Original Article

Estimation of Phenolic Compounds Present in the Plant Extracts Using High Pressure Liquid Chromatography, Antioxidant Properties and its Antibacterial Activity

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ABSTRACT

Objective: The aim of the present study was to evaluate total phenolic content of the prepared plant extracts and investigation of phenolic compounds present in the extract. **Background:** The method was done to evaluate the phenolic compounds and finding the components presence. **Methods:** The extraction was carried out using methanol as the solvent. Plants used in this analysis were *Acalypha indica, Azadirachta indica, Lawsonia inermis* and *Murraya konegii*. Qualitative estimation of total phenol content was done using Folin- Ciocalteu method in with gallic acid was used as standard. HPLC analysis was done to reveal the nature of phenolic compounds, C18 column was used. Antibacterial activity was carried out using agar diffusion method. Antioxidant studies were done using DPPH assay. **Results:** Antibacterial assay revels *Lawsonia inermis* showed highest zone of inhibition on *Proteus* sps. The high amount of phenolic content was obtained in *Lawsonia inermis* (5.2 μg/ml) and the lowest was *Murraya konegii* (2.3 μg/ml). HPLC of various extracts revel the presence of simple phenols, catechins, anthocyanins, aglycons, anthraquinones, glycosides form of flavones, flavonols, isoflavones and flavanones. DPPH assay revels that Lawsonia inermis has good antioxidant properties compared to other extracts.

Key words: HPLC, Folin- Ciocalteu, Extraction, Simple phenols, Catechins.

INTRODUCTION

Nature has been a source of medicinal agents from ancient time. Herbal medicine is still the most common source for primary health care constituting about 65-80% of the world's population, mainly in developing countries. Leaves, flowers, stems, roots, seeds, fruit and bark can be used as herbal medicines. The medicinal values of these plants lie in their phytochemical components which produce definite physiological actions on the human body.[1]

The synthesized aromatic substances by plants act as defensive agent against predation by microorganism, insects and herbivores.[2] The most important of these components are alkaloids, tannins, flavonoid and phenolic compounds.[3] Phytochemicals are extensively found at different levels in various medicinal plants and used in herbal medicine to treat diverse ailments such as cough, malaria, wounds, tooth ache and rheumatic diseases.[4] controlled release systems increases therapeutic activity reducing the number of drug administration.[5]

Phenolic compounds are usually found in both edible and non edible plants, and they have been reported to have multiple biological effects. Flavonoids and other phenolics have been suggested to play a preventive role in the development of cancer and heart disease.[6]

Submission Date: 20-07-2017;
Revision Date: 18-08-2017;
Accepted Date: 13-10-2017

DOI: 10.5530/ijper.52.2.37

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Polyphenols are a group of secondary metabolites involved in the hydrogen peroxide scavenging in plant cells. Interest in plant materials rich in polyphenolic compounds increase recently, due to their high antioxidant potency which may offer protection against chronic disease, such as cardiovascular diseases, neuronal disease, cataract and several forms of cancer.[7]

L. inermis is used worldwide as a cosmetic agent to dye hair, skin and nails and in traditional medicine for different pathologies such as inflammation, mycoses, skin eruption, headache and diseases of the digestive apparatus.[8] The leaves of *L.inermis* were also found to exhibit strong fungi toxicity and non-phytotoxicity.[9] *L. inermis* exhibited significant hepatoprotective, antioxidant, anti-inflammatory, antibacterial, analgesic and adaptogenic effects indicating that it is a safe substance to be used as a drug ordinarily.[10]

Murraya koenigii from family Rutaceae is rich source of carbazole alkaloids. Bioactive coumarins, acridine alkaloids and carbazole alkaloids. Leaves and roots are also used traditionally as bitter, anthelmintic, analgesic, curing piles, inflammation, itching and are useful in leucoderma and blood disorders.[11] Crude extracts of leaves of *Murraya Koenigii* were found to possess anti-diabetic, cholesterol reducing property, anti-diarrhea activity, cytotoxic activity antioxidant property, antiulcer activity antimicrobial, antibacterial potential and many more useful medicinal properties.[12]

Acalypha indica can be useful in treating pneumoniae, asthma, rheumatism and several other ailments.[13] The plant contains kaempferol, a cyanogenetic glucoside, a base, triacetonamine and an alkaloid acalyphine.[14] Recently, four kaempferol glycosides, mauritianin, clitorin, nicotiflorin and biorobin have also been isolated from the flowers and leaves of this plant.[15]

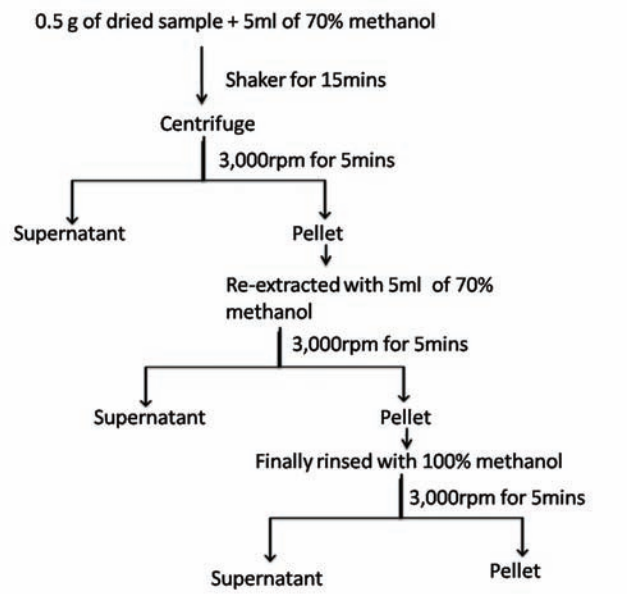
Chemical constituents of *Azadirachta indica* contain many biologically active compounds that can be extracted from neem, including alkaloids, flavonoids, triterpenoids, phenolic compounds, carotenoids, steroids and ketones.[16] One active components 'gedunin' gave significant control as effective as qunine on malaria. Several reports have also highlighted the pronounced antiviral efficacy of aqueous extract of neem leaves against small pox, fowl pox, polio and HSV as assessed by virus inhibition assay. Aqueous extract of neem leaf and fraction from neem oil have also been reported to suppress HIV and Polio viruses.[17]

HPLC is a sensitive and accurate tool that widely used for the quality assessment of plant extract and its derived product/formulation. RP-HPLC with C18 columns is the most popular technique for the analysis of polyphenols of the different food.[18]

In the present study, an attempt has been made to improve the understanding of antibacterial activity of plant extract against g positive and g negative bacteria. Further, to investigate and estimate total phenolic content using RP-HPLC.

MATERIALS AND METHODS

Extraction of Phenolic Compounds



These supernatants were pooled together before removal of methanol and the concentrated extract was dried and stored for further use.[19]

Qualitative analysis of total Phenolic content

Quantitative analysis of total phenolics in extracts was determined with the Folin- Ciocalteu reagent. Standard used for the analysis were gallic acid. Concentration of 0.01, 0.02, 0.03, 0.04 and 0.05 mg/ml of gallic acid were prepared in methanol. The standard graph was obtained for $Y=0.0061x + 0.0396$; $R^2=0.9991$. Concentration of 0.1 and 1mg/ml of plant extract were also prepared in methanol and 0.5ml of each sample were introduced into test tubes and mixed with 2.5ml of a 10 fold dilute Folin- Ciocalteu reagent and 2ml of 7.5% sodium carbonate. The tubes were covered and allowed to stand for 30 min at room temperature before the absorbance was at read at 760 nm spectrometrically. The Folin-Ciocalteu reagent is sensitive to reducing compounds including polyphenols, thereby producing a blue colour upon reaction. This blue colour is measured spectro-photometrically. Accordingly, total phenolic content can be determined.[20] All determination was performed in triplicate for all three extracts.

Test organisms

G positive organisms such as *Staphylococcus aureus* (MTCC 96), *Bacillus subtilis* (MTCC 441) and g negative organisms like *Klebsiella pneumonia* (MTCC109), *Proteus mirabilis* (MTCC 425), *Pseudomonas aeruginosa* (MTCC 424), *Enterobacter faecalis* (MTCC 459) *and Escherichia coli* (MTCC 443) were used.

Antibacterial Assay

Antibacterial assay was done according to agar diffusion method. The nutrient agar medium was prepared by weighing 0.5g of peptone, 0.3g of beef extract/yeast extract, 0.5g of sodium chloride and 1.5g of agar in 100ml of distilled water. The prepared media was auto-claved at 121°C for 15 min. The media was poured into the plates and allowed to solidify. Inoculum of 24 h culture was swabbed (rubbed) on the plate with the help of cotton swab. Wells were punched on each plate using sterile borer. Each plant extract were added to wells which were punched. The plates were incubated in an upright position at 37°C for 24 h in an incubator. Antibacterial activity was determined by measuring the diameter of zone of inhibition using Himedia zone measuring scale.

High Pressure Liquid Chromatographic analysis

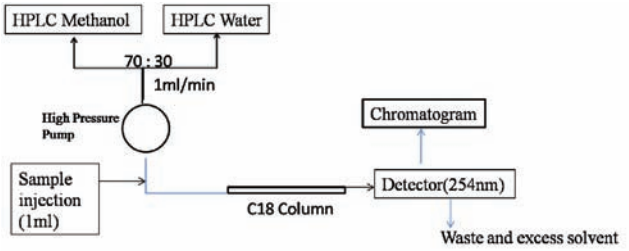
The extracted samples were subjected to HPLC analysis using Shimadzu HPLC system with C18 column, liquid pump LC-10 AT, system controller SCL-10A, UV-Vis detector SPD-10A and the detection at 254 nm with mobile phase methanol : HPLC grade water (70:30, v/v) flow rate 1ml/min. Identification of the peaks were done with references.[21]

Sample preparation for HPLC analysis

Previously obtained dried phenolic extracts was used for the sample preparation. 100mg of the sample was mixed with 10 ml HPLC grade methanol and dissolved without any agglomeration. From the 10ml, 1ml of the sample was injected into the HPLC and chromatog was obtained for each sample.

In-vitro antioxidant activity: DPPH free radical scavenging activity

DPPH stock was prepared by dissolving 0.2 mg of DPPH in 15.3 ml methanol it was protected from light by covering the test tubes with aluminum foil. 150 µl DPPH solution from stock was added to 3 ml methanol and absorbance was taken immediately at 516 nm for control reading. Different volume levels of test sample (50, 100, 150 and 200 µl) were made 200µl of each dose level by dilution with methanol. Diluted with methanol with up to 3 ml. 150 µl l DPPH solution was added to each test tube. Absorbance was taken at 516 nm in UV-visible spectrophotometer after 15 min using methanol as a blank. Scavenging activity was calculated by using following formula.[22]



Scheme 1: Diagmatic representation of HPLC

$$\text{Scavenging Activity \%} = \frac{(\text{Control absorbance} - \text{Sample absorbance})}{\text{Control absorbance}}$$

$$\frac{(\text{Control absorbance} - \text{Sample absorbance})}{\text{Control absorbance}} \times 100$$

Each experiment was carried out in triplicate and results are expressed as mean % scavenging activity.

RESULTS AND DISCUSSION

Determination of Total Phenolic Compounds

Phenolic compounds are known to act as antioxidants not only because of their ability to donate hydrogen or electrons but because they form stable radical intermediates, which prevent various food ingredients from oxidation.[23] The total phenolic compound of the plant extracts was determined using Folin- Ciocalteu method. Maximum phenolic content was obtained in *Lawsonia inermis* (5.2µg/ml) and Murraya konegii was the lowest phenolic content (2.3µg/ml). These results were tabulated (Table 1).

Components in the methanolic extract of Plants revealed by HPLC analysis

Phenolic compounds are commonly found in both edible and non edible plants, and they have been reported to have multiple biological effects, including antioxidant activity. HPLC analysis of phenolic compounds was done using methanol and water as mobile phase at 254 nm.

Table 1: Estimation of Total Phenolic Content.

Name of the plants	Total phenolic content µg/ml
Azadirachta indica	3.1
Acalypha indica	3.8
Lawsonia inermis	5.2
Murraya konegii	2.3

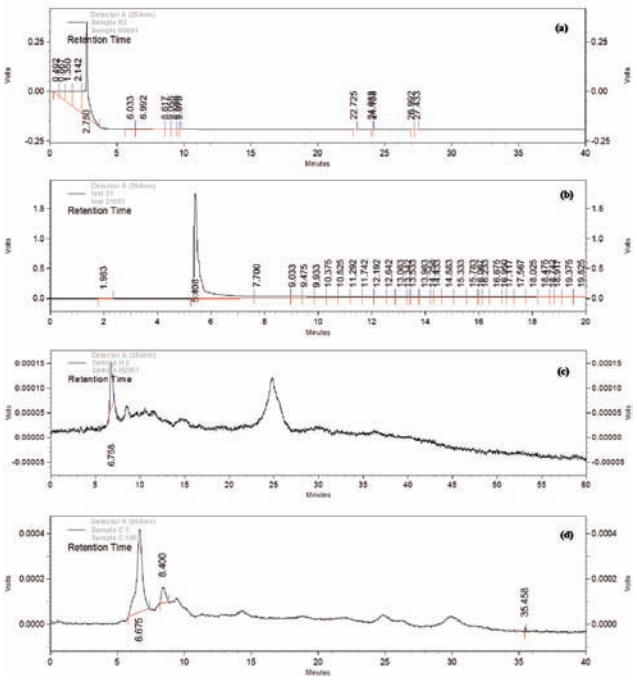


Figure 1: HPLC Analysis of (a) *Azadirachta indica* (b) *Acalypha indica* (c) *Lawsonia inermis* (d) *Murraya konegii*.

According to Hiroyuki.S et.al, (2003) simple phenols were eluted at the retention time between 5.8 and 34.3min, catechins at 8.1 and 26.1min, anthocyanins between 17.4 and 47.7 min, flavones, flavonols, isoflavones and flavanones in the form of glycoside were eluted between 20.1 and 61.4 and aglycons at retention time between 26.7 and 91.6min.[24]

In Azadirachta indica phenolic compound extract, 16 peaks were seen (Figure 1(a)) in different retention time from 0.491 to 27.433min. Simple polyphenols were eluted at retention time of 6.033min, catechins at 8.617, 9.058, 9.500 and 9.675 min. Glycosides form of flavones, flavonols, isoflavones and flavanones were seen at retention time 22.725, 24.033 and 24.158. At the retention time of 26.992 and 27.433 min aglycons were eluted.

Extract from *Acalypha indica* (Figure 1(b)) a showed 33 peaks. Herein, at 5.408 min simple polyphenols were eluted; catechins were eluted at 7.700min. Anthocyanins were eluted at 17.117 and 17.567 min. The glycoside forms of flavones, flavonols, isoflavones and flavanones were appeared at 19.375 and 19.825mins

Phenolic extract of Murraya konegii shows (Figure 1(d)) three peaks, at a retention time of 6.675min which were simple phenols. Catechins were eluted at 8.400 min where as at a retention time of 35.458 min aglycons were eluted. *Lawsonia inermis* extract showed (Figure 1(c)) single peak at 6.758 min which indicates the elution of only simple polyphenols

Antioxidant Activity of plant extracts

Natural antioxidants present in plants are responsible for inhibiting or preventing oxidative stress. In this study, evaluation of free radical scavenging percentage was determined.[25] The decrease in the absorbance of DPPH radical causes due to the reaction between antioxidant molecules and radical that result in the scavenging of radical by hydrogen donation.[22] The determined results were tabulated (Table 2).Among the four extracts *Lawsonia inermis* showed higher percentage of scavenging activity in minimum concentration.

Antibacterial Assay

Antibacterial study was done with the prepared extracts. It was observed that all the prepared plant extracts were effective inhibiting both g positive and g negative organisms. (Table 3). Among the four extracts *Lawsonia inermis* showed highest zone of inhibition on *Proteus sp* (32mm). *Azadirachta indica* shows maximum activity against *Proteus* and *Staphylococcus*. *Murraya konegii* showed mild activity against *Proteus* and *Pseudomonas*. *Acalypha indica* was found to inhibit *Enterococuss* and *Proteus*. Even though the extracts showed activity against g positive and negative organisms, g negative bacteria showed maximum level of inhibition. These variations of the antimicrobial study may be due to the differences in cell surface structures between g-negative and g-positive bacteria.[26] The cell wall of g positive bacteria is a coat of murein, whereas g negative bacteria possess a second lipid bilayer called outer membrane. Murein layer of g positive bacteria is generally much thicker than that of g negative. Hence the murein membrane acts as barrier for the phenolic compound to act against the g positive bacteria.

CONCLUSION

In conclusion, phenolic extract of the four plants were analyzed for total phenolic content by using Folin- Ciocalteu method and were tested for antibacterial assay. HPLC analysis was done to determine the various phenolic components present in the extract. From the results obtained it was observed that *Lawsonia inermis* showed higher total phenolic content and posses a

Table 2: DPPH antioxidant scavenging activity.

Extract	% Scavenging activity			
	50 ml	100 ml	150 ml	200 ml
Acalypha indica	42.40	65.72	73.45	76.70
Azadirachta indica	37.70	62.20	77.50	78.50
Lawsonia Inermis	44.20	67.80	82.53	86.11
Murraya konegii	36.80	59.40	73.40	80.45

Table 3: Determination of Antimicrobial activity.

Plant extracts	Zone of Inhibition (mm)						
	P.mirabilis	*K. pneumonia*	*P.aeruginosa*	*E. fecalis*	*E.coli*	*B.subtilis*	*S.aureus*
Acalypha indica	18	13	16	18	10	15	16
Azadirachta indica	28	23	17	20	18	20	26
Lawsonia inermis	32	24	18	16	13	17	20
Murraya konegii	21	20	24	19	17	19	24
Control	11	14	12	10	10	11	15

Note: Zone of inhibition for the extracts was calculated by subtracting the control value.

single peak for simple phenols as obtained from chromatog. Moreover, it exhibits maximum zone of inhibition on both g positive and g negative organisms. Since, the amount of phenolic compound was proportional to antimicrobial activity. The results imply that phenolic compound play an important role in inhibition of bacterial cells.

ACKNOLWEDGEMENT

The authors are thankful to the Department of Chemical Engineering, A.C.Tech, Anna University.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ABBREVIATIONS USED

HPLC: High Pressure Liquid Chromatography; **DPPH-** 2,2-Diphenyl-1-picrylhydrazyl.

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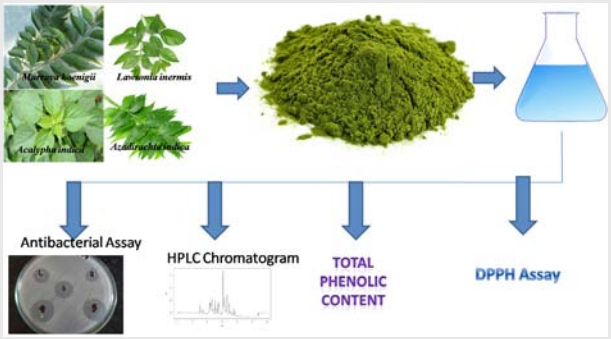
PICTORIAL ABSTRACT



SUMMARY

- The work was done to evaluate total phenolic content of prepared plant extracts and its antioxidant properties. Plant extracts were prepared using methanol as the solvent. Total phenolic content was estimated using Folin-Ciocalteu method and HPLC. The extracted samples were tested for antibacterial activities. As the phenolic compound has an antioxidant activity, DPPH assay was done to revel the extract with higher antioxidant active of samples. As the results obtained Lawsonia inermis has higher antioxidant properties as compared to other extracts.

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Cite this article: Supritha P and Radha KV. Estimation of Phenolic Compounds Present in the Plant Extracts Using High Pressure Liquid Chromatography, Antioxidant Properties and its Antibacterial Activity. Indian J of Pharmaceutical Education and Research. 2018;52(2):321-6.