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# Phytochemical, Proximate, Anti-Oxidant, and Anti-Inflammatory Properties of Aqueous and Methanol Extracts of Dried Shell of *Cajanus cajan* seed collected from a Rural Community in Nsukka, Enugu State, Nigeria

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**ABSTRACTS:** *Cajanus cajan*, commonly known as pigeon pea, is widely cultivated by farmers in Nsukka and other parts of Enugu State, Nigeria. The seeds have a long history of usage in the traditional food combinations of many families and are reportedly very nutritious. Hence, the objective of this paper is to investigate the phytochemical, proximate, anti-oxidant, and anti-inflammatory properties of aqueous and methanol extracts of the dried shell of Cajanus cajan seed collected from a rural community in Nsukka, Enugu State, Nigeria using appropriate standard techniques. Data obtained show that some of the analytical constituents were flavonoids (2,226.50 ± 47.350, Phenolics (6,294.65±117.35), Alkaloids (175.09±3.34), moisture (13.762%), ash (4.183%), and protein (9.01%). Some of the anti-oxidant properties - DPPH $IC_{50}$ of 25.07 ± 0.09 µg/ml, nitric oxide scavenging activity equivalent to 20.0mM/g, and a Ferric Reduction Antioxidant Property (FRAP) value of 2.53 compared, favorably with ascorbic acid standard. The anti-inflammatory inhibition of the extracts was 44.1ug/mL as against standard diclofenac 37.62ug/mL. The extracts have promising properties as raw materials for developing anti-scaling agents for pipeline rust inhibition and nutriceuticals to manage oxidative stress-induced functional diseases.

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The need to recycle agricultural biomass for sustainable economic values and environmental health cannot be overemphasized. Fractionation of the shell extract of *Cajanus cajan* for its possible industrial applications in food and health sectors holds a strong promise in this direction, owing to its global availability. *C. cajan* plant, commonly called pigeon pea, is mostly cultivated by farmers because of its edible seed which is very rich in proteins(A'yuni *et al*., 2022; Ekpo *et al*., 2023; Gargi *et al.*, 2022; Yang *et al*., 2020). In addition to its human nutritional values, the seed can be used for the composition of feed for some animals such as pigs, fish, and even fowl. The plant can equally be used environmentally to control erosion, for ornamental purposes, and biologically in fixing atmospheric nitrogen to the soil. *C. cajan* can be found in over 80 countries of the world, and beers different tribal names: Guandul, poroto guandul, porotoparaguayo, sachacafé, falso café, arveja (Argentina); pigeonpea

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(Australia); guando (Brazil); mu dou (Chinese); pigeonpea, congo pea, red gram (English); pois cajan, poisd'Angole, ambrevade (French); poisd'angole (French-speaking West Africa); straucherbse (German); Puerto Rican bean, pigeonpea (Hawaii); red gram, tur, arhar, dal (India); frijol de árbol (Mexico); Cumandái (Paraguay); ervilha do Congo, feijão, guandu, ervilha de Angola (Portuguese); cachito (spanish); mbaazi (Swahili); duvart (Swedish); pigeonpea, angola pea (United Kingdom); quinchoncho (Venezuela), Fio fio, agbugu (Nigeria Igbo) (Fuller *et al*., 2019). Studies(A'yuni *et al*., 2022; Fuller *et al*., 2019; Gargi *et al*., 2022; Mallikarjuna *et al*., 2011; Sameer Kumar *et al*., 2017; Yang *et al*., 2020) have shown that *C. cajan* is not harmful to man and other animals and can richly grow in Nigeria. While literature abounds on the nutritional, phytochemical, anti-inflammatory, and antioxidant properties of the seed extracts, there is still a paucity of information on the potential candidature of the shell extracts for developing anti-scaling agents and nutriceutical products. Nutraceutical is a broad umbrella term used to describe any product derived from food sources with extra health benefits in addition to the basic nutritional values found in foods. The processing of *C.cajanus* seed for food involves the generation of solid wastes in the form of shells, which has contributed to causing environmental fouls. Therefore, recycling the shell extracts into raw materials for the scaling inhibition, in the food and pharmaceutical industries will increase their economic value and help in controlling environmental cleanliness.(Orjiocha *et al*., 2024).

Phytochemical analysis remains the gold standard for evaluating the chemical components of plant materials that may have pharmacological actions and they include; alkaloids, phenolics (flavonoids and tannins), terpenoids, saponins, and steroids(Orjiocha et al., 2024). Similarly, proximate studies are used to determine the nutritional composition of the plant materials and involve such parameters as ash content, protein, and fat content. The anti-inflammatory and anti-oxidant studies of plant extracts provide useful information on the role of plant materials in the management of functional diseases as many natural substances found in plants which include flavonoids, terpenoids, alkaloids, and saponins, have been shown to have anti-inflammatory properties in vivo and in vitro.(Gulcin, 2020; Jaiswal et al., 2021; Kotha et al., 2022; Latief et al., 2023; Mucha et al., 2021; Odu et al., 2023). In the same way, the antioxidant properties of plant extracts have found good applications in numerous industrial operations. Antioxidants are sometimes introduced into industrial products such as polymers, fuels, and lubricants to improve their applications and lifetime. Antioxidants are equally introduced in food to prevent it from spoilage(Huang et al., 2005; Prior et al., 2005; Spiegel et al., 2020)

## MATERIALS AND METHODS

*Equipment and Reagents:* The following items of laboratory equipment and reagents were used: Analytical Balance (Mettler-Toledo, ATX224), UV/Visible spectrophotometer (Thermo scientific, evolution 160 UV-Vis), water bath (VWR WB05), manual single channel micropipette (Pipette-Lite XL), rotary evaporator (Heidolph laborota 4001 519-10000-01-5), laboratory glassware and Whatman No 1 filter paper.

*Reagents:* The following reagents were used; picric acid, Fehling's solutions A and B, Dragendorff's reagent, , rutin, methanol, concentrated sulphuric acid, concentrated ammonia (Merck KGa A, Darmstadt, Germany), gallic acid, potassium ferricyanide, NaOH, acetic anhydride, (Reagents, Charlotte, NC 28214, USA) ferric chloride (Xilong Scientific Co., Ltd, China) $Na_2CO_3$, (Zouping Zhijin New Material Technology Co., Ltd Shandong, China), Cholesterol (Fissions Chemicals, United Kingdom) atropine and vanillin (Sigma Chemical, USA), diosgenin (Xiangyang Wellbeing Pharmchem Co., Ltd, China), linalool (Basf Se, Belgium) 1. Sodium nitroprusside solution (10mM), Naphthyl ethylene diamine dihydrochloride (NEDD) (0.1%) solution, Sulphanilic acid (0.33% w/v) reagent, Phosphate buffer saline (PBS) pH.7, De-ionized Water.

*Preparation of Crude Methanol Extract:* The dried C. *cajan* shell, picked from the dump site at a rural community in Nsukka, Enugu State, Nigeria, was washed with running tap water, re-dried at room temperature for four days, and ground into a powder. The powdered plant material was extracted for 24 hours through cold maceration using methanol as solvent, amidst intermittent shaking to bring about rapid equilibrium between intra and extracellular fluids, thereby enhancing proper percolation of the men strum (methanol) into the particle surface for exhaustive extraction. The extract was filtered with Whatman filter paper No. 1 and concentrated using a rotary evaporator. The extract concentrate was used for the analysis.

*Phytochemical analyses of Crude Methanol Extract:* Qualitative and quantitative phytochemical analyses of samples were performed according to the methods of (Richardson and Harborne, 1990; and Herawati *et al*., 2022)**.** The proximate analysis was according to

the methods reported in (O. Aluge et al., 2016) and the Official Methods of Analyses of the Association of Association of Official Analytical Chemists (AOAC, 2005).

*Anti-oxidant Analyses of Crude Methanol Extract:* Antioxidants in food and biological systems are tested using a variety of techniques, which involve oxidizing a substrate under standard conditions and measuring the activity using different techniques to find out how much oxidation is inhibited. Free radical-trapping techniques are other protocols that are categorized and gauge how well antioxidants can capture free radicals. One of the most frequently used antioxidant assessment techniques is the ferric-reducing antioxidant power (FRAP) assay. The assay is based on the principle of reduction of ferric tripyridyltriazine ($Fe^{3+}$-TPTZ) complex to ferrous tripyridyltriazine ($Fe^{2+}$-TPTZ) by the antioxidants of a sample at low pH. The end product ($Fe^{2+}$-TPTZ) has a blue color with an absorption maximum of 593 nm and the change in absorbance is related to the antioxidant capacity of the sample. For calibration, aqueous solutions of known Fe ($FeSO_4.7H_2O$) concentration in the range of 100 to 1000 µmol/L are used, and the values are expressed as µmol/L $Fe^{2+}$.(Benzie & Strain, 1996). A potential antioxidant will reduce the ferric ion ($Fe^{3+}$) to the ferrous ion ($Fe^{2+}$); the latter forms a blue complex ($Fe^{2+}$/TPTZ), which increases the absorption at 593 nm. It is based on the reduction of a colorless $Fe^{3+}$-TPTZ complex into intense blue $Fe^{2+}$-TPTZ once it interacts with a potential Antioxidant. (Spiegel *et al*., 2020)

*Ferric Reducing Antioxidant Power (FRAP):* A slightly modified method of Spiegel *et al*, (2020) was used for the test. The modification involved the volume of extract/ standard used (0.1 ml instead of 0.3 ml). Lower concentrations of the sample as well as the standard were obtained by serial dilution of their respective (1mg/ml) stock solutions. Different concentrations (10 - 70µg/ml) of the extract in de-ionized water were placed in test tubes, the volume was made up to 1ml with water. This was followed by addition/mixing with 2ml of Ferric Reducing Antioxidant Power (FRAP) reagent prepared immediately before use. The absorbance was taken at 593 nm after 60 min. All determinations were performed in triplicate. The FRAP value of the sample was calculated from the $FeSO_4$ standard table.

*Nitric Oxide Radical Scavenging Assay (NORSA) of Crude Methanol Extract:* The method of Agarwal, (2013) was used on the principle that sodium nitroprusside in an aqueous solution at physiological pH spontaneously generated nitric oxide, which would interact with oxygen to produce nitrite, which would be estimated by the use of Griess illusory reagent. Briefly, the reaction mixture (6.0 ml) containing sodium nitroprusside (4.0 ml) phosphate buffer saline (PBS, 1.0 ml) and extract (1.0 ml at various concentrations) in methanol was incubated at 25 $^0$C for 15 minutes. Thereafter, 0.5 ml of the reaction mixture was removed, 1.0 ml of sulphanilic acid reagent was added, mixed well, and allowed to stand for 5 mins to complete diazotization. Then 1.0ml of N- (1-Naphthyl)-ethylenediamine-dihydrochloride (NEDD) was added and allowed to stand for 30 minutes in diffused light. A pink-colored chromophore formed was measured at 540nm against a corresponding blank solution. Ascorbic acid was used as standard. The linear standard curve was obtained by plotting the mean absorbance against the sodium nitrite concentrations. The standard curve was used to calculate the sodium nitrite (mM) equivalent activity in the test sample.

*DPPH Radical Scavenging Activity of Crude Methanol Extract:* The free radical scavenging activity of the extracts and ascorbic acid as a positive control was measured in terms of hydrogen donating or radical-scavenging ability using the stable radical DPPH. A stock solution of the extract containing 1 mg/ml of methanol was prepared. Lower concentrations of (5, 10, 20, 30, 40, 50, 60, and, 70 µg/ml) were obtained from the stock. 2 ml of freshly prepared DPPH solution (0.004%) in methanol was added to each.

The reaction mixture was incubated in the dark for 30 minutes, and the absorbance was recorded at 517 nm against the blank. For the control, a DPPH solution in methanol was used. The assay was carried out in triplicate. The degree of de-colorization of DPPH from purple to yellow indicated the scavenging efficiency of the extract. The free radical scavenging activities of the tested samples were expressed as a percentage of inhibition and were calculated according to the equation 1:

$$\text{PI} = \frac{A_{control} - A_{sample}}{A_{control}} x\ 100 \quad (1)$$

Where: PI = Percentage inhibition; $A_{Control}$ = Absorbance of control; $A_{sample}$ = Absorbance of a sample (extract/ascorbic acid). The amount of sample necessary to decrease the absorbance of DPPH by 50 % ($IC_{50}$) was calculated graphically for the extracts and the standard ascorbic acid. The extract was compared to the standard by calculating the concentration of the standard that gave the same absorbance value as the extract (an absorbance value

for a certain concentration was read (extrapolated) from the ascorbic acid standard curve to get the ascorbic acid equivalence.

*In-vitro Anti-inflammatory Activity of Crude Methanol Extract:* Methods of Mizushima and Kobayashi, (1968); and Juvekar *et al.*, (2009) were followed with minor modifications. Briefly, the reaction mixture consisted of test extracts and a 1% aqueous solution of bovine albumin fraction, and the pH of the reaction mixture was adjusted using a small amount of HCl. The sample extracts were incubated at 37 °C for 20 min and then heated to 51 °C for 20 min, cooled and the resultant turbidity sample was measured spectrophotometrically at 660 nm. The reference drug (diclofenac) was prepared at different concentrations (10, 20, 30, 40, 50, 60, 70µg/ml) and treated the same as the extract. The experiment was performed in triplicates. The inhibition percentage of protein denaturation was determined using the equation 2:

$$PI = 1 - \frac{A_C - A_S}{A_C} X\ 100 \quad (2)$$

Where, PI = Percentage inhibition of denaturation; $A_s$ = absorbance of the sample; $A_c$ = absorbance of control. The extract and the reference drug concentration for 50% inhibition ($IC_{50}$) were determined from the dose-response curve by plotting the inhibition percentage against concentration.

**Fig 1:** (a) Flowered *c. cajan*, (b). *C. cajan* with seed (c.) harvested seeds (d). Dry shells

## RESULTS AND DISCUSSION

The results of the study are presented in tables 1and 2. Table 1 represents the quantitative phytochemical and proximate compositions of the extracts. The following secondary metabolites were identified: Flavonoids, Phenolics, Tannins, Alakaloids, Saponins, Steroids, and Terpenoids. The quantitative values of the phyto-constituents ranged from 0.46±.06mg/100g in steroids to 6,294.65±117.35mg/100g in phenolics. The proximate value was dominantly solid. The gap between medicinal plants and plant foods is becoming much thinner because of the increasing knowledge of many applications of numerous food plants in modern medicine.

**Table 1:** Quantitative Phytochemical and Proximate Compositions of the Extracts

| Secondary Metabolite | Concentration (mg/100g) |
|---|---|
| Flavonoids | 2,226.50±47.35 |
| Phenolic | 6,294.65±117.35 |
| Tannins | 176.49±13.18 |
| Alkaloids | 175.09±3.34 |
| Saponins | 2.53±0.15 |
| Terpenoids | 989.87±26.72 |
| Steroids | 0.46±.06 |
| Cyanogenic compounds | |
| **Proximate** | |
| Moisture | 13.762 |
| Ash | 4.183 |
| Total solids | 86.238 |

Thus, neutraceuticals are products isolated from herbs, dietary supplements, and natural or processed

foods that have therapeutic potential and also prevent diseases. They are categorized into; functional foods, medicinal foods, and dietary supplements and are currently useful in the management of various chronic diseases, some of which are diabetes, hypertension, cancer, and other oxidative degenerative disorders. There is an increasing research and growing interest in the sourcing and development of Nutraceuticals. Nigeria is richly endowed with numerous foods and plants that are beneficial to human health and with the relative abundance of *C. cajan* in many parts of the country, harnessing the photocomposition of the plant's seed shell extract for nutriceutical products, and scale inhibiting agent is an important paradigm shift from waste to wealth. In the past two to three decades, several studies have shown that phytochemicals play an important role in preventing chronic diseases such as cancer, diabetes, and coronary heart disease. (Kurmukov, 2013) The major classes of phytochemicals with disease-preventing functions are antioxidants, anticancer, detoxifying agents, immunity-potentiating agents and neuropharmacological agents. Each class of these functional agents consists of a wide range of chemicals with differing potency (Kurmukov, 2013). Some of these phytochemicals have more than one function. In the present study some of the phytoconstituents identified fall within the class of phytochemicals reportedly useful in ethnomedicine, for example, as one of the major phytosterols, stigmasterol a steroid, is included among sterol compounds in the diet having the potential to reduce the risk of cardiovascular diseases (Ferreres *et al.*, 2017). Consumption of 2 grams per day of plant sterols is associated with a reduction in blood LDL cholesterol of 8 - 10%, possibly lowering cardiovascular disease risk (Ferreres *et al*., 2017). Stigmasterol has also been shown to exert anti-angiogenic and anti-cancer effects via the downregulation of Tumour Necrosis Factor-alpha (TNF-alpha) and Vascular endothelial growth factor receptor 2 - VEGFR-2 (Kangsamaksin *et al*., 2017). β-sitosterol, another steroid, is being studied for its potential to reduce benign prostatic hyperplasia (BPH) (Wilt *et al.*, 2000*)* and blood cholesterol levels (Glanville *et al.*, 2015). Ecdysteroids have a variety of physiological effects in mammals, including hepatoprotective, immune-modulatory, as well as hypoglycemic action. Notably, 20-Ecdysteroid (20E) affects certain major metabolic pathways such as protein synthesis, and lipid and carbohydrate metabolism (Bathori *et al*., 2008). That steroid was identified though without classification, albeit in small concentration, is a further indication of the potential of the extract to be useful in pharmaceutical formulations.

Carotenoids, otherwise called tetraterpenoids, are yellow, orange, and red organic pigments that are produced by plants and algae, as well as several bacteria, fungi, and egg yolk (Micronutrient Information Center, 2016). They include such macromolecules as lutein, zeaxanthin, and lycopene. Dietary carotenoids are obtained from several fruits and vegetables, such as green leafy vegetables, spinach, carrots, peaches, apricots, and sweet potatoes. Lutein and zeaxanthin are found abundantly in green leafy vegetables. These carotenoids are found in high concentrations in the macula of the eye, which is responsible for central vision. Macular degeneration is a common problem in the elderly and is among the four leading eye diseases found in this population. Reports of Ma *et al.,* (2012) and Mrowicka *et al*., (2022) point to the positive effects of lutein and zeaxanthin supplementation in the management of patients with early signs of macular degeneration. The relatively high abundance of terpenoids in the crude methanol extract of *C.cajan* holds good promise for use as a drug candidate for the formulation of ophthalmological drugs.

Polyphenols, naturally occurring compounds found largely in fruits, vegetables, legumes, cereals, and beverages (Cory *et al.*, 2018; Alqarni *et al.*, 2024) have been credited with much pharmacological importance. Basic research and epidemiological studies have shown an inverse association between the risk of degenerative diseases and the intake of a diet rich in polyphenols (Holst and Williamson, 2008). Several studies ((Jha and Subramanian, 2016; Pooja Singh *et al.*, 2017; Rani, 2017; Szymanska *et al.*, 2018; and Wojdylo *et al.*, 2007), provide convincing shreds of evidence that a diet rich in antioxidants is associated with a lower incidence of degenerative diseases. The extract is super rich in phenolics and therefore, points to the need to explore the extract for nutriceutical development. Table 2 represents the *in-vitro* antioxidant, DPPH radical scavenging, nitric oxide radical scavenging activities, and ferric-reducing antioxidant power (FRAP). The *in vitro* antioxidant result of the methanolic extracts of *C. cajan* showed DPPH $IC_{50}$ of 25.07 ± 0.09 µg/ml compared to 17.98µg/ml for ascorbic acid, with ascorbic acid equivalent values of 39.94 ± 3.09 of cajunu cajan extract. The extract had nitric oxide scavenging activity equivalent to 20.0mM/g, $IC_{50}$ 29.00 compared to 38.81mM/g for ascorbic acid, $IC_{50}$= 24.48 µg/ml and a FRAP value 2.53 respectively compared to 481.33 for the standard (Ascorbic acid)

**Table 2:** *In -vitro* Antioxidant, DPPH Radical Scavenging and Nitric Oxide Radical Scavenging Activities, Ferric Reducing Antioxidant Power and *In vitro* anti-inflammatory activity

| | Cajanus cajan | AA |
|---|---|---|
| DPPH radical scavenging activity $IC_{50}$ | 25.07 | 17.98 |
| Ascorbic acid equivalence µg* | 39.94 | 17.98 |
| Nitric oxide radical scavenging activity $EC_{50}$ $NaNO_2$ (mM)** | 2.02 | |
| Ascorbic acid equivalence µg* | 29.0 | 38.81 |
| Ferric reducing antioxidant power(FRAP) | 21.85 | |
| FRAP value (mMFe(11)/g) | 2.53 | 481.33 |
| Ascorbic acid equivalence µg* | 21 .85 | |
| In vitro anti-inflammatory activity ($IC_{50}$ (µg/ml)) | 44.10 | 37.62 (Diclofenac) |

**Amount of ascorbic acid with the same activity as 50 µg of extract, ** Amount of $NaNO_2$ with the same activity as 20 µg of extract*

Antioxidant agents found in foods such as β-carotene and vitamins A, C, and E are reportedly beneficial to human health (Pruteanu *et al*., 2023). Therefore, antioxidants naturally present in the body or supplied in the form of plant diets (phytonutrients) play important roles in controlling various diseases resulting from oxidative stress. The favorable comparison of the extract DPPH radical scavenging activity and nitric oxide radical scavenging activity with ascorbic acid makes it a good candidate for the development of dietary supplements with antioxidant properties. Normal biochemical reactions, increased exposure to the environment, and higher levels of dietary xenobiotics result in the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Snezhkina *et al.,* 2019). ROS and RNS are responsible for oxidative stress in different pathophysiological conditions (Di Meo *et al.,* 2016; Zarkovic, 2020). Cellular constituents of the human body are altered in oxidative stress conditions, resulting in various disease states. The oxidative stress can be effectively neutralized by enhancing cellular defenses in the form of antioxidants (Sharifi-Rad *et al*., 2020; Jena *et al*., 2023).

Certain compounds act as in vivo antioxidants by raising the levels of endogenous antioxidant defenses. It is believed, therefore, that diets rich in antioxidants, such as many bioactive polyphenol compounds found in fruits and vegetables will help to combat free radical damage and improve health. This belief is consistent with the emerging better health outcomes resulting from plant-based diets. Plant-based diets have positive effects on cancer, heart disease, and neurodegenerative diseases (Obrenovich et al., 2010; Gupta and Prakash, 2014). Several studies (Subramoniam, 2016; Koehnlein et al., 2016; and Renata *et al.,* 2018) provide convincing evidence that a diet rich in antioxidants, including polyphenols, is associated with a lower incidence of degenerative diseases. The *C.cajan* methanol extract is rich in polyphenol content, supporting its candidature for pharmaceutical and food industrial raw material.

Scale formation is the byproduct of oxidative reaction and can thus be prevented or ameliorated by any substance with antioxidant properties. The capacity of the extract to scavenge 2, 2-diphenyl-1-picrylhydrazl (DPPH), the nitric acid scavenging activity, and the Ferric Reduction Antioxidant Property (FRAP) are desirable attributes of scale inhibition agent.

*Conclusion:* The result of the study shows that the extracts have numerous phytochemical compounds with the potential for phytotherapeutic applications in ethnomedicines and as nutraceuticals. The antioxidant properties also point to strong scaling-inhibiting properties that favourably dispose of the extract as potential raw materials for the development of an oil pipeline scale-inhibiting agent. Thus, the study has shown the methanol extract of *c. cajan* shell at the trijunction of anti-scaling, food, and medicine.

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*Declaration of Conflict of Interest:* The authors declare no conflict of interest

*Data Availability Statement:* Data are available upon request from the first author or corresponding author or any of the other authors

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