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Original Research Article

DETECTION OF BIOACTIVE FRACTIONS OF *JUSTICIA ADHATODA* L. LEAVES

Faiza Rasheed[1*], Waqas Khan Kayani[2], Adeel Mahmood[3] and Muhammad Gulfraz[4]

1. [*]Department of Agro systems, P.O Box 104, The Swedish University of Agriculture Sciences, SE 230 53, Alnarp, Sweden
2. Molecular Biology Laboratory, Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, 45320, Pakistan
3. Department of Plant Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, 45320, Pakistan
4. Department of Biochemistry, PMAS Arid Agriculture University Rawalpindi.

ABSTRACT

In vitro antibacterial and antioxidant activities of various leaf extracts of *Justicia adhatoda* L. (locally known as Bhaikar) were assessed. The leaves were also subjected to various phytochemical analysis. Results revealed that leaves of *J. adhatoda* L. contain significant amount of total alkaloid, phenols flavonoid, saponins, tannins , protein, crude oil, dietary fiber, essential and non essential metal ions. The methanol, ethanol, butanol, chloroform and *n*-hexane *leaf extracts* of *J. adhatoda* significantly inhibited the growth of all bacteria tested as compared to standard antibiotic. However, acetone and aqueous leaf extracts of *J. adhaotda* were not effective against any bacteria. Methanol extract of *J. addhatoda* provide lowest Minimum Inhibitory Concentration (MIC) for *E. coli* (1.0 mg/ml) followed by *Klebsella. pneumoni* (1.2 mg/ml), where as MIC values of other solvent extracts were in the order of ethanol > chloroform>n-hexane > butanol. The order of antioxidant activities of various leaf extracts found in different solvent extractions was ethanol >methanol >chloroform> water> butanol>. This study will help to promote scientifically use of *J. adhatoda* in local medicine as well as its use as an important raw material for pharmaceutical industries.

Keywords**:** *Justicia adhatoda*, Phtochemicals, Antimicrobial compounds, Bioactivity

Corresponding Author: **Faiza Rasheed**[*] Department of Agro systems, P.O Box 104, The Swedish University of Agriculture Sciences, SE 230 53, Alnarp, Sweden. E.: faizi.uaar@gmail.com

INTRODUCTION

Plants produce a vast array of bioactive compounds that are involved in essential self-preserving functions, such as photosynthesis, respiration, protection from oxidative reactions and defense against microorganisms (Black *et al.*, 2008 ; Kumar *et al*., 2006). It has been shown by numerous research

groups that aromatic and medicinal plants are sources of diverse nutrient and non-nutrient compounds. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolic compounds. (Edeoga *et al*., 2005).

Justicia adhatoda Linn, syn. *Adhatoda zeylanica* Medic. *Adhatoda vasica* Nees. dominant vegetation of hilly areas of Rawalpindi, Islamabad and extended up to NWFP (Khattak and Gillani, 1985). It systematic position is family Acanthaceae, subclass Asteridae ana specie *Adhatoda*.

The wound healing and anti-diabetic properties of *J. adhatoda* L. were documented by different authors including Bruke *et al*. (2006). Antimicrobial activity of *A. zelanica* . was also reported by Madhu and Devi (2000) against several microorganisms including one gram positive *(Staphylococcus aureus*) and three gram-negative *(Escherichia coli, Pseudomonas aeruginosa* and *Shigella boydii*) ones Their results show significant activity against all the tested microorganisms except *E. coli.*

The antimicrobial activity of methanolic extracts of *J.adhatoda* L. against *E. coli, K. pneumoniae* and *S. aureus* and anti-inflammatory activity was reported by Juneja *et al.* (2007). They reported that powder made out of its leaves along with other plants (*Eclipta alba, Zingiber officinale, Azadirachta indica, Ocimum sanctum etc*) is used as a remedy for diseases like asthma bronchitis, chronic fever, cough, leucorrhoea, prostate enlargement and bleeding piles. Keeping in view the importance of *J. adhatoda* as important medicinal plant, present study was conducted to assess phytochemical analysis as well as antimicrobial and *in vitro* anti-oxidant activity of leaf extracts of *J. adhatoda.*

MATERIAL AND METHODS

Collection of Sample : Leaves sample of *J. adhatoda* L. was collected in fine plastic bags from surrounding area of Islamabad.

Preparation of Sample: Leaves samples of *J. adhatoda* L. were air dried followed by oven drying and ground to fine powder (80 mashes) with the help of pestle mortar and electric blender. Samples were saved in fine plastic bags and stored at 4ºC for further analysis.

Biochemical Analysis of *Justicia adhatoda*

Protein content (nitrogen × 6.25) was determined by micro-Kjeldahl nitrogen analysis by using AOAC 979.09 and 920.87 methods (AOAC, 1990). The oil contents were analyzed by AOAC method, 920.85 (AOAC, 1990) with Soxhlet apparatus. In the Soxhlet extraction procedure, 4 g of the powdered form of sample was packed in a thimble and the oil was extracted with diethyl ether for 4 hours. Ash and fibre content was determined by AOAC methods 942.05 and 962.09 respectively.

Analysis of fatty acids with gas chromatography (GC)

The leaves sample *J. adhatod* L. were mixed with boron trifluoride (BF_3)-methanol reagent (20%). The fatty acids were converted into the methyl ester derivatives using method of Morrison and Smith (1964). The methyl esters of the fatty acids were dissolved in chloroform ($CHCl_3$) and analyzed with the help of GC.

Determination of Phenolic Compounds: Total phenolic contents were extracted by boiling 2 gm of defatted sample with 50 mL of diethyl ether in water bath for 15 minutes as described by Lillian *et al*. (2007).

Determination of Flavonoid: Flavonoid contents were determined by dissolving 5 gm of sample in 50 mL of 80% aqueous ethanol and the whole mixture was left in shaker incubator for 24 hours.

Further analysis of flavonoid from leaves of *J.adhatoda* was performed by using method reported by Lillian *et al*. (2007).

Determination of Tannins: Tannins were extracted by dissolving 0.5 gm of sample in 100 mL of 70% acetone. Different concentrations of tannic acid (6.25 mg- 50 mg) were prepared by serial dilution from stock solution (50 mg/100 mL of 70% acetone). The absorbance was measured at 725 nm after the addition of 0.5 mL of folin-phenol reagent and 2.5 mL of Na_2CO_3 (Trease and Evans, 1989).

Determination of Alkaloids: The dried sample was dissolved in ethanol (1:10) and was left on shaking for 24 hours. Extract was concentrated near to dryness in oven and was re-dissolved in ethanol with addition of 1% HCl. The mixture was placed in refrigerator for three days. The solution was filtered and pH was maintained 8-10 and was extracted with chloroform by using separating funnel. Chloroform layer was recovered and ethanol layer was discarded where as the solution was heated in hot water bath for evaporation. After that the sample was dried in oven to constant weight. Alkaloid contents were calculated on the basis of weight obtained and weight used (Trease and Evans, 1989; Edeoga *et al*.,2005).

Determination of Saponins: The saponins were extracted by mixing 10 gm of dried sample with 50 mL of 20% aqueous ethanol. The mixture was heated over a shaking water bath at 55ºC for 4 hours and the amount of saponins in leaves of *J .adhatoda* L. was determined following method reported by Harbone (1973)

Determination of Metal ions: For metal ion detection, samples were digested by using dry digestion method. Total 1 gram of sample was placed in porcelain crucible and ashed at 450ºC for 18-20 hours. The ash was then dissolved in 1 mL concentrated nitric acid (HNO_3) and was evaporated to dryness Then it was heated again at 450ºC for 4 hours, treated with 1 mL concentrated H_2SO_4, 1mL HNO_3 and 1mL H_2O_2 and finally diluted with deionized water up to volume of 50 mL. Blank was also treated in the same way. Metal ions including Ca, Mg, Zn, Fe and Cu in the sample were determined by atomic absorbtion spectrophotometry while sodium and potassium were determined by flame atomic absorption spectroscopy.

Preparation plant extracts: The powder form of sample was extracted with different solvents (n-hexane, acetone, chloroform, butanol, ethanol, methanol and water) on the basis of their polarity. The sample was extracted by shaking with n-hexane (1:10) for 24 hours followed by centrifugation at 10,000 rpm for 15 minutes. Supernatant was then transferred to a pre-weighed falcon tube and residue was re-extracted with next solvent which was slightly polar then n-hexane. The same procedure was repeated with all solvents and all extracts were allowed to dryness in incubator. The dried extracts were dissolved in dimethylsulfoxide (DMSO) for antimicrobial assay.

Microorganisms Tested: Antimicrobial activity was tested against *Echerichia coli* (*E .coli*), *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*) *and Klebsella pneumonia* (*K. pneumonia*) by using agar well diffusion method. Inocula of all microbes were prepared in sterilized Lauria-Bertini media gL^{-1} (10 gm tryptophan, 10 gm NaCL, 5 gm yeast extract and distilled water) in separate test tubes which were then placed in shaker incubator at 37ºC for 24 hours to contain approximately 10^8 cfu/mL

Antimicrobial Activity: Antimicrobial activity was tested by agar well diffusion method as described by Rojas *et al* (2006).

Minimum inhibitory concentration: A quantity of 0.5g of each extract was dissolved in 4ml sterile Muller-Hinton broth which yields an initial concentration of 125 mg/l. Subsequently two fold serial dilutions were made from the stock of 4ml containing 125 mg/l. Muller-Hinton broth was used to obtain the following concentrations 125, 62.50, 31.250, 15.65, 7.83, 3.91, 1.95, 1.00, 0.50, 0.25 and 0.13 mg/l. One milliliter of a standardized inoculum of each test organisms was introduced into each extract-nutrient broth mixture and then incubated at 37 ˚C for 24 h. The lowest concentration of the extract that inhibited the test organisms was recorded as MIC values.

Antioxidant Activity: The ferric ion reducing power capability of samples was determined by using a modified method of Chen and Yen (1995). The extract (750 µl) of each sample was mixed with an equal amount of phosphate buffer (0.2 M, pH 6.6) and 1% potassium ferrciyanide (a source of ferric ions). The mixture was incubated at 50 ºC for 20 minutes followed by addition of an equal amount of trichloroacetic acid (10%) then centrifuged at 3000 rpm for 10 minutes. Upper layer (1.5 mL) was separated and mixed with an equal amount of any solvent and 0.1 mL $FeCl_3$ solution (0.1%). A blank was also prepared by using same procedure and the absorbance was measured at 700 nm as the reducing power (Alam *et al*.,2003).

RESULTS

Bioactive compound Determination

The results of phytochemicals analyzed from leaves of *J.adhatoda* are give in Tables 1 and 2. . The higher concentration of protein (21.33 %), ash (11.62 %), fiber (7.11 %), and crude oil (3.11 %) was obtained from leaves of *J. adhatoda* (Table 1). The data shows that alkaloid (71.5 mg/g), tannins (43.0 mg/g), flavonoids (130 mg/g), saponins (51.0 mg/g)and phenols (89.0 mg/g) were also present in leaves of *J. adhatoda* L. (Table 2).

Fatty Acid Composition of *J. adhatoda* L.

The result of gas chromatographic analysis of the leaves of *J. adhatoda* L. for fatty acid contents are shown in figure 4.1. According to results *J. adhatoda* L. contains the highest amount of linoleic acid (C: 18:2) and palmitic acid (C: 16:0) followed by oleic acid (C: 18:1) and stearic acid (C: 18:0).

Metal ion Analysis

Macro and microelements from leaves samples of *J. adhatoda* L. were analyzed by using Flame atomic absorption spectroscopy (FAAS). (Figs. 1 and 2). The concentration level of Ca (5.15%), Na (2.5%), K (1.19%) and Mg (2.65 %) was found. Where as higher concentrations of Fe (204.0 µg/g), Zn (80.0 µg/g) and Mn (56.0 µg/g) were found as compared to Cr (24.2 µg/g), Cu/ (11 µg/g), Ni (9.5 ug/g, Pb (8.43 µg/g) and Cd (1.8 µg/g) those were present with lower levels.

Table 1 Compositional analysis (%) of leaves of *J. adhatoda* L.

Chemical compounds	Amount in %age
Protein	21.33
Crude oil	3.01
Fiber	7.11
Ash	11.62

Antimicrobial Activity and MIC of Different Crude Extracts

Antimicrobial activities of *J.adhatoda* leaf extracts of *n-hexane*, chloroform, acetone, butanol, ethanol, methanol and water were tested against *E .coli, S .aureus, S. pyogenes* and *K. pneumoneae,* (Table 3). According to results *n-hexane*, chloroform, butanol, ethanol and methanol leaf extracts have shown inhibitory effect against the tested microorganisms. Where as water and acetone leaf extracts failed to inhibit growth of any microorganism tested. It was observed that *n-hexane* and chloroform extracts showed activity only against *E .coli* and *K. Pneumoniae* while all other microorganisms showed resistance against *n-hexane* and chloroform extracts. Butanol leaf extracts have shown high activity against *E .coli* and *S. aureus* but was inactive against the other two bacteria. The ethanol extract was highly effective against *E. coli, K. pneumineae and P .aeroginosa* but did not show any effect on *S.aureus*. However, methanol extract was very effective against all the microbes as methanol is considered as good solvents when plant extracts is subjected to antimicrobial testing (Katerere *et al*., 2008). The results are in accordance to those reported by Klausmyer *et al*. (2004), they studied the antimicrobial activity of different plants including *J .adhatoda* against a number of microorganisms. Furthermore lowest MIC values of Methanolic *leaf extracts* was obtained for *E.coli* (1.0 mg/ml) followed by *S.aureus* (1.1 mg/ml) and *K. pneumonia* (1.3 mg/ml) as compared to other solvents (Table 4).

Table 2. Phytochemical (mg/g) analysis of leaves of *J. adhatoda* L.

Phytochemicals	Concentration
Alkaloids	91.5
Tannins	43.0
Phenols	89.0
Flavonoids	130.0
Saponins	51.0

Table 3. Antimicrobial activity of different crude extracts of *J. adhatoda* L. Inhibitory zone (mm)

Extracts	*E. coli*	*S. aureus*	*K. pneumoniae*	*S. pyogenes*
n.hexane	10.0	_	7.0	_
Chloroform	11.0	_	13.7	_
Acetone	_	_	_	_
Butanol	11.5	12.0	_	_
Ethanol	12.0	15.0	5.0	16.0
Methanol	11.0	15.0	12.0	18.0
Water	_	_	_	_

Table 4: MIC value (mg/ml) Values of Extracts of *J. adhatoda* L.

Extracts	*E. coli*	*S. aureus*	*K .pneumoniae*	*S. pyognes*
n-hexane	3.0	-	5.0	-
Chloroform	2.5	-	1.5	-
Butanol	5.1	4.1	-	-
Ethanol	1.4	1.5	2.1	1.5
Methanol	1.0	1.1	1.2	1.3

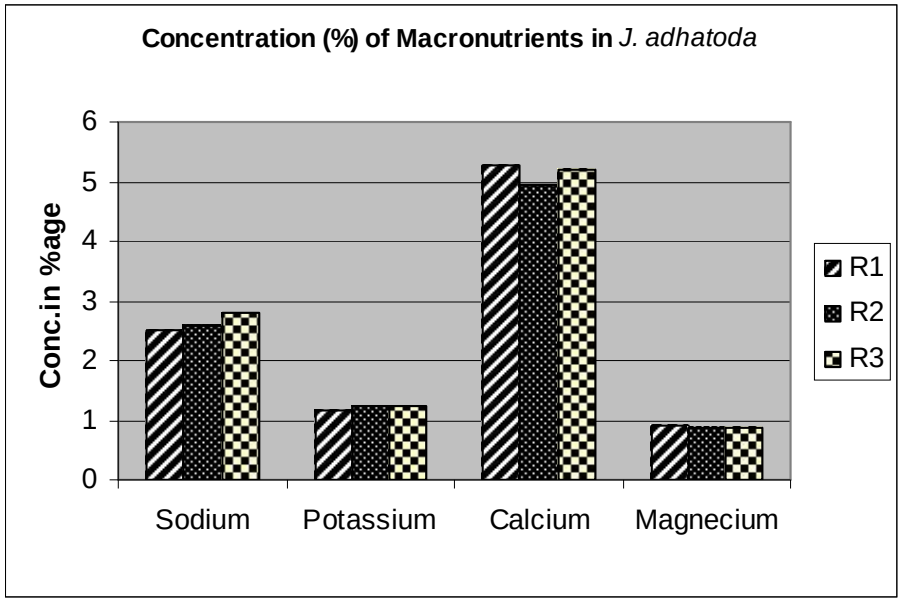

Fig. 1. Concentration (%) of macronutrients present in leaves of *J. adhatoda* L.

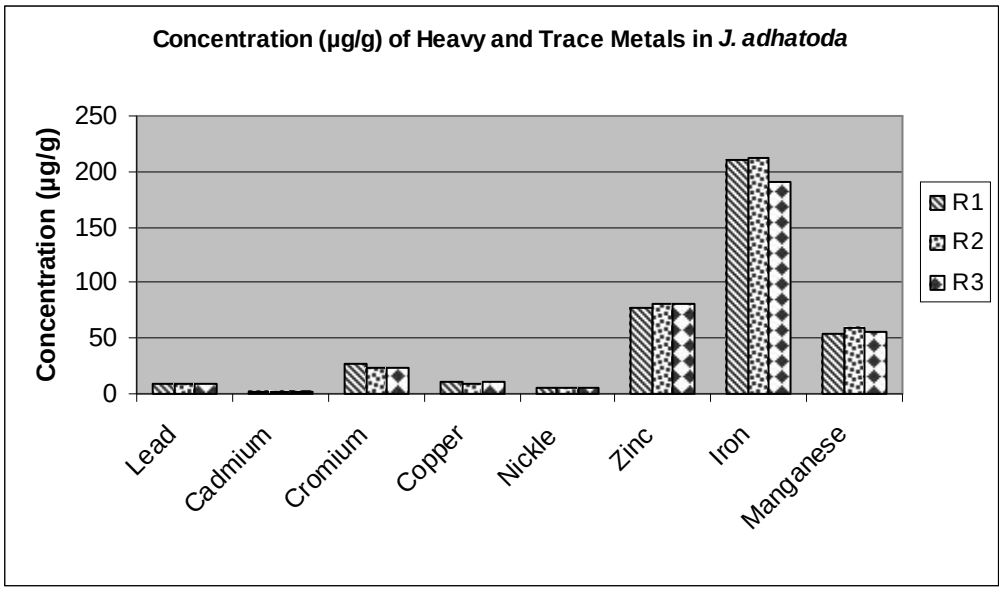

Fig. 2: Concentration of heavy and trace elements present in leaves of *J. adhatoda* L.

Antioxidant Activity of Different Crude Extracts

Antioxidant activities of ethanol, methanol, chloroform, water and butanol are shown in Fig 3. It was observed that ethanol leaf extracts have shown highest antioxidant activity followed by methanol, chloroform, water and butanol. Thus increase in absorbance was due to increase in antioxidant activity of plant extracts (Koleva *et al*., 2002). The similar results of antioxidant activity of *J. adhatoda* L. have also been reported by Ilango *et al.* (2009).

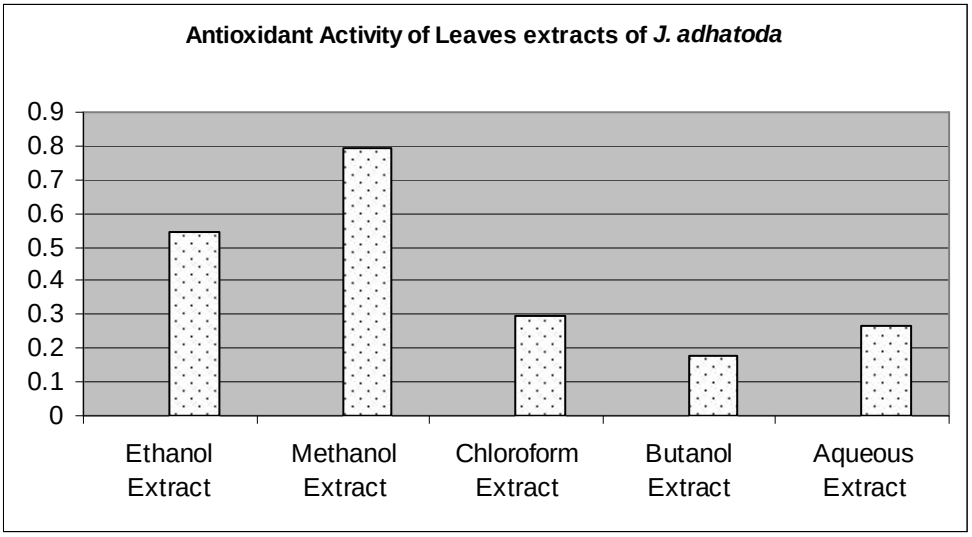

Fig 3. Antioxidant Activity of Leaf extractss of *J. adhatoda* L.

DISCUSSION

Most of medicinal properties of *J. adhatoda* L. including antimicrobial, antioxidant, hypocholesterolemic and anti-cancerous are found to be associated with these bioactive compounds mainly phenols and flavonoids (Bajpai *et al.*, 2005). These compounds have hydroxy group which is toxic to microbial enzymes and act as antimicrobial agents. It was reported somewhere else that hydrogen atom convert free radicals to their reduced form and act as good antioxidant. As *J. adhaatoda* contain lower amount of oil as compared to protein, therefore food material containing leaves of *J. adhatoda* can be considered as a beneficial food for persons suffering from cardiovascular diseases.

J. adhatoda contains fiber 7.11% and role of dietary fibers in diabetes has been studied by several workers. Nandini *et al*. (2003) has described that long term treatment with foods having high fiber contents may cause low-glycaemic index and help to maintain blood glucose level events in type I diabetic patients.

Metal ions composition of plants mainly dependent on their ecosystem and substrate composition. Sodium and potassium are very important metal ions and their presence in food help to maintain blood pressure. Potassium deficient diets may raises blood pressure in normal and healthy persons Results indicate that presence of significant amounts of calcium in *J. adhatoda* L. increases its nutritional value for individuals suffering from calcium deficiency (Alam *et al*., 2003). Pandit *et al*.

(2004) has also described the presence of metal ions Mg, Co, Cu, Mn and Cr in trace elements. These elements are also responsible for bioactivity of *J. adhatoda* L. (Pandit *et al*., 2004). Antimicrobial activity results of *J. adhatoda* extracts are in accordance to those reported by Panthi and Chaudhary (2006). They studied the antimicrobial activity of extract of *J. adhatoda* L. against a number of microorganisms (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Shigella boydii*) except methanolic extracts failed to show activity against *E. coli* whereas in present study both ethanolic and methanolic extracts showed activity against *E. coli* strains. The variation in results of two studies can be due to genotypic differences among the *E. coli* strains used. It can also be due to seasonal variation in phytochemicals of *J. adhatoda* L. like flavonoid or alkaloid contents. Moreover both the solvent and the extraction system might have an effect on the final results (Rios and Recio, 2005). Juneja *et al*. (2007) has also studied the anti inflammatory activity of ethanolic extracts of *J. adhatoda* L. They reported that active principles suspected to responsible for antiinflammatory activity are vasicinone, vasicine and vasicinol. According to Agoramoorthy *et al.* (2007) linoleic acid displayed antibacterial activity against *Bacillus subtilis*, *S. aureus*, *K. pneumoniae* and *E. coli*. Accordingly, the antimicrobial activity of *J. adhatoda* L. can be correlated with the presence of these fatty acids, particularly linoleic and palmitic acid.

The antioxidant activity of *J. adhatoda* L. has also been reported by Pandit *et al*. (2004). They evaluated the power of *J. adhatoda* L. to prevent carbon tetrachloride-induced hepatotoxicity in Rats and described that *J. adhatoda* L. possesses a high potential to prevent hepatotoxicity induced by carbon tetrachloride possibly through an antioxidant mechanism. They suggested that flavonoids, tannins and microelements act as antioxidants and exert their antioxidant activity by scavenging the lipid peroxidation (Pandit *et al*., 2004). The free radical scavenging property may be one of mechanisms by which the drug is effective as a traditional medicine. Most of tannins and flavonoids are phenolic compounds and may be responsible for antioxidant property of many plants. Chu (2000) evaluated the antioxidant power of *J. adhatoda* L. to prevent carbon tetrachloride-induced hepatotoxicity in Rats and described that *J. adhatoda* L. possess a high potential to prevent hepatotoxicity induced by carbon tetrachloride possibly through an antioxidant mechanism (Mantle *et al*.,2000). They suggested that flavonoids, tannins and some microelements act as antioxidants and exert their antioxidant activities by scavenging the lipid per oxidations. The similar results of antioxidant activity of *J. adhatoda* L. have also been reported by Ilango *et al.* (2009). Their results have also shown that methanol extracts possess highest antioxidant activity.

CONCLUSION

It is concluded that these scientific finding of phytochemicals, antimicrobial and antioxidant activities of *J. adhatoda* L. leaves will enhance use of this shrub in local medicine. It will promote proper and sustainable use of plant resources and awareness of local communities should enhance and traditional knowledge must be taken into consideration along with scientific findings.

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