OXYGEN REACTIVE SPECIES EFFECTIVELY SCAVENGED BY VARIOUS EXTRACTS OF LEAVES AND BARK OF *Eucalyptus globulus*

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ABSTRACT

Eucalyptus globulus is belong to a family of *Myrtaceae* and one of the important medicinal plants. Eucalyptus leaf extract is a used as an antioxidant in various food additives. With this in mind, fresh leaves and bark of eucalyptus were taken dried, grinded and extractive contents were measured. Various extracts of leaf and bark of *Eucalyptus globulus* were made and used for the estimation of phytochemicals, total flavonoids or phenolic content, and for determination of *in-vitro* antioxidant activity. The highest extractive contents of *E. globulus* leaf were 4.75±0.25% and bark 7.95±0.50% with water. Phytochemical tests showed that the water extracts of the leaves and bark contain alkaloids, flavonoids, glycosides, terpenoids, steroids, and saponins, while there are no cardiac glycosides and anthraquinones. Phenols and flavonoids studies have shown that leaves extract contain significant amounts of phenols and flavonoids (152.6±1.5 mg/g gallic acid equivalent; 42.30±0.7 mgQE/g dry weight) and 120.3±1.1 mg/g gallic acid equivalent 29.2±0.5 mgQE/g dry weight in water extract of bark. All extracts exhibited considerable DPPH (2,2-diphenyl-1-picrylhydrazyl), with the highest percentage inhibition in aqueous leaves extracts 87.41±1.2 (IC_{50} 58.88±2.15 µg/ml), while lower in chloroform 48.39±0.6 (IC_{50} 88.22±2.90 µg) /ml), than bark water extract: 80.15±1.1 (IC_{50} 63.43±2.25 µg/ml; chloroform extract: 42.02±0.5 (IC_{50} 90.10±2.95), While the percentage inhibition of BHT was 92.8±1.3, the IC_{50} was 29.70±1.55 µg/ml was detected at 250 µg/ml concentration. Reducing capacity when compared in leaves and bark, the following order was observed: water extract>ethanol extract>EtOH-H_2O>methanol extract>MeOH-H_2O extract>chloroform extract. This study has confirmed that water extracts of *E. globulus* leaves and bark have the highest antioxidant activity and may contain high amounts of flavonoids and phenolics.

Keywords: *Eucalyptus globulus*, leaves, bark, extractive contents, phytochemicals, total phenols, flavonoids, oxygen scavenging activity.

INTRODUCTION

Today, consumers have a huge demand for low-processed foods and free from synthetic chemical preservatives with natural feel. Therefore, the food industry faces enormous challenges in producing natural antioxidants to reduce the use of synthetic chemical preservatives. In addition, there is a growing trend towards the use of herbal lifestyle and dietary choices for the maintenance of human well-being and these natural products can help the whole body and improve immune status [1]. According to [2], approximately 52% of therapeutic medicines were prepared from natural sources such as wood which is a prime natural source of varied biomolecules e.g., secondary metabolites cumulated during the growth period of plant. Extract of bark used as a medicine and reported in traditional pharmacopeias treat several pathological conditions. [3-5]. Members of the *Myrtacaea* family *Eucalyptus globulus* are native to Australia and grown in multiple locations worldwide particularly in subtropical regions such as, southern Europe, America, Africa and South, Asia. Eucalyptus leaves were used in medicines to treat respiratory tracts, bronchitis inflammation and in symptomatic condition such as asthma, throat inflammation and fever. Various biological activities by eucalyptus were also reported such as antibacterial, [6-7] hypoglycemic, [8] analgesic and anti-inflammatory [9]. Essential oils have bactericidal, bactericidal, and insecticidal properties [10]. It's essential oils also have been reported to have antioxidant activity [11-13].

The antioxidant activity of its wood samples [14], stem bark [15] and leaves were also reported in given literature. [16, 17]. After normal cellular metabolism several reactive species were produced such as reactive oxygen, hydroxyl radicals ($^{\bullet}OH$), hydrogen peroxide (H_2O_2) and superoxide anion ($O^{\bullet-2}$). Those species are toxic enough to disturb the normal metabolism if not normalized by antioxidants. They activated several reactions to damage lipids, proteins and oxidation of DNA thus contributing to implications of serious pathologies such as cancer, neurodegeneration, atherosclerosis, and diabetes. Several risk factors are associated with its higher production such as environment pollutants, consumption of alcohol, inhaling tobacco smoke and synthetic pesticides. [18-21]. Natural antioxidants used as an alternative to synthetic antioxidants, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), due to their higher toxicity potential. As those synthetic substances impart carcinogenic effects and enhance hepatic complications. [22-23]. The preparation of antioxidants from natural sources like seeds, grains, fruits, and vegetables are encouraged by factors such as conflicting data on the safety of synthetic compounds, growing demand, and increasing consumer preference for natural food additives. Extraction of natural antioxidants by various employing various techniques, was focus of recent studies [24-26] and identified various compounds which can be used an alternative to synthetic food additives [27]. We performed the present work on *Eucalyptus globulus* leaves and bark which show that the regularly shed discarded of the plant can be used as a food dye with high antioxidant activity.

MATERIALS AND METHODS

Chemicals and Reagents

Folin Ciocalteu reagent, DPPH and gallic acid were purchased from Sigma Chemical Co. solvents (n-hexane, chloroform and methanol and reference compound BHT purchased from Merck. Chemicals and solvents are all are of analytical grade.

Plant material and preparation of different extracts

Eucalyptus leaves and stem bark were collected at the PCSIR, Labs Complex and Lahore sites during the summer of July 2021. Leaves and bark are dried at room temperature and were extracted using three different solvents: chloroform, methanol, methanol-water (50%), ethanol, 50% ethanol-water and water. 5 gm of leaf and stem bark were taken in each case and extracted with 250 ml of these solvents for 24 h in a shaker and incubated at 25 °C. Under reduced pressure filtered concentrated extract was dried [28].

Extractives Contents (Yield %)

All extracts of *E. globulus* leaves and bark were individually weighed by using a digital balance (OHAUS, USA) and extractive contents (% yield) were determined using the following formula [29].

Extractive content = (weight of particular crude extract/total amount of crushed powder) x 100.

Profiling of bioactive compounds

E. globulus leaves and bark aqueous extract was prepared to reveal bioactive compounds such as alkaloids, tannins, flavonoids, saponins, carbohydrates, glycosides, steroids, phenols, anthraquinones and reducing sugars. These were identified by characteristic changes using standard reporting methods [30-32].

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Total Phenolic Contents

Folin-Ciocalteu method after slight modification was employed to determine the total phenolic content of extract. Add 1mL of Folin-Cioalteu reagent and 0.8 mL of 7.5% sodium carbonate reagent in 200 µL of *E. globulus* leaves and bark extract [33-34]. Readings were taken at 760 nm on (UV-Vis: 1700, Shimazdu, Japan) after 30 minutes incubation. Standard, gallic acid was used to generate calibration curve. Standard curve for gallic acid was used to detect the concentration of polyphenols in extract and expressed as mg of gallic acid which is equivalents to per milligram (mg GAE/mg) [35].

Determination of flavonoids

Aluminium chloride colorimetric determination of flavonoids in *E. globulus* leaves and bark extracts was implemented according to the protocol described by Dewanto et al. [36] after modest modifications [37]. Approximately 250 µL of solvent extract was added in 1250 µL of distilled water and mixed the solution thoroughly following the addition of 275 µL of 5% sodium nitrite solution. For 6 minutes mixture was incubated at room temperature. After the addition of 10% aluminum trichloride solution ($AlCl_3$) and incubated for 5 minutes at room temperature. 500 µL of sodium hydroxide solution (1 M) solution dissolved in mixture and stand it at room temperature for 30 minutes. Using spectrophotometer (UV-Vis: 1700, Shimazdu, Japan) absorbance was taken at 510 nm and solvent used as blank. Reaction was carried out in triplicates. Standard compound quercetin was to estimate total flavonoid contents. Quantification was expressed as mg of quercetin equivalent to gram of extract.

DPPH Assay

Free radical scavenging activity of *E. globulus* leaves and bark extract was determined by DPPH assay developed by Brand-Williams 1995 [38], after some modifications (Saeed et al., 2021) [39]. Aliquots of about 0.1 mL extract solution (50-250 µL/mL) was mixed with 2.9 mL 2.9 mL of 0.04% of methanolic solution and mixed thoroughly. Absorbance of all extract was taken at 517 nm by using spectrophotometer UV-Vis (1700, Shimazdu, Japan). Estimation was expressed as percentage inhibition and calculated as.

$$\% \text{ Inhibition} = A_{control} - A_{sample}/A_{control} \times 100$$

Reducing power activity

E. globulus reducing capacity was determined by using method of Oyaizu, (1986) [40] after implementing slight modifications [41]. Phosphate buffer (pH 6.6, 0.2 mol/l) of 2.5 mL and 1% of 2.5 mL $K_3Fe(CN)_6$ was mixed with extract solutions of E. globulus. After incubation for 20 minutes at 50°C add 10% of 2.5 mL trichloroacetic acid as stopping agent. For 10 minutes centrifuge the mixture at 6000 g. After centrifugation 2.5 mL of upper mixture was mixed with 2.5 mL H_2O and 0.5 mL of 1% $FeCl_3$. Absorbance was taken at 700 nm on spectrophotometer.

Statistical analysis

One-way analysis of variance (ANOVA) was performed on all data to determine significance using STATISTICA 7.1. The standard deviation (±SD) of all data were calculated based on three replicates (n=3). Means were compared using Tukey's HSD test with a significance level of $P < 0.05$. IC50 was obtained using Origine Pro 8.5 software.

RESULTS AND DISCUSSION

Extractive contents

The yield percentage/extractive content of various extracts of *E. globulus* leaves were ranged from 1.9–4.75% and bark were 2.5–7.95% **(Fig 1A and 1B)**. The extractive content of *E. globulus* leaves and bark depends on the solvent used and to a lesser extent the method used for extraction [42–44]. The highest extractive contents of *E. globulus* leaves and bark were obtained with water extraction (4.75±0.25 % and 7.95±0.50 %, respectively), indicating the possible presence of large amounts of polar compounds such as phenolic compounds and flavonoids. The results obtained using extracts with different solvents like chloroform, methanol, ethanol, 50% water/methanol, 50% water/ethanol depend on the extraction time and the method used [45]. Numerous studies on several tropical species have reported that bark extracts are considerably higher than leaves [46-48].

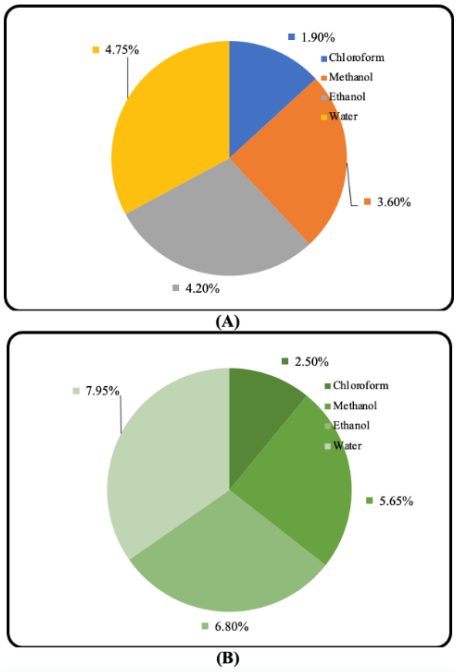

Figure 1. (A) Extractives Contents *of E. globulus* Leaves, (B) Extractives Contents *E. globulus* Bark

Phytochemical analysis

The phytochemical properties of *E. globulus* leaves and bark water extract are summarized in Table I. Qualitative phytochemical (bioactive compounds) testing indicated that aqueous extracts of *E. globulus* leaves and bark contain alkaloids, flavonoids, glycosides, terpenoids, steroids, saponins and reducing sugars. Aqueous extract eucalyptus leaves are free of cardiac glycosides and anthraquinones while aqueous bark extract also have no chlorophyll and carbohydrates. Our results are identical to those described by Mishra et al. in 2010 [49]. The presence of these phytochemical compounds in plant material suggests that their extracts have potential medicinal value in the prevention and/or treatment of specific diseases [50-51].

Table 1. The analysis of bioactive compounds in the water extracts of *E. globulus* leaves and bark.

Bioactive compounds	Test name	Observation	Qualitative Leaves	Result Bark
Alkaloids	Dragendorff's	Orange red precipitate	+	--
Flavonoids	General test	Reddish pink color	++	++
Glycoside	Liebermann test	Rose pink-red color in aq. layer	--	--
Phenols	Ferric chloride	Red or blue color	++	++
Saponins	Frothing test	A foam layer	+	+
Steroids	Salkowski test	Reddish brown coloration	--	--
Tannins	Ferric chloride	Blue-green coloration	++	+
Anthraquinones	Borntrager"s test	Pink or red coloration	--	--
Carbohydrates	Molisch"s test	Purple ring on the intercom	+	--
Chlorophyll Test	General Test	Deposition of red precipitate	++	--

"++" stands for the vastly presence, "+" for the presence and "--" indicates the absence of bioactive compounds.

Total Phenolic Content

Bark extract and *E. globulus* leaves extract total phenolic content was estimated by using Folin-Ciocalteu method and expressed as mg/g.Reagent constitute of polymeric ions produced from phosphotungstic heteropoly acid and phosphomolybdic acid and is of yellowish colour. It gives molybdenum-tungsten blue colour after oxidizing phenate and detected using spectrophotometrically at 760 nm [52]. The data in **Table 2** show that, on a gallic acid equivalent basis, the highest total phenol content was 152.6±1.5 mg/g obtained in polar water extracts, while the lowest total phenol content was 40.3 ± 0.7 mg/g on a gallic acid basis to obtain an equivalent amount in chloroform extract. The results were similar to those described by Bhuyan et al., 2016 [53], who reported TPC in aqueous

extracts (150.60±2.47 mg GAE/g). The results of this study showed a higher TPC compared to that of Luis et al. [54] who found the total phenolic content 218.67±4.52 mg/g dw plant material in a 75% ethanolic wood extract of *E. globulus*. These results also corroborate previous studies measuring the phenolic content of *E. globulus* plant leaves extracts [55, 56].

Flavonoids content

Extract flavonoid content was estimated and results were shown in Table 2. It was shown that aqueous extract of bark and E. globulus showing higher significantly ($p < 0.05$) total flavonoids contents of 42.30±0.7 mgQE/g which is higher as compared to solvent extract of about 29.2±0.5 mgQE/g. When comparing the values of flavonoids, the following order was observed in leaves and bark: water extract > ethanol extract > EtOH-H_2O > methanol extract > MeOH-H_2O extract > Chloroform extract. Regarding the total flavonoids of *E. globulus* leaves extract Hassine et al. [57] reported a significant reduction in flavonoids in ethanolic extracts (34.3±0.1 mg/g dw plant material), and Dezsi et al., 2015 [58] reported that *E. globulus* leaves in ethanolic extracts had 35.76±0.95 mg/g dw in plant material. Flavonoid content of plant was regulated several genetic factors and type of species, geographic location, soil type, harvesting season, type of herb prepared storage and drying method [59]. Herb antioxidant potency is related to TPC and TFC content [60-61].

Table 2. Total polyphenols and flavonoids of various extracts of *E. globulus* leaves and bark.

Extracts	*Eucalyptus globulus* Leaves		*Eucalyptus globulus* Bark	
	TPC (mgGAE/g)	TFC (mgGAE/g)	TPC (mgGAE/g)	TFC (mgGAE/g)
Chloroform ($CHCl_3$)	40.3 ± 0.7	06.2 ± 0.1	14.5 ± 0.2	02.1 ± 0.1
Methanol (MeOH)	95.8 ± 1.1	14.60 ± 0.4	67.4 ± 0.8	10.3 ± 0.2
Ethanol (EtOH)	138.7 ±1.3	31.40 ± 0.6	106.7 ± 1.0	24.6 ± 0.5
Water (H_2O)	152.6 ± 1.5	42.30 ± 0.7	120.3 ± 1.1	29.2 ± 0.5
MeOH + H_2O	83.9 ± 1.0	09.40 ± 0.3	50.8 ± 0.8	04.7 ± 0.1
EtOH + H_2O	122.8 ± 1.1	17.60 ± 0.4	80.2 ± 0.8	12.6 ± 0.2

Data are represented ± standard deviation.

DPPH radical scavenging activity

Free radical scavenging activity is easy, definitive reliable method used widely to assess the reactive oxygen species and is intuitive toward Lewis bases [62]. Principally it accomplished the detection by working in polar solvent and nonpolar solvent as electron donor and hydrogen atom donor, respectively [63-64]. Alteration of colour from violet to pale yellow DPPH indicated the presence of antioxidant [65]. In present study all extracts demonstrated highest antioxidant activity as indicated by lower absorbance also the percent inhibition indicated that our extract inhibited DPPH free radicals in a dose-dependent manner at all concentrations tested. Among all tested extracts, leaves exhibited higher free radical scavenging activity (48.39±0.6-87.41±1.2) than bark (42.02 ± 0.5-80.15±1.1), while BHT at 250 µg/ml concentration had 92.8 ±1.3 percent inhibition **(Figure 2)**.

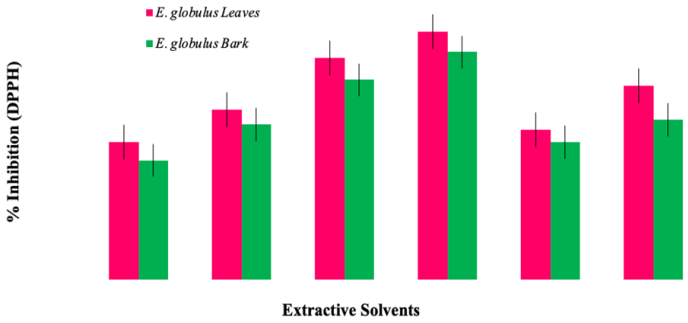

Figure 2. Free radical scavenging activity of various extract of *E. globulus* Leaves and Bark.

The leaves of various extracts of *E. globulus* have percentage inhibition (% I) ranges, namely: chloroform 7.20±0.25-48.39±0.95; methanol 16.25±0.40-59.97±1.15; ethanol 24.57±0.55-78.04±1.75; water 35.90±0.76-87.41± 2.50; MeOH-H_2O (50%) 12.35±0.30-52.85±1.02 and EtOH-H_2O (50%) 20.19±0.50-68.35±1.65 **(Figure 3)**.

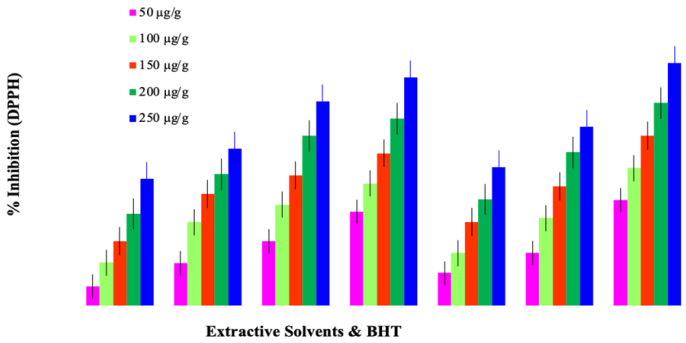

Figure 3. Free radical scavenging activity of various extract of *E. globulus* Leaves and BHT.

Similarly, the bark of various extracts of *E. globulus* have percentage inhibition ranges, namely: chloroform 4.50±0.15-42.02±0.80; methanol 12.15±0.30-54.65±0.95; ethanol 20.17±0.50-70.40±1.68; water 29.31 ±0.70-80.37±2.25; MeOH-water (50%) 9.52±0.28-48.50±0.95 and EtOH-water (50%) 14.71±0.40-56.30±1.50, while BHT inhibits DPPH 40.30-92.80% at concentration 50-250 µg/ml **(Figure 4)**. Therefore, in order to better compare the free radical activities of various extracts of leaves and bark of *E. Globulus* the IC50 was determined, and the result reported as IC_{50}; effective concentration value at which DPPH free radicals are scavenged by 50% [66].

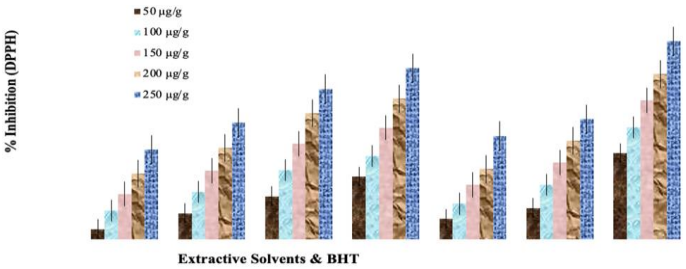

Figure 4. Free radical scavenging activity of various extract of *E. globulus* Bark and BHT.

According to the results obtained in **Table 3**, the leaves extract showed best IC_{50} values than the bark extract with values of 58.88±2.15 µg/ml (water), 68.51±2.70 µg/ml (ethanol), 70.49±2.60 µg/ml (ethanol-water; 50%), 72.90±2.80 (methanol), 76.63±2.85 µg/ml (methanol-water; 50%), 88.22±2.90 µg/ml (chloroform). While bark extracts have IC_{50} values ranging from 63.43 ± 2.25 µg/ml (water) to 90.10 ± 2.95 (chloroform) and the IC_{50} for BHT was 29.70±1.55 µg/ml. Preliminary studies of scavenging screening tests on eucalyptus leaves and bark are the first indications that these extracts have a potent scavenging activity. According to the results, all eucalyptus extracts cleared DPPH in a concentration-dependent manner. The scavenging or antioxidant activity correlated with the content of total phenolic compounds and flavonoids in investigated extracts [67]. This activity may also be due to the presence of hydrolysable tannins in both leaves and bark extracts and predominance in leaves extracts. Our results are consistent with those reported by Eyles et al [68-69].

Table 3. IC_{50} (DPPH) values of various extracts of *E. globulus* leaves and bark.

	Eucalyptus globulus Leaves IC_{50} µg/g (DPPH	*Eucalyptus globulus* Bark IC_{50} µg/g (DPPH
Chloroform ($CHCl_3$)	88.22 ± 2.90	90.10 ± 2.95
Methanol (MeOH)	72.90 ± 2.80	77.76 ± 2.85
Ethanol (EtOH)	68.51 ± 2.70	71.35 ± 2.60
Water (H_2O)	58.88 ± 2.15	63.43 ± 2.25
MeOH + H_2O	76.63 ± 2.85	80. 37 ± 2.85
EtOH + H_2O	70.49 ± 2.60	73.87 ± 2.65
BHT	29.70 ± 1.55	29.70 ± 1.55

Data are represented ± standard deviation.

Reducing power Assay

Figure 5 depicts the reducing capacity of different concentrations of *E. globulus* leaves extracts (50, 100, 150, 200, 250 and 300 µg/mL) compared to BHT. A part of the chloroform extract, the reducing capacity of all extracts were found to be significant and dose dependent. The best reducing power at 300 µg/mL came from the water extract of leaves (1.32±0.05), followed by ethanol (1.14±0.04), ethanol aqueous solution (0.99 ± 0.03), methanol (0.85 ± 0.02), methanol aqueous solution (0.73 ± 0.04) 0.02) and chloroform (0.55 ± 0.01) respectively.

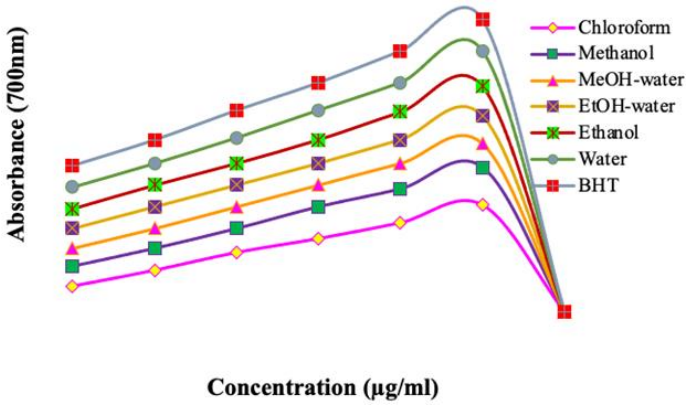

Figure 5. Reducing Power activity of various extract of *E. globulus* Leaves and BHT.

Similarly, the bark water extract and ethanol extract also showed potent reducing power (1.18±0.04; 1.08±0.02), followed by ethanol-water, methanol, methanol-water and chloroform **(Figure 6)**, while BHT have absorbance 1.48±0.06 at a concentration 50-300 µg/ml. Our results are consistent with those reported by Zakia Bey-Ould et al., 2016) [70].

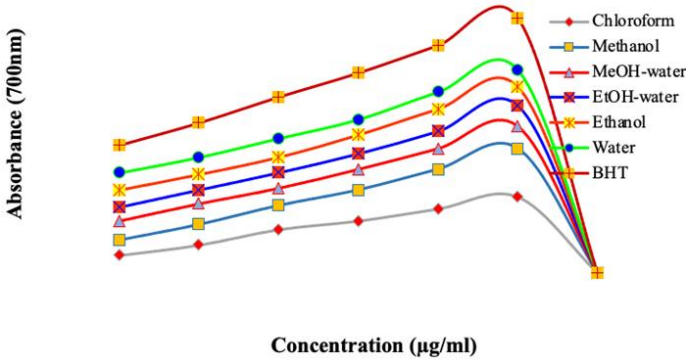

Figure 6. Reducing Power activity of various extract of *E. globulus* Bark and BHT

Antioxidant activity was reported to linked with the electron donating capacity of bioactive compounds. They are also referred as good reducing agents. Oxidative agents act as reducing agents as they induce redox reaction toward oxidants by reducing and oxidizing the reactive specie. Electron donating activity was indicated by the reduction of Fe^{3+} which described the antioxidant potency of phenols [71-84].

Antioxidant was shown to reduce Fe^{3+} to Fe^{2+} in a concentration dependent manner as validated by reducing assay [73]. Formation of Perl's Prussian blue $(Fe^{3+})_4[Fe^{2+}\ (CN^-)_6]^3$ was monitored at 670 nm and this complex aids in the transfer of electron. Higher reducing potential was indicated by higher absorbance value [86-89].

CONCLUSIONS

There has been an increased interest in natural phytochemicals and antioxidants that can be used to protect humans from oxidative stress damage. In our study, *E. globulus* leaves and bark were subjected to phytochemicals analysis and antioxidant testing using two different methods. Our results show *that E. globulus* leaves and bark are good sources of bioactive compounds, polyphenols, flavonoids and antioxidant activity in both assays. In addition, these extracts are a natural source of antioxidant substances and can be used as natural additives in the food and pharmaceutical industries.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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