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IN VITRO ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITY OF FRACTIONS FROM Alstonia scholaris Linn. R.Br.

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ABSTRACT: The present work is carried out to evaluate the antioxidant potential of fractions from ethanolic extract of *Alstonia scholaris* Linn. (Apocynaceae) using various *in vitro* tests including 1,1-diphenyl-2-picryl-hydrazil ($DPPH^{\cdot}$) free radical scavenging, metal ion chelating, hydrogen peroxide scavenging, superoxide anion radical scavenging, and ferric thiocyanate reducing ability. Dichloromethane and ethyl acetate fractions were found to have significant ($p<0.01$) free radical scavenging and metal ion chelating properties, whereas the petroleum ether and n-butanol fractions lack the in vitro antioxidant property. These various antioxidant activities were compared to standard antioxidants such as butylated hydroxyanisole (BHA) and l- ascorbic acid. These results indicate that dichlormethane and ethyl acetate fractions of *Alstonia scholaris* Linn. possess antioxidant property. The results observed were comparable to antioxidant properties of BHA and l-ascorbic acid.

KEY WORDS: *Alstonia scholaris*, antioxidant, radical scavenging.

INTRODUCTION

Alstonia scholaris is an antimalarial[1] drug used in the marketed Ayurveda preparation Ayush-64, NRDC, India. The plant *Alstonia scholaris* Linn. R.Br., belongs to the family Apocynaceae and is native to India. It grows throughout India, in deciduous and evergreen forests, also in plains[2].

The bark is bitter, astringent, acrid, thermogenic, digestive, laxative, anthelmintic, febrifuge, antipyretic, depurative, galactogogue, stomachic, cardiotonic and tonic[2]. It is useful in fevers, malarial fevers, abdominal disorders, dyspepsia, leprosy, skin diseases, pruritus, tumours, chronic and foul ulcers, asthma, bronchitis, cardiopathy, helminthiasis, agalactia and debility[2,3]. In folklore medicine, milky juice is applied on wounds, ulcers and rheumatic pains; mixed with oil and dropped into ear, it relieves ear ache. Juice of the leaves and tincture of the bark acts in certain cases as a powerful galactogogue. The drug is also used in cases of snake-bite[2].

The methanolic extract of this plant was found to exhibit pronounced antiplasmodial activity[4]. The plant is reported to have anti-mutagenic effect[5]. The bark extract of *Alstonia scholaris* has immunostimulating effect. The aqueous extract at low dose induced the cellular immune response while at high dose inhibited the delayed type of hypersensitivity reaction[6]. Echitamine chloride, an indole alkaloid, extracted from the bark of *Alstonia scholaris* has got high promising anticancer[7,8] effect against sarcoma – 180. The plant has hepatoprotective activity on liver injury induced by CCl_4, β-D-galactosamine, acetaminophen and ethanol[9]. Recently we have reported the wound healing and anti-inflammatory activities of *Alstonia scholaris* Linn.[10].

The ethanolic extract of leaves of *Alstonia scholaris* Linn. (R.Br.,) had a significant ($p<0.01$) in vitro antioxidant activity[11], which prompted us to fractionate the ethanolic extract and further screen the fractions to identify the promising fraction.

MATERIALS AND METHODS

Plant Material and Extraction:

The leaves of *Alstonia scholaris* (Family: Apocynaceae) were collected in the month of April May 2008 from hills of Sawantwadi, Maharashtra, India. The plant material was taxonomically identified by the Botany Survey of India (BSI), Pune and the voucher specimen AS-1 was retained in herbarium of BSI, Pune for future reference. The dried powder of the leaf (500 g) was subjected to extraction in a Soxhlet apparatus by using ethanol. The solvent was removed from the extract under reduced pressure. This extract was partitioned with petroleum ether (60 - 80º C), dichloromethane, ethylacetate and butanol in a separating funnel. The yields were as follows: Petroleum ether fraction (PE): 2.10 % w/w, Dichloromethane fraction (DCM) 1.20 % w/w, Ethyl acetate fraction (EA) 0.30 % w/w and n-butanol fraction (BUT) 2.62 % w/w.

Chemicals and Reagents:

Nicotinamide adenine dinucleotide (NADH), butylated hydroxyanisole (BHA), l- ascorbic acid, nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), the stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 3-(2-Pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), polyoxyethylenesorbitan monolaurate (Tween – 20) and trichloroacetic acid (TCA) were obtained from Sigma Aldrich, US. All other chemicals used were analytical grade and obtained from Merck, US.

DPPH (1,1-Diphenly-2-picryl-hydrazil$^{\cdot}$) Free Radical Scavenging Activity:

The free radical scavenging activity of the fractions of ethanolic extract of *Alstonia scholaris* was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH$^{\cdot}$) using the method described by Shimada[12]. Briefly 0.1 mM solution of DPPH in ethanol was prepared; 1 ml of the solution was added to 3 ml of the fraction in ethanol at different concentrations (25-500 µg/ml). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm by using a UV-Visible Spectrophotometer (Schimadzu UV-Vis 1700). Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The percent DPPH scavenging effect was calculated using the following equation:

DPPH$^{\cdot}$ scavenging effect (%) = $[(A_0-A_1)/A_0] \times 100$

where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the standard sample or fraction.

Ferrous Metal Ion Chelating Activity:

The ferrous ions chelating by the fractions and standards were estimated by the method of Dinis[13]. Briefly, the samples (25-500 µg/ml) were added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine and the mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was measured spectrophotometrically at 562 nm by using a UV-Visible Spectrophotometer (Schimadzu UV-Vis 1700). All test and analyses were run in triplicate and averaged. The percentage of inhibition of ferrozine$^{\cdot}$-Fe^{2+} complex formation was found using the formula: % inhibition = $[(A_0-A_1)/A_0] \times 100$

where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the standard sample or fraction. The control contains $FeCl_2$ and ferrozine complex formation molecules.

Scavenging of Hydrogen Peroxide:

The ability of the fractions to scavenge hydrogen peroxide was determined according to the method of Ruch[14]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4) and concentration was determined spectrophotometrically at 230 nm (Schimadzu UV-Vis 1700). Fractions in ethanol (25-500 µg/ml) was added to a hydrogen peroxide solution (0.6 ml, 40 mM) and the absorbance of hydrogen peroxide at 230 nm was determined after 19 min against a blank solution in phosphate buffer without hydrogen peroxide. The percentage of scavenging of hydrogen peroxide of fraction and standard compounds:

% scavenged $[H_2O_2] = [(A_0-A_1)/A_0] \times 100$

where A_0 was the absorbance of the control, and A_1 was the absorbance of fraction or standards.

Superoxide Anion Radicals Scavenging Activity:

Measurement of superoxide anion radicals scavenging activity was based on the method described by Liu[15]. Superoxide radicals are generated in PMS – NADH systems by oxidation of NADH and assayed by the reduction of NBT. In these experiments, the superoxide radicals were generated in 3 ml of Tris-HCl buffer (16 mM, pH 8.0) containing 1 ml of NBT (50 µM) solution, 1 ml NADH (78 µM) solution and sample solution of fraction (25 – 500 µg/ml) in ethanol. The reaction started by adding 1 ml of PMS solution (10 µM) to the mixture. The reaction mixture

was incubated at 25° C for 5 min, the absorbance was read at 560 nm by spectrophotometer (Schimadzu UV-Vis 1700) against blank samples using l- ascorbic acid as a control. Decreased absorbance of the reaction mixture indicated the increasing of superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

% inhibition = $[(A_0-A_1)/A_0] \times 100$

where A_0 was the absorbance of the control (l-ascorbic acid), and A_1 was the absorbance in the presence of fraction or standards.

Total Reduction Capability:

Total reduction capability of the fractions was estimated by using the method of Oyaizu[16]. The different concentration of fractions (25-75 µg/ml) in 1 ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide $[K_3Fe\text{-}(CN)_6]$ (2.5 ml, 1%). The mixture was incubated at 50° C for 20 min by adding a 2.5 ml of 10 % trichloroacetic acid. Then the mixture was centrifuged for 10 min at 1000 x g. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm by a spectrophotometer (Schimadzu UV-Vis 1700). Higher absorbance of the reaction mixture indicated greater reducing power.

Phytochemical Analysis:

Based on the *in vitro* antioxidant activity, the promising fractions were subjected to preliminary phytochemical evaluation using standard methods[17, 18].

Statistical Analysis:

Experimental results were mean ± SEM of three parallel measurements. Analysis of variance was performed by ANOVA followed by Newmans-Keul multiple comparison test. P values < 0.05 were regarded as significant.

RESULTS

Free Radical Scavenging Activity by DPPH Method:

A significant ($p<0.01$) decrease in the concentration of DPPH radical was observed due to the scavenging ability of the EA and DCM (Fig 1). There was a little or no difference between the control and PE or n-butanol fractions, which indicate the lack of PE and n-butanol fractions to have free radical scavenging activity. The scavenging effect of the EA, DCM and standards on the DPPH radical decreased in the order of EA > BHA > l-ascorbic acid > n-butanol > PE which were statistically significant ($p<0.01$) compared to the control. The free radical scavenging effect at 250 µg/ml reached a plateau as shown in Figure 1.

Ferrous Metal Ion Chelating Activity:

The formation of Fe^{2+} -ferrozine complex is not completed in the presence of EA, DCM and standards which indicate that EA and DCM chelate the iron (Figure 2). The absorbance of Fe^{2+}-ferrozine complex was dose dependent and linearly decreased. There was a significant ($p<0.01$) difference of EA and DCM when compared to the control, whereas there was no effect on ferrous metal ion chelation in presence of PE and n-butanol. fractions. The metal scavenging effect of EA, DCM and standards were decreased in the order of l- ascorbic acid > BHA > EA > DCM > n-Butanol > PE.

Scavenging of Hydrogen Peroxide:

EA and DCM had strong H_2O_2 scavenging activity as comparable to the doses of BHA and l- ascorbic acid. The H_2O_2 scavenging activity of DCM and EA was statistically significant ($p<0.01$) compared to the control (Figure 3), whereas there was no difference between the control and PE or n-butanol fractions.. These results showed that DCM and EA had effective H_2O_2 scavenging activity whereas the n-butanol and PE fractions lack H_2O_2 scavenging activity and it was in the following order: EA > DCM > l- ascorbic acid >BHA > n-butanol > PE.

Superoxide anion radical scavenging activity:

EA and DCM had a strong superoxide radical scavenging activity comparable to that of BHA and l-ascorbic acid (Figure 4). The superoxide scavenging activity of EA and DCM were statistically significant ($p<0.01$) from the control. Superoxide radical scavenging activity of these samples followed the order: l- ascorbic acid > BHA > EA > DCM > n-butanol > PE.

Total Reductive Capability by Potassium Ferricyanide Reduction Method:

The reducing power of EA and DCM increased with increasing concentration (Figure 5). The reduction power of EA and DCM were comparable to that of the BHA and l ascorbic acid. There was no statistical difference between the control and PE or n-butanol fractions. Reducing power of DCM, EA and standard compounds followed the order: BHA > EA > DCM > l-ascorbic acid > n –butanol > PE.

Phytochemical Analysis of DCM and EA:

The phytochemical analysis of DCM fraction revealed the presence of tannins, flavonoids, triterpenoids, alkaloids and sterols, whereas EA fraction revealed flavonoids, triterpenoids and alkaloids.

DISCUSSION

In our previous studies, the ethanolic extract of *Alstonia scholaris* was found to possess *in vitro* antioxidant activity[11] and it was also found to decrease the malondialdehyde level and prevent lipid peroxidation[10] *in vivo*. Prompted by these findings it was decided to evaluate the antioxidant ability of the

fractions responsible for the *in vitro* antioxidant assays.

There are numerous antioxidant methods for evaluation of antioxidant activity. For *in vitro* antioxidant screening, ($DPPH^{\cdot}$) free radical scavenging, metal ion chelating, hydrogen peroxide scavenging, superoxide anion radical scavenging and ferric thiocyanate reducing activities are most commonly used. However, the total antioxidant activity of an antioxidant cannot be evaluated by using one single method, due to oxidative processes. Therefore, at least two methods should be employed in order to evaluate the total antioxidant activity[19]. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecule and radical progresses, results in the scavenging of the radical by hydrogen donation[20]. It is visually noticeable as a discoloration from purple to yellow. Figure 1 indicates noticeable effect of DCM and EA on scavenging of free radicals. These results revealed that the DCM and EA is free radical inhibitor or scavenger acting possibly as primary antioxidants, which can be correlated with previous studies[21].

Iron can stimulate lipid peroxidation by the Fenton reaction and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation[22]. In the presence of chelating agents, the complex formation is disrupted with the result that the red colour of the complex is decreased. The data obtained from Figure 2 reveal that DCM and EA demonstrates a marked capacity for iron binding, suggesting that their action as peroxidation protector may be related to its iron binding capacity.

H_2O_2 is highly important because of its ability to penetrate biological membranes. H_2O_2 itself is not very reactive, but it can sometimes be toxic to cell because of it may give rise to hydroxyl radical in the cells. The results showed that DCM and EA had an effective H_2O_2 scavenging activity.

Superoxide anion radicals are produced endogenously by flavoenzymes like xanthine oxidase, which converts hypoxanthine to xanthine and subsequently to uric acid in ischemia-reperfusion. Superoxide is generated *in vivo* by several oxidative enzymes, including xanthine oxidase. In the PMS-NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT[19]. The decrease of absorbance at 560 nm with DCM, EA and antioxidants indicates the consumption of superoxide anion in the reaction mixture.

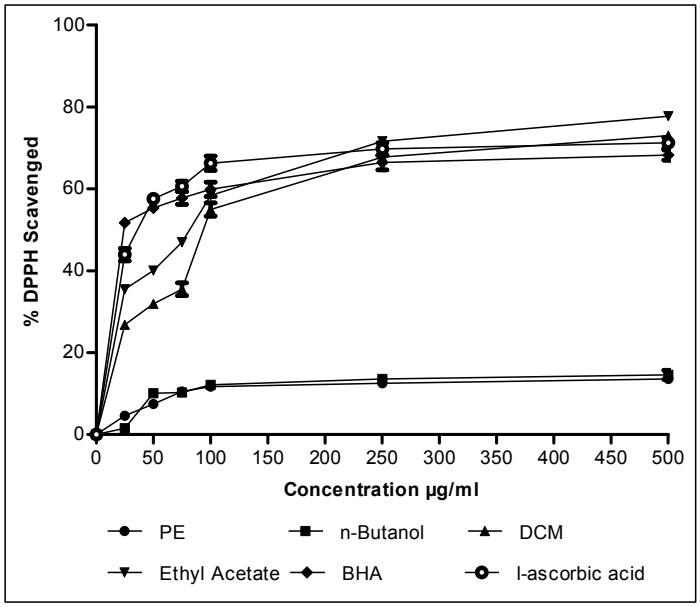
For the measurements of the reductive ability, the Fe^{3+}-Fe^{2+} transformation in the presence of DCM and EA was investigated and found to have significant reducing ability. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The antioxidant activity of an antioxidant compound has been attributed to various mechanisms among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging.

Generally flavonoids are the important class of antioxidants; hence the medicinal plants containing flavonoids and phenolic compounds are repeatedly screened for antioxidant activity. In addition to flavonoids and phenolic compounds, some of the alkaloids, saponins and triterpenoids are reported to possess antioxidant activity[23]. The presence of flavonoids, alkaloids and triterpenoids in DCM and EA has been reported[24] and the results of preliminary phytochemical investigation in the present study also further substantiate this. Hence, the observed *in vitro* antioxidant activity may be because of these phytoconstituents, which needs further investigation.

CONCLUSION

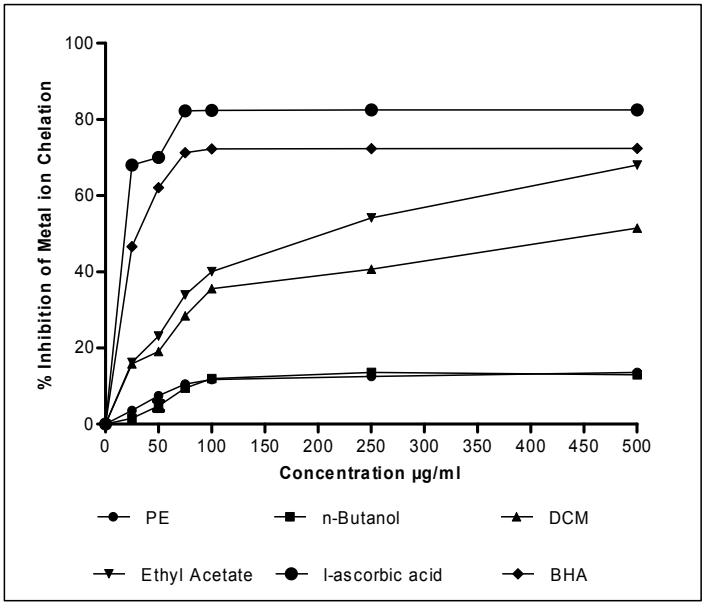
The results of the study clearly indicate that DCM and EA possess powerful *in vitro* antioxidant activity. The encouraging results of DCM and EA with the various *in vitro* antioxidant tests proved the plant as a reducing agent, metal chelator, its hydrogen donating ability and effectiveness as scavengers of hydrogen peroxide, superoxide, and free radicals. Hence, it is worthwhile to isolate and elucidate the bioactive principles that are responsible for the anti-oxidant activity that is underway.

Figure 1 Free radical scavenging activity of fractions from ethanolic extract of leaves of *Alstonia scholaris* Linn. (R.Br.,) on DPPH$^{\cdot}$



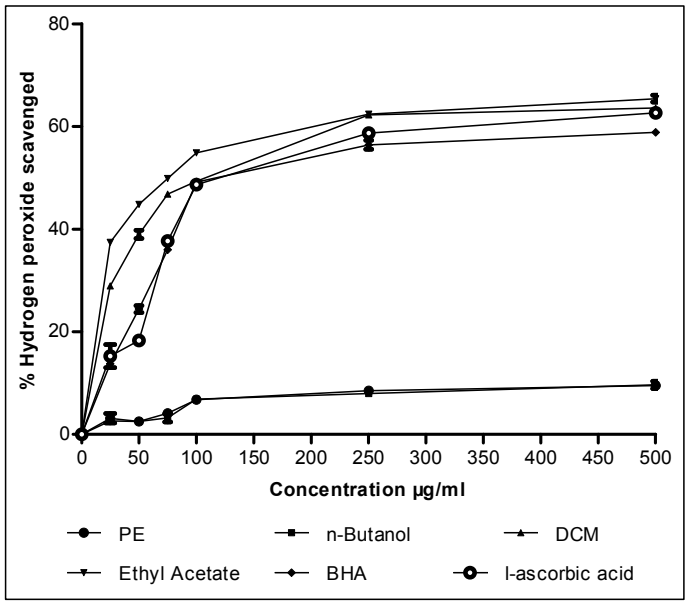
Values are expressed as mean ± SEM, n = 3
PE – Petroleum ether fraction
DCM – Dicholromethane fraction
EA – Ethyl acetate fraction
BHA – Butylated hydroxy anisole

Figure 2 Ferrous metal ion chelating activity of fractions from ethanolic extract of leaves of *Alstonia scholaris* Linn. (R.Br.,)



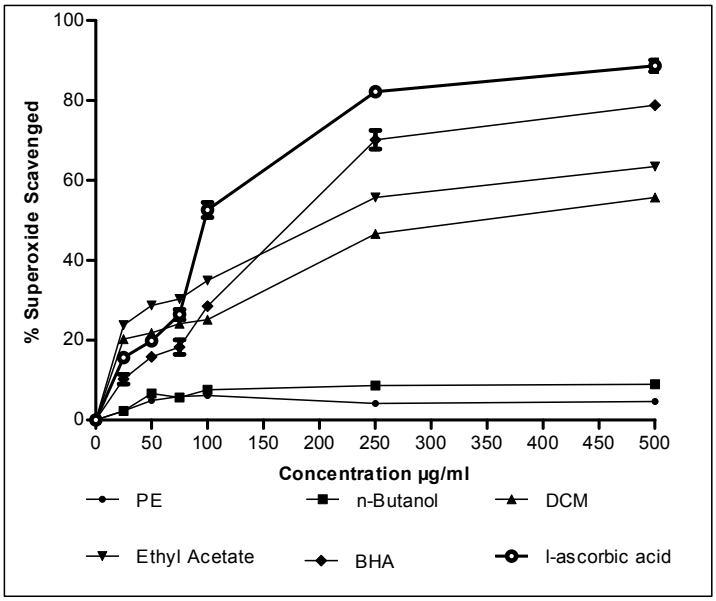
Values are expressed as mean ± SEM, n = 3
PE – Petroleum ether fraction
DCM – Dicholromethane fraction
EA – Ethyl acetate fraction
BHA – Butylated hydroxy anisole

Figure 3 Hydrogen peroxide scavenging activity of fractions from ethanolic extract of leaves of *Alstonia scholaris* Linn. (R.Br.,)



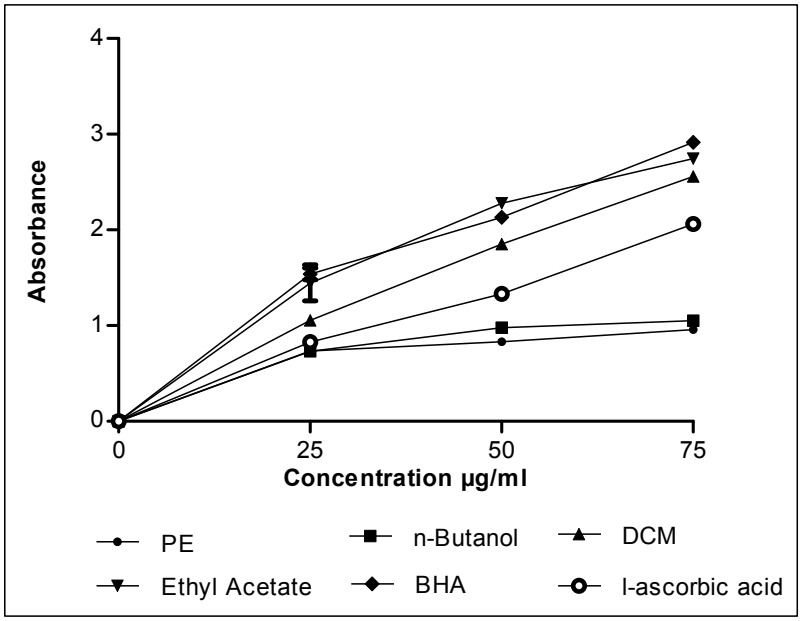
Values are expressed as mean ± SEM, n = 3
PE – Petroleum ether fraction
DCM – Dicholromethane fraction
EA – Ethyl acetate fraction
BHA – Butylated hydroxy anisole

Figure 4 Superoxide anion radical scavenging activity of fractions from ethanolic extract of leaves of *Alstonia scholaris* Linn. (R.Br.,)



Values are expressed as mean ± SEM, n = 3
PE – Petroleum ether fraction
DCM – Dicholromethane fraction
EA – Ethyl acetate fraction
BHA – Butylated hydroxy anisole

Figure 5 Total Reductive Capability of fractions from ethanolic extract of leaves of *Alstonia scholaris* Linn. (R.Br.,)



Values are expressed as mean ± SEM, n = 3
PE – Petroleum ether fraction
DCM – Dicholromethane fraction
EA – Ethyl acetate fraction
BHA – Butylated hydroxy anisole

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