 0.2 M phosphate buffer (0.2 M/l, pH 6.6) and 1% (w/v) potassium ferricyanide [K<sub>3</sub>Fe(CN)<sub>6</sub>]. The mixture was incubated at 30°C for 50 min. It was followed by addition of 400 µl of 10% (w/v) trichloroacetic acid (TCA) solution and centrifugation at 3000 rpm for 10 min. An aliquot of 150 µl was taken from the supernatant of each mixture and shifted to corresponding well in the micro-well plate. Lastly, 50 µl 0.1% (w/v) ferric chloride (FeCl<sub>3</sub>) solution was added to each well and absorbance was measured at 700 nm. Ascorbic acid (1 mg/ml DMSO) at final concentration of 6.25-100 µg/ml was used as a standard (positive control) and calibration curve was drawn ( $y = 0.038x + 0.748$ ), DMSO as a negative control. The assay was performed in triplicate and reducing power of the test samples was expressed as µg ascorbic acid equivalent (AAE) per mg DW.

### Antimicrobial assays

**Antibacterial assay:** Agar disc diffusion method was used for preliminary screening of antibacterial activity of the plant extracts (Sharma *et al.*, 2009). Seeding density of the refreshed bacterial cultures [gram positive (*Micrococcus luteus* ATCC#10240 and *Staphylococcus aureus* ATCC#6538) and gram negative (*Klebsiella pneumoniae* ATCC#1705 and *Salmonella typhimurium* ATCC#14028)] was adjusted as per the test requirement ( $1 \times 10^6$  CFU/ml) and a refreshed culture (50 µl) was used to prepare a lawn on nutrient agar plates. Test samples (5 µl from 20 mg/ml DMSO stock solution) in a final concentration of 100 µg/disc were applied on sterile filter paper discs and the discs were placed on the seeded agar plates after been properly labeled. Standard antibiotic (cefixime) served as positive control, while DMSO impregnated disc served as a negative control. The plates were incubated at 37°C for 24 hr. They were then examined for any appearance of zone of inhibition around the disc carrying test samples and controls. The diameter of the zone was measured to the

nearest mm. Samples which exhibited significant zones of inhibition i.e.  $\geq 12$  mm were then analyzed further for the determination of minimum inhibitory concentration (MIC). Standard microdilution method was used with slight modification (Galappathie *et al.*, 2014). Three folds serial dilution of 100  $\mu\text{g/ml}$  final concentration were prepared (33.33, 11.11 and 3.70  $\mu\text{g/ml}$ ). Each dilution was applied in triplicate. Bacterial inoculum (190  $\mu\text{l}$ ) prepared in nutrient broth was added to each well while inoculum size and the density was adjusted as per predetermined limits (up to  $5 \times 10^2$  CFU/ml). After incubating the plate at 37°C for 24 hr, absorbance was recorded at 630 nm. Percent growth inhibition was estimated using the following formula:

$$\% \text{ growth inhibition} = T_s / T_c \times 100$$

$T_s$  and  $T_c$  = turbidity of the sample and negative control respectively

Absorbance was measured with microplate reader and the minimum concentration which inhibited  $\geq 90\%$  bacterial growth was considered as MIC.

**Antifungal assay:** Agar disc diffusion method was chosen for preliminary screening of antifungal activity of the plant extracts (Sharma *et al.*, 2009). Separate petri plates having sterile Sabouraud Dextrose agar (SDA) (20-25 ml) were swabbed with an aliquot of 100  $\mu\text{l}$  spore suspension from each refreshed fungal strain [*Aspergillus flavus* (FCBP 0064), *Aspergillus fumigatus* (FCBP 66), *Aspergillus niger* (FCBP 0198), *Fusarium solani* (FCBP 0291) and *Mucor* species (FCBP 0300)] harvested in 0.02% (v/v) Tween 20 solution and the turbidity was adjusted as per McFarland 0.5 turbidity standard. Test samples (5  $\mu\text{l}$  from 20 mg/ml DMSO stock solution) were applied on sterile filter paper discs and the discs were placed on the seeded agar plates after been labeled accordingly. Standard antifungal (clotrimazole) was also employed in the test and a disc impregnated with clotrimazole served as positive control, while DMSO impregnated disc served as a negative control. The plates were incubated at 28°C for 24-48 hr. They were then examined for any appearance of zone of inhibition around the disc carrying test samples and controls. The diameter of the zone was measured to the nearest mm.

**Antileishmanial assay:** Crude extracts of selected plant were tested for potential antileishmanial activity using MTT colorimetric assay following a previously reported protocol (Khan *et al.*, 2015). The assay involved the use of 6–7 days incubated culture of *Leishmania tropica* kwh 23 promastigotes. Briefly, the parasites were cultured in 10% fetal bovine serum (FBS) supplemented Medium-199 at 24°C. Streptomycin sulphate (100  $\mu\text{g/ml}$ ) and penicillin G (100 IU/ml) were also added to the medium. Each well of 96 microwell plate received 180  $\mu\text{l}$  of the culture having a seeding density of  $1 \times 10^6$  promastigotes/ml, each well also contained 20  $\mu\text{l}$  of samples to be tested at 100  $\mu\text{g/ml}$  concentration, DMSO concentration was kept less than 1%. The positive and negative controls employed were amphotericin B (0.33-0.004  $\mu\text{g/ml}$ ) and 1% DMSO in PBS respectively. Following a 72 hours incubation period at 24 °C, filter sterilized MTT solution (20  $\mu\text{l}$  of 4mg/ml solution

in Dw) was added and the plate was incubated at 24°C for 4 hours. It was followed by cautious removal of the supernatant without unsettling the colored formazan sediments. The plate was kept for an hour after addition of 100  $\mu\text{l}$  of DMSO to each well in order to fully dissolve formazan sediments. A microplate reader was used to measure the absorbance at 540 nm.

### Cytotoxicity assay

**Brine shrimp lethality assay:** Previously reported protocol by Bibi *et al.*, (2011) was followed along with minor modifications. Briefly perforated, bi-compartment tank was filled with artificial sea water (34 g/l sea salt in distilled water, supplemented with 6 mg/l dried yeast), and brine shrimp (*Artemia salina*) eggs (Ocean 90, USA) were poured in sufficient amount and were allowed to hatch, the incubation temperature was 30°C. The compartment carrying the eggs was covered with an aluminium foil, after two days of incubation eggs were hatched and hatched shrimps move towards the light source placed over the uncovered compartment of the tank. Mature phototrophic nauplii were collected with the help of a pasteur pipette and were transferred to a beaker having artificial sea water where they were harvested. The test samples were analyzed at various final concentrations in order to determine their lethality (1000, 500 and 250  $\mu\text{g/ml}$ ). Each well to be used in the assay received 10 mature nauplii and 150  $\mu\text{l}$  of sea water which is supplemented with dried yeast in a concentration of 6 mg/l. Different dilutions of test samples were then introduced to the corresponding wells to have respective concentrations. Doxorubicin (4 mg/ml) at final concentration of 200, 100, and 50  $\mu\text{g/ml}$  served as a positive control while DMSO alone was used as a negative control. Lastly, sea water was used to make the final volume of each well up to 300  $\mu\text{l}$ . Following an incubation period of 24 hr at 37°C, the number of dead nauplii was counted in each well and samples showing  $\geq 50\%$  mortality were analyzed for their  $\text{LC}_{50}$  determination using Table Curve 2D v4 software. The experiment was run in triplicate and percent mortality was calculated using the following formula:

$$\% \text{ mortality} = \text{no. of dead shrimps} / \text{total no of shrimps} \times 100$$

**Cytotoxicity against Hep G2 cell line:** Crude extracts of selected plant were tested for cytotoxic activity against Hep G2 cancer cell line (RBRC-RCB1648) using SRB colorimetric assay following a previously reported protocol (Khan *et al.*, 2015). Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% v/v FBS, 100  $\mu\text{g/ml}$  streptomycin, 100 IU/ml penicillin G sodium and 0.25  $\mu\text{g/ml}$  amphotericin B was used to grow Hep G2 cells. Cytotoxicity was evaluated at 6 serial dilutions of *F.indica* extracts (20, 6.66, 2.22, 0.74, 0.24 and 0.08  $\mu\text{g/ml}$ ) and  $\text{IC}_{50}$  values of samples showing greater than 50% inhibition was evaluated. The cells were incubated at 37°C in a humidified atmosphere for 72 hours and the presence of 5%  $\text{CO}_2$  was ensured. After replacing the old medium with fresh one, cells were incubated again for 24 hours followed by their trypsonization and dilution to procure as assay density of  $1 \times 10^5$  cells/ml. Each well of 96 microwell plate received 180  $\mu\text{l}$  of the aforementioned

culture, the wells also contained 20 µl of samples to be tested at 20 µg/ml concentration, DMSO concentration was kept less than 1%. The positive and negative controls employed were doxorubicin (20-0.08 µg/ml) and 1% v/v DMSO in PBS respectively. Following a 72 hours incubation period at 37°C in CO<sub>2</sub> incubator, 20% w/v cold TCA was added and cell fixation was done for 1 hour at 4°C. Tap water was used for washing of fixed cells, which were then dried for 30 min at room temperature and stained with 50 µl of 0.057% w/v SRB in 1% w/v acetic acid. Each well was then washed 4 times with 1% v/v acetic acid and allowed to dry overnight. 10 mM Tris base (200 µl) having pH 10 was used to solubilize the bound dye for 1 hour. A microplate reader was used to measure optical density at 515 nm and percent survival was estimated. A zero day control was employed in each case by addition of comparable number of cells to not less than sixteen wells and incubation at 37°C for 1 hour, followed by similar procedure as defined earlier. Percent inhibition of cell growth was estimated using the following formula:

$$\% \text{ inhibition} = 100 - \frac{[(\text{OD}_{\text{cells}} + \text{samples} - \text{OD}_{\text{day 0}}) / (\text{OD}_{\text{cells}+1\% \text{ DMSO}} - \text{OD}_{\text{day 0}})] \times 100}$$

**Protein kinase inhibition assay:** The assay was performed in triplicate using *Streptomyces* 85E as a test strain (Khan *et al.*, 2015). Plates having ISP4 medium were seeded with inoculum of *Streptomyces* (100 µl). Spores of *Streptomyces* 85E strain were inoculated in sterile trypton soya broth (TSB), incubation was done at 37°C for 24 hr and turbidity was adjusted with sterile TSB as per McFarland 0.5 turbidity standard. Test samples (5 µl from 20 mg/ml DMSO stock solution) in a final concentration of 100µg/disc were applied on sterile filter paper discs and the discs were placed on the seeded agar plates. Disc impregnated with surfactin was used as a positive control, while DMSO impregnated disc served as a negative control. The plates were then incubated at 28°C for a period of 72 hr allowing the hyphae formation. The inhibition of phosphorylation and ultimately that of mycelia and spore formation was indicated by the appearance of clear or bald zone around the disc. A bald zone with a diameter of 12 mm or greater was considered as significant. The diameter of the zone was measured to the nearest mm.

#### Antidiabetic assay

**Alpha amylase inhibition assay:** Previously reported method by Khan *et al.*, (2015) was followed with slight modification. The activity was performed in 96 well microplate, and to each well included in the test, 15 µl phosphate buffer (pH 6.8), 25 µl alpha amylase enzyme [0.5 mg (28 U/mg) in 1 ml phosphate buffer, diluted further to get 0.12 U/ml], 10 µl of sample (4 mg/ml DMSO) and 40 µl starch (2 g/l phosphate buffer) was added in subsequent steps. Incubation was done at 50°C for 30 min; it was followed by addition of 20 µl of 1 M HCl and finally 90 µl iodine solution (5 mM I<sub>2</sub> (6.3 mg) and 5 mM KI (8.3 mg) per 10 ml Dw). Suitable wells were assigned for blank, positive and negative control. Blank contained buffer solution, starch and DMSO while negative and positive

control wells contained DMSO and acarbose respectively instead of crude plant extracts. Results were noted using a microplate reader and readings were taken at 540 nm. Percent enzyme inhibition was calculated using the following formula:

$$\% \text{ enzyme inhibition} = \frac{[(\text{OD}_x - \text{OD}_y) \div \text{OD}_z - \text{OD}_y]}{\text{OD}_z - \text{OD}_y} \times 100$$

OD<sub>x</sub>, OD<sub>y</sub> and OD<sub>z</sub> = absorbance value of sample, negative control and blank respectively.

#### Statistical analysis

Data is expressed as mean of triplicate analysis ± Standard deviation. LC50 values were calculated by table curve 2D Ver.4 software. Correlation analysis of the phytochemical activities was carried out using the correlation and regression by Microsoft Excel program.

#### Results and Discussion

**Percent extract recovery:** The complete pharmacological spectrum of *F. indica* was evaluated using a range of solvents with varying polarity. The extraction efficiency of each solvent was determined as shown in table 1. The highest extraction efficiency was observed in Dw with 25.75% (w/w) extraction yield. The yield obtained was least (1.25%) when nH+Ea was used as extraction solvent. The values obtained affirm the role of polarity in effecting the extraction efficiency. Choice of solvents, plant material and extraction methods are the three basic parameters which need optimization in order to get high quality results. Our results further strengthen the findings of previously reported studies where maximum extraction yield was reported with aqueous-methanol solvent (Sultana *et al.*, 2009) and the general acceptability of ethanol, methanol and water as the most commonly used solvents for preliminary investigation of plants for pharmacological activities (Salie *et al.*, 1996; Lourens *et al.*, 2004; Parekh *et al.*, 2006; Rojas *et al.*, 2006;). The presence of diverse compounds of complex chemical characteristics might be responsible for varied extraction efficiency with different solvents. The presence of sugars and oligosaccharides in plant material and their comparatively easy solubility in water might well be the reason of its highest extraction efficiency. The serial exhaustive extraction method is the ideal choice when complete pharmacological profile of the plant needs to be assessed (Green, 2004). Sonication was done in parallel and it offer distinct advantages i.e. improved extraction efficiency and extraction kinetics, fewer steps and minimum sample degradation (Vilegas *et al.*, 1997; Huie, 2002;).

#### Phytochemical Analysis

**Total phenolic content:** The analysis of phenolic content in various extracts of *F. indica* demonstrated that highest total phenolic content expressed as gallic acid equivalent (µg GAE/mg DW) was observed with M+Dw extract i.e. 25.59 ± 0.18 µg GAE/mg DW. It was followed by Ac+Dw with 24.43 ± 0.12 µg GAE/mg DW. Lowest phenolic content was observed in case of nH extract with 0.76 ± 0.12

µg GAE/mg DW. The total phenolic content in different extracts decreased with the following trend: M+Dw > Ac+Dw > Dw > Ac+M > M > Ac > M+Ch > E > Ea+M > nH+E > Ea > Ch > nH+Ea > nH (Fig.1). Plant phenolics have reported antioxidant properties which make them imperative in fighting different conditions like oxidative stress, cell death and cytotoxicity by virtue of their radical scavenging and trace elements chelating potential (Kumar *et al.*, 2013). The presence of hydroxyl, methoxy and ketonic functional group might attribute to the antioxidant properties of phenols (Afshar *et al.*, 2012). Free radical scavenging and inhibition of lipid peroxidation are considered as the most useful traits which make these compounds pharmacologically important

**Table 1. Percent extract recovery**

Solvent code	Approximate Polarity Index	% Extraction Yield
nH	0	3.25
nH+Ea	2.2	1.25
nH+E	2.6	5
Ch	4.1	2
Ea	4.4	2.24
M+Ch	4.6	8.75
Ea+M	4.75	8
Ac+M	5.1	10.75
M	5.1	10.75
Ac	5.1	9.25
E	5.2	8
Ac+Dw	7.05	19.75
M+Dw	7.05	21.25
Dw	9	25.75

**Total flavonoid content:** The results of total flavonoid content assay revealed that M+Dw extract of *F. indica* have the highest total flavonoid content expressed as Quercetin equivalent (µg QE/ mg DW), i.e.  $9.38 \pm 1 \mu\text{g QE/mg dry weight}$ . It was followed by Ac+Dw with  $4.99 \pm 0.44 \mu\text{g QE/mg DW}$ . Lowest reading was observed when nH was used as a solvent with only  $0.35 \pm 0.34 \mu\text{g QE/mg dry weight}$ . The total flavonoid content in different extracts decreased with the following trend: M+Dw > Ac+Dw > M > Ac+M > Dw > Ac > Ea+M > E > M+Ch > nH+E > Ea > nH+Ea > Ch > nH (Fig.1). Inside biological systems, phenolics and flavonoids have been reported to be associated with definite antioxidant action having free radical and singlet oxygen quenching ability (Kim *et al.*, 2006). Similarly, polyphenols and other oxygenated derivatives are known to have immense antioxidant potential (Devasagayam *et al.*, 2004).

Our study revealed significant results regarding the total flavonoid and total phenolic content of *F. indica* crude extracts. Significant flavonoid and phenolic contents were quantified in M+Dw which indicate its possible use as a natural antioxidant preparation.

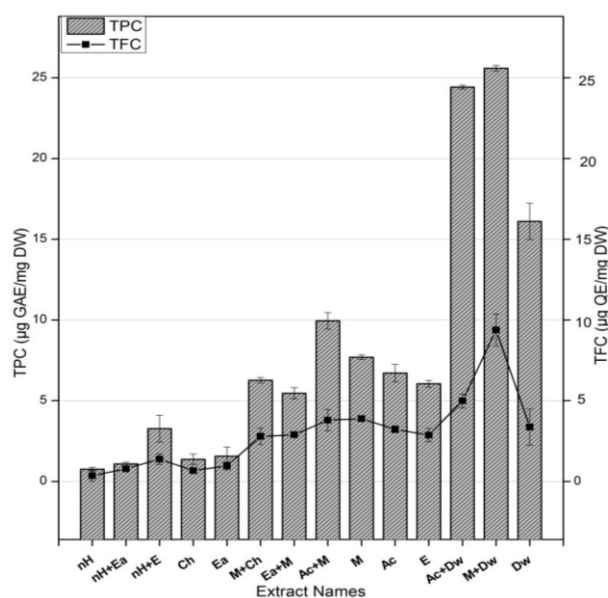
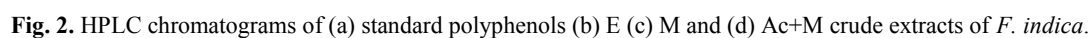


Fig. 1. Total phenolic content (TPC) and total flavonoid content (TFC) of extracts. Values presented are expressed as mean of triplicate  $\pm$  standard deviation.

**HPLC-DAD analysis:** The quantitative analysis of *F. indica* extracts was performed using reverse phase HPLC-DAD based profiling. The peaks were then compared with UV absorption spectra and retention time of standard/reference compounds including various important polyphenols, the results are summarized in Fig 2 and Table 2. Significant amount of apigenin, myricetin, quercetin and rutin were quantified in some of the analyzed extracts. Apigenin was found to be present in highest amount in Ac+M extract i.e.  $0.011 \mu\text{g/mg DW}$ , followed by E extract with  $0.008 \mu\text{g/mg DW}$ . Significant amount of myricetin was quantified in E extract with  $0.215 \mu\text{g/mg DW}$ , followed by Ac+M with  $0.189 \mu\text{g/mg DW}$ . Furthermore, M extract displayed highest quercetin content as compared to other solvent extracts i.e.  $0.030 \mu\text{g/mg DW}$ , followed by Ac+M with a value of  $0.0053 \mu\text{g/mg DW}$ . Rutin was observed to be highest in Ac+M extract with a value of  $0.185 \mu\text{g/mg DW}$  and the M extract followed with rutin contents of  $0.092 \mu\text{g/mg DW}$ .

The presence of apigenin, quercetin (flavone aglycons), myricetin (a flavonoid), and rutin (flavonoid glycosides) further signifies the medicinal worth of *F. indica*. The metabolites quantified have established clinical uses i.e. Apigenin have documented anticancer, anti-inflammatory properties (Singh *et al.*, 2004). Myricetin possess strong antioxidant (Roedig-Penman & Gordon, 1998), antimicrobial (Puupponen-Pimiä *et al.*, 2001) and TNF suppressive activity (Tsai *et al.*, 1999). Similarly, quercetin have reported antimicrobial (Lupaşcus *et al.*, 2010), antioxidant and cytotoxic activity (Velloso *et al.*, 2011). Rutin is known to have significant antioxidant, anticancerous, antidiabetic (Chua, 2013) and antimicrobial properties (Lupaşcus *et al.*, 2010). High performance thin layer chromatography (HPTLC) analysis of *F. indica* have previously confirmed the presence of phenolics (Rao *et al.*, 2007), which is in accordance with our findings. The presence of useful antioxidant constituents might be responsible for the clinical benefits associated with *F. indica*.

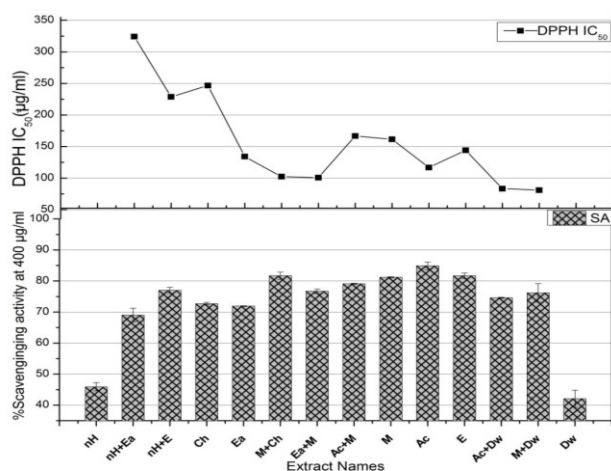
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## Biological Evaluation

**Free radical scavenging activity (DPPH assay):** The percent free radical scavenging activity (%FRSA) was detected for crude extracts based on the discoloration of purple colored methanolic DPPH solution. IC<sub>50</sub> values were calculated using Table Curve 2D v4 software. The results showed that M+Dw extract showed highest free radical scavenging activity with an IC<sub>50</sub> value of 81.03 µg/ml. It was followed by Ac+Dw and Ea+M extracts with IC<sub>50</sub> values of 83.32 and 100.76 µg/ml respectively. Least free radical quenching capacity was observed in case of nH and Dw extracts with IC<sub>50</sub> values greater than the highest concentration employed in the assay i.e. > 400 µg/ml. The activity detected for different crude extracts employed in the assay followed the mentioned trend: M+Dw > Ac+Dw > Ea+M > M+Ch > Ac > Ea > E > M > Ac+M > nH+E > Ch > nH+Ea > Dw > nH (Fig 3).

Current study revealed notable radical scavenging activity with significant IC<sub>50</sub> values exhibited by M+Dw and Ac+Dw extracts. The results obtained are somewhat identical to previously reported antiradical capacity of *F. indica* crude extract (Gupta *et al.*, 2012). Our study has documented greater free radical scavenging potential which might be attributed to the presence of important compounds like apigenin, myricetin, quercetin and rutin as confirmed by HPLC analysis of various crude extracts. The use of multiple solvents have revealed higher DPPH reducing potential in Ac, E and M crude extracts, previous studies have employed a different set of solvents with results slightly variable from our current study (Riaz *et al.*, 2012).

**Total antioxidant capacity:** The total antioxidant capacity of *F. indica* crude extracts was evaluated using phosphomolybdenum based method. The results of TAC assay demonstrated that M+Dw extract possess the highest activity (16.43 ± 2.16) expressed as ascorbic acid equivalent (µg AAE/mg DW). It was followed by Ac+Dw with 16.34 ± 0.14 µg AAE/ mg DW. Lowest antioxidant capacity was observed when nH was used as an extraction solvent with merely 0.73 ± 0.23 µg AAE/mg DW. The



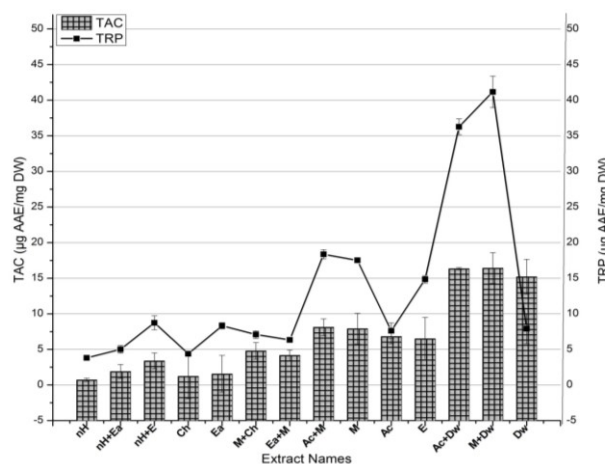
**Fig. 3.** DPPH radical scavenging activity of *F. indica* crude extracts. Values presented are mean of triplicate ± standard error.

total antioxidant potential of all the tested extracts followed the given order: M+Dw > Ac+Dw > Dw > Ac+M > M > Ac > E > M+Ch > Ea+M > nH+E > nH+Ea > Ea > Ch > nH (Fig 4).

Our results indicate the presence of appreciable antioxidant capacity in various crude extracts especially M+Dw and Ac+D. Least activity was shown by nH extract which is in accordance with previously reported studies using *F. indica*. However due to variation in solvent systems used, the maximum TAC reported in our study contradicts the previous findings (Riaz *et al.*, 2012).

**Total reducing power:** The reducing power of *F. indica* crude extracts was manifested by analyzing the conversion of ferric ion (Fe<sup>3+</sup>) to ferrous ion (Fe<sup>2+</sup>) in the presence of desired concentration of our test samples. The results showed that M+Dw extract showed highest reducing power with a value of 41.17 ± 2.22 µg AAE/mg DW followed by Ac+Dw with 36.26 ± 1.12 µg AAE/mg DW. On the contrary, lowest reducing power was demonstrated in case of nH extract with 3.82 ± 0.18 µg AAE/mg DW. The results of all the tested samples followed the mentioned order: M+Dw > Ac+Dw > Ac+M > M > E > nH+E > Ea > Dw > Ac > M+Ch > Ea+M > nH+Ea > Ch > nH (Fig 4).

The reducing power has been reported to be associated with phenolics (Shon *et al.*, 2003), HPLC-DAD analysis have quantified different phenolics in our test samples. The significant reducing potential of various crude extracts thus affirms presence of phenolic and flavonoid compounds. The polarity based increase in results is in accordance with the previously reported studies where a different solvent range of varying polarity was employed (Riaz *et al.*, 2012). The complete phytochemical screening and HPLC-DAD analysis of various extracts of *F. indica* revealed the significance of a few crude extracts which include; M+Dw, M, Ac+M, and Ac+Dw as potential candidates for natural antioxidant preparations.



**Fig. 4.** Total antioxidant capacity (TAC) and Total reducing power (TRP) of *F. indica* crude extracts. Values presented are mean of triplicate ± standard deviation.

**Table 3. Antibacterial activity and MIC values of extracts of *F. indica* tested against gram positive and negative bacterial strains.**

Extract Names	Antibacterial Assay							
	Diameter of zone of inhibition in mm (Mean $\pm$ SD)* (MIC: $\mu$ g/ml)							
	<i>K.pneumoniae</i>	MIC	<i>S. typhi</i>	MIC	<i>M. luteus</i>	MIC	<i>S. aureus</i>	MIC
nH	14 $\pm$ 0.23	33.33	11 $\pm$ 0.76	--	07 $\pm$ 0.12	--	09 $\pm$ 1.67	--
nH+Ea	12 $\pm$ 1.14	100	08 $\pm$ 1.21	--	10 $\pm$ 2.12	--	08 $\pm$ 0.13	--
nH+E	07 $\pm$ 0.78	--	09 $\pm$ 0.13	--	--	--	09 $\pm$ 0.67	--
Ch	08 $\pm$ 0.13	--	10 $\pm$ 1.87	--	22.5 $\pm$ 1.21	<3.70	8.5 $\pm$ 1.78	--
Ea	09 $\pm$ 0.45	--	09 $\pm$ 0.56	--	08 $\pm$ 1.12	--	09 $\pm$ 2.78	--
M+Ch	11.5 $\pm$ 0.23	--	11 $\pm$ 0.87	--	08 $\pm$ 0.34	--	07 $\pm$ 0.23	--
Ea+M	10 $\pm$ 1.21	--	13.5 $\pm$ 0.45	100	14 $\pm$ 1.21	33.33	09 $\pm$ 1.78	--
Ac+M	07 $\pm$ 0.87	--	08 $\pm$ 0.56	--	13.5 $\pm$ 1.45	100	12 $\pm$ 1.56	100
M	08 $\pm$ 1.67	--	11.5 $\pm$ 1.56	--	20 $\pm$ 1.23	<3.70	09 $\pm$ 2.09	--
Ac	07 $\pm$ 0.96	--	11 $\pm$ 0.23	--	15 $\pm$ 1.21	33.33	09 $\pm$ 1.34	--
E	8.5 $\pm$ 2.12	--	12 $\pm$ 2.22	33.33	24.5 $\pm$ 0.12	<3.70	10 $\pm$ 1.33	--
Ac+Dw	07 $\pm$ 0.16	--	14 $\pm$ 1.54	33.33	07 $\pm$ 0.12	--	09 $\pm$ 1.05	--
M+Dw	11 $\pm$ 1.21	--	11 $\pm$ 1.23	--	07 $\pm$ 0.56	--	08 $\pm$ 0.53	--
Dw	11.5 $\pm$ 2.45	--	10 $\pm$ 1.34	--	07 $\pm$ 1.56	--	11 $\pm$ 0.47	--
Cefixime	17 $\pm$ 1.6	0.06	10 $\pm$ 0.07	0.06	24 $\pm$ 0.95	0.8	15 $\pm$ 0.85	0.125
Cipro**	19.5 $\pm$ 1.3	0.2	21 $\pm$ 0.85	0.02	24.6 $\pm$ 0.69	0.8	22.5 $\pm$ 0.11	0.25
DMSO	--	--	--	--	--	--	--	--

\*The sample concentration was 100  $\mu$ g per disc. Values (mean  $\pm$  SD) are average of triplicate of each plant extract ( $n = 1 \times 3$ ). -- = No activity in disc diffusion assay or not applicable (zone < 12 mm) for MIC determination. Cefixime and \*\*Ciprofloxacin: Positive control (20  $\mu$ g/disc), Negative control: DMSO.

### Antimicrobial capability

**Antibacterial activity:** The antimicrobial properties of herbal products largely depend upon solvent used, organism tested and plant part used (Gul *et al.*, 2012). *F. indica* has reported folklore uses and the phytochemical analysis have revealed presence of useful components like phenolics and flavonoids. The antibacterial activity was determined using disc diffusion method and the results are presented in Table 3. Samples which illustrated significant antibacterial activity  $\geq 12$  mm zone were further analyzed at lower concentrations and their MICs were calculated using broth microdilution method. *M. luteus* was found to be most sensitive with E extract exhibiting maximum activity having an inhibitory zone of 24.5  $\pm$  0.12 mm and an MIC value of 3.70  $\mu$ g/ml. It was followed by M with 20  $\pm$  1.23 mm zone of inhibition and 3.70  $\mu$ g/ml MIC value. Notable activity was shown by Ac+M against the other gram positive strain used in the study i.e. *S. aureus*, with a 12  $\pm$  1.56 mm zone and an MIC value of 100  $\mu$ g/ml. Significant results were observed in case of *S. typhi*, with Ac+Dw showing maximum activity having a zone of inhibition equal to 14  $\pm$  1.54 mm and MIC of 33.33  $\mu$ g/ml. It was followed by E with 12  $\pm$  2.22 mm zone of inhibition and 33.33  $\mu$ g/ml MIC value. Moderate activity was noted against *K. pneumoniae* with largest inhibitory zone of 14  $\pm$  0.23 mm observed with nH extract and a resultant MIC of 33.33  $\mu$ g/ml. No activity was observed with negative control (DMSO) which confirmed its non-toxic effect on the tested strains.

The site and number of hydroxyl groups present in phenolics have been reported to be responsible for toxicity against microorganisms (Dixon *et al.*, 1983; Scalbert, 1991), similarly flavonoids have also been known to be effective against a large number of microbes *in vitro* (Bennett & Wallsgrove, 1994). The

mechanisms of their antibacterial action include inhibition of cytoplasmic membrane function, energy metabolism and nucleic acid synthesis (Cushnie & Lamb, 2005). The presence of antibacterial properties in some of the crude extracts is evident of the fact that they possess antimicrobial principles like phenolics, flavonoids as confirmed by various phytochemical assays and HPLC-DAD analysis. Our results are in agreement with previous studies regarding the use of comparatively polar solvents for better extraction of antimicrobial compounds (Bisignano *et al.*, 1996; Salie *et al.*, 1996; Lourens *et al.*, 2004; Parekh *et al.*, 2006; Rojas *et al.*, 2006; Addai *et al.*, 2013)

**Antifungal activity:** Crude extracts of *F. indica* were tested for their antifungal potential against five filamentous fungal strains. Disc diffusion method was employed and results revealed that none of the samples were active against the test strains and no significant zones of inhibition were seen. The standard drug used (clotrimazole) at concentration of 10  $\mu$ g/disc exhibited a zone of 28  $\pm$  1.42 mm. The results are in accordance with previously conducted antifungal analysis of *F. indica* (Khan *et al.*, 2013). The diverse nature of solvents in our study could not produce different results against the test strains which further affirm the findings of previous study. Other investigators reported antifungal potential of the under study plant against some fungal strains which were not part of our study (Gupta *et al.*, 2012a).

**Antileishmanial potential:** Crude extracts of *F. indica* were tested for their antileishmanial capability against *Leishmania tropica* kwh 23 promastigotes. The results revealed that the tested samples were not active against the parasite and no significant inhibition was reported with any of the sample. The positive control used was

amphotericin B and it exhibited an  $IC_{50}$  value of 0.01  $\mu\text{g/ml}$ . Other investigators have reported antileishmanial potential of compounds isolated from a different specie of the genus *Fumaria* (Jameel *et al.*, 2014). Leishmaniasis is a serious affliction specially in the developing countries and search of new, less toxic and effective antileishmanial agents is mandatory to cope with the compromised effectiveness, side effects and lack of any vaccines (Sadeghi-Nejad *et al.*, 2011).

### Cytotoxicity determination

**Brine Shrimp lethality assay:** The cytotoxicity profile of *F. indica* crude extracts was analyzed and verified using brine shrimp lethality assay, it has been considered as a useful probe for the biological activities possessed by plant extracts (Mayerhofer *et al.*, 1991). The samples showed remarkable cytotoxicity and among the 14 different extracts, nH proved to be the most potent with an  $LC_{50}$  value of 349.55  $\mu\text{g/ml}$ . It was followed by nH+Ea with  $LC_{50}$  value of 376.15  $\mu\text{g/ml}$ . M+Dw extract showed minimum toxicity with an  $LC_{50}$  value of 1000  $\mu\text{g/ml}$ . The cytotoxic potential of crude extracts exhibited the following order: nH > nH+Ea > M > Ac+M > Ch > Ac > nH+E > Ea > Ac+Dw > Ea+M > M+Ch > E > Dw > M+Dw (Table 4).

Shrimps larvae are suggested to behave similarly as mammalian carcinoma cells and the cytotoxic effects of tested samples might well illustrate their potential anticancer and antitumor activity (Ullah *et al.*, 2012). The results observed showed that the activity was concentration dependant and percent mortality decreased with a decrease in sample concentration which is in agreement with previous cytotoxic studies employing brine shrimp as test subject (Khan *et al.*, 2014). The  $LC_{50}$

of each sample was less than 1000  $\mu\text{g/ml}$  with the exception of M+Dw extract, it represents a prominent cytotoxic profile of a plant crude extract. The observed activity might be accredited to the presence of plant defense system in shape of secondary metabolites like phenols, flavonoids, alkaloids (Aziz *et al.*, 2013) and other constituents which are not detected in our phytochemical and HPLC analysis. The fractionation of crude extracts with least  $LC_{50}$  values might offer useful antitumor and/or cytotoxic principles and their further screening would be ideal for the validation of our pilot study results.

**Cytotoxicity against Hep G2 cell line:** The cytotoxicity profile of *F. indica* crude extracts was analyzed against Hep G2 human hepatoma cell line and the results were expressed as percent inhibition at 20  $\mu\text{g/ml}$  concentration. A few samples showed considerable antiproliferative activity with an inhibition range of  $85 \pm 1.21 - 6 \pm 0.32\%$ . Among the 14 different extracts, nH+Ea proved to be the most potent extract with  $85 \pm 1.21\%$  inhibition which was followed by Ea with  $48.85 \pm 0.9\%$ . The antiproliferative potential of crude extracts exhibited the following order: nH+Ea > Ea > M+Ch > Ac+Dw > M+Dw > M > Ch > Ac+M > nH+E > nH > E > Dw > Ac > Ea+M (Table 5).

Hepatoma is the most prevalent cancer in the world and it is amongst the two main forms of primary liver cancers which is preceded by events i.e. ROS generation and subsequent hepatocellular damage and chronic inflammation (Machana S *et al.*, 2012). Thus, crude extracts which have shown significant antiproliferative activity might offer a useful source of anticancer compounds and their screening and fractionation would be helpful for the validation of our observed results.

**Table 4. Brine shrimps lethality assay for the determination of cytotoxicity profile of crude extracts of *F.indica*.**

Extract Names	% Mortality (concentration: $\mu\text{g/ml}$ )			$LC_{50}$ $\mu\text{g/ml}$
	1000	500	250	
nH	100 $\pm$ 2.25	70 $\pm$ 4.12	30 $\pm$ 4.78	349.55
nH+Ea	100 $\pm$ 3.54	70 $\pm$ 0.98	20 $\pm$ 3.23	376.15
nH+E	100 $\pm$ 1.54	40 $\pm$ 0.34	20 $\pm$ 1.22	602.28
Ch	100 $\pm$ 0.87	50 $\pm$ 2.56	20 $\pm$ 1.78	499.24
Ea	90 $\pm$ 1.56	40 $\pm$ 3.17	30 $\pm$ 1.78	656.26
M+Ch	90 $\pm$ 4.23	20 $\pm$ 1.98	10 $\pm$ 1.34	810.02
Ea+M	100 $\pm$ 1.56	30 $\pm$ 0.87	20 $\pm$ 1.45	765.04
Ac+M	100 $\pm$ 0.87	60 $\pm$ 1.98	10 $\pm$ 1.23	419.01
M	90 $\pm$ 2.31	60 $\pm$ 1.78	20 $\pm$ 2.87	404.77
Ac	100 $\pm$ 1.87	50 $\pm$ 3.12	30 $\pm$ 3.23	499.50
E	80 $\pm$ 1.39	20 $\pm$ 1.98	10 $\pm$ 1.87	830.48
Ac+Dw	80 $\pm$ 2.21	30 $\pm$ 5.43	20 $\pm$ 1.22	762.80
M+Dw	50 $\pm$ 3.12	20 $\pm$ 3.89	10 $\pm$ 0.98	1000
Dw	50 $\pm$ 1.88	40 $\pm$ 4.34	20 $\pm$ 0.65	789.12

Values represented are the mean of triplicate  $\pm$  SD. DMSO was applied as negative control;  $LC_{50}$  of positive control (Doxorubicin) was 5.93  $\mu\text{g/ml}$ .

**Table 5. SRB assay for the determination of cytotoxicity profile of crude extracts of *F.indica* against Hep G2 cell line.**

Extracts	Hep G2 cytotoxicity (µg/ml)	
	% inhibition	IC <sub>50</sub>
nH	10 ± 0.19	> 20
nH+Ea	85 ± 1.21	8.45 ± 2.11
nH+E	11 ± 0.45	> 20
Ch	15 ± 1.63	> 20
Ea	48 ± 0.90	> 20
M+Ch	47 ± 1.54	> 20
Ea+M	6 ± 0.32	> 20
Ac+M	12 ± 2.01	> 20
M	23 ± 2.02	> 20
Ac	6 ± 1.05	> 20
E	9 ± 1.81	> 20
Ac+Dw	32 ± 2.42	> 20
M+Dw	31 ± 1.85	> 20
Dw	7 ± 1.76	> 20
Doxorubicin	98 ± 0.18	5.1
1% DMSO in PBS	--	--

Values are presented as mean ± standard deviation of triplicate analysis. --: no activity.

**Protein kinase inhibition assay:** The crude extracts of *F. indica* were screened for any protein kinase inhibition activity. Protein kinases play a key role in aerial hyphae formation and it acts as a principle behind the utility of this assay in the measurement of kinases inhibitory potential of test samples. The results are presented in Table 6. Among all the extracts, a noteworthy inhibition of hyphae formation i.e. 18 ± 0.23mm bald phenotype zone at 100 µg/disc was formed around the nH+E extract. Significant protein kinase inhibiting potential was also demonstrated by M extract presenting a bald zone of 15 ± 0.98 mm. The non-toxic effect of DMSO (negative control) was confirmed by the absence of growth inhibition zone around the disc whereas surfactin, the positive control showed 16 mm bald growth inhibition zone.

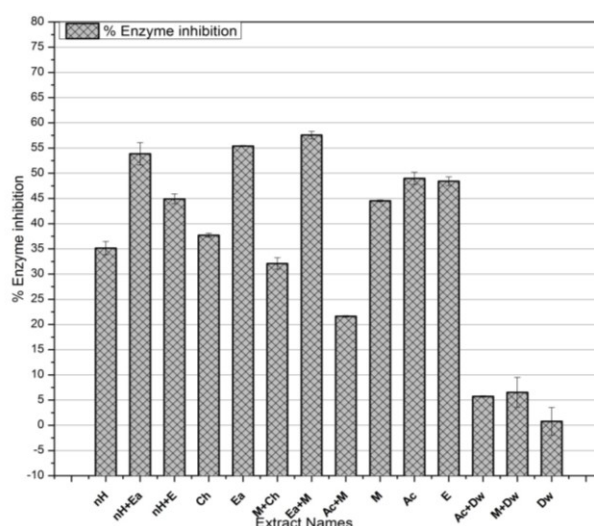
Protein kinases are important enzymes which are key regulators of numerous cellular processes and play a major role in cell growth and development as well as cell cycle progression and signals transduction across the nuclear membrane (Manning *et al.*, 2002; Pearson & Fabbro, 2004). Protein kinase inhibitors are considered as significant anticancer drugs as they represent a unique class of compounds specifically the ones which are responsible for inhibition of kinases which are oncogenic. The results indicated that crude extracts prepared in M and Ea solvents whether used alone or in combination has appreciable activity against the test strain. Moreover, nH and E extract showed excellent inhibitory activity. Our study indicate the polarity based variations in the extraction of

phytoconstituents which are potentially active protein kinase inhibitors and it is suggested that moderately polar solvents are better suitable in this regard, while the extremes of polarity i.e. nH and Dw extracts exhibited little or no activity. The solvent polarity based activity is supported by previous studies (Rodrik-Outmezguine *et al.*, 2011; Yang *et al.*, 2013). The activity shown by methanolic crude extracts might be due to the presence of multiple kinases inhibitory principle like quercetin (Boly *et al.*, 2011), as verified by HPLC-DAD analysis of the samples. Our results present valuable footprints for further evaluation and screening of the notified extracts for isolation and characterization of potential drug candidates possessing protein kinases inhibiting properties.

### Antidiabetic assay

**Alpha amylase inhibition assay:** The inhibition of key carbohydrate hydrolyzing enzymes i.e. alpha amylase and alpha glucosidase is an effective strategy to keep blood glucose level within permissible range (Nair *et al.*, 2013). Alpha amylase inhibition assay was carried out to screen the plant for any antidiabetic property. Our results revealed that maximum enzyme inhibition (57.5 ± 0.40%) was achieved with Ea+M extract. It was followed by Ea and nH+Ea extracts with 55.39 ± 0.74 and 53.86 ± 0.65% inhibition respectively. Activity was least in case of Dw extract with only 0.80 ± 0.05% inhibition. Results observed were in the following order: Ea+M > Ea > nH+Ea > Ac > E > nH+E > M > Ch > nH > M+Ch > Ac+M > M+Dw > Ac+Dw > Dw (Figure 5).

Extracts obtained with solvents of lesser polarity showed better enzyme inhibition potential as compared to highly polar solvents. Previous studies have established a positive correlation between the presence of phenolic and flavonoids and alpha amylase inhibition activity (Mai *et al.*, 2007; Ramkumar *et al.*, 2010). Further analysis and fractionation of the ethyl acetate extracts might offer useful natural compounds to be employed in management of diabetes.



**Fig. 5.** Alpha amylase inhibition assay of *F. indica* extracts. Experiment was performed in triplicate and values are presented as mean ± standard deviation.

Table 6. Protein kinase inhibition potential of various extracts of *F. indica*

Extract Names	Zone of growth inhibition in mm (Mean $\pm$ SD)* at 100 $\mu$ g/disc	
	Clear Zone	Bald Zone
nH		
nH+Ea	--	--
nH+E	--	10 $\pm$ 0.97
Ch	--	18 $\pm$ 0.23
Ea	--	12 $\pm$ 1.22
M+Ch	--	14 $\pm$ 0.34
Ea+M	--	--
Ac+M	--	14 $\pm$ 0.45
M	--	9 $\pm$ 1.23
Ac	--	15 $\pm$ 0.98
E	--	13 $\pm$ 1.24
Ac+Dw	--	10 $\pm$ 2.12
M+Dw	--	--
Dw	--	--

\*Values (mean  $\pm$  SD) are average of triplicate assays. DMSO: negative control, Surfactin: positive control (20  $\mu$ g/disc; 16 mm ZOI)

## Conclusion

Results of our detailed screening led us to the conclusion that extraction efficiency, biological efficacy and nature of pharmacological response depend upon the type of solvent employed and its polarity. The detection and quantification of important polyphenols in various crude extracts may well explain the reported actions and traditional uses of this plant. The current study reveals that the methanol + distilled water extract of *F. indica* might offer a potentially useful source of phytochemicals provoking significant antioxidant responses. The plant shows appreciable antibacterial properties against the tested Gram positive strains. Cytotoxicity assay confirms the least polar crude extract i.e. n-hexane to be highly proficient depicting a strong cytotoxic potential against brine shrimps, moreover, n-hexane in combination ethyl acetate showed remarkable cytotoxic activity against Hep G2 cell line while in combination with ethanol it is a significant tumorigenic kinase inhibitor. Ethyl acetate crude extracts showed appreciable alpha amylase inhibitory action. Our study affirmed the pharmacological significance of *F. indica* based on notable outcomes of a multitude of assays and highlighted the need of critical evaluation and expansion of our pilot study results

## Abbreviations

ATCC: American Type Culture Collection; DMSO: Dimethyl sulfoxide; DPPH: 2, 2-diphenyl-1-picrylhydrazyl; Dw: Distilled water; DW: Dry weight; FCBP: Fungal Culture Bank of Pakistan; IC<sub>50</sub>: 50 % inhibitory concentration; LC<sub>50</sub>: Lethal concentration causing 50 % mortality; TPC: Total phenolic content; TFC: Total flavonoid content; RSA: Radical scavenging activity; TRP: Total reducing power; TAC: Total antioxidant capacity; MIC: Minimum inhibitory concentration; ZOI: Zone of inhibition.

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