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Assessment of Antioxidant Activity of Ethanol and n-Hexane Seed Extracts of *Annona muricata*in Rats

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Abstract

The *in vitro* and *in vivo* antioxidant effects of *Annona muricata* seed extracts (n-hexane and ethanol extracts) were investigated using ascorbic acid as standard. Free radical scavenging activity in vitro was evaluated using 2,2-diphenyl-1-picryl- hydrazyl (DPPH). Lipid peroxidation was assayed using TBARS. Reduced glutathione and catalase activity were also investigated. Twenty-four male albino rats, divided into six groups were used for the *invivo* assay. Group A (control) received olive oil, group B and C received 200mg/kg n-hexane and ethanol extracts, respectively, group D received ascorbic acid, group E and F received 100mg/kg of n-hexane and ethanol extracts, respectively. Ethanol and n-hexane extracts at 100µg/ml and 20µg/ml, respectively, exhibited 49% and 32% inhibition of DPPH radical, respectively. Ascorbic acid (standard) exhibited upto96.9% inhibition of DPPH radical even at 20µg/ml. The extracts significantly increased catalase activity, glutathione levels and reduced the formation of malondialdehyde in the treated groups compared with the control especially in the heart and liver tissues. The results of the present study suggest that *Annona muricata*seed extracts could be a promising source of potent antioxidants that could inhibit lipid peroxidation in tissues as well as ameliorate oxidative stress.

Keywords: *Annona muricata*, Antioxidants, lipid peroxidation, 2,2- Diphenyl-1-picrylhadrazyl Ascorbic acid, Catalase Glutathione.

1. Introduction

The ability to utilize oxygen has provided humans with the benefit of metabolizing fats, proteins, and carbohydrates for energy; however, oxygen is a highly reactive atom that is capable of becoming part of potentially damaging molecules commonly called “free radicals.” These free radicals are capable of attacking healthy cells of the body, causing them to lose their structure and function (Carr and Frei, 1999).

Cell damage caused by free radicals appears to be a major contributor to aging, degenerative diseases, such as cancer, cardiovascular disease, cataracts, immune system decline, and brain dysfunction (Sieset al., 1992). Free radical formation is however controlled naturally by compounds known as antioxidants (Poongothaiet al.,2011). These includeα-Tocopherol, ascorbic acid, carotenoids, flavonoids and related polyphenols, α-lipoic acid, glutathione etc. (Devasagayamet al., 2004).

Anonnamuricata, family (Annonaceae), commonly known as ‘soursop’ or ‘Graviola’, is a deciduous, terrestrial, erect tree of about 5-8 meters in height. Although a native of America, ithas now been naturalized and is established in many tropical countries of the world. The plant is used medicinally in many tropical African countries for an array of human ailments, especially for parasitic infections and cancer(Adewoleet al., 2009). Preliminary studies have confirmed the antioxidant activity of Annona squasmosa– a differentspeciesin different in vitro models (Baskaret al., 2007). In continuation of the search for potential free radical scavenging agents (Kokate, 1999), the present study is designed to establish the antioxidant capacity of ethanol and n-hexane seed extractsof annonamuricatain albino rats.

2. Materials and Method

2.1. Preparation of Plant Sample

The matured fruits of *Anonnamuricata* were collected from Abocho in Dekina local government area,Kogi State, Nigeria. The fruits were identified by department of herbarium and ethnobotany, National Institute of Pharmaceutical Research and Development (NIPRD), Abuja Nigeria; with the identification number NIPRD/H/6164.

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The seeds were collected from the ripe fruits and air dried at room temperature, de-shelled to expose the inner fleshy core of the seeds, which were air dried and then blended, with a sterilized iron hand blender and later fine-blended using an electric blender.

2.2. Experimental Animals (albino rats)

Thirtyhealthy male albino rats weighing 129-250g were purchased from Salem University's animal house. The animals were housed in metallic cages under standard environmental conditions of light, temperature and relative humidity. They were allowed to acclimatize for one week, and were given free access to food (broiler's finisher) and water.

2.3. Animal Groupings

The rats were divided into six groups of fiveanimals each. They were given different doses of the ethanol and n-hexane extracts, reconstituted with olive oil. The different groups are as shown below:

Group A (control Olive oil)
Group B 200mg/kg (n-Hexane extract in olive oil)
Group C 200mg/kg (ethanol extract in olive oil)
Group D 200mg/kg ('Ascorbic acid' in distilled H_2O)
Group E 100mg/kg (n-Hexane extract in olive oil)
Group F 100mg/kg of (ethanol extract in oliveoil)

2.4. Preparation of Seed Extracts

About 50g of the blended seed powderwas extracted each time using 250ml n-hexane in a soxhlet extractor. The extraction was allowed to continue for about 2-3hours, after which the solvent was recovered and the extract further concentrated(using rotary evaporator, Model -ST15 OSA UK) followed by oven drying at 40^0C.The bulk extract, 1000mg/ml, was prepared from the dried extract using olive oil.This was stored in sample bottles and kept in a refrigerator for further use. This process was repeated using absolute ethanol as the extraction solvent.

2.5. Administration of Extract

The extracts were administered orally using acanula (intubator). The animals were treated with different doses of the extracts as shown in the animal groupings (100mg/kg- 200mg/kg) for a period of eight weeks. At the end of this period, the animals were fasted overnight prior to sacrifice. They were weighed and anesthetized using chloroform soaked in cotton wool and sacrificed by humane decapitation.

2.6. Chemicals

All chemicals were of analytical grade. These include, absolute ethanol, n- hexane (Sigma–Aldrich), 2,2. Diphenyl, 1- picryl hydrazine, Ascorbic acid, trichloroacetic acid, thiobarbituric acidand methanol.

2.7. Preparation of Serum

Blood was collected into non-heparinized tubes and centrifuged at 3000RPM (using a Microfield centrifuge, Model 90-2) for 10 minutes. The sera were then decanted into another sample tubes and stored in the refrigerator for subsequent use.

2.8. Preparation of Tissue Homogenates

Liver and heart tissues from each animal were rapidly excised during the sacrifice, washed with cold normal saline to remove excess blood, weighed and stored immediately at-4°C. Subsequently, they were homogenized individually (using a Bocsh PSB 570-2 homogenizer) in ice-cold phosphate buffer (pH 7.4). The homogenates were centrifuged at 3000 RPM for 15 minutes and the supernatant decanted into sample tubes and kept in the refrigeratorfor further use.

2.9.0. Assessment of Lipid Peroxidation

Thiobarbituric acid reacting substances (TBARS) in tissue were estimated by the method of (Torres *et al.*,2004).

Principle

At low pH and high temperature malondialdehyde binds TBARS to form a pink complex that can be measured at 535nm.

Procedure

One milliliter of thiobarbituricacid (TBA) and trichloro acetic acid (TCA) were added to 50µl of the tissue homogenates, respectively. The mixture was incubated for 30 minutes at 80^0C. The tubes were allowed to cool immediately under ice and centrifuged at 3000RPM for 15 minutes. The supernatant was measured using spectrophotometerat a wavelength of 535nm.The results are expressed as malonaldehyde concentration in µmol/ mg protein.

2.9.1.Determination of Catalase (CAT) Activity

The activity of CAT was measured spectrophotometrically as described by (Gott, 1991).

Procedure

About 100µl of liver and heart homogenates, respectively, were mixed with 500µl of hydrogen peroxide at 37°C for a minute. The catalase preparation was allowed to split hydrogen peroxide for different periods of time.The addition of 500µl of ammonium molybdate solution stopped the reaction with a formation of a yellow complex. The absorbance of the yellow complex formed between ammonium molybdate and hydrogen peroxide was then measured at a wavelength of 405nm using a spectrophotometer. One unit of catalase was defined as the amount of enzyme that catalyses a decomposition of 1micro mole of hydrogen perioxide per min.

2.9.2. Estimation of Reduced Glutathione (GSH) Level

The method of Beutler*et al*.,(1963) was used.

Procedure

An amount of four hundred and fifty microliters (450µl) of distilled water was added to 100µl of test sample and 1.5mlof sulphosalicyclic acid added

(deproteinization). The mixture was then centrifuged at 3000RPM for 10mins. 2ml of 0.1M phosphate buffer pH 7.4 and 2.25ml of Ellman's reagent was added to 0.25ml supernatant. Readings were taken within 5mins at 412nm.

2.9.3. Protein Determination

Principle

Under alkaline conditions, substances containing two or more peptide bonds form a purple complex with copper salts in the reagents.

Procedure

An amount of four hundred and fifty milliliter of distilled water was pipette into test-tubes (in duplicate) and 50µl of the sample was added after which 1.5ml of biuret reagent was added. The absorbance was read at 540nm.

2.9.4.In Vitro Antioxidant Assay

2,2- Diphenyl-1-picrylhadrazyl Radical Scavenging Activity

This was estimated according to the method described by Mensor*et al.,* 2001.

Procedure

Extracts (40-2000µg) in 4ml of distilled water wereadded to the methanolic solution of DPPH (1mM, 1ml). The mixture was shaken and left to stand at room temperature for 30mins. The absorbance of the resulting solution was measured spectrophotometrically at 517nm for 20, 100, 250, 500 and 1000 µg/ml of the extracts. The standard used was ascorbic acid dissolved in distilled H_2O.

Calculation

$\%I = [(A_{control} - A_{sample})/ A_{control}] \times 100$

where%I is inhibition of the DPPH free radicals in percentage; $A_{control}$ is the absorbance of the control reaction containing all reagents except the test compound and $A_{sample}$is the absorbance of the test compound.

2.9.5. Statistical Analysis

The results were expressed as mean ± SD of five animals in each group. The data were evaluated by one way ANOVA using SPSS version 20. P<0.05 was observed to be statistically significant.

3. Results

3.1. Body Weights of Treated Rats

Table 1 shows the effect of ethanol and n-hexane seed extracts on body weight of the treated animals. There was a relative increase in body weights of animals treated with ascorbic acid, and 100mg/kg of the extracts. However, 200mg/kg of both the ethanol and n-hexane seed extract caused a relative decrease in the final body weights of the treated animals.

Table 1.Effect of *Annona muricata*ethanol and n-hexane seed extract on body weights of rats

Groups	Ethanol extracts		n- Hexane extracts	
–	Initial weight(g)	Final weight (g)	Initial weight (g)	Final weight (g)
A	157.7±5.0	164.4±12.0	157.7±11.0	164.4±3.0
D	220.7±10.0	243.9±17.0	220.7±8.0	243.9±5.0
F	107.9±9.7	166.7±12.2	144.7±13.0	185.4±15.0
C	212.8±4.0	165.9±10.0	212.4±5.0	202.3±12.0

Keys: A (control), B-200mg/kg n-hexane extract, C-200mg/kg ethanol extract, D-200mg/kg ascorbic acid, E-100mg/kg n-hexane extract, F- 100mg/kg ethanol extract.

3.2. Effects on Lipid Peroxidation

Figure 1 shows the concentration of thiobarbituric reacting substances (TBARS) in the liver,heart and serum of the treated animals. There was a significant decrease (P<0.05) in TBARS concentration in the liver and serum of the treated animals compared to the control group.

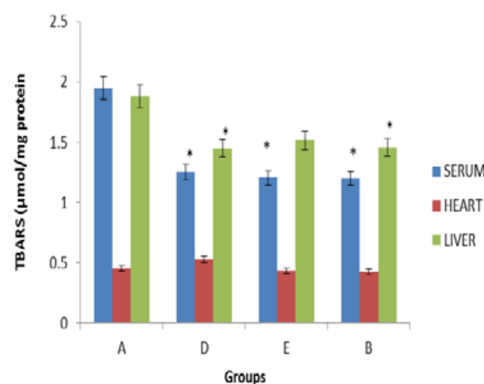

Figure1. Effect of n-hexane extracts of *Annona muricata* seeds on lipid peroxidation in the liver,serum and hearts of rats.

*S.D at (P<0.05) from control.

Keys: A (control), B-200mg/kg n-hexane extract, D-200mg/kg ascorbic acid, E-100mg/kg n-hexane extract.

Figure2 reveals the effect of ethanol extract of *A. muricata*seed on lipid peroxidation in liver heart and serum of rats.

There was a significant decrease (P<0.05) in TBARS concentration in the serum of animals treated with ascorbic acid and 100mg/kg extract compared with the control. There was also an observed decrease in the TBARS concentration in the heart of the animals treated with 100 and 200mg/kg of the ethanol extract compared with control.

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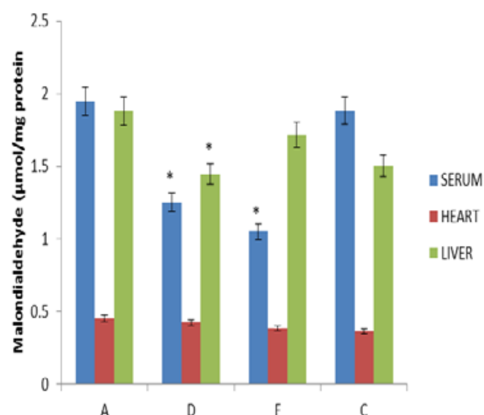

Figure 2. Effect of ethanol extract of *Annona muricata*seeds on lipid peroxidation in serum, liver and heart tissues of rats.
*S.D at (P<0.05) from control.
Keys: A (control), C-200mg/kg ethanol extract, D-200mg/kg ascorbic acid, F- 100mg/kg ethanol extract.

3.3. Effect on Catalase Activity

Figure 3 shows the effect of *Annona muricata*n-hexane seeds extract on catalase activity of the treated animals.

There was a significant difference (P<0.05) in catalase activity in the serum of animals treated with ascorbic acid and100mg/kg of the extract compared with the control. There was also an observed increase in catalase activity in the heart and liver of animals treated with 100 and 200mg/kg of the extracts compared to the control.

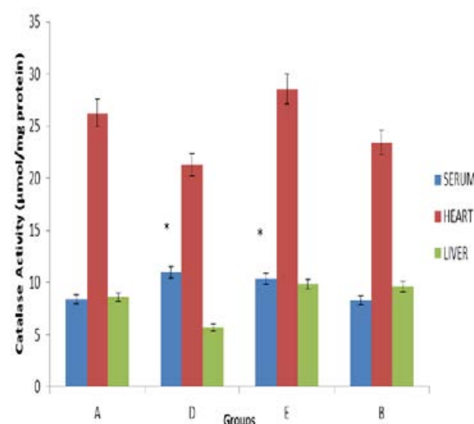

Figure 3. Effect of n-hexane extract of *Annona muricata*seeds on catalase activity of liver, serum and heart of rats.
*S.D at (P<0.05) from control.
Keys: A (control), B-200mg/kg n-hexane extract, D-200mg/kg ascorbic acid, E-100mg/kg n-hexane extract.

Figure 4 shows the effect of ethanol extract of *Annona muricata*seeds on catalase activity. There was a significant difference (P<0.05) in the catalase activity in the serum of animals treated with ascorbic acid and 100mg/kg extract compared with control. An increase was also observed in the catalase activity in the heart and liver of the animals treated with 200mg/kg of extract compared with control.

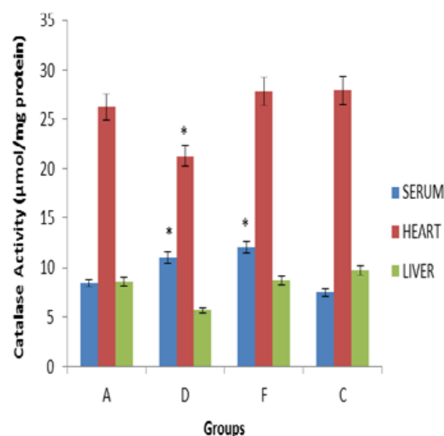

Figure 4. Effect of ethanol extract of *Annona muricata*seeds on catalase activity in the serum, liver and heart of albino rats.
*S.D at (P<0.05)
Keys: A (control), C-200mg/kg ethanol extract, D-200mg/kg ascorbic acid, F- 100mg/kg ethanol extract.

3.4. Assessment of Glutathione

Figure 5 depicts the effect of n-hexane extract of *Annona muricata*seed on glutathione.

The result revealed a significant increase (p<0.05) in glutathione activity in the heart of rats treated with100mg/kg extracts and ascorbic acid compared to the control group. There was also a significant increase (p< 0.05) in glutathione activity in the liver of animals treated with the standardand extracts compared to control.

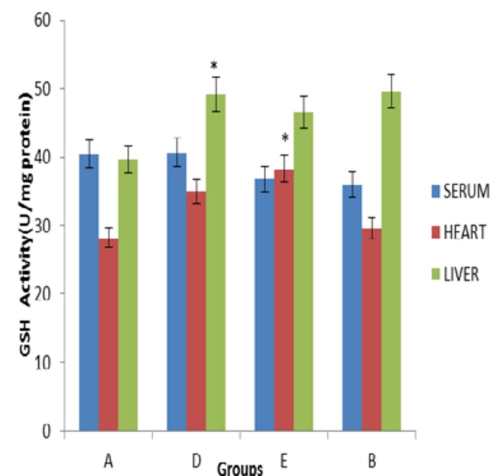

Figure 5. Effect of n-hexane extract of *Annona muricata*seeds on glutathione activity in rats.
*S.D at (P<0.05)

Keys: A (control), B-200mg/kg n-hexane extract, D-200mg/kg ascorbic acid, E-100mg/kg n-hexane extract.

Figure 6 shows the effect of ethanol extract of *Annona muricata*seeds on glutathione.There was a significant increase inglutathione activity in the liver of the rats treated with 200mg/kg extracts and standard and in the hearts of the treated groups compared with the control group.

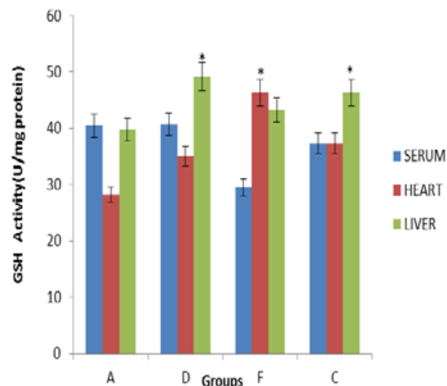

Figure 6. Effect of ethanol extract of *Annona muricata*seeds on glutathione activity in the liver serum and heart of rats.
*S.D at (P<0.05)

Keys: A (control), C-200mg/kg ethanol extract, D-200mg/kg ascorbic acid, F- 100mg/kg ethanol extract.

3.5. In VitroAntioxidant Activity

Figure 7 shows the percentage inhibition of ethanol extract of *A. muricata*seeds on DPPH. Percentage inhibition was observed to be highest at a concentration of 100µg/ml

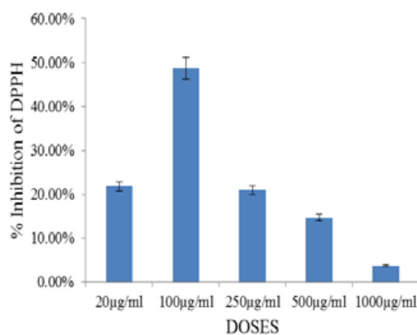

Figure 7. Percentage inhibition of ethanol extract of *Annona muricata*seeds onDPPH
*S.D at (P<0.05)

Figure 8 shows the percentage inhibition of n-hexane extract of *Annona muricata* seeds on DPPH.The percentage inhibition was observed to be highest at a concentration of 20µg/ml of n-hexane extract.

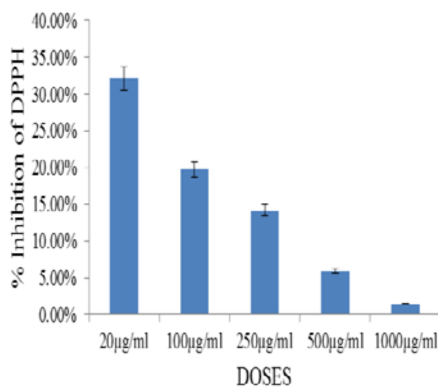

Figure 8. Percentage inhibition of n-hexane extract of *Annona muricata*seeds on DPPH.
*S.D at (P<0.05)

Figure 9 shows the scavenging effect of ascorbic acid on DPPH. The scavenging effect of Ascorbic acid seemed to be decreasing as the concentration was increasing.

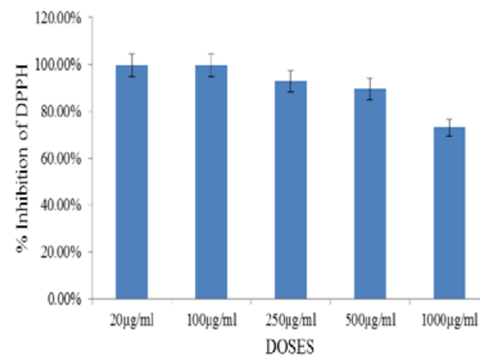

Figure 9. Percentage inhibition of Ascorbic Acid on DPPH
*S.D at (P<0.05)

4. Discussion

Plants constitute a reservoir of potentially useful chemical compounds which serve as drugs, as well as provide newer leads and clues for modern drug design and synthesis (Arun*et al.*, 2011). Experimental evidence suggests that Free Radicals (FR) and Reactive Oxygen Species (ROS) are involved in quite a number of diseases (Sajeed*et al.*, 2011). Many studies investigated the role of antioxidant drugs and plant-derived compounds in the prevention of oxidative stress (Madhavan*et al.*,2010). Thus the role of plants and plant products cannot be over emphasized in this area.

In the present study, a decrease in the final body weight was observed in rats treated with a higher dose of both n-hexane and ethanol extracts (200mg/kg). This decrease could be a result of the enhancing activities of the bioactive compounds(phenols, flavonoids, saponins alkaloids etc.) which have been found to be present in the administered extracts(yet to be published article) on the lipolytic enzymes. This is an indication that these extracts could be useful as a weight reduction agent especially at higher doses.

Lipid peroxidation involves the formation of lipid radicals which lead to membrane damage (Baskar*et al.*, 2007). Increased lipid peroxidative status in membranes indicates changes in the membrane bilayer as a result of the formation of reactive oxygen species (ROS), thereby impairing membrane functions by decreasing membrane fluidity and changing the activity of the membrane-bound enzymes and receptors.

Research findings (yet to be published)revealed the presence of relativelyhigh levels of phenols, flavonoids andsaponins in both the n-hexane and ethanol although much higher in the ethanol seed extracts. Flavonoids are known to be potent antioxidants found in most plant species and accounts for significant percentage of chemical

constituents in vegetables, fruits and seeds (Mamta*et al*., 2013).

In the present study, there was a significant decrease in the concentration of thiobarbituric acid reactive substances in the serum, heart and liver of the treated animals especially for those treated with 100mg/kg of the extracts. This is a pointer to the protective effect of these extracts on oxidative stress and membrane damage. This resultis similar to that obtained byAdewole*et al.* (2009) who reported a similar reduction in nitric oxide(NO) and Malonaldehyde levels in rats treated with *A.muricata*leaf extracts.

Oxidative stress has been shown to be characterized by altered non-enzymatic and enzymatic antioxidant systems (Zima *et al.,*2001). Oxidative stress also plays a key role in aging and the pathogenesis of many diseases (including kwashiorkor, seizure, alzheimers's disease, parkinson's disease, liver disease, cystic fibrosis, sickle cell anemia, HIV, cancer, heart attacks, stroke and diabetes) (Wu *et al.*, 2004). Catalase activity as observed in the present studywas relatively high in the heart and liver of the treated animals;moreso, in the serum of the n-hexane extract treated group. Increased catalase activity could be an indication of the chemopreventive activity of the extracts as observed by (Ojo and Ladeji, 2005) in a similar study of the black tea in rats. Likewise, glutathione activity in the tissues and serum of the treated groups were significantly ($p<0.05$) high. Annona muricata seed extractstherefore could be a good source of potent natural antioxidants that could ammeliorate oxidative stress.

2,2-diphenyl-1-picryl hydrazyl (DPPH) stable free radical method is an easy, rapid and sensitive way to survey the *invitro*antioxidant activity of a specific compound or plant extracts (Koleva*et al.*, 2002). In the presentinvestigation, the antioxidants which were present in the seed extracts and ascorbic acid reacted with DPPH which was measured at 517nm. The n-hexane extract at 20µg/ml, ethanol extract at 100µg/ml and ascorbic acid 20µg/ml and 100µg/ml produced percentage inhibition of DPPH, 32% 49% and 96.9%,respectively. Although, the percentage inhibition of the extracts were lower than that of the standard (ascorbic acid); they are however, significant. This free radical scavenging effect of the extracts could possibly be due to the presence of antioxidant phytochemicals(phenols, flavonoids etc.) present in the extracts.

According to Carr and Frei (1999), antioxidantsinhibit the growth of transformedcellsdecreasingtheirintercellular communication by the oxidative protective mechanism. *Annona muricata* seed extracts therefore could be a promising source of potent antioxidants and may be efficient as a preventive and management agent in some degenerative diseases caused by oxidative stress.

However, further research is required to fractionate this extracts in order to determine the fraction with the highest antioxidant activity.

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