Activity guided assessment of in-vitro antioxidant potential of Annona reticulata leaves extract and fractions

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ABSTRACT

Annona reticulata (*Annonaceae*) is a tree with numerous glabrous lateral branches. Its traditional and folklore usage motivates further investigation on its pharmacognostic parameters and pharmacological potential. Therefore, in order to establish its activity guided with antioxidant potential, DPPH radical scavenging (DPPH assay), ferric reducing ability (FRAP assay), superoxide scavenging (SOD assay) and total antioxidant potential were determined for hydro-alcoholic extract (ARCE) of flowering tops of *A.*reticulata and its ethyl acetate (AREAF) and diethyl ether (ARDEE) fractions.

For DPPH, SOD and total antioxidant assays, ascorbic acid was used as standard antioxidant compound. IC_{50} for DPPH radical scavenging assay were determined as 102.51, 79.13, 88.36 µg /ml respectively for ARCE, ARDEE and AREAF as compared to 21.80 µg /ml for ascorbic acid standard. FRAP vlaue for FRAP assay were determined as 129.98±11.36, 452.61±26.34 and 326.91±28.47 µg[Fe(II)/g extract] respectively for ARCE, ARDEE and AREAF. Superoxide scavenging potential, in term of SOD (expressed as IC_{50}), was 143.69 µg /ml for complete extract (ARCE), 63.63 µg /ml for ARDEE and 56.570 µg /ml for AREAF in contrast to 132.41 for standard ascorbic acid. Total antioxidant capacity was found to be 146.33 mg Ascorbic acid Eq /g, 426.33 mg Ascorbic acid Eq /g and 242.33 mg Ascorbic acid Eq /g. Results indicated the scavenging capacity for oxidative radicals were found even better with diethyl ether and ethyl acetate fractions. Diethyl ether and ethyl acetate fractions showed comparatively better results, which indicates that hydroalcoholic extract might be having some components that are having antioxidant power and are present in much higher amount in its fractions with the said solvents. Further studies are required to be carried out on other fractions for activity guided pharmacological investigations.

Keywords: Annona, DPPH, FRAP, SOD, superoxide, antioxidant, ascorbic acid
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INTRODUCTION

Folklore usage of herbs in various ailments motivates research of traditional drugs in modern system. Indigenous medical system is much more explored to develop drugs from plants.[1] Traditional use of *Annona reticulata* leaves in is the base of present study. Leaves are used to prepare tea for relieving colic. (Dule et al, 1993) It has also been reported against dysentery, cardiac problems, epilepsy, constipation, dysurea, hemorrhage, fever, ulcer, bacterial infections etc. Leaves has been reported to be used as anthelmintic and bark as powerful astringent and tonic. (2) Bhaskar (2007) reported antioxidant potential of leaf extracts in different *in-vitro* models. (Bhaskar, 2007)

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Oblong-lanceolate leaves are 10-20 cm long, 2-5 cm wide, deciduous, alternate, membranous, rounded or curate at the base and acute at apex. Ventral surfaces are glabrous, while lower surface have very few and spreading trichomes. (3–5)

The present study was undertaken to evaluate the extract and its fractions for their involvement in scavenging of oxidative radicals. In this order, their DPPH radical, superoxide radical scavenging, FRAP assay and total antioxidant capacity were evaluated.

EXPERIMENTAL

Materials:Absolute ethanol, acetate buffer (pH 3.6) (SD finechem), ascorbic acid (Himedia), DMSO (Rankem), DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) (Himedia), EDTA (SD finechem), ferric chloride (SD finechem), FRAP reagent containing TPTZ (2,4,6-tripyridyl-s-triazine) and $FeCl_3$.6H2O (Himedia), Malondialdehyde (MDA) (Himedia), methanol, Mueller-Hinton (Himdeia), nitroblue tetrazolium (NBT) (Himedia), Ortho-Phenanthroline (Rankem), phenazine methosulfate (PMS) (Himedia), Sabouraud dextrose media (Himedia), sodium dodecyl sulfate (SDS) (Rankem), sulphuric acid (Rankem), Thio barbituric Acid (TBA) (Rankem), Tris–HCl buffer (16 mM, pH 8.0) (Himedia)(6,7)

Methods:

Collection and extraction:

Annona reticulata leaves were collected from periphery of Jabalpur, Madhya Pradesh and authentication was done at "Department of Botany, University of Rajasthan, Jaipur" (Authentication certificate ***Ref. no.: Bot/2017/5424*** dated 13/02/2017).

Hydro-alcoholic (50-50) extract was prepared from air dried plant materials using maceration method.(6,8) Fractionation was carried out by first defatting and then by solvents of increasing polarity (dielectric constant). Solvents used for this purpose were petroleum ether, diethyl ether, ethyl acetate, benzene, acetone, and ethanol. Ethyl acetate (AREAF) and diethyl ether (ARDEE) fractions were further used to assess antioxidant potential.(9–11)

Preparation of stock solutions:

Stock solutions of extracts and standard-ascorbic acid were prepared in concentration of 1000µg/ml in methanol. From the stock solutions, serial dilutions of the samples and standard were prepared to obtain different concentration in methanol and used for antioxidant studies.(6,12)

DPPH radical scavenging

1 ml of methanolic extract of various concentrations was taken in test tube with 1ml of DPPH solution 0.1 mM (0.39 mg in 10ml methanol). Control was prepared with an equal amount of methanol and DPPH. Ascorbic acid was used as the standard to compare. All samples were incubated in dark for 20 minutes and absorbance was recorded at 517 nm in UV spectrophotometer. Experiment was performed in triplicate. (13,14)

The ferric reducing ability of plasma (FRAP) assay

The FRAP assay was carried out as per the process used by Benzie and Strain (15) with slight modification. The FRAP assay depends upon the reduction of ferric salt of tripyridyltriazine (Fe (III)-TPTZ) to ferrous salt (Fe (II)-TPTZ) by a reductant at low pH. Ferrous tripyridyltriazine (Fe (II)-TPTZ) has an intensive blue colour and can be monitored at 593 nm in UV spectrophotometer.(16,17)

The FRAP reagent was prepared using an acetate buffer (pH 3.6), 10 mM ferric tripyridyltriazine (Fe (III)-TPTZ) solution in 40 mM hydrochloric acid (HCl) and 20 mM iron

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(III) chloride solution in a proportion of 10:1:1 (v/v). 25 µl of sample were added to 175 µl of the FRAP reagent. Reaction mixture was allowed to stand for 5 minutes and its absorbance was recorded in UV spectrophotometer at 593 nm. Iron (II) sulphate was used as standard and calibration curve was prepared at various concentrations (40–0.078 µg/mL). Using this curve amount of Fe(II) was determined and the results were expressed as µg Fe (II)/g of extract. All the measurements were taken in triplicate and the mean values were calculated.(12,14,18,19)

Superoxide Scavenging:

Different concentrations of extracts were prepared. Alkaline DMSO (1 ml DMSO containing 5 mM NaOH in 0.1 ml water) and nitro blue tetrazolium (NBT) 20 mM (50 mg NBT in 10ml phosphate buffer pH 7.4) solutions were prepared. 1.5 ml of sample of different concentrations was taken and 2 ml alkaline DMSO was mixed and vortexed with it. To this mixture 0.6ml NBT reagent solution was added and vortexed. Final mixture was measured for absorbance @560 nm under UV spectrophotometer. (20) (21)

RESULTS AND DISCUSSIONS

Scavenging of superoxide free radicals by extracts and fractions was calculated using following formula as % scavenging:

$$\%\ Scavenging = 100 - \left(\frac{Abs.of\ control - Abs\ of\ sample}{Abs\ of\ control}\right) x100$$

Total Antioxidant:

10mg/ml stock solutions of extracts were prepared in water. 0.1ml of extract solution was mixed with 1 ml of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Test tubes were covered from top and incubated at 95°C for 90 min. After cooling to room temperature, the absorbance was measured at 695 nm in UV spectrophotometer (Shimadzu). Ascorbic acid was used as standard and calibration curve was prepared which was used to calculate total antioxidant activity in terms of number of equivalents of ascorbic acid per gram extract. (22,23)

Statistical Analysis

All results are expressed as mean ± S.E.M. Linear regression analysis was used to calculate the IC_{50} values when required.

DPPH Radical Scavenging

Table 1: Inhibition (%) of DPPH free radical by extracts

Sample type	Conc. (µg/ml)				
	20	40	60	80	100
ARCE	29.72 ± 0.13	33.27 ± 0.26	38.89 ± 0.15	44.36 ± 0.13	49.73 ± 0.24
ARDEE	31.58 ± 0.16	37.39 ± 0.18	42.73 ± 0.27	49.81 ± 0.14	57.69 ± 0.12
AREAF	29.97 ± 0.17	34.68 ± 0.12	40.51 ± 0.15	47.64 ± 0.21	53.99 ± 0.18
Ascorbic acid	48.57 ± 1.98	57.76 ± 1.81	65.94 ± 0.55	72.53 ± 0.55	80.63 ± 1.51

Table 2: Inhibitory concentration (IC50) values for DPPH scavenging by extracts

Sample type	Linear equation	Correlation co-efficient (R2)	IC_{50}
ARCE	y = 0.255x + 23.86	0.994	102.51

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ARDEE	y = 0.323x + 24.44	0.993	79.13
AREAF	y = 0.305x + 23.05	0.994	88.36
Ascorbic acid	y = 0.394x + 41.41	0.997	21.80

AR- Annona reticulata, suffixed with CE- complete extract, DEE- diethyl ether, EAF- ethyl acetate fraction, Ascorbic acid used as standard to compare

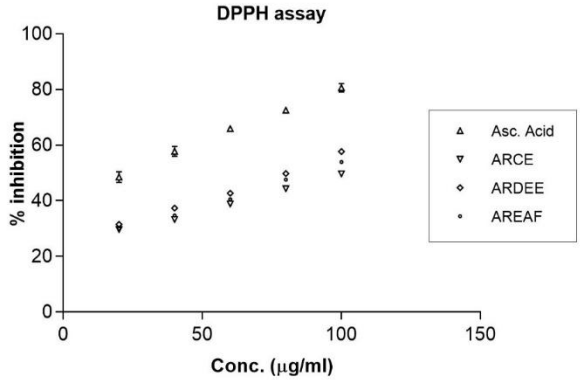

Figure 1: DPPH assay of various extracts and fractions

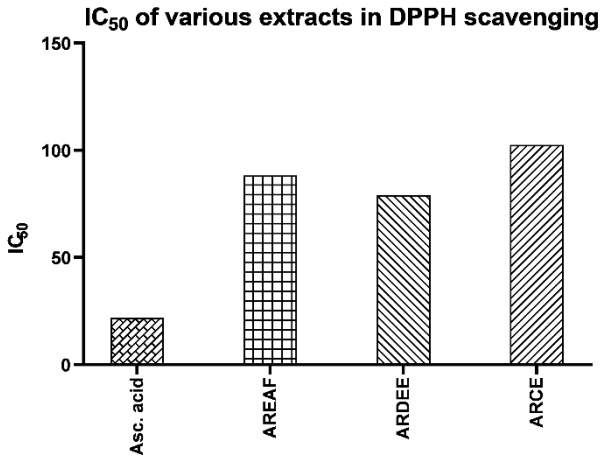

Figure 2: IC_{50} of various extracts and fractions in DPPH scavenging

DPPH radical scavenging of the fractions was compared with standard ascorbic acid in terms of IC_{50} value (*table-1,2, figure-1,2*). Based on the IC_{50} value obtained by fractions and comparing with standard ascorbic acid, DPPH radical scavenging was found maximum with complete hydro-alcoholic extract (ARCE)- 102.51 µg/ml, followed by ethyl acetate fraction (AREAF)- 88.36 µg/ml and diethyl ether fraction (ARDEE)- 79.13 µg/ml. This denotes that there might be some compounds in the diethyl ether fraction which are phenolic in nature and possess the radical scavenging potential, thereby showing greater activity. These compounds may be some type of flavonoids where are concentrated in diethyl ether fraction.

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4.1.1.1. FRAP Assay

Table 3: Frap values of the extracts and fractions in terms of Fe (II)

	ARCE	ARDEE	AREAF
FRAP Values (µg Fe(II)/g extract)	129.98±11.36	452.61±26.34	326.91±28.47

AR- Annona reticulata, suffixed with CE- complete extract, DEE- diethyl ether, EAF- ethyl acetate fraction

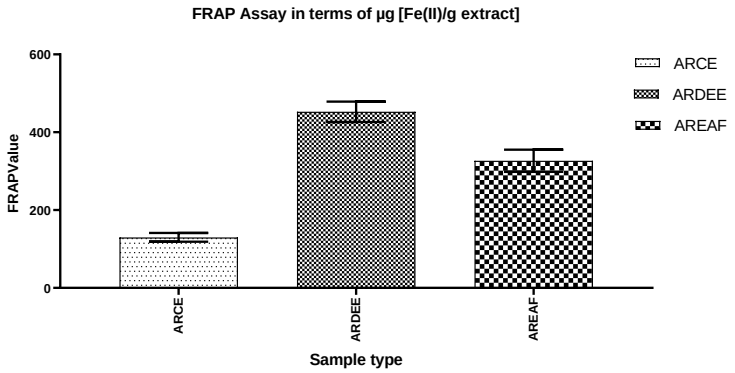

Figure 3: FRAP values as IC_{50} in terms of (µg Fe(II)/g extract)

FRAP assay indicates the ferric reducing capacity of the sample in test and denoted as equivalent to µg Fe(II)/g extract (*table-3, figure-3*). Diethyl ether fraction (ARDEE) showed maximum ferric reducing capacity equivalent to FRAP value 452.61±26.34 µg Fe(II)/g extract followed by ethyl acetate fraction- equivalent to FRAP value 326.91±28.47 µg Fe(II)/g extract and then least by complete hydroalcoholic extract- equivalent to FRAP value 129.98±11.36 µg Fe(II)/g extract. These result supported the DPPH radical scavenging and same type of compounds might be responsible for both type of scavenging viz. DPPH and ferric reducing capacity (FRAP). This denotes that there might be some compounds in the complete extract which are non-phenolic as well as phenolic in nature and possess the radical scavenging potential, thereby showing greater activity.

4.1.1.2. Superoxide Scavenging

Table 4: Superoxide scavenging- absorbance under UV-spectrophotometer

	Absorbance at 560 nm			
Conc. (µg/ml)	**Ascorbic acid**	**ARCE**	**ARDEE**	**AREAF**
31.125	0.055	0.201	0.286	0.277
62.500	0.131	0.253	0.325	0.332
125.000	0.309	0.300	0.428	0.496
150.000	0.412	0.348	0.536	0.546
250.000	0.671	0.474	0.672	0.677
Control	**0.678**			

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Scavenging of superoxide free radicals by extracts and fractions was calculated using following formula as % scavenging:

$$\%\ \boldsymbol{Scavenging} = \mathbf{100} - \left(\frac{\boldsymbol{Abs.of\ control - Abs\ of\ sample}}{\boldsymbol{Abs\ of\ control}}\right)\boldsymbol{x}100$$

Table Error! No text of specified style in document.**: Superoxide scavenging (%) of various extracts**

	% Scavenging			
Conc. (µg/ml)	**Ascorbic acid**	**ARCE**	**ARDEE**	**AREAF**
31.125	8.112	29.648	42.183	40.855
62.500	19.322	37.346	47.935	48.968
125.000	45.575	44.234	63.127	73.156
150.000	60.767	51.335	79.056	80.531
250.000	98.968	69.875	99.115	99.853

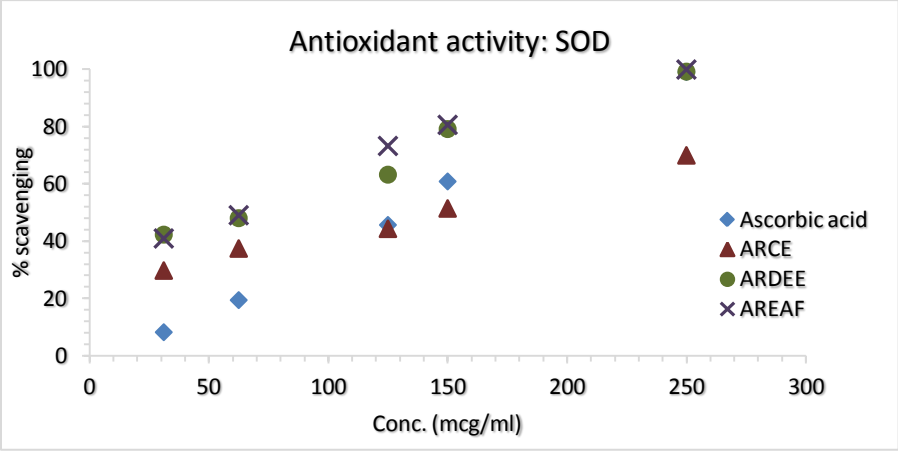

Figure 4: Comparative Superoxide scavenging activity of various fractions

Table 6: Superoxide scavenging in terms of Eq SOD for various fractions

Sample type	**Linear Equation**	**Correlation co-efficient (R^2)**	**IC_{50} (µg/ml) ≡ 1 unit of SOD***	**Eq SOD units/mg ext. or std.**
Ascorbic acid	y = 0.421x - 5.746	0.988	132.413	7.552
ARCE	y = 0.179x + 24.28	0.984	143.687	6.960
ARDEE	y = 0.270x + 32.82	0.976	63.630	15.716
AREAF	y = 0.277x + 34.33	0.971	56.570	17.677
***IC_{50} value is equivalent to 1 unit of SOD**				

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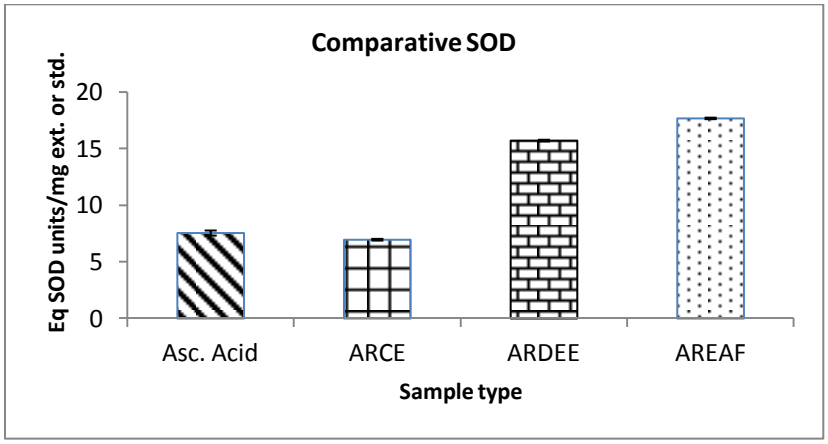

Figure 5: Comparative SOD of various extracts and fractions

Superoxide scavenging was determined using NBT method and its outcomes were denoted as Eq. SOD units/mg ext. or std. (*table-4-6, figure-4,5*). Ethyl acetate fraction (AREAF) showed maximum SOD value as 17.68 Eq. SOD units/mg ext. as compared to 7.552 Eq. SOD units/mg std. ascorbic acid. Very high SOD value shown by ARDEE and AREAF denotes the presence of highly active antioxidant polyphenolics and flavonoids which may be responsible their extremely high SOD activity and shows their relevance in use for pharmaceutical formulation purposes.(6,7,24–26)

4.1.1.3. Total Antioxidant

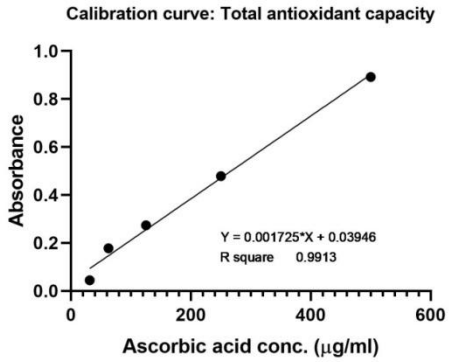

Figure 6: Total antioxidant- calibration curve for standard ascorbic acid

Table 7: Total antioxidant capacity of various extracts and fractions

AR Fraction	ARCE			ARDEE			AREAF		
	Mean	±	SD	Mean	±	SD	Mean	±	SD
mg Ascorbic acid E/g e	146.33	±	8.622	426.33	±	7.767	242.33	±	12.6

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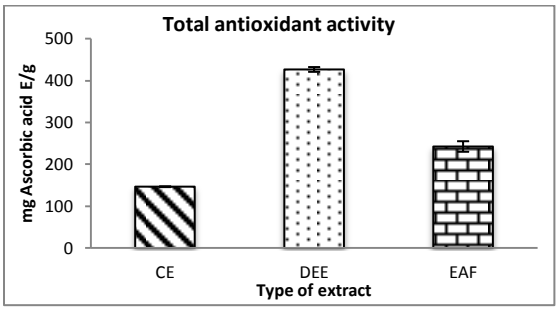

*CE=complete extract; DEE= diethyl ether fraction; EAF= ethyl acetate fraction; AR are extract of plants respectively

Figure 7: Comparative total antioxidant capacity of various extracts and fractions

Total antioxidant capacity of the extract and fractions were recorded as equivalent to ascorbic acid per gram of extract or fractions (mg Ascorbic acid Eq/g) (*table-7, figure-6,7*). Diethyl ether fraction of *Annona reticulata (ARDEE)* had shown maximal total antioxidant capacity (426.33 mg Ascorbic acid Eq/g) followed by ethyl acetate fraction (AREAF) (242.33 mg Ascorbic acid Eq/g) and complete hydroalcoholic extract (ARCE) (146.33 mg Ascorbic acid Eq/g). Higher SOD and total antioxidant capacity in diethyl ether and ethyl acetate fractions were due to the fact that diethyl ether and ethyl acetate contain more of polyphenolics which are, by nature, powerful antioxidant. Whereby, diethyl ether fraction contains more powerful antioxidant as compared to ethyl acetate fraction.

CONCLUSION

From the results of present study, this can be concluded that extracts in study possess powerful antioxidants which are more firmly distributed in diethyl ether and ethyl acetate fractions. These fractions may be used further for formulation as nutraceuticals and thereby preventive measures. The possibility of counteracting oxidative stress by a pool of proper antioxidants plus an appropriate diet, mainly in patients whose blood antioxidant deficiencies can be easily rebalanced, may have real health benefit and represent a promising way of inhibiting the progression of disease.

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