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Nutraceutical Composition and Antimicrobial Activity of *Cyperus Esculentus* (Tiger Nut) Against Urinary Tract Infection Pathogens

Imaobong E. Daniel and Etukudo Edigeal D.

Department of Chemistry, University of UYO, PMB 1017, UYO, Akwa Ibom State

Abstract

Phytochemical composition, antioxidant vitamins (vitamins A, C and E) and antimicrobial properties of methanol and ethyl acetate extracts of *Cyperus esculentus* (tiger nut) activity against human urinary tract infection pathogens (*Escherichia coli, Staphylococcus aureus and Klebsiella pneumonia*) were evaluated to validate the medicinal potential of this nut. The total polyphenol, flavonoid and alkaloid contents were determined according to Folin-Ciocalteu method, Aluminum colorimetric method and Harborne method respectively. Antioxidant vitamins were determined using standard procedures while the Disc diffusion method was employed in evaluating the antimicrobial activity of the extracts. The results indicated that the bioactive compounds (total phenol, flavonoids and alkaloids) determined quantitatively were present in appreciable concentration in both extracts. However, the concentration of these bioactive compounds determined was significantly higher in the methanol extract than the ethyl acetate extract. This implied that methanol may be a better solvent for extraction. Antioxidant vitamins composition in the different extracts indicated that tiger nut contained appreciable amount of these vitamins. However, the concentrations of these vitamins were considerably higher in the methanol extract, with vitamin E exceeding the daily recommended intake by international standards in both extracts. The susceptibility of these isolates towards the tiger nut extracts was compared with each other and with gentamycin, which was used as a positive control. All plant extracts showed antimicrobial activities against the selected microorganisms at various concentrations and the methanol extract was found to be most effective compared to ethyl acetate extract. Methanol extract exhibited maximum antibacterial activity against *E.coli* (12.0±1.0 at 100mg/ml) while ethyl acetate extract exhibited maximum antimicrobial activity against *Klebsiella pneumonia* (11.0 ±1.2 mm at 100mg/ml). The results obtained in this study clearly demonstrated higher and broad spectrum antibacterial activity of tiger nut extracts on all three UTI isolates and this which may be attributed to the presence of phenolic compounds and other phytochemicals. These results further authenticates the medicinal importance of tiger nut as it vast and diverse bioactive compounds of immense pharmaceutical importance.

Keywords: *Cyperus esculentus*, Folin-Ciocalteu method, Aluminum colorimetric method, Alkaloids, Gentamycin.

1.Introduction

Urinary tract infections (UTI) are the most ubiquitous extra intestinal diseases caused by pathogenic bacteria. They affect any part of the urinary tract which could be the kidney, ureter, bladder and urethra. The causes of UTIs include sexual intercourse with infected persons, poor hygiene, holding urine longer than necessary, using diaphragm singly or with spermicides or condoms, underlying kidney stones, diabetes, loss of oestrogen and catheter [1]. Most common pathogens remain *Esherichia coli* while others include *Staphylococcus species, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella* etc. [2]. All over the world, millions of people are diagnosed with urinary tract infections (UTI) every year [3]. It is estimated that about 35% of healthy individuals suffer from symptoms of UTI at some stages in their lives, with incidences occurring mostly in women than men.

Corresponding address: *e-mail*: imaudoekwere@gmail.com. Phone number: +2348052412933

This may be attributed to anatomical predisposition or urothelial mucosal adherence to the mucopolysaccharide lining or other host factors [4, 5]. When antioxidant capacity is limited, the lifespan of immune cells is reduced and an infection can become established or severity of an infection can increase [6]. Urinary tract infections are usually treated with broad spectrum antibiotics, which in most cases ensure short and long term cure. Unfortunately, these pathogens have gradually developed resistance to these drugs due to indiscriminate and improper use of most commercial antimicrobial drugs commonly used in the treatment of infectious diseases [7]. The increasing prevalence of antibiotic resistant bacteria, escalating costs of antibiotic therapy and unsatisfactory therapeutic alternatives in recurrent UTIs have stimulated an interest in novel, non-antibiotic based methods for preventing and controlling UTIs [8, 9]. As a result of the emergence of multi drug resistant strains of pathogenic bacteria, efforts are ongoing to check the potential of medicinal plants so as to combat with these pathogenic agents [10, 11]. Many epidemiological studies show that consumption of fruits, nuts and vegetables is able to reduce the risk for some major human chronic diseases due to the presence of phytochemicals and antioxidants such as ascorbic acid (hydrophilic antioxidants), tocopherols, carotenoids (lipophilic antioxidants), certain minerals, such as zinc and selenium, phenolic compounds and anthocyanins [12, 13, 14]. Among these bioactivities, the antibacterial activities have attracted much interest due to the potential in dealing with the drug-resistant bacteria that are insensitive to conventional antibiotics [15].

These phytochemicals possibly act by directly reacting with and quenching free radicals, chelating transition metals, reducing peroxides, and/or stimulating the antioxidative defence enzyme system therefore promoting health [16]. Polyphenols have been reported to exhibit antibacterial activities with distinguished characteristics in their reactivity with proteins related polyamides polymers [17]. Flavonoid belongs to the family of polyphenols. They are water insoluble and are found in most plant materials. Flavonoids are well known for their anti-oxidants, anti-carcinogenic, anti-microbial and anti-tumor properties [18]. Epidemiological studies have demonstrated that heart diseases are inversely related to flavonoid intake [19].

Studies have shown that flavonoids prevent the oxidation of low density lipoprotein thereby reducing the risk for the development of atherosclerosis. Vitamin C, also known as ascorbic acid, is a water soluble vitamin and is not easily stored in the body. It is a well-known antioxidant that efficiently scavenges free oxygen radicals The presence of an enediol group in the structure of vitamin C allows electron availability and makes it a member of an oxidation-reduction system with electron donating or accepting potential [20, 21]. Vitamin E is a fat-soluble vitamin which include four tocopherols (α, β, δ, γ) and four tocotrienols (α, β, δ, γ) Vitamin E has proved to be effective in preventing lipid peroxidation and other radical driven oxidative events. In the membrane-water interphase, the reaction between vitamin E and lipid radical takes place and vitamin E donates a hydrogen ion to lipid radical resulting in tocopheroxy radical formation which is regenerated back to its reduced form by vitamin C, reduced glutathione and coenzyme Q [22, 23]. Vitamin E is synthesized only by plants therefore, it is found primarily in plant products, the richest sources being vegetable oils and to a lesser extent, seeds, nuts and cereal grains. A wide variety of secondary metabolites, which are used either directly as precursors or as compounds in the pharmaceutical industry are produced by plants. It is expected that other than plant used by antibiotics, plant extracts showing target sites will be more active against drug-resistant microbial pathogens [24]. Vitamin A is a natural antioxidant that inhibits free radicals and is essential for normal vision, gene expression, growth and immune function by its maintenance of epithelial cell functions [25].

Cyperus esculentus is a monocotyledonous plant and belongs to the family *Cyperaceae* which is made up of over 4000 species [26]. Its common names include; tiger nut, Aya, chufa sedge, yellow nut sedge and earth almond. It is a perennial grass that grows in wet areas and often occurs as a weed especially on farmlands used for cultivation of vegetables [27]. In Africa, tiger nut is mostly cultivated in the west, Ivory Coast, Ghana, Mali, Niger, Nigeria, Senegal and Togo where they are used primarily uncooked as a side dish [28]. It has long been recognised as one of the best nutritional crops used to augment diets, since a substantial intake decreased reported cases of various health related conditions such as cardiovascular disease, diabetes, cancer, and obesity, and also ideal for

children, older persons and sportsmen [29], as well as an excellent source of iron and calcium for body growth and development [30]

Cyperus esculentus is valued for the highly nutritional starch content, dietary fibre and digestible carbohydrate of monosaccharide, disaccharides and polysaccharides [31]. The nut was also reported to be rich in sucrose, protein, minerals and fat, which are resistant to peroxidation [32]. Belewu and Belewu [33] reported that *Cyperus esculentus* can be taken by diabetics mainly for its sucrose and starch and for its high content of arginine which is reported to stimulate the production of insulin. It is believed that they help prevent thrombosis/cancer and activates blood circulation. They are equally thought to be beneficial in cholesterol lowering activities and as such implicated in the reduction of colon cancer [34]. It is also responsible for preventing and treating urinary tract and bacterial infection. Apart from the ethnopharmaceutical documentations of the uses of tigernut in the treatment of diseases, there have been numerous scientific researches and studies on the nutraceutical and benefits of consuming this nut. [35] investigated the phytochemical, proximate composition, amino acid profile and the effects of the extracts of two varieties of *Cyperus esculentus* (tiger nuts) on sickle cell blood. The results obtained indicated that the two varieties of *Cyperus esculentus* studied are very nutritious and exhibited high antisickling effectiveness by inhibiting sickle cell haemoglobin gelation and improving the oxidant status of the erythrocytes. [36] equally determined the proximate composition as well as in – vitro antisickling property of tiger nut. Three concentrations (100%, 50% and 20%) of the extracts were used. Both extracts demonstrated pronounced anti-sickling activity [through inhibition of haemoglobin-S (HbS) gelation]. The methanol extract, however, showed more pronounced inhibition of HbS. [37] determined the antioxidative and antimicrobial activities of the phenolic extract of tiger nut tubers. The results showed that that tiger nut phenolic extract can be used as natural antioxidants against oxidative rancidity in corn oil. Also, this extract possessed remarkable activity as antimicrobial agents against some microorganisms. [38] investigated the chemical constituents of *Cyperus esculentus* seed oil evaluated its potential antimicrobial activity. 21 components were detected by GC-MS analysis. Major constituents are: 9-octadecenoic acid (46.24%), hexadecanoic acid (19.27%), 9,12-octadecadienoic acid(13.62%), and methyl stearate (10.88%), Butylated hydroxytoluene, a potent antioxidant, was detected as a minor constituent (0.10%). The oil showed different antimicrobial responses against test organisms. It gave significant activity against the fungus: Candida *albicans* and partial activity against *Staphylococcus aureus*. The effect of aqueous extract of *cyperus esculentus* on some Liver functional indices in rabbit studied by [39] indicated that *Cyperus esculentus* may have some clearly definable hepatotoxic properties, probably, if taken in large quantities and for a prolonged period. According to different researchers who determined the fatty acid profile of tigernut oil, the consumption of the oil from *Cyperus esculentus* tuber decreases the risk of cardiovascular diseases because of the low content of saturated fatty acid [40, 41]. It was also reported to help in preventing thrombosis, and the tuber was also implicated in the reduction of colon cancer [42].

2. Materials and Methods

Fresh Tiger nuts (*Cyperus esculentus*) were obtained from Itam local market, Uyo, Akwa Ibom State. The nuts were thoroughly washed and dried in hot air oven at 40^0C for 72 h, after which they were ground into fine powder using electric blender and stored in an Air-tight container at room temperature prior to extraction. Approximately 107.42g each of the powdered material was extracted by cold maceration method with methanol and ethyl acetate and left for 72 hours with intermittent shaking. The extracts was filtered and then concentrated using rotary evaporator at 40 ^{o}C, and each extract was transferred into well labelled sterile glass vials and stored at 4 ^{o}C before use.

*Determination of Polyphenols:*The total phenolic content in the extracts were determined by the modified Folin-Ciocalteu method as described by [43] and modified by [44]. Sample extract was dissolved in methanol (1 mg/ml). An aliquot of 0.5 ml of each plant extract (1 mg/ml) was mixed with 5 ml of Folin- Ciocalteu reagent which was previously diluted with distilled water (1:10 v/v). The mixture was shaken slightly and allowed to stand at 22 °C for 5mins. After, 4 ml (75 g/L) of sodium carbonate (Na_2CO_3) was added, and the tubes containing the mixtures were allowed to stand for 30 min at 40 °C to develop colour.

Absorbance was then read at 765 nm using the spectrophotometer. Results were expressed as Gallic acid equivalent in (mg/g) of extracts. All samples were analyzed in triplicate.

*Determination of total flavonoids:*Total flavonoid contents were determined using the method of [45]. A volume of 0.5 ml of 2% $AlCl_3$ ethanol solution was added to 0.5 ml of sample solution. After one hour at room temperature, the absorbance was measured at 420 nm. A yellow color indicated the presence of flavonoids. Extract samples were evaluated at a final concentration of 0.1 mg/ml. Total flavonoid content were calculated as quercetin equivalent (mg/g).

Determination of total alkaloids: Total alkaloids were determined according to standard method as described by [46]. 5 g of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.

Determination of Vitamin A (carotenoids): Vitamin A was determined using Antimony trichloride method by [47]. About 0.5 g of each extract was taken into a beaker. 10 mL of chloroform was added to the extract and tested with 1 ml antimony trichloride reagent to develop blue colour. Reading was done using UV spectrophotometer at 620 nm.

Determination of Vitamin C: The extraction of the ascorbic acid was carried out with 0.5 g of each sample in 50 ml of oxalic acid. The mixture was filtered to remove suspended particles. 0.1 g of diatomaceous earth was added to the filtrate and agitated for 15 minutes. The mixture was filtered and colour development was done with 2, 4 – dinitrophenyl hydrazine and absorbance read at 520 nm.

*Determination of Vitamin E (Tocopherol):*This was determined spectrophotometrically using a modified standard method of [47]. 0.5 g of each of the plant extracts was extracted with 0.5 ml of ethanol and shaken for 1 minute. 3 ml of xylene was then added and centrifuged to separate extract. 1 ml of the extract was added to 1 ml.

Antimicrobial Susceptibility Assay: Disc diffusion assay as described by [48] was the key process used in evaluating the antibacterial potential of tiger nut extracts. The clinical isolates used (*Escherichia coli*, *Staphylococcus aureus and Klebsiella pneumonia*) were sub- cultured and incubated at 37^0C for 24hrs. Different concentrations (100, 50, 25 and 12.5mg/ml) of the extracts were prepared and kept in corked test tubes. The antibiotic media Mueller Hinton Agar (Oxoid, UK) was prepared and poured into Petri dishes. The sub- cultured isolates were then seeded on the solidified agar. Pre sterilized filter paper disc was soaked in the various concentrations for 30 minutes and allowed to dry. The impregnated disc was transferred aseptically to the seeded plates. A control antibiotic disc (Gentamycin) was transferred aseptically to the centre of the seeded plates. The seeded plates were kept for 30 mins before incubating in an upright position for 24 hrs. Zones of inhibition were measured and recorded for the isolates susceptible to the extracts.

Total phenols: The concentration of antioxidant components in the different extracts of tiger nut are shown in Table 1. Phenolic contents are expressed as mg Gallic acid equivalent (GAE)/mg of plant extract. From the results obtained, there was significant amount of total phenols present in both extracts. However, the concentration of total phenols was significantly higher in the methanol extract comparable to ethyl acetate extracts. This may be attributed to the polarities of different solvents as well as the chemical nature of the endogenous extractable compounds. Phenolic compounds are hydroxylated phenolic compounds which are synthesized in plants. Examples include hydroxycoumarins, hydroxycinnamate derivatives, flavanoids, flavonols, flavanones, flavones, anthocyanins, proanthocyanidins (tannins), hydroxystilbenes, aurones, etc. Polyphenols are well known to exhibit broad spectrum antimicrobial activities against a huge number of pathogenic bacteria [49, 50, 51]. The mechanism of polyphenols against microbes may be related to inhibition of hydrolytic enzymes (proteases and carbohydrolases) or other interactions to inactivate microbial adhesins, cell envelope transport proteins and non specific interactions with carbohydrates, etc [50].

In addition, these compounds have anti-inflammatory, anti-carcinogenic and anti-atherosclerotic activities, showing a broad field of application for the phenolics in these plants [52, 53]. Phenolic compounds are also natural antioxidants that have the ability to act as efficient radical scavengers, metal chelators, hydrogen donors and singlet oxygen quenchers [54, 55, 56, 57, 58]. This study indicates that there may be a strong linear relationship between the concentration of polyphenols and the underlying principle behind medicinal importance of tiger nut.

Table1. Antioxidant compounds in extracts of tiger nut

Phenolic phytochemicals	Methanol	Ethyl acetate
Total phenol (mgGAE/mg)	122±1.23	71.21±0.25
Total flavonoids (mgQE/g)	82.40±0.03	56.15 ±0.06
Alkaloids	21.4 ±0.01	23.14±1.4
Vitamin A(mg/100g)	4.1±0.01	0.79±0.01
Vitamin C (mg/100g)	18.37±1.3	12.4±0.71
Vitamin E (mg/100g)	183.4 ±1.34	132.2±0.01

Table 2. Antibacterial Activities of methanol Extract of tiger nut.

Methanol extract concentration(mg/ml)	Zone of inhibition in mm. Bacterial pathogens		
	Escherichia coli	*Staphylococcus aureus*	*Klebsiella pnuemoniae*
12.5	00	00	00
25.0	00	8.0±1.5	00
50.0	8.0±2.0	9.0±1.5	9.0±1.2
100.0	12.0±1.0	11.0±1.3	10.0±1.5
Gentamycin	25.0±1.0	22.0±0.5	24.0±1.0

Table 3. Antibacterial Activities of ethyl acetate Extract of tiger nut

Ethyl acetate extract concentration(mg/ml)	Zone of inhibition in mm. Bacterial pathogens.		
	Escherichia coli	*Staphylococcus aureus*	*Klebsiella pnuemoniae*
12.5	00	00	00
25.0	00	00	00
50.0	00	8.0±1.5	8.0±1.2
100.0	9.0 ±2.2	10.0 ±0.8	11.0 ±1.2
Gentamycin	25.0±1.0	22.0±0.5	24.0±1.0

Total flavonoids: This study showed varying concentration of flavonoids in methanol and ethyl acetate extracts. Methanol extract contained significantly high flavonoid content when compared to that of ethyl acetate. Comparing the flavonoid content of the different extracts of tiger nut with the phenolic content, it was observed that methanol also extracted the highest phenol. Their antimicrobial properties of flavonoids are probably because they form complexes with both extracellular and soluble proteins, as well as bacterial cell wall. They could also disrupt cell membranes if lipophilic enough [50]. Flavonoids are equally free radical scavengers that prevent oxidative cell damage, have strong anticancer, antiallergic, and anti-inflammatory activities [59, 60]. It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The presence of flavonoids in extracts of tiger nuts studied enhances its antimicrobial and antioxidant capacity.

Alkaloids: The presence of alkaloids in the different extracts of tiger nut in appreciable amounts indicate that tiger nut possess a strong antibacterial activity. Alkaloids, comprising a large group of nitrogenous compounds are widely used as cancer chemotherapeutic agents [61, 62]. Alkaloids also interfere with cell division; hence the presence of alkaloids in tiger nut could account for its use as an antimicrobial agent [63]. Alkaloids have been reported to possess various pharmacological activities including antihypertensive effects, antiarrhythmic effect, antimalarial and anticancer

activity [64]. Pure isolated alkaloids and their synthetic compounds have been used in medicine as an analgesic, antispasmodic and bactericidal agents [65, 66].

Vitamins: The results as presented in Table 1 indicated that tiger nut extracts contained a reasonable concentration of both water soluble Vitamins. (Vitamin C) and fat soluble vitamins (vitamins A and E). The concentration of vitamin A in methanol and ethyl acetate was the lowest while vitamin E recorded the highest concentration in both extracts. The concentration of vitamin C was higher in the methanolic extract compared to the ethyl acetate extract. It has been observed that the medicinal activity of plant extracts is not limited to only phenolics, but may come from the presence of other secondary metabolites such as volatile oils, carotenoids and vitamins [67].

Vitamin A is an essential nutrient needed for normal growth, reproduction, embryonic development, vision and immune function [68]. Vitamin A deficiency can lead to two major disorders like: night blindness and drying of the skin. In this study, the concentration of this vitamin A in both extracts was below the recommended dietary allowance (RDA) for vitamin A which is 900 µg,

Vitamin C is very important for cardiovascular health and reducing free radicals in the cells.

This study revealed that both extracts contained an appreciable amount of vitamin C. According to WHO [69], the recommended dietary allowance of vitamin C is 45mg per day. The result in this study indicated that the level of vitamin C in both extracts were below the recommended dietary allowance by WHO. It should be noted that despite the low levels of Vitamins A and C in both extracts when compared to international standards, consumption of tiger nuts will contribute in meeting the daily vitamin requirement as stipulated for healthy adults. It has been cited that vitamin C is capable of regenerating vitamin E and also facilitates the absorption of dietary iron from the intestine [70, 71].

The concentrations of vitamin E in this study exceeded the recommended dietary allowance of vitamin E which is15 mg/day of α – tocopherol. Vitamin E serves as the 1st line of defense against peroxidation of phospholipids [72].

It is especially effective in protecting low density lipoproteins (LDL) from oxidation. It corroborates with vitamin C to slow progression of cardiovascular diseases and protects the double bonds of beta carotene from oxidation and thus exhibits a sparing effect [73]. Fat soluble vitamin is considered the main antioxidant in biological membranes and many of its beneficial effects in immunoregulation are attributed to its radical scavenging activity in lipophilic environments, resulting in the stabilization of polyunsaturated fatty acids in membrane lipids [67, 74].

Biologically active compounds from natural sources have always been a great interest for scientists working on infectious diseases [75]. It is also worthy to note that many plants have been evaluated not only for their inherent antimicrobial activity, but also for their action as a resistance-modifying agent [76].

Antimicrobial activity of methanol and ethyl acetate extracts of tiger nut on different urinary tract infection organisms (E*scherichia coli*, *Staphylococcus aureus and Klebsiella pneumonia*) was determined and results presented in Tables 2 and 3. Methanol was selected as polar solvent and ethyl acetate was chosen as a semi polar solvent. The results presented in Tables 2 and 3 show that the extracts exhibited marked antimicrobial activities.The result of the antimicrobial activities of tiger nuts extracts revealed that the ethyl acetate and Methanol extracts of tiger nut at different concentrations (100, 50, 25 and 12.5 mg/ml) showed varying degree of inhibition on the different test isolates, with more significant inhibition seen with a higher extract concentration. In the present study, methanolic extract was found to be more resistant to all the organisms than the ethyl acetate extract. E*scherichia coli* showed the maximum inhibition (12.0±1.0mm) at 100mg/ml while *Klebsiella pneumonia* showed the least sensitivity (10.0±1.5mm) at 100mg/ml in methanol extract. It is worthy of note that *Staphylococcus aureus* was susceptible at all concentrations except at 25mg/ml, with inhibition ranging from 8.0 ±1.5mm to 11.0±1.3mm However, for ethyl acetate, the most susceptible bacteria at 100mg/ml was *Klebsiella pneumonia* (11.0 ±1.2mm). Inhibition of test bacteria by gentamycin was higher when compared to that of the extracts.

The variation among the zone of inhibition for the various bacteria isolates tested could be associated to the cell wall characteristics of the organisms,

physiology, metabolism, nutrition and biochemistry of the various bacteria isolates [77, 78, 79].

According to Junior and Zanil, [80], diameter of the inhibition zone: <9 mm is inactive; 9-12 mm, partially active; 13-18 mm, active; >18 mm, very active. Thomas *et al*., [81] equally suggested that the plant extract disc showing an inhibitory zone greater that (8mm) were considered as sensitive to the particular pathogen. From the results obtained, methanol and ethyl acetate extracts of tiger nut showed significant antimicrobial activity at higher concentrations against the test isolates based on the criteria suggested by Junior and [80] and [81]. On the other hand, the efficiency of these extracts against the bacteria could in part be because of their phenolic composition [82, 83]. The inhibitory effect of phenolic compounds could be explained by adsorption to cell membranes, interaction with enzymes, substrate and metal ion deprivation [49]. However, further purification and isolation of the active compounds in these extracts are required for pharmacological evaluation because tiger nut may be a potential source of a new type of antibiotic.

4. Conclusion

Methanol and ethyl acetate extracts of tiger nut were obtained and used in this study. The results revealed that these extracts were rich sources of polyphenols, flavonoids and alkaloids. These extracts equally contained appreciable concentration of antioxidant vitamins such as vitamins A, C and E. It is worthy of note that all the extracts in this study exhibited a broad spectrum antibacterial activity on UTI causing bacteria with highest activity recorded for methanolic extract. This may be attributed to its vast concentration of phytochemical compounds. Further studies should be carried out on tiger nuts for comprehensive structural elucidation of bioactive compounds in order to formulate a new drug to treat urinary tract infections.

Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human / or animal subjects (if exist) respect the specific regulation and standards.

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