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Determination of the Phytochemical Constituents and Antifungal Properties of *Annona senegalensis* Leaves (African Custard Apple)

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ABSTRACT

Phytochemical screening of *Annona senegalensis* leaves was carried out in order to identify and to quantify the bioactive compounds that are present in the plant. Methanol extracts and aqueous extracts of the plant leaves were screened for the presence of ten metabolites. The qualitative study indicated the presence of alkaloids, flavonoids, polyphenols, steroids, cardiac glycoside and carbohydrates for both the aqueous extracts and the methanol leaves extract. Tannins and terpenoids were only present in the aqueous extract and saponins were only present in the methanol extract. Anthraquinones were absent in both of the extracts. Quantitative analysis showed the amount of alkaloid that was present in both the leaves extract of *Annona senegalensis.* Antifungal activities of the methanol extracts and the aqueous extracts of *Annona senegalensis* were investigated and assessed against cultured *Trichoderma spp* fungus. The susceptibility of the tested fungus on the extracts was determined by measuring the diameter of the inhibition zones formed around the well. The highest anti-fungal activity was observed in the aqueous extracts of the leaf. This gave an inhibition zone of 14.5mm. The methanol extract of the leaf gave the lowest inhibition zone of 8.3mm.

Keywords: *Annona senegalensis*, bioactive, extracts, metabolites, leaves, phytochemical, antifungal

INTRODUCTION

Annona senegalensis (Annonaceae) is a multipurpose plant with high traditional and medicinal uses for the maintenance of free health life (Aiyeloja and Bello 2006). Traditionally, the plant is used as stimulant or as, - a pain reliever (and so on). Several uses of the plant species were reported to have anti-oxidant, antimicrobial, anti-diarrhea, anti-inflammatory, anti-parasitic, anti-convulsant, anti-malarial, anti-trypanosonal, anti-snake venom anti-nociceptive and many other biomedical properties of pharmaceutical relevance (Noumi and Safiatou 2015). These properties that the plant possesses are due to its important phytochemical constituents like triterpenes, flavonoids and alkaloids. (Samuel *et al*., 2016). The leaves of *Annona senegalensis* have been used in treating yellow fever, tuberculosis, and small pox (Ajaiyeoba *et al*., 2006). The stem bark of *Annona senegalensis* has been used in snakebite and hernia treatment (Dambatta and Aliyu, 2011). The root of *Annona senegalensis* has also been reportedly used in the treatment of conditions such as difficulty in swallowing, gastritis, snake bites, male sexual impotence, erectile dysfunction, tuberculosis, and as an antidote for necrotizing toxins. The roots bark has been effectively used against infectious diseases (Ofukwu *et al*., 2008). The juice from the tree is used in the treatment of chicken pox (Faleyimu and Akinyemi, 2010). Many of the plant parts are used as antidotes for venomous bites and in the management of diabetes (Ogoli *et al*., 2011). In Guinea, *Annona senegalensis* has been employed in the treatment of malaria (Traore *et al*., 2013). Among the Igede people of Benue State in North Central Nigeria, the plant is used in combination with *Ageratum conyzoides* for the treatment of diarrhea and in combination with *Nauclea latifolia* against dysentery (Igoli *et al*., 2005).

Phytochemicals are bioactive components, being non-nutrient plant chemical compounds, which are responsible for protecting the plant against microbial infections or infestation by pest (Mboh, 2001). The study of natural products on the other hand is called phytochemistry (Doughari *et al*., 2009). While it is well known that plants produce Phytochemicals in order to protect themselves, some researches have shown that many phytochemicals can also protect humans against diseases (Narasinga Rao, 2003). Phytochemicals are found in different parts of plants (Costa *et al*., 1999). Some phytochemicals, for example; phenols, tannins, flavonoids, saponins, carbohydrates, alkaloids and phytosterols are known to reveal medicinal and physiological activities which include anti-bacterial, anti-fungal, anti-oxidant, anti-inflammatory effects (Yadav *et*

al., 2017). The medicinal values of plants lie in possessing chemical substances that produce a definite physiological action on the human body (Hill, 1952).

Antifungal drugs are any drugs that can inhibit the growth of fungi and are used to treat fungal infections or any material that contains fungicides in it. *Tinea versicolor* (also known as *Dermatomycosis furfuracea, Pityriasis versicolor*, or *Tinea flava*) is one of the most common skin fungal infections that are found in East African regions, particularly the coastal regions. It is also found in hot and humid climates and sometimes on people who sweat profusely. This condition is caused by the *Melassezia globosa fungus* which gives the infected tissue on the skin some pigment change (hypo pigment) (Anthony *et al.*, 2017). The treatment of the infected skin has been a pain staking process, since the skin takes time to heal and the condition can recur post-treatment (Bakarnga-via *et al.*, 2016). Some people experience skin irritation upon applying antifungal ointments, while others believe that this infection is going against the taboos. Thus, they prefer the use of *Annona senegalensis* plant tissues and consider these best alternative treatments in contrast to pharmaceutically manufactured medicines (Anthony *et al.*, 2017).

Annona senegalensis is a tropical plant species that is also known as 'wild custard apple'. It is a shrub (2–6 m) or can be small tree (11m) under some suitable ecological conditions. The bark of the plant is smooth, being silver grey or grey-brown. The leaves of this plant are alternate, simple, oblong, ovate or elliptic, green to bluish green and, - mostly lack hairs on the upper surface, but with brownish hairs on the lower surface. Flowers are up to 3 cm in diameter on stalks that are 2 cm long, being solitary or in groups of 2–4, arising above the leaf axils. The fruits of the plant are formed from many fused carpels, being fleshy, lumpy, egg shaped, 2.5–5.0 by 2.5–4.0 cm, ovoid or globose, -. When unripe, the fruits are green, turning yellow to orange on ripening. Wild fruit trees of this species are found in semi-arid to sub-humid regions of Africa (Orwa *et al*., 2009). The plant is native to tropical east and northeast, west and west- central, and southern Africa, as well as southern subtropical Africa. It is also common on islands in the western Indian Ocean. The plant species occur along river banks. They fallow land, swamp, and forests, and could be found at the coast. The species commonly grow as a single plant in the understorey of the savannah woodlands (Orwa *et al*., 2009).

In addition to ethno-medicinal uses *Annona senegalensis*, the plant is also used as food and as food additives (Orwa *et al*., 2009). The leaves are sometimes used as vegetables, while the edible pulp of the ripe fruit has a pleasant pineapple-like taste. Its flowers serve as a spice for various meals (Orwa *et al*., 2009). The use of *Annona senegalensis* for pest control in Nigeria and Tanzania has also been reported (Igoli *et al.*, 2005). In the Republic of Benin, the fresh leaves are spread in poultry houses and are left until they are dried. This practice is repeated once or twice a week, in order to achieve the control of parasites such as fleas and lice (Salifou *et al*., 2012).

Therefore, the objectives of this study were to qualitatively determine the phytochemicals content and the antifungal properties of *Annona senegalensis* aqueous leaves extracts and methanol leaves extracts and to quantitatively determine the phytochemicals content of *Annona senegalensis* aqueous leaves extracts and methanol leaves extracts.

MATERIALS AND METHODS

Solvents

Distilled water and analytical grade (Sigma, Germany) methanol were used for the extraction. Solvents were used without further purification.

Reagents and chemicals

Reagents and other chemicals used in the phytochemical test include: potassium mercuric iodide solution (Mayer's reagent), 0.5 M ammonium hydroxide solution, 5% concentrated sulphuric acid, 0.1 % ferric chloride, 5% ferric chloride solution, 5% dilute hydrochloric acid, 1M ammonia solution, analytical grade chloroform, 2% glacial acetic acid, 3% acetic anhydride, Benedict's reagent, Keller – Killani reagent, Salkowski reagent and Froth reagent (Mon Scientific Nigeria Limited, Lagos).

Sample collection and identification

The fresh leaves of the *Annona senegalensis* (*Annonaceae*) plant used in this study were collected in July 2019 from Kofar Kaura layout, Katsina state Nigreria. The leaves were identified at the Plant Science Department, Bayero University Kano, Nigeria.

Sample preparation and treatment

The leaves samples of *Annona Senegalensis* were subjected to shed-drying for two weeks. The dried leaves were then pounded into a homogenous powder, using mortar and pestle. The powdered sample was then stored in specimen bottles until further use.

Plant extraction and analysis

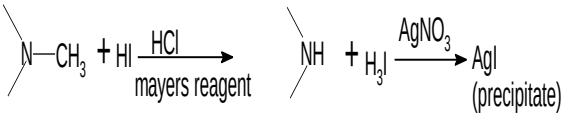
Two different solvents (methanol and distilled water) were used for the extraction procedures. 100 g portions of the powdered sample were separately macerated in 1000 ml of methanol and in distilled water, using a maceration method. Both mixtures were stirred using magnetic stirrer, for 24hours, in order to dissolve the powders. The two mixtures were then filtered using filter papers and were then subjected to heating in a water bath,

for 4 hours. The dried extracts were then analyzed further.

QUALITATIVE ANALYSIS

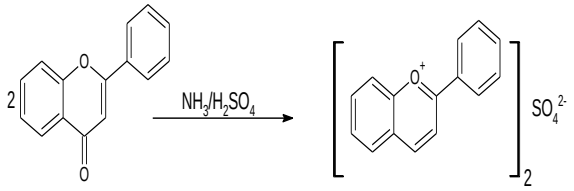
Test for alkaloids (Mayer's Test)

0.5g of the extract was weighed and transferred into a watch glass before, - 1ml of 5% hydrochloric acid and 2 drops of Mayer's reagents were added (Evans, 1997).



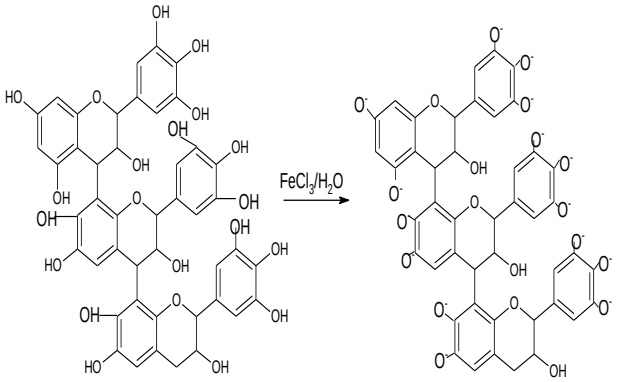
Test for flavonoids

5ml of 1M ammonia solution were added to a portion of the extract sample followed by addition of 1ml of 5% H_2SO_4 (Velavan, 2015).



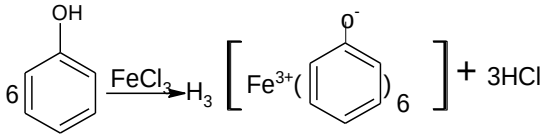
Test for tannins (Braymer's Test)

0.5g of the dried, powdered sample was boiled in 20ml of distilled water in a test tube. The resulting material was then filtered. 2 drops of 0.1% ferric chloride solution was added (Velavan, 2015).



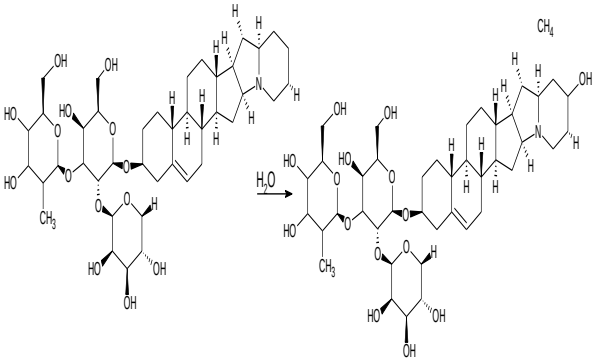
Test for polyphenols

The extracts were dissolved in 5ml of distilled water. 2 drops of 5% ferric chloride solution was added (Sahira and Cathrine, 2015).



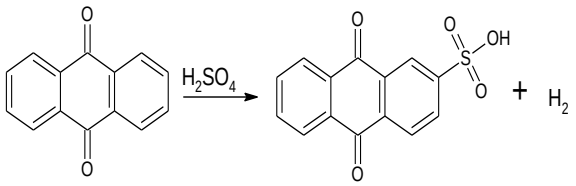
Test for saponins (Froth Test)

The extracts were diluted with distilled water to 20ml and were shaken in a graduated cylinder for 15 minutes (Kokate, 1999).



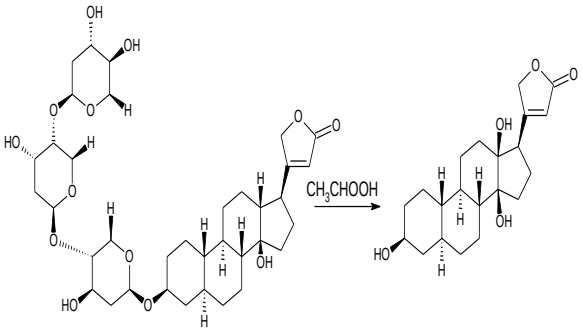
Test for anthraquinones

The extracts were diluted with distilled water to 20ml, 5ml of the filtrates were hydrolysed with 1ml of 5% H_2SO_4. 1ml of 1M ammonia solution was added (Velavan, 2015).



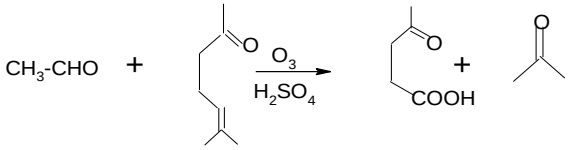
Test for cardiac glycosides (Keller-Killani test)

5ml of the filtrate was treated with 4ml of 2% glacial acetic acid containing one drop of 2% ferric chloride solution. This was then treated with 1ml of 5% sulphuric acid (Velavan, 2015).



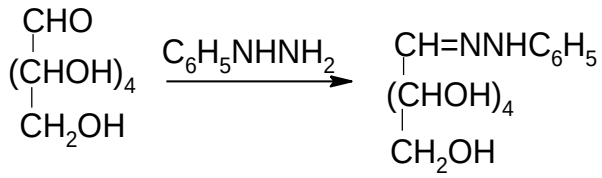
Test for terpenoids (Salkowski Test)

5ml of the filtrate was mixed with 2ml of chloroform, - 3ml of 5% H_2SO_4 was carefully added to form a layer (Velavan, 2015).



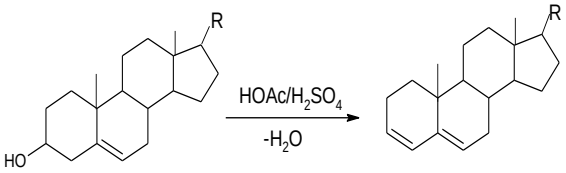
Test for carbohydrates (Benedict's Test)

To a 0.5ml of the filtrate, 0.5ml of the Benedict's reagent was added. The mixture was then heated in a water bath for 2 minutes (Sahira and Cathrine, 2015).



Test for steroids

2ml of 3% acetic anhydride was added into 0.5g of the extract, 2ml of 5% H_2SO_4 was also added (Velavan, 2015).



QUANTITATIVE ANALYSIS

Determination of alkaloids

5g of the sample was weighed and transferred into a 250ml beaker, 200ml of 10% acetic acid in ethanol was then added following which the beaker was covered and allowed to stand for 4hrs. This was then filtered and the filtrate was concentrated on a water bath to one quarter of the original volume. 0.5 M ammonium hydroxide solution was added drop wise until precipitation was complete. The whole solution was allowed to settle. The precipitate was collected and washed with dilute ammonium hydroxide solution and then filtered (Velavan, 2015)

$$\% \text{ Alkaloid } = \frac{\text{weight of alkaloid}}{\text{weight of sample}} \text{ x } 100$$

ANTIFUNGAL SCREENING

Culturing of potatoes dextrose agar (PDA)

7 petri dishes and watch glasses were cleaned and disinfected in the autoclave for two hours. 5.46g of potatoes dextrose agar was dissolved in 140ml of distilled water and then placed in the autoclave for 15 minutes, at 121ºC and at 15 lbs pressures. The agar solution was then removed from the autoclave, poured into the 7 petri dishes and smeared with the potatoes dextrose agar using a sterilized cotton wool.

Fungus inoculation and well-diffusion method

Different solutions were made using a serial dilution method and four (4) Bijou bottles were used for each extract (aqueous and methanol). 0.5g of the extracts were dissolved in 2ml of dimethylsulphoxide (DMSO), in a bottle at 500000 µg/ml loading. 1ml of the solution was transferred from the first bottle to the second bottle containing 1ml of DMSO at a concentration of 250000 µg/ml. A separate 1ml was transferred from the second bottle to the third bottle containing 1ml of DMSO, at 125000 µg/ml. 1ml of the solution was also transferred from the third bottle to the fourth bottle at a concentration of 62500 µg/ml. *Trichoderma spp* was inoculated into 6 petri dishes containing the media, using swab sticks. 4 zones of inhibition were made using the agar well diffusion method, to apply the plant extracts into the inoculated fungi. The zones of inhibition were measured in mm, after 24hours.

Determination of zone of inhibition:

The antifungal activity was evaluated by measuring zone of inhibition using Hi Media zone scale. The diameters of the inhibition zones were measured in mm. Ketoconazole (200mg/ml) was used as a positive control and dimethylsulphoxide (DMSO) was used as a negative control.

Determination of minimum inhibitory concentrations

To determine the minimum inhibitory concentrations of the aqueous extract and the methanol extract of *Annona senegalensis* leaves, 6 test tubes, for each extract were washed and dried. 0.5g of the extract and 0.5ml of the fungi in a test tube at 62.500mg/ml were dissolved in 2ml of the potato dextrose broth (PDB).1ml of the mixture was transferred to the second test tube that contains 0.5ml of the fungi and 1ml of PDB, at a concentration of 31.25mg/ml. 1ml of the mixture was also transferred from the second test tube to the third test tube which contains 0.5 ml of the fungi and 1ml of PDB, at 15.625 mg/ml. Another 1ml of the mixture was transferred from the third test tube to the fourth test tube containing 1ml of PDB and 0.5ml of the fungi, at 7.813mg/ml loading. 0.5 ml of the fungi and 1ml of PDB were poured into the fifth test tube which serves as the positive control (no extract). 1ml of the mixture was transferred from the fourth test tube to the sixth test tube containing 1ml of PDB, with this serving as the negative control (no fungi). Cotton wool was used to cover all the test tubes and results arising from observations on the test tubes were taken after 48hours.

RESULTS AND DISCUSSION

Table 1 shows the physical properties of the extracts (aqueous and methanol) of the *Annona senegalensis* leave. In this, the methanol extract shows higher quantity of the extract than the aqueous extract. A deep brown coloration was observed in the aqueous extract while a brown colored material was observed in the methanol extract. Non-citrus fruity and resinous smells were perceived from both the methanol and the aqueous extracts.

Table 2 shows the qualitative phytochemical screening of the aqueous extract and

the methanol extracts of the *Annona senegalensis* leaves. The data indicates the presence or absence of ten metabolites that were studied. The results showed that among the ten metabolites, six were positive for both the aqueous extract and the methanol extract. While only two were positive for the aqueous extract. One is positive for the methanol extract and one is neither positive for both the extracts.

It was reported that, the phytochemical screening of the leaves of *Annona senegalensis* revealed the presence of various secondary metabolites including tannins (Jada *et al.,* 2014), flavonoid (Jada *et al.,* 2015), saponins (Afolabi and Afolabi 2013), alkaloids (You *et al.,* 1995), glycosides, steroids (Ijaiya *et al.,* 2014), volatile oil (Ngamo *et al.,* 2007), anthocyanins (Mpiana *et al.,* 2012), triterpenes and coumarins.

Table 3 shows the alkaloid content of the aqueous extract and the methanol extract of the *Annona senegalensis* leaves, the data obtained showed that, the aqueous extract contain higher alkaloid content of 1.49g while, the methanol extract showed the lowest alkaloid content of 1.42g.

Phytoconstituents such as alkaloids cardiac glycosides, carotenoids, flavonoids, tannins, phlobatanin and saponins form the basis of natural plant antifungals. These phytoconstiuents have also been identified in Annona senegalensis (Gbeassor *et al.,* 1996). More recent studies also revealed that the methanol extract of the leaves have multiple phtyochemical constituents including: proteins, aminoacids, sterol, terpenoids, acetogenins (Bamba *et al.,* 1984), The leaf extract also contains anthraquinones (Gbeassor *et al.,* 1996) which is known for laxative effects.

Table 4 shows the antifungal activities of the aqueous extract and the methanol extracts of the leaves of *Annona senegalensis* against *Trichoderma spp*. This also shows the zones of inhibition results. The antifungal activity was evaluated by measuring the zone of inhibition using a Hi Media zone scale. The evaluations were carried out in triplicate for both the extracts; with the mean of the data reported in Table 4. At a concentration of 250000µg/ml, the aqueous extract shows the highest value of 14.5mm and the lowest value of 8.5mm at 31250µg/ml. The methanol extract shows the highest value of 13.3mm at 250000µg/ml and a lowest value of 8.3mm at 31250µg/ml. Moreover, persistence and recurrence of coetaneous fungal infections (e.g., tinea versicolor) after antifungal treatment, has been a growing problem contributing to unregulated use of medication and growth of resistance (Levy, 2002). Thus, the leaf extracts of *Annona senegalensis* were shown to have highest concentration of phytochemicals, thus; supporting its medicinal efficacy incomparable to other parts of the plant (McLaughlin *et al.,* 1991). The high antifungal activity of the aqueous extract and the methanol extracts of the leaves of *Annona senegalensis* may give an alternative approach to combat resistant pathogens (Van Vuuren, 2011).

Table 5 shows the minimum inhibitory concentrations of the aqueous extract and the methanol extracts of the *Annona senegalensis* leaves. From the result of the aqueous extract, it was observed that the extract inhibits the growth of the fungi in the first test tube at a minimum concentration of 62.500mg/ml while there is growth of fungi in the second, third and fourth test tubes. The result of the methanol extract shows that the extract inhibits the growth of the fungi in the first and second test tubes at 31.250mg/ml but there is growth in the third and fourth test tubes.

Currently, there are only three classes of marketed antifungal drugs: amphotericin B and its various formulations that target the cell membrane; azoles (e.g., fluconazole, itraconazole, posaconazole, and voriconazole) that block ergosterol biosynthesis; and echinocandins (e.g., caspofungin, micafungin, and anidulafungin), which target cell wall biosynthesis (Ostrosky-Zeichner *et al.,* 2010) This current study confirms that these plant extracts can offer an alternative antifungal medicine, particularly against the stubborn Malassezia globosa fungus.

Table 1: Physical characteristics of the aqueous extract and the methanol extract of *Annona senegalensis* leaves

S/No	Plant sample (Solvent)	Quantity of plant used (g)	Quantity of solvent used (ml)	Quantity of extract obtained (g)	Colour	Odour	Texture
1.	*Annona Senegalensis* (aqueous)	100	1000	7.8	Deep brown	Resinous	Dried
2.	*Annona Senegalensis* (methanol)	100	1000	8.3	Brown	Non -itrus fruity	Dried

Table 2: Physical screening of aqueous extracts and the methanol extract of *Annona senegalensis* leaves.

Plant sample (solvent)	Saponins	Tannins	Cardiac glycoside	Steroids	Alkaloids	Flavonoids	Polyphenols	Terpenoids	Anthraquinones	Carbohydrates
Annona Senegalensis (aqueous)	-	+	+	+	+	+	+	+	-	+
Annona Senegalensis (methanol)	+	-	+	+	+	+	+	-	-	+

Table 3: Alkaloid content of the aqueous extract and the methanol extract of the *Annona senegalensis* leaves

S/No	Plant sample (Solvent)	Quantity of alkaloid (g)
1.	*Annona Senegalensis* (aqueous)	1.49
2.	*Annona Senegalensis* (methanol)	1.42

Table 4: Anti- fungal activities of the aqueous and methanol extracts of *Annona senegalensis* against *Trichoderma spp* (zone of inhibition in mm).

Plant sample (solvent)	**Plant extracts concentrations in $\mu g/ml$**					
	250,000	**125,000**	**62,500**	**31,250**	**Positive control (ketoconazole 200mg/ml)**	**Negative control(DMSO)**
Annona Senegalensis (aqueous)	14.5	12	9.5	8.5	36	0
Annona Senegalensis (methanol)	13.3	10.6	10	8.3	36	0

Table 5: Minimum inhibitory concentrations of the aqueous and methanol extracts of *Annona senegalensis* leaves.

Plant extract (solvent)	**MIC**
Annona Senegalensis (aqueous)	62.500mg/ml
Annona Senegalensis (methanol)	31.250mg/ml

CONCLUSIONS

Annona senegalensis or African custard-apple is a potent medicinal plant that is generally used traditionally in the treatment of many diseases. Many researchers have been isolating bioactive compounds from the plant. The results obtained from this study have shown that the phytochemical screening of the aqueous extracts and the methanol extracts of the *Annona senegalensis* leaves revealed the presence of metabolites such as alkaloids, flavonoids, polyphenols, cardiac glycoside, carbohydrates and steroids. The aqueous extracts and the methanol extracts arising from the plant leaves have demonstrated significant antifungal activities against *Trichoderma spp* and were found to be useful in inhibiting the growth of *Trichoderma spp* at evaluated concentrations. The presence of bioactive compounds in the plants and its known pharmacological activities have proved the potency of the plant in the development of anti – fungal materials.

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