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ANTIMICROBIAL PROPERTIES OF *AFRAMOMUM MELEGUETA* SEEDS ON *STAPHYLOCOCCUS AUREUS* AND *STREPTOCOCCUS* SPECIES

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ABSTRACT
Plants sources have been investigated for their antimicrobials in the search for novel compounds for the treatment and control of diseases. This study therefore investigated the potential of *Aframomum melegueta* seeds which have been long known for the ethno-botanical uses, as a source of antimicrobials. Analysis of the seeds used in this study showed that the ethanolic extract had the highest antimicrobial effect on *Staphylococcus aureus* at 0.6g/ml with the highest zone of inhibition at 18mm. It was also observed that the ethanolic extract had the highest antimicrobial effect on *Streptococcus* species with the concentration 0.6g/ml giving the highest zone of inhibition at 15.0mm. Petroleum ether extract of the seeds had lesser antimicrobial effect while the water extract had the least antimicrobial effect on *Streptococcus* species. Phytochemical screening indicated the presence of tannin, phenol, alkaloid, flavonoids, oxalate, saponin and phytate; some of which are recognised as bioactive components while the mineral components (per 100g) were sodium (Na), potassium (K), calcium (C), magnesium (Mg), zinc (Zn), iron (Fe), lead (Pb), copper (Cu), manganese (Mn) and phosphorus (P). This study highlights the antimicrobial property of *Aframomum melegueta* seeds and demonstrates the potential for the control of disease agents such as *Staphylococus aureus* and *Streptococcus* spp. It is expected that the information obtained will be helpful in medicinal formulations.

KEYWORDS: *Aframomum melegueta;* Seeds; Antimicrobial properties; *Staphylococus aureus*; *Streptococcus* species.

INTRODUCTION

Alligator pepper (*Aframomum melegueta*) has vast medicinal value in treating and managing some chronic diseases characterized by inflammatory conditions (Kumar *et al.*, 2015). It belongs to the family Zingiberaceae. The plant is herb native to the tropics commonly found in swampy habitats of Nigeria, Uganda, Angola, Benin, The Gambia, Ghana, Guinea, Cote d'Ivoire (Ivory Coast), Liberia, Sierra Leone, Togo, Cameroon, Congo, Gabon, Guinea-Bissau in West Africa and as far as the Democratic Republic of the Congo. It is also widely cultivated in other parts tropical Africa and South America. It possesses tufted leafy stem that can be up to 1.5m high. The leaves are simple, alternate and lanceolate. The mature leaves can grow as long as 40cm and 12cm-15cm wide. It produces purple coloured flowers which develop into pods that can be as long as 8cm and about 3cm wide. The pod contains numerous reddish-brown seeds (can be as many as 300 seeds in one pod) (Anjah *et al.*, 2015, Tropilab INC. (2015). The sharp and peppery taste of the seeds is caused by the aromatic ketones; 6-paradol, 6-gingerol and 6-shogaol present in it (Sugita *et al.*, 2013). The fruits are fleshy, indehiscent and produce spikes.

Alligator pepper is widely used by many cultures in Nigeria for the treatment of various types of diseases including the aetiologic agents of enteric diseases like *Escherichia coli*, as demonstrated by Kigigha, *et al.*, (2015). Socially, it is served along with kolanuts (*Kola* spp.) to guests for entertainment, and for religious rites by diviners for invoking spirits. It is a common ingredient in pepper soup, a spicy delight in many parts of West Africa. Concoctions made of alligator pepper are often used by traditional doctors as medications for various ailments (*personal communication*). Pregnant women are not excluded from eating this widely used substance. The constituents of an essential oil, extractable by hydro-distillation from the seeds of *Aframomum melegueta* include two sesquiterpene hydrocarbons, humelene and caryophyllene, their oxides and a few non-terpenoids. This oils are partly beneficial

to repel some destructive insect pest of stored grains like *Rhyzopertha dominica* (Fabricius) (Coleoptera:Bostrichidae) (Ukeh, 2008) and against some inflammatory conditions (Doherty *et al.,* 2010; Ilic *et al.,* 2014).

The spice is used in West Africa for the purposes of alleviating stomach ache and diarrhoea (Ilic, *et al.,* 2010), as well as hypertension, diabetes, tuberculosis and a remedy for snakebites and scorpion stings (Kumar *et al.,* 2015; Lans, 2001). These seeds are also used for culinary reasons (due to the pungency of the seeds, they are common used as food seasoning).

In the development of herbal medicines, the quality of base material used for formulating the products is a prerequisite. Since the materials used in herbal drugs are traded mostly as roots, bark, twigs, flowers, leaves, fruits and seeds, visible authentication of the material used is difficult and this has led to a high level of adulteration. To identify and authenticate the materials, the availability of detailed morphological, histological and pharmacognostic information is essential. Standardisation of natural products is a complex task due to their heterogeneous composition, which is in the form of whole plants, plant parts or extracts obtained thereof. To ensure quality reproduction of herbal products, proper control of starting material is essential. The first step towards ensuring quality of starting material is authentication. Thus, in recent years there has been a rapid increase in the standardisation of selected medicinal plants of potential therapeutic significance (Reddy *et al.,* 1999; Venkatesh *et al.,* 2004). This study therefore set out to evaluate the phytochemical properties, pharmaceutical values and the effectiveness of *Aframomum melegueta* on two common pathogens, *Staphylococcus aureus* and *Streptococcus* spp.

MATERIALS AND METHODS

Source of plant materials

Seeds of Alligator pepper (*Aframomum melegueta)* were obtained from the local market of Akungba-Akoko, Ondo State, Nigeria. The plant seeds were authenticated at the Department of Plant Science and Biotechnology, Adekunle Ajasin University, Akungba-Akoko, Nigeria.

Source of Micro-organisms

The test organisms, *Staphylococcus aureus* and *Streptococcus* spp., were obtained from the stock culture of previously identified isolates of the Microbiology Laboratory of the University. The bacteria were maintained on nutrient agar slant and stored in the refrigerator at 4^{o}C for further tests.

Extraction Methods

The seeds of *Aframomum melegueta* were washed with distilled water, dried ground to powder. Two hundred grammes (200g) of the powder were separately soaked in 400ml of 95% ethanol, distilled water and petroleum ether in a 500ml reagent bottle and stoppered. This was allowed to stand for 14 days to permit full extraction of the active ingredients. The fluids were then filtered using Whatman No.1 filter paper. The extracts were dried in a rotary evaporator to obtain the concentrate and kept in the refrigerator prior to use. A 2.0µg/ml solution of each extract (Brian LaBreque, Horizon Technology, Inc., Salem, NH, 2016; Naseer *et al.,* 2016). was prepared with DMSO (dimethyl sulfoxide) and fractionated into 0.6µg/ml, 0.4µg/ml and 0.2µg/ml concentrations needed for the bioassay.

Sterility Test of the Plant Extracts

The aqueous and the ethanolic extracts were tested for growth or contamination by inoculating 1ml each on nutrient agar and incubated at 37^{o}C for 24hours. The plates were observed for growth. No growth in the extract after incubation indicated that the extracts were sterile. The extracts were then assessed for antimicrobial activity.

Antimicrobial Assay

The antimicrobial properties of the extracts were determined using the agar diffusion method and the diffusion disc method (CLSI, 2012; Kaiser, 2012). In the agar diffusion method, twenty-four hour old broth cultures of the test organisms were swabbed onto a sterile Mueller Hinton agar in Petri dishes using sterile cotton swab. A sterile cork borer of 6mm diameter was used to punch wells on the agar on each of the Petri dishes. The holes were filled with 0.5ml of the extracts. Control experiments were also carried out where the holes were filled with 0.5ml metronidazole. Each hole was labelled representing a particular concentration (Mohanty *et al.,* 2010).

In the disc diffusion method, the Petri dishes containing Mueller Hinton agar were seeded throughout with the twenty-four hour old test organisms. Diffusion discs used were then impregnated with corresponding concentrations to that of extracts on the agar diffusion method which is 0.6µg/ml, 0.4µg/ml and 0.2µg/ml and also with 0.5ml of metronidazole as the control. The discs were then evenly dispensed and lightly pressed onto the agar surface. The process was carried out for each extract and the inoculated Petri dishes were left for few minutes for extract to diffuse into agar. The plates were incubated at 37^{0}C for 24 hours (not done in an inverted position) after which the zones of inhibition (if any) were measured (Mohanty *et al.,* 2010).

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration was determined against bacteria after the antimicrobial test had been performed. Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. The MIC was determined using Mueller Hinton agar based on the modified agar diffusion method described by Kirby and Bauer (Kaiser,

2012). Sterile cork borer of diameter 6mm was used to bore holes on the plates after seeding the plates with the bacterial strain concerned, left for one hour at room temperature and incubated at 37^{o}C. Results were read after 24hours of incubation using a calibrated ruler to measure the zone of inhibition.

Qualitative Method of Analyses

Filtrates were prepared by boiling 20g of the fresh plant in distilled water for 5 mins. The solution was filtered through a vacuum pump. The filtrates were used for screening for flavonoids, tannins, saponins, alkaloids, reducing sugars, anthraquinones and anthocyanosides.

(i)Test for Alkaloids

About 0.2ml of filterate was warmed with 2% of H_2SO_4 for two minutes, filtered and few drops of Dragendoff's reagent were added. Orange red precipitate indicated the presence of alkaloids (Trease and Evans, 1989; Evans, 2002).

(ii) Test for Tannins

One millilitre of the filtrate was mixed with 2m1 of FeCl. A dark green colour indicated the presence of tannins.

(iii) Test for Saponins

One millilitre of each filtrate was diluted with 2ml of distilled water and the mixture was vigorously shaken and left to stand for 10mins during which time, the development of foam on the surface of the mixture lasting for more than 10mm, indicates the presence of saponins.

(iv) Test for Anthraquinones

A Borntranger test (Indigo Pharma, 2009), was performed whereby one millilitre of the plant filtrate was shaken with 10ml of benzene; the mixture was filtered and 5 ml of 10% (v/v) ammonia solutions was added shaken (Mohammed et al., 2014). A pinkish or violet solution indicated a positive test (Indigo Pharma, 2009).

(v) Test for Anthocyanosides

One millilitre of the plant filtrate was mixed with 5m1 of dilute HCl; a pale pink colour indicated a positive test.

(vi) Test for Flavonoids

One millilitre of filtrate was mixed with 2m1 of 10% lead acetate; a brownish precipitate indicated a positive test for phenolic flavonoids. For Flavonoids, 1m1 of the filtrate was mixed with 2m1 of dilute NaOH; a golden yellow colour indicated the presence of flavonoids (Edeoga *et al.*, 2005).

(vii)Test for Reducing Sugars

Two solutions are needed for this purpose: Fehling's "A" uses 7 g CuSO4.5H2O dissolved in distilled water containing 2 drops of dilute sulfuric acid. Fehling's "B" uses 35g of potassium tartrate and 12g of NaOH in 100 ml of distilled water. One millilitre of the filtrate was mixed with Fehling's A and Fehling's B separately; a brown colour with Fehling's B and a green colour with Fehling's A indicated the presence of reducing sugars. (Brilliant biology Student, 2015).

(viii) Test for Cyanogenic glucosides

This was carried out adding 0.5g of the extract to 10ml sterile water. This is filtered and sodium picrate was added to the filtrate that is heated to boiling point.

(ix) Test for Cardiac glucosides

The killer-kiliani and legal test and was adopted for the purpose of cadiac glucosides examination. 0.5g of the extract was added to 2ml of acetic anhydrate plus H_2S0_4.

Quantitative Method of Analyses

(i) Saponins

a) 1 ml solution of extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. Development of stable foam suggests the presence of saponins.

b) 1ml extract was treated with 1% lead acetate solution. Formation of white precipitates indicates the presence of saponins (Devmurari, 2010). Alternatively, Twenty grammes each of dried plant samples were ground and, put into conical flasks after which 100ml of 20% aqueous ethanol was added. Each mixture was heated in a water bath at about 55^{O}C for 4hours with continuous stirring, filtered and the residue re-extracted with a further 200ml of 20% ethanol. The combined extracts were reduced to 40 ml over a water bath at about 90°C. Each concentrate was transferred into a 250ml separatory funnel and 20ml of diethyl ether was added and vigorously shaken. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated three times and 60ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10m1 of 5% aqueous sodium chloride and the remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight; the saponin content was calculated as percentage of the starting material.

(ii) Flavonoids

Ten (10) grammes of each plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol, at room temperature and the solution was filtered through Whatman's filter paper No 42. The filtrates were later transferred into a crucible and evaporated into dryness over a water bath; the resultant content was dried to a constant weight.

(iii) Cardiac glucosides

Legal test and the keller-Kiliani was adopted, 0.5g of the extract were added to 2ml of acetic anhydrate plus H_2S0_4 (Trease and Evans, 1989; Evans, 2002).

(iv) Tannins

About 500mg of the plant sample was weighed into a 50ml plastic bottle. Fifty milliliters of distilled water was added and shaken for 1 hour on a mechanical shaker. This was filtered into a 50ml volumetric flask and made up to the marked level. Five milliliters (5ml) of the filtrate was transferred into a test tube and mixed with 2ml of 0.1 M FeCl in 0.1 M HCl and 0.008 M potassium ferrocyanide. The absorbance was measured at 120nm within 10minutes. The tannins content was calculated using a standard curve.

(v) Alkaloids

Five grammes (5g) of the plant sample was weighed into a 250ml beaker into which 200ml of 10% acetic acid in ethanol was added. The reaction mixture was covered and allowed to stand for 4 hours. 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, washed with dilute ammonium hydroxide and then filtered. The residue being the alkaloid, was dried to a constant mass and weighed (Trease and Evans, 1989; Evans, 2002).

(vi) Phlobatannins

About 0.5grams of each *Aframomum* spp., seed extracts was dissolved in distilled water and filtered. The filtrate was boiled in 2% HCl, red precipitate show the present of phlobatannins. (Adegoke and Adebayo-tayo, 2009)

RESULTS

This study elucidated the activity of *Aframomum melegueta* on *Staphylococcus aureus* and *Streptococcus* spp. which are known to be specific aetiologic microbes. The ethanolic extract of *Aframomum melegueta* had highest antimicrobial effect on *Staphylococcus aureus* (Table 1) with the 0.6g/ml concentration of the extract giving the highest zone of inhibition (18.0mm). Petroleum ether extract, although effective, had lesser antimicrobial effect while the aqueous extract of had the least antimicrobial effect on *Staphylococcus aureus*. In the same vein, the ethanolic extract of *Aframomum melegueta* had the highest antimicrobial effect on *Streptococcus* species (Table 2) with 0.6g/ml concentration giving the highest zone of inhibition (15.0mm). Petroleum ether extract also had lesser antimicrobial effect while the aqueous extract had the least antimicrobial effect on *Streptococcus* species. Qualitative analysis showed that *Aframomum melegueta* is composed of various phytochemicals such as Alkaloids, Cyanogenic Glucosides, Steroids, Anthraquinones, Phenols, Tannins, Saponins and Flavonoids as presented in Table 3.

Quantitative Analyses of mineral elements (Table 4) showed the presence of Sodium (Na), Potassium (K), Calcium (Ca), Magnesium (Mg), Zinc (Zn), Iron (Fe), Copper (Cu), Manganese (Mn) and Phosphorus (P) but Lead (Pb) was not detected. Data presented show that phosphorus content was highest in comparison with other minerals analysed. Analyses also indicated that the plant material had all the anti-nutrients presented in Table 5, with saponin having the highest percentage composition. The results presented in Table 6 also show that the seeds were composed largely of carbohydrates.

Table 1: **Antibacterial effect of Aligator Pepper (*Aframomum melegueta*) seed extracts on *Staphylococcus aureus* (in mm inhibition zone).**

	0.2g/ml	0.4g/ml	0.6g/ml	Control
Aframomum melegueta **Ethanolic extract (mm)**	14.0	13.0	18.0	12.0
Aqueous extract (mm)	5.0	3.0	6.0	8.0
Petroleum ether extract (mm)	11.0	12.0	12.0	10.0

Table 2: Antibacterial effect of *Aligator Pepper (Aframomum melegueta) seed extracts* on *Streptococcus* species (in mm inhibition zone).

	0.2g/ml	0.4g/ml	0.6g/ml	Control
Ethanolic extract (mm)	5.0	8.0	7.0	15.0
Aqueous extract (mm)	3.0	1.0	3.0	10.0
Petroleum ether extract (mm)	5.0	4.0	4.0	12.0

Table 3: **Phytochemical Screening of *Aframomum melegueta***

Samples	Alkaloid	C. Glucoside	Steroid	Anthraquinone	Phenol	Tannins	Saponins	Flavonoids
	+ve	+ve	±ve	+ve	+ve	+ve	+ve	+ve

Keys: +ve= Presence of constituents, -ve =Absence of constituents , ±ve = Slightly present.

Table 4: Quantitative Analyses of *Aframomum melegueta* minerals composition (mg/100g)

	Na	K	Ca	Mg	Zn	Fe	Pb	Cu	Mn	P
Composition (mg/100g)	21.37	30.54	23.55	19.67	17.58	10.21	ND	0.03	25.37	97.65

Key: ND- Not Detected

Table 5: Quantitative Analyses of Anti–nutrients present in Plant Extracts (%)

Anti–nutrients	% Composition
Tannins	2.10
Phenols	3.41
Phylates	1.87
Oxalates	2.09
Saponins	4.00
Flavonoids	3.89
Alkaloids	3.00

Table 6: Quantitative analyses of proximate nutrient composition of plant extracts (%)

	Ash	Moisture Content	Crude Protein	Fat	Fibre	Carbohydrate
% Composition	10.67	8.00	11.93	5.92	9.68	40.56

DISCUSSION

The prevalence of antibiotic resistance has become problematic in disease management hence the widespread search for novel compounds. Usually, natural sources are preferred hence the need to screen plant resources for beneficial activity against common disease causing agents. This study has confirmed the potential of *Aframomum melegueta* as a source of antimicrobials as previously reported (Ramadurai *et al.*, 1999). The results show that the mode of extraction is crucial in the efficacy of the extract. As presented in Table 1, it was observed that the ethanolic extract of *Aframomum melegueta* had the highest antimicrobial effect on *Staphylococcus aureus*. Petroleum ether extract also had antimicrobial effect but was observed to be less efficacious and the aqueous extract had the least antimicrobial effect. This corroborates the findings of Kigigha, *et al.* (2015) who reported low antimicrobial effect on some *Staphylococcus aureus* strains compared with relatively high activity on *Escherichia coli* and *Bacillus* species in their study. The aqueous extract also had very low antimicrobial effect on *Streptococcus* species (Table 2). Analysis of the phytochemical constituents of *Aframomum melegueta* confirmed the presence of Alkaloids, Cyanogenic Glucoside, Steroids, Anthraquinone, Phenols, Tannins, Saponins and Flavonoids (Table 3) and these could act as bioactive agents (Devmurari, 2010). Analysis of mineral elements (Table 4) indicated the presence of Sodium (Na), Potasium (K), Calcium (Ca), Magnesium (Mg), Zinc (Zn), Iron (Fe), Copper (Cu), Manganese (Mn) and Phosphorus (P), with phosphorus being the most prevalent while Lead (Pb) was not detected.

Quantitative Analyses of Anti–nutrients (Table 5) indicated the presence of Saponins, Tannins, Phylates, Oxalates, Flavonoids, Phenols and Alkaloids, with Saponins having the highest percentage. Similarly, as shown in Table 6, the seeds were made up of Ash, Crude Protein, Fat, Fibre and Carbohydrate with carbohydrates having the highest percentage composition.

This study confirms the potential of *Aframomum melegueta* in the development of new chemotherapeutic agents. The first step towards this goal is the *in vitro* antibacterial activity assay (Tona *et al.*, 1998; Samy and Ignacimuthu, 2000; Palombo, and Semple, 2001). Some of these observations have helped in identifying the active ingredients responsible for such activities as a prelude to the development of drugs for therapeutic use in humans. However, there are limited reports on the exploitation of the antifungal or antibacterial properties of plant extracts in the development of commercial formulations for crop protection. Adopting this approach may therefore be valuable in the treatment of plant diseases too. The major benefit of this study however is that it will be helpful in developing valuable medications that can be incorporated into products for sustainable treatment of skin and intestinal aetiologic diseases whose causative agents are of *Staphylococcus aureus* and *Streptococcus* spp. related origin.

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