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COMPARATIVE STUDIES ON THE ANTIMICROBIAL PROPERTIES OF SEED AND BARK OF WALNUT

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Abstract

Walnut (*Juglans regia*) is widely distributed over the world. This work compares the antimicrobial activity of seed and bark ethanol extracts as well as the n-hexane extracts of walnut (*Juglans Regia*) against two clinical strains of one Gram positive (*Staphylococcus aureus*), and one Gram negative (*Escherichia coli*) by agar well diffusion method using ciprofloxacin as the standard antibiotic. Ethanolic and n-hexane extracts of the seed and bark of walnut were used in varying concentrations of 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml and 3.125 mg/ml respectively. The four extracts (n-hexane and ethanol extract of the seed and bark of walnut, separately) were found to show a moderate antimicrobial activity though the ethanolic extracts possessed more activity than the n-hexane. The minimum inhibitory concentration (MIC) was found to range from 1.25 mg/ml to 6.25 for the four extracts respectively. When compared with standard antibiotics, the ethanolic and n-hexane extracts showed a moderate inhibition on these organisms. Seed and bark of walnut are therefore justified for the treatment of human infections associated with these organisms.

Keywords: *Antimicrobial, Ethanol, n-hexane, Mueller hinton agar*

Introduction

The threat of antimicrobial resistance (AMR) is growing fast and the situation is troublesome in developing countries due to gross abuse in the use of antimicrobials [1]. It should be stressed, however, that antimicrobial resistance is also evident in other microorganisms namely, parasites, fungi and viruses [2]. It is well known that any use of antimicrobials however appropriate and justified, contributes to the development of resistance, but widespread unnecessary and excessive use makes the situation worse [3]. Medicinal plants are variety of plants that have medicinal properties or activities [4]. Non-compliance in the use of antimicrobials has many repercussions upon resistance and poverty is a major root factor of antimicrobial misuse in developing countries [5].

Resistance to antibiotic is one of the highest problems that is being faced by public health [6]. Antimicrobial resistance is not new, but the number of resistant organisms, the geographic locations affected by drug resistance, and the breadth of resistance in single organisms are unequalled and rising [7]. In this review, we focus on the underlying principles and In view of this, there is an urgent need to find the alternative to chemotherapeutic drugs in disease treatment particularly those of plants origin which are easily available and have considerably less side effects [8].

In the past, action humans used plant to treat common infectious diseases and even long before mankind discovered the existence of microbes; the idea that certain plant had healing potential was well accepted [9]. Specifically, the medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human or animal body [10]. The most important of these bioactive constituents which are mainly secondary metabolites are alkaloids, flavonoids, tannins and phenolic compounds [11]. These phytochemicals are toxic to microbial cells. Medicinal plants generally contain a number of compounds which may be potential natural antibacterial for the treatment of common bacterial infections [12]. In order to find novel agents with new modes of, plants like walnut (tetracarpidium conophorum) has been explored as a source for the identification of new and effective antimicrobial.

Walnut is found mostly in mountain area. Green walnuts, shells, kernels and seeds, bark and leaves are used in the pharmaceutical and cosmetics industries [13]. Besides this walnut species are important sources of nuts and timbers in the temperate zones across the world [14]. In China, *Juglans* (Juglandaceae) is not only an agricultural commodity, but its leaves, barks, stems, pericarps, fruits, flowers and ligneous membranes are all applied for different medicinal use [15]. In fact, walnut leaves are considered to be a source of healthcare compounds and have been intensively used in traditional medicine for the treatment of venous insufficiency, hemorrhoids, hypoglycemia, diarrhoea, and fungal or microbial infections [16]. Phenolic compounds, natural antioxidants, are found in walnut trees. These compounds are of much importance due to their benefits in improving health and decreasing the risk of degenerative diseases [17]. A lot of work has been done to determine antifungal, antibacterial and oxidative stability of food components of this plant species [18]. Linoleic acid is the major fatty acid, followed by oleic, linolenic, palmitic and stearic [19]. In addition, walnuts have other components that may be beneficial for health including plant protein, dietary fibre, and melatonin. Walnut is common fruit which is used as food supplement all over the world. All the plant parts of walnut are used as medicine in the treatment of various diseases and can be used to fight against bacterial infecion [20].

Some authors also found that walnut oil has a great number of phytochemicals fully armed with the potential to reduce Fe^{3+} of FRAP reagent to Fe^{2+} [21]. The extent of reduction was taken as the power of neutralizing free radicals in the body. Thus the walnut oil has great anti-microbial activity and high antioxidant potential [22]. Medicinal value of black walnut was explored as it creates toxicity on rates and can be used to control the rate population [23]. Antimicrobial property of black walnut juice of unripe hull was studied and found that the presence of a naphthaquinone (juglone) had a great antimicrobialactivity [24]. Black walnut has a great antioxidant property. Due to this, it is used as food to reduce the cardiovascular diseases [25].

Methods

Processing of Plant Samples

Walnuts were bought from Eke Agbani in Enugu State Nigeria. The walnuts were authenticated by a Botanist from the Department of Applied Biology and Biotechnology, ESUT. The walnuts were washed in tap water and rinsed in sterile distilled water. The bark of the walnuts were removed to get the seed, both the bark and seed were dried under room temperature.

Preparation of Extracts

The air-dried plant materials (seed and bark of walnut) were each ground with a hammer mill to a coarse powder. 73 grams of powdered bark of walnut were weighed into a conical flask and 100 ml of extranctant (ethanol and n-hexane) were added and left to extract at room temperature. Also, 167 grams of powdered seed of walnut were weighed into a conical flask and 200ml of extranctant (n-hexane and ethanol) were added and left to extract at room temperature. The extract solutions were filtered separately using a watt-man No 1 filter paper. The filtrate were placed into an evaporator at room temperature to drive-off the extractants and stored at 4°C.

Microorganism Preparation

The test organisms used were all food samples and were isolated from soya milk. They are one species of gram positive bacteria (*Staphylococcus aureus*) and one species of gram-negative bacteria (Escherichia *coli*). They were identified and confirmed based on standard microbiological method. They were stored on a nutrient agar slant in the Department of Applied Microbiology and Brewing, Faculty of Natural Sciences, ESUT, where they were kept at stock cultures at 4°C. Biochemical characterisation was carried out on each of the test organisms for confirmation.

Antimicrobial Assay

The antimicrobial assay of ethanol and n-hexane extracts of seed and bark of walnut were performed by agar well diffusion method. To test antibacterial activity of seed and bark of walnut extract, 0.4 g of each extract was dissolved in DMSO and then varying concentrations of the extracts (100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, and 3.125 mg/ml) were obtained according to Maragathavalli *et al.* [26]. A standard inoculum of 1.5x108 cells which matched 0.5 McFarland standards was spread on the surface of sterile Muller Hinton agar plates in duplicates. A sterile 8 mm cork borer was used to make a hole on the Muller Hinton agar plates in which 0.1 ml of each of the plant extracts were added. The plates were incubated at 37°C for 24 h. The antimicrobial activity was detected by measuring zones of inhibition in millimetres. To test antibacterial activity of the synthetic antibiotics, standardized discs were also used and zones of inhibition were determined.

Determination of the Minimum Inhibitory Concentration (MIC)

This was determined using broth dilution method as described by Maragathavalli *et al.* [26]. The minimum inhibitory concentration was defined as the lowest concentration of the compound to inhibit the growth of microorganisms [27]. Varying concentrations of the extracts (100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, and 3.125 mg/ml) were prepared. 0.1 ml of standardized test organisms was inoculated into the tubes containing the different concentrations of the extracts and controls were equally setup by using solvents and test organisms without extract. These were incubated for 24 h at 37°C. The tube with least concentration of extract without growth after incubation was taken and recorded as the minimum inhibitory concentration.

Results

Table 1 shows the antibacterial activity of ethanolic extract of Walnut seed measured as diameter (mm) of zone of inhibition. The zone of inhibition for the standard is high when compared with the ethanolic extract. The ethanolic extract had high zone of inhibition in *S. aureus* compared to *E. coli* in the study.

Table 2 shows the antibacterial activity of n-Hexane extract of Walnut seed measured as diameter (mm) of zone of inhibition. The zone of inhibition for the standard is high when compared with the n-Hexane extract. The n-Hexane extract

had high zone of inhibition in *S. aureus* compared to *E. coli* in the study.

Table 3 shows the antibacterial activity of ethanolic extract of Walnut bark measured as diameter (mm) of zone of inhibition. The zone of inhibition for the standard is high when compared with the ethanolic extract. The ethanolic extract had high zone of inhibition in *S. aureus* compared to *E. coli* in the study.

Table 4 shows the antibacterial activity of n-Hexane extract of Walnut bark measured as diameter (mm) of zone of inhibition. The zone of inhibition for the standard is high when compared with the n-Hexane extract. The n-Hexane extract had high zone of inhibition in *S. aureus* compared to *E. coli* in the study.

Table 5 and Table 6 shows the MIC values of seed and bark of walnut extracts against the two test organisms ranged from 2.50 mg/ml to 6.25 mg/ml. From Table 5, the ethanolic extracts of the Walnut seed had MIC values of 2.50 mg/ml and 4.25 mg/ml for the two tested organisms, respectively. The n-hexane extract of Walnut seed showed lower MIC value of 3.25 mg/ml for *E. coli* compared to MIC value of 4.99 mg/ml for *S. Aureus*.

Table 6 showed that the MIC values of the ethanolic extracts of the Walnut seed had MIC values of 6.25 mg/ml for the two tested organisms.The n-hexane extracts of Walnut bark were 6.25 mg/ml and 6.00 mg/ml for the two tested organisms, respectively.

Discussion

Over the past few decades there has been much interest in natural products as sources of new antimicrobial agents. Different extracts from traditional medicinal plants have been tested. Many reports show the effectiveness of traditional herbs against microorganisms. As a result, plants have become one of the bases of modern medicine [28]. In the present study, powder of seed and bark of walnut were extracted successively with n-hexane and ethanol. The extracts were subjected to antimicrobial screening against one Gram positive bacteria (*Staphylococcus aureus*) and one gram negative (*Escherichia coli*) by agar well diffusion method using Ciprofloxacin as a standard antibiotic. The result of the antimicrobial activity of the test organism as shown in table 1, 2, 3 and 4. The plant extracts showed different degrees of inhibitions on the test organism. The ethanol extract was found to be more effective than n-hexane extracts which indicates the potency of the bioactive components of the plant against all the test species. The result obtained in the present study clearly showed that the anti-bacterial activity decreases with decreasing concentration of the extract in all the test organisms. All the extracts of seed and bark of walnut had highest values for the gram +ve (S. aureus) than that of the gram –ve (E. coli). All the extracts of bark of walnut exhibited promising moderate activities against the two organisms.

This study is in agreement with the works of Hala *et al.* [29] who stated that bark of walnut has inhibitory effect on *E. coli* and *S. aerus.* The result in Table 5 and 6 have shown that the MIC values of seed and bark of walnut extracts against the two test organisms ranged from 2.50 mg/ml to 6.25 mg/ml. The extracts of the walnut seed showed lower MIC values than that of the extracts of the bark of walnut tested against the microorganisms. The lowest MIC value for E. coli 2.50 mg/ml and S. aureus 4.25 mg/ml was observed with the extracts of walnut seed and the lowest MIC value for E. Coli 6.25 mg/ml and S. aureus 6.00 mg/ml was also observed with the extracts of walnut bark.

In conclusion, the results of this study have provided some justification for the therapeutic potential of medicinal plants. The practice of using these plants as supplementary or alternative medicine in developing countries like Nigeria will not only reduce the clinical burden of drug resistance development but also the side effects and cost of treatment with modern medicine. However, further clinical evaluation of medicinal plants in vivo experiments is required to be carried out to enable low cost treatment with little or no side effects and for the prevention of recurring infections.

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Table 1. Inhibition Zone Diameter (mm) of Ethanolic Extract of Walnut Seed

Test Organism	Concentration of Extract (mg/ml)						Standard
	100	50	25	12.5	6.25	3.125	Ciprofloxacin (10µg)
E.coli (-ve)	10	7	6	5	4	4	14
S. aureus (+ve)	12	10	8	7	5	5	15

Table 2. Inhibition Zone Diameter (mm) of n-Hexane Extract of Walnut Seed

Test Organism	Concentration of Extract (mg/ml)						Standard
	100	50	25	12.5	6.25	3.125	Ciprofloxacin (10µg)
E.coli (-ve)	8	7	6	5	4	4	14
S. aureus (+ve)	10	10	8	7	5	5	15

Table 3. Inhibition Zone Diameter (mm) of Ethanolic Extract of Walnut bark

Test Organism	Concentration of Extract (mg/ml)						Standard
	100	50	25	12.5	6.25	3.125	Ciprofloxacin (10µg)
E. coli (-ve)	9	7	6	5	4	4	14
S. aureus (+ve)	11	10	8	7	5	5	15

Table 4. Inhibition Zone Diameter (mm) of n-Hexane Extract of Walnut bark

Test Organism	Concentration of Extract (mg/ml)						Standard
	100	50	25	12.5	6.25	3.125	Ciprofloxacin (10µg)
E. coli (-ve)	8	6	6	5	4	4	14
S. aureus (+ve)	10	8	8	6	5	5	15

Table 5. Minimum Inhibitory Concentration (mg/ml) of the extract of Walnut seed

Bacteria strain	Ethanol Extract	n-Hexane extract	Standard Ciprofloxacin (10µg)
E. coli (-ve)	2.50	3.25	4.39
S. aureus (+ve)	4.25	4.99	5.66

Table 6. Minimum Inhibitory Concentration (mg/ml) of the extract of Walnut bark

Bacteria strain	Ethanol Extract	n-Hexane extract	Standard Ciprofloxacin (10µg)
E. coli (-ve)	6.25	6.25	4.39
S. aureus (+ve)	6.25	6.00	5.66