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Evaluation of Antioxidant and Antimicrobial potential of Leucas urticaefolia (Lamiaceae)

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ABSTRACT

The present study was designed to screen phytochemical constituents with antioxidant and antimicrobial activity of 50% EtOH extract of *Leucas urticaefolia*. Antimicrobial activity was tested against *Staphylococcus epidermidis*, *Salmonella typhi*, *Salmonella typhimurium*, *Candida krusei* and *Aspergillus fumigatus* by disc diffusion method. DPPH free radical scavenging assay and ferric reducing assay were used for the determination of antioxidant activity. Qualitative and quantitave analysis of polyphenoles was performed by HPLC-UV. Remarkable antimicrobial potential was exhibited in concentration dependent mode against *S. epidermidis*, *S. typhi* and *C. krusei*. However, *S. typhimurium* and *A. fumigatus* showed resistance at lowest concentrations but higher concentrations were effective in inhibiting both microorganisms. Total phelnolic and flavonoid content were found to be 0.71335±0.025% and 0.2594±0.028% respectively. Different concentrations of extract showed dose dependent reducing power and scavenging of DPPH radicals with IC_{50} 149.59±0.24 µg/mL. A marked correlation was observed between antioxidant and antimicrobial activity of 50% EtOH extract of *L. urticaefolia*. HPLC analysis showed the presence of important polyphenoles and may be attributed to antimicrobial and antioxidant activity of the extract. The observations provided sufficient evidences that *L. urticaefolia* might indeed be potential sources of natural bioactive agent, if further investigated.

INTRODUCTION

Medicinal plants have long been used as source of remedies and represent a rich source for potential alternatives antimicrobial agents, which may be used for the treatment of many infectious diseases. The increasing antibiotic resistance and side effects of synthetic drugs have led to the screening for more effective, less toxic and cost effective antioxidants and antimicrobials from natural sources (Bhatt and Neggi, 2012; Elizabeth, 2005). The recent studies suggested that plant products are rich source of many biologically active phenolic compounds which have been found to possess potential antioxidant as well as antimicrobial activity (Kaneria *et al*., 2012). Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide of lipid hydroxyl thus; inhibit the oxidative mechanisms that lead to degenerative diseases (Subramanion *et al*., 2011). Many of these phenolic compounds also possess other activities like antimicrobial, anti-inflammatory, hypocholestemic, antiplatelet aggregation properties, etc. *Leucas* one of largest genus of the family Lamiaceae and have great medicinal potential (Singh, 2001). Every species of this genus has unique medicinal value and widely used by traditional healers in India as well as in other countries (Chouhan and Singh, 2012). *L. urticaefolia* (Vahl.) R. Br. is known as Goma or Guldora (Kiritikar and Basu, 2005). The plant is used for the treatment of diarrhoea, dysentery, uterine hemorrhages, dropsy, gravel, cystitis, calculus, bronchial catarrh, skin diseases, fever and mental disorder (Watt, 1890; Mhaskar *et al*., 1935).

Infusion of the flowers is given in cold and cough; leaf decoction is used to cure fever and roasted leaves are bandaged on swollen parts (Katewa and Galav, 2005), decoction of the leaves and apical shoots with gur is used locally as an abortifacient up to three months of pregnancy (Jafri, 1966).

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The studies revealed the presence of triterpenes (Leucisterol, β-sitosterol, and ursolic acid), acids and esters (urticic acid, methoxybenzyl benzoate, 4-hydroxy benzoic acid) (Habib *et al*., 2008), diterpene (Momilactone-A) (Fatima *et al*., 2008). The flavonoidal glucosides (leufolins A and B) of *L. urticaefolia* reported to have significant inhibitory potential against the enzyme butyrylcholinesterase (Noor *et al.*, 2007). In addition, aqueous extract of leaves exhibited potential wound healing activity (Suthar *et al.*, 2011).

Due to its high value medicinal usage, the present study was performed to establish the quality parameters of the herb, however, the medicinal values of *L. urticaefolia* pertaining to antioxidant and antimicrobial activity have not yet been reported. This study was designed to explore antioxidant and antimicrobial activity of 50% EtOH extract *L. urticaefolia* against some human pathogens with HPLC analysis.

MATERIAL AND METHODS

Chemicals and microorganisms

Sodium acetate, potassium chloride, 2-diphenyl-1-picrylhydrazyl-hydrate (DPPH), Folin–Ciocalteu reagent, catechol, beta carotene, Quercetin, Gallic acid, Tween-20, sodium carbonate, sodium hydroxide, acetone, butanol, chloroform, ethanol and aluminium chloride were obtained from Merck India, Mumbai. Whatman No.1 filter paper and disc were used for filtration of the samples and antimicrobial assay respectively. Microorganisms such as *Salmonella enterica* ser. *typhi* (MTCC-733), *Salmonella enterica* subsp. *enterica* ser. *typhimurium* (MTCC-3224), *Staphylococcus epidermidis* (MTCC-3382), *Aspergillus fumigatus* (MTCC-10561) and *Candida krusei* (MTCC-9215) were obtained from the Microbial Type Culture Collection & Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh. Various Media for analysis were purchased from Hi-media laboratories Pvt. Ltd., Mumbai. All the chemicals used for analysis were of analytical grade.

Collection of plant material

The whole plant of the *L. urticaefolia* was collected from the local areas of Mandav, Indore, Madhya Pradesh, India in the month of October, 2014. For future reference, voucher specimens (Collection No.:260619) were deposited in the LWG herbarium, CSIR-NBRI, Lucknow.

Preparation of crude extract and phytochemical screening:

The material was shaded dried and pulverized to a coarse powder. The powdered material was passed through a 40 mesh sieve and extracted with of 50% (v/v) EtOH at 39^0C on using Soxhlet extractor for 24 h. The extract was filtered and concentrated under reduced pressure in a rotavapour (Buchi R-200 USA) below 40^0C, further dried in desiccator to obtain cream of extract. The resulting crude extract was then stored at 4^0C. 50% EtOH extract of *L. urticaefolia* was subjected for preliminary phytochemical screening (Trease, 1989 and Harborne, 1993).

Determination of total phenolic content

Total phenolic content (TPC) was calculated according to the Folin–Ciocalteu method (Bray & Thrope, 1954) with some modifications. A stock solution (1mg/ml) of 50% EtOH extract of *L. urticaefolia* using extract solvent was prepared. 0.5ml stock solution was taken in the test tube and added 10ml distil water and 1.5ml Folin–Ciocalteu reagent, stand for 5 minutes then added 4ml 20% Na_2CO_3 make the volume upto 25 ml with distil water, and stand for 30 minute. The OD (optical density) was taken at 765nm. Gallic acid of different concentration was used as standard. Total phenolic content were calculated by the following formula,

Total Phenolic content= Conc. in 1ml x Extr value % /.02x1000.

Determination of total flavonoid content

The amount of total flavonoid was calculated according to Woisky and Salatino (1998) with some modifications, stock solution (1mg/ml) of 50% EtOH extract of *L. urticaefolia* using extract solvent was prepared, 0.5ml stock solution was taken in a test tube and added 0.5ml 2% methanolic $AlCl_3$ and volume made up to 5ml with methanol. Yellow colour indicated the presence of flavonoid. The optical density (OD) was noted at 420 nm. Quercetin solution used as standard in serial dilutions of Percent flavonoid was calculated by the given formula. Percent flavonoid= conc. In 1ml x Ext. value x 100/1000

2, 2'-diphenyl-1-picrylhydrazyl (DPPH$^{\circ}$) radical scavenging assay

The capacity to scavenge the stable free radical DPPH was evaluated according to the method of Blois (1958) with some modifications. Various concentrations (50-200µg/mL) of extract were prepared using 50% EtOH and were mixed with equal volume of ethanolic solution containing DPPH radicals (0.135mM).

The mixture was shaken vigorously and reaction mixture was left to react in the dark for 30 minutes at room temperature (until stable absorption values were obtained). The reduction of the DPPH radical was determined by measuring the absorption at 517 nm. The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: % RSA = [Absorbance of DPPH - Absorbance of test Sample /Absorbance of DPPH] X 100. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of RSA percentage against extract concentration. Ascorbic acid was used as standard.

Reducing power assay

The reducing power of the sample was determined by the method of Oyaizu (1986) with some modifications. An aliquot of the sample (1.0 mL) at various concentrations (50-200µg/mL) was mixed with phosphate buffer (0.2 M, pH 6.6, 2.5 mL) and 1% potassium ferricyanide (2.5 mL), the mixture was incubated at 50 °C for 20 min., after 2.5 ml of 10% trichloroacetic acid (w/v)

were added, the mixture was centrifuged at 650 rpm for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and 0.1% iron (III) chloride (0.5 mL), and the absorbance was measured at 700 nm using an appropriate blank; higher absorbance indicates higher reducing power.

Antimicrobial Assay

Agar disc diffusion method was performed to evaluate antimicrobial activity of 50% EtOH extract of *L. urticaefolia* (Ahlam *et al*., 2013). Briefly all concentrations of extract were prepared using dimethyl sulphoxide (DMSO). For the inoculums (10^8 cfu/mL), test bacteria and fungi were grown in sterile Muller–Hinton broth and Sabouraud dextrose broth tubes respectively overnight.

The inoculums of bacteria and fungi were then aseptically plated using sterile cotton swabs into petri dishes with Muller–Hinton agar and Sabouraud dextrose agar respectively. Filter paper disc were impregnated with different concentrations to obtain 50, 100, 150, 200 µg/disc samples and placed on prepared agar surface. The petri dishes were pre-incubated at room temperature, allowing complete diffusion of the samples and incubated at 37°C for 24 hours (for bacteria) and 48 hours (for fungi).

Tetracyclin (20 µg/disc) and nystatin (20 µg/disc) were used as standard antibacterial, antifungal antibiotics respectively. The experiments were performed in triplicate. After incubation the inhibition potential of extract was quantified by measuring the diameter of the zone of inhibition in mm. Antimicrobial activity was assessed using the parameters according to Quinto & Santos (2005): inhibition zone <10 mm, inactive; 10-13 mm, partially active; 14-19 mm, active; >19 mm, very active.

HPLC studies

1mg/ml stock solution of 50% EtOH extract of *L. urticaefolia* was prepared in 50% (v/v) methanol. Qualitative and quantitave analysis of sample extract and standard polyphenoles (Gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, ruitn, ferulic acid, quercetin, kaempferol) was performed by HPLC-UV (Niranjan *et al*., 2009).

RESULTS

The percentage yield was found to be 12.97%. The preliminary phytochemical studies showed that the 50% EtOH extracts of *L. urticaefolia* gives positive results for alkaloids, carbohydrates, tannins, flavonoides, glycosides, proteins. After estimation of the 50% EtOH extract of *L. urticaefolia* was found to contain 0.71335±0.025% of total phenolic and 0.2594±0.028% of total flavonoid content.

2, 2'-diphenyl-1-picrylhydrazyl (DPPH˚) radical scavenging assay

The DPPH test provided information on the reactivity of test compounds with a stable free radical. Because of its odd electron, 2, 2-Diphenyl-Picryl Hydrazyl (DPPH) radical gives a strong absorption band at 517 nm in visible spectroscopy (deep violet color). In the present study, **Fig. 1** showed significant DPPH radical scavenging activity of *L. urticaefolia* with IC_{50} values 149.59±0.24 µg/mL compared to ascorbic acid (IC_{50} 42.23µg/mL).

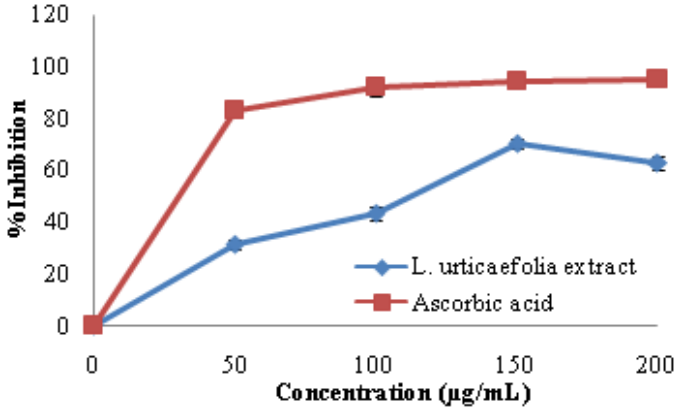

Fig. 1: DPPH radical scavenging activity of 50% EtOH extract of *L. urticaefolia.*

Reducing power assay

Reducing power is to measure the reductive ability of antioxidant, and it is evaluated by the transformation of Fe (III) to Fe (II) in the presence of the sample extracts (Gülcin *et al.*, 2003). The reducing power of 50% EtOH extract of *L. urticaefolia* is shown in **Fig. 2**, which depicted that, reducing power increased with an increase in extract concentration.

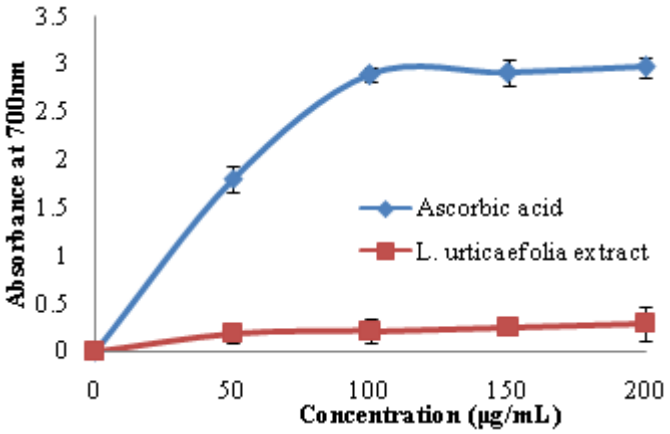

Fig. 2: Reducing power of 50% EtOH extract of *L. urticaefolia.*

Antimicrobial assay

Antimicrobial activity of the different concentration of extracts was evaluated by agar disc diffusion method against five pathogens and expressed in terms of inhibition zone (mm). The inhibition zone of extracts against different pathogens varied depending on pathogens and concentration of extract. The 50% EtOH extract of *L. urticaefolia* was found to be most effective against the gram negative bacteria *Salmonella typhi* and showed maximum (inhibition zone, 17.68 mm) activity. In case of gram

positive bacteria *Staphylococcus epidermidis* exhibited partially active to active antimicrobial activity (inhibition zone, 8.14-15.18 mm). Similarly, all the concentrations showed inhibition zone in concentration dependent manner (8.13-16.52 mm) against fungal strain *Candida krusei*. *Salmonella typhimurium* and *Aspergillus fumigatus* showed no activity at lower concentration, however, at higher concentrations the extract exhibited partially active to active antimicrobial activity and showed 16.12 mm and 15.21mm, zone of inhibition at highest concentrations (**Fig. 3**).

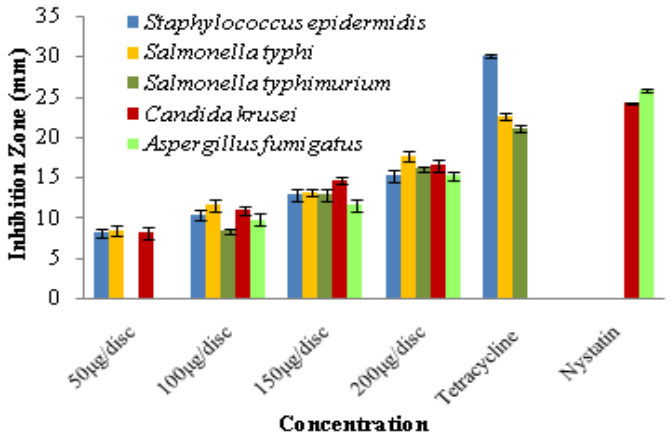

Fig. 3: Antimicrobial activity of 50% EtOH extract of *L. urticaefolia.*

HPLC studies

From the HPLC study chromatogram (**Fig. 4**) of distinct peaks for all seven polyphenols of following compounds have been identified in the total cell-free 50 EtOH extract of *L. urticaefolia*. The amount of compounds present in extract of *L. urticaefolia* were: gallic acid (0.22%), protocatechuic acid (0.273%), chlorogenic acid (0.077%), caffeic acid (0.096%), rutin (0.078%), ferulic acid (0.052%) and kaempferol (0.021%).

DISCUSSION

Phytoconstituents such as alkaloids, flavonoids, tannins, phenolics, saponins, terpenoids and other aromatic compounds are secondary metabolites that are produced in plants as a response to stress or as a part of their defence mechanism against prediction by many microorganisms, insects and other herbivores (Bonjar *et al.*, 2004).

Phenolic compounds are a class of antioxidant agents which act as free radical terminators (Shahidi, 1992) and plant extracts containing high phenolic content exhibited stronger scavenging and reducing capacity (Rekha *et al.*, 2012, Dileep *et al*., 2012). The protective effects of flavonoids in biological systems are ascribed to their capacity to transfer electron free radicals, chelate metal catalysts (Ferrali, 1997), activate antioxidant enzymes (Elliott, 1992), reduce alpha-tocopherol radicals (Hirano, 2001), and inhibit oxidases (Cos, 1998). The principle of DPPH method based on production of free radical (Hossain and Rehman, 2011) and the effect of antioxidants on DPPH radical scavenging is due to their hydrogen donating ability. The results indicated that, total phenolic and flavonoid content in 50% EtOH extract of *L. urticaefolia* was responsible for its antioxidant and antimicrobial activity. Earlier numerous studies also suggested that total phenolic, flavonoid content and antioxidant activity have a strong relationship (Ghasemzadeh *et al.*, 2010; Dorman *et al*., 2003).

Direct reduction of Fe^{+3} to Fe^{+2} was assessed in order to estimate the reducing power of 50% EtOH extract of *L. urticaefolia*. An increase in the absorbance at 700 nm with increasing concentrations indicated potential reducing capacity of *L. urticaefolia*. The reducing properties of antioxidants are generally associated with the presence of reductones. Many researchers have already used this assay to evaluate antioxidant activity of medicinal plants (Yuan *et al*., 2005; Kim *et al*., 2006; Rekha *et al*., 2012; Junaid *et al*., 2013).

Continuous evolution of microbial resistance to existing synthetic antimicrobial agents and side effects of these drugs have necessitated the development of new, noval and effective natural antimicrobial compounds. Most of the phyto-constituents are extensively used as medicinal compounds for treatment of various ailments all over the world (Cowan, 1999). Earlier, Ojala *et al*., 2000 reported that higher plants represent a promising source of new antimicrobial compounds with no adverse effect. In the present study different concentrations of 50% EtOH extract *L. urticaefolia* exhibited strong antimicrobial activity against bacterial as well as fungal strains, which showed its broad spectrum antimicrobial activity. However, all the concentrations exhibited moderate antimicrobial activity against *S. typhimurium* and *A. fumigatus*.

The results showed that the mean zone of inhibition produced by positive controls (tetracyclin and nystatin), was larger than those produced by all concentrations of 50% EtOH extract. This may be attributed to the fact that plant extract being in crude form contain smaller concentration of bioactive compounds (Chew *et al*., 2012).

The HPLC analysis revealed the presence of gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, rutin, ferulic acid and kaempferol in the 50% EtOH extract of *L. urticaefolia*. Earlier, It was reported that majority of polyphenoles having antimicrobial property in some extent. Mary and Merina (2014) reported the antimicrobial activity of kaempferol content. Chlorogenic acid exhibited antibacterial as well as antifungal activity by disrupting the structure of the cell membrane (Lou *et al.*, 2011; Sung and Lee, 2010). Rutin itself is not reported to possess anti bacterial property, however, it enhances the anti bacterial potentials of flavonoids (Arima *et al.*, 2002). Protocatechuic and caffeic acids have also exhibited inhibitory effect against the enterobacterial microorganisms (Almeida *et al.*, 2006). Ferulic and gallic acids show antibacterial activity against gram positive and gram negative bacteria (Borges *et al.*, 2013).

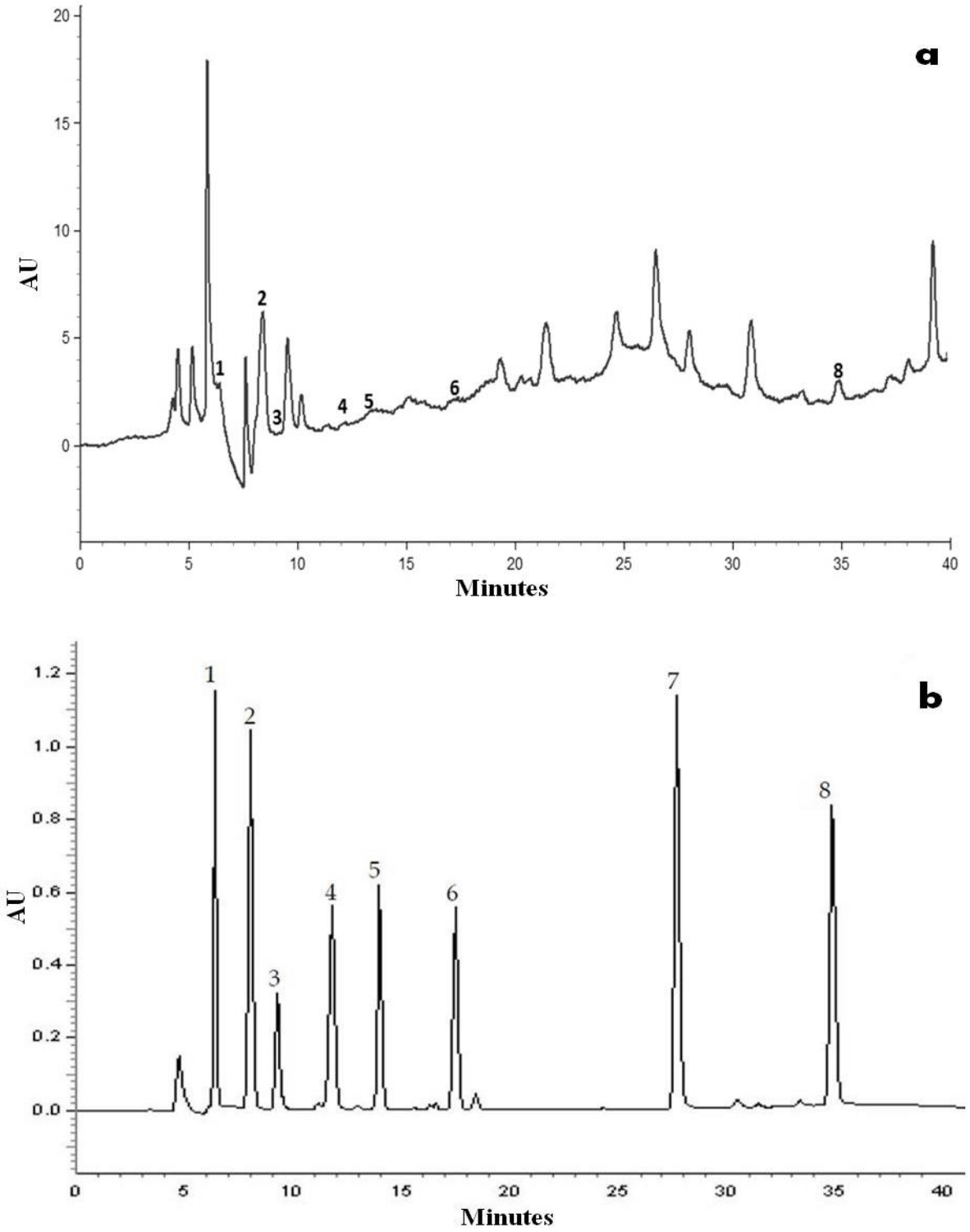

Fig. 4: HPLC chromatogram obtained from *L. urticaefolia* (a) and standard (b) at 254nm: 1. gallic acid; 2. protocatechuic acid; 3. chlorogenic acid; 4. caffeic acid; 5. rutin; 6. ferulic acid; 7. quericitin; 8. kaempferol

CONCLUSION

The present study provided the fact, that *L. urticaefolia* has potential antimicrobial and antioxidant activity and also revealed the relationship between total phenolic, flavonoid content and biological activities of the plant extract. The results suggested that plant based phytomedicines are cheap and affordable option to treat microbial infection due to their lesser adverse effect and readily availability compared to current synthetic medication. The obtained results might be sufficient to provide a baseline data for further studies such as, isolation and identification of the biologically active constituents and its mode of action, which is responsible for the antioxidant and antimicrobial activity of *L. urticaefolia*.

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