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**Secondary metabolites and *In-vitro* Antioxidant Properties of the Methanol Extracts of Fruits of *Annona senegalensis*, *Curcubita pepo L, Cucumi melo inodorous* and *Sarcocephalus latifoliu*s.**

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**Abstract**

*The Methanolic extracts of fruits of Annona senegalensis, Curcubita pepoL., Cucumi melo inodorous and Sarcocephalus latifolius were screened for their phytochemical constituents and in-vitro antioxidant properties using standard methods. Results revealed the presence of alkaloids, cardiac glycosides, tannins, saponins, flavoniods and phenols in all the fruits studied. Quantitative phytochemical determination of the fruits showed that alkaloid content of the extract ranged between 8.89 ± 0.30 to 193.47± 0.30mg/g while tannin content was in the range of 89.45 ± 1.23 mg/g and 392.71 ± 0.23 mg/g, saponins between10.50 ± 0.50 mg/g and 97.31 ± 0.61 mg/g, total phenols between 75.25 ± 1.85 and 374.52 mg/g, and total flavonoid content between 5.24 ± 0.44 mg/g and 57.00± 10.50mg/g. All the fruits studied showed antioxidant activity in a concentration dependent manner. Annona senegalensis extract showed the highest 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity (38.43-77.15%) while Curcubita pepoL showed the least DPPH scavenging ability (1.00- 3.8%) compared to other extracts. The highest reducing power was also observed in Annona senegalensis extract compared to other fruits extract. These fruits can serve as good sources of antioxidants which can be used in the management of degenerative diseases.*

Key words: DPPH Free radical, reductive power, *Annona senegalensis*, *Curcubita pepo L, Cucumis melo inodorous,* *Sarcocephalus latifolius*

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**Introduction**

Reactive oxygen species (ROS) including superoxide radicals, hydroxyl radicals and non-radicals such as hydrogen peroxide and hypochloride ions are often generated as byproducts of biological processes or from exogenous factors (Kikuzaki *et al*., 1994).An imbalance between these reactive oxygen species and endogenous antioxidant system result in oxidative stress. Oxidative stress has been implicated in the etiology of several human diseases including inflammation, metabolic disorders, cellular aging and atherosclerosis, heart disease, stroke, diabetes mellitus, cancer, malaria, rheumatoid arthritis and HIV/AIDS (Alho and Leinonen, 1999; Olukemi *et al.,* 2005). Antioxidants are molecules which when present at low concentrations compared with those of an oxidizable substrate are capable of neutralizing the harmful effects of ROS through the endogenous enzymatic defense system such as the superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) in human system (Aliyu *et al*., 2009). .However, with the increasing damaging environmental factors such as cigarette smoke, UV rays, radiation and toxic chemicals, the endogenous defense system is weakened resulting to an imbalance in the equilibrium status of pro-oxidant/antioxidants reactions in living systems.

A broad spectrum of medicinal plants, vegetables and fruits have been shown to contain antioxidants which are capable of serving as preventive intervention for free radical mediated cellular damage and diseases ( Zahin *et al.*, 2009; Hamzah *et al.,* 2013). There is also substantial amount of evidence showing an association between individuals who have a diet rich in fresh fruits and the decreased risk of cardiovascular diseases and certain forms of cancer (Hertog *et al.,* 1997, Salah *et al*., 1995). Currently, immense interest is in the use of natural antioxidants in the prevention and possible treatment of many degenerative diseases because of the adverse effects associated with synthetic antioxidants and drugs.

Although different parts of *Annona senegalensis*, *Curcubita pepo L, Cucumis melo inodorous and Sarcocepahlus latifoliu*s have been shown to possess various medicinal effects, only scanty information has been reported on the antioxidant properties of their fruits. .For Instance leaves and root extracts of *Annona sengenalensis* have been shown to possess antioxidant,, hepato-protective (Ajiboye *et al*., 2010), antimalarial (Ajaiyeoba *et al.*, 2006) and anti convulsant effects (Konate *et al*., 2012); while the methanolic seed extract *of Cucumis melo* has been reported to possess significant antioxidant, anti-inflammatory and analgesic properties (Arora *et al.* 2011). Also pharmacological tests have shown that *Cucubita pepo* leaves possess antibacterial, antiviral, anti-inflammatory and analgesic effects (Wang *et al*., 2007). The leaves of *S.latifolius* have been shown to paralyse *Trichostrongylus columbriformis* larvae in a concentration dependent manner (Asuzu and Njoku, 1996) and lower blood glucose levels in normal and alloxan induced rats (Gidado et al. 2005). This study was thus carried out to elucidate the phytochemical and *in-vitro* antioxidant properties of methanolic extracts of fruits of *Anonna senegalensis, Cucumis melo, Curcubita pepo* and *Sarcocephalus latifolius.*

**Materials and Methods**

**Collection and Identification of Fruit Species**

Fresh fruits of *Sarcocephalus lafifolius (OG), Annona Senegalensis (AS), Cucumis melo(SM)*  and *Curcubita pepo* used for the present study were collected at different locations in Minna, Nigeria between the month of April and September, 2013. They were identified by a Botanist, Mr. Muhammad C. Dagana of Biological Science Department, Federal University of Technology Minna, Nigeria.

**Sample preparation and Extraction**

Fresh fruits of *Sarcocephalus lafifolius (OG), Annona Senegalensis (AS), Cucumis melo(SM)*  and *Curcubita pepo* were removed from the pod and separated from the seed. They were blended into a paste and extraction was done according to the method of Ogbadoyi *et al.,* (2011). Fifty grammes of the sample were weighed into a round bottom flask to which 400 ml of methanol was added. A reflux condenser was then used to extract crude compounds at 64ºC for 2h after which it was filtered .The filtrate was evaporated using a rotary evaporator. The extract was collected and stored in the freezer until required for use.

**Qualitative Phytochemical Screening**

The extracts were screened for qualitative phytochemical constituents using standard methods (Sofowora, 1993).

**Quantitative Determination of the Chemical Constituents in Samples**

**Total flavonoid determination**

Aluminium chloride colorimetric method was used for flavonoid determination Chang *et al.* (2002). Each fruit extracts (0.5 ml) was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M sodium acetate and 2.8 ml of distilled water. This was left at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with a double beam Perkin Elmer (Waltham, MA, USA) UV-visible spectrophotometer. The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100 µg ml-1 in methanol.

**Determination of total phenol content**

The total phenol content of the extracts was determined using the method reported by Singleton *et al.*, (1999). Appropriate dilutions of the extracts (0.5ml) was oxidized with 2.5mL of 10% Folin–Ciocalteau’s reagent (v/v) and neutralized by 2.0 ml of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at 45°C and the absorbance measured at 765 nm in the spectrophotometer. The total phenol content was subsequently calculated using gallic acid as standard.

**Determination of alkaloids**

Exactly 0.5 g of the sample was dissolved in 5ml of 96% ethanol -20% H2 SO4 (1:1). Then 1 ml of the filtrate was added to 5 ml of 60% tetraoxosulphate (VI) and allowed to stand for 5 min. Then, 5 ml of 0.5% formaldehyde was added and allowed to stand for 3 h. The reading was then taken at absorbance of 565 nm (Oloyed, 2005). Vincristine was used as the standard for the preparation of standard curve.

**Saponin Determination**

Exactly 0.5 g of the extract was added to 20 ml of 1NHCl and boiled for 4 h. After cooling it was filtered and 50 ml of petroleum ether was added to the filtrate for ether layer and evaporated to dryness. Five milliliter of acetone ethanol was added to the residue and 0.4mls of each taken into 3 different test tubes. Ferrous sulphate reagent (6ml) was then added each followed by 2 ml of conc H2SO4. This was thoroughly mixed after 10 min and the absorbance taken at 490 nm Oloyed, (2005). The absorbance of saponin standard solution was read after color development at same wavelength of 490nm.

**Tannin Determination**.

Sample (0.2g) was measured into a 50ml beaker. Then 20ml of 50%methanol was added and covered with para film and placed in a water bath at 77-80o for one hour. It was shaken thoroughly to ensure a uniform mixing .The extract was quantitatively filtered using a double layered whatman NO 41 filter paper into a 100ml volumetric flask. 20mls of water, 2.5 ml folin-Denis reagent and 10ml of 17% Na2CO3 was then added and mixed properly. The mixture was then made up to mark with water, mixed well and allowed to stand for 20min.A bluish-green colour was developed at the end of range 0-10ppm.The absorbance of tannin acid standard solution as well as sample shall be read after color development on a spectrophotometer at wave length of 760nm (AOAC, 1984).

***In vitro* Antioxidant Determinations**

**Free radical scavenging activity (DPPH Method).**

The free-radical-scavenging ability of the extracts against DPPH (1, 1-diphenyl-2 picrylhydrazyl) free radical was evaluated as described by Gyamfi *et al.* (1999). Briefly, appropriate dilution of the extracts (1 ml) was mixed with 1 ml, 0.4 mM methanolic solution containing DPPH radicals; the mixture was left in the dark for 30 min and the absorbance was taken at 516 nm. The DPPH free-radical-scavenging ability was subsequently calculated. Methanol was used to blank the spectrophotometer while the control contained 1ml of methanol and 1ml of DPPH. DPPH scavenging activity was calculated as:

% scavenging activity

 = AbsorbanceControl- AbsorbanceSample X 100

 AbsorbanceControl

**Determination of reducing property**

The reducing property of the extracts was determined by assessing the ability of the extracts to reduce FeCl3 solution as described Oyaizu (1986). In total, 2.5 ml aliquot was mixed with 2.5 ml 200mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50oC for 20 min and then 2.5 ml 10% trichloroacetic acid was added. This mixture was centrifuged at 650 rpm for 10 min when necessary. Five milliliters of the supernatant was mixed with an equal volume of water and 1 ml 0.1% ferric chloride. The absorbance was measured at 700 nm and the ferric reducing antioxidant property was subsequently calculated.

**Statistical Analysis**

All values were expressed as Mean ± SEM. Statistical analyses were performed by one-way Analysis of Variance (ANOVA) and individual comparisons of the group mean values were done using Duncan multiple range test.

**Results**

**Qualitative Phytochemical Screening**

The result on the qualitative phytochemical screening of the methanol extract of the selected fruits in this study showed that alkaloids, flavonoids, phenols, cardiac glycosides, Saponins and tannin were observed to be present in all fruits extract at varying strength (Table 1). Terpenoids and steroids were found to be present in all fruits extracts except in *curcubitapepo L* while phlobatannin was found to be present in the methanolic extact of *Annona Senegalensis* and *Sarcocephalus latifolius* (pulp only and whole fruit). Athraquinone on the other hand were found in all fruits extract except that *Curcubita pepo p., Cucumis melo inodorous*.

Table 1: Phytochemical constituents of Methanolic extract of Fruits of *Annona senegalensis*, *Curcubita pepo L , Cucumimelo inodorous* and *Sarcocepahlus latifoliu*s

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Phytochemicals | *Annona**senegalensis* | *Curcubita**pepo L.* | Cucumismelo inodorous | *Sarcocephalus**latifolius (pulp only)* | *Sarcocephalus**latifolius* |
| Alkaloids | + + + | + | + | + | ++ |
| Anthraquinone | + + | - | - | + | + |
| Cardiac glycosides | + + + | + | + + + | + + + | + |
| Flavonoids  | + + | ++ | + | +++ | + |
| Phenols | + + + | ++ | ++ | ++ | +++ |
| Phlobatannins | + + | - | - | + | ++ |
| Saponins | + + | + | ++ | +++ | ++ |
| Steroids | +++ | - | +++ | +++ | ++ |
| Tannins | + + + | ++ | ++ | ++ | ++ |
| Terpenoids | + + | - | +++ | + | + |

KEY -Absent, + Faintly present, ++ Moderately present, +++ Highly present

**Quantitative Phytochemical Analysis**

Quantitative phytochemical analysis result (Table 2) on the selected fruits extract shows that alkaloid content in *Anonna senegalensis* (193.47µg /g) was most significant (P < 0.05) compared to other fruits while Saponin content was highest in Cucumis*melo inodorous* extract (97.31mg/g). *Sarcocephalus latifolius (AP)* had the highest amount of flavonoids (57.00mg/g)*,* tannin and phenol content was found also to be significantly highest in *Sarcocephalus latifolius* (392.71mg/g) and *Anonna senegalensis* (374.52mg/g) respectively.

Table 2: Quantitative Phytochemicals of the Methanolic extract of fruits of *Annona senegalensis*, *Curcubita pepo L , Cucumimelo inodorous* and *Sarcocepahlus latifoliu*s

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Phytochemicals | *Annona**senegalensis* | *Curcubita**pepo L* | *Sarcocephalus latifolius**(OG)* | *Sarcocephalus latifolius**(pulp)( AP)* | *Cucumismelo inodorous* |
| Alkaloids (µg /g) | 193.47± 0.30e | 9.12±0.00a | 126.99±1.59d | 51.47±0.37c | 8.8850±0.30b |
| Saponins (mg /g) | 33.67±3.64c | 10.50±0.50a | 18.08±0.64b | 62.55±11.08d | 97.31±0.61e |
| Tannins (mg /g) | 335.43±9.77d | 120.66±2.00b | 392.71±0.23e | 129.12±0.84c | 89.45±1.23a |
| Flavonoids (mg /g) | 23.96±0.34bc | 30.88± 0.13c | 19.13±0.50b | 57.00±10.50d | 5.24±0.44a |
| Phenols (mg /g) | 374.52±0.00e | 75.25±1.85a | 285.00±0.41d | 194.08±4.19c | 84.00±0.23b |

Results are presented as mean ± SEM. Letters represent the level of significance.

**DPPH Free Radical Scavenginging Activities**

DPPH scavenging results on the selected fruits extract shows that all fruits extract and the standard(gallic acid) scavenge DPPH radicals in a dose dependent manner however the methanolic extract of *Annona seneganlensis* and *Sarcocephalusl atifolius* showed a significantly (P < 0.05) higher DPPH scavenging activities when compared to other extracts.

Fig 1: DPPH Scavenging Activity of methanolic extract of Fruits of *Annonasenegalensis*, *Curcubita pepo L , Cucumimelo inodorous* and *Sarcocepahlus latifoliu*s

**Reductive Power**

The reductive power of the fruits extracts increases as the concentration increases (Fig 2) however *Annona senegalensis* had a better reductive powers(absorbance of 0.540 at 1000µg/ml ) than all other fruits extract in a dose dependent manner.

Figure 2: Reductive power of the Methanolic extract of Fruits of *Annona senegalensis*, *Curcubita pepo L, Cucumis melo inodorous* and *Sarcocepahlus latifoliu*s

**Discussion**

The phytochemical analysis conducted on the fruits *Annona senegalensis*, *Curcubita pepo L , melo inodorous* and *Sarcocepahlus latifoliu*s extract revealed the presence of alkaloids, saponins, tannins Phenols and flavonoids in all fruits. These phytoconstituents are known to exhibit physiological effects and thus can be used for various therapeutic purposes. Certain alkaloids have been reported to possess analgesic (Okwu and Okwu, 2004), antisplasmodic and antibacterial (Nyarko and Addy, 1990) properties. Saponins have been reported to produce inhibitory effects on inflammation (Just, 1998) and have the property of precipitating and coagulating red blood cells. Some of the characteristics of saponins include formation of foams in aqueous solutions, hemolytic activity, cholesterol binding properties and bitterness (Sodipo, 2006).

Tannins are known to be useful in the treatment of inflamed or ulcerated tissues and they have been shown to have remarkable activity in cancer prevention and treatment **(**Aiyegoro and Okoh, 2010). Thus these fruit extracts can be used in the formulation of food supplements or drugs for prevention and probable treatment of ulcer and cancer.

The significantly high amount of phenol in *Annona senegalensis* (374.52mg/g) and *Sarcocephalus* *latifolius* (285.00mg/g) was found to be higher than that reported in previous report in which *Solanum* fruit had flavonoid content of 53.60 mg/g and phenol content of 24.68 mg/g (Sudha *et al.,* 2011). Phenolics constitute a major group of compounds that act as primary antioxidants (Hatano *et al*., 1989). They have high redox potentials which allow them act as reducing agents, hydrogen donors and singlet oxygen quenchers (Kahkonen *et al*., 1999). Flavonoids have also been shown to possess a broad spectrum of chemical and biological activities including radical scavenging properties, antiallergenic, antiviral, antinflammatory, and vasodilating actions (Pereira *et al.,* 2009; Parajuli *et al.,* 2012). Flavonoids are highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various other free radicals implicated in several diseases (Bravo, 1998). They suppress reactive oxygen formation, chelate trace elements involved in free-radical production, up-regulate and protect antioxidant defenses (Agati *et al.,* 2012). The pharmacological properties of the fruits of *Annona senegalensis*, *Curcubita pepo L ,Cucumi melo inodorous* and *Sarcocephalus latifoliu*s extract may be due to their phytochemical constituents.

The free radical scavenging activity of the extracts was evaluated based on the ability to scavenge the synthetic diphenylpicrylhydrazyl (DPPH) radical. DPPH is a stable nitrogen- free radical which produces violet colour in methanol solution. It was reduced to a yellow coloured product diphenylpicrylhydrazine with the addition of the methanolic fruits extract in a concentration dependent manner. The high DPPH scavenging activity in *Annona seneganlensis* and *Sarcocephalus latifolius* could be correlated with high phenolic content. Literature review revealed high levels of phenolic content in plants showed higher DPPH radical scavenging ability by such plants (Dastmalchi *et al.,* 2007; Hamzah *et al* 2013). This was also shown in the work of Ajiboye *et al*., (2010) where antioxidant activity and drug detoxification activity of *Annona senegalensis* leaf in carbon tetrachloride-induced hepatocellular damage in rats using 2, 2-diphenyl-1-picrylhydrazyl (DPPH), superoxide ion, hydrogen peroxide (H2O2), 2, 2'-azinobis-(3- ethylbenzthiazoline-6-sulfonate) (ABTS) and ferric ion models decreased significantly.

Reducing power assays measure the electron-donating capacity of antioxidants (Yen and Chen, 1995). Presence of reductones in the samples would result in the reduction of Fe3+l/ferricyanide complex to ferrous Fe2+ form which serves as a significant indicator of their antioxidant activity (Yildrim *et al*., 2000). Reductive power of methanol extract of selected the fruits increased as the concentration increased however *Annona senegalensis* had a better reductive power than all other fruit extracts in a dose dependent manner.

From the above results, it can be concluded that the methanol extract of the fruit *Annona senegalensis* showed more potent *in vitro* antioxidant activity, with higher free radical scavenging ability and reducing properties than the other fruits extract. This fruit can therefore be harnessed as a source of natural antioxidants which can be formulated into supplements for the prevention and management of oxidative-induced diseases

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