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Microbiological and Chemical Indicator of Multicomponent Nature of Cashew Nut Shell Hot Water Extract

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ABSTRACT: Dark brown hot water extract of cashew nut shell was found to be partly colonized by molds that were identified to be *aspergillus* and *penicillum* when left in the open laboratory for several days. Test revealed that though the extract contained tannin but not of the depside bond type and so the nutrient used by the mold must have been derived from other carbon nutrient sources. The nutrient was found to be provided by soluble carbohydrate present in the extract. Further examination revealed that many more components were extracted into hot water and that the soluble sugar in the extract is the main source of food for the sustenance of the identified mold found in the extract.

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Cashew (*Anacardium Occidentale* L.), is a well-known species of the *Anacardiceae* family Subbarao *et al*., (2011), a tropical plant (shrub) found within the region between 23 °N and 23 °S of the equator. Because it has extensive root system it is adaptable to a wide range of moisture levels and soil types Olife *et al*., (2013). If grown in plantation it thrives best grown on well-drained, sandy loam soils. The tree fetches financial reward to farmers and the local buyers in Nigeria annually Elijah, (2015). The cashew tree consists of the cashew nut fruit (which is a curved edible seed, housed in a honeycomb-like shell), the apple, leaf and bark. The fruit consists of an outer shell, inner shell and the kernel. The thickness of the cashew nut shell is about 0.32 cm Patela *et al*., (2005). The soft honeycomb matrix, in between the outer and inner shell, contains a dark brown liquid, which is known as cashew nut shell liquid (CNSL) Patela *et al*., (2005). In cashew plant valuable products that are used in food, medicine, chemical and allied industries are obtained. It provides shade and serves as ornamental plant that is generally used to control soil erosion Elijah, (2015). The cashew apple which is about 10 cm long is attached to the externally born nut by a stem. Its color ranges from yellow to red, fibrous in nature, very juicy, sweet, pungent and high in vitamins A and C. The kidney shaped nut bears a leathery ash colored shell. The nut contains a kernel that is covered by a thin layer of brown testa. The cashew nut shell (CNS) contains the vesicant oily liquid (CNSL) Elijah, (2015). Basic physical operations before extraction are required on the samples of cashew nuts. These operations include washing, drying, shelling and size reduction Elijah, (2015). Sometimes, washing may involve the use of detergents to remove likely contaminants. Drying is done purposely to make the nuts moisture-free using either sun or oven-drying. Size reduction creates a better contacting surface area for the shell and solvent to enhance removal of the CNSL Elijah, (2015). Traditionally, the kernel is removed for the CNS manually. However, to improve the de-shelling process, several methods have been adopted Subbarao *et al*., 2011; Ojewola *et al*, 2004; Sue, 2001, which include, among others, soaking the nuts in water to improve the moisture content thereby reducing the scorching and cracking tendencies during roasting. Roasting the nuts makes the shell brittle and loosens the kernel from the shell easily. Also, CNSL is released during the roasting Sue, (2001).

The extraction processes can be classified into two basic types: those that involve heating and those that are done in cold or room temperature. The heating process (roasting) can be achieved by open recipients or drums Patel *et al*., (2006). In a thermo-mechanic (hot oil) process, the cashews can be heated by the actual CNSL Mazzetto *et al.,* (2009). In the cold process, the CNSL can be obtained by extrusion in solvents or by pressing. The cashew's liquid so obtained is denoted as natural CNSL and that extracted

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by the hot method is called technical CNSL. Rodrigues *et al*., (2011), Das *et al*., (2004) and Tsamba *et al*., (2006) reported CNSL extraction through pyrolysis. The extraction of CNSL using supercritical carbon dioxide has also been reported by Shobha and Ravindranath, (1991) and Smith *et al*., (2003). Solute extracted using supercritical carbon dioxide in the extraction process is generally significantly different from those obtained by other conventional alternative processes Elijah, (2015).

Cashew tree plant has become an important tree of commerce and therefore, research that is to do with better ways of extracting the constituent parts of the shell for research and commercial interest will continue to grow in the foresee able future. This work is hoped to revive and redirect research focus to this effort.Therefore the objective of this paper is to report the microbiological and chemical indicator of multicomponent nature of cashew nut shell hot water extract

MATERIALS AND METHODS

Water, grated cashew nut shell, serviette, autoclave, Potato Dextrose Agar, filter paper, cotton wool, Petri dish, lactophenol cotton blue, slide, cover slip, stirring rod, marker, masking tape, teasing needle, inoculating loop, streptomycin.

Extraction procedure*:* 50 g of grated cashew nut shell was wrapped in a serviette and inserted in a 500 ml beaker containing 200 ml water. The beaker and the content was immersed in an autoclave and heated for 3 hour. A part of the extract was transferred into a flask while hot and was tightly sealed, while the other part was kept in the evaporating dish on the laboratory desk. This sample was used for mold identification.

Macroscopy: Using the procedure of Ogbo, (2005), 3.9 g of PDA was weighed and dissolved in 100 ml of distilled water, boiled on heating mantle with constant stirring to have a clear solution. The medium was autoclaved at 121 ^{0}C for 15 min. and allowed to cool to about 47 ^{0}C. Streptomycin was added and was poured into Petri dishes and allowed to gel. The molds were inoculated into the medium and incubated at 37 ^{0}C for 72 hrs.

Microscopy: Each of the molds was picked with teasing needle placed on the slide and teased out well. A drop of lactophenol cotton blue was added with cover slip placed on top, and then it was observed using x10 and x40 objectives Ogbo, (2005).

*Determination of the soluble carbohydrate and tannin***:** Five gram (5 g) of the protected hot water extract was wash with n-hexane (4 x 20 ml), by filtration using filter paper. The residue was again washed with 80% ethanol (4 x 20 ml). The n-hexane soluble fraction was concentrated for subsequent tests. The combined ethanol filtrate was equally concentrated for the determination of carbohydrates and tannin.

Fehling's Test: To 1 ml of the heated filtrate placed in a test tube, 1 ml of *Fehling's* reagent was added and kept in a boiling water bath for about three minutes.

Barfoed's Test: To 1 ml of the filtrate placed in a test tube, 3 ml of *Barfoed's* reagent were added. The solution was then heated in a boiling water bath for three minutes.

Bial's Test: To 2 ml of the filtrate placed in a test tube, 2 ml of *Bial's* reagent was added. The solution was then heated gently in a boiling water bath.

Seliwanoff's Test: To 0.5 ml of the filtrate in a test tube, 2 ml of *Seliwanoff's* reagent was added. The solution was then heated in a boiling water bath for two minutes.

Test for tannin: Ferric chloride test: Ten milliliters (10 ml) of the ethanol extract was transferred to a test tube and few drops of 0.1 % $FeCl_3$ solution added to it. Formation of blue black precipitation is an indication of the presence of tannin.

Test for chlorogenic acid: Aqueous ammonia was added to an extract containing 2 ml chlorogenic acid and exposed to air to see if a green color gradually develops.

Shinoda test: To 5 ml of the ethanol extract was added few drops of concentrated hydrochloric acid and was observed for intense cherry red or magenta color.

Bromine water test: Add bromine water to an aqueous extract of the drug. - Condensed tannins is present if this test gives buff-colored precipitate, hydrolysable tannins do not form any precipitate.

Reactions of tannin extract with hexamethylenetetramine: To 2 ml of tannin extract containing 2 ml of (2 %) hexamethylenetetramine was added 2 ml conc. ammonia solution and observed for formation of precipitate or any other sign of reaction. The above procedure was repeated but this time sodium hydroxide was used in the place of ammonia. Again the above procedure was repeated but acetic acid was used in place of ammonia.

Reactions of CNSL with hexamethylenetetramine: Experiment 1 to 3 were repeated but tannin extract was replaced with CNSL

RESULTS AND DISCUSSION

Cashew nut shell hot water extract is viscous dark brown homogenous liquid that looks uniform throughout its mass. However, leaving a sample of the extract in the air for prolonged period of time, molds grew on it. The growth pattern of the mold was in patches, that is, they do not spread over the entire mass. That mold grew on the extract was not expected because CNSL is considered to be toxic to molds. However the pattern of growth of the mold on the extract provoked further examination of the extract to determine the source of nutrients to the mold. Hydrolysable tanning containing depside bonds are said to be metabolizable by fungi using tannase, hence the examination of the extract for this tannin type was called for. The result of our examination showed that this tannin type was absent. The identified molds could also use simple sugars and so the extract was examined for six carbon sugars which were found. The n-hexane and ethanol extracts proved to be reactive to hexamethylenetetramine. The ethanol extract was done to remove the extractives which were not soluble in n-hexane. These n-hexane insoluble extracts were later confirmed to be tannin (of the condensed type) and soluble carbohydrates. In table 1 the blue mold showed septate conidiophore (*Penicillium*), while the green mold consist branching hyphae with conidia born in chains (*Aspergillus*).

Table 1: Microbiological examination of the ubiquitous mold on CNS hot water extract

S.No	Microbiological tests	Observations
1	Macroscopy	Green and blue molds were observed in the plates.
2	Microscopy	*Hyphae* and the spores were observed. The green mold had branching hyphae with conidia borne in chains.

Table 2 present result of the qualitative test for the soluble carbohydrate determined from the defatted cashew nut shell. The soluble carbohydrate was positive to *Fehling's* and *Barfoed's* tests. The *Fehling's* test indicates the presence of reducing sugar due to the formation of cuprous oxide by the reducing action of the sugar. The *Barfoed's* test also indicated the presence of reducing sugar but in this case it was indicative of the presence of reducing monosaccharide which caused the reduction of copper (II) oxide to copper (I) oxide. *Bial's* test and *Seliwanoff's* tests gave negative results which meant that the soluble carbohydrate was neither a pentose nor fructose respectively (See Table 2). Similar report has been made by Salehdeen *et al.,* (2018).

Table 2: Chemical examination of carbon source of nutrient to ubiquitous mold

S.No.	Tests	Observations
1	Fehling's	Brown color formed
2	Bafoed's	Brown color formed
3	Bial's	No observed reaction
4	Seliwanoff's	No observed reaction

Table 3: Chemical examination of the presence of tannin and its nature

S.No.	Test	Observation
1	Iron III Chloride	Formed bluish green precipitate
2	Chlorogenic acid	No reaction observed
3	Shinoda's	Formed pink color
4	Bromine water	Formed

Test with Iron III chloride revealed that the ethanol soluble fraction of the hot water extract contain phenolic. This prompted us to find out the nature of the phenolics by carrying out chlorogenic acid and Shinoda's test to see if the phenolic is of the pseudo type.

Test showed that the extract does not contain chlorogenic acid but confirmed the presence of catechin. The catechin was eventually confirmed to be in form of true tannin using bromine water test (Table 3).

We observed that literatures on chemical test for distinction between the two groups of compounds are rare, but we found out that acetic acid will dissolve tannin but not CNSL. Both tannin and CNSL contain phenolic hydroxyl group and are known to be reactive towards hexamethylenetetramine. Our finding showed that a mixture of tannin /hexamethylenetetramine in ammonia and sodium hydroxide do not show noticeable reaction immediately. Similar mixture with acetic acid gave copious precipitate almost immediately (Table 4).

Table 4: Reactions of tannin with Sodium hydroxide and Ammonia solution

Composition	Reagents	Observation
Tannin + Hexamine	NH_4	No precipitate formed
Tannin + Hexamine	NaOH	No precipitate formed
Tannin + Hexamine	Acetic acid	Formed precipitate

Table 5: Reactions of CNSL with Sodium hydroxide and Ammonia Solution

Composition	Reagents	Observation
CNSL + Hexamine	NH_4	Dark brown viscous mass formed which foams in excess ammonia
CNSL + Hexamine	NaOH	Dark brown viscous mass formed which foams in excess sodium hydroxide
CNSL + Hexamine	Acetic acid	No reaction. CNSL is insoluble in acetic acid
CNSL + Hexamine + Methanol	Acetic acid	Brown precipitate/emulsion formed

However, the mixture of CNSL/hexamethylenetetramine in ammonia, sodium hydroxide gave dark brown, viscous mass (not precipitate). In acetic acid CNSL is not soluble and hence do not react. The mixture only reacts to form precipitate when methanol was added (Table 5).

*Conclusion***:** The use of hot water as an initial step in biomaterial utilization of cashew nut shell may draw down on the overall amount of volatile solvent in current use. More, this approach may provide many components of cashew nut shell that are traditionally burnt after the extraction of the CNSL, for technological applications. Closer attention to this line of investigation will reveal further valuable potentials of cashew nut shell and unravel a world of technological value to be exploited to the advantage of man.

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