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Research Article

IN VITRO ANTI-INFLAMMATORY, ANTIOXIDANT AND NEPHROPROTECTIVE STUDIES ON LEAVES OF *AEGLE MARMELOS* AND *OCIMUM SANCTUM*

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ABSTRACT

Objective: To study the *in vitro* anti-inflammatory, antioxidant and nephroprotective studies on leaves of *Aegle marmelos* and *Ocimum sanctum*.

Materials and Methods: *In vitro* nephroprotective screening of aqueous extract of leaves were performed employing cytoprotective assay using epiflourescence dual staining assay was performed against normal kidney cell treated with gentamicin. *In vitro* anti-inflammatory studies were also performed for the extracts using protease inhibition activity, membrane stabilization and protein denaturation inhibition assays. *In vitro* nephroprotective activity was conducted using gentamicin induced normal rat kidney cells. *In silico* modeling and docking studies were also performed and *in vitro* cytoprotective assay was performed to evaluate cell cytotoxic efficacy.

Results: Leaf extracts clearly revealed the dose-dependent increase in nephroprotection. Similarly *in vitro* antioxidant and anti-inflammatory activities proved scientifically the ethnomedicinal claim of these selected plant drugs. Aegelin, marmin and marmesin identified from *A. marmelos* extract, showed good binding affinity towards the active site of monocyte chemotactic protein 1 and neutrophil gelatinase associated lipocalin protein. *In silico* modeling and docking studies were also performed to suggest the probable mechanism of action of these plant extracts. *In vitro* cytoprotective assay showed its potent cell cytotoxic efficacy.

Conclusion: The present study showed *in vitro* antioxidant and anti-inflammatory activities along with nephroprotective property, which scientifically prove the ethnomedicinal claim of these selected plant drugs.

Keywords: *Aegle marmelos*, *Ocimum sanctum*, Antioxidant, Anti-inflammatory, Cytoprotective, Nephroprotective, *In silico*, Ethnomedicinal.

INTRODUCTION

Traditionally *Aegle marmelos* and *Ocimum sanctum* are considered as sacred and are widely used to treat various ailments. Parts such as stem, leaves, flower, fruit, (or) whole part of *A. marmelos* were used as cardioactive [1], antihyperglycemic [2], antiulcer [2], antiseptic [3] and antibacterial agent [4]. Whole of the *O. sanctum* is used in the treatment of gastric disorders [5], hepatic problems [6], cold [6] and act as a prophylactic against malaria [6]. The phenols, proanthocyanidins and flavonoids present in these plants are largely responsible for imparting various therapeutic potential of these plants [7]. Antioxidants are chemical compounds that can scavenge free radicals that are formed in the body due to normal physiological process. These free radicals then initiate a chain reaction which leads to the formation of various other free radicals leading to oxidative stress which in turn results in the productivity of reactive oxygen species and reactive nitrogen species causing lipid peroxidation (LPO) and cellular damage. These free radicals are also capable of disintegrating various biomolecules such as nucleic acids, proteins and lipids. Since there is no report on the nephroprotective efficacy of the selected plants, cell-based cytotoxicity assay, epiflourescence staining was performed. Results provide strong evidences for the use of these plants in the management of nephritis and inflammation.

MATERIALS AND METHODS

Chemicals

Folin ciocalteau reagent, sodium carbonate, methanol, aluminum chloride, potassium acetate, vanillin, hydrochloric acid, bovine serum albumin, acetyl salicylic acid, trypsin, 1,1-diphenyl picrylhyrazyl (DPPH), butylated hydroxyl toluene (BHT), ferricyanide, trichloroacetic acid (TCA), ferric chloride, nicotinamide adenine dehydrogenase (NADH), hydrogen peroxide, ferrous sulfate, ascorbic acid, ethylene diamine tetraacetic acid (EDTA), thiobarbituric acid (TBA), sodium nitroprusside, Griess reagent, gentamicin, vitamin E, MTT, agarose, acridine orange, ethidium bromide from Merck were used. Milli Q Type I water grade was used for all the assays.

Collection of plant

The selected plants were collected from the surroundings of SASTRA University, Thanjavur and were identified by Dr. N Ravichandran, Department of CARISM, SASTRA University. Further authenticated with the help of herbarium specimens deposited at Rapinat Herbarium, St. Joseph College Trichy. Fresh leaves were shade dried coarsely powdered.

Preparation of extract

Coarsely powdered leaf were kept in water for 48 hr and then filtered. The obtained filtrate was then concentrated and used for all studies. 1 kg of leaf powder of the plants was used for the crude extract preparation.

Phytochemical analysis of the extract

Determination of total phenolic content

Total phenolic content was determined using Folin-Ciocalteau method [8] with slight modification. A volume of 550 µL of 10% Folin-Ciocalteau reagent was added to the 2% of 400 µL of sodium carbonate, which was added to the 50 µL of both the extracts selected for the study. This was incubated for 15 minutes at 45°C with shaking. The absorbance was recorded at 765 nm against blank. Total amount of phenol was calculated and measured in mg/mL.

Estimation of total flavonoid

Total flavonoid content was determined by alumium chloride colorimetric method [9] with slight modification. 200 µL of the aqueous extract of selected plants were added to 600 µL of methanol, 40 µL of

10% aluminum chloride, 40 µL of 1 M potassium acetate and 1.12 mL of milli-Q water. This was kept at room temperature for 30 minutes. The absorbance was recorded at 420 nm against blank. The content was determined by extrapolation of the calibration curve, which was made using quercetin as standard. Flavonoid concentration was expressed in mg/mL.

Estimation of total proanthocyanidins

Total proanthocyanidin content was determined as per the procedure of Sun *et al.* [10]. 600 µL of vanillin methanol (4% v/v), 300 µL of hydrochloric acid was added to 100 µL of aqueous extract (1 mg/mL). The resulting mixture was incubated at room temperature for 15 minutes. Absorbance of the solution was read at 500 nm against blank. Total amount of proanthocyanidin was measured.

AQ2

Anti-inflammatory activity

Inhibition of protein denaturation

Inhibition of protein denaturation was evaluated by the method of Mizushima and Kobayashi [11] and Sakat *et al*. [12] with slight modification. 500 µL of 1% bovine serum albumin was added to 100 µL of plant extract. This mixture was kept at room temperature for 10 minutes, followed by heating at 51°C for 20 minutes. The resulting solution was cooled down to room temperature and absorbance was recorded at 660 nm. Acetyl salicylic acid was taken as a positive control. The experiment was carried out in triplicates and percent inhibition for protein denaturation was calculated using:

$$\% \text{ Inhibition} = 100 - ((A_1 - A_2)/A_0) * 100)$$

Where A_1 is the absorbance of the sample, A_2 is the absorbance of the product control and A_0 is the absorbance of the positive control.

Membrane stabilization

Preparation of red blood cell (RBC) suspension

Anticoagulated human blood was collected and centrifuged at 3000 rpm for 10 minutes. The obtained solution was washed three times with saline. RBC layer was collected and diluted to make 10% v/v using ×1 phosphate buffer saline (PBS) (Sadique *et al*., 1989 [13]; Saket *et al*., 20107 [14]).

Heat induced hemolysis

A volume of 100 µL of 10% RBC was added to 100 µL of the extract. The resulting solution was heated at 56°C for 30 minutes followed by centrifugation at 2500 rpm for 10 minutes at room temperature. Supernatant was collected, and absorbance was read at 560 nm. Acetyl salicylic acid was used as a positive control. Percent membrane stabilization was calculated by the method of Shinde *et al*. (1999) [15]; Saket *et al*. (2010) [14].

$$\% \text{ Inhibition} = 100 - ((A_1 - A_2)/A_0) * 100)$$

Where A_1 is the absorbance of the sample, A_2 is the absorbance of the product control and A_0 is the absorbance of the positive control.

Protease inhibition assay

Inhibition of trypsin was evaluated by the method of Oyedepo and Femurewas (1965) [16] Sakat *et al*. (2010) [14]. 100 mL of bovine serum albumin was added to 100 µL of extract. This was incubated at room temperature for 5 minutes. Reaction was inhibited by the addition of 250 µL of trypsin followed by centrifugation. The supernatant was collected, and absorbance was observed at 210 nm. Acetyl salicylic acid was used as a positive control. The experiment was carried out in triplicates and percent inhibition of protease inhibition was calculated.

$$\% \text{ Inhibition} = 100 - ((A_1 - A_2)/A_0) * 100)$$

Where A_1 is the absorbance of the sample, A_2 is the absorbance of the product control and A_0 is the absorbance of the positive control.

Determination of antioxidant efficacy

DPPH radical assay

The DPPH free radical scavenging assay was performed by Liyana-Pathirana and Shahidi method [17]. 200 µL of 0.1 mM DPPH prepared in methanol was added to 100 µL of the plant extract. The resulting mixture was incubated at room temperature in the dark for 15 minutes. Absorbance was observed at 517 nm. BHT was taken as a positive control. The experiment was carried out in triplicates and percentage inhibition of the DPPH radical scavenging activity was calculated.

$$\% \text{ Inhibition} = ((A_0 - A_1)/A_0) * 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

Estimation of reducing power

The reducing power of the plant extract was estimated by the method of Oyaizu [18]. 500 µL of 0.2 M phosphate buffer (pH - 6.6), 500 µL of ferricyanide (1% w/v) and 200 µL of both the extract selected for the study was added. The above prepared mixture was incubated at 50°C for 20 minutes followed by the addition of 500 µL of TCA (10% w/v). The resulting mixture was then centrifuged at 3000 rpm for 10 minutes. 500 µL of the supernatant was collected and to it 500 µL of milli-Q water was added along with 100 µL of ferric chloride (0.1% w/v). Absorbance of the solution was observed at 700 nm against blank. Percentage inhibition was calculated for the determining reducing power.

$$\% \text{ Inhibition} = ((A_0 - A_1)/A_0) * 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

Scavenging activity of superoxide anion

This activity was carried out by the method of Yen and Chen [19]. 200 µL of the aqueous extract was added to 200 µL of ferricyanide and 200 µL of NADH prepared in phosphate buffer. The resulting solution was incubated at 25°C for 5 minutes, and absorbance was taken at 560 nm. BHT was used as a positive control and the reaction was carried out in triplicates. Percentage inhibition of scavenging activity of superoxide anion was calculated.

$$\% \text{ Inhibition} = ((A_0 - A_1)/A_0) * 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

Hydrogen peroxide scavenging activity

This activity of the plant was evaluated by the method of Ruch *et al*. [20]. 850 µL of the aqueous plant extract was added to 150 µL of 4 mM hydrogen peroxide solution prepared in phosphate buffer (0.1 M, pH-7.4). This was incubated for 10 minutes, and absorbance was read at 230 nm. BHT was taken as a positive control and the reaction was carried out in triplicates. Percent inhibition of the assay was calculated.

$$\% \text{ Inhibition} = 100 - ((A_0 - A_1)/A_0) * 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

LPO assay

LPO assay was carried out by the method of Ruberto *et al.* (2000) [21]. RBC's were collected freshly and was added to 1% solution in phosphate buffer at a pH of 7.2 10 µL of aqueous extract of the test sample was added to 20 µL of ferrous sulfate, 20 µL of ascorbic acid and 150 µL of RBC. The solution was kept at incubation for half an hour at room temperature. The reaction was inhibited by the addition of 200 µL of EDTA. This solution was then mixed with 1 mL of TBA and boiled for 15 minutes. This resulted in the formation of a stable colored derivative with MDA. Absorbance of the resulting solution was read at

517 nm. Butylated hydroxy toluene was used as a positive control. The experiment was carried out in triplicates and percentage inhibition was calculated:

$$\% \text{ Inhibition} = 100 - ((A_0 - A_1)/A_0) * 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

TBA method

The reaction of 2 TBA and malonedialdehyde (MDA) is used widely to determine the extent of oxidative deterioration of lipids, and it was carried out by the method of Liu *et al* (1997) [22]. The sample is quantified by its reaction with TBA to form a colored complex with a maximum absorbance at 552 nm. TBA-MDA complex and other TBA reactive species (TBARS) may also contribute to the absorbance at 552 nm. The assay is carried out by mixing 400 µL of 20% TCA, 400 µL of 0.67% 2 TBA and 200 µL of sample. This mixture was kept for boiling on a water bath for 10 minutes and then it was cooled at room temperature, this is followed by centrifugation at 3000 rpm for 20 minutes. Supernatant was collected, and absorbance at 552 nm was recorded. The experiment was performed in triplicates and percent inhibition was calculated.

$$\% \text{ Inhibition} = 100 - ((A_0 - A_1)/A_0) * 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

Nitric oxide (NO) scavenging activity

NO scavenging activity [23] of sample was determined by adding 400 µL of 100 mM sodium nitroprusside, 100 µL of PBS (pH - 7.4) and 100 µL of different concentration of plant extract. This reaction mixture was kept for incubation at 25°C for 150 minutes. To 0.5 mL of above solution, 0.5 mL of Griess reagent was added (0.1 mL of sulfanilic acid and 200 µL naphthylethylenediamine dichloride (0.1%) w/v)). This was kept on incubation at room temperature for 30 minutes, and finally absorbance is observed at 540 nm. All the reactions were performed in triplicates, and their percentage inhibition was calculated by the following formula:

$$\% \text{ Inhibition} = ((A_0 - A_1)/A_0) * 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

Nephroprotective studies

Epiflourescence staining

Healthy normal kidney cells were used for the assay. Extracts of 500 µg/mL and dyes such as ethidium bromide and acrydine orange were added to the cell followed by the incubation for 30 minute in the dark. After incubation, the resulting solution was centrifuged, and the pellet was viewed under fluorescence microscope.

Cytoprotective assay

Fresh normal rat kidney cells were collected during experimental necropsy of rats. Tissue was mashed and washed with RPMI 1640 culture medium and made into single-cell suspension and seeded into a 96-well flat bottom plate with 0.1×10^6 cells per well. After 24 hr incubation, 0.5 mL of gentamicin (50 mg/mL) was added to each well followed by the addition of diluted extract to the appropriate wells and the plates were incubated for further 48 hr at 37°C in a humidified incubator with 5% CO_2. Supernatant was removed from each well, and 10 µL of MTT (0.5 mg/mL) was added. MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored (dark purple) formazan product. The cells are then solubilized with an organic solvent (e.g. isopropanol) and the released, solubilized formazan reagent is measured spectrophotometrically. Cytotoxicity of extracts was tested using the method of Mosmann [25] and the readings were obtained at 630 nm.

***In silico* studies**

The docking analysis was carried out by means of Maestro 9.3, Schrodinger suite. All ligands such as aegelin, marmesin and marmin were obtained from the plants by the means of LC-MSMS and were docked to the active site of the monocyte chemotactic protein 1 (MCP1) and neutrophil gelatinase associated lipocalin (NGAL) protein. Binding site of MCP1 protein comprised of serine, threonine, arginine, proline, valine and isoleucine. Binding site of NGAL protein comprised of serine, threonine, phenyl alanine, valine, glycine, alanine, arginine, and glutamine. Glide score and maximum volume for each protein-ligand interaction was recorded.

Statistical analysis

All *in vitro* assays data signify the mean ± standard deviation of triplicates and IC_{50} was calculated using Graphpad prism 5.

RESULTS AND DISCUSSION

Phytochemical screening

It was reported that phenolic compounds were associated with antioxidant activity. The total phenolic content of aqueous leaf extracts of *A. marmelos* and *O. sanctum* were 14 mg gallic acid equivalent (GAE)/g and 12 mg GAE/g respectively which also support the possible bioactivity of these plant extracts. The total flavonoid content of *A. marmelos* and *O. sanctum* were 35 mg QE/g and 15 mg QE/g respectively. The total proanthocyanidin content of *A. marmelos* and *O. sanctum* were 22 mg GAE/g and 7 mg GAE/g respectively (Tables 1 and 2).

Anti-inflammatory studies

Inhibition of albumin denaturation

Denaturation of proteins is the main cause of inflammation. As part of the investigation on the mechanism of the anti-inflammatory activity, ability of the extract to inhibit protein denaturation was studied. Selected extracts were effective in inhibiting heat induced albumin denaturation. IC_{50} of *A. marmelos* and *O. sanctum* were observed as 95.64 µg/mL and 42.17 µg/mL, respectively. Aspirin was used as a standard anti-inflammation drug. Results are presented in Fig. 1a and a'.

Membrane stabilization effect

Stabilization of RBCs membrane was studied to further establish the mechanism of anti-inflammatory action of aqueous extract of *A. marmelos* and *O. sanctum.* Both the extracts effectively inhibited the heat induced hemolysis. The results provide evidences for membrane stabilization effect of the selected plant drug as an additional mechanism for their anti-inflammatory effect. Due to the resemblance of RBC membrane with lysosomal membrane, this effect may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation. The extracts inhibited the heat induced hemolysis of RBCs. IC_{50} of *A. marmelos* and *O. sanctum* were observed as 405.6 µg/mL and 58.64 µg/mL respectively. Results are presented in Fig. 1b and b'.

Table 1: Phytochemical constituents of *A. marmelos*

Phytochemical constituents of *A. marmelos*	Amount
Total phenol content	14 mg GAE/g
Total flavonoid content	35 mg QE/g
Total proanthocyanidin content	22 mg GAE/g

GAE: Gallic acid equivalent, QE: Quercetin equivalent, *A. marmelos: Aegle marmelos*

Table 2: Phytochemical constituents of *O. sanctum*

Phytochemical constituents of *O. sanctum*	Amount
Total phenol content	12 mg GAE/g
Total flavonoid content	15 mg QE/g
Total proanthocyanidin content	7 mg GAE/g

GAE: Gallic acid equivalent, QE: Quercetin equivalent, *O. sanctum: Ocimum sanctum*

Proteinase inhibitory activity

The aqueous extract of leaves of *A. marmelos* and *O. sanctum* exhibited significant antiproteinase activity. Maximum inhibition was observed in *A. marmelos* leaf extract. The standard aspirin (92.87%) drug showed maximum proteinase inhibitory action. IC_{50} of *A. marmelos* and *O. sanctum* were observed as 74.45 µg/mL and 49.70 µg/mL respectively. Results are shown in Fig. 1c and c'.

Antioxidant studies

DPPH scavenging activity

The result of DPPH scavenging assay carried out in the present study indicated that both the plants were potentially active. This suggests that both the plants extract do contain compounds that are capable of donating hydrogen to a free radical in order to remove odd electron, which are responsible for radical's reactivity. This implies that the plant extracts could be useful for treating radical related pathological damage, especially at higher concentration. IC_{50} of *A. marmelos* and *O. sanctum* were observed as 132.1 µg/mL and 184.6 µg/mL respectively. Results are shown in Fig. 2a and b.

Reducing power capacity

The reducing power of a compound is related to the electron transfer ability of the test drugs and may serve as a significant indicator of their potential antioxidant activity. In this assay, the yellow color of the test solution changes to green and blue depending on the reducing power of test specimen. Greater absorbance at 700 nm indicated greater reducing power. In the concentration range investigated, all the extracts demonstrated reducing power that increased linearly with concentration. The reducing power of the extract might be due to their hydrogen-donating ability. Possibly, *A. marmelos* and *O. sanctum* leaves contain high amounts of reductone, which could react with radicals to stabilize and terminate radical chain reactions. IC_{50} of *A. marmelos* and *O. sanctum* were observed as 17.84 µg/mL and 11.40 µg/mL respectively. Results are shown in Fig. 3a and b.

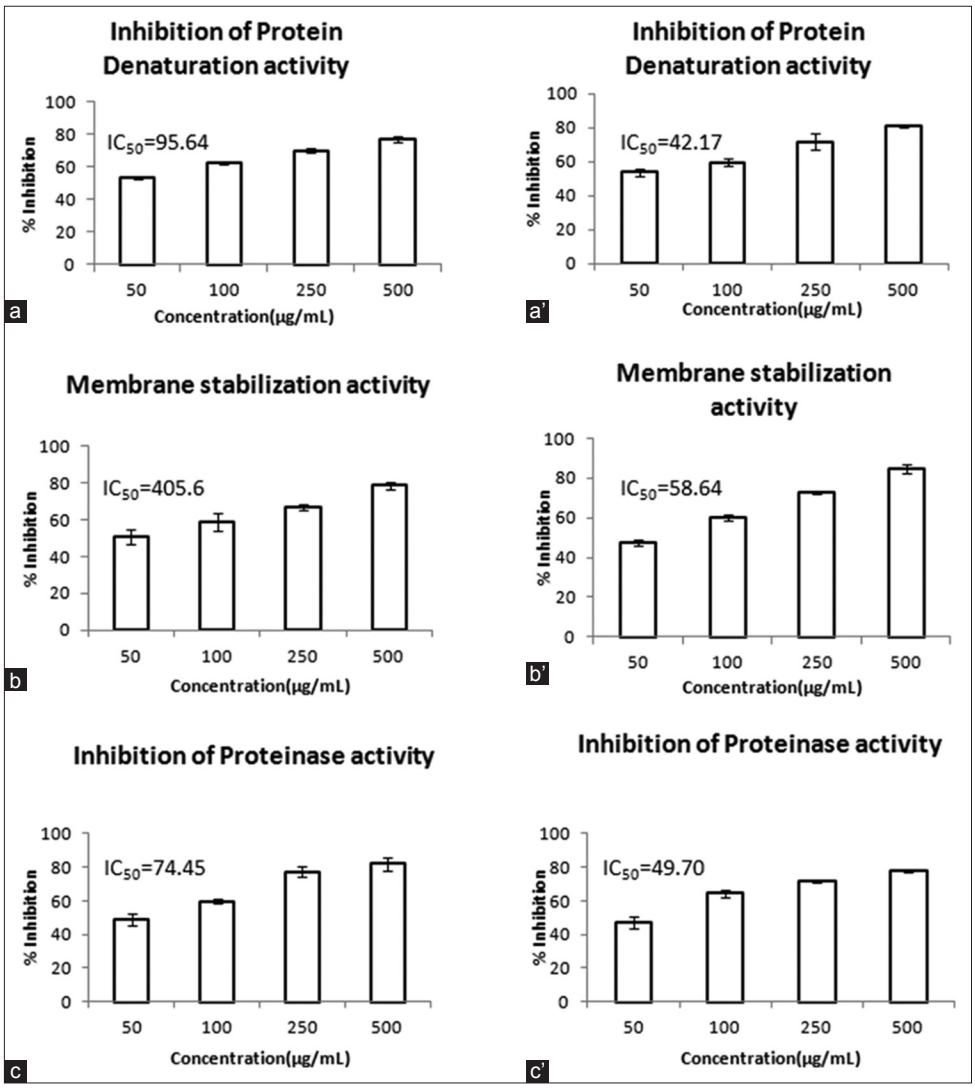

Fig. 1: Anti-inflammatory assays of *Aegle marmelos* (a-c) and *Ocimum sanctum* (a'-c'). (a) The inhibition of protein denaturation of *A. marmelos* determined in various concentrations varying from 50 µg/mL to 500 µg/mL; (a') the inhibition of protein denaturation of *O. sanctum* was evaluated in concentration varying from 50 to 500 µg/mL. A gradual increase in scavenging potential of the extract was obtained with an increase in concentration. IC_{50} of *A. marmelos* and *O. sanctum* for inhibition of protein denaturation was found to be 95.64 µg/mL and 42.17 respectively; (b) the membrane stabilization potential of *A. marmelos*; (b') the membrane stabilization potential of *O. sanctum*. A gradual increase in scavenging potential of the extract was obtained with an increase in concentration. IC_{50} of *A. marmelos* and *O. sanctum* for membrane stabilization was found to be 405.6 µg/mL and 58.64 µg/mL, respectively; (c) the inhibition of proteinase activity of *A. marmelos* was determined; (c') the inhibition of proteinase activity of *O. sanctum* was determined. A gradual increase in scavenging potential of the extract was obtained with an increase in concentration. IC_{50} of *A. marmelos* and *O. sanctum* for inhibition of proteinase as found to be 74.45 µg/mL and 49.70 µg/mL respectively; n=3 (mean±standard deviation)

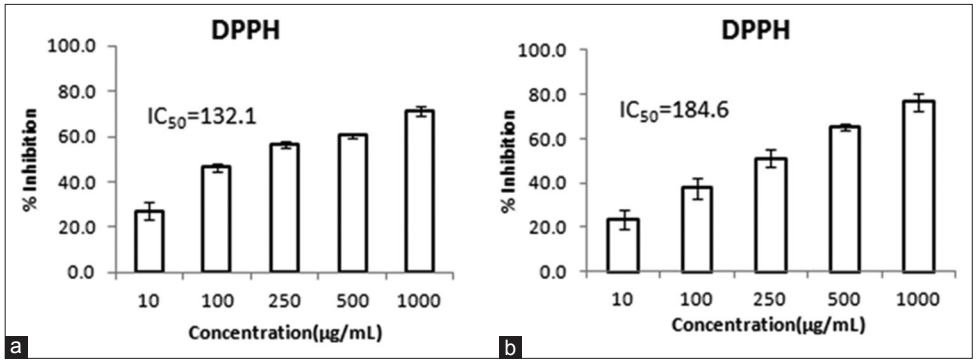

Fig. 2: 1,1-diphenyl picrylhyrazyl (DPPH) assay of *Aegle marmelos* (a) and *Ocimum sanctum* (b). (a) The DPPH scavenging activity of *A. marmelos* at various concentrations varying from 10 to 1000 µg/mL was evaluated; (b) the DPPH scavenging activity of *Ocimum sanctum* at various concentrations varying from 10 to 1000 µg/mL was evaluated. A gradual increase in scavenging potential of the extract was obtained with an increase in concentration. IC_{50} of *A. marmelos* and *O. sanctum* was found to be 132.1 µg/mL and 184.6 µg/mL respectively; n=3 (mean±standard deviation)

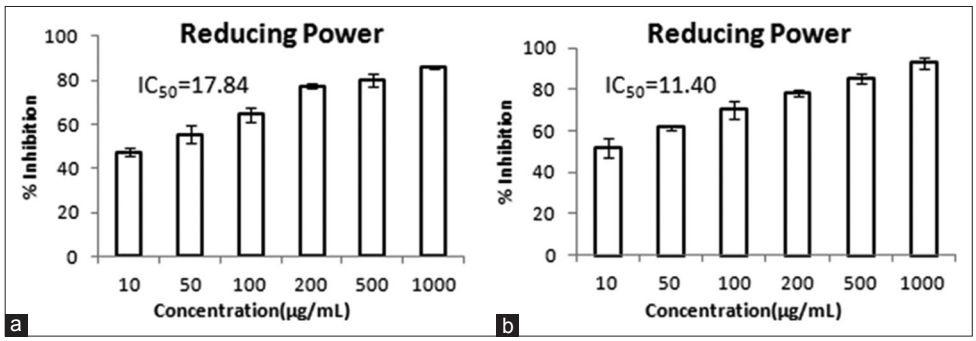

Fig. 3: Reducing power of *Aegle marmelos* (a) and *Ocimum sanctum* (b). (a) The reducing power of *A. marmelos* was determined at various concentrations varying from 10 to 1000 µg/mL; (b) The reducing power of *O. sanctum* was determined at various concentration varying from 10 to 1000 µg/mL. A gradual increase in scavenging potential of the extract was obtained with an increase in concentration. IC_{50} of *A. marmelos* and *O. sanctum* was found to be 17.84 µg/mL and 11.40 µg/mL respectively; n=3 (mean±standard deviation)

Superoxide ion scavenging activity

Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals that are generated. The scavenging activity of this radical by the plant extracts was compared with the standard reagents such as gallic acid to establish that the plant is also a potent scavenger of superoxide radical. Hydrogen peroxide is an important reactive oxygen species because of its ability to penetrate biological membranes. However, it may be toxic if converted to hydroxyl radical in the cell. Scavenging of H_2O_2 by the plant extracts may be attributed to the presence of phenolics, which donate electron to H_2O_2, thus reducing it to water. The extract was capable of scavenging hydrogen peroxide in a dose-dependent manner. IC_{50} of *A. marmelos* and *O. sanctum* was observed as 24.89 µg/mL and 49.48 µg/mL respectively. Results are shown in Fig. 4a and b.

Hydrogen peroxide

Hydrogen peroxide is an important reactive oxygen species because of its ability to penetrate biological membranes. However, it may be toxic if converted to hydroxyl radical in the cell. Scavenging of H_2O_2 by the plant extracts may be attributed to their phenolics, which donate electron to H_2O_2, thus reducing it to water. The extract was capable of scavenging hydrogen peroxide in a concentration dependent manner. IC_{50} of *A. marmelos* and *O. sanctum* were observed as 85.70 µg/mL and 157.7 µg/mL respectively. Results are shown in Fig. 5a and b.

LPO

During LPO, low molecular weight end products, generally malonaldehyde, are formed by the oxidation of polyunsaturated fatty acids that may react with two molecules of TBA to give a pinkish-red chromogen which was then colorimetrically determined. Selected plant extracts prevent the oxidation of the lipid layer of the cell membrane hence preventing the formation of maloaldehyde. IC_{50} of *A. marmelos* and *O. sanctum* was observed as 58.16 µg/mL and 52.97 µg/mL respectively. Results are shown in Fig. 6a and b.

TBA

Linolenic acid from the C_{18} family was subjected to the TBA assay to elucidate how polyunsaturated fatty acid characteristic affect the assay. The purpose of this study was to standardize the TBA assay by measuring the *in vitro* LPO from polyunsaturated fatty acid. TBA reacts to give multiple biomolecular breakdown products that undergo free radical attack to form TBARS. The TBA assay is not specific for MDA, one of the breakdown products of degraded fatty acids. The non-specificity probably results from the acid-heating step of the TBA assay that causes the formation of artifactual TBA/MDA-like derivatives. IC_{50} of *A. marmelos* and *O. sanctum* was observed as 38.39 µg/mL and 41.53 µg/mL respectively. Results are shown in Fig. 7a and b.

NO scavenging activity

NO is a reactive free radical produced by phagocytes and endothelial cells, to yield more reactive species such as peroxynitrite which can be decomposed to form OH radical. The level of NO was significantly reduced in this study by the crude extract. Since NO plays a crucial role in the pathogenesis of inflammation, this may explain the use of *A. marmelos* and *O. sanctum* in the treatment of inflammation. Plants with antioxidant activity have been reported to possess free radical scavenging activity. Free radicals are known as major contributors to several clinical disorders such as diabetes mellitus, cancer, liver diseases, renal failure and degenerative diseases, as a result, of deficient natural antioxidant defense mechanism. IC_{50} of *A. marmelos* and

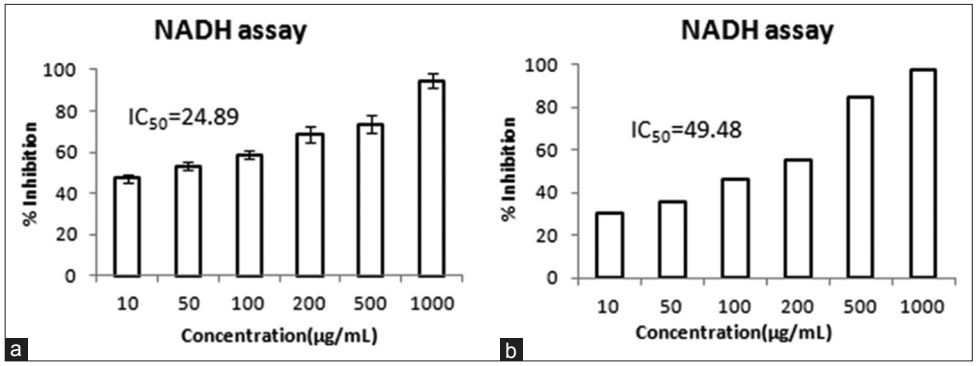

Fig. 4: Superoxide anion scavenging activity of *Aegle marmelos* (a) and *Ocimum sanctum* (b). (a) The superoxide anion scavenging activity of *A. marmelos* was determined at various concentration varying from 10 to 1000 µg/mL; (b) the superoxide anion scavenging activity of *O. sanctum* was determined at various concentration varying from 10 to 1000 µg/mL. A gradual increase in scavenging potential of the extract was obtained with an increase in concentration. IC_{50} of *A. marmelos* and *O. sanctum* was found to be 24.89 µg/mL and 49.48 µg/mL respectively; n=3 (mean±standard deviation)

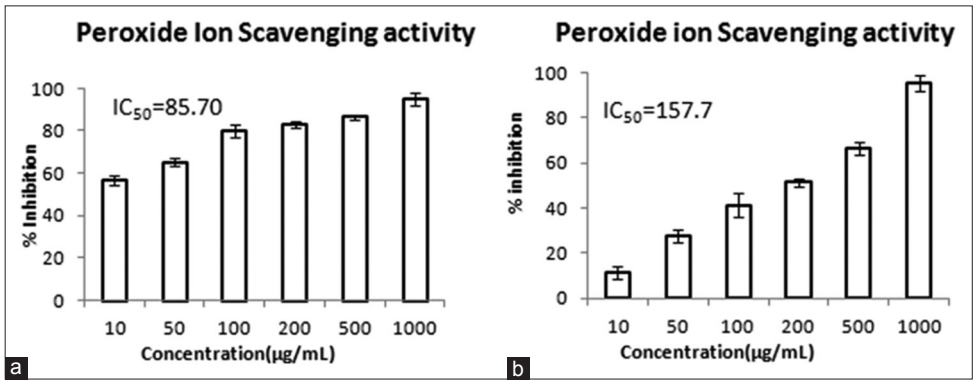

Fig. 5: Peroxide anion scavenging activity of *Aegle marmelos* (a) and *Ocimum sanctum* (b). (a) The peroxide ion scavenging activity of *A. marmelos* was determined at various concentration varying from 10 µg/mL to 1000 µg/mL; (b) the peroxide ion scavenging activity of *O. sanctum* was determined at various concentration varying from 10 to 1000 µg/mL. A gradual increase in scavenging potential of the extract was obtained with an increase in concentration. IC_{50} of *A. marmelos* and *O. sanctum* was found to be 85.70 µg/mL and 157.7 µg/mL respectively; n=3 (mean±standard deviation)

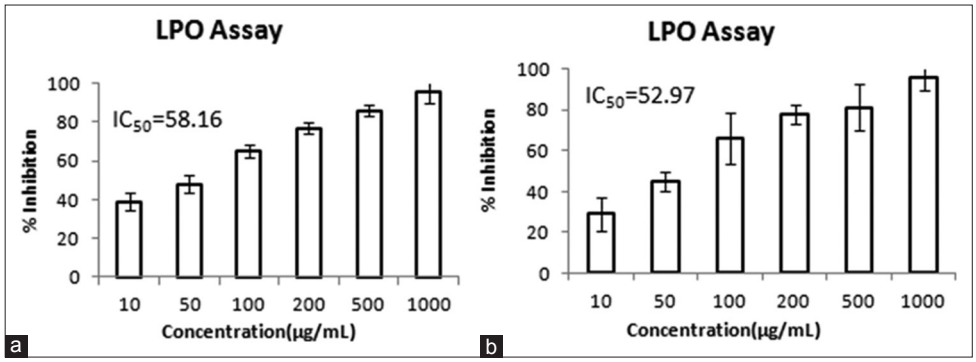

Fig. 6: Lipid peroxidation (LPO) potential of *Aegle marmelos* (a) and *Ocimum sanctum* (b). (a) represents the LPO potential of *A. marmelos* was determined at various concentration varying from 10 to 1000 µg/mL; (b) the LPO potential of *Ocimum sanctum* was determined at various concentration varying from 10 to 1000 µg/mL. A gradual increase in scavenging potential of the extract was obtained with an increase in concentration. IC_{50} of *A. marmelos* and *O. sanctum* was found to be 58.16 µg/mL and 52.97 µg/mL respectively; n=3 (mean±standard deviation)

O. sanctum was observed as 29.06 µg/mL and 44.10 µg/mL respectively. Results are shown in Fig. 8a and b.

Nephroprotective studies

Epiflourescence staining

Epiflourescence staining of healthy kidney cells treated with gentamicin and treated with *A. marmelos.* (a) The live cells stained with acrydine orange; (b) cells treated with gentamicin and stained with ethidium bromide showing dead cells as orange spots; (a') the live cells treated with genramicin and treated with vitamin E and stained with acrydine orange; (b') the cytotoxic effects of *A. marmelos* on cells treated with gentamicin and stained with acrydine orange showing the live cells; (c) the cytotoxic effects of *O. sanctum* treated on gentamicin treated kidney cells and stained with acrydine orange. Epifluorescence staining

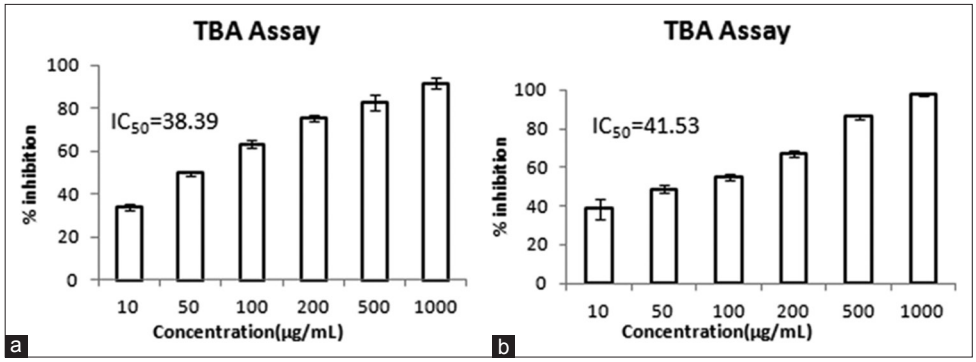

Fig. 7: Thiobarbituric acid (TBA) potential of *Aegle marmelos* (a) and *Ocimum sanctum* (b). (a) The TBA potential of *A. marmelos* was determined at various concentration varying from 10 to 1000 µg/mL; (b) the TBA potential of *O. sanctum* was determined at various concentration varying from 10 to 1000 µg/mL. A gradual increase in scavenging potential of the extract was obtained with an increase in concentration. IC_{50} of *A. marmelos* and *O. sanctum* was found to be 38.39 µg/mL and 41.53 µg/mL respectively; n=3 (mean±standard deviation)

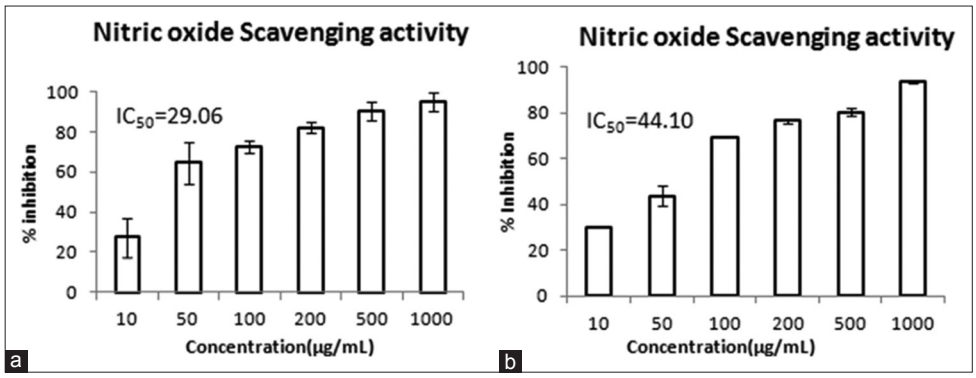

Fig. 8. Nitric oxide (NO) scavenging activity of *Aegle marmelos* (a) and *Ocimum sanctum* (b). (a) The NO scavenging activity of *A. marmelos* was determined at various concentration varying from 10 to 1000 µg/mL; (b) the NO scavenging activity of *O. sanctum* was determined at various concentration varying from 10 to 1000 µg/mL. A gradual increase in scavenging potential of the extract was obtained with an increase in concentration. IC_{50} of *A. marmelos* and *O. sanctum* was found to be 29.06 µg/mL and 44.10 µg/mL respectively; n=3 (mean±standard deviation)

is carried out to evaluate the potential of extract on gentamicin treated kidney cells. Ethidium bromide is used for staining as it stains the dead cells by entering into the nucleus and makes it appear red in the fluorescence microscope, whereas acrydine orange being permeable to the cell membrane, enters and makes it appear green which denotes live cells. Hence live cells and dead cells can be differentiated by epifluorescence staining. Both the extracts showed better activity against EAC cells. Results obtained are shown in Fig. 9a, a', b, b'and c.

***In silico* studies**

MCP1 and NGAL protein binding to ligand. (a) The binding of MCP1 protein with aegelin molecule, with an binding efficiency of −2.80; (c) the binding of MCP1 with marmin with a glide score of −3.401; (a') the binding of NGAL protein with aegelin with a glide score of −5.173; (b') the binding of NGAL with marmesin with a glide score of −4.719; (c') the binding of NGAL with marmin with a glide score of −5.433. *A. marmelos* shows better antioxidant potential; hence molecules derived from *A. marmelos* are used for *in silico* studies. MCP1 protein and NGAL protein are considered for this study as targets to bind with the ligands identified in LC-MSMS such as aegelin, marmesin and marmin. MCP1 protein is secreted by monocytes, memory T-cells and dendritic cells at the site of inflammation produced by either tissue injury or infection. Binding of ligands to this protein reduces inflammation. NGAL protein is a biomarker for acute renal injury. It is involved in innate immunity by sequestering iron that in turn limits bacterial growth. NGAL levels is proportional to the severity of nephritis. Hence, high-affinity binding of the ligand with NGAL protein result in a decrease in the nephritis. Marmin showed better binding to NGAL protein with a glide score of −5.433, hence marmin obtained from *A. marmelos* can reduce the expression levels of NGAL protein thereby preventing nephritis. Docking results are shown in Fig. 10a, a', b, b' and c', supported by Tables 3 and 4 represent IC_{50} values of *A. marmelos* and *O. sanctum*.

CONCLUSION

Antioxidant potentials of aqueous extract obtained from leaves of *A. marmelos* and *O. sanctum* were evaluated. The extracts were found to possess radical scavenging activity, anti-inflammatory, antioxidant and nephroprotective effects, as determined by protein denaturation, membrane stabilization, protease inhibition assay, scavenging effect on the DPPH, reducing power, superoxide anion, peroxide anion, LPO, TBA, NO, anti-mitotic and cell-based cytotoxicity activity suggested the nephroprotective efficacy. In general, results indicated that the extracts possess potent bioactivities. In the present study, it is found that the aqueous extract of leaves of *A. marmelos* and *O. sanctum* contains a substantial amount of phenolics and flavonoids, and it is the presence of phenolics present in these extracts that are responsible for their marked antioxidant activity. *In silico,* results show a prominent binding of the ligand with anti-inflammatory targets. Thus, it can be concluded that aqueous extract of leaves of *A. marmelos* and *O. sanctum* can be used as an antioxidant, anti-inflammatory and nephroprotective agent. Anti-inflammatory potentials of the selected extracts were depicted clearly in protein denaturation, membrane stabilization and protease inhibition assay. Antioxidant and free radical scavenging potential is clearly revealed in DPPH assay, reducing power assay, superoxide anion assay, peroxide anion assay, TBA assay and LPO assay.

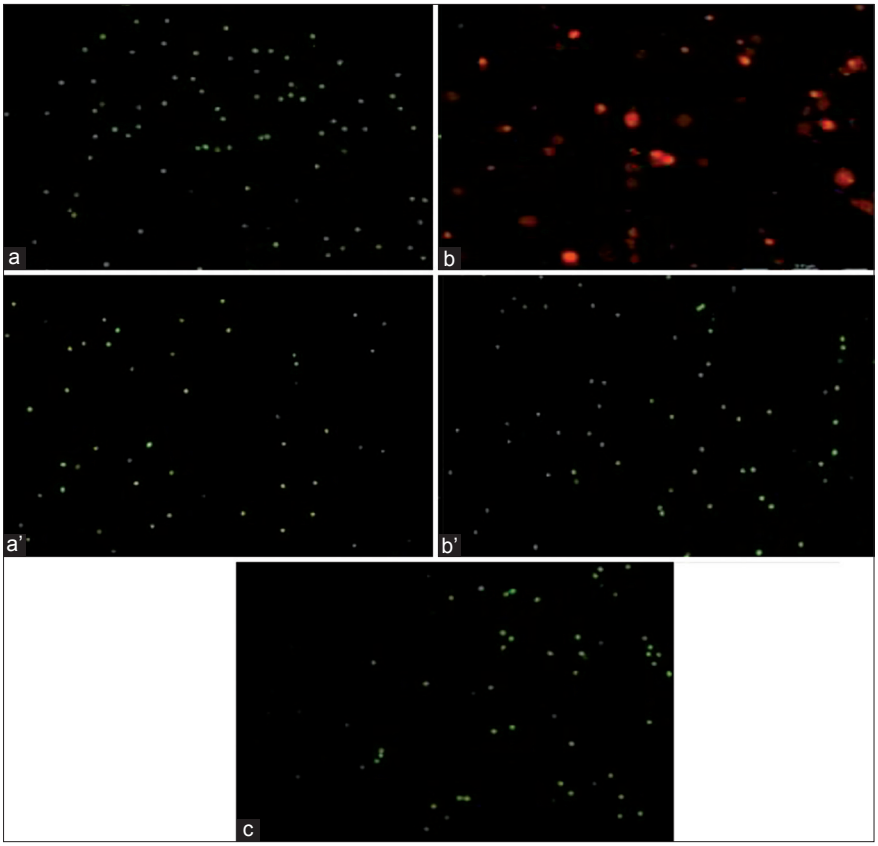

Fig. 9: Epiflourescence staining of normal kidney cells treated with gentamicin and treated with *Aegle marmelos* (a and b) and *Ocimum sanctum* (a' and b')

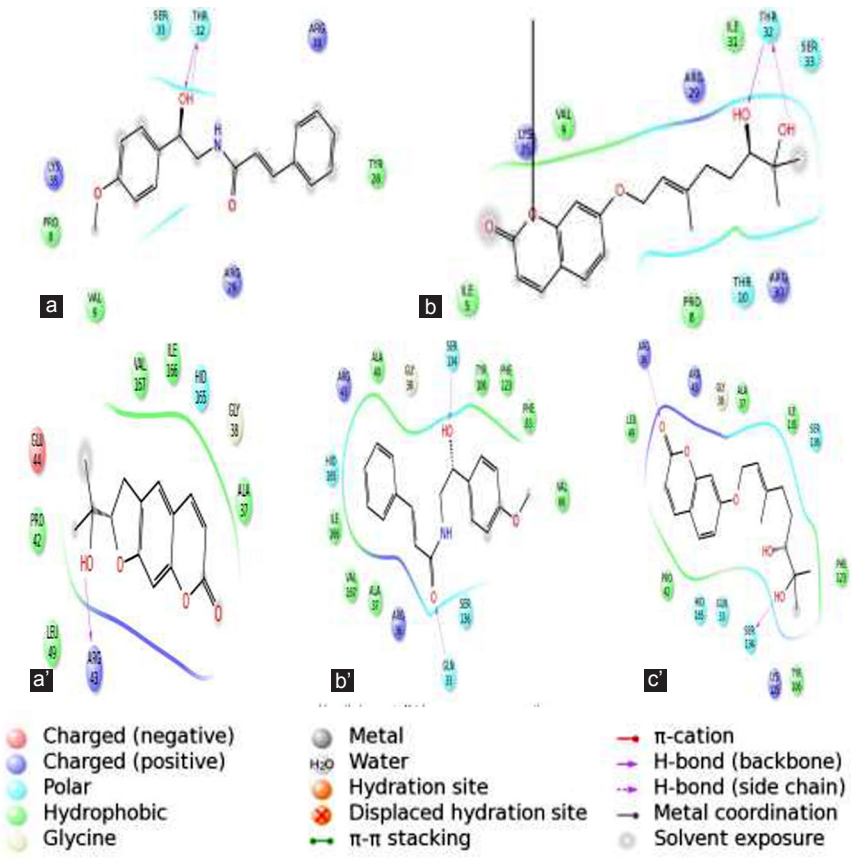

Fig. 10: Molecular docking of monocyte chemotactic protein 1 and neutrophil gelatinase-associated lipocalin protein with ligands

Table 3: IC_{50} values of anti-inflammatory assays of *A. marmelos* and *O. sanctum*

Anti-inflammatory assays	*A. marmelos* (IC_{50}) µg/mL	*O. sanctum* (IC_{50}) µg/mL
Inhibition of protein denaturation	95.64	42.17
Membrane stabilization activity	405.6	58.64
Inhibition of proteinase activity	74.45	49.70

A. marmelos: Aegle marmelos, O. sanctum: Ocimum sanctum

Table 4: IC_{50} values of antioxidant assays of *A. marmelos* and *O. sanctum*

Antioxidant assays	*A. marmelos* (IC_{50}) µg/mL	*O. sanctum* (IC_{50}) µg/mL
DPPH scavenging activity	132.1	184.6
Reducing power activity	17.84	11.40
Superoxide anion scavenging activity	24.89	49.48
Peroxide ion scavenging activity	85.70	157.7
LPO assay	58.16	52.97
TBA assay	38.39	41.53
Nitric oxide scavenging activity	29.06	44.10

DPPH: 1,1-diphenyl picrylhydrazyl, LPO: Lipid peroxidation, TBA: Thiobarbituric acid, *A. marmelos: Aegle marmelos, O. sanctum: Ocimum sanctum*

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