

# Phenolic acid content, antioxidant properties, and antibacterial potential of flowers and fruits from selected Pakistani indigenous medicinal plants

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**ABSTRACT:** Biological evaluation of *Lantana camara* and *Justicia adhatoda*, belonging to the medicinal plant families Verbenaceae and Acanthaceae, respectively, was performed. Both plants contain a very good range of phenolic compounds ( $20.5 \pm 1.9$ – $80.9 \pm 4.1$  mg GAE/g of dry extract). HPLC analysis of *Justicia adhatoda* flower and fruit extract showed a broad profile of phenolic acids such as *p*-hydroxy benzoic acid, syringic acid, gallic acid, and quercetin in substantial amounts. The antioxidant study showed moderate to good ability to inhibit linoleic acid peroxidation and to scavenge DPPH free radicals ( $67.5 \pm 4.7$  to  $80.4 \pm 3.0\%$  and  $71.3 \pm 3.8$  to  $85.9 \pm 2.0\%$ , respectively). The antibacterial study reflected good antibacterial potential. This study showed that extracts of flowers and fruits of both plants grown in Soon Skaser Valley, Pakistan have a strong potential to scavenge free radicals and may be used to develop functional food and herbal medicines to treat oxidative stress diseases and bacterial infections.

**KEYWORDS:** reactive oxygen species, lipid peroxidation, Folin-Ciocalteu reagent, *Bacillus* sp., *Staphylococcus* sp.

## INTRODUCTION

Several research groups have explored and reported that the presence of reactive oxygen species (ROS) enhances the oxidative stress in a living body which triggers chronic diseases such as asthma, diabetes, cardiovascular diseases, and cancer<sup>1–3</sup>. ROS include a wide range of chemicals, prominently nitric oxide, hydrogen peroxide, superoxide anion, hydroxyl radicals, peroxynitrite anions, alkylperoxyl and hydroperoxyl radicals that are actual cause of degenerative processes in human body<sup>4</sup>. The formation of ROS, an oxidant by-product of normal metabolism, cannot be stopped to form in human body due to inhaling extensive amount of oxygen daily<sup>5</sup>. ROS work in two directions; on one side ROS and immune system work collectively to kill pathogens and on the other side, which is dominant in our environmental conditions, they cause chronic illnesses. However, we can protect

human body by creating balance between antioxidants and oxidants. Human defensive system fights against ROS to protect the body from degenerative processes but, when it becomes overwhelmed by an extensive generation of oxidants, our body remains no longer safe from tissue injury via oxidative damage of the proteins, nucleic acids, and lipids. Numerous studies have indicated that daily uptake of antioxidants offer a healthy lifestyle<sup>6</sup> and impart strength to defensive system of living organisms to nullify the effect of oxidants, by-products of normal body metabolism. In addition to natural foods, it has also been reported that wild plants and agricultural wastes such as olive waste and grape seed extracts have also been successfully applied for the isolation of natural antioxidants on industrial scale<sup>7,8</sup>.

In the present study we have selected two wild medicinal plants grown in valley Soon Skaser of Pakistan, *Lantana camara* L. and *Justicia adhatoda* which

belong to family Verbenaceae and Acanthaceae, respectively. The reason to select these plants of different families is that they grow widely in the valley of Soon Skaser and are extensively used in a traditional way to treat ailments. *L. camara* is a common wild shrub which can grow 2–4 m in height, native to the American tropics<sup>9</sup> and extensively planted as an ornamental and hedge plant in Asia. In Pakistan, it commonly grows at 1500 m altitudes and is known to be used in folk medicines<sup>10</sup>. *J. adhatoda* grows wild in abundance all over India, Nepal and Pakistan<sup>11</sup>. It is commonly known as Vasaka or Malabar nut with unpleasant smell and bitter taste<sup>12</sup>. The medicinal importance of *J. adhatoda* to treat respiratory disorder can be well understood from the Asian saying “No man suffering from phthisis need despair as long as the *Vasaka* plant exists”<sup>13</sup>. However, limited studies have been conducted in order to explore the antioxidant and antibacterial activities of its flowers and fruit extracts.

Our aim in this study was to explore the phenolic compounds present in flowers and fruits of *J. adhatoda* through state-of-the-art technique HPLC, antioxidant, and antibacterial assays of methanolic and ethanolic extracts of flowers and fruits of both plants.

## MATERIALS AND METHODS

### Materials

All chemical and reagents used in this work were of reagent grade. Folin-Ciocalteu's phenol reagent, ascorbic acid, linoleic acid, 1,1-diphenyl-2-picryl hydrazyl (DPPH), trichloroacetic acid, butyl hydroxyl toluene (BHT), gallic acid, butylated hydroxyanisole (BHA) and all phenolic standards were purchased from Sigma-Aldrich Chemical Co. Millipore type filters (pore size 0.22  $\mu\text{m}$  and 0.45  $\mu\text{m}$ ) were obtained from Sartorius, USA. UV-Vis absorption was measured using a double beam U-2800 UV-Vis spectrometer (HITACHI).

### Extraction

Fully matured flowers and fruits of *L. camara* and *J. adhatoda* were collected from Soon Sakesar valley situated in the central Punjab, Pakistan and specimens were identified at the Department of Botany, Government College University, Faisalabad. Voucher Specimens were deposited at the Department of Botany, Government College University, Faisalabad. The fruits and flowers were manually separated from plants and carefully washed with distilled water to remove unwanted materials and dried in shade. Dried material was ground to obtain 80 mesh size powders.

The powder of fruits and flowers (20 g) was extracted by mixing 200 ml of 80% methanol or ethanol using orbital shaker at 200 rpm. The extract was filtered using filter paper (Whatman No. 41) and evaporated at low pressure to get dried material. All dried samples were stored at  $-4^{\circ}\text{C}$  until further analysis.

### Determination of total phenolic compounds

The total concentration of phenolic compounds in the fruits and flowers extracts was determined according to the method reported by Bozin et al<sup>14</sup> with slight modification and results were expressed as gallic acid equivalents (GAE). The extracts of fruits and flowers were dissolved (w/v, 0.05 g/2 ml) in ethanol. Samples (1 ml) were mixed with 15-fold diluted solution of Folin-Ciocalteu reagent. The mixture was kept at room temperature for 10 min followed by the addition of 0.75 ml of 20%  $\text{Na}_2\text{CO}_3$  (w/v). After subjected to heat in a water bath at  $40^{\circ}\text{C}$  for 20 min, the mixture was allowed to cool in an ice-bath at  $4^{\circ}\text{C}$ . The absorbance of cooled mixture was noted at 755 nm using double beam U-2800 UV-Vis spectrometer. Amount of TPC was calculated using a calibrated curve for gallic acid (10–130 ppm) in triplicate and results were averaged.

### Determination of total flavonoid

Total flavonoid contents were determined following the procedure described by Bozin et al<sup>14</sup>. One millilitre of solution containing 0.01 g/ml of dry crude extract followed by the addition of 5 ml of distilled water and 0.3 ml of 5%  $\text{NaNO}_2$  was placed in a 10 ml volumetric flask. The mixture was incubated for 5 min and then 0.6 ml of 10%  $\text{AlCl}_3$  and 2 ml of 1 M NaOH were added. The total volume was made up to mark with distilled water. Absorbance was measured at 510 nm. TFC were expressed as catechin equivalents mg/100 g of dry extract.

### HPLC analysis for phenolic compounds

Phenolic acids were determined by HPLC (Shimadzu) analysis. The extracted sample (0.025 g) was dissolved in 1 ml methanol followed by passing through membrane filter with 0.45  $\mu\text{m}$  pore size and 20  $\mu\text{l}$  of filtered solution was then injected into HPLC through sample injection valve. The HPLC system is equipped with a diode array detector ( $\lambda_{\text{max}} = 278 \text{ nm}$ ) and EclipseXDB-C18 column (Agilent) (dimension: 250 mm  $\times$  4.60 mm, 5  $\mu\text{m}$ ) operated at  $30^{\circ}\text{C}$ . The elution solvents were 100% acetic acid (A) and 100% acetonitrile (B). The sample was analysed for phenolic compounds using the following gradient elution: 99.2% A in B for 0.1 min and 90% A in B for 1.0 min

**Table 1** Extraction yield, total phenolic, and total flavonoid content of methanolic and ethanolic extract of flower and fruits of *L. camara* and *J. adhatoda*.

Sr. no.	Plant part	Yield (g/100 g dry extract)		Total phenolic contents (mg/100 g dry extract)		Total flavonoid contents (mg/100 g dry extract)	
		methanol	ethanol	methanol	ethanol	methanol	ethanol
Flowers							
1	<i>L. camara</i>	10.21	5.32	50.2 ± 2.2	30.6 ± 2.1	20.1 ± 1.9	10.1 ± 1.2
2	<i>J. adhatoda</i>	14.66	13.33	30.4 ± 3.3	40.8 ± 3.2	11.0 ± 1.2	40.8 ± 4.1
Fruits							
1	<i>L. camara</i>	12.66	11.33	80.9 ± 4.1	20.5 ± 1.9	10.1 ± 1.2	10.6 ± 1.6
2	<i>J. adhatoda</i>	14.12	11.89	40.2 ± 3.0	50.8 ± 3.4	10.9 ± 1.4	10.7 ± 1.1

Values are presented as mean ± S.D. ( $n = 3$ ).

as initial conditions; 70% A in B for another 1 min followed by 50% A in B for 35 min, 20% A in B 5 min, and finally 0% A in B for 5 min. The flow rate of elution was kept at 1 ml/min. Peaks were identified according to the standards run under same conditions.

#### DPPH free radical scavenging activity

The potential of scavenging DPPH free radical was also studied using the method reported by Bozin et al<sup>14</sup> with slight change. Briefly, 2 ml solution of extract in methanol (w/v, 10 mg per 10 ml methanol) was taken in test tube followed by the addition of equal volume of DPPH solution. All test tubes were put in dark for 30 min. At the end of incubation time the absorbance was noted at 515 nm using a UV-Vis spectrophotometer. Scavenging activity was calculated as (Absorbance of DPPH solution – Sample absorbance)/(Absorbance of DPPH solution).

#### Determination of % inhibition of peroxidation in linoleic acid system

This study was performed following the method reported by Marxen et al<sup>15</sup> with slight modifications. A 20 mg sample was mixed to a solution containing linoleic acid (0.10 ml), 99.5% ethanol (8 ml), 0.2 M Na<sub>3</sub>PO<sub>4</sub> buffer of pH 7.0 (8 ml), and the solution was diluted to 20 ml with distilled water followed by incubation for 75 h at 40 °C. At the end of incubation period the extent of oxidation was measured following thiocyanate method reported by Yen et al<sup>16</sup>. Briefly, 10 ml 75% ethanol, 0.2 ml 30% of an aqueous solution of ammonium thiocyanate, 0.2 ml of sample solution, and 20 mM ferrous chloride (FeCl<sub>2</sub>) solution in 4% HCl were mixed sequentially. The solution was then stirred for 3 min and absorbance at 500 nm was monitored. A blank sample with linoleic acid but without extract sample was taken as negative control while BHT was used as a positive control. The

minimum peroxidation level was measured after 75 h (3 days) in the blank sample. The inhibition of linoleic acid per oxidation was calculated as 1 – (Abs. increase of sample at 75 h)/(Abs. increase of control at 75 h).

#### Antibacterial potential assay

Antibacterial potential of each extract was determined by following a reported method<sup>17</sup>. For inoculation, liquid culture of 4 selected bacteria (100 µl) with optical density value 1.4–1.6 at 600 nm was used to inoculate 10 ml LB medium having no extract (0 µg as control), 12.5 µg, 25 µg, 37.5 µg, and 50 µg of each flower and fruit extract. In order to grow all four bacterial strains, all tubes were incubated at 37 °C for 24 h. On completion of the incubation period, the optical density of each growing strain was recorded with a UV-Vis spectrophotometer at 600 nm.

## RESULTS

#### Extract yield, phenolic acid and flavonoid compounds determination

Table 1 shows the yields, total phenolic acids and total flavonoid compounds of methanolic and ethanolic extracts of flowers and fruits. The amounts of extractable substances were expressed as percentage by weight of dried flowers and fruits. Total phenolic and total flavonoid contents were also present in appreciable quantity and were expressed as GAE.

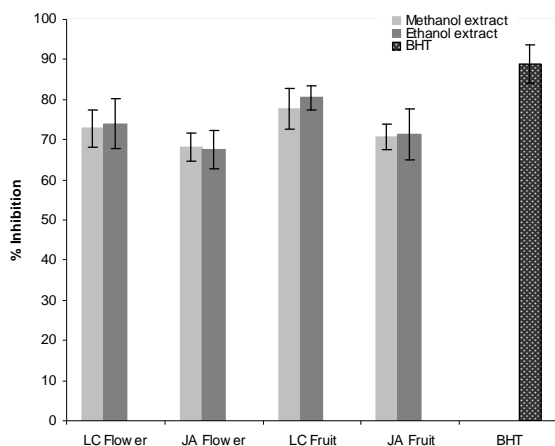
#### HPLC analysis for phenolic acids

The HPLC patterns of different phenolic acid present in extracts of *J. adhatoda* flowers and fruits were analysed by comparing the peaks of standard phenolic compounds. Comparison was performed with eleven biologically important phenolic acids and the results were summarized in Table 2.

**Table 2** HPLC analysis of methanolic extracts of flowers and fruits of *J. adhatoda* for phenolic acids.

No.	Standards	Retention time (min)	Flowers		Fruits	
			Retention time (min)	Concentration (mg/100 g)	Retention time (min)	Concentration (mg/100 g)
1	vanillic acid	5.343	5.33 ± 0.03	10.50 ± 0.32	5.30 ± 0.05	2.68 ± 0.25
2	tannic acid	6.000	5.901 ± 0.012	1.33 ± 0.21	5.93 ± 0.08	1.17 ± 0.12
3	ellagic acid	6.436	—	ND	6.50 ± 0.09	2.15 ± 0.14
4	3,4-dihydroxy benzoic acid	9.178	9.26 ± 0.06	1.98 ± 0.11	—	ND
5	syringic acid	10.401	10.52 ± 0.04	3.13 ± 0.19	10.38 ± 0.06	5.64 ± 0.41
6	<i>p</i> -hydroxy benzoic acid	11.707	11.77 ± 0.08	6.84 ± 0.25	11.80 ± 0.09	372.1 ± 4.7
7	ferulic acid	15.754	—	ND	15.81 ± 0.08	1.54 ± 0.13
8	quercetin	22.345	—	ND	22.42 ± 0.10	1.65 ± 0.19
9	caffeic acid	38.151	—	ND	—	ND
10	gallic acid	48.797	48.69 ± 0.04	2.89 ± 0.17	—	ND
11	<i>p</i> -coumaric acid	49.112	49.10 ± 0.04	1.80 ± 0.09	—	ND

The values are presented as mean ± S.D ( $n = 3$ ); ND = not detected.



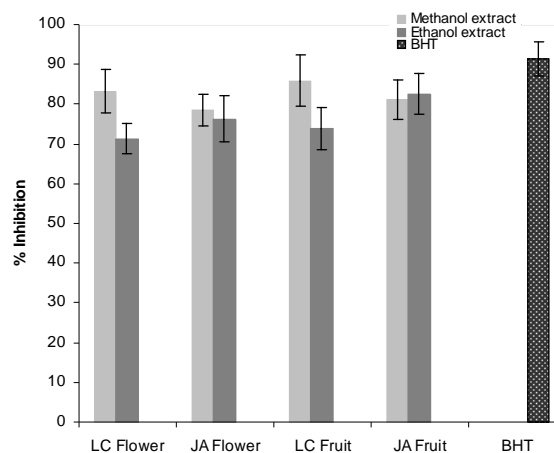
**Fig. 1** DPPH free radical scavenging potential of phenolic acids in the methanol and ethanol extracts of flowers and fruits of *L. camara* and *J. adhatoda*.

### DPP free radical scavenging activity

Fig. 1 explained the DPPH free radical scavenging ability of flowers and fruits of both plants. Methanol extract of *L. camara* flower and *J. adhatoda* fruit showed  $83.2 \pm 3.6$  and  $85.9 \pm 2\%$  scavenging activity, respectively. However, ethanolic extract contained less phenolic acids and less free radical scavenging potential as compared to methanolic extract except *J. adhatoda* fruit extract that showed  $82.6 \pm 4\%$  activity.

### Inhibition of linoleic acid peroxide

The data obtained by performing linoleic acid peroxide inhibition was shown in Fig. 2. All the extracts except that of *J. adhatoda* flower showed promising



**Fig. 2** Percent inhibition of linoleic acid oxidation by methanol and ethanol extracts of flowers and fruits of *L. camara* and *J. adhatoda*.

inhibition of linoleic acid peroxidation. However the observed % inhibition of linoleic acid peroxidation for all extracts was less than for the reference compound (BHT).

### Antibacterial activity

In the antibacterial assays, extracts of *L. camara* and *J. adhatoda* flowers and fruits were also evaluated for their antibacterial activities against four bacterial strains, viz., *Bacillus* sp., *Staphylococcus* sp., *Streptococcus* sp., and *E. coli*. Overall all extracts showed biological activity in terms of 50% inhibition of bacterial growth with reference to control (devoid of plant extract) in concentration range 25.0  $\mu$ g to 37.5  $\mu$ g (Table 3).

**Table 3** Antibacterial activity in terms of IC<sub>50</sub> values of methanolic and ethanolic extracts of flowers and fruits of *L. camara* and *J. adhatoda*.

Microorganism	<i>L. camara</i> IC <sub>50</sub> (µg)				<i>J. adhatoda</i> IC <sub>50</sub> (µg)			
	flower extract		fruit extract		flower extract		fruit extract	
	MeOH	EtOH	MeOH	EtOH	MeOH	EtOH	MeOH	EtOH
<i>Bacillus</i> sp.	25.0	37.5	37.5	50.0	37.5	25.0	50.0	50.0
<i>Staphylococcus</i> sp.	37.5	37.5	50.0	37.5	25.0	25.0	37.5	37.5
<i>Streptococcus</i> sp.	25.0	25.0	50.0	50.0	25.0	25.0	50.0	50.0
<i>E. coli</i>	37.5	37.5	37.5	37.5	37.5	37.5	50.0	50.0

MeOH = methanol, EtOH = ethanol.

## DISCUSSION

The yields of extracts obtained from flower and fruits of both plants were in good quantity (5% to 15%). Methanol was found to be the solvent of choice to extract plant contents better than ethanol. Methanol extract of *L. camara* exhibited significant phenolic acid contents in case of flowers and fruits while flower and fruits of *J. adhatoda* showed lowest TPC in case of methanol extract and higher in the case of ethanol extracts. From the data obtained, methanol was found to be the effective solvent for the extraction of phenolic components. Previously reported data also recommend methanol as the better solvent for phenolic compounds extraction<sup>18,19</sup>. However, TFC was obtained in good yield in the ethanol extract of *J. adhatoda* flower (40.8 ± 4.1 mg/100 g) followed by the methanol extract of *L. camara* flower (20.1 ± 1.9 mg/100 g). All other extracts exhibited low quantity of TFC (Table 1). In order to detect phenolic acids, eleven phenolic acids in extracted samples were run as reference standards on HPLC before analyzing the flower and fruits extract of *J. adhatoda*. The analysis showed that *p*-hydroxy benzoic acid was present in significant amount (372.1 mg/100 g dry extract) in *J. adhatoda* fruit extract but in small quantity (11.77 mg/100 g dry extract) in *J. adhatoda* flower extract. Other important phenolic acids such as syringic acid, *p*-hydroxybenzoic acid, quercetin, gallic acid and *p*-coumaric acid were also detected in both sample extracts (Table 2).

Plants may carry number of classes of biological active compounds but the presence of phenolic compounds in plants defines their medicinal importance<sup>20</sup>. Phenolic acids are predominantly associated with antioxidant activities predominantly by scavenging free radicals produced in the human body. For in vitro free radical scavenging model, DPPH free radical is widely used to investigate antioxidant potential of natural material extracts<sup>21</sup>. Methanol extracts of

*L. camara* showed significant DPPH free radical scavenging ability (> 85%) as compared to *J. adhatoda* extracts (< 80%). Anyhow, all extracts showed good DPPH free radical scavenging activity.

Further confirmation of antioxidant activity was assessed using inhibition of linoleic acid peroxidation. Ethanol extract of *L. camara* fruit showed > 80% inhibition of linoleic acid peroxidation, followed by its methanol fruit extract. Other extracts showed inhibition in the range of 67–73% (Fig. 2). However, overall antioxidant results demonstrated good correlation between phenolic compounds and antioxidant activity. The closely related correlation has also been reported earlier in other reports<sup>22–24</sup>.

Antibacterial activity of all extracts showed 50% inhibition of bacterial growth in the range of 25–50 µg (Table 3). Flower extracts in methanol and ethanol of both plants showed good inhibition against bacterial growth. *Bacillus* sp. and *Streptococcus* sp. were more susceptible to *L. camara* flower extracts while *Staphylococcus* sp. and *Streptococcus* sp. to *J. adhatoda* flower extracts. However the fruit extracts showed relatively low antibacterial activity (IC<sub>50</sub> = 37.5–50.0 µg). Most of them showed IC<sub>50</sub> = 50.0 µg, which exhibited weak antibacterial effect. Our findings are in good agreement with the antibacterial potential of extracts of both parts of *L. camara* and *J. adhatoda* against different strains of bacteria<sup>12,25,26</sup>.

## CONCLUSIONS

Biological evaluation of flowers and fruits of medicinal important shrubs *L. camara* and *J. adhatoda* in term of antioxidant and antibacterial potential revealed that the extract could be used to prepare herbal medicines. Further the isolation of individual components could be used to derive more active compound to combat diseases caused by oxidative stress and bacterial infection.

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