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Therapeutic Potentials of *Oroxylum indicum* Bark Extracts

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[ABSTRACT] AIM: Diverse therapeutic potentials of methanolic and aqueous extracts of *Oroxylum indicum* (L.) Vent. bark, including antioxidant property, cytotoxicity and protection against oxidative DNA damage were investigated in this study. **METHODS:** Total phenolics in the extracts were determined by spectrophotometric method. Ferric reducing antioxidant property (FRAP), free radical ($DPPH^{\bullet}$ and $^{\bullet}OH$) scavenging activities, as well as inhibitory effect on lipid peroxidation have been investigated. Cytotoxicity of the extracts was investigated by XTT assay in MDA-MB-435S and Hep3B cell lines. Protection of DNA by the extracts against oxidative damage by UV-photolysis of H_2O_2 was studied. **RESULTS:** Total phenolic content and inhibition of lipid peroxidation in both extracts were found to be dosage-dependent. Moreover, both extracts exhibited considerable free radical scavenging and ferric reducing abilities. The extracts demonstrated extensive cytotoxicity in both tested cell lines, with the methanolic extract showing greater cytotoxic potential. Both extracts exhibited moderate levels of DNA protection against oxidative stress. Total phenolic content was noted to have significant positive correlations with free radical scavenging and reducing power, as well as lipid peroxidation inhibition ($P < 0.05$). **CONCLUSIONS:** These results indicate that *O. indicum* bark extracts possess diverse biological activities *in vitro* and could render itself as a prospective candidate for drug research.

[KEY WORDS] Total phenolics; DPPH; FRAP; Lipid peroxidation inhibition; Cytotoxicity; DNA protection; *Oroxylum indicum*

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1 Introduction

Natural agents derived from plants have been the recent focus of many researchers due to their various health-promoting effects and their preventive effects towards many diseases including cancer. Many phytotherapies have been used traditionally to combat diseases for thousands of years [1]. Plants are known to possess various chemopreventive agents like phenolic compounds (e.g. phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant activity [2-5]. A number of chemopreventive phytochemicals have been identified in plants [6] and these phytochemicals react with prooxidants and prevent damages to DNA thereby preventing the onset of various diseases.

Reactive oxygen species (ROS) such as hydrogen peroxide, singlet oxygen and hydroxyl radical are well known prooxidants [7-8]. For maintaining health, a proper balance of prooxidants and antioxidants has to be maintained. Excessive prooxidants result in cellular stress that aggravates progression of cancer and other diseases related to aging, inflammation, cardiovascular and neuronal systems [9].

Oroxylum indicum (L.) Vent. (Bignoniaceae) is a medium-sized deciduous tree well known for its medicinal properties. There are reports that the bark decoction of *O. indicum* can be used effectively in treating cancer [10]. The plant is also used for the treatment of various other ailments like gastric ulcer, scabies and tonsil pain. *O. indicum* bark extracts are reported to have antiproliferative activity on human breast cancer cell lines [11]. The bark extracts are found to possess anti-inflammatory activity [12] and anti- microbial activity [13]. However, information on the potential of the bark extracts as a source for clinical therapeutics is still scarce.

Hence, in the present study, therapeutic properties of methanolic and aqueous extracts of the bark of *O. indicum* (L.) Vent. have been investigated.

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2. Experimental

2.1 *Chemicals*

2, 2-diphenyl-1-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), ascorbic acid, trichloroacetic acid (TCA), phenazine methosulfate (PMS) (also known as *N*-methyl-phenazonium methosulfate), dimethyl sulfoxide (DMSO), L-15 (Leibovitz) cell culture medium (with *L*-glutamine), MEM (minimal essential medium) cell culture medium (with Earle's salt, NEAA and *L*-glutamine) and 2, 4, 6-tripyridyl-*S*-triazine (TPTZ) were purchased from Himedia Labor- atories Pvt. Ltd. (India). XTT {2, 3-bis (2-methoxy- 4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide} was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Folin-Ciocalteau reagent was procured from Sisco Research Lab (India). The remaining chemicals and solvents used were of standard analytical grade and HPLC grade respectively. pBR322 was purchased from Medox Biotech India Pvt. Ltd. (India). MDA-MB-435S (human breast carcinoma) and Hep3B (human liver carcinoma) cell lines were obtained from National Centre for Cell Science (Pune, India).

2.2 *Plant material*

O. indicum bark was collected in December, 2007, from their natural habitat in the Mundoor forest range near Kanjikode (10° 47' North, 76° 47' East; 120 m above sea level), Palakkad district, Kerala, India. The plant was identified by Prof. R. V. Nair, Senior Botanist, Centre for Indian Medical Heritage (CIMH), Kanjikode, Palakkad, Kerala, India (Ref: CIMH/MP/2019/2007). The collected specimens were shade dried, powdered and extracted. Vouchers specimens are maintained in our laboratory for future reference.

2.3 *Extraction*

The powder was serially extracted with methanol and water using a Soxhlet apparatus in a ratio of 1∶6. The extract obtained was evaporated to dryness at 40 °C in reduced pressure (methanol: 337 mbar and Aqueous: 72 mbar) in a rotary evaporator (BÜCHI Labortechnik AG, Switzerland). The dried samples of each extract were weighed to determine the yield of soluble constituents and stored in a vacuum desiccator.

2.4 *Determination of total phenolic compounds*

The method described by Lister and Wilson (2001) [14] was followed to determine the total phenolic compounds of each extracts. 50, 100, 150, 200 and 250 μg of the extracts were taken and made up to 0.5 mL with distilled water. 2.5 mL of Folin-Ciocalteau reagent (1∶10 dilution) was added, followed by the addition of 2 mL of sodium carbonate (7.5% *W*/*V*). Samples were incubated at 45 ºC for 15 min, and then absorbance was read at 765 nm using a Cary 50 UV-Vis Spectrophotometer (Varian, Inc., CA, USA). The results were expressed in terms of gallic acid equivalence (GAE) in μg.

2.5 *Ferric reducing antioxidant property (FRAP)*

FRAP assay was done according to the protocol of Benzie and Strain (1996) [15] with some modifications. The stock solutions were 300 mmol acetate buffer (3.1 g $C_2H_3NaO_2{\cdot}3H_2O$ and 16 mL $C_2H_4O_2$; pH 3.6), TPTZ solution (10 mmol TPTZ in 40 mmol HCl) and 20 mmol $FeCl_3{\cdot}6H_2O$ solution. Working FRAP solution was prepared freshly by mixing 25 mL of acetate buffer, 2.5 mL TPTZ solution and 2.5 mL of $FeCl_3{\cdot}6H_2O$ solution, and then warmed to 37 °C before use. 150 μL of individual extract solutions (containing 25, 50, 100 and 200 μg of extracts, respectively) was allowed to react with 2.85 mL of FRAP solution for 30 min in dark. Absorbance was read at 593 nm. Percentage Fe^{3+} reduction (to Fe^{2+}) was calculated by a $FeSO_4$ standard calibration curve. Percentage scavenging was also evaluated in ascorbic acid equivalence (AAE) (in μg).

2.6 *Tests for free radical scavenging*

2.6.1 *DPPH Radical Scavenging Test*

The ability of the extracts to scavenge free radicals was tested by DPPH radical scavenging test as described by Blois (1958) [16]. The basic principle of the assay involves the reduction of DPPH$^{\bullet}$ which is related to the antioxidant potential of the sample. 20, 40, 60, 80 and 100 μg of the extracts were used for the assay. The volume was made up to 0.5 mL with double distilled water and 3 mL of 0.1 mmol DPPH$^{\bullet}$ in ethanol was added. Samples were incubated for 30 min at room temperature in dark. The absorbance was then read at 517 nm. Percentage inhibition (I/%) was calculated using the formula,

$$\mathbf{I/\% = [(}A_C - A_S\mathbf{) / }A_C\mathbf{]} \times \mathbf{100}$$

whrere A_C = control absorbance, A_S = sample absorbance.

Results were also presented in ascorbic acid equivalence (AAE) in μg.

2.6.2 *Hydroxyl radical scavenging activity (HRSA)*

$^{\bullet}$OH radical scavenging activity of the extracts was estimated by the method of Klein *et al.* (1981) [17]. 50, 100, 150 and 200 μg of the extracts were taken in test tubes. 1 mL of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 mL of 0.018% EDTA and 1 mL of 0.85% (*V*/*V*) DMSO (in 0.1 M phosphate buffer, pH 7.4) were added followed by 0.5 mL of 0.22% (*W*/*V*) ascorbic acid. The tubes were capped tightly and incubated on a water bath at 85 ˚C for 15 min. Post incubation, the test tubes were uncapped and ice-cold trichloroacetic acid (17.5% *W*/*V*) was added in each immediately. 3 mL of Nash reagent (7.5 g of ammonium acetate, 300 μL glacial acetic acid and 200 μL acetyl acetone were mixed and made up to 100 mL with distilled water) was added to all the tubes and incubated at room temperature for 15 min. Absorbance was measured at 412 nm. Percentage hydroxyl radical scavenging activity (HRSA/%) was calculated by the following formula:

$$\mathbf{HRSA/\% = [(}A_c - A\mathbf{)/}A_c\mathbf{]} \times \mathbf{100}$$

where A_c is the absorbance of the control and A is the absorbance of the extract.

2.7 *Test for inhibition of lipid peroxidation*

Lipid peroxidation inhibition activity of the extracts was

determined by thiobarbituric acid (TBA) assay of Halliwell and Gutteridge (1999) [18]. Lipid peroxidation by reactive oxygen species (ROS) is known to be involved in the damaging mechanism of cells and tissues. This assay is based on the reaction of the end product of lipid peroxidation, malondialdehyde (MDA) with TBA to form an adduct, a pink chromogen. The liver for the preparation of homogenate used in this assay was obtained from Wistar strain Albino rats after the approval of the Institutional Animal Ethical Committee (PSGIMSR/27.02.2008) and was performed in accordance to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised in 1985) [19]. 15, 30, 45 and 60 μg of the extracts were used for the assay. The extracts were taken in test tubes and were evaporated to dryness. 1 mL of 0.15 mol·L^{-1} potassium chloride was added followed by 0.5 mL of rat liver homogenate (10% *W*/*V* in PBS). Peroxidation was initiated by the addition of 100 μL of 2 mmol·L^{-1} ferric chloride, and incubated at 37 ºC for 30 min. The reaction was then stopped by adding 2 mL of ice cold HCl (0.25 mol·L^{-1}) containing 15% TCA and 0.38% TBA. The tubes were kept at 80 ºC for 1 h. Samples were then cooled and centrifuged at 7 500 r·min^{-1}. Absorbance of the supernatants was read at 532 nm. The lipid peroxidation inhibition (LPI/%) was calculated using the formula:

$$\mathbf{LPI/\%= [(}A_C - A_S\mathbf{) / }A_C\mathbf{] \times 100}$$

where A_C = control absorbance, A_S = sample absorbance.

Results were also presented in butylated hydroxy toluene (BHT) equivalence in μg.

2.8 *Cytotoxicity testing by XTT assay*

XTT assay was performed on MDA-MB-435S (in L-15 medium) and Hep3B (in MEM medium with 1mmol·L^{-1} sodium pyruvate) cell lines as described by Weislow *et al*. (1989) [20]. 6 × 10^3 cells (of each cell type) were seeded on a 96-well plate and were supplemented with 200 μL of culture media for a period of 24 h. The media were then removed, and 200 μL of fresh media containing varying concentrations of the extracts (15.625, 31.25, 62.5 and 125 μg·mL^{-1}) were added to the cells in the exponential growing phase and the plate was incubated in a CO_2 incubator for 24 hours. At the end of the incubation period, the media were removed and fresh media added. 50 μL XTT reagent prepared in medium (0.6 mg·mL^{-1}) containing 25 μmol·L^{-1} of PMS was then added and the plates were wrapped in an aluminum foil and incubated in a humidified atmosphere at 37 ºC for 4 h. After the incubation period, the orange colored complex formed was read at 450 nm using a Dynex Opsys MR^{TM} Microplate Reader (Dynex Technologies, VA, USA) using a reference filter of 630 nm. Wells containing only media and XTT reagent were used to blank the plate reader. Wells containing cells grown on media and no extract treatments served as the control. The percentage cytotoxicity was calculated by using the formula,

$$\mathbf{Cytotoxicity/\%= [(}A_C - A_S\mathbf{) / }A_C\mathbf{] \times 100}$$

where A_C = control absorbance, A= sample absorbance.

2.9 *DNA damage protective activity*

DNA damage protective activities of the extracts were determined as described by Russo et al. (2001) [21] with few modifications, by observing the protection of pBR322 DNA (200 μg·mL^{-1}) from damage by hydroxyl radicals generated from UV-photolysed H_2O_2. The experiment was performed with 1 μL of pBR322 DNA and 50 μg of the plant extracts. Prior to irradiation with UV, 3% H_2O_2 was added. The tubes were then placed directly on the surface of a UV trans- illuminator (8 000 μW/cm^{-2}) at 300 nm and irradiated for 15 min. All DNAs were run on 1% agarose gel and photographed on Lourmat Gel Imaging System (Vilbar, France). Plasmid DNA with and without H_2O_2 and UV irradiation was also included as negative and positive control groups respectively.

2.10 *Statistical analysis*

All data were recorded as $\bar{x} \pm s$ deviation of triplicate measurements. Significant differences among sample groups were determined by one-way ANOVA at $P < 0.05$. MATLAB ver. 7.0 (Natick, MA, USA), SPSS ver. 9.05 (Chicago, IL, USA) and Microsoft Excel 2007 (Roselle, IL, USA) were used for the statistical and graphical evaluations.

3 Results and Discussion

3.1 *Yield of extracts*

50 g of bark powder yielded 1.92 g of methanolic crude extract and 10.46 g of aqueous crude extract.

3.2 *Total Phenolic compounds*

According to Velioglu et al. (1998) [5], phenolic compounds are one of the most effective kinds antioxidants present in plants. Hence, the total phenolic content in methanolic and aqueous extracts of *O. indicum* bark was quantified and expressed in GAE (Table 1). The methanolic extract showed high phenolic constitution as compared to the aqueous extract. This may be due to the differences in the polarity of the extracting solvents which elute diverse types of components differentially].

Table 1 Total phenolic contents of methanolic and aqueous extracts of *Oroxylum indicum*

Concentration (μg)	GAE ± s (in μg) [a]	
	Methanolic extract	Aqueous extract
50	9.69 ± 0.22	9.17 ± 0.06
100	13.10 ± 0.14	12.50 ± 0.20
150	17.00 ± 0.08	15.58 ± 0.08
200	21.76 ± 0.38	15.86 ± 0.36
250	25.56 ± 0.10	16.17 ± 0.13

[a] GAE ± s at 95% confidence interval

3.3 *FRAP*

Many studies have indicated that the electron donation capacity (reflecting the reducing power) of compounds is associated with their antioxidant activity [22]. The ability of the extracts to reduce Fe^{3+} to Fe^{2+} was determined and compared to that of ascorbic acid (Fig. 1). Both extracts of *O. indicum* showed a concentration-dependent reducing effect.

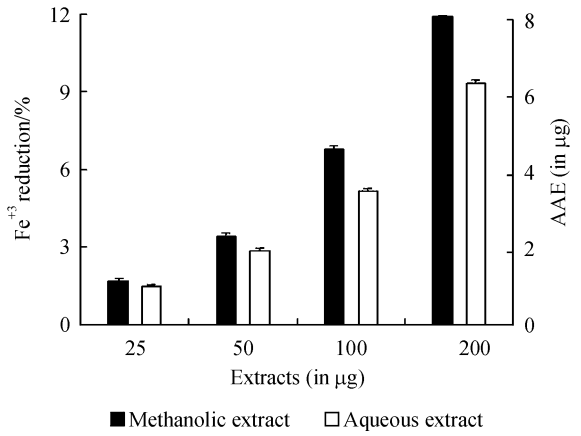

Fig. 1 Reducing power of methanolic and aqueous extracts of *O. indicum* bark along with AAE (ascorbic acid equivalence) in μg determined by FRAP assay. Data expressed as $\bar{x} \pm s$ of $n = 3$ samples ($P < 0.05$).

3.4 *Free radical scavenging*

3.4.1 *DPPH radical scavenging activity*

DPPH scavenging activities of the extracts were found to be concentration-dependent. Such results have also been reported by many other investigators [23]. Percentage inhibition (I/%) of DPPH$^{\bullet}$ radical is presented in Fig. 2. I/%, as observed, demonstrated statistically significant diversity between the methanolic and aqueous extract at $P < 0.05$.

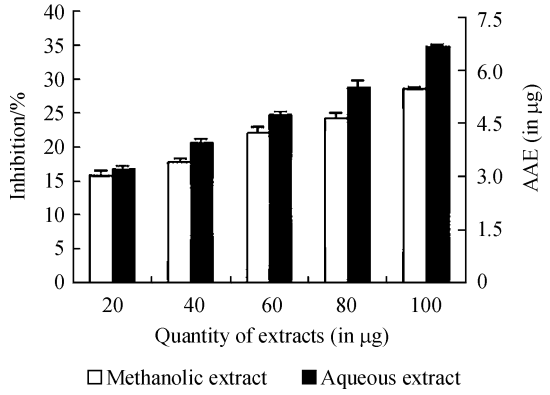

Fig. 2 DPPH scavenging activities of methanolic and aqueous extracts of *O. indicum* bark with AAE (ascorbic acid equivalence) in μg. Data expressed as $\bar{x} \pm s$ of $n = 3$ samples ($P < 0.05$)

3.4.2 *HRSA*

Fig. 3 represents the $^{\bullet}$OH radical scavenging efficacy of the extracts. Both extracts showed significant HRSA/% at $P < 0.05$.

3.5 *Inhibition of lipid peroxidation*

Both extracts of *O. indicum* bark displayed comprehensive dosage-dependence in inhibiting lipid peroxidation in the range of concentrations tested. The results are illustrated in Fig. 4 with BHT equivalence.

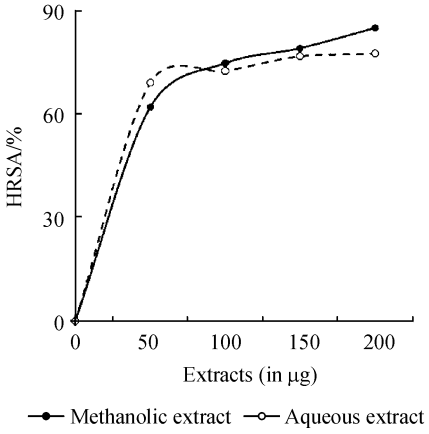

Fig. 3 Hydroxyl radical scavenging efficacy of methanolic and aqueous extracts of *O. indicum* bark

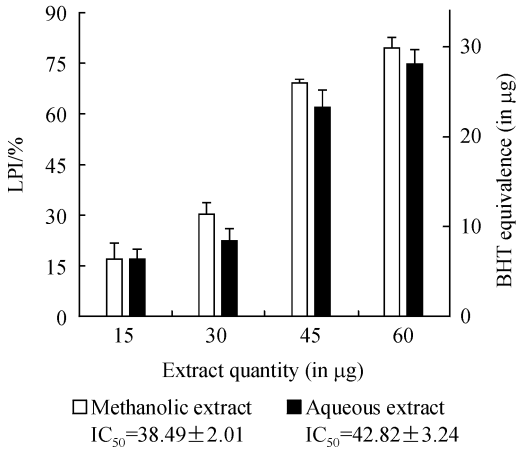

Fig. 4 Lipid peroxidation inhibition activities of methanolic and aqueous extracts of *O. indicum* bark with BHT equivalence in μg. Data expressed as $\bar{x} \pm s$ ($n = 3$, $P < 0.05$).

3.6 *Cytotoxicity in MDA-MB-435S and Hep3B cell lines*

Cytotoxicity of the extracts as determined by XTT assay in MDA-MB-435S and Hep3B cell lines is presented in Fig. 5. Both extracts showed cytotoxic effect in inhibiting the proliferation and survival of the carcinoma cells.

3.7 *DNA damage protective activity*

The electrophoresis pattern of DNA after UV-photolysis of 3% H_2O_2 in the absence and presence of the methanolic and aqueous extracts of *O. indicum* bark is shown in Fig. 6. 50 μg of both extracts were tested for their protective activity. pBR322 DNA without UV and H_2O_2 treatment acted as the control (Lane 1). Lanes 2 represented irradiated control, and lanes 3 and 4 were extract treated irradiated samples. Untreated pBR322 run on a 1% agarose gel formed faint open circular DNA (OC) and distinct super coiled DNA (SC) bands which were identified with their respective electrophoretic mobility. UV induced photolysis of H_2O_2 led to

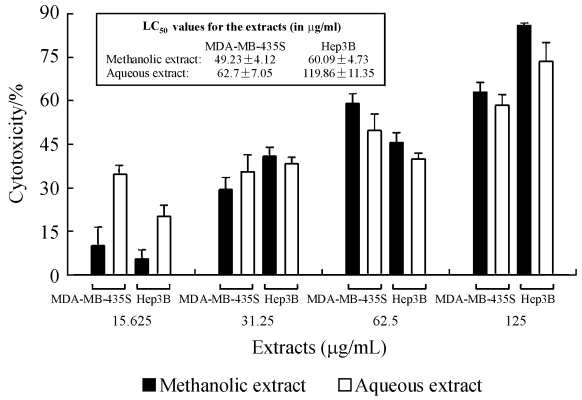

Fig. 5 Percentage cytotoxicity of methanolic and aqueous extracts of bark of *O. indicum* in MDA-MB-435S and Hep3B cells. Data expressed as $\bar{x} \pm s$ of $n = 3$ samples ($P < 0.05$).

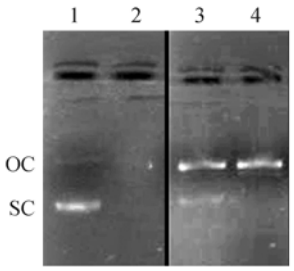

Fig. 6 Effect of methanolic and aqueous extracts of *O. indicum* bark at 50 μg concentration, on the protection of DNA against $OH^{\bullet}$ radicals generated by photolysis of H_2O_2. Lane 1: untreated DNA (control); lane 2: 3% H_2O_2 + DNA; lane 3: Aqueous extract + H_2O_2 + DNA; lane 4: Methanolic extract + H_2O_2 + DNA. Samples in lanes 2-4 were UV-irradiated.

complete obliteration of both bands in UV-photolysed control, thereby indicating total destruction of the DNA. Aqueous extract displayed a significant protective activity in comparison to methanolic extract.

Recent reports have recommended using at least two test systems for the determination of antioxidant activity owing to the complex nature of phytochemicals present in plants [24]. Accordingly, this study holistically makes the antioxidant capacity of the extracts evident through multi-model testing.

Correlations (significance at $P < 0.05$) were analyzed between different parameters investigated in this study. Table 2 presents r^2 (coefficient of determination) values for the correlations extrapolated from the observed results.

4. Conclusions

It was concluded that *O. indicum* bark bears diverse therapeutic potential in crude methanolic and aqueous extract forms, and possesses compounds which might be exploited to develop precursors of drugs that might act as antithesis of free radical damage in biological systems. Further studies on the isolation and characterization of these compounds are in prospect.

Table 2 Correlations between experimental results (of total phenolic estimation, FRAP, DPPH, hydroxyl and TBA assays) tested for significance at $P < 0.05$. r^2 denotes coefficient of determination.

Correlations	Extracts	r^2 ($P < 0.05$)
Total phenolics and FRAP	Methanolic	0.997 4
	Aqueous	0.995 2
Total phenolics and DPPH scavenging	Methanolic	0.982 9
	Aqueous	0.992 4
Total phenolics and $^{\bullet}OH$ scavenging	Methanolic	0.985 8
	Aqueous	0.962 1
Total phenolics and LPI/%	Methanolic	0.931 8
	Aqueous	0.966 8
FRAP and DPPH scavenging	Methanolic	0.968 5
	Aqueous	0.976 2
FRAP and $^{\bullet}OH$ scavenging	Methanolic	0.973 3
	Aqueous	0.935 6
FRAP and LPI/%	Methanolic	0.975 1
	Aqueous	0.960 1
DPPH scavenging and $^{\bullet}OH$ scavenging	Methanolic	0.999 5
	Aqueous	0.986 8
DPPH scavenging and LPI/%	Methanolic	0.901 0
	Aqueous	Not significant
$^{\bullet}OH$ scavenging and	Methanolic	0.916 1
LPI/%	Aqueous	Not significant

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