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ANTIMICROBIAL, PHYTOCHEMICAL AND ANTIOXIDANT POTENTIAL OF *LAMIACEAE* FAMILY PLANT: *L. ASPERA* (WILLD.) LINN.

Reena Antil, Lalita Singh, Dheeraj K Gahlawat and Pushpa Dahiya*

Department of Botany, M.D. University Rohtak-124001, India.

Abstract

Plant extracts of *L.aspera* prepared in six different solvents were analysed for antimicrobial activity using disc diffusion assay against six bacterial and four fungal strains. Higehst activity was observed in the methanol extract. Qualitative phytochemcial test were analyses for all extracts. The total phenolic and flavonoid content was determined quantitatively. Out of the six plant extracts, methanolic extract showed the highest amount of phenolic (124.62 ±.552 mg GAE/g) and flavonoid (98.23 ±0.41 mg QE/g) contents after quantitative observation and it positively correlated with antimicrobial potential of the extract. Radical scavenging potential was evaluated by using non-enymatic (DPPH, ABTS assay) and enzymatic (SOD,Catalase, GST) assays. All other extracts showed moderate antimicrobial, antioxidant and phytochemical activity. Besides, methanolic extract showed the highest amount of phenolic (124.62 ±.552 mg GAE/g) and flavonoid (98.23 ±0.41 mg QE/g) contents after quantitative observation. The methanolic extract also showed highest antioxidant activity with IC_{50} value (40.79µg/ml) against DPPH radical and IC_{50} value (46.1 µg/ml) against ABTS radicals. The FTIR and GC-MS analysis of methanolic extract exhibited main functional group and important phyto-compounds showed higher percentage of phenolic compound and nine other bioactive compounds by GC-MS analysis and depicted acceptable antibacterial, anti-oxidant as well as phytochemical activity possessed by *L. aspera*.

***Key words*:** *L. aspera*, Antioxidant, Antimicrobial, Phytochemicals, FTIR, GC-MS.

Introduction

Plants have extensive recognition throughout the world being the richest source of bioactive compounds. Due to multi-drug resistivity to synthetic drugs and their side effects, there has been an increased interest in plant based herbal medicines (Nair R. *et al*., 2006). A diverse range of phytochemicals and their promising biological activities raise the significance of medicinal plants and promoted for further research (Nascimento G.G. *et al*., 2000). Since ancient era, plants have shown traditional medicinal properties throughout the globe to treat many infectious diseases and to provide many remedies to humankind. Secondary metabolites such as phenols, alkaloids, terpenoids, flavonoids, glycosides and steroids present in medicinal plants has defensive mechanism and increase survivability (Acamovic *et al*., 2005, Edwards N.J. *et al*., 2000). The radical scavenging activity plays a potent role in medicinal properties of plants (Ahmad *et al*., 2008, Krinsky N.I. *et al*., 2001, Giardino I. *et al*., 1998). As a result, there is upsurge of interest in therapeutic potential of plants for their use to treat various infectious diseases (Cowan M.M. *et al*., 1999). The antioxidants and antimicrobial properties of various plants have been explored (Mahboubi *et al*., 2015, Edeoga *et al*., 2005, Greathead *et al*., 2003, Parekh *et al*., 2007, Akinmoladun *et al*., 2007).

Leucas aspera (commonly known as 'Thumbai') of Lamiaceae family is an aromatic herb distributed throughout Asian and African countries. It is found in India from the Himalayas range to Ceylon region**.** It grows as competitive weed in crop fields, homesteads, fallow lands and along roadsides. Traditionally, it has been used to cure abdominal pain, cough, and cold, skin infection. The paste of leaves of *L.aspera* are applied on snake bite, psoriasis, used in respiratory disease, gastrointestinal disorders, heart disorder, and jaundice (Dutta *et al*., 1998, Nawaz *et al*., 2009, Sadhu *et al*., 2003). Flowers of *L.*

***Author for correspondence :** E-mail: pushpa.dahiya@hotmail.com

aspera are used to reduce fever (Kannappa Reddy M. *et al*., 1993).

Keeping in view the medicinal importance, *L. aspera was* selected to evaluate the biological properties of whole plant and preparation of extracts in different solvents such as *i.e*. methanol, ethanol, aqueous, acetone and chloroform and petroleum ether. Phyto-constituents of *Leucas aspera* was also determined by using Gas Chromatography and Mass spectrum.

Materials and Methods

Preparation of plant extract

Aerial parts of *L. aspera* were collected from Samar Gobalpur village near Rohtak, Haryana, India. The collected plant material was washed and shade air dried for 7-10 days. The dried material was then grinned into fine powder. Extracts of *L.aspera* were prepared in six different solvent *viz*. methanol, ethanol, aqueous, acetone, chloroform and petroleum ether. 50grams of grinned powder was taken in Soxhlet extractor with 250ml of solvent and after 25-30 cycles, the extract was prepared by evaporating the extracting material using rotary evaporator. Dried extracts were stored at 4°C for further use.

Phytochemical analysis

Phytochemical analysis of extracts of *L.aspera* was performed for the detection of alkaloids, steroids, glycosides, terpenoids, saponins, flavonoids and phenols. The methodology used for the qualitative analysis of different phytoconstituents is given below:

Alkaloids: 1ml of extract was stirred with 2 ml of 1% HCL and heated gently for few minutes after it, 2-3 drops of Mayer's reagent (Harbone J.B. *et al*., 1998) and Wagner's reagent (Nair R. *et al*., 2006) were added. Turbidity of precipitate appeared indicating the presence of alkaloids.

Steroids: 2ml of conc. H_2SO_4 and 2ml of methanol were added in all different extracts with volume of 1ml. The appearance of layer of red color in the lower side of test tube indicated the presence steroid (Khandelwal K.R. *et al*., 2008).

Glycosides: For glycosides two tests were performed:

1. *Salkowski test*: 2ml of chloroform was added in 1 ml of plant extract and few drops of conc. H_2SO_4 were added from the side of test tube. A reddish brown colored ring on the test tube indicated the presence of glycosides (Evans W.C. *et al*., 1997).

2. Kellar killani test: Glacial acetic acid (2ml) was added along with 1-2 drops of 2% $FeCl_3$ solution were added in the 1ml of all different extracts. The mixture of solution was poured into test tube contained 2 ml of conc. H_2SO_4. Brown color ring appeared at interface showed the presence of glycosides (Onwukaeme *et al*., 2007).

Saponins: In a different test tubes, 5ml of ds H_2O were mixed with, 1ml of extracts and shaken vigoursly. A stable soap like foam indicated the presence of Saponins (Parekh and Chanda *et al*., 2007).

Terpenoids: 2 ml of methanol was mixed with 1ml of extracts and evaporated to dry. After few minutes, in the test tube 3 ml of conc. H_2SO_4 added. A reddish brown color appeared at interface (Edeoga *et al*., 2005).

Flavonoids: By alkaline reagent test, 2-3ml of dil. Sodium hydroxide was added to 1ml of extract. A yellow color appeared followed by addition of 3-4 ml dilute HCl, turn yellow color to colorless showed the presence of Flavonoids (Onwukaeme *et al*., 2007).

Phenols: Ferric Chloride test: 2-3 drops of $FeCl_3$ was added directly in plant extracts. Appearance of violet-blue color indicated the presence of phenols (Kumar *et al*., 2007).

$$\text{Total Phenolic content} = C \times \frac{V}{m} \quad (1)$$

Total phenolic content was carried out by Folin-Ciocalteu method as followed by Aiyegroro and Okoh, 2010. In the alkaline medium, phenol was reacted with phosphomolybdic acid of Folin-Ciocalteau reagent that produced a molybdenum blue-coloured complex that was spectrophotometrically estimated at 650 nm. 1ml of plant extract was added in 5ml of 10% v/v Folin-Ciocalteu and 2ml of Na_2CO_3. The mixture was vortexed for 2minutes and incubated for 15 minutes in dark condition. Blank solution consisted of 5ml Folin-Ciocalteu, 1ml of solvent and 2ml of Na_2CO_3 was added. Gallic acid was used as standard of 10ug/ml-100ug/ml range from stock of 1mg/ml. By using calibration curve, the unknown concentration of samples was obtained and results were expressed in terms of milligrams of Gallic acid equivalent (mg of GAE) gm of dry weight. All tests were performed in triplicates. Total phenolic content was calculated as per formula given below equation 1:

Total phenolic content = $C \times (V/m)$

Where *'V'* is the volume of extract in ml, *'C'* is the concentration (mg/ml) and *'m'* is the weight (g) of plant extract.

$$\text{Total flavonoid content} = C \times \frac{V}{m} \quad (2)$$

Flavonoid content was estimated by using aluminium chloride method spectrophotometrically with some modifications (Aiyegroro O.A. *et al*., 2010). From stock solution (1mg/ml) of plant extract, 1ml of extract was added in 3ml of methanol with 0.2ml of (10% w/v) aluminum chloride, 0.2ml of 1M sodium acetate and the total volume was raised to 10ml by using distilled water. Samples were allowed to stand for 30 minutes at room temperature. The absorbance was measured at 420nm. Quercetin was used as standard with different concentration (10ug/ml-100ug/ml). All the measurement was carried out in triplicates. The unknown concentrations were determined by calibration curve and results were expressed as Quercetin equivalent (mg/g of extracted compound). Total flavonoid content was calculated as below in equation 2:

Total flavonoid content = $C \times (V/m)$

Where *'V'* is the volume of extract in ml, *'C'* is the concentration (mg/ml) and *'m'* is the weight (g) of plant extract.

Antioxident Aasay

(DPPH Assay)

The antioxidant activity of different plant extracts was analysed by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) assay **(**Mensor *et al*., 2001). To 2.5ml of different concentrations (10µg/ml to 100µg/ml) of plant extract, 1ml of 0.3mM DPPH solution was added and incubated in dark for 30 minutes. Ascorbic acid used as standard and DPPH solution without plant extract was used as control. The decrease in absorbance of sample from 10µg/ml to 100µg/ml was measured at 517nm by using UV-Vis spectrophotometer. All the measurements were carried out in triplicates. The absorbance was converted into percentage antioxidant activity by using following formula: Eq. 03:

$$Free radical scavenging activity(\%) = \frac{Abs(control) - Abs(sample)}{Abs(control)} \times 100 \quad (3)$$

ABTS assay

ABTS **(**2, 2'-azino-bis, 3-ethylbenzothiazoline-6-sulphonic acid**)** assay was done as per Shirwaikar *et al*., 2006. 7mM ABTS solution was mixed with 2.45 mM ammonium per sulphate. Before use the mixture was allowed to stand at room temperature in dark condition for 12-16 h. ABTS solution was diluted with ethanol till to get 0.700 optical density and the absorbance was measured at 745nm. A stock solution of plant extract was prepared to 1mg/ml in their respective solvent. Each stock solution diluted from 10-100 µg/ml. One ml solution of ABTS was added to 1ml of sample solution of different concentration. Ascorbic acid was used as standard with same concentration (10-100µg/ml). Antioxidant activity was calculated with the help of following equation 4:

$$Free radical scavenging activity(\%) = \frac{Abs(control) - Abs(sample)}{Abs(control)} \times 100 \quad (4)$$

IC_{50} value

IC_{50} value (mg/ml) was determined by plotting graph of concentration different plant extracts against percentage inhibition which is defined as amount of plant extract having antioxidants quench 50% free radicals of DPPH.

Extract for enzymatic Assays: From the freshly collected plant sample (0.5gm), the plant extract was prepared by homogenization in 5ml of cold extraction buffer containing 100mM potassium phosphate buffer (pH 7.0) and 0.1mM sodium ethylene diamine tetraacetic acid (EDTA). The homogenized extract was centrifuged at 14000 rpm for 20 minute at 4°C and supernatant was directly used for all the enzymatic assays.

Catalase assay: Catalase activity was estimated by the method of Aebi *et al.*, 1984. Total 3ml reaction mixture contained 100µL of plant extract, 10 mM H_2O_2 and 50 mM (pH-7.0) potassium phosphate buffer. Absorbance was measured at 240 nm. In every 30 sec, for 5minutes there is decrease in absorbance. Activity of plant extract enzyme was expressed as µmoles of H_2O_2 decomposed mg^{-1} fresh weight min^{-1}. One unit of catalase will decompose 1.0 µmole of H_2O_2 per minute at pH 7.0 at 25°C under the assay condition (EC of H_2O_2 is 39.4 mM^{-1} cm^{-1}).

SOD assay

Superoxide dismutase (SOD) activity was determined by Misra *et al*., 1972. 3ml reaction mixture contained 100µl plant extract, 75 mM NBT, 50mM potassium phosphate buffer, 13 mM methionine, 2µM riboflavin and 0.1 mM EDTA. After shaking of reaction mixture, the tubes were placed in the light intensity of 5000 lux for 25 minutes. The absorbance was measured at 560 nm. Reaction mixture placed in dark did not produce any colour, served as the control. One unit activity was measured by amount of the enzyme required to causes 50% inhibition of the reduction of NBT per unit time. That was measured at 560 nm.

GST assay

Glutathione S-Transferase activity was estimated by

Habig *et al*., (1974). 3 ml reaction mixture of 97mM potassium phosphate buffer (pH-6.5), 1mM EDTA (in DW), 30mM CDNB (prepared in 95% ethanol) from 4°C and GSH 75 mM (in Buffer) from -20°C freezer were taken. Absorbance was measured at 340 nm for every 30 sec. upto 5 minutes**.** One unit of activity was calculated by the amount of enzyme which produces 1.0 µmol of GS-DNB conjugate/min under the conditions of the assay. **(**EC of GS-DNB conjugate at 340 nm is *9.6 mM*-1cm-1).

In vitro* antimicrobial potential of *L.aspera

Test organisms:

The antimicrobial activities of extracts of *L.aspera* were assessed against three gram negative: *Klebsiella pneumonia* (MTCC109), *Pseudomonas aeruginosa* (MTCC2453) and gram positive: *Staphylococcus aureus* (MTCC96), *Bacillus subtilis* (MTCC2057) and *Mycobacterium smegmatis* (MTCC992), *Chromobacterium violaceum* (MTCC4322). The bacterial strains were procured from IMTech, Chandigarh, India. The bacterial strains were cultured in nutrient broth and incubated at 37°C overnight. Suspension of the bacterial strains was further diluted with sterile peptone water and checked the turbidity according to Mcfarland standard until 10^8 CFU/mL (turbidity = McFarland barium sulfate standard 0.5) and the absorbance was obtained at 600nm. Potential of the plant extracts also assessed against four fungal strains namely *Aspergillus niger* (MTCC514), *Fusarium oxysporum* (MTCC7392), *Rhizopus oryzae* (MTCC-262) and *Penicillium expansum* (MTCC2818).

DISC diffusion assay: The antimicrobial potential of plant extract was determined by Disc diffusion assay (CLSI, 2015). The sterile disc of 6mm diameter of what man paper 1 were soaked with 15µl of plant extracts having various concentrations (100mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml). The discs of different concentration were placed on agar plates, dried and incubated at 37°C overnight for 18 hours. Ampicillin (100µg/ml) (Hi-Media Laboratories Pvt. Ltd. India) was taken as positive control and the different solvents (used for preparation of plants extract) were expressed as negative control. After incubation, evaluation was done by measurement of the diameter of the inhibited zone (IZ) in mm using a plastic ruler. All measurements were repeated thrice and the mean was taken by statistical analysis and standard deviation in MS Excel program.

Antifungal activity was also determined by disc diffusion assay (Bauer *et al*.,1966, Sarkar *et al*., 2007) as described above. The fungal culture was grown on Czapex dox broth (Hi-media) for *A. niger* and potato dextrose broth (Himedia) for *F. oxysporum, R.oryzae* and *P. expansum*. Inoculums were applied on the surface of the Czapek's dox agar and Potato Dextrose agar plates and spread out. The sterile disc (6mm diameter) was soaked with different concentration of extracts (200ug/ml, 100ug/ml and 50ug/ml) and incubated for 48 hrs at 28°C.Miconazol (Hi-Media Laboratories Pvt. Ltd. India) antibiotic was used as positive control and different solvents as negative control.

Determination of MIC: The MIC of *L.aspera* was determined by using micro dilution assay (Sarkaer *et al*., 2007). MIC of different plant extracts was evaluated against six bacterial strains in 96 well microtiter plates with addition of 100 µL of nutrient broth in each well. 100 µl plant extract were serially diluted in each well and 10 µl of test organism suspension was added to each well containing 10 µl of rezazurin dye. MIC plates were incubated at 37°C for 24hrs. The lowest extract concentration that inhibited the test organism was recorded as MIC. For antifungal activity, Czapek's dox broth was used for *A. niger* and potato dextrose broth was used for *F. Oxysporum, P. expansum* and *R. oryzae.* The incubation of plates at 28°C for 48 hrs. and the inhibitions of fungal strains were directly visualized without any coloring agent.

FTIR Analysis

Fourier Transform Infrared Spectrophotometer (BRUKER, ALFA MODAL, ABS /Transmitance) absorb light in infra-red region with wavelength of 4000-400 cm^{-1} range and 4 cm^{-1} of resolution. FTIR was used to find out the functional groups of compounds present in the methanolic extract. The methanolic extract of plant was directly loaded in FTIR spectrometer.

GC-MS Analysis

Extract of *L.aspera* in methanol was exposed for phytochemical analysis using GCMS analyzer (BRUKER SCION 436-GC SQ). Extracts were dissolved in methanol (HPLC grade, HiMedia Laboratories Pvt.Ltd.India) and filtered by Whatman TMFILTER DEVICE (0.2 µm). 99.99% of helium gas was used as carrier, with flow rate of 1 ml/min. RESTEK Rtx®-5 [Crossbond® with 5% , biphenyl and dimethicone (l/95%)] with 30metre length, 0.25 µmdf and 0.25 mm ID column was used for separation of phytochemicals with injected temperature 280°C. 2 µl of sample was injected in the column. Initial temperature of oven is 70°C that hold for 2 min. and slowly raised the temperature with 7°C rate per minute up to 320°C and Ion source

temperature was sustained at 250°C. Spectrum was obtained at 70eV by electron ionization and the detection of compound by detector conduct in mode of scan (30–500 Da atomic units). 38.71 min including 3 min solvent delay was total running time. The phytoconstituents identification was verified based on the relative retention time.

Statistical analysis

All the experiments were carried out in triplicates and expressed as average ± standard deviation. Correlation between antioxidant potential (IC_{50} value), phenols and flavonoids of all the extract of plant was find out with the help of SPSS software (version 16.0).

Results

Plant extracts of *L.aspera* prepared in six different solvents were evaluated for their biological activity. The results obtained are given below:

Phytochemical Analysis

Phytochemical analysis of extracts of *L.aspera* was carried out as per standard methods and results obtained are presented in table 1. Out of the seven phytocompounds, flavonoids and phenols were present in all the extracts, whereas glycosides were also detected in all extracts except chloroform extract. Alkaloids were detected in extracts prepared in methanol, ethanol, aqueous and acetone whereas steroids were present in methanolic, ethanolic and aqueous extracts. Saponins were present only in acetone and chloroform extract and terpenoids were detected in methanol, chloroform and petroleum ether extract.

Total phenolic and flavonoid content

When the plant extracts were analysed for the total phenolic and flavonoid content, it was found that there is a significantly ($p<0.05$) difference in their amount different extracts (Fig. 1 a, b, c and d). Methanolic extract showed highest phenolic content (124.62 ±.552 mg GAE/g) followed by ethanolic (105.2 ± 0.232 mg GAE/g), acetonic (97.33±1.15 mg GAE/g), aqueous (82.7 ±0.467 mg GAE/g) and chloroform (72.3± 0.57 mg GAE/g). However the lowest was depicted in petroleum ether (64.32 ± 1 mg GAE/g). Similarly Maximum TFC (98.23 ±0.41 mg QE/g) was observed in methanolic extract followed by ethanolic (76.63 ± 0.35 mg QE/g), aqueous (68.36 ± 0.56 mg QE/g), acetonic extract (55.74 ± 0.61 mg QE/g) and chloroform (42.89±0.33 mg QE/g). The lowest value was calculated for petroleum ether extract (37.5 ±0.45 mg QE/g).

Radical Scavenging Activity

The antioxidant properties of *L.aspera* were evaluated by DPPH and ABTS assay. The antioxidant activity of plant extract of *L.aspera* was determined with the range of different concentration between 10µg/ml to 100 µg/ml and that were depicted as percentage inhibition calculated with unknown concentration represented in fig. 2a.

When all extracts of *L.aspera* were compared out of six extracts for DPPH assay, the percentage inhibition of *L.aspera* was range between 4.4% (10µg/ml) to 93.29% (100µg/ml). Petroleum ether extract exhibited least percentage scavenging activity. The percent inhibition of methanol extract was higher that range between 14.14% (10µg/ml) to 90.21% (100µg/ml) where as lowest inhibition was observed in the petroleum ether extract that range between 8.31% (10µg/ml) to 93.29 % (100µg/ml). IC_{50} is the concentration of a substance needed to reduce the rate of any chemical process by 50%. IC_{50} value was calculated from percentage scavenging activity of all the extracts and results obtained are represented in fig. 2b. IC_{50} value of the plant extracts ranged between 40.75 µg/ml to 83.52 µg/ml. The highest antioxidant activity was shown by methanolic extract as it showed least IC_{50} value (40.75µg/ml) followed by ethanolic extract (51.27µg/ml), acetonic extract (64.92µg/ml) and aqueous extract (68.07µg/ml), chloroform extract (75.59µg/ml) and highest IC_{50} value (83.52µg/ml) was observed in petroleum ether extract.

Antioxidant activity of all extracts of *L.aspera* was demonstrating by using ABTS assay fig. 2c. The plant extracts have strong ability to decolorize the radical of ABTS. All the extracts were compared with standard

Table 1: Phytochemical analysis of all extracts of *L.aspera*

Solvent	Steroid	Terpenoid	Alkaloids	Saponins	Glycosides	Phenols	Flavonoids
Methanol	+	+	+	-	+	+	+
Ethanol	+	-	+	-	+	+	+
Aqueous	+	-	+	-	+	+	+
Acetone	-	-	+	+	+	+	+
Chloroform	-	+	-	+	-	+	+
Petroleum ether	-	+	-	-	+	+	+

(+) indicates presence and (-) indicates absence of compound

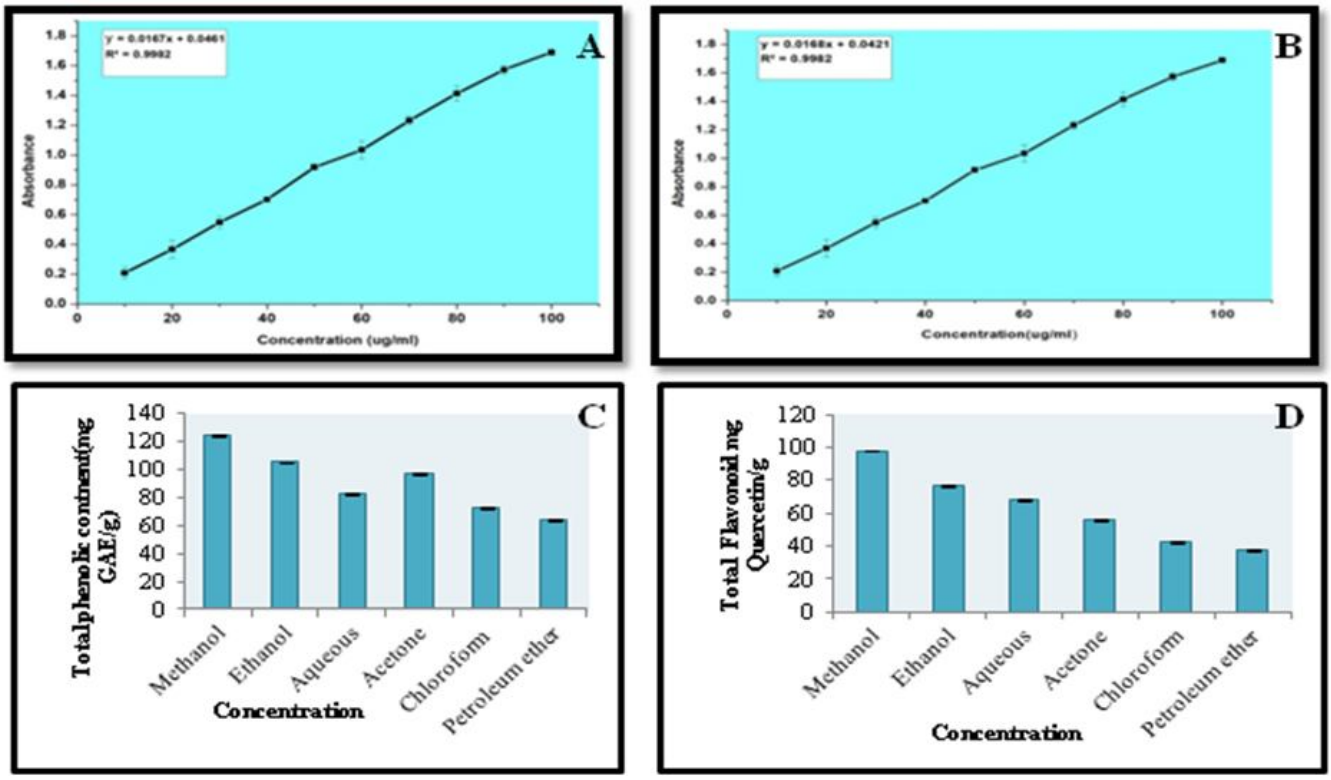

Fig. 1: (a) Phenolic content of standard gallic acid. (b) Flavonoid content of standard quercetin. (c) Total phenolic content of all extracts in different solvents. (d) Total flavonoid content of all extracts in different solvents.

i.e. ascorbic acid and range between 6.62(10µg/ml) to 94.45% (100µg/ml). Radical scavenging activity of methanolic extract showed high inhibition with range between 15.26% to 94.45% followed by ethanolic, acetonic, aqueous and chloroform. Lowest inhibition was observed in petroleum ether extract range between 9.33 % (10µg/ml) to 66.32% (100µg/ml). IC_{50} value was calculated for all extract of *L.aspera* and represented in the fig. 2d. Methanolic extract showed the least value of IC_{50} (46.1µg/ml), followed by ethanolic extract (53.31 µg/ml), the acetonic extract (60.86µg/ml) and aqueous extract (63.71µg/ml). Chloroform extract (72.05µg/ml) and Petroleum ether extract (78.67µg/ml) showed low antioxidant activity.

Table 2: enzyme activities of *L.aspera*

Enzyme	Activity
Catalase	9.09±0.021
Glutathione-S- Transferase	17.33±0.40
Superoxide dismutase	25.5±0.44

Values are expressed as mean (n=3) ±SD
Units of enzyme activities are expressed as:
CAT- One unit of catalase will decompose 1.0 µmole of H_2O_2 per minute at pH 7.0 at 25°C under the assay condition.
SOD- One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT per unit time as monitored at 560 nm.
GST-One unit of GST activity is the amount of enzyme which produces 1.0 µmol of GS-DNB conjugate/min under the conditions of the assay.

Enzymatic assay

The anti-oxidative potential also evaluated by using enzymatic assay. The results obtained with three different enzymes are given in table 2.

Activity of enzyme was found to be 9.09±0.021µmole of H_2O_2 per minute FW for Catalase, 17.33±0.40µmol of GS-DNB conjugate/min FW for Glutathione-S-Transferase and 25.5±0.44 SOD (Unit) FW for Superoxide dismutase.

Correlation between IC_{50} value, total phenolic and total flavonoid content

The antioxidant potential of plant extract was represented by DPPH and ABTS assay. These two assays were correlated with phenolic and flavonoid contents. Correlation coefficient tests were also

Table 3: Correlation between IC_{50} value of DPPH and ABTS was depicted with phenolic and flavonoid content.

Plant	*Leucas aspera*
TPC & IC_{50} (ABTS)	-0.860
TFC & IC_{50} (ABTS)	-0.718
TPC & IC_{50} (DPPH)	-0.900
TFC & IC_{50} (DPPH)	-0.892
TPC & TFC	0.926

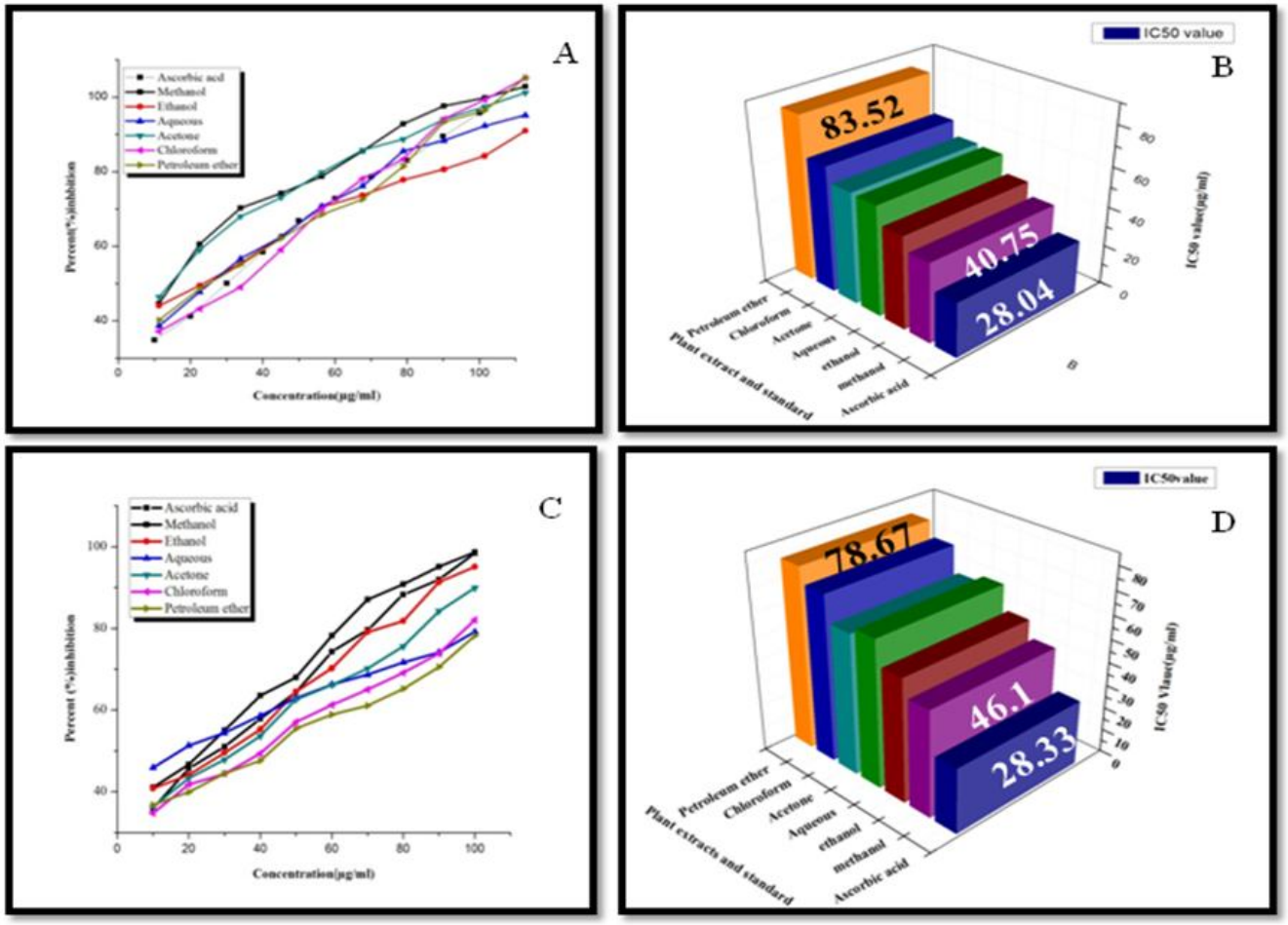

Fig. 2: (a) Represent the % Inhibition of DPPH radical. (b) IC_{50} value of DPPH. (c) Percent Inhibition of ABTS radicals. (d) IC_{50} value of ABTS.

accomplished between total phenolic content and total flavonoid content of plant extracts. The results obtained are given below in table 3. Total phenolic and flavonoid content and IC_{50} value of different plant extracts, all are negatively correlated. Negative correlation signified that all plant extract contained high phenolic and flavonoid content that exhibited relatively lower IC_{50} value. Lower IC_{50} value represents high antioxidant potential. The two variables TPC and TFC are positively correlated to each other for all the plant extracts. The correlation (linear regression coefficients, R^2) between TFC and IC_{50} (antioxidant activity) was found to be -0.718 (ABTS) and -0.892(DPPH) at significant level of 0.015. The correlation between TPC and IC_{50} was -0.860(ABTS) and -0.900(DPPH) and the significant level were 0.046.

Antibacterial activity of *L.aspera*

Activity of six extracts of *L.aspera* was assessed

Table 4: MIC values (mg/ml) of plant extracts of *L.aspera* against different bacterial strains, (Unit: mg/ml).

Plant extract	*Klebsiella pneumonia*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Micobacterium smegmatis*	*Bacillus subtilis*	*Chromobacterium violaceum*
			Leucas aspera			
Methanol	1.56	**0.19**	**0.19**	**0.19**	**0.19**	**0.39**
Ethanol	3.125	12.5	0.19	3.125	0.19	0.19
Aqueous	25	25	12.5	25	25	12.5
Acetone	6.25	12.5	0.78	3.125	1.56	6.25
Chloroform	6.25	12.5	3.125	1.56	6.25	3.125
Petroleum ether	6.25	6.25	12.5	3.125	6.25	3.125
Ampicillin	**0.0195**	NA	**0.0195**	0.039	0.0156	0.078
DMSO	NA	NA	NA	NA	NA	NA

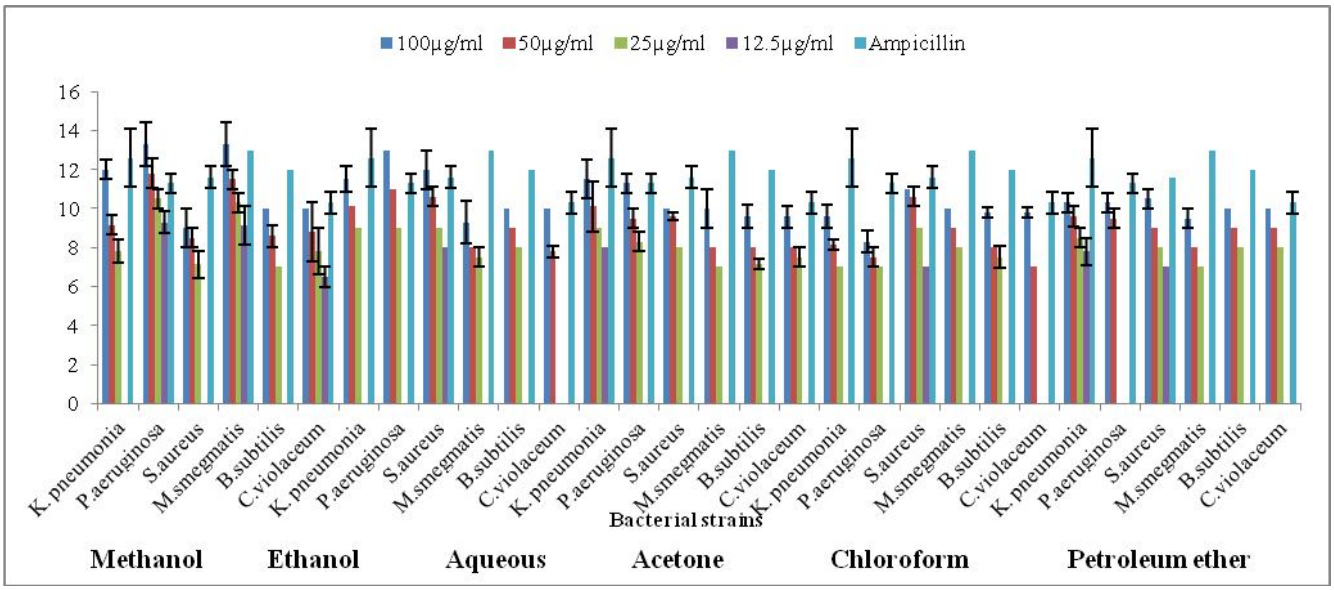

Fig. 3: Disc diffusion assay of six different *L.aspera* extracts, ZOI showed as mean ± standard deviation (n=3), *K. pneumonia: Klebsiella pneumonia, P.aeruginosa: P.seudomonas aeruginosa, S. aureus: Staphylococcus aureus, M. smegmatis: Mycobacterium smegmatis, B. subtilis: Bacillus subtilis, C.violaceum: Chromobacterium violaceum.*

against six different bacterial strains by using disc diffusion method and Micro-dilution method. The results obtained are shown graphically in fig. 3. The range of zone of inhibition (ZOI) all extract of *L.aspera* was observed the range between 12.8±0.28mm to 8.3±0.28mm. Plant extract prepared in methanol showed highest ZOI (12.8±0.28mm) against *P.aeruginosa* similar ZOI (12.8±0.28mm) was observed against *M. smegmatis*. While ZOI (12.0±0mm) against *K. pneumonia* higher than *B.subtilis* (10±0mm) and *C. violaceum* (10±0mm). Less inhibition was depicted against *S.aureus* (9±0mm). The ethanolic extract exhibited maximum inhbition *P.aeruginosa* (12.5±0.5mm) that proceed by *S.aureus* (12±0mm), *K. pneumonia* (11.5±0.5mm). ZOI against *Bacillus subtilis* (10±0mm) and *C. violaceum*(10±0mm) was similar while least activity was observed against *M. smegmatis* (9.3±0mm). The aqueous extract of *L.aspera* did not showed any activity against all bacterial strains while choloroform and petroleum extract was found to be least effective against all the six bacterial strains as they exhibited poor ZOI. Chloroform extract expressed similar activity against *S.aureus* (10±0mm), *M. smegmatis* (10±0mm) and *B.subtilis* (9.8±0.28mm), *C. violaceum* (9.8±0.28mm). The extract was less resistant to *K. pneumonia* (9.6±0.57mm) and *P.aeruginosa* (8.3±0.28mm). The petroleum ether extract was exhibited least activity against *S.aureus* (10.5 ± 0.5mm), whereas similar results was observed against *K. pneumonia* (10.3±0.57mm), *P.aeruginosa* (10.3±0.57mm) and *B. subtilis* (10±0mm), *C. violaceum* (10±0mm). The petroleum ether showed least activity was against *M. smegmatis* (9.5±0.57mm). Maximum ZOI recorded with the positive controls i.e. ampicillin against *M.smegmatis* (13±0mm) and *K.pneumonia* (12.6±.57mm) whereas *S.aureus* and *P. aeruginosa* were found to be resistant against ampicillin. Negative control (DMSO) didn't show any activity against any of the bacterial strain used in the study.

Microbroth Dilution Assay

MIC values of extracts of *L.aspera* obtained against different bacterial strains were range between 0.19 mg/ml to 0.25mg/ml and represented in table 4. For the methanolic extract MIC range from 0.19 mg/ml to 1.56 mg/ml and lowest and similar value (0.19mg/ml) were observed against four bacterial strains *M.smegmatis*, *P.aeruginosa*, *B.subtilis*, *S.aureus.* Ethanolic extract also showed same value against *B.subtilis*, *S.aureus, C. violaceum* while maximum MIC (12.5mg/ml) observed in *P.aeruginosa.* The plant extract prepared in acetone showed MIC values in range of 0.78mg/ml to 12.5mg/ml, least MIC (0.78 mg/ml) was observed in case of *S.aureus* followed by *B.subtilis* (3.125 mg/ml), *K. pneumonia* (6.25 mg/ml) *C.violaceum* (6.25 mg/ml). Highest MIC value (12.5 mg/ml) was observed in *P.aeruginosa*. MIC value range obtained in chloroform were from 1.56 mg/ml to 12.5mg/ml. Maximum inhibition was found against (1.56 mg/ml) *M.smegmatis* and minimum inhibition was against (12.5 mg/ml) *P.aeruginosa.* In case of petroleum ether extract minimum MIC value (3.125 mg/ml) was depicted against two bacteria *M.smegmatis* and *C.violaceum* followed by same value (6.25 mg/ml) in three bacteria that were *K. pneumonia,*

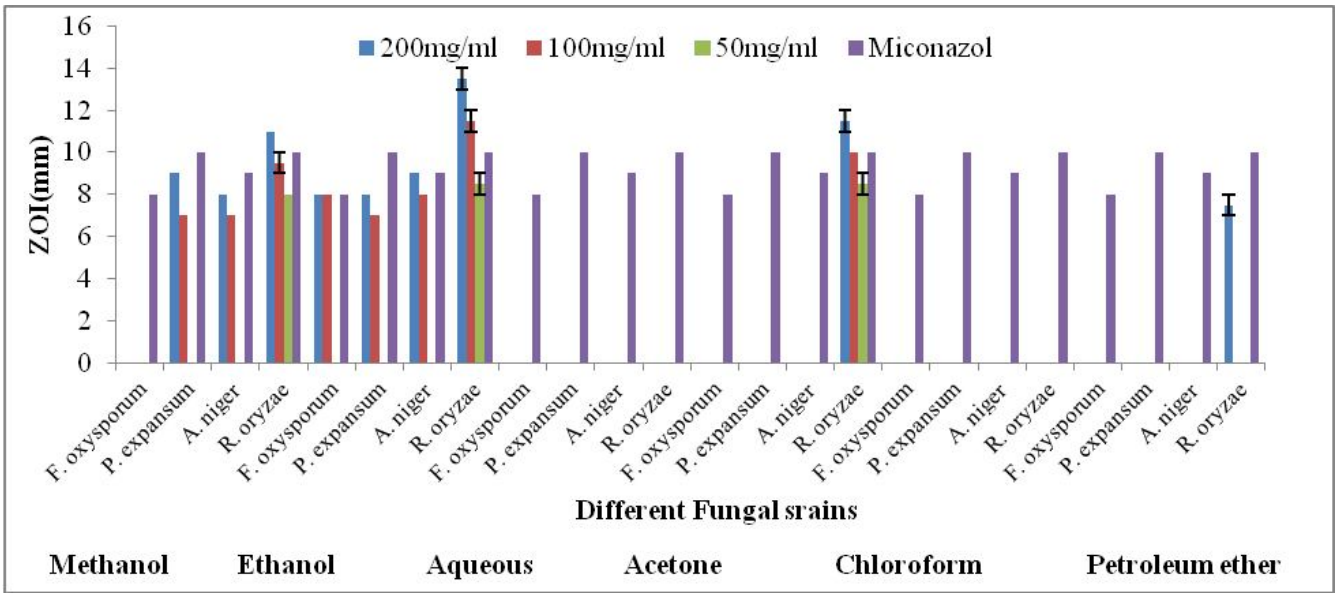

Fig. 4: Disc diffusion assay of three *L.aspera* extracts, *F. oxysporum*: *Fusarium oxysporum, P.expansum: Penecillum expansum, A. niger: Aspergillus niger, R. oryzae: Rhizopus oryzae.*

P.aeruginosa and *B.subtilis*. Aqueous extract showed least inhibition against all bacteria that were range from 12.5 mg/ml to 25mg/ml.

Disc Diffusion Assay

Antifungal activity of *L.aspera* is not so much effective against fungal strains. Methanol and ethanol extract showed significant ZOI (Fig. 4) against all the strains whereas all other extract exhibited activity against *R.oryzae* except aqueous extract. Methanolic extract showed highest ZOI against *R.oryzae* (11±0mm) followed by *P. expansum* (9±0mm) and *A. niger* (8±0mm). Methanolic extrarct did not show any activity against *F. oxysporum.* In case ethanolic extract highest ZOI was observed against *R.oryzae* (13.5±0.5mm) followed by *A.niger* (9±0mm). The ethanolic extract showed similar activity against *F. oxysporum* (8±0mm) and *P. expansum* (8±0mm). Aqueous and chloroform extract did not show any activity against all fungal strains where as other two extracts acetone (11.5±0.5mm) and petroleum ether (7.5±0.5mm) showed activity against *R.oryzae.*

Microbroth Dilution Assay

Methanolic extract of *L.aspera* was exhibited minimum MIC value against the *R.oryzae* (0.78 mg/ml) followed by *A.niger* (6.25mg/ml) and *F. oxysporum* (6.25mg/ml), least inhibition was observed against P *expansum* (12.5mg/ml). In case of ethanolic extract minimum MIC values was obtained for *R.oryzae* (1.56 mg/ml) followed by same value (12.5mg/ml) against three fungal strains that were *A.niger, P. expansum* and *F. oxysporum*. The range of MIC values for acetonic extract was 3.125-25mg/ml. Maximum inhibition was depicted against *R.oryzae* (3.125mg/ml) followed by *A.niger* (12.5mg/ml), *P. expansum* (12.5mg/ml) and *F. oxysporum* (25mg/ml). In case of chloroform and petroleum ether extract the MIC value against all fungal strains were same. Least value was observed against *F. oxysporum* (12.5mg/ml). Aqueous extract showed minimum inhibition against *R.oryzae* (50mg/ml) and *A.niger* (25mg/ml). There was no activity of aqueous extract against *P.expansum* and *F. oxysporum*. The positive control miconazol showed minimum MIC value (0.019mg/ml) against *R.oryzae* followed by *F. oxysporum, P.expansum* (0.078mg/ml) *and A.niger* (1.56mg/ml).

FT-IR spectroscopy analysis of *L.aspera*

The FT-IR spectrum of methanolic extract of *L.aspera* is shown in fig. 5. The FT-IR analysis exhibit the presence of phenols, alcohols, amides, vinyl compound, ester, alkyl halide, β-lactones, unsaturated hydrocarbons and carbonyl group. Major FT-IR peaks were observed at 538-1732 cm^{-1} and 2200-3430 cm^{-1}. These peaks (3429cm^{-1} due to –OH stretching, 2914cm^{-1} –C-H stretching, 1620cm^{-1} –C=O, 1436 –OH bending, 1616cm^{-1} –C=C stretching) indicated the presence of alcohols, alkanes, alkyl halides, unsaturated hydrocarbons, vinyl compounds that were present in secondary metabolites such as flavonoids having –OH group, –C=O group, phenolic compounds having –OH functional group. Methanolic extract showed different peak of alkyl halide from 699 cm^{-1} to 538 cm^{-1}.

Table 5: MIC values (mg) of different extracts of *L.aspera* against different fungal strains.

Plant extract	*Fusarium oxysporum*	*Penicillum expansum*	*Aspergilus niger*	*Rhizopus oryzae*
Leucas aspera				
Methanol	6.125	12.5	6.25	**0.78**
Ethanol	12.5	12.5	12.5	1.56
Aqueous	NA	NA	25	50
Acetone	25	12.5	12.5	3.12
Chloroform	12.5	25	25	25
Petroleum ether	12.5	25	25	25
Miconazol	0.078	1.56	0.078	0.019
DMSO	NA	NA	NA	NA

The MIC values are showed as mean (n=3), *F.oxysporum*: *Fusarium oxysporum, P.expansum: Penecillum expansum, A.niger: Aspergillus niger.*

GC-MS analysis

The bioactive compounds of methanolic extract were detected by GC-MS analysis and shown in table 6. The results showed methanolic extract to be a mixture of 12 identified bioactive compounds. Out of 12 compound, the % total of Phenol- 2, 4-bis (1,1-dimethylethyl)- was (64.353) higher followed by caryophyllene (12.397), cyclohexane, 1-ethyl-1-methyl-2,4-bis (1-methylethenyl- (8.954), tricontane (4.122), 2-Nephthalenemethanol, decahydroalpha, alpha., 4a-trimethyl-8-methylene (2.284), Heptadecane, 2,6,10,15-tetramethyl (2.017), Hentriacontane (1.734), Humulene (1.571), Cyclohexane, nonadecyl (1.323) and Nonane, 2, 2, 4, 4, 6, 8, 8- heptamethyl (1.246).

Discussion

L.aspera is a common weed growing in tropical regions. It used orally as stimulant, anthelmintic, laxative, and diaphoretic also used to treat inflammation, dyspepsia, and jaundice. It is also used orally for the treatment of headache, asthma, and bronchitis. Keeping in view all the ethanomedicinal properties of *L.aspera* our present investigation efforts have been made to evaluate phytochemical, antioxidant and antimicrobial activity of *L.aspera* extract prepared in different solvents.

Phytochemical and antioxidative potential

When the plant extracts were screened for their phytoconstituents, methanolic extract of *L.aspera* showed the presence of all phytocompounds for which they were tested except for saponins by ethanolic and aqueous extract have all phytochemical except saponins and terpenoid. In case of acetonic extract steroid and terpenoids were absent. Our results have similarity with Tahareen S. *et al*., 2016 showed that all phytochemicals were present in order of methanol>Aqueous. Petroleum ether extract showed flavonoid, phenols whereas steroids and alkaloid were absent. Similar observation was also made in petroleum ether extract of *Ficus* palmate (Abdul Wadood *et al.,* 2013). Flavonoids, phenols, alkaloids and steroids have been shown to possess efficacy for antimicrobial, anti-oxidative and anti-inflammatory properties (Anhwange *et al*., 2010, Rai V. *et al*., 2005, Kadiyala Gopi *et al*., 2014). Hence, presence of these compounds in plant extracts suggests the medicinal value of these extracts.

Methanol extracts of *Leucas aspera* were analysed with highest amount of TPC (124.62 ±.552 mg GAE/g)

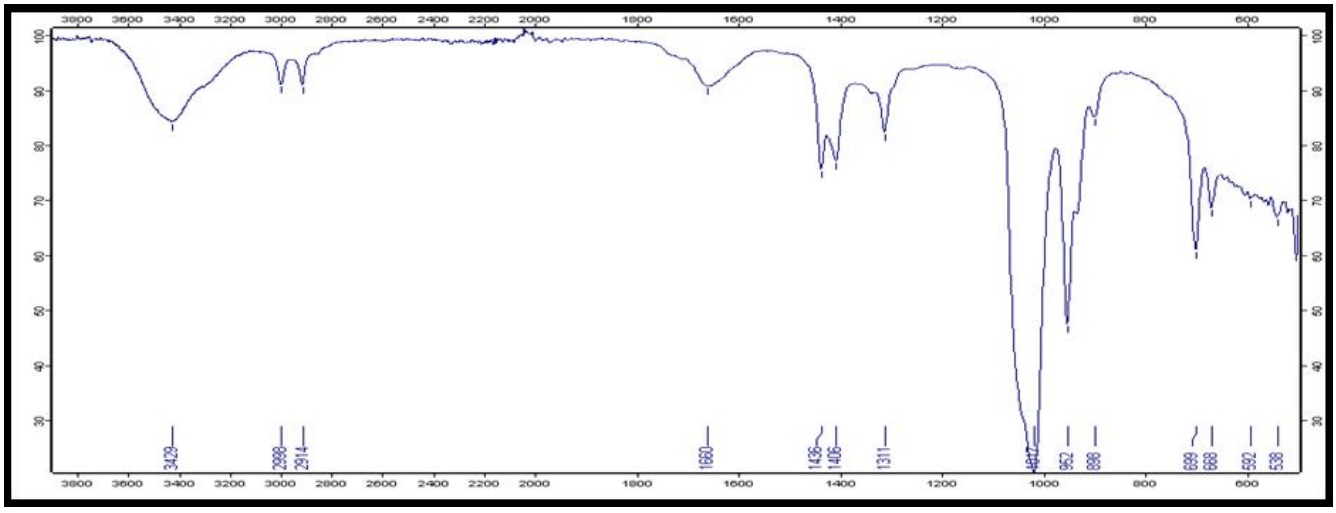

Fig. 5: FTIR of methanolic extract of *L. aspera*

Table 6: Bioactive compounds after GC-MS anaylsis of methanolic extract of *L.aspera* with area, formula and % total.

Name of compound	Area	% of Total	Formula
Cyclohexane, 1-ethyl-1-methyl-2, 4-bis (1-methylethenyl)	2.511e+8	8.954	$C_{15}H_{24}$
Caryophyllene	3.477e+8	12.397	$C_{15}H_{24}$
Humulene	4.406e+7	1.571	$C_{15}H_{24}$
Phenol- 2,4-bis (1,1-dimethylethyl)	1.805e+9	64.353	$C_{14}H_{22}O$
Nonane, 2,2,4,4,6,8,8- heptamethyl	3.494e+7	1.246	$C_{16}H_{34}$
2-Nephthalenemethanol, decahydro-alpha, alpha.., 4a-trimethyl-8-methylene	6.405e+7	2.284	$C_{15}H_{26}O$
Heptadecane, 2,6,10,15-tetramethyl	5.656e+7	2.017	$C_{21}H_{44}$
Hentriacontane	4.863e+7	1.734	$C_{31}H_{64}$
Triacontane	1.156e+8	4.122	$C_{30}H_{62}$
Cyclohexane, nonadecyl	3.709e+7	1.323	$C_{25}H_{5}$

and TFC (98.23±0.41 mg QE/g) that were followed by ethanolic, acetonic, aqueous and chloroform. The lowest value of TPC and TFC was calculated for petroleum ether extract (64.32 ± 1 mg GAE/g) and (37.5±0.45 mg QE/g). Our results are promising results than Latha. B, 2013 showed the total phenolic contents 15.36 ±0.512 GAE/g dry weight of extract. Other previous studies has shown the phenolic and flavonoid content in *L.aspera* was found to be 16 µg and 43.08 µg (Ramandeep Kaur, 2016). According to Meghashri S., 2010 quantity of phenol and flavonoid was found to be 2.31 µg per miligram and 1.88 µg per miligram of leaves extract. Similar observations were also made by Mahboubi *et al*., (2015) in the plant extracts of *Punica granatum* prepared in ethanol solvent (1.1 mg RE/g).

Antioxidative potential of *L.aspera* was evaluated by DPPH and ABTS non-enzymatic assay. Antioxidant activity of plant extracts increases with increase in their concentration. Observation of our finding is similar with earlier findings of Motalleb *et al*., 2005 depicted the antioxidant effect of the extract on the DPPH and ABTS radical. Among all the extract the methanolic extract exhibits maximum antioxidant potential followed by ethanolic extract, aqueous, acetonic and chloroform. Plant extract prepared in petroleum ether showed least amount of activity. Correspondingly, the lowest IC_{50} value (49.7mg/ml) was obtained with DPPH radical by methanolic extract followed by ethanolic extract (51.27mg/ml), acetonic extract (64.9mg/ml) and chloroform extract (68.07 mg/ml), petroleum ether (75.59mg/ml) and highest IC_{50} value (78.74 mg/ml) was observed in aqueous extract. Similar observation was depicted in ABTS assay except the petroleum ether extract showed high IC_{50} value. Meghashri S, 2010 shown the IC_{50} value of extract of *L.aspera* higher (85.57) represented that our extract has higher antioxidative potential. However, Rahman *et al*., 2007 showed lower IC_{50} value of *L.aspera* extract and lower than IC_{50} value observed by Ai Lan Chew (2012) in extract of *L.aspera*. The differences in the observations could be due to the plant part selected in the study. According Ramandeep Kaur, 2016, *L.aspera* plant can be used for medicinal purpose. A good correlation was observed between TPC, TFC and antioxidant potential of the *L.aspera* in our study which is in conformity with other studies. Similar to our observation workers from other parts of the country have also reported *L.aspera* to exhibit good antioxidant properties carried out by non enzymatic assays (Prajapati M.S. *et al*., 2010, Rahman M.S. *et al*., 2007, Md. Sekendar Ali *et al*., 2013). According to the present investigation of enzyme activity, we can conclude that *L.aspera* has significant antioxidant capacity. The enzymatic assays carried out with the plant extracts possessed significant enzymatic activity. The results are in conformity with the non enzymatic assay thus suggesting the antioxidant potential of the plant extracts. Plant produces enzymes like SOD, CAT, GST, glutathione peroxidase and reductase to combat the free radical produced during metabolic reactions to detoxify or degrade ROS (Shaaltiel Y. *et al*., 1988, Sudipta *et al*., 2014). Enzyme Catalase detoxifies hydrogen peroxide (H_2O_2) into molecular oxygen (O_2) and water (H_2O) (Hunt C.R. *et al*., 1998, Olson *et al*., 2017). Catalase is the second most important enzyme (after SOD), which reduce the effects of ROS (Vendemiale *et al*., 1999). The extracts of *L. aspera* were investigated for the presence of these enzymes**,** significantly higher SOD activity was observed. The results of enzymatic assays showed good antioxidant potential of the plant extracts.

Antimicrobial assay

The collective analysis of antimicrobial activity of *L.aspera* indicated range of zone of inhibition from 10mm to 13.3 mm against six bacterial strains. Out of six extracts, methanolic extract exhibited higher zone of inhibition against three bacteria *i.e. P. aeruginosa*, *M. smagmatis* and *K.pneumonia.* It may be attributed that the crude extracts contain very small concentration of bioactive compounds (Zuraini Z. *et al*., 2007). In antimicrobial activity, generally expected that greater number of bioactive compounds present in the extracts

would be effective against gram positive bacteria than gram negative (Joshi B. *et al*., 2011). In our study, the plant extract showed higher range of ZOI against gram negative bacteria as compared to gram positive. The activity against both kinds of bacteria indicated that extract is having more bioactive compounds (Srinivasan D. *et al*., 2001). Methanolic extract showed highest activity against *P.aeruginosa* (13.3±1.15) which is in conformity with the observations made by Atiarrahman *et al*., (2013). Mangathayaru. *et al*., (2005) reported that methanolic extract of *L.aspera* was effective against *S.aureus, E.coli, P.aeruginosa* which were similar to our study. However, Ai Lan Chew *et al.,* 2012, exhibited poor activity of methanolic extract of *L.aspera* against both gram negative and gram positive bacteria.

MIC

In our results minimum inhibitory concentration 0.19µg/ml exhibited by methanolic extract against *M.smegmatis*, *P.aeruginosa*, *B.subtilis*, *S.aureus* indicated that methanolic extract have high antimicrobial activity. The plant extract of *Leucas inflate* also showed less MIC value (0.81 mg/mL) against *Bacillus subtilis* and *Staphylococcus aureus* (Mothana *et al*., 2017). According to Vagionas *et al*., (2007) the essential oil of *L. glabrata* has a strong antimicrobial activity against 2 Gram-positive and 4 Gram-negative bacteria (MIC values 0.45–1.14 mg/mL). Present study revealed that methanolic extract possesses strong antimicrobial activity against all three gram positive and gram negative bacteria.

Antifungal assay

Extracts of *L.aspera* were not found to be effective against four fungal strains used in the study.

Generally expected that higher number of phytocompounds present in the plant extract, there will be high antimicrobial potential (Joshi B. *et al*., 2011). The activity of extracts of *L.aspera* is not so much affective against all four fungal strains although methanolic and ethanolic extract showed significant ZOI. It has been reported that fungal strains are more resistant to plant extracts due to presence of more complex cell wall, presence of some extracellular enzymes that metabolize some bioactive compounds of plants (Tortora *et al*., 2005, Yam *et al*., 1997, Nostro *et al*., 2000, Duarte *et al*., 2005).

FTIR

The spectrum of FTIR was analyse for the functional group of the phyto-components present in the methanolic extract, depends on the peaks observed in infrared radiation region. From the spectral data, presence of C=O, C-H, C=O, C-O, C-C functional groups were identified, that exhibited the presence of phenols, alcohols, amides, vinyl compound, ester, alkyl halide, unsaturated hydrocarbons, and carbonyl group. Earlier studies of *L. aspera*s howed the presence of flavonoids, phenolic contents, alkaloids, Saponins, Glycosides, carbohydrate and terpenoids etc. (Aanandan *et al*., 2012).

GC-MS analysis

Phenolic compounds are the secondary metabolite which shows remarkable higher activity against microbial pathogens (Alasalvar *et al*., 2001, Acamovic and broker *et al*., 2005, Edreva *et al*., 2008). Previous studies have been reported that the role of phenolic compounds in anti-oxidative property, antiaging, anti-inflammatory and as anti-proferative agents (Shukitt-Hale B. *et al*., 2005, Moo-Huchin *et al*., 2015). So there may be possibilities that antimicrobial potential of *L. aspera* extracts is due to finding of phenolic compounds. A part from phenolic compound, Caryphyllene are also found in methanolic extract of *L. aspera.* It is natural sesquiterpenes having various biological activities such as antimicrobial, antioxidative, anti-inflammatory and especially it is anticarcinogenic and antianalegesic in nature (Klaudyna F. *et al*., 2016). Another compound, Humulene also possess antibacterial, antitumor and both kind of typical and systemic anti-inflammatory properties (Fernandes *et al*., 2017, Chaves *et al*., 2008). Interestingly, humulene was shown to increase secretion of IL-8 which is helpful in wound healing (Satsu *et al*., 2004). Hentriacontane, Triacontane and Heptadecane are the triterpene hydrocarbons which provide defence system to plant (Ananthakrishnan T.N. *et al*., 1991, Padmavathi C.H. *et al*., 1998).

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