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Evaluation of Antioxidant and Anti-Inflammatory Activities of *Justicia neesii* Ramam Whole Plant Extract

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Abstract: The plant *Justicia neesii* is rich in polyphenolic compounds like lignans which can exhibit anti-inflammatory properties. In this study ethanolic extracts of *Justicia neesii* was screened for elucidating its antioxidant potential using various methods. In all the methods it has shown good antioxidant nature and reducing ability. The IC_{50} values against DPPH, H_2O_2, NO and lipid peroxidases were found to be 55.72µg/mL, 133.35µg/mL, 29.65µg/mL and 271.02µg/mL respectively. The total phenolic content in the ethanolic extract was found to be 99.8mg per gram dry plant in gallic acid equivalents. The anti-inflammatory nature of *Justicia neesii* was tested using carrageenan induced paw edema model in rats. The extract has shown significant inhibition of paw volume at all the three gradient doses of the extract. The percent inhibition of paw volume is correlating with the standard diclofinac sodium. From this study we concluded that *Justicia neesii* is a plant with potent anti-inflammatory molecules.

Keywords: *Justicia neesii*, Ethanolic extract, Antioxidant, Anti-inflammatory.

1. INTRODUCTION

When the antioxidant enzymes present in the body are insufficient to neutralize the stress an external supply of antioxidants are given. But, some invitro experimental studies emphasized the deleterious effect of synthetic antioxidants on human health (Wang, 2011).The natural antioxidants are

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gaining huge importance in recent era because of their possible synergistic effects with endogenous antioxidants in defending oxidative stress caused by reactive oxygen species. The prolonged inflammation will cause harmful effects to the host and contributing to the pathogenesis of many disease states like rheumatoid arthritis, atherosclerosis and asthma (Pascual, 2006). The adverse reactions associated with the NSAIDs are decreasing the value of these drugs in regular prescription. So the researchers are concentrating in developing new NSAIDs with less adverse reactions.

Justicia neesii is a plant belonging to family Acanthaceae, growing in tropical regions of India as a perennial herb. The ethno pharmacological information suggested that the plants belonging to *Justicia* genera can be used for treating variety of diseases including cancer, diabetes, fever, headache, inflammation, arthritis and different gastrointestinal disorders (Geone M Correa *et al*, 2012). The review of the scientific literature did not reveal any significant information on antioxidant and anti-inflammatory properties of *Justicia neesii*. So it was considered worthwhile to elucidate the antioxidant and anti-inflammatory properties of *Justicia neesii.*

2. MATERIALS AND METHODS

2.1 Collection and identification of plant

Plant material was collected from different areas of East Godavari district, Andhra Pradesh during the month of February 2014 on day time. The plant was taxonomically identified by the experts of Botanical Survey of India, Hyderabad (BSI/DRC/2013-14/Tech./915-A).

2.2 Extraction of plant material

Whole plant parts including leaves, stem, twigs, flowers, seeds, roots were separated and made free from soil matter. They were dried and powdered by using hand pulveriser to a coarse powder. Then the powder was extracted with ethanol by using sohxlet apparatus at a temperature of 50-55ºC for 8h. The extracts were concentrated using vacuum evaporator and the semisolid mass was dried in vacuum desiccators. The yield of plant extract was found to be 10.58 percent.

2.3 Experimental animals

Adult albino rats of either sex weighing between 200-250g were procured from authorized venders (Mahaveera Enterprises, Hyderabad). They are maintained in polypropylene cages at a temperature of 25±2°C and relative humidity 45-55% with 12:12 light and dark cycle. Animals are given with standard pellet

diet and water *ad libitum*. Animals are acclimatized to the laboratory conditions one week before the experiment. Animals were fasted over night before the experiment. Experimental protocol was approved by the Institutional Animal Ethical Committee which was approved by CPCSEA (1047/ac/07/CPCSEA).

2.4 Acute toxicity study

The toxicity of plant extract on experimental animal was tested according to the OECD-423 (Organisation of Economic Co-operation and Development) guideline. Adult nulliparous and non-pregnant female albino rats were selected for the toxicity study, as female rats are more sensitive. Six animals were assigned to each group and fasted overnight prior to the administration of oral doses of test substances at a concentration of 5, 50, 300 and 2000 mg/Kg body weight. All the test concentrations are adjusted to below 2mL volume and administered using oral gavages. The animals were observed for first 30min and periodically for 24h. Mortality was not observed at any dose level. The observation was continued for 14 days for toxic signs.

2.5 Antioxidant activity

2.5.1 Reducing ability

The molecules with reducing potential will reduce the potassium ferricyanide (Fe^{3+}) to potassium ferrocyanide (Fe^{2+}). The reduced form will react with the ferric chloride and form a ferric-ferrous complex which is in Perl's Prussian blue color (Oyaizu, 1986). To the 1mL of 50, 100, 150, 200, 250 and 300µg concentrations of the test extract 2.5mL of 6.6 phosphate buffer and 2.5mL of 1% potassium ferricyanide were added and incubated at 50°C for 30min. Then 2.5mL of 10% trichloro acetic acid was added and centrifuged at 3000 rpm for 10min. About 2.5mL of the supernatant was mixed with 2.5mL of water and 0.5mL of 0.1% ferric chloride solution and the absorbance was measured at 700nm. The increase in absorbance is proportional to the reducing potency of extract. Incubation mixture without test compound was used as blank and α- tocopherol was used as standard.

2.5.2 **DPPH radical scavenging assay**

The in vitro free radical scavenging activity was assessed by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) reagent (Brand Williams, 1995). DPPH solution of 0.1mM concentration was prepared using methanol. To the 20, 40, 60, 80 and 100µg concentrations of test extract 1mL of DPPH solution was added and the final volume was made up to 3mL using deionised water. The prepared solutions were kept in dark for 30min. The color change from violet to yellow

was measured using spectrometer at 517nm. Butylated hydroxyl toluene (BHT) was used as standard. The intensity of the yellow color is proportional to the presence of radical scavengers. The percentage of scavenging was calculated by following equation.

$$\text{Percentage scavenging [DPPH]} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

Where, $A_{control}$ is absorbance of control and A_{sample} is absorbance of sample.

2.5.3 Hydrogen peroxide scavenging assay

Hydrogen peroxide (H_2O_2) solution of 40mM concentration was prepared in 7.4 phosphate buffer. To the 50, 100, 150, 200 and 250µg concentrations of test extracts 0.6mL of prepared H_2O_2 solution was added and the final volume was made up to 3mL using deionised water. The solutions are incubated for 10min and the absorbance was measured at 230nm using spectrophotometer (Ruch, 1984). Phosphate buffer without H_2O_2 solution was used as blank and α-tocopherol was used as standard. The percentage of scavenging was calculated by following equation.

$$\text{Percentage scavenging } [H_2O_2] = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

2.5.4 Nitric oxide scavenging assay

At physiological pH, aqueous solution of sodium nirtoprusside will spontaneously generate nitric oxide. This nitric oxide will be oxidized in the presence of oxygen and form nitrite ions. The nitrogen scavengers will compete with the oxygen and reduce production of nitrite ions. The concentration of nitrite ions produced can be measured by using Griess reagent (1% Sulphanilamide, 2% ortho phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride) (Marcocci *et al*, 1994). Sodium nirtoprusside solution of 10mM concentration was prepared using 7.4 phosphate buffer. To the 1mL of 20, 40, 60, 80 and 100µg concentrations of test extract 1mL of sodium nirtoprusside solution was added and incubated at 25°C temperature for 150min. Then 1mL of Griess reagent was added to 1mL incubated mixture and the absorbance of purple azo dye was measured at 546nm. Ascorbic acid was used as standard.

$$\text{Percentage scavenging [NO]} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

2.5.5 Total phenolic content

The total phenolic content present in the extract was estimated by using Folin-ciocalteu method (Slinkard and Singleton, 1977). Gallic acid stock solution

was prepared by dissolving 250/mg of gallic acid in 1mL of ethanol and diluted to 500 mL with water to give 0.5mg/mL solution of gallic acid. From this solution 50, 100, 150, 250 and 500µg/mL concentrations were prepared and used for establishing calibration curve. To 1 mL of the each solution, 2 mL of 2% sodium carbonate was added. After 2min diluted folins reagent was added to the test extracts and incubated at 40°C for 30min. The absorbance was measured at 765nm using spectrophotometer. The above procedure was repeated for one mL of 1mg/mL solution of plant extract and absorbance value was noted. The phenolic content present in the extract was expressed as gallic acid equivalents.

2.5.6 Lipid peroxidation assay

For the determination of degree of lipid peroxidation, rat liver homogenate was used as a source of polyunsaturated fatty acids and thiobarbituric acid reactive substances were measured using standard method (Ohkawa *et al*, 1979). The rat liver was collected immediately after sacrifice and homogenized using 0.15M Tris-HCl buffer (pH 7.4). The tissue homogenate was centrifuged at 3000 rpm for 20 min. To 1 mL of 50, 100, 200, 400 and 800 µg concentrations of the test extract 0.5 mL of supernatant was added. Then 100 µL each of 15 mM $FeSO_4$, 0.5M KCl and 6 mM ascorbic acid was added and incubated at 37°C for 60min. The addition of $FeSO_4$ will initiate the lipid peroxidation process. After incubation, 1 mL of 10% TCA was added to each mixture and centrifuged at 3000rpm for 20min at 4°C to remove insoluble proteins. To the 2mL supernatant, 1mL of 0.8% TBA was added and the mixture heated at 90°C for 20 min in a water bath. The reactants were cooled and the pink colored complex was extracted with 2mL butanol. The absorbance was measured at 532nm using spectrophotometer. Ascorbic acid was taken as standard.

$$\text{Percentage inhibition of peroxidation} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

2.6 Anti-inflammatory activity (Carrageenan induced acute inflammation)

The anti-inflammatory activity of plant extract was assessed by using carrageenan induced paw oedema model using adult albino rats of either sex weighing between 200-250g. Carrageenan is a sulphated polysaccharide and phlogistic agent which will produce oedema when injected into the sub plantar surface of hind paw of the rat (Winter, 1962). For quantitative measurement of the oedema produced we use mercury plethysmograph and measure the distance of mercury column dislocated, in millimetres. Rats are divided into five groups of six animals each. First group serve as negative control and receives 1% Tween 80 in

distilled water, 10 mL/Kg body weight. Group -2, Group-3 and Group-4 receives 100, 200 and 400 mg/ Kg body weight of ethanolic extracts. Group-5 serves as positive control and receives 10mg/ Kg of standard drug diclofinac sodium. All the drugs are given orally using gavages 60 min before the administration of 0.1mL of 1% carrageenan through subplantar route. The volume of the paw was measured using plethysmograph at 0h, 1h, 2h, 3h and 4h.

$$\text{Percentage inhibition of edema} = [(A_{control} - A_{treatment}) / A_{control}] \times 100$$

Where, $V_{control}$ is paw volume of control group and $V_{treatment}$ is the paw volume of treatment group.

2.7 Statistical Analysis

Data was represented as mean ±SEM and analyzed by one- way Analysis of variance (ANOVA) followed by Dunnett's multiple compression. $P < 0.05$ was considered as significant.

3. RESULTS

3.1 Acute toxicity study

The acute toxicity studies conducted on plant extract did not reveal any toxic signs even at 2000mg/Kg concentration. So, the ethanolic extract of *Justicia neesii* was found to be safe for internal administration.

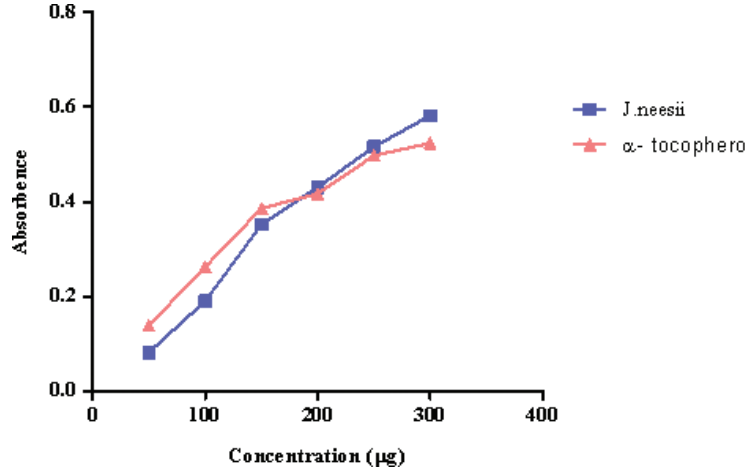

Figure 1: Reducing ability of *Justicia neesii.*

Table 1: Standard and test absorbance values in determination reducing ability of *J.neesii.*

Concentration (µg)	Absorbance	
	α- tocopherol	*J. neesii* extract
50	0.139	0.082
100	0.262	0.190
150	0.386	0.352
200	0.417	0.431
250	0.498	0.516
300	0.524	0.582

3.2 Antioxidant activity

3.2.1 Reducing ability

The antioxidant property of a compound is due to its ability of donating a hydrogen atom to reduce the reactive oxygen species. The plant extract has shown its reducing ability in dose dependent manner (Figure 1) and found to be significant compared to standard α- tocopherol. The 300µg concentration of plant extract has shown highest absorbance value (Table 1). The reducing ability in turn represents the presence of phenolic compounds, which contain hydroxyl radicals that can act as hydrogen donors (Dreosti, 2000).

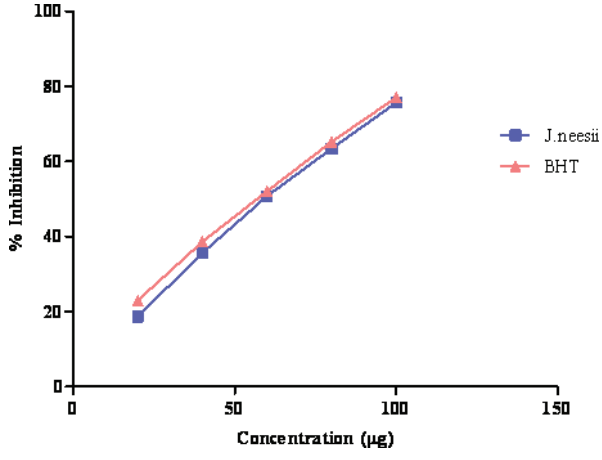

Figure 2: DPPH radical scavenging activity of *Justicia neesii.*

Table 2: Percentage inhibition of DPPH radicals by test and standard.

Concentration (µg)	BHT		*J. neesii* extract	
	Absorbance	% inhibition	Absorbance	% inhibition
20	0.854	22.99	0.902	18.66
40	0.681	38.59	0.714	35.62
60	0.532	52.03	0.546	50.77
80	0.386	65.19	0.406	63.39
100	0.255	77.01	0.270	75.65
A_0 = 1.109; IC_{50} = 55.72 µg/mL				

3.2.2 DPPH radical scavenging assay

DPPH assay is the mostly used method for studying antioxidant potentials. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) is violet colored free stable radical, first reported by Goldschmidt and renn in 1922 during oxidation of hydroquinone to benzoquinone. Upon reduction DPPH converts to 2, 2-diphenyl-1-picrylhydrazine (DPPH-H), which is orange-yellow, colored (Goldschmidt and renn, 1922). The test extract has shown its ability of reducing DPPH to the corresponding hydrazine by converting unpaired electrons to paired electrons. The reduction was done in stoichometric manner and has shown comparable values with standard BHT (Figure 2). The IC_{50} value was found to be 55.72µg/mL (Table 2).

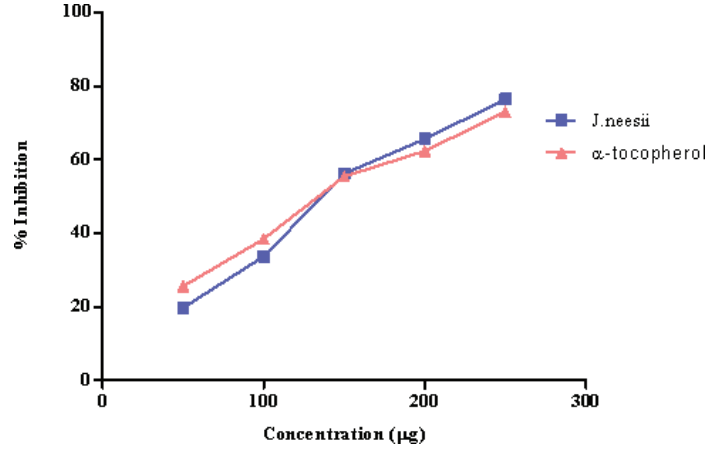

Figure 3: Hydrogen peroxide radical scavenging activity of *Justicia neesii*.

Table 3: Percentage inhibition of hydrogen peroxide radicals by test and standard.

Concentration (µg)	α- tocopherol		*J. neesii* extract	
	Absorbance	% inhibition	Absorbance	% inhibition
50	0.486	25.57	0.524	19.75
100	0.402	38.44	0.433	33.69
150	0.291	55.44	0.286	56.20
200	0.246	62.33	0.225	65.54
250	0.176	73.05	0.154	76.42
A_0 = 0.653; IC_{50} = 133.35 µg/mL				

3.2.3 Hydrogen peroxide scavenging assay

Hydrogen peroxide is a non-toxic molecule which is produced in the body by superoxide dismutase. It can easily cross the biological membranes and has the ability to produce hydroxyl radicals, which can destruct the biomolecules and cause toxic effects (Kerr *et al*, 1991). The plant extract has shown significant scavenging effect compared to the standard α- tocopherol (Figure 3). The IC_{50} value was found to be 133.35µg/mL (Table 3).

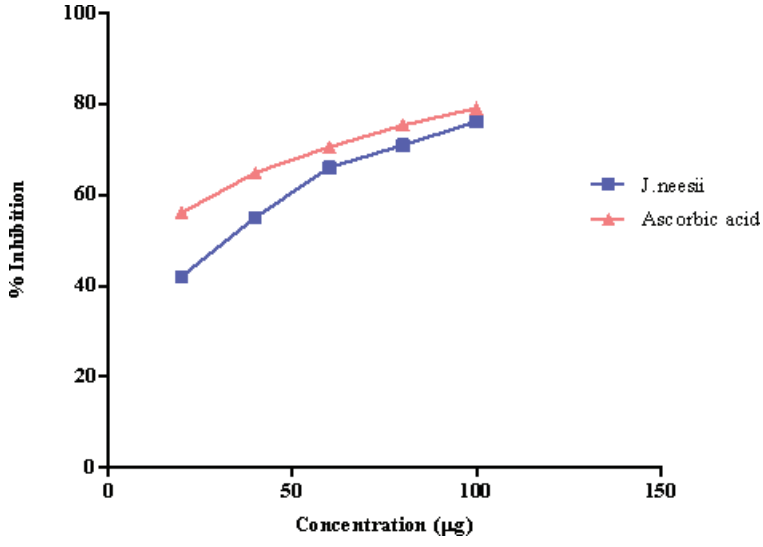

Figure 4: Nitric oxide radical scavenging activity of *Justicia neesii*.

Table 4: Percentage inhibition of nitric oxide radicals by test and standard.

Concentration (µg)	Ascorbic acid		*J. neesii* extract	
	Absorbance	% inhibition	Absorbance	% inhibition
20	0.854	56.02	1.126	42.02
40	0.682	64.88	0.873	55.05
60	0.573	70.49	0.661	65.96
80	0.480	75.28	0.564	70.96
100	0.407	79.04	0.465	76.06

A_0 = 1.942; IC_{50} = 29.65 µg/mL

3.2.3 Nitric oxide scavenging assay

Nitric oxide is a chemical mediator produced from endothelial cells, macrophages and neurons etc., which involves in several physiological functions and also associated with several pathological conditions (Lata, 2003). Nitric oxide and TNF-α are the molecules which will play an important role in immune-inflammatory mechanisms. Excessive production of Nitric oxide can also cause tissue damage as they readily react with superoxides and form peroxynitrites. So the nitric oxide scavengers can also support as anti-inflammatory agents (Anjaneya S Ravipati *et al*, 2012). The extract has shown the inhibition of anion generation by competing with the oxygen to react with excess nitric oxide and given good inhibitory results (Figure 4) with low IC_{50} value of 29.65µg/mL (Table 4).

Table 5: Standard and test absorbance values in determination of total phenolic content.

Treatment	Concentration (µg/ mL)	Absorbance
Galic acid	0	0.000
	50	0.060
	100	0.120
	150	0.170
	250	0.250
	500	0.520
J. neesii extract	1000	0.108

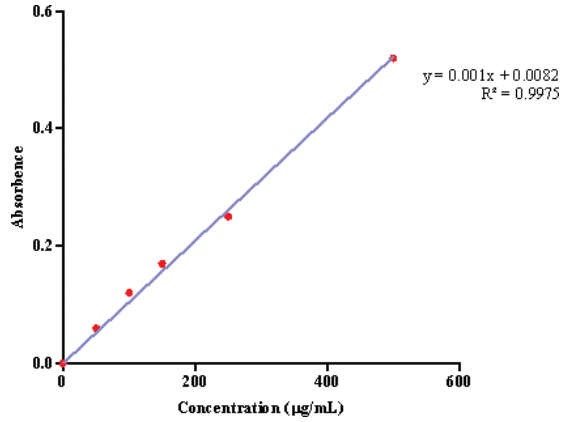

Figure 5: Calibration curve of gallic acid.

3.2.4 Total phenolic content

The redox potential of phenolic compounds will enable the reducing, hydrogen donating, metal chelating and singlet oxygen quenching properties, therefore act as strong antioxidants. The presence of conjugated ring structures and hydroxyl groups are helpful to the phenolic compounds in conjugation and hydroxylation reactions and so useful in stabilizing the free radicals (Kaviarasan, 2007). A series of concentrations of gallic acid was analyzed to plot a linear calibration curve (y = 0.001x+0.0082) with 0.9975 coefficient of correlation (Table 5; Figure 5). Form this equation the phenolic content was found to be 99.8mg per gram dried plant material in gallic acid equivalents. Instead of their high antioxidant potentials phenolics and flavonoids are also reported to have good anti-inflammatory properties (Zhang, 2011).

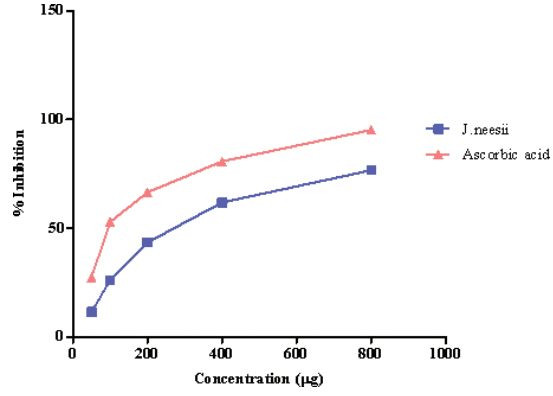

Figure 6: Lipid peroxidation nature of *Justicia neesii*.

Table 6: Percentage inhibition of lipid peroxidation by test and standard.

Concentration (µg)	Ascorbic acid		*J. neesii* extract	
	Absorbance	% inhibition	Absorbance	% inhibition
50	0.702	27.40	0.856	11.48
100	0.456	52.84	0.714	26.16
200	0.324	66.49	0.548	43.43
400	0.186	80.76	0.369	61.84
800	0.046	95.24	0.226	76.63
$A_0 = 0.967$; $IC_{50} = 271.02$ µg/mL				

3.2.5 Lipid peroxidation assay

The anti lipid peroxidation nature of antioxidants is applied in food industry for preservation. Butylated hydroxyanisole, butylated hydroxytoluene are some classical preservatives used for this purpose. But, the matter of safety and toxicity decreases the use of these antioxidants in food products (Pradyuti Dash, 2013). The lipid peroxidation of radicals can be mainly observed in high polyunsaturated lipid containing areas like brain and liver. The plant extract has shown significant inhibition of lipid peroxides along with ascorbic acid (Figure 6). The IC_{50} value was found to be 271.02µg/mL (Table 6).

3.3 Anti-inflammatory activity

It was reported that inflammation induced by carrageenan will be proceed in two pathological phases. The first phase is dominated by release of histamines and serotonins. In the second phase inflammation is mediated by bradykinins, proteases, lysosomes, prostaglandins and other slow reacting substances which give a threshold of inflammation at nearly three hours time. Third phase was also observed, but it was not fully characterized (Vinegar *et al*, 1969; Crunkhorn *et al*, 1971).

In present study all the three concentrations of plant extract have shown significant (P<0.05) reduction in inflammation up to a period of 4h (Table 7), indicating that plant extracts are active in both prostaglandin phase and amplification phase of edema. Much significant values (P<0.001) are observed for 400mg/Kg dose and comparable with the standard diclofenac sodium (Figure 7). Percentage reduction in paw edema shows significance dose dependent nature of plant extract in reducing inflammation (Figure 8).

Table 7: Anti-inflammatory activity of *J.neesii* on Carrageenan induced paw edema in rats.

Group	Treatment	Dose mg/Kg	Carrageenan induced paw edema (Volume in mL) at				
			0h	1h	2h	3h	4h
I	Control	-	0.13± 0.02	0.33± 0.02	0.37± 0.02	0.42± 0.02	0.43± 0.03
II	*J. neesii* ethanolic extract	100	0.12± 0.02	0.25± 0.02*	0.23± 0.02*	0.22± 0.02*	0.18± 0.03*
III	*J. neesii* ethanolic extract	200	0.12± 0.02	0.22± 0.03**	0.20± 0.03**	0.17± 0.02**	0.15± 0.02**
IV	*J. neesii* ethanolic extract	400	0.12± 0.02	0.20± 0.03***	0.17± 0.02***	0.13± 0.02***	0.12± 0.02***
V	Diclofinac sodium	10	0.12± 0.02	0.17± 0.02***	0.15± 0.02***	0.12± 0.02***	0.12± 0.02***

Values are expressed in mean±SEM, n=6; *P<0.05, **P<0.01, ***P<0.001 statistically significant compared to control group

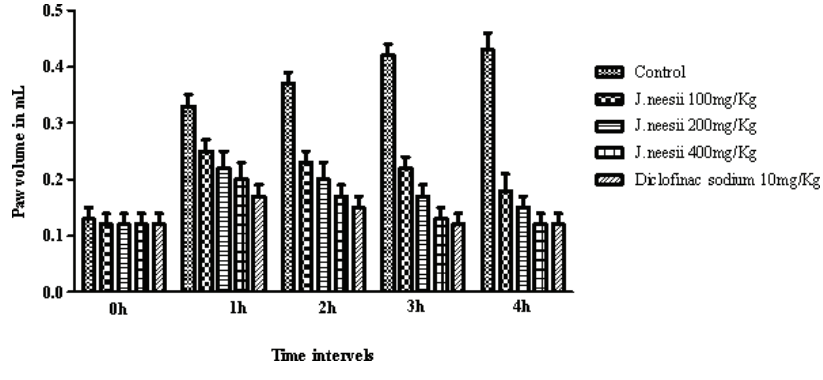

Figure 7: Anti-inflammatory potential of *Justicia neesii*.

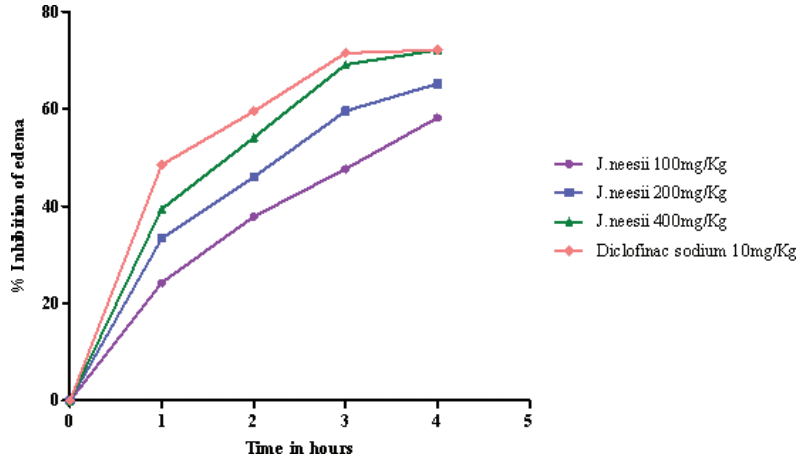

Figure 8: Comparative percentage reduction in paw volume by test and standard doses.

4. DISCUSSION

Previous studies on this plant reported the presence of various types of lignans. Three β-apolignans including 1,4-Dihydotaiwanin C, Jusneesiin, Jusneesiinol (Dodda Rajasekhar *et al*, 1998) and two arylnaphthalide lignans including jusmicranthin and justirumalin are found to be present (Dodda Rajasekhar *et al*, 1999; Kovuru Gopalaiah *et al*, 2001). The plant was also found to contain diphyllin glycosides like Neesiinoside A and Neesiinoside B (Gottumukkala V Subbaraju *et al*, 2001). It was reported that lignans can exhibit anti-inflammatory activity (Navarro *et al*, 2001; Day *et al*, 2000). So, the presence of lignans and antioxidant potential to the extracts may be reason behind good anti-inflammatory activity of extract.

In recent times researchers are elevating the importance of antioxidants with anti-inflammatory potentials (Filomena Conforti, 2008; Shingu, 1982; Pandey, 2010; Sridhar, 2013). There is a mutual and directly proportional relationship between the inflammation injury and production of reactive oxygen species. The inflammatory process will precipitate reactive oxygen species from macrophages and neutrophils, which are multiplied by the oxidative chain reactions and degenerate macromolecule present in body. These radicals also provoke release of cytokines like interleukin-l, tumor necrosis factor- α and interferon- γ which will further trigger the macrophages and neutrophils (Filomena Conforti, 2008). It was also reported that these cytokines will provoke them self and the promoted levels of cytokines will leads to oxidative

stress, causing chronic inflammation (Venkatachalam *et al*, 2013). The present study supported use potential antioxidants for anti-inflammatory properties.

It is well known that prostaglandins are having significant role in mediating inflammation, nociception and pyrexia. Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly used drugs in these clinical complications, which are having evidentiary mechanisms in blocking the prostaglandin synthesis. But these drugs are associated with serious adverse effects. The results of present experiment show a parallel pharmacological relationship between the test extract and standard NSAID. So, we are expecting that the active principles present in test drug is having potential of inhibiting prostaglandin actions and can serve as anti-inflammatory medicine. However, the exact phytochemicals responsible for these effects of *Justicia neesii* have to be identified and further pharmacological studies may be taken up in developing lead compounds and to overcome the limitations of current work.

5. CONCLUSION

From the present study we can conclude that the ethanolic extract of *Justicia neesii* is having good antioxidant and anti-inflammatory properties. We can suggest this plant as a choice for developing anti-inflammatory drugs with fewer side effects.

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