International Scholars Journals

International Journal of Medicinal Plants Research ISSN 2169-303X Vol. 6 (5), pp. 346-354, May, 2017. Available online at www.internationalscholarsjournals.org © International Scholars Journals

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Full Length Research Paper

Antidiabetic activity guided screening and characterization of *fagonia indica*

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Accepted 11 June, 2017

The present work was carried out to investigate the antidiabetic and antioxidant activities of *Fagonia Indica* through *In-vitro* assay. Extraction was done by methanolic solvent while fractionation was performed by n-hexane, chloroform, ethyl acetate and aqueous solvent on the basis of their polarity. Antidiabetic activity was performed by inhibition of α-amylase enzyme while antioxidant activity by DPPH scavenging free radical assay. Total phenolic and flavonoid contents were also studied via spectrophotometric assay by Follen Ciocalteu and Aluminum chloride reagents respectively. Highest antidiabetic activity (59.52±0.75%) was shown by aqueous fraction while lowest by n-hexane solvent (31.55±0.84%). Aqueous fraction showed highest antioxidant activity (74.70±1.20%) while n-hexane fraction exhibited lowest activity (43.16±0.99 %). Higher phenolic and flavonoids contents (81.2±1.2% and 67.4±2.23% respectively) were exhibited by aqueous fractions. Statistical analysis by one way ANOVA showed that all the activities performed significant results ($p<0.05$). Results of Antibacterial activity against gram positive and gram negative bacteria and cytotoxicity potential was also found significant. The major bioactive compounds such as trans 4-hydroxy-3-methoxy cinamic acid, chlorogenic acid, gallic acid, syringic acid, chromatotropic acid and vinillic acid were found in crude extract and various fractions by liquid chromatography.

Keywords: *Fagonia indica*, fractionations, antidiabetic activity, antioxidant activity, HPLC analysis.

INTRODUCTION

Fagonia indica is a flowering plant, belonging to genus of wild family *Ztgophyllales.* This family has almost 34 species, commonly found in different areas of Pakistan, India, Mid East, Saudi Arabia, Cyprus, Tunisia, Morocco, Egypt, Algeria, Africa and America (Ali *et al.*, 2008). *F.indica* has tough woody and cylindrically roots; procumbent, solid and woody stem and spiny, glabrous and glandular branches. The leaves are trifoliolate, imparipinnate compound, opposite and cauline. Height of this plant is around 30cm. Central part of leaf is about narrowly elliptic, glandular and glabrous, and is greater than the lateral part which is linearly narrowly elliptic. The petals (if present) are long in the range of 2-16mm and stipulate which are modified into slender thorns, sharp and may be rarely longer or shorter than peroile but always smaller than leaf (Beier, 2005). The flowers of *F. indica* are pedicillate, solitary, polypetalous and polysepalous. The color of petals are pinkish purple and

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there are two collateral ovules in each carpel. The fruits of *F. indica* are like a capsule. Various phytochemical such are vitamins, lignans, saponins, lignins, cyanogenic glycosides, phenolic compounds, tannins, alkaloids and flavonoids are generally founds in medicinal plants. The herbal medicine practitioners can gain scientific basic data from the knowledge of quantitative phytochemical analysis. The crude extract were found to contain total protein content, pectin, phenolic content, glycosides, flavonoids and alkaloids. Three important vitamins including ascorbic acid, niacin and riboflavin were also found through quantitative analysis (Hussain *et al*., 2011; Hossen et al., 2016). Shaukat *et al*. (1981) found total protein contents including glutamate, serine, aspartate, threonine and lysine. In Pakistan, *F. indica* is widely used for the treatment of various diseases including gynaecological problems (Iqbal *et al.,* 2011), gastric troubles (Badshah and Hussain 2011; Aslam et al., 2016), skin allergies (Qureshi *et al*., 2010; Liaqat et al., 2016), digestive disorders, piles (Marwat *et al*. 2008; Akhtar and Begum 2009), liver troubles, bronchitis, asthma, urinary discharges, scabies, Typhoid and fever (Ahmad, 2007). It is also used for the treatment of cancer.

MATERIALS AND METHODS

The analytical grade chemicals used for the present study including Phosphate buffer (pH 6.9)**,** DNSA (3,5-Dinitrosalicylic acid)**,** Starch**,** Alpha amylase, DPPH,Acrobase, Ascarbic acid, Methanol, N-hexane, Chloroform, Ethyl acetate, Hydrochloric acid, Folin-Ciocalteu reagent.

Collection of Plant Materials

The fresh sample of *F. indica* was collected from area of Bhagwal, District Chakwal-Pakistan. The plant sample was identified from the Department of Botany, University of Agriculture, Faisalabad-Pakistan and got voucher number 232-1-2017. The collected plant sample was washed with distilled water for the removal of dust particles and other impurities and was dried under shade. The dried sample was then subjected to grinding to get fine powder and stored in opaque screw-capped containers at room temperature.

Preparation of plant extracts

The plant sample in fine powder form (2.1 kg) of *F. indica* was macerated in methanol solvent for 15 days. After the maceration, the soluble fractions in methanol were filtered and the filtrate was concentrated at 40^0C by using rotatory evaporator to give crude extract (yield,173gm). The 50 gm crude extract was then dissolved in 150 mL distilled water and sequentially portioned with 150 mL of n-hexane, 150 mL of chloroform and 150 mL of ethyl acetate. The n-hexane, chloroform, ethyl acetate and aqueous solutions yielded 5, 6.8, 6.9 and 7.5gm of fractions respectively. All obtained methanolic crude extract and fractions were stored in refrigerator at 15°C (Hussain *et al*., 2010). Various parameter such as solvent used, method of extraction, amount and parts of the plant material and the nature of the plant material can affect the yield of extraction (Kumar *et al*., 2008).

Alpha amylase inhibition assay against different fractions of plants extracts

The 500µL of α-amylase solution was added to 500µL of plant extract and various fractions and incubated at 25˚C for 10 minutes, then dissolved 500µL of starch solution and incubated at the same conditions. 1 mL of Dinitric salicylic acid (DNSA) was added and the solution was kept in water bath for 5 minutes and cooled at 25C. The 20 mL water was added in solution. The absorbance was taken at 540 nm. Following formula is used for the calculation of percentage inhibition.

$I\ \% = \{(A_{blank}-A_{sample})/A_{blank}\} \times 100$

Where $A_{sample}$and A_{blank} indicated the absorbance of the tested sample and control reaction respectively (Ali *et al.,* 2006).

Determination of antioxidant activity by DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay

Antioxidant activity of extract and fractions of *F. indica* and the standard ascorbic acid were investigated by using 2, 2-Diphenyl-1-picryl hydrazyl free radical activity (Jain & Jain, 2011; Chikhi, *et al*., 2012). A weighed amount of the methanolic crude extract and various fractions was dissolved in methanol (HPLC grade) for the stock solution of 500 ppm. Different (500, 250, 100, 50, 40, 20, 10 1ppm) concentrations of solutions of extract and its various polar fractions were prepared in methanol. The methanol solvent was used for the preparation of DPPH (0.002%) solution. 2 mL of DPPH solution was dissolved in 2 ml of each concentration of sample as well as standard solution (ascorbic acid) separately. The obtained mixtures were incubated for 90 minutes. The measurement of absorbance was done at 517 nm. The methanol was used as blank. The calculation of percentage inhibition was done by using the following formula:

$I\ \% = \{(A_{blank}-A_{sample})/A_{blank}\} \times 100$

Where $A_{sample}$and A_{blank} indicated the absorbance of the tested sample and control reaction respectively.

Total Phenolics contents

Total phenolic contents were assayed according to Chaovanalikit and Wrolstad (2004). For this assay 1 mL of crude extract and various fractions of all different concentrations (1-500 ppm) were taken in separate test tubes and then added 7.5 mL distilled water followed by the addition of 0.5 mL Folin-Ciocalteu reagent and incubated the solution for 90 minutes. The absorbance was taken at 765 nm. The calibration curve for Gallic acid gram equivalent (1-500 ppm) was used for the estimation of amount of total phenolic contents. The gallic acid equivalents mg GAE/g unit was used for the expression of the obtained results.

Total flavonoids contents

1mL of extract solution (1mg/mL) or standard solution was added to 10mL volumetric flask followed by the addition of 4mL dd H_2O. 0.3mL of 5% $NaNO_2$ was added to flask. After 5min. 0.3mL of 10% $AlCl_3$ was added. At the sixth minute, 2mL of 1M NaOH was added and total volume was made 10mL of dd H_2O. Solution was mixed well and absorbance was measured at 415nm (Javid *et al.*, 2016).

RP- HPLC analysis for the determination of Phenolic compounds

The crude extract and various fractions with highest activities were analyzed through HPLC analysis for the determination of phenolic compounds. The sample for HPLC analysis was prepared by dissolving 50 mg of each concentration sample in 24 mL methanol and homogenized. Then 16 mL distilled water was added followed by 10 mL of 6M HCl. Then the homogenized mixture was kept in oven at 95°C for 2 hours. The filtration of final solution was performed by using 0.45 µm filter paper prior to HPLC (Dek *et al.*, 2011).

Liquid chromatoghraph (LC-10SHIMADZU, JAPAN) with 5 µm column and UV- visible detector (λ max 280 nm) was performed for such analysis. The chromatographic separation was carried out using as mobile phase gradient: A (H_2O: Acetic acid-94:6, pH = 2.27), B (acetonitrile 100%). The gradient used was 15% solvent B (0-15 min), 45% solvent B (15-30 min) and 100% solvent B (35-45 min) with 1 mL/min flow rate.

Antibacterial activity of crude extract and its polar fractions by disc diffusion method:

The strains *E. coli* and *S. aureous* were characterized from the Institute of Veterinary Microbiology, University of Agriculture Faisalabad. These pathogenic strains were used to determine the antimicrobial activity of the crude extract and its polar fractions of selected medicinal plant. Pure cultures were maintained on nutrient agar medium in the petri plates. For the inoculums preparation 13g/L of nutrient broth (Oxoid, UK) was suspended in distilled water, mixed well and distributed homogenously. The medium was autoclaved at 121˚C for 15 min. Loop full of pure culture of a bacterial strain was mixed in the medium and placed in shaker for 24 hours at 37˚C. The inoculums were stored at 4˚C. The inoculums with 1×10^8 CFU/mL were used for further analysis.

Nutrient agar (Oxoid) 28 g/L was suspended in distilled water, mixed well and distributed homogeneously. The medium was sterilized by autoclaving at 121˚C for 15 min. Before the medium was transferred to sterilized Petri plates; inoculation (100 µL/100 mL) was added to the medium and poured in sterilized petri plates. After this, small filter paper discs were laid flat on growth medium containing 100 µL of crude methanol extract and ethyl acetate and n-butanol fractions of crude methanol extract of selected plant.

The petri plates were then incubated at 37˚C for 24 hours, for the growth of bacteria. Crude methanol extract and ethyl acetate and n-butanol fractions of crude methanol extract of selected plant having antibacterial activity inhibited the bacterial growth and clear zones were formed. The zones of inhibition were measured in millimeters using zone reader (Afzal *et al.*, 2014).

Statistical Analysis

The data obtained by all the assayed from methanolic crude extract and it various fractions was performed in triplicate and represented in mean and standard error. One way Analysis of variance (ANOVA) technique was used for the statistical analysis by statistica 8.1.

RESULTS AND DISCUSSION

This research work was conducted to investigate the antidiabetic activity, total phenolic and flavonoids contents and scavenging capability by DPPH assay of whole plant of *F. indica.* The antidiabetic activity of various fractions at different concentrations was determined by inhibition of alpha amylase enzyme assay while antioxidant activity was determined by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay.

Alpha amylase inhibitory activity of *F. indica*

Methanolic crude extract and n-hexane, chloroform, ethylacetate and aqueous fractions of *F. indica* were investigated for their potential against the inhibition of α-

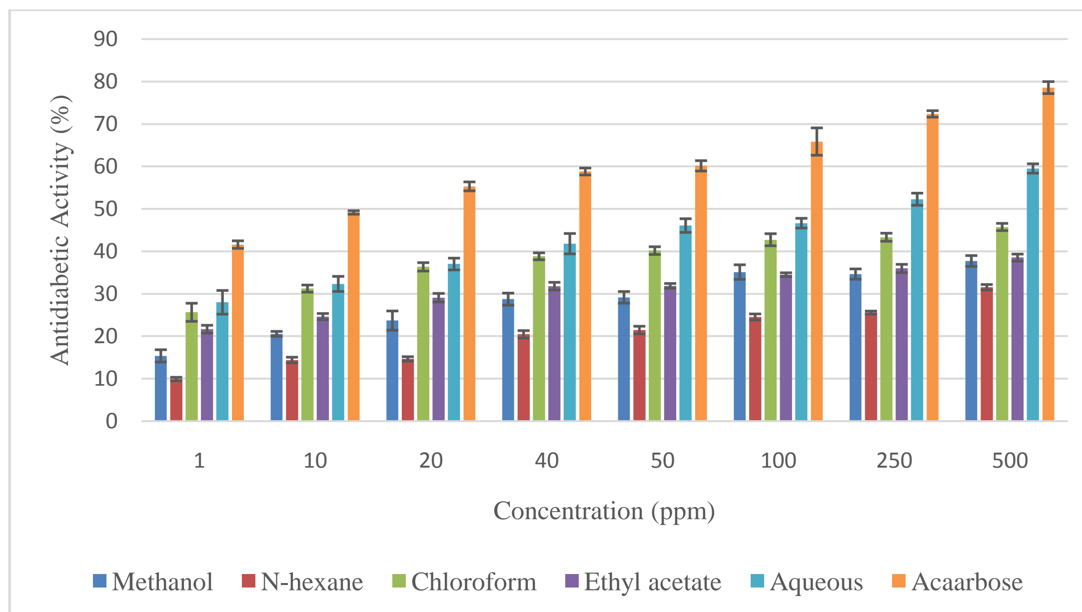

Fig. 1. Alpha amylase inhibitory activity of extract and its polar fractions of *F. indica.*

amylase enzymes. The acarbose was used as a standard for the comparison of the inhibitory activities shown by methanolic crude extract and various fractions at different concentration 1, 10, 20, 40, 50,100, 250 and 500 ppm separately tested for such activity (Fig. 1). Highest antidiabetic activity by inhibition of alpha amylase enzyme (59.52±0.75%) was shown by the aqueous fraction of *F. indica* at its higher concentration followed by chloroform fraction (45.75±0.45%) while n-hexane fraction showed lowest antidiabetic activity (31.55±0.84%). Methanolic crude extract and ethyl acetate fraction also showed significant inhibition of alpha amylase enzyme (37.73% ±0.38 and 38.54±0.94%) respectively at 500ppm concentration. Both these extract and fraction showed almost similar antidiabetic activity. Acarbose (dose dependent manner) was used as a standard for comparison of obtained results which revealed highest antidiabetic activity (78.57±0.48%) at 500 ppm, these results showed highest antidiabetic activity among all tested extract and fractions. These results exhibited decreased activity with decrease in the concentration. A linear trend occurs between the antidiabetic activity and concentration of extract and its various fractions. Statistical analysis also showed the significance of antidiabetic results as $p<0.05$. The α-amylase inhibition activity shown by methanolic extract and various fractions could be due to the presence of polyphenols, flavonoids and their glycosides, which would likely to offer more therapeutic methodologies for the treatment of postprandial hyperglycemia. Our results were comparable with Subramanian *et al.*, 2008 who investigated the inhibition of α-amylase enzyme of fractionated *P. virgatus* extracts which confirmed the highest inhibition of α-amylase shown by methanolic extract (Hashim *et al.*, 2013). These results revealed that more polar constituents of *P. virgatus* have more potential to inhibit the porcine pancreatic α-amylase.

Antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl assay

The antioxidant activity of methanolic crude extract and its subsequent various fractions of *F. indica* were studied by 2,2-diphenyl-1-picrylhydrazyl radicals which contain nitrogen free radicals. DPPH is stable at room temperature. DPPH method is used for investigation of scavenging activity of free radicals of natural product. DPPH has purple color which turns yellowish by receiving proton from natural product during chemical reaction (Sultana *et al.,* 2007; Jaz et al., 2016). It is used for the determination of antioxidant activity of natural products (Prabhune *et al.,* 2013). DPPH assay is more preferred due to its fair stability, sensitivity, feasibility and simplicity. The aqueous fraction showed the highest DPPH scavenging activity (74.70±1.20%) followed by the methanolic extract and ethyl acetate fraction which showed highest DPPH scavenging activity (64.18±0.98% and 58.45±1.15% respectively) at 500 concentration while lowest activity (43.16±0.99%) was shown by chloroform fraction. N-hexane fraction also showed significant antioxidant activity

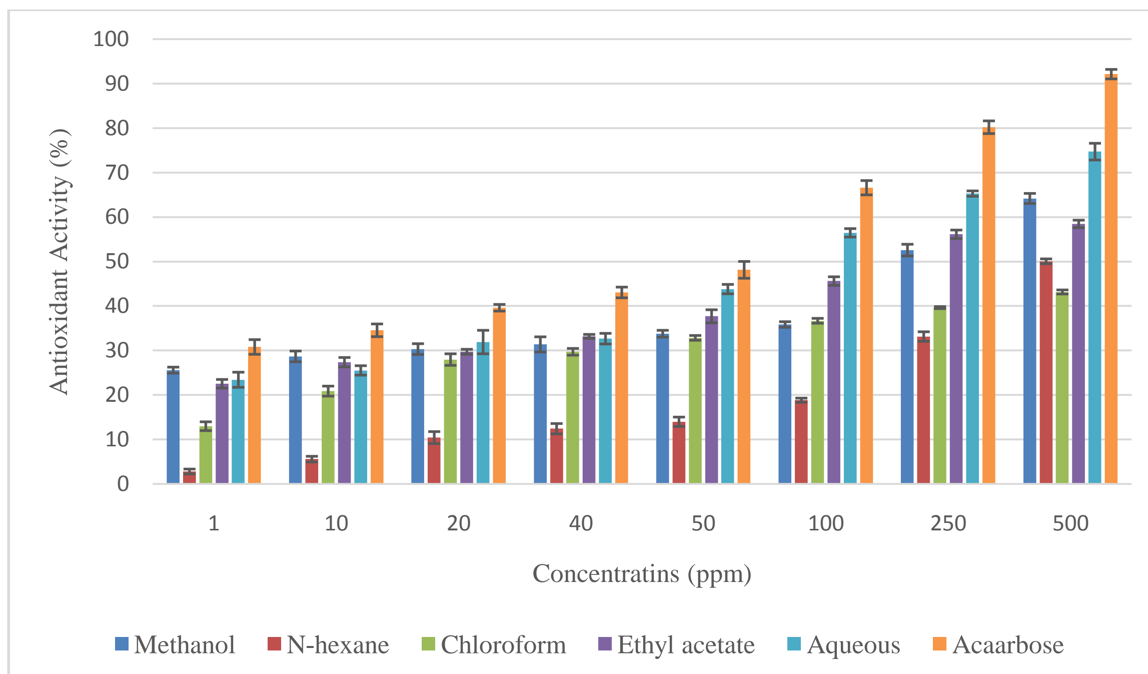

Fig. 2. Antioxidant activity of extract and its polar fractions of *F. indica.*

(50.06±0.45%) as shown in fig (2). Statistical analysis by one way analysis of variance also suggested the significance of results ($p<0.05$).

Total phenolic and Flavonoids contents by spectrophotometric assay

The spectrophotometric assay was used for the determination of total phenolic and flavonoids contents. The data of test was collected and statistical analysis was performed by one way analysis of variance at 5% significance level. The statistical analysis revealed that aqueous fraction contained highest phenolic contents (81.2 ±1.2mg/g equ G.A) as well as flavonoids contents (67.4±2.23 mg/g equ Q.E) followed by methanolic extract and chloroform fraction with 72.29±1.94 mg/g equ G.A, 62.01±1.97mg/g equ Q.E and 65.87±2.25 mg/g equ G.A and 54.03± 1.55mg/g equ Q.E phenolic and flavonoids contents respectively. While the lowest contents was found in n-hexane fraction having (32.74±0.93 mg/g equ G.A and 22.09±1.47mg/g equ Q.E). The ethyl acetate fraction also found significant amount of phenolic content (58.76±1.92 mg/g equ Q.E) as shown in fig (3). Satpute *et al.,* (2012) found TPC of methanolic extract of *Fagonia arabica* to be 47.4 ± 5.1 mg GAE/ g dry weight of plant. Eman *et al.,* (2012) reported very low amounts of flavonoids in *Fagonia indica* (0.78 mg QE/g of extract). This might be due to difference in the agro-climatic conditions of both areas under study.

RP-HPLC Analysis

The most suitable tool for chemical characterization of phenolic compounds from natural products is the HPLC analysis (Cimpan and Gocan, 2002). Different phenolic compounds are known for their biological activity such as gallic acid is reported for antibacterial and anti-oxidant (Noor *et al*., 2014) and caffeic acid with antibacterial, antifungal and anti-inflammatory activity (Kausar *et al.*, 2014). The presence of secondary metabolites in large amounts in several plants were reported to possess anti-inflammatory, antidiabetic, antioxidant and several others therapeutic activities.

The HPLC analysis of the crude extract and its polar fractions of *F. indica* were found to contain several types of the phenolic compounds such as Trans 4-hydroxy-3-methoxy cinamic acid, Vanillic acid, Gallic acid, p-coumeric acid, Caffeic acid, m-coumeric acid, Syringic acid, Ferulic acid, Sinapic acid, 4-hydroxy-3-methoxy benzoic acid and Chlorogenic acid in varying amount (Table. 1).

Vannilc acid (531.03±0.12 µg/g of dry plant material) and gallic acid (507.9±0.02 µg/g of dry plant material) were found major compounds in the methanolic extract of *F. indica* followed by cinamic acid (279.37±0.05 µg/g of dry plant material), chlorogenic acid (197.3±0.08 µg/g of dry

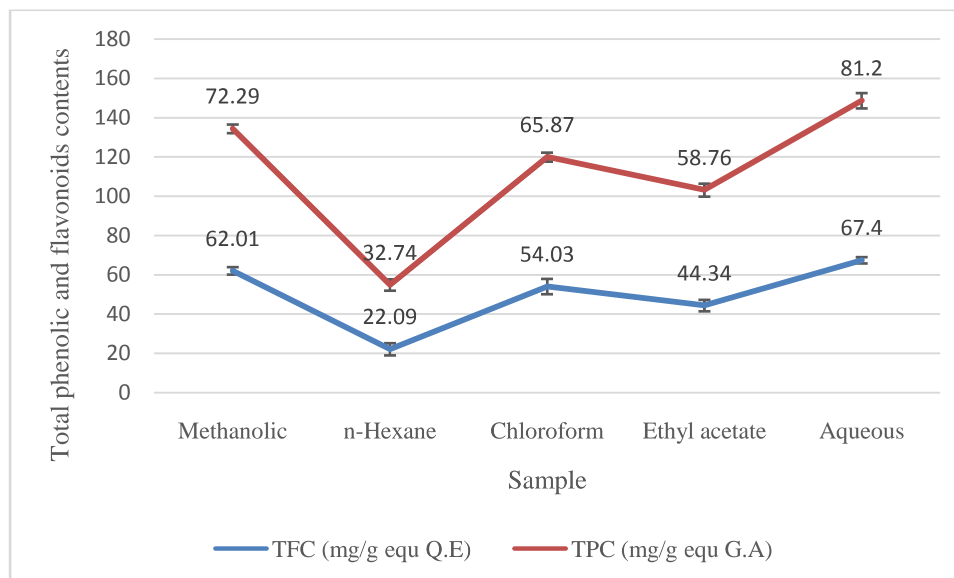

Fig.3. Total phenolic and Flavonoids contents of *F. indica* by spectrophotometric assay.

Table 1. Determination of phenolic compounds from *F. indica.*

Compounds	Methanol	n-Hexane	chloroform	Ethyl Acetate	Aqueous
Quercitin (Flavonoids)	145.6±0.04	4.71±0.04	8.12±0.09	1.07±0.05	7.12±0.08
Gallic acid	507.99±0.02	16.77±0.12	46.76±0.04	12.78±0.7	106.38±0.32
Chromatotropic acid	9.33±0.076	n.d	n.d	8.56±0.34	n.d
Syrinic acid	84.24±0.03	2.72±0.01	8.02±0.06	n.d	n.d
Caffeic acid	65.98±0.09	27.64±0.9	46.69±0.21	n.d	7.72±0.08
Benzoic acid	48.34±0.11	9.11±0.04	30.28±0.24	n.d	n.d
Ferulic acid	38.43±0.03	23.42±0.03	10.08±0.18	n.d	n.d
Cinamic acid	279.37±0.05	n.d	19.07±0.12	n.d	n.d
m-coumeric acid	5.65±0.14	n.d	n.d	n.d	3.42±0.02
Sinapic acid	12.43±0.1	n.d	9.06±0.09	5.59±0.5	3.87±0.05
Vitamin C	54.3±0.06	11.68±0.21	n.d	8.14±0.03	9.14±0.09
Chlorogenic acid	197.3±0.08	n.d	n.d	n.d	152.72±0.24
Vannilc acid	531.03±0.12	n.d	n.d	13.75±0.6	n.d

n.d= Not detected. Data are shown as Mean ± SD of triplicate determinations.

plant material), quercitine (145±0.04 µg/g of dry plant material), syrinic acid (84.24±0.03 µg/g of dry plant material). Some other phenolic compounds such as chromatotropic acid, caffeic acid, benzoic acid, ferulic acid, m-coumeric acid, sinapic acid and vitamin C were also identified in the methanolic extract in minor quantity. The n-hexane fraction of *F. indica* showed the presences of caffeic acid (27.64±0.9 µg/g of dry plant material), ferulic acid (23.42±0.03 µg/g of dry plant material), gallic acid (16.77±0.12 µg/g of dry plant material), vitamin C (11.68±0.21 µg/g of dry plant material), benzoic acid (9.11±0.04 µg/g of dry plant material), quercitin (4.71±0.04 µg/g of dry plant material) and syrinic acid (2.72±0.01 µg/g of dry plant material) as major phenolic compounds. Whereas gallic acid (46.76±0.04µg/g of dry plant material), caffeic acid (46.69±0.21µg/g of dry plant material), benzoic acid (30.28±0.24 µg/g of dry plant material), cinamic acid (19.07±0.12 µg/g of dry plant material), ferulic acid (10.08±0.18 µg/g of dry plant material), quercitin (8.12±0.09 µg/g of dry plant material), syrinic acid (8.02±0.06 µg/g of dry plant material), sinapic acid (9.06±0.09 µg/g of dry plant material) were found in the chloroform fraction. The ethyl acetate fraction was found to possess phenolic compounds including gallic

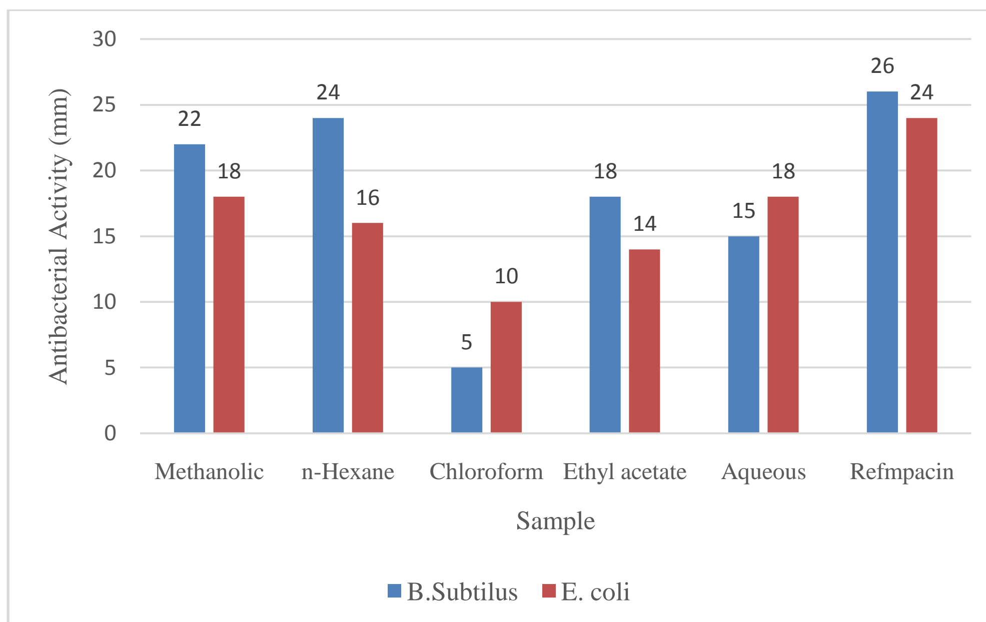

Fig. 4. Antibacterial activity of *F. indica*by well diffusion assay.

acid (12.78±0.70 µg/g of dry plant material), vannilc acid (12.78±0.60 µg/g of dry plant material), chromatotropic acid (8.56±0.34 µg/g of dry plant material), vitamin C (8.14±0.03 µg/g of dry plant material), sinapic acid (5.59±0.50 µg/g of dry plant material) and quercitin (1.07±0.05 µg/g of dry plant material). While seven phenolic compounds such as chlorogenic acid (152.72±0.24 µg/g of dry plant material), gallic acid (106.38±0.32 µg/g of dry plant material), vitamin C (9.14±0.09 µg/g of dry plant material), caffeic acid (7.72±0.08µg/g of dry plant material), quercitin(7.12±0.08 µg/g of dry plant material),sinapic acid (3.87±0.05 µg/g of dry plant material) and m-coumeric acid (3.42±0.02 µg/g of dry plant material) were identified in the aqueous fraction.

Antibacterial activity by well diffusion assay

The methanolic extracts and its polarities based various fractions *F. indica* were subjected to study their antibacterial potential against both gram positive and gram negative bacteria. *Bacilus subtillis* bacterial strain was used as gram positive while *Escherichia coli* was used as gram negative bacteria. All the plant extracts showed significant antibacterial activity as $p<0.05$ which was shown by fig (4). It was found that n-hexane fraction and methanolic crude extract showed highest antibacterial activity (24 mm and 22 mm) against *B. subtillis* followed by ethyl acetate extract of (18 mm). The lowest antibacterial activity was shown by aqueous and chloroform fraction (15 mm and 05mm). While methanolic extract and aqueous fraction possess same antibacterial activity (18mm) against *E. coli* which was found higher than all the other fractions, these results were followed by n-hexane (16mm). Ethyl acetate and chloroform fraction (16mm and 14mm) revealed the least inhibition potential of *E. coli*. The antibacterial potential of these plants extracts were compared with Rifmpacin which possess antibacterial activity 26 mm against *B. subtillis* and 24mm against *E. coli*.

CONCLUSION

Out of various fractions of *F. indica*, aqueous fraction showed the highest total phenolic contents 81.2±1.2mg GAE/g while the lowest 32.74±0.93 mg GAE/g were shown by the n-hexane fraction. All the fractions were found to have effective antidiabetic and antioxidant activities. The aqueous fraction showed highest antioxidant activity 74.70±1.20 %, the fractions also showed the significant antioxidant activity. All the fractions were found to possess lower antioxidative potential than that of the standard; ascorbic acid. The highest antidiabetic activity(59,52±0.75%) was shown by aqueous fraction followed by chloroform fraction (45.75±0.45%) whereas the lowest antidiabetic activity was depicted by n-hexane (31.55±0.84%) fraction. The effective antidiabetic potential might be attributed to the presence of higher phenolic compounds in various fractions and methanolic extract.

Acknowledgement

The authors thankfully acknowledge the cooperation extended by staff of Central Hi-Tech Laboratory, University of Agriculture, Faisalabad, Pakistan.

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