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Phytochemical and Antioxidant Screening of Seed Extracts of Kenyan Custard Apple (*Annona squamosa*)

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ABSTRACT: Medicinal plants have been the mainstay of traditional herbal medicine amongst rural dwellers worldwide since antiquity for their preventive and curative properties. Investigation of plants for phytochemicals and medicinal activities is imperative for the discovery of prospective novel molecules for medicinal use. This current analysis was aimed at screening the seed extracts of *Annona squamosa* for the presence of phytochemicals and their antioxidant activities. Extraction of the active components was through organic solvents; methanol, dichloromethane and methanol: dichloromethane blend in Soxhlet extractor and concentrated in a rotary evaporator. Investigation of phytochemicals was through standard qualitative screening procedures while antioxidant activities of the extract were evaluated through the radical scavenging effect of DPPH, H_2O_2 and the FRAP assay. Methanol was the most effective solvent in capturing phytochemicals including tannins, alkaloids, flavonoids, fixed oils and phenols. Dichloromethane captured only alkaloids and fixed oils. The antioxidant data obtained was assayed against that of the standard (Ascorbic acid). The methanolic extract was found to be the most potent extract with highest antioxidant activities followed by methanol: dichloromethane blend while dichloromethane portrayed the least potency. The highest DPPH radical scavenging was at 500mg/ml, while for H_2O_2 was at 1mg/ml while the highest FRAP activity was at 1mg/ml. Methanolic extracts were the most potent while dichloromethane extracts showed the least DPPH and H2O2 radical scavenging activities. Phenols appear have significantly contributed towards the antioxidant activity of the methanolic extracts and the alkaloids in dichloromethane extracts. Most of the inedible parts of commonly consumed fruits have not been studied for their antioxidant activity but the current assay demonstrates that seeds may be a promising source of antioxidants. This study shows that the seed extracts of *Annona squamosa* may be utilised therapeutically in the management of oxidative stress related disorders.

Keywords*:Annona squamosa*, Phytochemical, Antioxidant, Radical Scavenging, diphenyl-picrylhydrazyl (DPPH), Hydrogen Peroxide (H_2O_2) and Ferrous Reducing Antioxidant Power (FRAP)

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I. INTRODUCTION

Free radicals which play twin role of being both harmful and useful to natural systems include Reactive oxygen species (ROS) and Reactive nitrogen species(RNS) (Rudolf, 2001).These highly reactive molecules are indicated in various disorders in the human body in addition to having a role in lipids autoxidation which leads to spoilage of foodstuff (Matthaus, 2002). Besides, ROS and RNS interact with each other leading to nitrosative stress. Reactive nitrogen species (RNS) are usually propagated in the cells in response to stressors or during respiration (Pauly *et al*., 2006). The harmful effect of free ROS and RNS radicals causing potential biological damage is termed oxidative stress and nitrosative stress, respectively (Kovacic *et al*,.2001; Valko *et al*.,2001).This is evident in biological systems when there is either an excessive production of ROS/RNS and/or a deficiency of enzymatic and non-enzymatic antioxidants. The redox stress/oxidative stress is a complex process. Its impact on the organism depends on the type of oxidant, on the site and intensity of its production, on the composition and activities of various antioxidants, and on the ability of repair systems (Ďuračková, 2007).

Once systemic manifestation of highly reactive species exceeds natural mechanisms' capacity to neutralise them, subsequent harm is reflected by oxidative stress (Chandra *et al.*, 2000). When the levels of unstable molecules are raised, different compounds in the body are oxidised causing neurodegenerative and cardiovascular disorders (Nijs *et al*., 2006; Haider *et al*., 2011; Patel *et al*., 2011). Depending on the type and level of ROS and RNS, duration of exposure, antioxidant status of tissues, exposure to free radicals and their metabolites leads to different responses; increased proliferation, interrupted cell cycle, apoptosis, or necrosis (Halliwell, 2008).

To counteract the harmful effects taking place in the cell due to oxidative stress, cellular system has evolved itself with some strategies for prevention of damage, repair mechanism to alleviate the oxidative damages, physical protection mechanism against damage, and the finally the most significant is the antioxidant defence mechanisms (Ďuračková, 2010). Natural enzymatic alongside non-enzymatic antioxidant defences in the human body are some of the complex systems that counteract the damaging effects of oxidants including reactive species (Mantovani, Giovanni, *et al*., 2003). Antioxidants prevent or interrupt the reaction leading to oxidation of other compounds by obstructing the commencement of oxidising pathways (Lü *et al*., 2010). These compounds cushion the cells from reacting with the reactive species (ROS/RNS). They slow down the progression of various disorders and peroxidation of lipids (Gülçin *et al*., 2010).Naturally occurring antioxidants represent a promising method to shield the cells from the damage on their components caused by free radicals (Roy *et al*.,2011).Ample intake of dietary supplements with antioxidant properties can enhance protection against free radicals (Fang *et al.*, 2002). Also, antioxidants are used as food additives to enhance colour, taste, and smell and enhance shelf life (Nakatani and Nobuji, 1992).

The routine synthetic antioxidant agents include Propyl gallate, Butylated Hydroxyanisole and Butylated hydroxytoluene (Koksal and Gülçin, 2008).Nevertheless, many conventional antioxidants have been shown to possess lethal and/or carcinogenic effects, and this finding has promoted research into the properties of naturally occurring antioxidants (Fejes *et al*., 2000). Subsequent studies have established that conventional antioxidants may be harmful to some extent and as a result, restrictions have been imposed on their use. Scientists have consequently dedicated their research on herbal antioxidants (Kulisic *et al*., 2004). Antioxidants, especially polyphenols which are plant-derived have gained significant applications due to their would-be health benefits. Phytochemical molecules act as antioxidants due to their ability to, act as metal chelators hydrogen donors and also as singlet oxygen quenchers (Huang *et al*., 2003, Sikwese and Duodu, 2007).

Plant-derived antioxidants are regarded as the novel complementary and alternative antioxidants in the management of oxidative stress-related disorders. Studies have shown that antioxidant effect produced by carotenoids, beta- carotene and lycopene is by stabilising the free radicals and prevent cellular damage (Arulselvan *et al*., 2012). A lot of considerations have been made of secondary metabolites as well as plant-derived molecules capable of providing stable antioxidant supply. The synergistic and cumulative effects of plant food are accountable for their elevated antioxidant properties (Pellegrin *et al*., 2006). One of the most abundant natural flavonoids present in a large number of fruits and vegetables is quercetin (3,5,7,3′,4′, Penta-hydroxy flavone) which prevents oxidative injury and cell death by scavenging free radicals, donating hydrogen compound, quenching singlet oxygen, and preventing lipid peroxidation or chelating metal ions (Lee *et al*.,2010)

Annona squamosa is a shrub or a branchy tree of *Annonaceae* family which bears sugar-apples fruits. Globally Sugar-apple is cultivated in plains of tropical climates including Africa, Australia, Indonesia, North, Central and South America *Annona squamosa* Vernacular names include English Custard apple, sugar- apple, sweetsop and Hindi –Sitafal while in Kenya it is referred to as Matomoko (Pareek *et al*., 2011). Literature from several studies reveals that every part of *A. squamosa* possesses medicinal property (Muthu *et al*., 2006).Seed extracts are very toxic and have the insecticidal activity they also contain saponins that haemolyses erythrocytes and is poisonous to fish (Saluja and Santani, 1990). The seeds are also said to be abortifacient and used to destroy lice in hair in Yunani medicine. Seed yields oil and resin which acts as a detergent and their powder is mixed with gram-flour in hair wash. Seeds are a powerful irritant of the conjunctiva and produce ulcers in the eye.Two acetogenins, annoreticuin and isoannoreticuin, isolated from the leaves, were found to be selectively cytotoxic to certain human tumours. The leaves and stems also gave alkaloids dopamine, salsolinol and coclaurine (Saha and Rajsekhar, 2011)

Table I: Taxonomy

Kingdom	Plantae.
Order	Magnoliales
Family	Annonaceae
Genus	*Annona*
Species	*squamosa*

II:MATERIALS AND METHOD

Area of study, collection and preparation of plant materials

The study was conducted at Kenyatta University is located 19.3 km from Nairobi cityoff Nairobi-Thika Highway.The ripe fruits of *Annona squamosa* were obtained from local farms at Mwea in Wang'uru Sub-county, Kirinyaga County of Kenya with the help of a local herbalist. The seeds were then separated from the pulp and dried up in a shady area at room temperature to guarantee total desiccation and to prevent the breakdown of pharmacological elements

Extraction

For each sample, 150 mg of the powdered material was leached separately, in 300 ml of the different solvents that included methanol, dichloromethane and blend of DCM and MeOH in a ratio of 1:1 for 48 hours to allow the bioactive compounds to dissolve. The concoction was allowed to settle then filtered using Whatman's filter paper (185 mm) followed by steeping of the residual in the solvent for 48 hours. The procedure was repeated two times and then concentrated at low pressure using a rotary evaporator at 45ºC. Finally, the extracts were preserved in sterile conditions in tightly sealed bottles at 5ºC for future use in the antioxidant assay.

In vitro DPPH radical scavenging assay

The organic seed extracts were tested for the DPPH free radical scavenging activity using a method defined by Ngonda, (2013). A solution of 0.135 mM of DPPH radical with methanol was prepared and one millilitre of this solution mixed with one millilitre of each extract in the range of 0.03 to 1.00 mg/ml. The L-ascorbic acid was used as the reference in the same concentration range as that of extracts. The blend was then vortexed exhaustively and then put in a dark place at 23 °C for 30 minutes.

The solution's absorbance was measured spectrophotometrically at 517 nm. The blank used on the spectrophotometer was methanol while a preparation of DPPH and methanol was used as a reference. Any change in absorbance was compared to that of the reference. The ability to mop up the radicals by the extracts was then considered by use of the following equation;

$$\text{DPPH scavenging activity (\%)} = \frac{\text{Ac} - \text{As}}{\text{Ac}} \times 100$$

Where; Ac = Absorbance of DPPH + methanol
As = Absorbance of DPPH radical + test (sample or standard).

In vitro Hydrogen peroxide (H_2O_2) scavenging activity

The method described by Ngonda (2013), was used to determine hydrogen peroxide (H_2O_2) free radical scavenging activity of *A. squamosa* seed extracts. A solution of 40 mM of H_2O_2 was prepared in phosphate buffer pH 7.4 and one millilitre of the mixture added to 1.0 ml of each extract at a concentration range of 0.031 to 1.00 mg/ml. The L-ascorbic acid was used as the reference at the same concentration with the extracts. The absorbance of the extract and H_2O_2 was taken at 560 nm using a spectrophotometer. Phosphate buffer solution devoid of $H_2O_2$was used as the blank. The percentage H_2O_2 scavenging activities of the extracts, as well as that of the reference, were calculated using the formula below;

$$\text{Hydrogen peroxide scavenging activity (\%)} = \frac{\text{Ac} - \text{As}}{\text{Ac}} \times 100$$

Where; Ac = Absorbance of H_2O_2 + phosphate buffer
As = Absorbance of H_2O_2 + test (sample or standard).

In vitro reducing power assay

The Ferric Reducing Antioxidant Power of the organic seed extracts of *Annona squamosa* were evaluated using methodologies as described by Oyaizu (1986), and Dehpour *et al*. (2009). Different extracts dilutions in a range of 0.0625 to 1.00 mg/ml in one millilitre of distilled water were added to 2.5 ml phosphate

buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide [$K_3Fe(CN)_6$] (1%w/v). The mixture was then put in an incubator for 20 minutes at 50°C. A solution of Trichloroacetic acid (10%w/v) (2.5 ml) was added to halt the reaction. Centrifugation was followed at 3000 rpm for 10 minutes and 2.5 ml of the supernatant mixed with 2.5 ml of distilled water together with 0.5 ml (0.1%) $FeCl_3$. The absorbance of the mixture was measured at 700 nm using a spectrophotometer. Ascorbic acid of equal concentrations to those of extracts was used as the reference. Phosphate buffer was used as the blank solution at pH of 6.6. All the tests were carried out in triplicates for reproducibility.

Data Management and Statistical Analysis

Raw data on scavenging effects and reduction power were tabulated on Ms Excel spread sheets and organized for statistical analysis. The data was then exported to Minitab statistical software version 17.0 (Minitab Inc., Pennsylvania) for analysis. The data was subjected to descriptive statistics and the results expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to determine whether there were significant differences between the means of different groups. This was followed by Tukey's post hoc tests for pairwise separation and comparison of means. The values of $p \leq 0.05$ were considered significant. The data was presented in tables

II. RESULTS

Table II: *In vitro* DPPH scavenging activities

	Percentage DPPPH scavenging activity			
Concentration	**Ascorbic Acid**	**MeOH extract**	**DCM Extract**	**DCM: MeOH Extract**
7.8125µg/ml	35.85±0.52[a]	26.07±0.52[b]	12.01±0.69[d]	16.98±0.75[c]
31.25µg/ml	41.57±0.60[a]	33.28±0.52[b]	19.04±0.62[d]	24.70±0.52[c]
62.5 µg/ml	52.54±0.36[a]	42.02±0.69[b]	26.13±0.60[d]	35.51±0.62[c]
125 µg/ml	67.07±0.52[a]	57.86±0.60[b]	31.90±0.79[d]	47.97±0.43[c]
250 µg/ml	81.48±0.62[a]	70.79±0.10[b]	45.17±0.26[d]	64.84±0.36[c]
500 µg/ml	89.65±0.36[a]	82.62±0.36[b]	62.09±0.45[d]	75.36±0.95[c]
IC_{50}	7.30	64.97	145.25	105.25

Values expressed as the mean ± standard deviation for three replicates. Values with the same superscript letter are not significantly different along the row by one-way ANOVA followed by Tukey's post hoc test ($p>0.05$).

Table III: *In vitro* Hydrogen Peroxide Scavenging Activities

	Percentage H_2O_2 scavenging activity			
Concentration	**Ascorbic acid**	**MeOH extract**	**DCM extract**	**DCM: MeOH Extract**
0.0625mg/ml	39.27±0.72[a]	30.30±0.68[b]	21.34±0.67[d]	26.20±0.58[c]
0.125mg/ml	45.46±0.57[a]	38.51±0.77[b]	29.04±0.67[d]	34.34±0.48[b]
0.25mg/ml	54.10±0.85[a]	50.01±0.83[b]	38.45±0.87[d]	44.38±0.85[c]
0.5mg/ml	74.50±0.93[a]	67.24±0.96[b]	53.03±0.19[d]	59.91±0.22[c]
1mg/ml	85.86±0.77[a]	77.72±0.29[b]	64.52±0.85[d]	71.97±0.50[c]
IC_{50}	0.05	0.10	0.18	0.14

Values expressed as the mean ± standard deviation for three replicates. Values with the same superscript letter are not significantly different along the row by one-way ANOVA followed by Tukey's post hoc test ($p>0.05$).

Table IV: *In vitro* Ferric Reducing Antioxidant Power (FRAP)

	Absorbance at 700nm			
Concentration	**Ascorbic Acid**	**MeOH extract**	**DCM extract**	**MeOH: DCM extract**
0.0625mg/ml	1.073±0.001[a]	0.911±0.002[b]	0.637±0.001[d]	0.770±0.002[c]
0.125mg/ml	1.403±0.001[a]	1.057±0.001[b]	0.757±0.002[d]	0.897±0.001[c]
0.25mg/ml	1.474±0.002[a]	1.239±0.002[b]	0.825±0.002[d]	0.955±0.002[c]
0.5mg/ml	1.590±0.001[a]	1.350±0.002[b]	0.907±0.001[d]	1.073±0.001[c]
1mg/ml	1.744±0.002[a]	1.431±0.001[b]	1.063±0.004[d]	1.206±0.003[c]

Values expressed as the mean ± standard deviation for three replicates. Values with the same superscript letter are not significantly different along the row by one-way ANOVA followed by Tukey's post hoc test ($p>0.05$).

III. DISCUSSION

Continual interactions with the environment, consumption of xenobiotics and normal biochemical reactions have the consequence of the generation of reactive nitrogen species (RNS) and reactive oxygen species (ROS) (Nimse and Pal, 2015). These reactive species are responsible for different disease conditions in the oxidative stress (Kim and Byzova, 2014). Enhancement of cellular defences can effectively neutralise oxidative stress by the formation of antioxidants (Sies, 1997). Some molecules function as antioxidants within a living organism by elevating the intensities of internal antioxidant defence (Lü et al., 2010). One of the leading causes of pathological conditions is oxidative stress which can be managed by the use of antioxidants. Synthetic antioxidants are associated with toxic and/or mutagenic effects (Kadhum *et al*., 2011). This has led to a shift of attention in the direction of the naturally occurring compounds with antioxidants activities (Yao *et al*., 2004).

Antioxidants are the compounds responsible for the protection of living organism from the damage caused by the abnormal production of reactive oxygen species concomitant lipid peroxidation, protein damages and others including DNA strand breaking. Antioxidants are widely believed to be an essential line of protection from oxidative damage, which has been indicated in a range of degenerative disorders (Kehrer, 2008). Biological antioxidants are generally categorized into inhibitors of radical formation, such as Fenton reaction inhibitor; free radical quenching agents, such as alpha- tocopherol (Vitamins) and enzymes, such as superoxide dismutase. On the other hand, phenolic are good sources antioxidant molecules because of their good redox potentials and stability of the Aryloxy radical (Ho *et al*., 1994).Ethnomedical literature has revealed a large number of plants like *Annona squamosa* which can be used against diabetes, in which the free radicals and ROS plays an important role in the management of oxidative stress related disorders.

Antioxidant activities ought not to be established based on a single antioxidant experimental model. In practice, a number of *in vitro* examination techniques are taken into consideration for assessing antioxidant activities. Moreover, antioxidant test models are not same and for this reason, it is hard to compare one method to another (Badarinath *et al*. 2010). In general, *in vitro*, antioxidant experiments by means of free radical scavengers are relatively direct to carry out (Alam *et al*., 2013). This study used three models and it showed that the phytochemicals and antioxidant activity of different solvent extracts of A.squamosa seed extracts were effective in radical scavenging. The methanolic extract had the highest DPPH and H2O2 scavenging activity of >82% and >77% respectively and ferrous reducing power. In the current investigation, seed extracts of *A.squamosa* exhibited significant scavenging effects on DPPH and H_2O_2 radicals.it was also observed that the seed extracts contained a high level of flavonoids and phenols that are indicated for their antioxidant potential. The higher half maximal inhibitory concentration (IC50) as matched to that of reference (Ascorbic acid) suggested that the crude extracts were not as effective as the standard in free radical scavenging.

IV. CONCLUSION

The study showed that the organic seed extracts of *A. squamosa* contain phytochemical associated with antioxidants effects.The study revealed that the seeds of *A. squamosa* contain a considerable high level of phenolic flavonoid compoundthat was believed to be the major contributor to their antioxidant activities. Therefore, it is not surprising that *A. squamosa* has increasingly found immense use in Kirinyaga County as a remedy against oxidative stress-related disorders.

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