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Evaluation of antioxidant and free radical scavenging activity of *Annona muricata*

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ABSTRACT

The use of plant based remedies and their derived substances has been integral part of traditional medicine throughout the world with the discovery of new therapeutic agents and versatile applications. *Annona muricata* fruits commonly known as soursop have highest economic value and popularity. In the present study, 50% ethanolic extract prepared from the fresh leaves of soursop was screened for its antioxidant and free radical scavenging potential.. Tannins (0.44 ± 0.0013 mg/g), flavanoids (1.92 ± 0.02 mg/g), phenolics (104.43 ± 0.013 mg/g), carotenoids (0.302 ± 0.001 mg/g), α -tocopherol (14.80 ± 0.02 mg/g), reduced glutathione (7.4 ± 0.01 mg/g), lycopene (0.34 ± 0.01 mg/g) and Vit C (1.98 ± 0.011 mg/g) contents were found to be present. Glutathione-S-transferase (0.740 ± 0.0031 U/g), Peroxidase (6.8 ± 0.101 U/g), Superoxide dismutase (104.70 ± 1.88 U/g), Catalase (2.57 ± 0.02 U/g), Polyphenol oxidase (0.33 ± 0.002 U/g), and Glutathione reductase (35.89 ± 0.103 U/g) activities were found. In addition, total antioxidant capacity (TAC) was found with IC_{50} values of 44.2474 μ g/ml. Radical scavenging potentials against superoxide, nitric oxide, hydroxyl, and hydrogen peroxide were found to be effective with IC_{50} values 59.05 ± 0.103 , 70.12 ± 0.023 , 134.21 ± 0.063 and 43.4 ± 0.102 μ g/ml respectively. The finding of the study reveals that hydroalcoholic extract of *Annona muricata* leaves could be considered as nutraceutical with potent source of antioxidants and radical scavenging activity suitable for prevention of human diseases caused by oxidative stress.

Keywords: SOD, oxidation, *Annona muricata*, Total antioxidant capacity, OH radicals

INTRODUCTION

Bioactive principles present in the medicinal plants attribute to the therapeutic efficacy and it can be incorporated into modern medicine system for the development of newer drug formulation for therapeutic ailments [1]. Enzymic antioxidants play an important role in cellular defense against reactive oxygen species (ROS) [2]. This is accomplished by a set of endogenous antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), peroxidase (Px) and polyphenol oxidase (PPO). Nonenzymic antioxidants also play an important role in second line defense mechanism against damage induced by oxidative stress [3]. Antioxidant vitamins that are derived from the diet can be considered under nonenzymic antioxidants. Reactive oxygen species like hydroxyl radicals (OH^{\bullet}) are produced as the byproduct of normal cell metabolism and environmental stress [4]. Antioxidant and free radical scavenging potential have been reported to be rich in the fresh leaf than dry leaf [5].

Annona muricata L is an undersized, deciduous commonly known as Graviola and Soursop belongs to custard apple family and roundish canopy-like tree. Height of this fruit bearing tree is measured to be in the range between 5 and 8 m [6]. Fruits were consumed for reducing fever and improving mother's milk secretion. The seed extracts are used to kill external parasites, head lice, and worms [7]. The phytoconstituents that are naturally present in the plant exhibit disease preventive properties, though they are not essential nutrients to human health. Annonaceous acetogenins, lactones and isoquinoline, alkaloids, tannins, and coumarins are some of bioactive compounds present in the *Annona muricata* leaves. Hence, a study was designed to investigate the quantification of selected antioxidants and evaluation of free radical scavenging potential to validate the therapeutic efficacy of *Annona muricata* fresh leaf extract.

MATERIALS AND METHODS

Preparation of plant extract

Leaves of *Annona muricata* were authenticated by botanical survey of India, TNAU campus, Coimbatore. The fresh leaves were washed thoroughly and immediately crushed with 50% ethanol to prepare extract. The extract was filtered twice and used for further studies.

Qualitative phytochemical screening

The different phytochemicals such as Alkaloids, Flavonoids, Glycosides, Steroids, Tannins, Phenols, Proteins, Saponins were qualitatively tested in the 50% ethanolic extract of fresh leaves [8-9].

Quantification of selected phytoconstituents

Enzymic antioxidants

Glutathione - S – transferase

GST (CDNB) and GST (DCNB) activities were determined spectrophotometrically as described by Habig *et al.* (1974) with modifications. For GST (CDNB) assays, the reaction medium contained 0.1 m potassium phosphate buffer Ph 7.5 or 6.5, 1.0 mm GSH, 1.0 mm CDNB, 1% absolute ethanol, and protein in a total volume of 1.0 mL. For GST (DCNB) assays, the reaction medium contained 0.1 m potassium phosphate buffer, pH 7.5, 5.0 or 1.0 mm GSH, 1.0 mm DCNB, 1% absolute ethanol, and protein in a total volume of 1.0 mL. The reaction, conducted at 25°C, was initiated by the addition of CDNB or DCNB, and the change in A340 or A345, respectively, was monitored for 120 seconds with a spectrophotometer [10].

Estimation of glutathione reductase

The assay of glutathione reductase (GR) was done according to the procedure of David and Richard (1983) [19]. To 0.1 ml of sample, 1 ml of Potassium buffer (0.12M pH 7.2), 0.1ml of EDTA, 0.1ml of Sodium azide and 0.1 ml of oxidized glutathione were added and the volume was made up to 2 ml with water. The mixture was kept at room temperature for three minutes and 0.1ml of NADPH was added. The absorbance at 340nm was recorded at intervals of 15seconds for 2 to 3 minutes. One unit of GR is expressed as μM of NADPH oxidized/ minute/gram [11].

Estimation of catalase activity

The method of Luck was adopted to measure the activity of catalase. The enzyme extract (0.1ml) was added to the reaction mixture containing 3ml of H₂O₂ and 0.01M phosphate buffer (pH 7.0) and the OD change was measured at 240nm, the time taken for decrease in the absorbance from 0.45 to 0.4 is noted as ΔT . The activity of the enzyme is expressed in the terms of μmole of H₂O₂ consumed/ min/ mg protein [12].

Estimation of superoxide dismutase (SOD) activity

Activity of superoxide dismutase enzyme was evaluated based on the ability of SOD to inhibit the oxidation [13].

Estimation of peroxidase activity

Peroxidase catalyses the oxidation of a variety of electron donors with the help of H₂O₂ thus scavenging the endogenous H₂O₂. 3 ml of pyrogallol solution (0.05M pyrogallol in 0.05M phosphate buffer, pH 6.5) and 0.05 to 0.1 ml of enzyme extract were pipetted out into cuvettes. 0.5 ml of 1% H₂O₂ was added in the test cuvettes. Recorded the change in absorbance every 30 seconds up to 3 minutes at 430nm [14].

Polyphenol oxidase activity

The polyphenol oxidases (PPO) comprise of catechol oxidase and laccase. Homogenized the sample in a blender with 50mM Tris-HCl (pH 7.2), 0.4M sorbitol and 10mM NaCl and centrifuged for 10 minutes. The supernatant was used for the assay. Took 2.5 ml of 0.1M phosphate buffer (pH 6.5) and 0.3 ml of catechol solution (0.01M) in a cuvette and set the spectrophotometer at 495 nm. Now added 0.2 ml of the enzyme extract and started recording the change in the absorbance every 30 seconds upto 5 minutes. One unit of either, catechol oxidase or laccase is defined as the amount of enzyme that transforms 1 μ M of dihydrophenol to 1 μ M of quinone per minute under the assay conditions [15].

Estimation of Nonenzymic antioxidants

Non enzymic antioxidant such as ascorbic acid [16], α -tocopherol [17] total carotenoids and lycopene [18], flavonoids [9], tannins [19], total phenols [8], and reduced glutathione [20] were estimated in the fresh 50% ethanolic extract of *Annona muricata* leaves.

Hydroxyl radical scavenging assay

The assay is based on the qualification of degradation product of 2-deoxy ribose by condensation with TBA. Hydroxyl radical was generated by Fe³⁺ - Ascorbate -EDTA-H₂O₂ system (Fenton reaction). The reaction mixture contained 0.1 ml of deoxyribose (2.8 mM), 0.1 ml EDTA (0.1mM), 0.1 ml H₂O₂ (1mM), 0.1 ml ascorbate (0.1mM), 0.1 ml KH₂PO₄-KOH buffer, pH 7.4 (20mM) and various concentration of plant extract in a final volume of 1 ml. the reaction mixture was incubated for 1 hour at 37^oC. Deoxyribose degradation was measured as TBARS and the percentage inhibition was calculated [21].

RESULTS AND DISCUSSION**Preliminary phytochemical screening of *Annona muricata***

The qualitative screening of phytochemical constituents exhibited the presence of bioactive compounds in the 50% ethanolic extract of *Annona muricata* fresh leaves (Alkaloids, flavanoids, glycosides, tannins, steroids, phenolics, proteins, and saponins). Secondary metabolites such as phenolics and alkaloids are attributed to the therapeutic activities in the biological systems [22-23]. Tannins and flavanoids have been reported to possess biological activities that lead to prevention and management of many ailments [24]. Tannins are polymeric phenolic substances that are capable of tanning leather and precipitating gelatin from solution. They are well known for their astringent property, antioxidant potential and antimicrobial property at low concentration [25]. Flavanoids containing plants are reported to have antioxidant, antifungal, antiinflammatory, antiallergenic, anticarcinogenic and hepatoprotective activities [26-28]. Saponins are shown to have beneficial effects on the blood cholesterol level, cancer therapy, immune system and also have antiviral property. Plant glycosides are found to play essential role in the treatment of heart disorders and are known to possess beneficial effects on cardiac arrhythmias [29]. These qualitative observations offer a scientific validation for the lead compound identification and for further *in vitro* and *in vivo* studies.

Enzymic antioxidants

Catalase (CAT) and Superoxide dismutase (SOD) activities were observed to be 2.57 \pm 0.02 U/g and 104.70 \pm 1.88 U/g activities respectively (Table 1). Superoxide dismutase serves as a natural antioxidant that protects tissues from the damage induced by reactive oxygen species [30-31]. Peroxidase (POD) activity was found to be 6.8 \pm 0.101 U/g in. In plants, antioxidant enzymes namely catalase and peroxidase have been showed to increase during oxidative stress condition [32]. Peroxidase enzyme catalyses the oxidation of a wide variety of electron donors with the help of H₂O₂ and thereby scavenges the endogenous H₂O₂ [33]. Polyphenol oxidase (PPO) and glutathione reductase activity were found to be 0.33 \pm 0.002U/g and 35.89 \pm 0.103 U/g units. Plant polyphenol oxidase is reported to be very sensitive to environmental stress conditions and has a rapid turnover rate [34]. It has been reported that PPO isoenzymes are actively involved in scavenging oxidant radicals. Some isoenzymes of peroxidases (POD) are reported to exhibit PPO activity. PPO and POD are among the most studied enzymes in fruits and vegetables and many reports suggest their role in antioxidation [35-36].

Table 1: Level of antioxidant enzymes in 50% ethanolic extract of *Annona murica*

Activity of enzymes (U/g)					
G-S-T	Peroxidase activity	SOD	Catalase	Polyphenol oxidase	Glutathione reductase
0.740±0.0031	6.8±0.101	104.70 ± 1.88	2.57 ± 0.02	0.33 ± 0.002	35.89±0.103

#Values are Mean ± Standard deviation of triplicates

Non-enzymic antioxidants

A spectrum of non-enzymic antioxidants namely ascorbic acid, α -tocopherol, total carotenoids, lycopene, flavonoids, tannins, total phenols and reduced glutathione are essential for the cellular systems in curtailing reactive oxygen species (ROS) [37]. The levels of non-enzymic antioxidants analyzed in fresh leaf of *Annona muricata* are presented in Table 2. 1.98 ± 0.11 mg/g ascorbic acid content was found to be present in the extract. Ascorbic acid is distributed in both intracellular and extracellular fluid and is shown to function as scavenger for many free radicals [38]. α -tocopherol content was detected to be 14.80 ± 0.02 mg/g. Extensive levels of α -tocopherol have been documented in plant tissues and its concentration was shown to be varied among different plant tissues from 5 mg/g fresh weight in potato tubers to 200 mg/g in palm leaflets [39]. Tocopherol is a protective agent that can act against the toxic effects of oxygen radicals within the membrane and can act as an excellent inhibitor of lipid peroxidation [40].

Table 2: Level of non-enzymic antioxidants in 50% ethanolic extract (mg/g) of *Annona muricata*

level of antioxidants in <i>Annona muricata</i> 50% ethanolic extract(mg/g)							
Tannin	Flavonoids	Total phenolics	Vit C	Total Carotenoids	Lycopene	α -tocopherol	Reduced glutathione
0.44±0.0013	1.92±0.02	104.43±0.013	1.98±0.011	0.302±0.001	0.34±0.01	14.80±0.02	7.4±0.01

#Values are Mean ± Standard deviation of triplicates

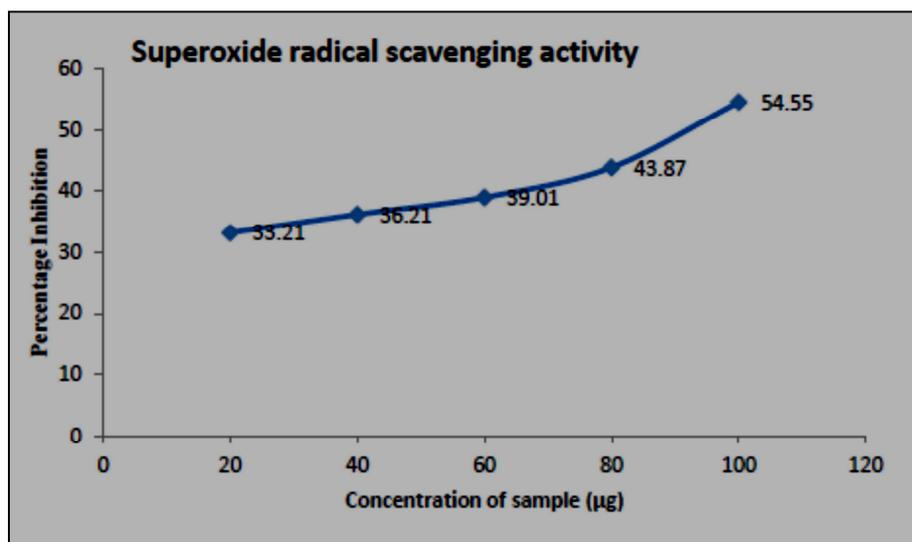
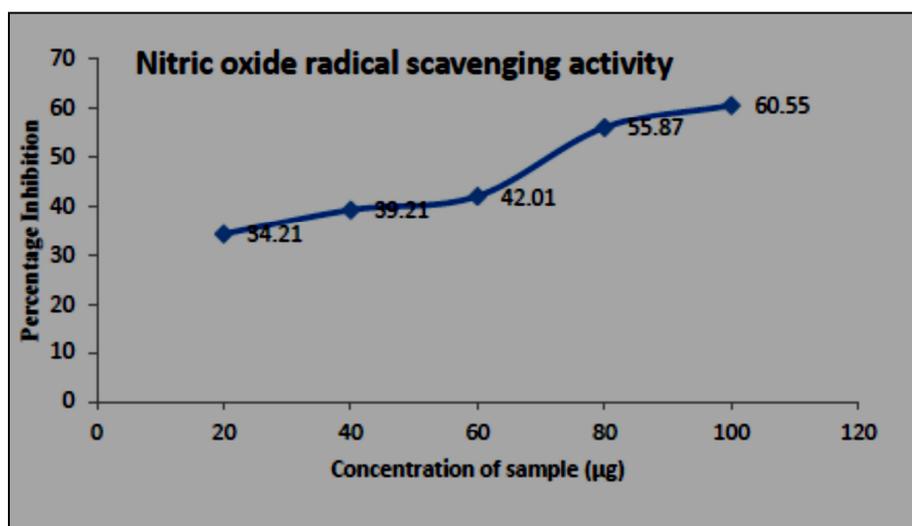
Total carotenoid and lycopene contents were found to be 0.302 ± 0.001 mg/g and 0.34 ± 0.01 mg/g respectively. Carotenoids and lycopene have been identified as phytochemicals with potential health benefits. Studies have indicated that the cellular content of carotenoids and other related carotenoids with proposed beneficial health effects can be increased through prolonged supplementation. Carotenoids exhibit a central role against cancer, cardiovascular disease, HIV infection and other age related disorders. Recent report suggests that all the carotenoids except β -carotene are very efficient antioxidants [40-41]. Flavonoids and total phenols contents were found to be 1.92 ± 0.02 and 104.43 ± 0.013 mg/g. Polyphenolic compounds have been evolved as a means to render protection and defense to plants against pathogens and antioxidant roles [42-43]. It is reported that natural polyphenolic compounds exhibit cellular defense mechanism and have strong antioxidant and immunomodulatory activity [44]. Reduced Glutathione level was found to be 7.4 ± 0.01 mg/g. In plants, glutathione concentration is proven to be higher in chloroplast and it plays a role as radical scavenger, membrane stabilizer and precursor of heavy metal binding peptide which are well documented [44]. The findings of the present study revealed that 50% ethanolic extract of *Annona muricata* fresh leaves possesses rich medicinal properties and can be much effective in treating diseases caused by oxidative stress.

Inhibition of superoxide (SO) radical production

The maximum superoxide radical quenching effect of the extract at 100 μ g/ml was observed to be 84.67%. The lowest concentration of the extract exhibited 82.89% inhibition on SO radical generation, which possibly reveals its powerful antioxidant capacity (Figure 1). IC_{50} value was found to be 59.05 ± 0.103 μ g/ml. SO radicals damage biomolecules directly or indirectly by producing H_2O_2 , peroxy nitrate, and other singlet reactive oxygen species thereby causing aging and pathological conditions. Lipid peroxidation also was found during the SO radical generation. The inhibitory effect of *Annona muricata* has revealed its therapeutic efficacy [45].

Inhibition of Nitric oxide (NO) radical generation

Though nitric oxide is involved in host defense system and beneficial effects, over production of these radicals contribute to the pathogenesis of some inflammatory diseases. The percentage inhibition of NO radical by the 50% ethanolic extract was found to be concentration dependant manner with highest inhibition of 63.23% at 100 μ g/ml (Figure 2). IC_{50} value 70.12 μ g/ml is the concentration which can exert 50% inhibition on NO radical generation [46].

Figure 1: Superoxide radical scavenging activity of *Annona muricata*Figure 2: Nitric oxide radical scavenging activity of *Annona muricata*

Hydroxyl Radical scavenging activity

The extract exerted concentrated dependent scavenging activity against hydroxyl radical with maximum inhibition of 54.21% at 100 µg/ml concentration. IC_{50} value was found to be 134.21 ± 0.063 µg/ml in (Figure 3). Hydroxyl radical is widely accepted as the most toxic factor leading to tissue injury. It is produced when H_2O_2 , a metabolite existing in tissues, reacts with Fe^{2+} through Fenton reaction. The hydroxyl radical (OH) thus produced may attack the sugar of DNA base causing sugar fragmentation; base loss and DNA stand breakage. Hence hydroxyl radical system helps to understand the antiperoxidation mechanism of the compound. Hydroxyl radical promotes lipid peroxidation, denatures certain enzymes and nucleoproteins. Therefore removal of hydroxyl radicals is probably one of the most effective defense mechanisms of a living body against diseases [47].

Hydrogen peroxide (H_2O_2) scavenging activity

H_2O_2 scavenging effect of *Annona muricata* extract was found to be dose dependant with maximum inhibition of 86.4% at 100 µg/ml concentration. IC_{50} value of the H_2O_2 inhibition is 43.4 ± 0.102 µg/ml (Figure 4). Hydrogen peroxide is a weak oxidizing agent and tends to inactivate few enzymes in the cells through oxidation of essential thiol (-SH) group. It further crosses the cell membrane rapidly. Inside the cell, the H_2O_2 reacts with Fe^{2+} and Cu^{2+}

ions to form hydroxyl radicals and this leads to toxic effects. Hence, *Annona muricata* extract has been found to show promising effect against hydrogen peroxide [48].

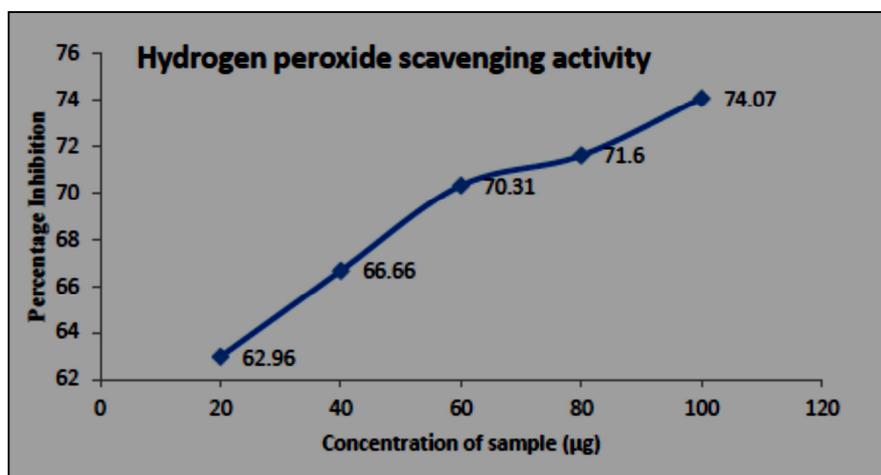


Figure 3: Hydrogen peroxide scavenging activity of *Annona muricata*

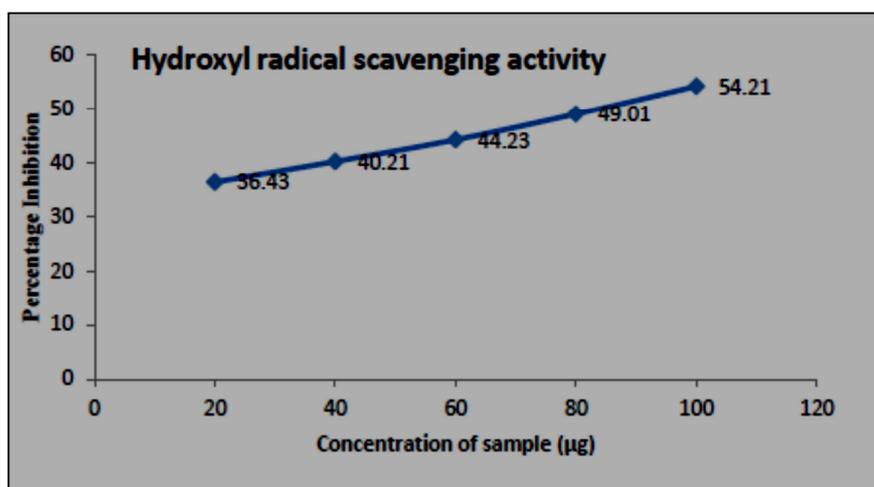


Figure 4: Superoxide radical scavenging activity of *Annona muricata*

CONCLUSION

In conclusion, 50% ethanolic extract prepared from the *Annona muricata* leaves contains phytochemicals such as tannins, flavanoids, ascorbic acid, carotenoids, alkaloids, and other nutrients that exhibit biological activities in various ailments. Several in vitro models were adopted to evaluate and estimate the enzymic and non-enzymic antioxidants in the extract. Further, total antioxidant capacity and free radical scavenging potentials were demonstrated to be effective against $SO^{\cdot-}$, $NO^{\cdot-}$, $OH^{\cdot-}$, H_2O_2 radical species. Experiment results showed that *Annona muricata* is good antioxidant reserves and exhibits dose dependent antioxidant activity which could be considered for further investigation towards therapeutic application.

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