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ANTIOXIDANT ACTIVITY OF UNRIPE PERICARP EXTRACTS OF *ANNONA RETICULATA* LINN.

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ABSTRACT

The total phenol and flavanoids were measured in ethanol, ethyl acetate and water extracts of unripe pericarp of fruits of *Annona reticulata* Linn. Ethanol extract showed high phenol and flavonoid contents. The ethanolic crude extract is also screened for its free radical scavenging properties using various *in vitro* assays like DPPH radical scavenging assay, hydroxyl radical scavenging assay, nitric oxide radical scavenging assay, hydrogen peroxide radical scavenging assay and superoxide radical scavenging assay. Ethanol extract exhibited the strongest antioxidant capacity in all used assays and its effect is comparable to the synthetic antioxidant Ascorbate. The total phenol content (TPC) showed a very strong positive significant ($P<0.05$) correlation with DPPH ($R= 0.999$). Similarly, a positive correlation was observed between TPC and NO radical scavenging ($R=0.904$), hydroxyl radical scavenging ($R=0.997$), superoxide radical scavenging ($R=0.959$) and hydrogen peroxide radical scavenging ($R=0.973$) assays. This suggested that the phenols are the main compounds responsible for antioxidant activity of investigated samples. Data from this study could be used for developing natural antioxidants for promoting health.

Key words: flavonoids, phenols, antioxidant, correlation, free radicals

INTRODUCTION:

The excess production of free radicals results in abnormal physiological conditions like oxidative stress that impedes various cellular and metabolic functions leading to neurodegenerative diseases, gastroduodenal disorders, cancer, cataracts, premature ageing, inflammation, cardiovascular diseases [5], chronic kidney ailments [41] and metabolic impairments [31]. The rising incidence of such diseases is alarming and becoming a serious public health problem. Many synthetic drugs confer protection against oxidative damage but

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they have adverse side effects. An alternative solution to the problems was to consume natural antioxidants from food supplements and conventional medicines. Lately, many natural antioxidants have been isolated from different medicinal [11, 38]. The antioxidant activity of natural and synthetic compounds is attributed to various mechanisms such as hindrance of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen removal and radical scavenging that hinder the consequences of oxidative damage [5, 19]. Plant products have been traditionally used for the treatment of various diseases due to their free radical scavenging and antioxidant properties. Phytochemicals like carotenoids, tocopherols, ascorbates, lipoic acids, flavonoids and related polyphenols are potent natural antioxidants with strong antioxidant capacity that offers protection against oxidative deterioration by the radicals [12, 32]. Plants contain antioxidant principles that can explain and justify their use in traditional medicine in the past as well as in the present.

The present study is aimed to evaluate the antioxidant activity of the ethanol extract of the unripe pericarp of fruits of *Annona reticulata* through various *in vitro* models like DPPH radical scavenging assay, hydroxyl radical scavenging assay, nitric oxide radical scavenging assay, hydrogen peroxide radical scavenging assay and superoxide radical scavenging assay. It also used to establish the relationship between antioxidant activity and phenolic compounds present in the extract to confirm that phenolic constituents are responsible for the antioxidant activity of the plants.

MATERIALS AND METHODS:

Collection of plant parts

Fresh unripe fruits of An*nona reticulata* were collected from Thuckalay, Kanyakumari district, Tamil Nadu, Southern India in the month of January 2015. The taxonomical identity of the plant was confirmed by Dr. A. G. Pandurangan, Director, Jawaharlal Nehru Tropical Botanical Garden and Research Institute (TBGRI), Palode, Thiruvananthapuram, India. A voucher specimen of the leaves bearing collection number 76877 was deposited at the herbarium in TBGRI.

Preparation of unripe pericarp extracts

The skin of the unripe and ripe fruits was peeled off. The pericarp (both unripe and ripe) of fruits was separated from the seeds. These were then cut into small pieces with a razor and shade dried. During shade drying, utmost care is taken to prevent the infection of fruits with bacteria and more importantly fungi. The shade dried plant materials (100 gms each) were powdered using mixer grinder and subjected to sequential extraction process [25].

Estimation of total phenolic content - Folin method

A dilute extract of isolated samples in different solvents (0.5 ml of 1:10 dilution) or gallic acid (used as standard of phenolic compound) was mixed with FC reagent (2N) (5 ml, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4 ml, 1 M) was added. The mixtures were allowed to stand for 15 min and the total phenols were determined at 765 nm.

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Quantification was done with respect to the standard curve of gallic acid. The standard curve was prepared using 0.8, 1.6, 3.12, 6.25, 12.5 and 25 mg/L solutions of gallic acid in methanol: water (50:50, v/v). Total phenol values were expressed in terms of gallic acid equivalent (GAE) (mg/g of dry mass), used as reference compound. The OD was measured at 765 nm using a Systronics double beam UV-VIS -180 model spectrophotometer [17]. The concentration of phenolic compounds was calculated according to the following eq. (1) obtained from the standard gallic acid curve.

$$y = 0.005 \text{ x GAE in } \mu g + 0.040 \ (R^2 = 0.998) \qquad (1)$$

where y is absorbance at 760 nm and x is total phenolic content in the different extracts of *Annona reticulata* Linn. expressed in mg/g.

3.2.4.6 Estimation of total flavonoid content – aluminium chloride colorimetric assay method

Plant extracts (0.5ml of 1:10 dilution) were taken in methanol and were separately mixed with 1.5 ml of methanol, 0.1 ml of 10 % $AlCl_3$, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It was kept at room temperature for 30 minutes and the absorbance of the reaction mixture was measured at 415 nm. The calibration curve was prepared by preparing quercetin solutions at concentrations 0.5, 1, 2, 4, 6, and 8 mg/L in methanol. Total flavanoid values were expressed in terms of quercetin equivalent (QE) (mg/g of dry mass), used as reference compound [8].

The concentration of flavonoid compound was calculated according to the following eq. (2) obtained from the standard quercetin curve.

$$y = 0.010 \text{ x QE in } \mu g + 0.041 \ (R^2 = 0.951) \qquad (1)$$

where y is absorbance at 415 nm and x is total flavanoid content in the different extracts of *Annona reticulata* expressed in mg/g.

Evaluation of antioxidant activity by *in vitro* techniques

DPPH (2, 2-diphenyl-1-picrylhydrazyl radical) free radical scavenging assay

The methanol DPPH solution (0.15%) was mixed with serial dilutions (20 - 100 μg/ml) of the fractions and after 10 minutes, the absorbance was read at 515 nm. The antiradical activity was expressed as IC_{50} (μg/ml). Vitamin C was used as standard [29, 45]. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = \frac{\text{Absorbance of control} - (\text{Sample with DPPH} - \text{sample without DPPH}}{\text{Absorbance of control}} \text{x100}$$

Nitric oxide radical scavenging assay

The reaction mixture (3 ml) containing sodium nitroprusside (10 mM, 2 ml), phosphate buffer saline (0.5 ml) and different concentration of major fractions from unripe pericarp ethanol extracts (20 - 100μg/ml) and standard solution (0.5 ml) were incubated at 25˚C for 150 minutes. After incubation, 0.5 ml of the reaction mixture containing nitrite was pipetted and mixed with 1ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 minutes for completing diazotization. Then, 1ml of naphthylethylene diamine dihydrochloride (1%) was added, mixed and allowed to stand for 30 minutes. A pink coloured chromophore was formed in diffused light. The absorbance of chromophore formed was measured at 540 nm against the corresponding blank. Ascorbic acid was used as a positive control [18]. The scavenging activity was calculated using the formula:

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$$\text{Nitric oxide radical scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Hydroxyl radical scavenging assay

Stock solutions of EDTA (1 mM) were prepared in DMSO and $FeCl_3$ (10 mM), ascorbic acid (1 mM), hydrogen peroxide (10 mM) and deoxyribose (10 mM) were prepared in distilled deionized water. The method was carried out by adding 100 µl of EDTA, 10 µl of $FeCl_3$, 100 µl of H_2O_2, 360 µl of deoxyribose, 1000 µl of the extract (100 to 500 µg/ml) dissolved in distilled water, 330 µl of phosphate buffer (50 mM, pH 7.4) and 100 µl of ascorbic acid. This mixture was then incubated at 37°C for 1 hour. About 1 ml of incubated mixture was mixed with 1 ml of 10% TCA and 1 ml of 0.5% TBA and measured spectrophotometrically at 532 nm. Vitamin C was used as a positive control [22]. The hydroxyl radical scavenging activities was calculated by using the formula:

$$\text{Hydroxyl radical scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Superoxide radical scavenging assay

Tris HCl buffer (3 ml, 16 mM, pH 8.0) containing 1 ml NBT (50 µM) solution, 1 ml NADH (78 µM) solution and a sample solution of extract (20-100 µg/ml) in water were mixed. The reaction was started when 1 ml of phenazinemethosulfate (PMS) solution (10 µM) was added to the mixture. The reaction mixture was incubated at 25°C for 5 minutes and read at 560 nm against the corresponding blank samples. Quercetin was used as the reference compound [30]. The scavenging effect was calculated using the formula:

$$\text{Superoxide free radical scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Hydrogen peroxide radical scavenging assay

A solution of H_2O_2 (0.2 M) was prepared in phosphate buffer (pH 7.4). 1 ml extract of different concentrations (20, 40, 60, 80 and 100 µg /ml) was added to 0.6 ml solution of 40 mM H_2O_2 solution. The absorbance of the mixture was measured at 230 nm using UV–Visible spectrophotometer (Shimadzu Kyoto1800) against a blank solution containing phosphate buffer solution without H_2O_2. A known antioxidant such as ascorbic acid was used as positive control [19]. The percentage of H_2O_2 scavenging was calculated by following formula:

$$\text{Hydrogen peroxide scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Statistical analysis

All data were expressed as mean value ± standard deviation (SD) of the number of experiments (n=3). Linear regression analysis is used to calculate the IC_{50} values. To determine whether there were any differences among the means, one - way analysis of variance (One – way – ANOVA) and Duncan's Multiple Range Test (DMRT) were applied to the results. The Pearson correlation analysis was performed to assess the correlation between the antioxidant activities (using different assays) and total phenol and flavonoid content. P - values <0.05 were regarded to be significant. All statistical analysis was carried out using Statistical Package for Social Science 10.0 (SPSS) software package.

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RESULTS AND DISCUSSION:

Natural antioxidants are considered to be multifunctional and are of high interest as alternatives to synthetic antioxidants to reduce oxidation from complex food systems. Owing to the complex reactive nature of phytochemicals, the antioxidant activities of plant extracts cannot be assessed by just a solitary technique, but at least two test systems have been recommended for the determination of antioxidant activity to establish authenticity [7, 40]. Oxygen is essential for survival but certain abnormal physiological conditions convert some of the oxygen to ROS by univalent reactions that make them highly reactive [32]. Lipids, proteins and DNA are all susceptible to attack by free radicals [10]. They are also involved in autoimmune disorders like rheumatoid arthritis, etc.. [35]. These free radicals also regulate critical aspects of cellular physiology like signal transduction, gene transcription, and regulation of soluble guanylate cyclase activity [15]. Antioxidant compounds may function as free radical scavengers, initiators of the complexes of peroxidant metals, reducing agents and quenchers of singlet oxygen formation [3]. The total phenol and flavonoid content of unripe pericarp extracts are presented in Table 1. Phenolic compounds and flavonoids are major constituents of most of the plants reported to possess antioxidant and free radical scavenging activity [9]. Therefore, the importance of the search for natural antioxidants has increased in the recent years and so many researchers focused the same (Jayaprakasha *et al*., 2003).

The results of the study showed that a maximum DPPH scavenging activity of 68.55 ± 1.28% was exhibited by the ethanol extract at 50 µg/ml followed by ethyl acetate extract (46.93 ± 1.29) and water extract (34.75 ± 0.97). Thus, among the five fractions ethanol extract was observed to have the lowest IC_{50} value (25.8 µg/ml) (Tab: 2, Fig 1a). The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of the extracts was found to be dose - dependent and the scavenging activity of extracts on DPPH radicals increased with increasing concentrations of the extracts. Phenols and flavonoids increases DPPH radical by their hydrogen donating ability [46]. Also it is proved that a higher total phenol and flavonoid contents lead to better DPPH scavenging activity [14]. The result obtained in this investigation thus revealed that the DPPH radical scavenging activities of the unripe pericarp of *A. reticulata* might be attributed to the hydrogen donating ability of phenols and flavonoids present in the extracts.

Studies in animal models have suggested the role for NO in the pathogenesis of inflammation and pain [26]. Excess concentration of nitric oxide is associated with several diseases [26]. Oxygen reacts with excess nitric oxide to generate nitrite and peroxy nitrite anions, which act as free radicals [10, 37]. So, it is worthful to investigate the NO scavenging potential of the plant extract. In the present investigation, Ethanol extract showed maximum nitric oxide scavenging activity (29.09 µg/ml) followed by ethyl acetate (44.6 µg/ml) and water (108.35 µg/ml) extracts (Tab: 3, Fig 1b). It has been already proved through various studies that plant products have the property to counteract the effect of nitric oxide formation and, in turn, may be of considerable interest in relation to preventing the ill effects of excessive NO generation in the human system. Moreover, the scavenging activity of the plant products may also help to arrest the chain of reactions, initiated by excess generation of NO, that are detrimental to human health [4].

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In the present study, IC50 value for hydroxy radical scavenging activity was observed to be low for ethanol extract (20.27 µg/ml) followed by Ethyl acetate (47.71 µg/ml) and water (74.04 µg/ml) extracts. Thus, ethanol extract extract showed good hydroxy radical scavenging activity (Tab: 4, Fig 1c). Hydroxyl radicals are the most deleterious and reactive among the ROS and it bears the shortest half-life compared with other free radicals. Hydroxy radical causes lipid peroxidation by abstracting hydrogen atoms from unsaturated fatty acids and enormous biological damage [44]. The hydroxyl radical has the capacity to join nucleotides in DNA and cause strand breakage, which contributes to carcinogenesis, mutagenesis and cytotoxicity. OH- radicals react with an extensive variety of molecules, such as sugars, amino acids, lipids, and nucleotides, present in living entities [43]. Hence removal of OH^- ion is very important for the protection of living systems.

Superoxides are highly reactive molecules produced from molecular oxygen due to oxidative enzymes [37]. Also, the concentration of superoxide increases under conditions of oxidative stress and related situations [27]. Moreover, superoxide anions act as a precursor for the production of other kinds of cell-damaging free radicals and oxidising agents [21, 23]. Superoxide anions can result in the formation of H_2O_2 via a dismutation reaction. In the present study, A maximum scavenging activity of 79.37 ± 1.04% was exhibited by the ethanol extract at 50 µg/ml (IC_{50}= 34.21), followed by water (61.12 µg/ml) and ethyl acetate (46.66 µg/ml) extracts (Tab: 5, Fig 1d). The probable mechanism of scavenging the superoxide anions may be due to the inhibitory effect of the extract towards the generation of superoxides in the *in vitro* reaction mixture. This inhibitory ability of plant extract might primarily be due to the presence of phenols and flavonoids which possess hydroxyl groups.

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by the oxidation of essential thio (-SH) groups [20]. It rapidly crosses the cell membrane and reacts with Fe^{2+}, Cu^{2+} which in turn generates hydroxyl radicals which are the most reactive and cytotoxic among the oxygen radicals [2]. The hydrogen peroxide radical scavenging activity of ethyl acetate, ethanol and water extracts were observed as 64.63 ± 1.22, 82.50 ± 0.98 and 57.88 ± 1.59 respectively (Tab: 6, Fig 1e).

A correlation analysis was performed using Pearson correlation coefficient to check the linear correlation among the assays and total phenol and flavonoid content of the different unripe pericarp extracts (Tab: 7). The TPC showed a very strong positive significant ($P<0.05$) correlation with DPPH (R= 0.999). Similarly, a positive correlation was observed between TPC and NO radical scavenging (R=0.904), hydroxyl radical scavenging (R=0.997), superoxide radical scavenging (R=0.959) and hydrogen peroxide radical scavenging (R=0.973) assays. This suggests that phenols are the main compounds responsible for antioxidant activity detected by all scavenging assays performed in *Annona reticulata* unripe pericarp extracts. Amongst the five antioxidant assays, DPPH Vs OH has a strong positive correlation (R=0.999). A strong correlation was also found between DPPH and other assays. Flavonoids also exhibited a positive correlation between different assays employed (Tab: 7, 8). In the present study, highest phenolic content was observed in the ethanolic extracts. Numerous investigations of qualitative composition of plant extracts also revealed the presence of high concentrations of phenols in the extracts obtained using polar solvents [6]. Several types of researches have

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proved that there is a strong positive correlation between antioxidant activity and TPC [16, 28, 42]. This confirms the importance of polyphenols as a potential antioxidant biomolecule [1,13 34]. The extracts that perform the highest antioxidant activity have the highest concentration of phenols as recorded in the present work. The fact that phenolic compounds possess a high potential to scavenge radicals can be explained by their ability to donate a hydrogen atom from their phenolic hydroxyl groups [39].

The positive significant relationship observed between TFC and NO ($r=1.000$, $p<0.05$) indicates that flavonoid also plays a major role in the antioxidant activity However, the correlations between flavonoid content and different assays were significantly lower than those of total phenolics.

Tab: 1 Total phenolic and flavonoid content in unripe pericarp extracts of *Annona reticulata*

Extract prepared in	Total phenolic content (mg GAE/g of extract)	Total flavonoid content (mg QE/g of extract)
Water	58.49 ± 1.07^b	22.29 ± 0.70^b
Ethanol	88.50 ± 0.64^e	77.46 ± 2.07^e
Ethyl acetate	61.93 ± 1.28^c	34.36 ± 1.22^c

Tab: 2 DPPH scavenging assay of unripe pericarp extracts of *Annona reticulata*

Concentration (µg/ml)	Inhibition (%)			
	Ethyl	Ethanol	Water	Ascorbate
20	14.99 ± 1.09^a	37.29 ± 1.04^a	11.71 ± 1.69^a	29.88 ± 0.68
40	23.67 ± 1.13^b	46.11 ± 2.50^b	17.22 ± 0.78^b	44.21 ± 0.85
60	30.58 ± 1.54^c	53.66 ± 1.46^c	24.82 ± 1.03^c	59.29 ± 0.76
80	35.28 ± 0.77^d	60.55 ± 1.43^d	30.21 ± 1.05^d	68.36 ± 0.83
100	46.93 ± 1.29^e	68.55 ± 1.28^e	34.75 ± 0.97^e	81.67 ± 1.26
IC_{50}	56.11	25.8	74.45	24.77

Each value is the mean ± SD of triplicate analysis. Values followed by different superscript letters along the same column are significantly different from each other (One-Way ANOVA test; $P < 0.05$ and subsequent post hoc multiple comparison with Duncan's test)

Tab: 3 NO scavenging assay of unripe pericarp extracts of *Annona reticulata*

Concentration (µg/ml)	Inhibition (%)			
	Ethyl acetate	Ethanol	Water	Ascorbate
20	11.57 ± 0.71^a	26.90 ± 1.74^a	8.27 ± 0.91^a	34.62 ± 1.75

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40	28.33 ± 0.73^{b}	39.22 ± 0.78^{b}	15.24 ± 1.04^{b}	40.83 ± 1.48
60	39.09 ± 1.49^{c}	53.23 ± 1.92^{c}	19.52 ± 0.75^{c}	57.30 ± 0.93
80	47.25 ± 1.12^{d}	63.64 ± 1.37^{d}	21.34 ± 0.92^{d}	65.63 ± 1.24
100	51.57 ± 1.58^{e}	72.22 ± 1.00^{e}	25.64 ± 1.24^{e}	76.83 ± 1.21
IC_{50}	44.6	29.09	108.35	25.38

Each value is the mean ± SD of triplicate analysis. Values followed by different superscript letters along the same column are significantly different from each other (One-Way ANOVA test; $P < 0.05$ and subsequent post hoc multiple comparison with Duncan's test)

Tab: 4 Hydroxy radical scavenging activity of unripe pericarp extracts of *Annona reticulata*

Concentration (µg/ml)	Inhibition (%)			
	Ethyl	Ethanol	Water	Ascorbate
20	44.69 ± 0.87^{a}	20.17 ± 1.85^{a}	10.84 ± 2.07^{a}	48.32 ± 1.14
40	49.33 ± 0.93^{b}	33.97 ± 1.99^{b}	15.23 ± 2.17^{b}	55.53 ± 1.20
60	55.16 ± 0.97^{c}	40.02 ± 1.52^{c}	21.41 ± 1.91^{c}	62.26 ± 1.08
80	61.51 ± 0.77^{d}	45.69 ±1.32^{d}	29.33 ± 1.68^{d}	74.51 ± 0.90
100	64.71 ± 1.45^{e}	48.90 ± 1.53^{e}	35.14 ± 0.97^{e}	79.95 ± 0.85
IC_{50}	47.71	20.27	74.04	12.84

Each value is the mean ± SD of triplicate analysis. Values followed by different superscript letters along the same column are significantly different from each other (One-Way ANOVA test; $P < 0.05$ and subsequent post hoc multiple comparison with Duncan's test)

Tab: 5 Superoxide scavenging effect of unripe pericarp extracts of *Annona reticulata*

Concentration (µg/ml)	Inhibition (%)			
	Ethyl acetate	Ethanol	Water	Ascorbate
20	17.18 ± 0.84^{a}	17.73 ± 1.70^{a}	10.08 ± 1.97^{a}	47.52 ± 1.67
40	22.3± 0.92^{b}	28.44 ± 1.37^{b}	16.57 ± 1.56^{b}	55.82 ± 0.60
60	31.56 ± 1.38^{c}	37.55± 1.34^{c}	24.06 ± 1.73^{c}	52.86 ± 0.85
80	41.30 ± 1.10^{d}	49.14 ± 1.08^{d}	35.35 ±0.77^{d}	76.37 ± 1.39
100	56.44 ± 1.59^{e}	79.37 ± 1.04^{e}	40.39 ± 1.17^{e}	85.03 ± 0.97
IC_{50}	46.66	34.21	61.12	15.85

Each value is the mean ± SD of triplicate analysis. Values followed by different superscript letters along the same column are significantly different from each other (One-Way ANOVA test; $P < 0.05$ and subsequent post hoc multiple comparison with Duncan's test)

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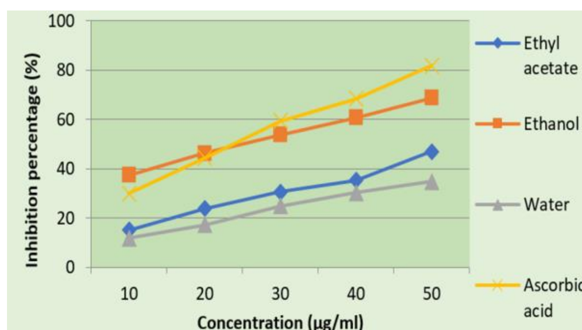
Tab: 6 Hydrogen peroxide scavenging activity of unripe pericarp extracts of *Annona reticulata*

Concentration (µg/ml)	Inhibition (%)			
	Ethyl acetate	Ethanol	Water	Ascorbate
20	31.53 ± 1.00^{a}	39.38 ± 1.04^{a}	11.45 ± 0.73^{a}	45.10 ± 1.62
40	41.67 ± 1.43^{b}	53.17 ± 0.78^{b}	24.85 ± 0.79^{b}	57.50 ± 0.74
60	50.64 ± 0.70^{c}	63.48 ± 1.02^{c}	36.06 ± 0.93^{c}	65.40 ± 1.21
80	57.66 ± 0.82^{d}	73.66 ± 0.69^{d}	47.03 ± 1.91^{d}	79.97 ± 0.80
100	64.63 ± 1.22^{e}	82.50 ± 0.98^{e}	57.88 ± 1.59^{e}	87.18 ± 1.74
IC_{50}	55.28	18.35	42.64	14.03

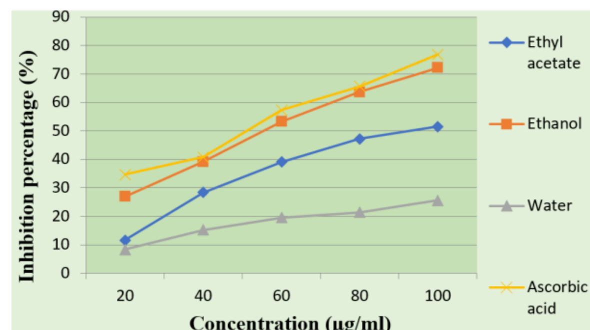
Each value is the mean ± SD of triplicate analysis. Values followed by different superscript letters along the same column are significantly different from each other (One-Way ANOVA test; $P < 0.05$ and subsequent post hoc multiple comparison with Duncan's test)

The ability of unripe pericarp extracts to scavenge hydrogen peroxide is shown in Table

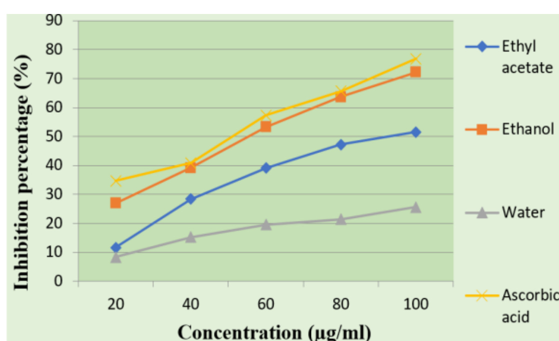
Fig: 1 Antioxidant assays of unripe pericarp extracts of *Annona reticulata*



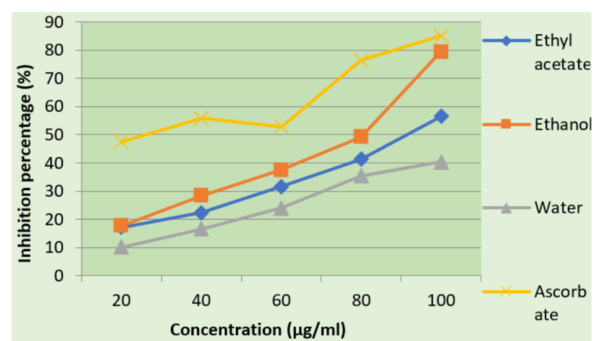
(a) DPPH Scavenging effect



(b) Nitric oxide scavenging assay



(c) Hydroxy radical scavenging assay



(d) Superoxide scavenging effect

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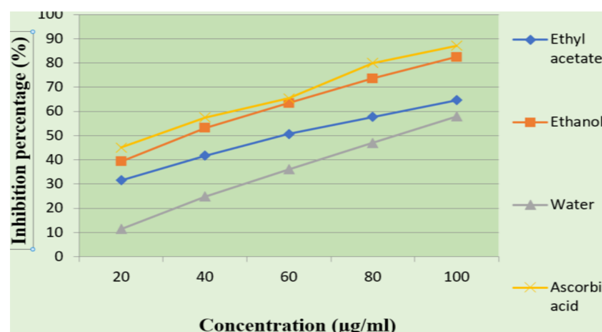

(e) Hydrogen peroxide scavenging effect

Tab: 7 Correlation between total phenolic content, total flavonoid content, and antioxidant assays

	TPC	TFC	DPPH	NO	OH	SO	HP
TPC	1						
TFC	0.895 NS	1					
DPPH	0.999* S	0.914 NS	1				
NO	0.904 NS	1.000* S	0.922 NS	1			
OH	0.997 NS	0.928 NS	0.999* NS	0.935 NS	1		
SO	0.959 NS	0.985NS	0.971 NS	0.988 NS	0.979 NS	1	
HP	0.973 NS	0.768NS	0.962 NS	0.781 NS	0.951 NS	0.868 NS	1

*Correlation is significant at the 0.05 level (2-tailed); S- Significant; NS- Non significant; TPC = Total phenol content; TFC = Total flavonoid content; DPPH: Scavenging of diphenyl –picrylhydrazyl radical; NO: Nitric oxide radical scavenging assay; OH: Hydroxy radical scavenging assay; SO: Superoxide radical scavenging assay; HP: Hydrogen peroxide radical scavenging assay

Tab: 8 Correlation coefficients between different parameters analyzed

Correlation	Correlation coefficient (r)	Correlation	Correlation coefficient (r)
DPPH Vs. TPC	0.999*	OH Vs. TPC	0.997
DPPH Vs. TFC	0.914	OH Vs. TFC	0.928
DPPH Vs. NO	0.922	OH Vs. SO	0.979
DPPH Vs. OH	0.999*	OH Vs HP	0.951
DPPH Vs. SO	0.971	SO Vs. TPC	0.959

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DPPH Vs. HP	0.962	SO Vs.TFC	0.985
NO Vs. TPC	0.904	SO Vs. HP	0.868
NO Vs. TFC	1.000*	HP Vs. TPC	0.973
NO Vs. OH	0.935	HP Vs. TFC	0.768
NO Vs SO	0.988	TPC Vs. TFC	0.895
NO Vs. HP	0.781		

DPPH: Scavenging of diphenyl –picrylhydrazyl radical; NO: Nitric oxide radical scavenging assay; OH: Hydroxy radical scavenging assay; SO: Superoxide radical scavenging assay; HP: Hydrogen peroxide radical scavenging assay; TPC: Total Phenol Content; TFC: Total Flavanoid Content; Asc: Ascorbate; * Correlation is significant at the 0.05 level; **Correlation is significant at the 0.01 level

CONCLUSION

Ethanol extracts of unripe pericarp obtained from the fruits of *A. reticulata* showed very strong *in vitro* antioxidative activity against all the five assay methods used, and its activity is comparable to the reference antioxidant, Ascorbate. All tested extracts, especially those of the ethanol extract, exhibited a high content of phenols and flavonoids. Comparisons of the antioxidant activities with the phenolic and flavonoid contents suggest that these compounds are responsible for the antioxidant activity. The TFC and the antioxidant activity of the unripe pericarp extracts also showed a positive correlation. Correlation between total phenol content and antioxidant activity was also very high suggesting that the phenolic and flavonoid compounds are the most responsible compounds contributing to the antioxidant activity of investigated samples. The results of this study revealed the unripe pericarp of *Annona reticulata* fruits are a rich source of antioxidants, with potential application in the protection and preservation of certain foods, as well as a central role in fighting cancer. Moreover, its value as a nutraceutical cannot be underestimated.

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