Original Research Article

IN VIVO AND IN VITRO ANTI OXIDANT ACTIVITY OF ETHANOLIC EXTRACTION OF *JUSTICIA GENDARUSSA* BURM LEAVES

ABSTRACT

Oxidation is very essential to many living organisms for the production of energy to fuel biological processes. The free radicals and other reactive oxygen species (ROS), which are continuously, produced in vivo, responsible for oxidation. The aim of the present study was to investigate in vitro and in vivo antioxidant potential of ethanolic extraction of *justicia gendarussa* burm leaves [EEJG]. The study was done by using various *in vitro and In vivo* methods such as Hydroxyl Radical scavenging activity, Determination of Reducing Power, Metal chelating activity, Carbon tetrachloride (CCl_4) induced lipid peroxidation and Inhibitory Test on Protein Oxidative Modification and in vivo Lipid peroxidation, reduced glutathione and Catalase was studied. The ethyl acetate extract showed the highest total phenolic content and total flavonoid content among other extracts of *justicia gendarussa* burm leaves. The percentage inhibition and IC_{50} value of all the extracts were followed dose-dependency and found significant ($P < 0.01$) as compared to standard (ascorbic acid). The oxidative stress markers as Lipid peroxidation (LPO) (CAT) and reduced glutathione (GSH) were increased significantly ($P < 0.01$) at 250 and 500 mg/kg of EEJG treated animals and decreased significantly the thiobarbituric acid reactive substances [TBARS] level at 500 mg/kg of EEJG as compared to control group. These results revealed that the ethanol extract of *justicia gendarussa* burm leaves exhibits both *in vitro* antioxidant activity against DPPH and *in vivo* antioxidant activity by modulating brain enzymes in the rat. This could be further correlated with its potential to neuroprotective activity due to the presence of flavonoids and phenolic contents in the extract.

Keywords: *Justicia gendarussa* Burm, anti oxidant, Radaical scavenging activity, Determination of Reducing Power

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1.0 INTRODUCTION

The traditional medicine all over the world is nowadays revealed by an extensive activity of researches on different plant species and their therapeutic principles. Plants contain phytochemicals with various bioactivities including antioxidant, anti inflammatory and anticancer activities. Currently, about 25% of the active component was identified from plants that are used as prescribed medicines [1]. Reactive oxygen species (ROS) exert oxidative damaging effects by reacting with nearly every molecules found in living cells including protein, lipid, amino acids and DNA, if excess ROS are not eliminated by antioxidant system. They play important roles in aging and in the pathogenesis of age related disorders such as cancer, hypertension, atherogenesis, Alzheimers disease and Parkinsons disease [2]. The most practical way to fight degenerative diseases is to increase antioxidant activity in our body and that could be achieved by consumption of vegetables, fruits or edible plants [3]. There is an increasing interest in natural antioxidants *e.g.* polyphenols, present in medicinal and dietary plants, which might help prevent oxidative damage [4]. Natural antioxidants increase the antioxidant capacity of the plasma and reduce the risk of diseases [5]. Different parts such as seeds, leaves and bark of stem and root known to contain substantial amounts of phytoconstituents such as phenolics, flavonoids, tannins having the ability to inhibit the free radicals that are excessively produced, hence can act as antioxidant [5]. The continued search among plant secondary metabolites for natural antioxidants has gained importance in recent years because of the increasing awareness of herbal remedies as potential sources of phenolic oxidants [6].

Justicia gendarussa Burm F. (Family: *Acanthaceae*) is a shade-loving, quick-growing, evergreen plant mostly found in moist areas [7]. It is believed to be native to China and is distributed widely across India, Sri Lanka, and Malaysia. In Indian and Chinese traditional medicine, the leaf of the plant is recommended to treat ailments such as fever, hemiplegia, rheumatism, arthritis, headache, earache, muscle pain, respiratory disorders, and digestive trouble [8]. However, to our knowledge, there are no published scientific studies on the anti-arthritic activities of the leaves of *J. gendarussa* or its potential toxicity. Therefore, the objective of this study is to examine *in vitro* and *in vivo* antioxidant potential of ethanolic extraction of *justicia gendarussa* burm leaves).

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2.0 MATERIALS AND METHODS:

2.1 Plant collection and authentication

Leaves of *Justicia gendarussa Burm* were obtained from the local places of Tirupati, AP. Plant was authenticated by Dr. K. Madhava Chetty, M.Sc., M.Ed., M.Phil., Ph.D., PG DPD., Assistant Professor, Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh.

2.2 Extraction by Maceration

Fresh leaves were washed with water to get rid of contaminants like dirt and other impurities and were shade-dried. These dried leaves were ground and sieved to get a uniform, coarse powder. Powdered plant material was weighed (1Kg) and is immersed in ethanol [9] and kept for maceration [10] for a period of 7 days with occasional stirring. On the 8^{th} day, the solvent was filtered by pressing with a muslin cloth and was evaporated in a rotary evaporator at 40^{o}C. The resultant extract was put in a desiccator to remove any ethanol left in it. The dried ethanolic extract of *Justicia gendarussa Burm*. (EEJG) was packed in an air-tight bottle and put in a dry place for further studies.

2.3 Qualitative evaluation of phytoconstituents

The EEJG was screened for the presence of various phytoconstituents like carbohydrates, flavonoids, polyphenolic compounds, saponins, tannins, triterpenoids, etc.

2.4 Animals

Albino rats (175-225gm) of either sex and of approximate same age used in the present studies were procured from Central Animal facility, CMR college of Pharmacy, Hyderabad, India. The animal was fed with standard pellet diet and water *ad libitum*. All the animals were housed in polypropylene cages. The animals were kept under alternate cycle of 12 hours in darkness and light. The animals were acclimatized to the laboratory condition for a one week before starting the experiment. The experiment protocols were approved by Institutional Animal Ethics committee after securitization **(IAEC No: CPCSEA/1657/IAEC/CMRCP/COL-19/67).** The animal received the drug treatment by oral gavage tube.

2.5 *In vitro* antioxidant studies

2.5.1 Hydroxyl Radaical scavenging activity

The hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the extract for hydroxyl radicals generated from the Fe^{3+} / ascorbate / EDTA / H_2O_2 system. The hydroxyl radicals attack deoxyribose, which eventually results in TBARS

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formation. The reaction mixture contained deoxyribose (2.8mM), $fecl_3$ (0.1mM), H_2O_2 (1mm), ascorbate (0.1mM), KH_2PO_4-KOH buffer (20mM, pH 7.4) and various concentrations (EEJG 100, 200, and 300 µg/ml and standard Mannitol 100µg/ml) of the drug in a final volume of 1 ml. The reaction mixture was incubated for 1 hr at 37^oC. Deoxyribose degradation was measured at 532nm [11].

2.5.2 Determination of Reducing Power

The reducing power of EEJG was determined according to the following method. Various concentrations (125,250,175 and 500µg/ml) of extract of EEJG in 1 ml of distilled water was mixed with phosphate buffer (2.5ml, 0.2M, pH 6.6) and potassium ferricyanide (K_3Fe (CN) $_6$) (2.5ml, 1%).The mixture was incubated at 50^oC for 20 min. A portion (2.5ml) of trichloroacetic acid (15%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5ml) was mixed with distilled water (2.5ml) and ferric chloride (0.5ml, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates increased reducing power [12].

2.5.3 Metal chelating activity

Metal chelation property for Ferric ion (Fe^{3+}) was estimated by using thiocynate method. Here different ratio of the extract (4:1) was mixed with a fixed concentration of ferric chloride (10µg). The mixture was incubated for 30 min. At the end of the incubation, 1ml of potassium thiocyanate (25%) was added and absorbance of ferric-thiocyanate complex (reddish brown complex) was measured at 460 nm. The results were compared with EDTA (1:10). Metal chelation property for ferrous ion (Fe^{2+}) was estimated by using 2, 2-bipyridyl method. Here different concentrations of the extract were mixed with a fixed concentration of ferrous sulphate (10µg). The mixture was incubated for 30 min. At the end of the incubation, 2ml of 2, 2-bipyridyl (1mM) was added and absorbance of ferrous –bipyridyl complex (pink-colored complex) was measured at 525 nm. The results were compared with EDTA [13].

2.5.4 Carbon tetrachloride (ccl_4) induced lipid peroxidation

Rat liver (30%w/v) homogenate in ice – cold 0.15 M potassium chloride was prepared in homogenizer. Aliquots of 0.5 ml of homogenate were taken in different small conical flasks. These flasks were incubated at 37^0C in a constant shaker bath (150 cycles/min) for 45 min with 1.5 ml of potassium phosphate buffer (pH 7.4), 2ml of 0.15 M potassium chloride–, EEJG at (25,50,100,200 and 300 µg/ml) and Vitamin – E 100 µg/ml in different flasks and finally 10 µl

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of carbon tetrachloride (CCl_4) was added. In case of control, both CCl_4 and drugs were not added and in some flasks only drug was excluded. The reaction was stopped by the addition of 4 ml of 10%(w/v) tri chloro acetic acid and after incubation, the contents were centrifuged at 4000 rpm for 10 min and about 2ml of clear supernatant was transferred to a graduated tube and 2 ml of 0.67%w/v of thiobarbituric acid was added and heated in a boiling water bath for 15 min. The tubes were cooled, bringing the mixture to pH 12-12.5 with potassium hydroxide, stabilized the colour developed, and the absorbency was measured at 543nm [14].

2.5.5 Inhibitory Test on Protein Oxidative Modification

Albumin oxidative modification by copper was performed by the following method. The test sample (EEJG 100-1000 µg/ml) and Vitamin- E (100-1000µg/mi), was added to the reaction mixture containing albumin (10µg/ml) and 100 µM $CuCl_2$ in 50 mM Tris- HCl buffer (pH 7.4) in a total volume 0.3 ml. The mixture was incubated at 37^{o}C for 2 hr. Next 1.6ml of 0.125 M phosphate buffer (PH 8.0) containing 12.5 mM EDTA and 10.0 M urea, and 0.1 ml of 50 mm phosphate buffer (pH 7.0) containing 10mM DTNB were added to the reaction mixture. This solution was allowed to stand at room temperature for 5 min. The absorbency was read at 412 nm as cysteine-SH residue [15].

2.6 *In vivo* anti-oxidant studies

2.6.1 Animal treatment

Twenty-four male albino rats (n = 6) were divided into four different groups. Group I served as a control group and treated with vehicle only (0.5% carboxymethylcellulose sodium). Group II served as disease control, Group III served as standard control Group IV and V animals were administered orally with 250 and 500 mg/kg of EEJG, respectively, for 7 days. At the end of 7th day, the animals were sacrificed by cervical dislocation and each brain was excised, rinsed in ice-cold normal saline and followed by 0.15 M Tris-hydrochloride. The homogenates were centrifuged at 15,000 × g for 10 min. The supernatants were employed for the following assays.

2.6.2 Lipid peroxidation assay (thiobarbituric acid reactive substances)

It was evaluated by TBARS tests during an acid-heating reaction. Aliquots of samples were incubated with 15% trichloroacetic acid and 0.38% thiobarbituric acid. The mixture was heated (1 h) in a boiling water bath. TBARS was determined by reading the absorbance of the pink-colored complex formed in a spectrophotometer at 532 nm [16].

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2.6.3 Catalase assay

It was determined with reaction solution contained 2.5 mL of 0.05 M phosphate buffers (pH 8.3), 0.7 mL of 0.2 M H_2O_2 and 0.1 mL of tissue homogenate. Changes in absorbance of the reaction solution at 570 nm were determined after 1 min. Results were expressed in units/mg protein [17].

2.6.4 Reduced glutathione assay

This was estimated by using dithiobisnitro-benzoate as a substrate. The yellow color developed and read immediately at an absorbance of 412 nm and expressed as μM GSH/g protein.

2.7 Statistical analysis

The values were expressed in mean ± standard error of the mean. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple comparison test versus control. $P < 0.05$ and $P < 0.01$ were considered as significant [18].

3.0 RESULTS

3.1 Hydroxyl radical scavenging activity

The EEJG (at all tested doses 100μg, 200μg and 300μg) significantly (P<0.001) scavenged the hydroxyl radicals generated by the EDTA/H_2O_2) system, when compared with that of control. The percentage scavenging of OH radicals by EEJG increased in a dose depended manner. Results were comparable standard (Mannitol100ug), (P<0.001). Results were shown in **Table 1.**

Table 1: Hydroxyl radical scavenging activity EEJG and Mannitol

S. No	Concentration (μg/ml)	% Inhibition Of Hydroxyl Radical
1.	Control	-
2.	EEJG (200)	75.9±11.172*
3.	EEJG (400)	81.43±14.156*
4.	Standard (Mannitol 100μg)	81.62±12.177*

Statistical significant test for comparison was done by ANOVA, followed by Dunnet's "t' test

*p< 0.001, when test and standard are compared against control.

Values are Mean± SEM.

3.2 Determination of reducing power

The reducing power of EEJG increased with increasing concentration of EEJG. All the tested concentrations of EEJG showed significant (P< 0.001) activity than control. Results were comparable with the standard (BHT) (P<0.001). Results were shown in **Table 2.**

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Table 2: Determination of reducing power of EEJG and BHT

S. No	Concentration (µg/ml)	Absorbance (OD)
1	**Control**	0.086± 0.000392
2	**EEJG (500)**	1.0111±0.00054*
3	**EEJG (375)**	0.9578±0.00074*
4	**EEJG (250)**	0.5361±0.00087*
5	**EEJG (125)**	0.3328±0.00070*
6	**BHT(500)**	0.6288±0.00070*
7	**BHT(375)**	0.4935±0.0037*
8	**BHT(250)**	0.389±0.00073*
9	**BHT(125)**	0.300±0.00110*

Statistical significant test for comparison was done by ANOVA, followed by Dunnet's't' test *p< 0.001, when compared against control. Spectro photometric deduction of the Fe^{3+} - Fe^{2+} transformation. Values are Mean± SEM

3.3 Effect of EEJG on fe^{2+} and fe^{3+} metal chelation

EEJG chelated Fe^{2+} (53.08%) and Fe^{3+} (55.19) significantly (P<0.001) at 1:10 ratio of iron : EEJG and chelating ability for mental transition ions (Fe^{2+},Fe^{3+}) increased in a dose dependent manner respectively. EEJG at all tested concentrations exhibited significant (P<0.001) chelation, when compared against control. In similar conditions, EDTA exhibited 78.64% chelation for $Fe^{2+}$and 85.42% for Fe^{3+} respectively, which is significant (P<0.001) when compared with the control. Results were shown in **Table 3.**

Table 3: Effect of EEJG and EDTA on Fe^{2+}/ Fe^{3+} metal chelation

Iron : Drug	OD at 525 nm	% Chelation of Fe^{2+}	OD at 460nm	% Chelation of Fe^{3+}
1:00(control)	0.308	0	1.021	0
1:0.25 EEJG	0.246	20.26±6.1948*	0.884	13.44±0.093*
1:0.5 EEJG	0.208	32.69±4.308*	0.793	22.33±2.171*
1:1 EEJG	0.200	35.02±7.259*	0.716	29.94±2.006*

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1:2.5 EEJG	0.195	36.84±8.260*	0.697	31.78±6.003*
1:5 EEJG	0.163	47.25±11.177*	0.654	36.01±5.006*
1:10 EEJG	0.145	53.08±9.433*	0.458	55.19±10.005*
(1:10)Standard (EDTA)	0.067	78.64±10.204*	0.149	85.42±18.006*

Statistical significant test for comparison was done by ANOVA, followed by Dunnet 's 't' test

Fe^{2+} and Fe^{3+} were quantitated by Fe2+ -dipyridyl complex (525 nm) and

Fe^{3+} - thiocynate complex (460nm), respectively.

*p< 0.001, when test and standard are compared against control.

EDTA: Ethylene diamine tetra acetic acid

3.4 Lipid Peroxidation Induced By CCl_4

Lipid peroxide formation from CCl_4 was significantly (P<0.001) inhibited by EEJG at all tested dose levels (25µg, 50µg, 100µg, 200µg and 300µg) when compared with that of control. The percentage inhibitions of peroxide formation increased in a dose dependent manner–. Results were comparable with that of standard. Results were shown in **Table 4.**

Table 4: Inhibition of lipid peroxidation–induction by CCl_4 system of EEJG and Vitamin-E

S. No	Concentration (µg/ml)	% Inhibition
1	**Control**	-
2	**EEJG (25)**	32.59±2.577*
3	**EEJG (50)**	42.15±3.413*
4	**EEJG (100)**	52.99±9.211*
5	**EEJG (200)**	60.37±12.552*
6	**EEJG (300)**	68.90±14.565*
7	**Standard (Vitamin- E)**	66.27±8.205*

Statistical significant test for comparison was done by ANOVA, followed by Dunnet 's 't' test (n=6), *p< 0.001, when test and standard are compared against control, Values are Mean± SEM.

3.5 Inhibitory test on protein oxidative modification

The inhibitory ratio of EEJG on albumin oxidative modification was as high as 78.94 at a concentration of 1000µg/ml and increased in a concentration dependent manner. The EC50 of EEJG was found to be 416.86± 0.351µg/ml. The results were comparable with the standard

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(Mannitol), with percentage inhibitory ratio of 81.99% at a concentration of 1000µg/ml. The IC_{50} of Mannitol was found to be 263.35 ±7.41 µg/ml. Results were shown in **Table 5.**

Table 5: Inhibitory test on protein oxidative modification of EEJG and Vitamin-E

S. No	Concentration of (µg/ml)	% inhibition	IC_{50} Value (µg/ml)
1	**EEJG(100)**	22.46±0.380	416.86±0.351
2	**EEJG (200)**	41.34±0.2374	
3	**EEJG (400)**	55.24±0.174	
4	**EEJG (600)**	64.10±0.248	
5	**EEJG (800)**	70.93±0.165	
6	**EEJG (1000)**	78.94±0.130	
7	**Standard (Vitamin-E100)**	32.15±0.079	263.35±7.47
8	**Vitamin-E(200)**	51.68±0.242	
9	**Vitamin-E(400)**	63.22±0.042	
10	**Vitamin-E(600)**	72.18±0.052	
11	**Vitamin-E(800)**	80.26±0.106	
12	**Vitamin-E(1000)**	81.99±0.055	

Values are Mean± SEM.

3.6 *In vivo* anti oxidant study

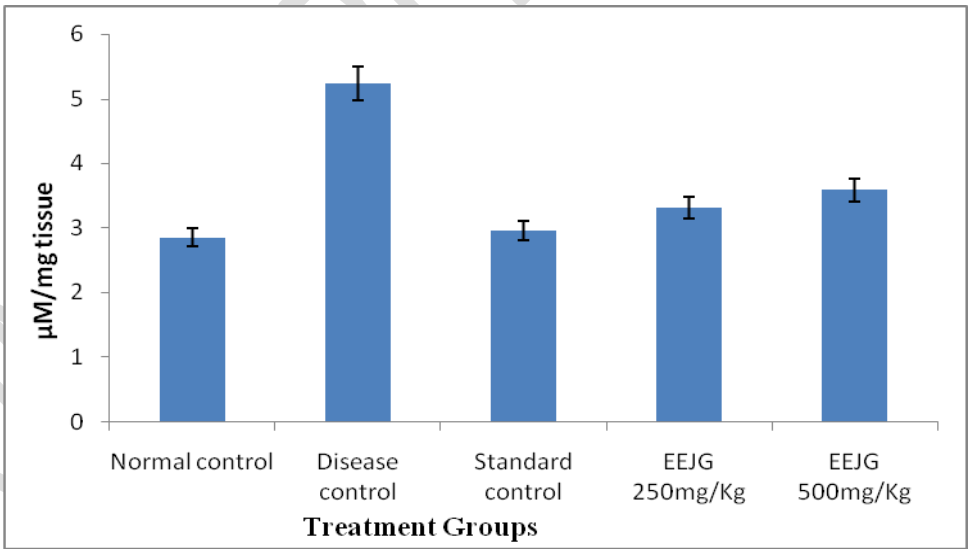

Fig. 1: Effect of EEJG on LPO levels

The significant values of LPO levels of normal, disease, standard, EEJG 100mg/Kg and EEJG 200mg/Kg were found to be 2.85±0.08, 5.23±0.21, 2.96±0.16, 3.31±0.08, and 3.59±0.08

respectively on Day 29. There is a significant decrease in LPO levels of animals treated with EEJG 250mg/Kg and 500mg/Kg compared to disease control. Results were showed in **Fig. 1**

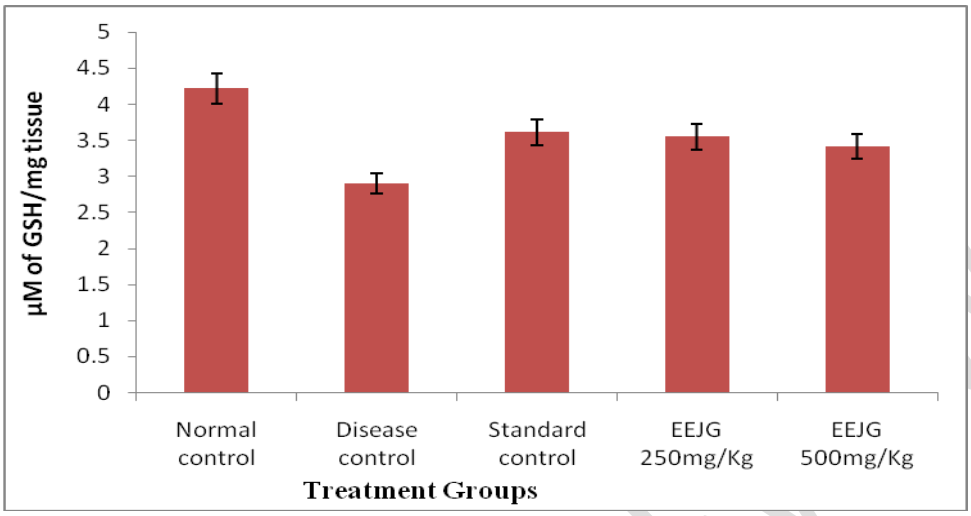

Fig. 2: Effect of EEJG on GSH levels

The significant values of GSH levels of normal, disease, standard, EEJG 100mg/Kg and EEJG 200mg/Kg were found to be 4.22±0.12, 2.90±0.13, 3.61±0.08, 3.55±0.10 and 3.41±0.08 respectively on Day 29. There is a significant increase in GSH levels of animals treated with EEJG 250mg/Kg and 500mg/Kg compared to disease control.

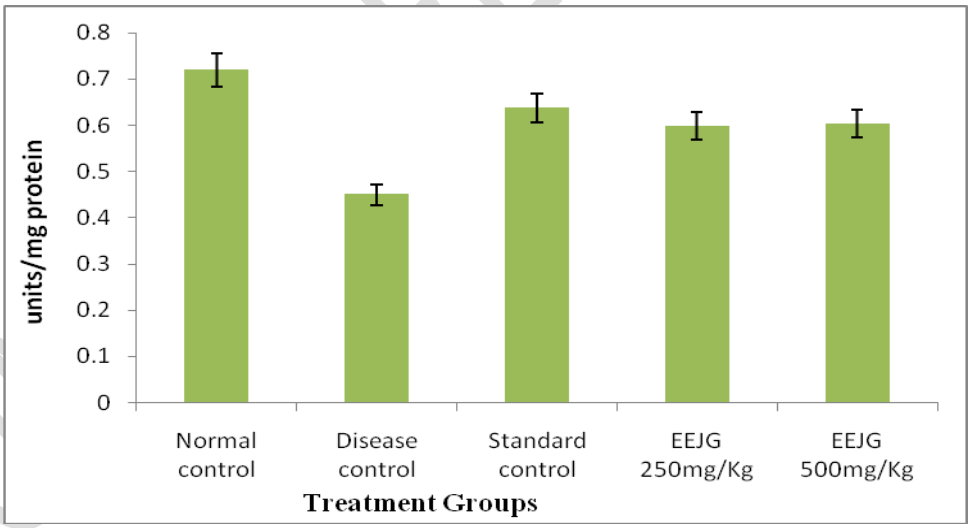

Fig. 3: Effect of EEJG on CAT levels

The significant values of CAT levels of normal, disease, standard, EEJG 100mg/Kg and EEJG 200mg/Kg were found to be 0.72±0.04, 0.45±0.03, 0.63±0.02, 0.59±0.03, and 0.60±0.05 respectively on Day 29. There is a significant increase in CAT levels of animals treated with EEJG 250mg/Kg and 500mg/Kg compared to disease control.

4.0 DISCUSSION

The free radicals are very unstable chemical species containing one or more unpaired electrons that causes damage to other molecules by removing electrons from them with a specific end goal to achieve stability. Free radicals assume the double part as both pernicious and valuable species since they can be either harmful or beneficial to the living system [19]. It is apparent from this study, that EEJG possesses effective antioxidant activity. In vitro and In vivo antioxidant assay of the EEJG was explored in the present study by total flavonoids content, total phenolic content, nitric oxide radical, DPPH• radical, hydrogen peroxide radical and superoxide anion scavenging activities, ABTS•+ radical cation de-colourisation, Fe^{2+} chelating activity assay and ferric reducing power [20]. The antioxidant activities of plant extracts can be ascribed to the presence of respective phytochemicals like phenolics, flavonoids, alkaloids, saponins, steroids, glycosides, tannins, protein and amino acids in that species [21, 22]. Reactive oxygen species (ROS) readily combine and oxidize biomolecules thus making them inactive subsequently causing damage to cells, tissues and organs. Antioxidants mainly work by neutralizing the free radicals produced in the biological systems [23,24].

Higher phenolic contents such as flavonoids, polyphenols and mono-phenols are some features of medicinal plants [25]. The flavonoid and phenolic compounds are responsible for the antioxidant ability of the medicinal plants [26] and these are abundant in some vegetables, fruits and flowers [27]. Antioxidants, derived from plant origin, especially flavonoids and polyphenols have been used to treat various disease such as aging, diabetic, cancer and prevention of cardiovascular diseases [28].

The EEJG (at all tested doses 100µg, 200µg and 300µg) significantly ($P<0.001$) scavenged the hydroxyl radicals generated by the EDTA/H_2O_2) system, when compared with that of control. The percentage scavenging of OH radicals by EEJG increased in a dose depended manner. Results were comparable standard (Mannitol100ug), ($P<0.001$).

The reducing power of EEJG increased with increasing concentration of EEJG. All the tested concentrations of EEJG showed significant ($P< 0.001$) activity than control. Results were comparable with the standard (BHT) ($P<0.001$). EEJG chelated Fe^{2+} (53.08%) and Fe^{3+} (55.19) significantly ($P<0.001$) at 1:10 ratio of iron–: EEJG and chelating ability for mental transition ions (Fe^{2+},Fe^{3+}) increased in a dose dependent manner respectively. EEJG at all tested concentrations exhibited significant ($P<0.001$) chelation, when compared against control. In

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similar conditions, EDTA exhibited 78.64% chelation for $Fe^{2+}$and 85.42% for Fe^{3+} respectively, which is significant ($P<0.001$) when compared with the control. Lipid peroxide formation from CCl_4 was significantly ($P<0.001$) inhibited by EEJG at all tested dose levels (25µg, 50µg, 100µg, 200µg and 300µg) when compared with that of control. The percentage inhibitions of peroxide formation increased in a dose dependent manner. Results were comparable with that of standard. The inhibitory ratio of EEJG on albumin oxidative modification was as high as 78.94 at a concentration of 1000µg/ml and increased in a concentration dependent manner. The EC50 of EEJG was found to be 416.86± 0.351µg/ml. The results were comparable with the standard (Mannitol), with percentage inhibitory ratio of 81.99% at a concentration of 1000µg/ml. The IC_{50} of Mannitol was found to be 263.35 ±7.41µg/ml.

5.0 CONCLUSIONS

Antioxidants are important in the prevention of human diseases. Compounds with antioxidants activity may function as free radical scavengers, complexers of pro-oxidants metals, reducing agents, and quenchers of single-oxygen formation or reactive oxygen species, thereby protecting the body from degenerative diseases such as cancer. The reactive oxygen species (ROS) are harmful byproducts generated during normal cellular metabolism or from toxic insult. They lead to a state of oxidative stress that contributes to the pathogenesis of a number of human diseases by damaging lipids, proteins and DNA. This has inspired much interest in antioxidant activity of phytochemicals. In this study, the choice of ethanol as an extract was because of its availability and affordability at the time of this study. Our results have shown ethanol extracts from *Justicia gendarussa Burm* displayed strong antioxidant activity.

ABBREVIATIONS

EEJG: Ethanolic extract of *Justicia gendarussa Burm*.; DPPH: 2, 2-diphenyl-1-picryl-hydrazyl-hydrate; ROS: Reactive oxygen species; CCl_4: carbon tetrachloride; LPO: Lipid peroxidation GSH: Reduced glutathione; CAT: Catalase

6.0 REFERENCES:

1. Gill NS, Bajwa J, Sharma P, Dhiman K, Sood S. Evaluation of antioxidant and antiulcer activity of traditionally consumed *Cucumis melo* seeds. J Pharmacol Toxicol. 2011; 6:82–89.
2. Graf BA, Milbury PE, Blumberg JB. Flavonols, flavonones, flavanones and human health: Epidemological evidence. J Med Food. 2005;8:281–290.

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3. Adefegha SA, Oboh G. Cooking enhance the antioxidant properties of some tropical green leafy vegetables. Afr J Biotechnol. 2011; 10(4):632–639.
4. Arts ICW, Hollman PCH. Polyphenols and disease risk in epidemiologic studies. Am J Clin Nutr. 2005; 81:317–325.
5. Shekhar HU, Goto M, Watanabe J, Konishide-Mikami I, Bari ML, Takano-Ishikawa Y. Multi food functionalities of Kalmi Shak (Ipomoea aquatica) grown in Bangladesh. Agric Food Anal Bacteriol. 2011; 1(1):24–32.
6. Samatha T, Acharya RS, Srinivas P, Ramaswamy N. Quantification of total phenolic and total flavonoid contents in extracts of Oroxylum indicum L. Kurz. Asian J Pharm Clin Res. 2012; 5(4):177–179.
7.](Ratnasooriya, Wanigasekara & Deraniyagala, Srianthie & Dehigaspitiya, Dilani.. Antinociceptive activity and toxicological study of aqueous leaf extract of Justicia gendarussa Burm. F. in rats. Phcog Mag. 2007; 3. 145-155.
8. Aliyu AB, Ibrahim H, Musa AM, Ibrahim MA, Oyevale AO, Amupitan JO. In vitro evaluation of antioxidant activity of Anisopus amannii N.E. Br. Afr J Biotechnol. 2010; 9(16): 2437–2441.
9. B.N. Sastri. Wealth of India: Raw materials, Vol. V,(Council of Scientific and Industrial Research, new Delhi, 1959; 312-3,
10. Saxena K. Analytical and medicinal properties of ethanolic extract of leaves of carissa carandas. World Journal of Pharmacy and Pharmaceutical Sciences. 2016; 5(4):1683-1690.
11. Bint-e-Sadek Y, Choudhury N, Shahriar M. Biological investigations of the leaf extracts of Carissa Carandas. International Journal of Pharmaceutical Research and Technology. 2013; 5:97-105.
12. Mary NK, Shylesh BS, Babu BH, Padikkala J. Antioxidant, and hypolipidaemic activity of a herbal formulation-liposem. Ind J Exp Biol. 2002; 40:901-904.
13. Gulcin I,Oktay M, Kufrevioglu OI, Aslan A. Determination of antioxidant activity of lichen *Cetraria islandica* (L) Ach. J Ethnopharmacol. 2002; 79: 325 - 329.
14. Tripati YB, Singh AV, Dubey GP. Antioxidant property of the bulb of *Scilla indica*. Science 2001; 80:1267-1269.
15. Comporti M. Three models of free radical-induced cell injury. 1989; 72:1-56.

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16. Toda S, Shirataki Y. Inhibitory effects of *Astragali Radix*, a crude drug in Oriental medicines on lipid peroxidation and protein oxidative modification by copper. J Ethnopharmacol. 1999; 68:331-333.
17. Satoh K. Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method. Clin Chim Acta. 1978; 90:37–43
18. Maehly AC, Chance B. I. New York: Interscience; 1954. Methods of Biochemical Analysis; pp. 357–8.
19. (Droge W. Free radicals in the physiological control of cell function. Review. Physiol. Rev. 2002; 82:47–95.
20. Matill HA. Antioxidants. Annu Rev Biochem. 1947;16:177–92
21. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys. 1959; 82:70–7.
22. Akter R., Raquibul Hasan S.M., Mokarram Hossain M., Jamila M., Sultana S., Chowdhury Mazumder M.E.H., and Rahman S., Antidiarrhoeal and antioxidant properties of Curcuma alismatifolia leaves, Australian Journal of Basic and Applied Sciences, 2010; 4(3): 450-456.
23. ArmoskaiteV., Ramanauskiene K., Maruska A., Razukas A., Dagilyte A., Baranauskas A., and Briedis V., The analysis of quality and antioxidant activity of green tea extracts, Journal of Medicinal Plants Research. 2011; 5(5): 811-816.
24. Siddique A. N., Mujeeb M., Kalam Najmi A., and Akram M., Evaluation of antioxidant activity, quantitative estimation of phenols and flavonoids in different parts of Aeglemarmelos, African Journal of Plant Science. 2010; 4(1): 1-5.
25. Koksal E., Bursal E., Dikici E., Tozoglu F., and Gulcin I., Antioxidant activity of Melissa officinalis leaves, Journal of Medicinal Plants Research. 2011; 5(2): 217-222.
26. Zhao-Jian G., Jian-Bing L., and Xing-Guo X., Purification and characterization of polyphenol oxidase from leaves of Cleome gynandra L, Food Chem. 2011; 129(3): 1012-1018.
27. Sangilimuthu A., Lukmanul H.F., and Sathish K.R., Antioxidant activity of Withania somnifera (L.) Dunal by different solvent extraction methods, J Pharm Res. 2011; 4(5): 1428-1430.
28. Onanong K., Sirithon S., Natthida W., and Naret M., Phenolic compounds and antioxidant activities of edible flowers from Thailand, J Funct Food. 2011; 3: 88-99.

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