

RESEARCH

Antioxidant and Anti-inflammatory Activities of Polysaccharides from the Coat of Fermented and Unfermented Sugar Apple (*Annona squamosa* L.) Seed

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(R-3148-2016)

ABSTRACT

This study explored the effect of fermentation on the antioxidant and anti-inflammatory activities of polysaccharides from seed coat of fermented and unfermented *Annona squamosa* seed. Fresh and ripe sugar apple fruits were collected from a tree in Ota-Efun, Osogbo, Nigeria (07° 32' 30.2496" N, 04° 31' 41.7036" E) and their identities were verified at IFE Herbarium, Department of Botany, Obafemi Awolowo, University, Ile-Ife, Nigeria. The seeds were collected and divided into two portions: fermented and unfermented. The coats of both the fermented and unfermented seeds were defatted with n-hexane separately. Polysaccharides were extracted from the defatted samples using cold and hot water procedure according to standard methods to give fermented seed coat polysaccharides and unfermented seed coat polysaccharides. Antioxidant and anti-inflammatory activities of the polysaccharides were investigated using standard methods. The reducing power, metal chelating, DPPH radical scavenging, inhibition of albumin denaturation, membrane stability potentials of the polysaccharides revealed the efficacy of the polysaccharides to take care of free radicals and maintain the integrity of the cell membrane and the fermented seed coat polysaccharide was the best. This study concluded that the polysaccharides from *A. squamosa* seed coat have great potential as antioxidant and anti-inflammatory agents to combat diseases related to oxidative stress, and fermentation enhanced the bioactivity of the polysaccharides.

KEYWORDS

Fermentation, reactive species, oxidative stress

INTRODUCTION

Reactive oxygen and nitrogen species (ROS and RNS) are known to be a contributing factor in the development of several human pathologies, including cancer, stroke, neurological diseases, and many other illnesses [1]. Antioxidants are thought to mitigate the negative effects of ROS and RNS, and so alleviate illnesses brought on by oxidative stress. Given that most diseases are mediated by ROS and that oxidative stressors have significantly increased due to rapid advancements in civilisation, industrialisation, and overpopulation, an antioxidant approach to disease management shows promise [2]. According to epidemiological studies, foods rich in antioxidants and scavengers may have a preventive impact against illnesses brought on by ROS. By strengthening the

body's natural antioxidant defences or taking dietary antioxidant supplements, many chronic diseases can be avoided and their course halted [3].

Polysaccharides are polymeric carbohydrates made up of long chains of monosaccharide units linked together by glycosidic bonds. When polysaccharides are hydrolysed, the constituent monosaccharides or oligosaccharides are released [4]. Numerous plant-derived polysaccharides have demonstrated strong antioxidant and anti-inflammatory properties, and could be investigated as potential antioxidants. Indeed, numerous scientific investigations have been made to identify natural compounds that may have antioxidant properties in order to enhance the healing of wounds during the repair process [5]. Multiple studies have also shown the value of natural antioxidants in defending the body against free radicals and lowering the

risk of illnesses including arthritis, cancer, and cardiovascular diseases as well as other age-related and chronic conditions [6,7].

Fermentation has been in practice around the world for centuries because of its beneficial values of preserving foods. Fermentation as a process also increases the nutritional values and shelf-life of foods and enhances the appearance, aroma, flavour, and other organoleptic characteristics of foods [8].

Annona squamosa (sugar apple), belonging to the family *Annonaceae*, is often found throughout India and grown in Thailand. It comes from Southern America and West Indies. It is usually cultivated in gardens because of its fruits and decorative appeal [9]. Various phytochemicals have been extracted from this plant but nothing has been done on the polysaccharides and this study seeks to provide information on that [10,11].

EXPERIMENTAL

Materials

Collection and identification of *Annona squamosa* fruits

Sugar apples that were mature, fresh, and juicy were harvested from an orchard at Ota-Efun, Olorunda Local Government, Osogbo, Osun State, Nigeria (07°32' 30.25" N, 04° 31' 41.70" E). The fruits were recognised and verified at the IFE Herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria where the specimen copy was lodged and voucher number (IFE-17805) was acquired.

Reagents and chemicals

The analytical grade chemicals and reagents utilised in this study were all bought from different sources.

Methods

Fermentation and processing of sugar apple seeds

The *A. squamosa* seeds were removed from the matured, ripe, soft fruits into clean containers and divided into two portions. A portion was fermented traditionally. The traditional fermentation method involved enclosing the seeds in banana leaves, storing them in a dark closet for seven days, and then drying them in an oven at 40°C [12]. The second portion was washed with clean water to remove the pulp and also oven dried at 40°C as unfermented seeds. Both fermented and unfermented seeds' cotyledons were removed from the seed coat and the seed coat was pulverised separately in a grinder. The powdered materials were termed fermented seed coat (FSC), and unfermented seed coat (USC) respectively. The powdered cotyledon samples were defatted with n-hexane, extracted with 80% (v/v) ethanol for 24 hr to remove organic compounds, and the residues were air-dried.

Preparation of polysaccharides

The preparation of polysaccharides of FSC and USC was done by using a protocol based on previously described

scientific methods [13,14] (Fig. 1). The precipitates were labelled as fermented seed coat polysaccharide (FSCP) and unfermented seed coat polysaccharide (USCP) respectively.

Quantification of total soluble sugar concentration of the polysaccharides

The quantification of the polysaccharides' total soluble sugar concentration was done according to the method described by Dubois *et al.* [15]. The standard calibration curve was prepared by pipetting varying concentrations of standard glucose solution. The amount of the polysaccharides was interpolated from the standard calibration curve and written as mg/g of sample.

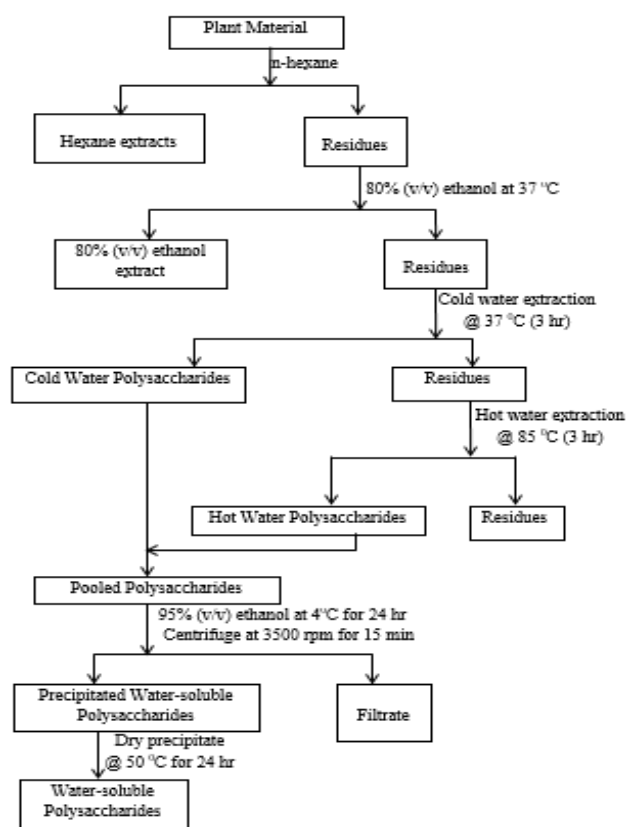


Fig. 1. Fractionation Scheme of *Annona squamosa* Fermented and Unfermented Seed Coat.

Estimation of total hexosamines contents

This was done according to the method of Blumenkrantz and Asboe-Hansen [16]. Glucosamine working standard was used. Absorbance was read on UV-Vis Spectrophotometer at 415 nm. Total hexosamines in the purified polysaccharides (FSCP and USCP) was interpolated from the standard calibration curve and written as µg/g of sample.

Estimation of the concentration of uronic acids

This was analysed according to m-hydroxydiphenyl reaction method by Meseguer *et al.* [17]. Standard

galacturonic acid solution was used. The absorbance of the sample and standard solutions were read at 520 nm. The total uronic acid concentration in the purified polysaccharides was interpolated from the standard calibration curve and the concentration was written as µg/g of sample.

HPLC analysis of the polysaccharides

HPLC was used to analyse the isolated polysaccharides' composition [18].

Assessment of total antioxidant capability of the polysaccharides

The total antioxidant capacity (TAC) of *A. squamosa* polysaccharides (FSCP and USCP) was spectrophotometrically evaluated using phosphomolybdenum assay as reported [19]. The TAC was calculated as ascorbic acid equivalents (mg AAE/g of the dry sample).

Assay of DPPH radical scavenging capacity

The radical scavenging activities of FSCP and USCP against DPPH radical were assessed by the protocol described by Bouhlali *et. al.* [20] with little modifications.

The inhibition of DPPH radical was calculated as a percentage from the expression:

$$\text{Percentage inhibition} = \frac{Abs_{\text{control}} - Abs_{\text{sample}}}{Abs_{\text{control}}} \times 100$$

where Abs_{control} = absorbance without extract; Abs_{sample} = absorbance of the extract or standard.

Assessment of reducing power

The procedure of Oyaizu [21] was used for the assay of reducing power of polysaccharides (fermented and unfermented) of cotyledon to reduce iron from Fe^{3+} to the form Fe^{2+} . The absorbance was plotted against corresponding concentrations of the polysaccharides and ascorbic acid standard. The absorbance is a direct measurement of the reducing potential of the polysaccharides and standard.

Assessment of metal chelating potential of the polysaccharides

The assay procedure followed the protocol described by Dinis *et. al.* [22]. To determine how well the sample chelated ferrous ion in comparison to the control, this formula was employed:

$$\text{Percentage chelating effect (\%)} = \frac{A_c - A_s}{A_c} \times 100$$

where A_c is absorbance of control, A_s is absorbance of sample

Assay of membrane stability

The bovine red blood cell (RBC) was prepared according to the earlier reported methods [23]. It was kept undisturbed at 4°C in the refrigerator to prevent lysis of the RBC.

The RBC membrane stability assay was carried out using a slightly modified procedure by Oyedapo *et. al.* [23].

Percentage membrane stability by extract/drug =

$$100 - \left[\frac{Abs_{\text{test}} - Abs_{\text{drug control}}}{Abs_{\text{control}} - Abs_{\text{drug control}}} \right] \times 100$$

Evaluation of albumin denaturation inhibition

This was evaluated as described by Chandra *et. al.* with slight modification [24]. Diclofenac was used as standard. The percentage inhibition (IP%) of albumin denaturation was calculated using the formula:

$$\text{Percentage Inhibition} = \frac{Abs_{\text{control}} - Abs_{\text{sample}}}{Abs_{\text{control}}} \times 100$$

Abs_{control} = absorbance without polysaccharide; Abs_{sample} = absorbance of the mixture with polysaccharide or standard.

Statistical analyses

The results of the analyses were written as Mean ± SEM, n = 3 or 5 readings. Except where otherwise indicated, Tukey multiple comparison test using GraphPad Prism 5 was used to make distinctions between the average values of the control and treated groups. Differences at p < 0.05 were considered to be significant.

RESULTS AND DISCUSSION

The fermented seed coat of sugar apple is quite different from the unfermented one. The seed coat had a dull or light brown colour (Plate 1a). It has a very pleasant, fruity characteristic like that of brewed red wine. The unfermented seed coat was dark brown (Plate 1b), having a smooth shining seed coat and a non-attracting characteristic smell.



Plate 1: (a) Fermented Seed Coat (b) Unfermented Seed Coat

The spectrophotometric quantification of the total hexosamines, total uronic acids, and total soluble sugar in the fermented and unfermented seed coat showed that the unfermented had higher quantities of those parameters which are significantly different (p < 0.05) in comparison with the fermented (Table 2), and all the increases were significant at p < 0.05. The increase in these parameters in unfermented could be a result of an increase in α-amylase and α-glucosidase activities which have been reported to accompany fermentation [28].

Monosaccharide and derived-sugars analyses of the two purified polysaccharides on HPLC produced results typical for pectic polysaccharides (Appendices 1 to 6 in supporting information) due to the high uronic acids

content of the polysaccharides. Ridley *et al.* [29] reported that uronic acid is found in hemicelluloses, gums, and pectin but the content of uronic acids is low in hemicelluloses and gum while uronic acid units are high in pectic acids polysaccharide chain. The presence of various sugars like rhamnose, glucose, fructose, arabinose, xylose, hexosamines and uronic acids in the polysaccharides was in agreement with the sugar composition of water-soluble pectin from the fruit of *Annona chemorila* Mill as reported by Brito *et al.* [30].

The total antioxidant capacity (TAC) of the purified polysaccharides showed that the fermented and unfermented coat polysaccharides of *A. squamosa* seed had antioxidant activities (Table 1). However, the fermented seed coat polysaccharides (FSCP) exhibited the highest TAC value. According to Wang *et al.* [31], the fermentation of oolong tea improved the conjugation of tea polysaccharides (TPS) and protein, and the polysaccharides isolated from the highly fermented tea had elevated antioxidant activity.

Table 1. Total Antioxidant Capacity.

Polysaccharides	(mg Ascorbic Acid/g of the dry sample)
FSCP	162.50 ± 2.50
USCP	117.500 ± 17.50 ^a

Each value represented Mean ± SEM of n = 3 replicates. The values with alphabet superscripts are statistically significant at p < 0.05, a compares FSCP with USCP.

FSCP = fermented seed coat polysaccharides, USCP = unfermented seed coat polysaccharides

Polysaccharides have protective properties for cells and can scavenge free radicals as antioxidants. They can also lessen oxidative cell damage. The antioxidant potential of polysaccharides from many sources, including plants, fungi, bacteria, algae, and animals, has been detailed in the literature, for either their capacity to scavenge free radicals or to guard against oxidative stress in living organisms [32]. The antioxidant activities of the two polysaccharides extracted in this study thus position them as major remediators of free radical-mediated diseases like diabetes, hypertension, cancer, etc.

In this study, a dose-dependent relationship was observed in the abilities of the fermented and unfermented coat polysaccharides of *A. squamosa* seed to scavenge DPPH radical. This implied that as the concentration increased, the activity increased. The activity was assessed by the ability of the extracted polysaccharides to reduce the free radical from DPPH which is detected by a decrease in absorbance of DPPH (purple) which is reduced to yellow-coloured diphenylpicrylhydrazine (DPPH-H). The quantity of an antioxidant required to achieve a 50% reduction in the initial DPPH concentration (IC₅₀) (Table 2) was used to measure the antioxidant potential of the extracted polysaccharides (Fig. 2). The results implied that the

purified polysaccharides (FSCP, USCP) were capable of neutralising free radicals and transferring enough hydrogen atoms or electrons. It's been demonstrated that polysaccharides could provide solitary electron or hydrogen atom to stop free radical chain reactions and accomplish radical scavenging activity [33,34].

Moreover, these findings are similar to previous observations of the abilities of polysaccharides from plant sources to scavenge DPPH radical. Khaskheli *et al.* [35] reported the ability of polysaccharides from fresh and pickled *Auricularia auricular* seed to scavenge DPPH radicals. Luo *et al.* [36] study on water-soluble polysaccharides showed that the carbonyl groups (C=O of the glycosidic bond) from these polysaccharides can function as a quencher of free radicals. To this end, FSCP, and USCP can act as natural antioxidants and replace synthetic antioxidants in food preparation.

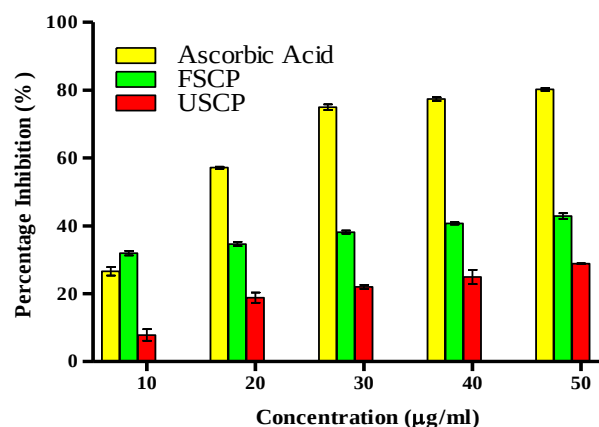


Fig. 2. DPPH Radical Scavenging Activity of the Polysaccharides and Standard Ascorbic Acid.

Each value represented Mean ± SEM of n = 3 replicates
FSCP = fermented seed coat polysaccharides, USCP = unfermented seed coat polysaccharides

Table 2. IC₅₀ Values for DPPH Radical Scavenging Potential of the Polysaccharides.

Sample	IC ₅₀ (µg/ml)
Ascorbic Acid	3.231 ± 0.210
FSCP	79.420 ± 9.236 ^a
USCP	105.645 ± 2.822 ^a

Each value represented Mean ± SEM of n = 3 replicates. The values with alphabet superscripts are statistically significant at p < 0.05, a compares Ascorbic acid with the rest, b compares FSCP with the rest, and c compares USCP with the rest.

FSCP = fermented seed coat polysaccharides, USCP = unfermented seed coat polysaccharides

The assessment of the ability of the purified polysaccharides to reduce Fe³⁺ to Fe²⁺ using ascorbic acid as the standard drug was carried out. The reducing strength of the polysaccharides and standard ascorbic acid followed

a concentration-dependent pattern (Fig. 3). All the extracted polysaccharides exhibited reducing power at various degrees and the fermented cotyledon polysaccharides had the highest reducing strength. An indicator of a substance's potential as an antioxidant could be its ability to reduce other substances [37]. The purified polysaccharides could be said to behave as electron donors (reductants/antioxidants) and reacted with radicals, converted them to non-radical products, and terminated radicals chain reaction. Sun *et al.* [38] also demonstrated the strong reducing power of four tea polysaccharides (TPS0, TPS1, TPS2, TPS3).

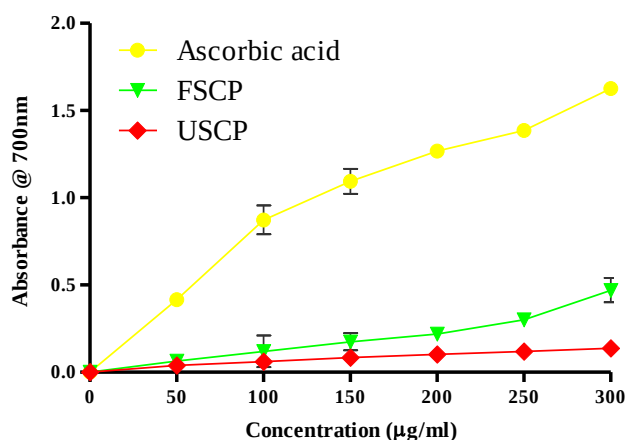


Fig. 3. Reducing Power of the Polysaccharides and Standard Ascorbic Acid.

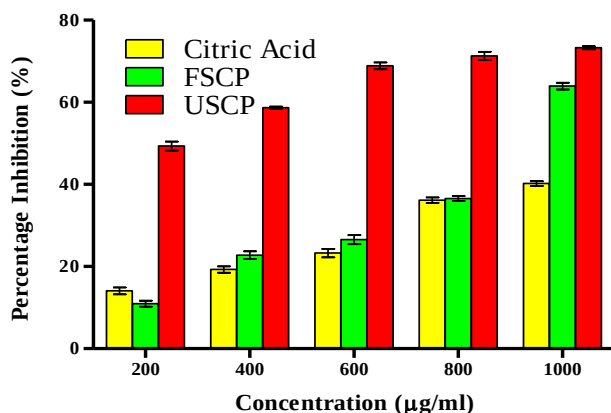


Fig. 4. Metal Chelating Ability of the Polysaccharides and Standard Citric Acid.

Each value represented Mean $\pm$ SEM of $n = 3$ replicates
FSCP = fermented seed coat polysaccharides, USCP = unfermented seed coat polysaccharides

The competitive production of the ferrozine- Fe^{2+} complex, which exhibits a strong band in the range of 400 to 650 nm with maximum absorption at 562 nm, was where the chelating activity of the isolated polysaccharides was detected [39]. The ferrozine- Fe^{2+} complex formation occurs rapidly (< 2 min) but a notable decrease in the complex concentration was observed with the introduction of the

polysaccharides. The metal chelating potential of the fermented and unfermented coat polysaccharides of *Annona squamosa* seed significantly ($p < 0.05$) increased with increased concentration of the polysaccharides (concentration-dependent) and was higher than that of citric acid standard (Fig. 4). It is thought that polysaccharides have antioxidant mechanisms that allowed them to chelate metal ions, which are necessary for the production of free radicals, or to trap free radicals created in lipid peroxidation. The reduction of hydroxyl radical production by the chelating effect has generally been shown to boost the antioxidant effects of polysaccharides derived from marine algae [40]. Barahona *et al.* [41] reported the concentration-dependent metal chelating activity of the polysaccharide from *Lesonia vadosa*.

Each value represented Mean $\pm$ SEM of $n = 3$ replicates
FSCP = fermented seed coat polysaccharides, USCP = unfermented seed coat polysaccharides

Vascular tissues' complex biochemical reaction to damaging stimuli is inflammation. Additionally, it represents an effort on the part of organisms to defend themselves by getting rid of harmful stimuli and starting the recovery process [42]. In this study, the extracted polysaccharides protected the bovine erythrocyte membrane against both heat- and hypotonic-induced lyses at concentrations of 0-350 $\mu\text{g/ml}$. The fermented and unfermented coat polysaccharides of *A. squamosa* seed exhibited a concentration-dependent inhibition (monophasic) to effect membrane stabilization whereas the standard ibuprofen exhibited a biphasic mode of action (not concentration-dependent) (Fig. 5). The extracted polysaccharides exhibited better membrane stabilising activity than the ibuprofen standard used at the highest concentration used (350 $\mu\text{g/ml}$) and the fermented seed coat polysaccharide (FSCP) had the best membrane stabilizing power among the extracted polysaccharides. Bovine erythrocyte membranes are analogous to the lysosomal membrane [43], thus the inhibition of hypotonic-induced haemolysis was employed in assessing the anti-inflammatory activities of the extracted polysaccharides [44].

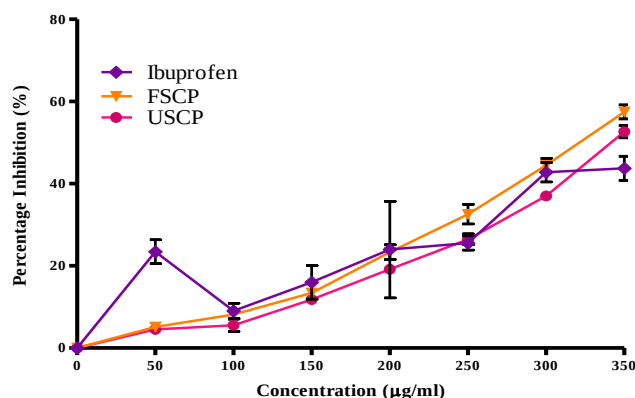


Fig. 5. Membrane Stability Profile of the Polysaccharides and Ibuprofen (Standard).

Each value represented Mean $\pm$ SEM of n = 3 replicates
FSCP = fermented seed coat polysaccharides, USCP = unfermented seed coat polysaccharides

It is widely known that the formation of auto-antigens by tissue protein denaturation contributes to the development of inflammatory and arthritic disorders. The propensity of plant matter to prevent protein denaturation may be useful in treating inflammatory disorders. According to the findings of this investigation, protein denaturation was inhibited by pure polysaccharides in a concentration-dependent manner (**Fig. 6**) with the fermented cotyledon polysaccharides (FCP) as the best inhibitor of albumin denaturation. Elrayess *et al.* [45] also reported that albumin denaturation inhibition was dose-dependent with some inflammatory drugs (1,2,4-triazole Schiff bases scaffold with aryl and heteroaryl, etc.).

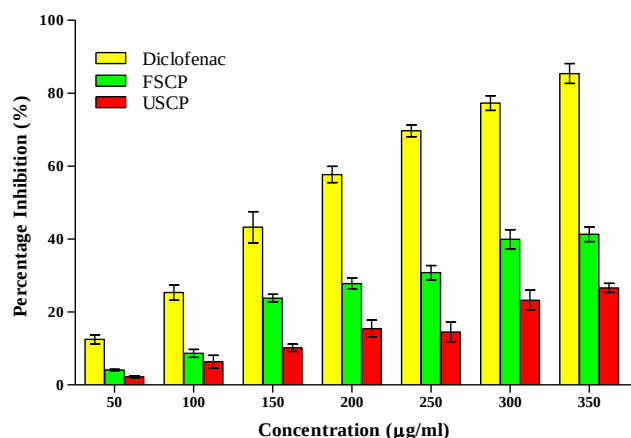


Fig. 6. Albumin Denaturation Inhibition Potential of the Polysaccharides and Diclofenac (Standard).

Each value represented Mean $\pm$ SEM of n = 3 replicates
FSCP = fermented seed coat polysaccharides, USCP = unfermented seed coat polysaccharides

CONCLUSION

Although data on the bioactivities of polysaccharides from various plants are available, there is a dearth of data on the effects of fermentation on the bioactivity of polysaccharides. This study reported on the bioactivity attributes of extracted polysaccharides from fermented and unfermented *Annona squamosa* seed coat. The results of this investigation demonstrated scientifically the superior anti-inflammatory and antioxidant properties of the polysaccharides from fermented and unfermented coats of *A. squamosa* seed. The polysaccharides could be recommended as great candidates as antioxidant agents to take care of oxidative stress and its accompanying metabolic diseases. However, fermentation enhanced the activities of the polysaccharides in both the cotyledon and the seed coat. Further research will be conducted on establishing the type of glycosidic bonds and applications

of the antioxidants and anti-inflammatory strength of the polysaccharides.

ACKNOWLEDGEMENTS

The funding for this study came from institution-based grants rather than any specific grants from funding organizations in the public, private, or not-for-profit sectors.

CONFLICTS OF INTEREST

There are no conflicts to declare.

SUPPORTING INFORMATION

Supporting pieces of information are available online at the journal website.

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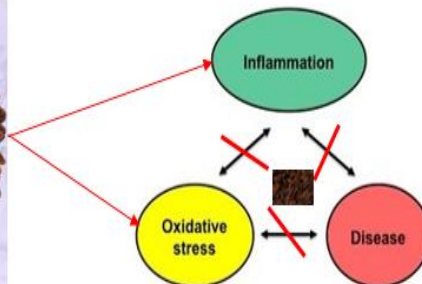


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GRAPHICAL ABSTRACT



Ammonia squamosa Seed
Coat Polysaccharides



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