# ANTIOXIDANT ACTIVITY AND EFFICACY OF NUTMEG EXTRACTS AGAINST URINARY TRACT INFECTION PATHOGENS

**Imaobong,** E. Daniel[1], **Chinemerem,** P. Chukwudire[1]

[1]*Department of Chemistry, University of UYO, PMB 1017, UYO, AKWA IBOM STATE.*
E-mail address: imaudoekwere@gmail.com

***Abstract***
*Methanol and ethyl acetate extracts of nutmeg (Myristica fragrans) seeds were investigated for their antioxidant capacity and antimicrobial activity against human urinary tract infection pathogens (Escherichia coli, Staphylococcus aureus and Klebsiella pneumonia). The antioxidant activities of these extracts were evaluated using the vitro by 2, 2'-diphenyl-1-picrylhydrazyl (DPPH method) and Ferric reducing/antioxidant power (FRAP) assay while the antimicrobial activity was determined using Disc Diffusion method.Folin‑Ciocalteu reagent method was employed for the determination of total phenolic contents. The result obtained revealed that the extracts showed dose dependent scavenging of DPPH as well as ability of the extracts to reduce $FeCl_3$ solution, with methanol exhibiting the highest scavenging and reducing capacity.The susceptibility of these isolates towards the seed extracts was compared with each other and with gentamycin, which was used as a positive control. Results obtained showed that these extracts were able to inhibit the growth of the isolates at various concentrations. It was also observed that methanol extract exhibited a stronger antibacterial activity against all isolated than the ethyl acetate extracts. On comparing the zones of inhibition of the extracts with that of the standard (Gentamycin), the results showed that the zone of inhibition of the standard for all the tested isolates was greater than that of the extracts.The phenolic content in the methanol extract was higher, thus establishing the fact that the antioxidant and antimicrobial activity of these extracts is directly proportional to the phenolic content present.This research further corroborates the potential of nutmeg as a broad spectrum antimicrobial agent, which may be considered as an alternative to common antibiotics to treat infectious diseases.*

**Keywords:** Urinary Tract Infection, Gentamycin, Disc Diffusion Method, Antimicrobial Activity, Folin‑Ciocalteu reagent Ferric reducing/antioxidant power (FRAP) assay.

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## INTRODUCTION

Human beings are continually exposed to potential harmful pathogens throughout their life which result in various diseases and have a great impact on their health. As a result of these, many potent antibiotics had been discovered and used over time. Despite the efforts at curbing most of the infections, bacterial pathogens have developed numerous defence mechanisms against antimicrobial agents; hence resistance to old and newly produced drugs is on the rise. A urinary tract infection (UTI) is a bacterial infection that affects any part ofthe urinary tract which could be the kidney, ureter, bladder and urethra. It is the second most common infection next to respiratory tract infection in the human body, and it remains one of the most common infections globally with no less than half of all women having at least one episode of UTIs during their life time (Luthje and Brauner, 2016; Bashir *et al.*, 2008). 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 (Barini-Garcia and Whitmore, 2008). Most common pathogens remain *Esherichia coli* while others include *Staphylococcus species, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella* etc (Luthje and Brauner, 2016).It is recognized that in some developing countries, plants are the main medicinal source to treat various infectious diseases in view of the fact that the antibiotics are sometimes associated with adverse side effects to the host including hypersensitivity, immunosuppressive and allergic reactions. Free radicals and other reactive oxygen species are produced in the human body during various physiological and biochemical processes. Oxidative stress causes

damage to cell components, such as proteins, lipids and nucleic acids, eventually leading to many chronic diseases, such as cardiovascular diseases, cancer, atherosclerosis, diabetes, aging, and other degenerative diseases in humans and cell death (Vaz *et al.,* 2011:Rahimi *et al.,* 2005; Wright *et al*., 2006; Gladine *et al*., 2007: NazIroglu *et al*., 2004; Emekli-Alturfan *et al*.,2009).There are a number of endogenous antioxidant enzymes, such as glutathione peroxidase, catalase and superoxide dismutase, which are capable of deactivating free radicals and therefore maintaining optimal cellular functions, however, endogenous antioxidants may not be sufficient to maintain optimal cellular functions under increased oxidative stress Furthermore, studies have shown that there is decline in viability and potency of the human's antioxidants as individual ages (Sanz andStefanatos 2008; Liochev 2013). As a result of these, dietary antioxidants may be necessary (Rahman 2006: Kurutas 2016).However, under the conditions of prolonged oxidative stress an exogenous supply of antioxidants is warranted in order to maintain the redox homeostasis and keep the debilitating diseases in check. Researches over the years have produced convincing evidence towards application of natural antioxidants in place of the synthetic molecules as the later have associated toxicities (Moure *et al.,* 2001; Tseng, 2006). Consequently, there is an urgent need to look for alternative to synthetic antibiotics and other alternate source of drugs. As a part of the growing consciousness of dietaryhabits, herbs and spices are becoming an important sourceof natural antioxidantswhich have been reported to have antioxidant and antimicrobial properties besides their numerous folk medicinal usage(Hinneburg *et al.,* 2006; Yano *et al.,* 2006: Gutteridge and Halliwell 1994: Schuler 1990: Kanner *et al*., 1994).

Although the primary purpose of spices is to impart food flavour, the antimicrobial and antioxidant properties have also been exploited, and are generally considered safe and proved to be effective against certain diseases(Souza, *et al.,* 2005).

*Myristica fragrans* is a tropical plant from the family of *Myristicaceae.* The fruit of this plant is well known as nutmeg, a yellow colour fruit that almost looks like an apricot. Nutmeg has a characteristic pleasant fragrance and has a slightly warm taste. It is used to flavour many kinds of baked goods, confections, pudding, meats, sausages, sauces, vegetables and beverages (Panayotopoulos and Chisholm, 1970). In alternative medicine, nutmeg is used for treating diarrhoea, mouth sores, insomnia, as aphrodisiac, memory enhancer, antidiarrhoeal, anti-inflammatory and anticancer drug. (Somani and Singhai, 2008; Tajuddin *et al.,* 2003; Grover *et al.,* 2002; Olajide, *et al.,* 1999).

The essential oil of nutmeg is used externally for rheumatism and possesses analgesic and anti-inflammatory properties (Santos *et al.,* 1997; Olajide *et al.,* 1999).Nutmeg extracts have a potential use as antifungal and antibacterial (Indu *et al.,* 2006; Takikawa *et al.,* 20002; El malti *et al.,* 2008). This later antibacterial effect is interesting, as it appears to single out pathogenic bacteria while leaving normal flora unharmed. For example, the *157 E coli* strain is sensitive to nutmeg extract while the non-pathogenic strains of *E. coli* are not. A similar phenomenon happens in the mouth. *Streptococcus mutant*, the bacteria that cause cavities, is killed by nutmeg extract but the harmless bacteria are unaffected.

The chloroform extract of nutmeg inhibited the carrageenan-induced rat and produced a reduction in writhing induced by acetic acid in mice (Olajide *et al.,* 1999).The trimyristin and the acetone insoluble fraction of the hexane extract of nutmeg seed demonstrated anxiogenic and antidepressant activities in mice (Sonavane *et al.,* 2001; Dhingra and Sharma 2006). According to Gupta *et al.,* (2013), nutmeg seeds extracted with acetone, ethanol, methanol, butanol and water showed significant antioxidant and antimicrobial activities against *B. Subtilis, Staphylococcus aureus, P.putida, P. aeruginosa, A. fumigates, A. niger and A. flavus.* Study carried out by Has *et al.,* (2014) indicated that the essential oil of nutmeg may be used in the treatment of

epilepsy as evidenced it its use traditionally. Anandharaj and Varghese (2015) determined the spice extract sensitivity patterns of urinary tract pathogens. The pathogens used were *Escherichia coli, Klebsiella pneumoniae, Citrobacter diverses, Pseudomonas aeruginosa and Proteus vulgaris.* From these Findings it was observed that *Eugenia caryophyllus, Cinnamomum zeylanicum and Myristica fragrans* had the maximum antibacterial activity against all the five urinary pathogens. Antioxidant and antimicrobial activities of nutmeg (*Myristica fragrans* Houtt) seed extracts were evaluated by Gupta *et al.*, (2013). Seeds were extracted with acetone, ethanol, methanol, butanol and water. The results obtained showed that all the extracts have significant antioxidant and antimicrobial activities against the tested microorganisms. Shafiei *et al.*,(2012) evaluated the bactericidal potential of ethyl acetate and ethanol extracts of flesh, mace and seed of *Myristica fragrans* against three Gram-positive cariogenic bacteria (*Streptococcus mutans* ATCC 25175, *Streptococcus mitis* ATCC 6249, and *Streptococcus salivarius* ATCC 13419) and three Gram negative periodontopathic bacteria (*Aggregatibacter actinomycetemcomitans* ATCC 29522, *Porphyromonas gingivalis* ATCC 33277 and *Fusobacterium nucleatum* ATCC 25586). Based on the findings, it was proved that bothethyl acetate and ethanol crude extracts from flesh, seed, and mace of *Myristica fragrans* exhibited good potential against oral pathogens. Sunder et *al.*, (2014) evaluated the antimicrobial activity of Nutmeg (*Myristica fragrans* Houtt) seed extract against the lower respiratory pathogen (*Acinetobacter baumannii*). Results showed that toluene, tetrahydrofuran and methanol extracts of the seed exhibited antibacterial activity against the bacterial isolates.

As a result of increase in resistance of disease causing pathogens to antibiotics, the aim of this work is to evaluate the antioxidant properties as well as the efficacy of the different extracts of nutmeg against different pathogens that cause urinary tract infection.

## MATERIALS AND METHODS

Dry nutmeg (*Myristica fragrans*) seeds were obtained from Itam local market, Uyo, Akwa Ibom State. The seeds were thoroughly washed and dried in hot air oven at $40^0$C 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 seed 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 by Singleton *et al.*, (1999) and Ayoola *et al* (2008).Hydro alcoholic extracts of the sample in different concentrations ranging from 10µg/ml - 50µg/ml were prepared by dissolving the sample extract in methanol. An aliquot of each plant extract was mixed with 5 ml of Folin- Ciocalteu reagent which was previously diluted with distilled water (1:10 v/v) and 4 ml (75 g/l) of sodium carbonate ($Na_2CO_3$). 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.

### Antioxidant activity by 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) method

The free radical scavenging activity of the extract was measured *invitro* by 2, 2'-diphenyl-1-picrylhydrazyl (DPPH method) as described by Brand-Williams *et al.*, (1995).DPPH solution was mixed with sample solutions at different concentrations (10 - 50. µg/ml). A control (AbsControl) containing methanol and DPPH solution was also realized. All solutions obtained were then incubated for 1 hour at room temperature. Absorbance was measured at 517 nm. Vitamin C was used as standard and

the same concentrations of it were prepared as the test solutions. The percentage of inhibition of samples was calculated from obtained absorbance by the equation:
% inhibition = ((Abs control-Abs test)/Abs control) × 100. A percent inhibition versus concentration curve was plotted and the concentration of sample required for 50% inhibition was determined and represented as $IC_{50}$value for each of the test solutions.

**Ferric reducing/antioxidant power (FRAP) assay**

The reducing property of the extract was determined by assessing the ability of the extracts to reduce $FeCl_3$ solution (Jayaprakash *et al*., 2001). Briefly appropriate concentrations of the extracts were mixed with 2.5 ml of 200 mM of sodium phosphate buffer (pH 6.6)and 2.5 ml of 1% potassium ferrocyanide. The mixture wasincubated at 50°C for 20 min after which 2.5 ml of 10%trichloroacetic acid was added. The mixture was then centrifuged at650 rpm for 10 min. Supernatant(The upper layer) (5 ml) was mixed with equal volume of deionized water and 1 ml of 0.1% ferric chloride, and the absorbance was measured at 700 nm. A higher absorbance indicates a higher reducing power (Jayaprakash *et al.,* 2001). Ascorbic acid was used as positive reference. The experiment was done in triplicate.

**Antimicrobial Susceptibility Assay**

The antimicrobial activity of the different extracts of nutmeg against UTI was carried out using the method described by Akinjogunla, (2016). Disc diffusion assay was the key process used in evaluating the antibacterial potential of nutmeg extracts. The clinical isolates used (*Escherichia coli, Staphylococcus aureus and Klebsiella pneumonia*) were sub-cultured and incubated at $37^0$C 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.

**Statistical analysis**

Data were analyzed using Microsoft Excel and reportedas mean ± standard deviation of triplicate determination.

## RESULTS AND DISCUSSION

**Percentage yield**

About 107.42g each of the dried seed of nutmeg was extracted with ethyl acetate and methanol solvents using maceration method in other to obtain semi polar and polar extracts respectively. The percentage yield of the crude extracts is presented in Table 1. The results obtained revealed that ethyl acetate extract had the highest yield of 19.48%.

**Total phenolic content**

Many phenolic compounds have been reported topossess potent antioxidant activity due to their redox properties that allow them to act as reducing agents, hydrogen donors and metal chelators. Besides their role as antioxidants, these compounds present a wide spectrum of medicinal properties, such as anti-cancer, antiallergic, anti-viral, anti-inflammatory, anti-bacterial and anti-thrombotic, plus present cardio protective and vasodilator effects, showing a broad field of application for the phenolics in these plants (Chung *et al.,* 1998; Cassidy *et al.,* 2000; Gao *et al*., 2000; Tapiero *et al.,* 2002; Balasundram *et al.,* 2006).

**Table 1.** Percentage yield of crude extracts of *Myristica fragrans*

| Weight of raw material (g) | Solvent used | Weight of extract (g) | Percentage yield (%) |
|---|---|---|---|
| 107.42 | Methanol | 14.85 | 13.82 |
| 107.42 | Ethyl acetate | 20.92 | 19.48 |

Polyphenols have been reported to exhibit antibacterial activities with distinguished characteristics in their reactivity with proteins related polyamides polymers (Haslam, 1996). Total phenolic content of nutmeg extracts was determined according to the Folin–Ciocalteu method and expressed as mg GAE/100 g dry weight of plant material and are presented in Table 4. The results obtained in this study showed a significant level of phenolic compounds in both extracts. However, methanol extract contained the highest phenolic content when compared to that of ethyl acetate. The extraction procedures and solvents maybe responsible for dissolving the endogenous compounds of the plants Moreover, plant components can be polar or non-polar in nature. Phenolic compounds are more soluble in polar organic solvents due to the presence of a hydroxyl group; hence the high phenolic content in methanol extract. (Siddhuraju and Becker, 2003: Weller, 2006).

**Antioxidant activity**

**1. 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity**

The result of the antioxidant activity of the samples varies according to the nature of the solvent used and particularly to the methods of analysis. Recent studies have shown that there is no universal method to evaluate antioxidant activity quantitatively and accurately, therefore, the antioxidant activity of plants is evaluated using several methods (Prior *et al.*, 2005).

DPPH stable free radical method is a sensitive way to determine the antioxidant activity of plant extracts (Koleva e*t al*., 2002; Suresh *et al*., 2008).The assay is based on the reduction of DPPH radicals in methanol which causes an absorbance to drop at 515 nm. The DPPH free radical scavenging activity of the different extracts of nutmeg at different concentrations are presented in Figure 1. From the results obtained, a dose dependent relationship was observed, where methanol and ethyl acetate extract inhibited DPPH radical by 71.6% and 58.2% respectively at 50µg/ml compared to the standard vitamin C (76.8%). The results of the DPPH also showed that the scavenging ability of standard drug (vitamin C) was not significantly different from that of the extracts. All of the assessed extracts of Nutmeg were able to reduce the stable, purple-colored radical DPPH to the yellow colored DPPH-H form. However, methanolic extract have prominent antioxidant activity; the presence of phenolic compounds are mainly found in this extract and could be attributable to the antiradical properties of this extract. The $IC_{50}$ (50% of inhibition) value was calculated following a linear regression analysis of the observed inhibition percentage *versus* concentration, where a lower $IC_{50}$ value shows higher antioxidant activity. With reference to the positive control ascorbic acid, the results revealed that both extracts have very remarkable antioxidant capacity.

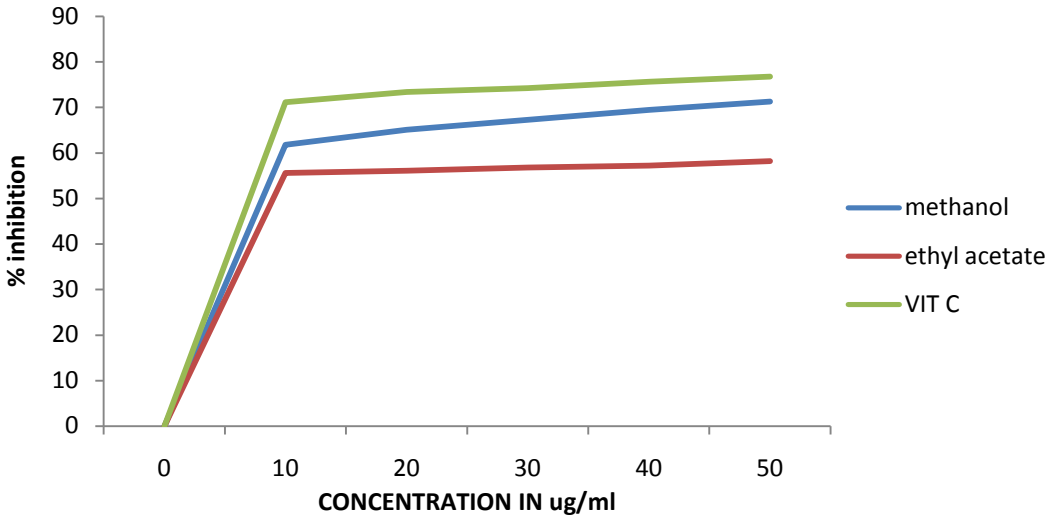


**Figure 1:** Inhibition of DPPH radical by various extracts of nutmeg. Vitamin C was used as positive reference.

**Ferric reducing power activity (FRAP) assay**

The direct reduction of $Fe^{3+}$ to $Fe^{2+}$ was assessed in order to evaluate the reducing potential of the different extracts of nutmeg under study. The reducing properties of antioxidants are generally associated with the presence of reductones, which acts by donating electrons. (Chung *et al*., 2006). The reducing nature of a compound may serve as a significant indicator of its potent antioxidant activity (Hsu *et al*., 2006). The Dose response curves for the reducing powers of the extracts of nutmeg are shown in Figure 2. In the present study, it was observed that the reducing powers of all extracts were directly proportional to their concentrations. As in case of DPPH assay, the extracts containing high phenolic contents displayed greater reducing power. From the results obtained, it can be inferred that the extracts studied possess reducing power and therefore, could serve as electron donors, terminating the radical chain reactions.

**Antimicrobial activities of nutmeg extracts**

The threat of antibiotic resistance by common pathogens has led to increased search of medicinal plants as alternatives. It is also reported that the synergistic effect of the mixture of phytochemicals play important role in the use plant extracts as antimicrobial agents (Amit Kumar *et al*, 2012).Extracts of nutmeg were evaluated for antimicrobial activity against urinary tract infection pathogens (*Escherichia coli, Staphylococcus aureus and Klebsiella pneumonia*) and diameter of the zone of inhibition of the methanol and ethyl acetate extracts of nutmeg are shown in Table 2 and Table 3 respectively.

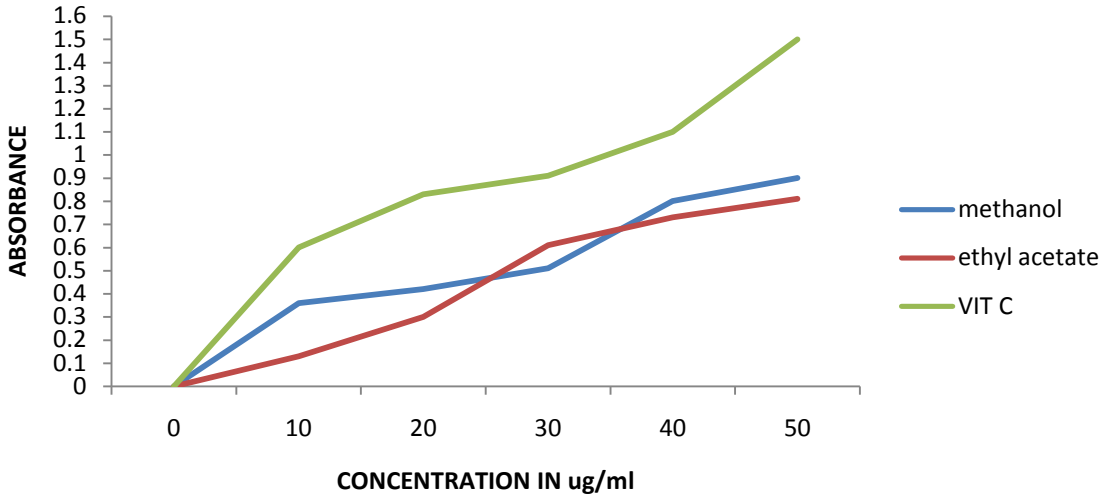


**Figure 2:** Ferric reducing power activity of nutmeg extracts

**Table 2.** Antimicrobial Activity of ethyl acetate Extract of nutmeg

| Methanol extract concentration(mg/ml) | Zone of inhibition in mm. Bacterial pathogens. | | |
|---|---|---|---|
| | *E. coli* | *Staph. aureus* | *K.pneumoniae* |
| 12.5 | 0.00 | 0.00 | 0.00 |
| 25.0 | 0.00 | 0.00 | 0.00 |
| 50.0 | 7.0±0.2 | 0.00 | 0.00 |
| 100.0 | 8.0±0.5 | 9.0±0.25 | 9.0±0.23 |
| Gentamycin | 25.0±1.0 | 22.0±0.5 | 24.0±1.0 |

**Table 3.** Antimicrobial Activity of methanol Extract of nutmeg

| Methanol extract concentration(mg/ml) | Zone of inhibition in mm. | | |
|---|---|---|---|
| | Bacterial pathogens. | | |
| | *E. coli* | *Staph. aureus* | *K.pneumoniae* |
| 12.5 | 6.0±0.20 | 0.00 | 0.00 |
| 25.0 | 7.0±0.19 | 0.00 | 0.00 |
| 50.0 | 7.0±0.2 | 8.0±0.11 | 0.00 |
| 100.0 | 10.0±0.17 | 10.0±0.2 | 11.0±0.20 |
| Gentamycin | 25.0±1.0 | 22.0±0.5 | 24.0±1.0 |

**Table 4.** Total phenolic content and $LC_{50}$ of different extracts of nutmeg

| **Extracts** | **Total phenolics (mg GAE/100 g)** | **$LC_{50}$ (µg/ml) DPPH** | **$LC_{50}$ (µg/ml) FRAP** |
|---|---|---|---|
| VIT C | N/A | 2.40 | 7.2 |
| METHANOL | 78.23±0.02 | 2.96 | 23.3 |
| ETHYLACETATE | 62.15±0.04 | 3.84 | 35.1 |

Methanol was selected as polar solvent and ethyl acetate was chosen as a semipolar solvent. The result of the antimicrobial activities of nutmeg extracts indicated that the ethyl acetate and Methanol extracts of nutmeg 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. For ethyl acetate extract, *Staphylococcus aureus and K. pneumoniae* exhibited the widest zone of inhibition and susceptibility of 9.0 ±0.25 mm and 9.0±0.23 respectively at a concentration of 100 mg/ml, while *Escherichia coli,* exhibited zones of inhibition of 7.0±0.2 and8.0±0.5mm at 50mg/ml and 100mg/ml respectively. Methanol extract of nutmeg exhibited maximum antimicrobial activities against *Escherichia coli* at all concentrations, with the highest inhibition (10.0±0.17mm) at 100mg/ml whereas *Staphylococcus aureus and K. Pneumoniae* were susceptible to the extract at 50mg/ml and 100mg/ml. The result obtained in this study indicated that Methanol extract of nutmeg showed maximum antimicrobial activity than the ethyl acetate extract against the test pathogens used in this study. This may be attributed to the occurrence, solubility and concentration of various bioactive substances present in the extract. Since several plant antimicrobials contain different functional groups in their structure, their antimicrobial activity is attributed to multiple mechanisms (Burt, 2004). Therefore, unlike antibiotics, the potential for bacteria to develop resistance to plant antimicrobials is relatively smaller (Ohno *et al.*, 2003). Studies also show that Phytochemicals present in these extracts exert these antimicrobial activities through different mechanisms such as iron deprivation, hydrogen bonding or non-specific interactions with vital proteins such as enzymes (Scalbert, 1991).The results obtained also indicated that antibacterial activity of plant extracts was more susceptible to *E. coli* when compared to other isolates. On comparing the efficacy of the extracts with that of antibiotic gentamycin, which is one of the drugs used in treating urinary tract infection, it was observed that the zone of inhibition of these extracts was small compared to that of the drug. According to Junior and Zanil, (2000), 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*., (1996) 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 nutmeg showed significant antimicrobial activity at higher concentrations against the test isolates based on the criteria suggested by Junior and Zanil (2000) and Thomas et al., (1996). This

research further corroborates the potential of nutmeg as a broad spectrum antimicrobial agent, which may be considered as an alternative to common antibiotics to treat infectious diseases.

## CONCLUSION

The results of the antioxidant and antimicrobial activity of the methanol and ethyl acetate extracts of nutmeg indicated that these extracts contains significant amount of polyphenolic compounds and exhibits significant antioxidant activity. It can be inferred that these extracts showed considerable broad spectrum antimicrobial activity at higher concentration against bacterial UTI isolates with highest activity recorded for methanol extracts, thereby validating the ethno-pharmacological importance of nutmeg.

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