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Full Length Research Paper

PHYTOCHEMICAL, PROXIMATE AND ANTIINFLAMMATORY EVALUATION OF AQUEOUS ETHANOL SEED EXTRACT OF *AFRAMOMUM MELEGUETA*

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ABSTRACT
This study analysed the phytochemical and proximate compositions of the aqueous ethanol extract of *Aframomum melegueta* (Paradise) seeds. The antiinflammatory activity was also investigated using inhibition of agar-induced rat-paw edema and stabilization of bovine erythrocyte membrane as the indices. The extract (1000.0 and 1500.0 mg/kg body wt), standard drug (indomethacin, 5.0mg/kg body wt.) and control (distilled water, 1.0 ml/kg wt.) were administered orally to four different groups of animals (A, B, C, and D respectively, n=5). The effect of the extract on cell membrane integrity was compared to that of ibuprofen and indomethacin. Phytochemical and proximate analyses were carried out using conventional methods. Results showed high content of phytosterols (360.15 mg/100g), alkaloids (227.0mg/100g) and flavonoids (64.86 mg CE/100g) in *A. melegueta* seeds. It also contained moisture (3.47%), crude protein (4.93%), total lipids (2.60%), ash (2.54%), crude fibre (14.01%) and total carbohydrates (72.45%). Maximum reduction (62%) in rat-paw edema was observed at the sixth hour of the inflammatory response at both doses of the extract tested. Membrane stabilization ability was demonstrated at 5.0 and 10.0mg/ml of the extract. The extract was more efficacious at lower dose (i.e 77.9% inhibition of membrane lysis). The standard drug, ibuprofen, was more potent in stabilizing erythrocyte membrane than indomethacin and the extract. These findings therefore validate the efficacy of *A. melegueta* seed extract as an antiinflammatory agent. Stabilization of lysosomal membrane could be one of its mechanisms of action. This biological activity may lie in its phytosterol content.

Keywords: *Aframomum melegueta*, Inflammation, Phytosterols, Antihaemolysis, Agar-induced edema.

INTRODUCTION

Advances have been recorded in the application of medicinal plants in drug discovery, development of new synthetic drugs, and dietary supplements for the treatment and management of several blood and organ related diseases. The roots, bark, leaves, flowers and seeds of these plants are the principal sources of the bioactive ingredients used in ethnomedical practices (Nwahujor *et al*., 2014).

Aframomum melegueta (ziniberaceae) commonly known as alligator pepper is widely used in

alternative medicine. The seeds are well known for their strong aromatic and pungent aroma, peppered and slightly bitter taste (Kokou *et al*., 2013). They are effective against cardiovascular diseases, diabetes mellitus, arthritis, inflammation, stomach ache and diarrhoea (Ilic*et al*., 2010).

In Eastern part of Nigeria, the seed is chewed with kolanut inorder to enhance the aroma and reduce the bitter taste of kolanut, whereas in the Northern part of the country, it is well recognized as a spice rich in micronutrients (calcium, potassium and iron) and vitamins (thiamine, riboflavin, niacin and ascorbic acid) (Edeoga *et al*., 2005). Essential oils such as gingerol, shogoal, paradol, gingeredrone and zingerone have also been extracted from *A. melegueta* seeds and these may confer antiinflammatory property to this plant (Umukoro and Ashorobi, 2001). The broad-spectrum antimicrobial activity against tested organisms have also been reported by Doherty *et al*., 2010; Chiejina and Ukeh, 2012).

Inflammation could be described as the body tissues' response to trauma, damage, irritating or destructive stimuli such as foreign organisms or exogenous substances (Ferrero-milieni *et al*., 2007, Abbas and Lichtman, 2009). Characterized by heat, pain, redness and swelling (localized edema), this complex biological response has been implicated in arthritis, atherosclerosis, diabetes mellitus, cardiovascular diseases, cancers and Acquired Immunodeficiency Syndrome (AIDS). Failure of the body to eliminate the causative agent of the inflammation may result to chronic inflammatory reaction (Hautz *et al*., 2012).

This study was therefore designed to evaluate the effect of aqueous ethanol extract of *A. melegueta* seed on experimental inflammatory response induced with agar using albino rat (*Ratus novegicus*) as experimental animal model.

MATERIALS AND METHODS

Plant Materials

A. melegueta fruit was purchased from Eke-Awka Market, Awka, Anambra State, Nigeria, in July, 2016. It was identified and authenticated by a taxonomist, Mr P.O.Ugwuozor, in the Department of Botany, Nnamdi Azikiwe University, Awka. The viable seeds were picked and dried at the laboratory temperature for three weeks.

Experimental Animals

Apparently healthy adult Wistar albino rats were purchased from ChrisFarms, Awka, Anambra State and used for the study. The animals were kept in standard animal cages in The Department of Applied Biochemistry Animal House under room temperature with free access to commercial animal pellets (Guinea Animal Feeds, Benin, Edo State, Nigeria) and drinkable water. The animals were allowed seven days of acclimatization.

Extraction Procedure

One kilogramme quantity of the dried *A. melegueta* seeds was dehulled and pulverized. The powdered seed was macerated in 70% (v/v) aqueous ethanol for 24hours. The extract was filtered through cheese cloth and Whatman No. 1 filter paper and the filtrate evaporated with the aid of a rotary evaporator (Rotary Evaporator RE52-2 SEARCHTECH INSTRUMENT).

Chemicals and Reagents

All the chemicals and reagents used were of analytical grade and manufactured by BDH chemicals (UK) except where otherwise stated.

Determination of Total Saponin Content

The method adopted was that of Obadoni and Ochuko, (2001). Pulverized sample (5.0g) was put into a 250 ml cornical flask and 20% aqueous ethanol (25.0ml) added to extract the saponins.

The sample was heated in a hot water bath at 55°C for 4hrs. The extract was filtered and the residue re-extracted with 20% ethanol (50.0 ml). The combined extract was concentrated to 10.0ml in a water bath at 90°C. The concentrate was then transfused into a 1.25L separating funnel and diethyl ether (5.0ml) added and shaken rigorously. The aqueous layer was recovered while the ether layer was discarded. Fifteen millilitres of n-butanol was added to the extracts and washed twice with 5% aqueous sodium chloride solution (2.5 ml). After purification, the solution was discharged into a pre-weighed evaporating dish and heated in a water bath to dryness. The evaporating dish was dried in an oven (60°C) to a constant weight and the percentage saponin content was calculated as follows:

$$\frac{W_2 - W_1}{W_0} X \frac{100}{1} = \text{PercentageSaponin}$$

Where,
W_0 = Weight of sample
W_1 = Weight of evaporating dish
W_2 = Weight of evaporating dish + dried extract.

Determination of Total Flavonoids

The method according to Barros and Baptista et al., 2008 was employed. The extract (250ml) was mixed with distilled water (1.25ml) and 5.0% $NaN0_2$ solution (75ml) and left to stand undisturbed for 5 minutes. A solution of 10% $ALCl_3.H_20$ was then added and allowed to stand for 6 minutes. NaOH (IM, 500.0ml) and distilled water (275ml) were added to the mixture. After mixing, the intensity of the pink colour was measured at 510 nm with Ultrospec 3100 pro Amersham Biosciences, Biochrom Ltd, England.

Catechin was used as the standard and the results were expressed as mg of Catechin equivalent (CEs) per gramme of extract.

Determination of Total Tannins

One gramme of the fat-free sample was extracted with distilled water (10.0ml) and centrifuged for 10min at 1000g. The extract was mixed with distilled water (4.5ml), $FeCl_3$ (0.1M, 0.5ml) and potassium ferrocyanate (0.1M, 0.3 ml). Finally, the volume of the mixture was made up to 11.8ml with distilled water and the absorbance taken at 720 nm with Ultrospec 3100 pro Amersham Biosciences, Biochrom Ltd, England. Tannic acid was used as standard (Harbone, 2000).

Determination of Total Alkaloids

The method of Harbone, 1995 was used. Five grammes (5g) of the sample was weighed into a 250.0ml beaker and 20% acetic acid (200.0ml) in ethanol was added. The beaker was covered and allowed to stand for 4hours at 25°C. This was filtered with Whatman No. 42 of the original volume using a water bath. Concentrated NH_4OH was added dropwise to the extract until the precipitate is formed. The precipitate was collected and washed with dilute NH_4OH (1%), filtered out with a pre weighed filter paper. The alkaloid residue on the filter paper was dried in the oven (precision electro-thermal model BNP 9052, England) at 80°C and the alkaloid content calculated and expressed a percentage of the weight of the sample.

Determination of Total Phytosterol Content

The total phytosterol content of *A. melegueta* was determined according to the method of Larissa *et al*., 2013. Two grammes of the powdered sample was soaked in 70% ethanol (100.0l) for 2hr. The alcoholic extract (100.0 ml) was filtered and dried in a water bath at 60°C. The residue was re-suspended in

chloroform (20.0ml) and the volume adjusted to 50.0 ml with the same solvent. Aliquot (50.0 ml) was transferred to 100.0 ml volumetric flask and 2.0 ml of Liebermann-Burchard reagent was added and final volume adjusted to 80 ml with chloroform. The absorbance was measured in a spectrophotometer at 625nm 5 minutes after the addition of the reagent. Chloroform served as the blank while stigmasterol was used as the standard.

Proximate Analysis.

Determination of moisture content

Moisture content was determined using the method of (AOAC, 1990). Two (2.0) grammes of the sample was weighed into a dried crucible of known weight. The sample was heated to a constant weight in a moisture extraction oven for 3hr at a temperature of 105°C. The dried sample was transferred into a desicator, allowed to cool and then reweighed. Percentage moisture content was calculated using the formula:

$$\text{Percentage moisture} = \frac{W_2 - W_1 \times 100}{W_2 - W_3 \times 1}$$

Where,
W_1 = Weight of crucible
W_2 = Weight of crucible + sample
W_3 = Weight of crucible + dried sample.

Determination of Ash Content

Two (2.0) grammes of the sample was heated in a moisture extraction oven for 3h at 105°C. The sample was incinerated in a muffle furnace maintained at a temperature of 550°C until a residual grey ash of constant weight was obtained. Percentage ash content of sample was then calculated (AOAC,1990).

$$\text{Percentage Ash} = \frac{\text{Weight of Ash}}{\text{Initial Weight of Sample}} \times \frac{100}{1}$$

Crude Protein Determination

The microkjeldahl method of AOAC, 1990 was adopted. Two (2.0) grammes of sample was digested in 10.0 ml of concentrated H_2SO_4 using selenium as the catalyst. The digestion was carried out by heating in a fume cupboard at 410°C for 1h. After cooling, 10.0ml aliquot of the digest was mixed with an equal volume of 45% NaOH and then distilled in a kjeldahl distillation apparatus. The distillate was collected into 4% boric acid solution and titrated against 0.2M hydrochloric acid with 3 drops of methyl red as indicator.

Triplicate samples were used and the average value taken. The nitrogen content was calculated and crude protein estimated by multiplying the nitrogen content by 6.25.

$$\text{Percentage Nitrogen} = \frac{(100 \times N \times 14 \times VF)\ T}{100 \times V_a}$$

Where,
N = Normality of the titrate
VF = Total volume of the digest
T = Titre value
V_a = Aliquot volume distilled

Crude Fat Determination

Total fat was determined according to AOAC, 1990 by running the sample through a soxhlet extraction unit. Two grammes of the sample wrapped with a Whatman no.1 filter paper was loaded into the thimble fitted to a clean, dry and weighed sound bottom flask containing 120.0 ml of petroleum ether. The sample was then heated and allowed to reflux for 5h. The heating was stopped and the thimbles and its content cooled and reweighted. The percentage fat content was then calculated as:

$$\frac{W_2 - W_1}{W_3} \times \frac{100}{1}$$

Where,
W_1 = Weight of the empty extraction flask

W_2 = Weight of the flask and extracted
W_3 = Weight of the sample.

Crude Fibre Determination

Crude fibre content was determined (AOAC,1990) by digesting 2.0 g of the sample and 1.0 g of asbestos in 200.0 ml of 1.25% of H_2SO_4 for 30 minutes. The digest was then filtered through Buchner funnel equipped with a muslin cloth and secured with an elastic band. The residue was similarly treated with 1.25% of NAOH. The final residue was then washed twice with ethanol, thrice with petroleum ether, and dried to a constant weight in the moisture extraction oven. The dry residue was weighed and difference in weight expressed in percentage crude fibre as:

$$\frac{W_2 - W_1}{W_3} \times \frac{100}{1}$$

Where,
W_1 = Weight of sample before incineration
W_2 = Weight of sample after incineration (Wt of crucible and Dietary Fibre) – (Wt of crucible) x 100] / wt. of sample

Determination of Carbohydrate Content

Percentage carbohydrate content of the nitrogen free extract (NFE) was calculated as the difference between 100 and the sum of other proximate parameters (AOAC, 1990).
Percentage carbohydrate = 100 – (M + P + F_1 + A + F_2)
Where,
M = Moisture
P = Protein
F_1 = Fat
A = Ash
F_2 = Crude fibre

Antiinflammatory Activity Studies
Agar-induced Ratpaw oedema

Twenty (20) healthy adult Wistar albino rats (150 – 180g.wt) were selected and divided into four groups of five animals per group. The control rats were given 1.0ml / kg wt. of distilled water, whereas the reference group of rats received the standard anti-inflammatory drug indomethacin (5.0 mg/kg wt). The test group A and B were treated with 1000 and 1500 mg/kg of extract respectively. Water, indomethacin, and extract were administered orally to the animals. Indomethacin and extract were solubilized in distilled water. After 1hr, 0.05ml of agar (1%) was injected subcutaneously into the planter surface of the left hand paw of all the animals(Ezekwesili *et al*., 2000). Paw volume of the injected left hind paw of each rat was measured using the mercury displacement method. The paw volumes were measured 1hr prior to the injection of agar and 1,2,3,4,5, and 6hr after the injection of agar (Gupta *et al*., 2007, Mahat and Patil, 2007).

The oedema inhibitory activity was calculated as follows:

$$(\text{Control}_{\Delta V} - \text{Test}_{\Delta V} / \text{Control}_{\Delta V}) \times 100$$

Where,
$\text{Control}_{\Delta V}$ = Change in paw volume for the control
$\text{Test}_{\Delta V}$ = Change in paw volume for the treated group

Membrane stability of Red Blood Cell

The membrane stabilization property of *A. melegueta* seeds was investigated using the method of Oyedapo *et al*., 2004.

Fresh bovine blood sample was collected into an anticoagulant (10% sodium citrate) container and centrifuged at 3000 rpm at room temperature. The supernatant containing plasma and leucocytes was carefully separated from the packed red blood cells using a Pasteur pipette. The packed red blood cells werewashed repeatedly until a clear supernatant was obtained. Bovine erythrocytes (2% v/v) was prepared by suspending the erythrocytes in normal

saline, and used for the determination of the membrane stabilizing activity. Ibuprofen and indomethacin were used as standard drugs.

The assay mixtures (4.5 ml) consisted of hyposaline (0.25% w/v sodium chloride, 2.0ml), 0.15M sodium phosphate buffer, $_{P}$H4.0 (1.0ml), bovine erythrocyte suspension (2% v/v, 0.5 ml), drug (which was either standard drug or extract, 0.5ml), and finally isosaline (0.5ml).

Blood control did not contain any drug, while the drug controls lacked the erythrocyte suspension (Abiodun*et al*., 2014). Reaction mixtures were then incubated in a water bath at 56^{o}C for 30minutes, followed by centrifugation at 5000 rpm for 10minutes at the laboratory temperature. The absorbance of the haemoglobin released from the erythrocytes was finally read at 560nm and percentage membrane stability calculated using the formula.

$$100 - \frac{[\text{Test Drug abs} - \text{Drug Control abs}] \times 100}{\text{Blood Control abs}}$$

Where, abs = absorbance

The erythrocyte membrane represent 100% lysis or zero percentage stability.

RESULTS AND DISCUSSION

Results of the phytochemcial analysis summarized in Table 1 revealed high content of alkaloids (227.0mg/100g), phytosterols (360.15 mg/100g), and flavonoids (64.86 mgCE/100g). Saponins (22.0 mg/100g) and tannins (0.86mg / 100g) were also detected. These phytochemical are well known for their biological activities and they support the medicinal properties of herbal drugs. Plant sterols have been shown to reduce blood cholesterol (Abumweis *et al*., 2008; Demonty *et al*., 2009; Rouyanne *et al*., 2014) and inhibit the inflammatory process (Othman and Moghadasian, 2011; Liz *et al*., 2011).

The European Foods Safety Authority, EFS, in 2002 concluded that the blood cholesterol can be reduced (by up to 10.5%) by a daily intake of 1.5 to 2.4 grams of plant sterols and stanols for about 2 – 3 weeks. Antimicrobial, antidiarrhoeal and antihypertensive effects of alkaloids and alkaloidal substances have been documented (Usunobun and Okolie, 2015; Achinewu *et al*., 1995).

Flavonoids are highly water soluble polyphenolic substances with antidiabetic, antihypertensive, antioxidant properties. The strong free radical scavenging property of flavonoids has also been linked to their anticancer activity due to their ability to prevent DNA and oxidative cell damage responsible for several cases of cancers. These phytochemicals may therefore be responsible for many of the established medicinal properties of *A. melegueta* seeds.

Table 1: Phytochemical Composition of *A. melegueta* seed

Phytochemical	Concentration
Saponin	22.0mg/100g
Flavonoids	64.86mgCE/100g
Tannin	0.86mg/100g
Alkaloid	227.0mg/100g
Sterols	360.15mg/100g

Proximate analyses showed that these seeds are rich in carbohydrates and crude fibre, whereas proteins, fat and ash are relatively low (Table 2).

Table 2: Proximate Composition of *A. melegueta* Seed

Parameter	Percentage composition
Moisture	3.47
Crude Protein	4.93

Total Fat	2.60
Total Carbohydrates	72.45
Crude Fibre	14.01
Ash	2.54

Inflammation could be defined as localized responses of body tissues to injury which includes physical trauma, radiation, chemical compounds, infection by viruses, bacteria and fungi, etc.

This natural phenomenon is characterized by redness, pains, heat and edema (i.e swelling). A study of the effect of the extract on the inflammatory process using rat-paw edema and erythrocyte membrane stabilization as models revealed that *A. melegueta* seed extract at 1000.0 and 1500.0 mg/kg wt. protected against agar-induced edema by 62% (Table 3b). The potency of the extract did not vary with increase in dose. Findings also showed that the extract exhibited a higher activity against the later phase of the inflammatory reactions than the earlier phase, unlike the NSA1D, indomethacin, which remarkably prevented both phases of inflammation.

Table 3a: Effect of Aqueous Ethanol Extract of *A. melegueta* on Agar-induced Edema in Albino Rats

Treatment	Dose (mg/kg)	Initial paw volume	1h	2h	3h	4h	5h	6h
A. melegueta	1500.0	0.30±0.01	0.58±0.01	0.57±0.16	0.90 ± 0.02	0.73 ± 0.10	0.60 ± 0.20	0.50 ± 0.04
A. melegueta	1000.0	0.25±0.03	0.61±0.02	0.87±0.20	0.90 ± 0.15	0.70 ± 0.12	0.57 ± 0.09	0.45 ± 0.09
Indomethacin	5.0	0.31±0.02	0.65±0.06	0.45 ±0.08	0.55 ± 0.11	0.51 ± 0.21	0.52 ± 0.08	0.43 ± 0.06
Water (ml/kg)	10.0	0.31±0.06	0.70±0.10	0.78 ±0.09	1.16 ± 0.06	1.18 ± 0.04	1.01 ± 0.05	0.85 ± 0.12

Each value represents mean ± SEM Paw volume (ml). n = 5

Table 3b: Percentage Inhibition of Rat Paw Edema

Treatment	Doses (mg/kg)	1h	2h	3h	4h	5h	6h
A. melegueta	1000.0	7%	-7%	23%	48%	54%	62%
A. melegueta	1500.0	28%	42%	29%	44%	57%	62%
Indomethacin	5.0	12%	70%	71%	77%	70%	77%

Ethanol extract of *A. melegueta* seed reportedly inhibited carragenan-induced rat-paw edema (Ilic *et al*., 2014). Xylene-induced topical edema induced in mouse ear and egg albumin-induced edema of rat paw were also inhibited by the methanol extract of this seed (Okoli *et al*., 2007).

The antiinflammatory effect could be ascribed to the high level of plant sterols detected (See Table 1). Inhibitory action of the ethanol fraction of *A. melegueta* grain extract against cyclooxygenase, the key enzyme catalysing the biosynthesis of prostaglandins which are chemical mediators of inflammatory response, has been documented (Ilic *et al*., 2014). The principal components of this fraction were paradol, gingerol, shogoal, gingeredrone and zingerone which are all plant sterols.

During inflammation, the lysosomes exocytose in the presence of calcium ion, releasing their enzymes, and chemical mediators of the inflammatory reactions such as prostaglandins and bradykinins (GeW *et al*., 2015). Bioactive compounds that stabilize lysosomal membranes have been found to

demonstrate antiinflammatory effects (Chayen and Bitensky, 1971)

Table 4: Effect of *A. melegueta* Extract on Membrane Stability of Bovine Red Blood Cell.

Fraction	Dose (mg/ml)	Value (%)	Inhibition%
A .melegueta	5.0	22.1	77.9
A .melegueta	10.0	50.5	49.6
Indomethacin	10.0	68.9	31.2
Ibuprofen	10.0	11.1	88.9

Each value represents mean ± SEM. n = 5

Result presented in Table 4 indicated that *A. melegueta* seed extract, at 5.0 and 10.0 mg/ml, also prevented the haemolysis of bovine red blood cells by 77.9% and 49.6% respectively, showing that the extract was more potent at a lower dose. Umukoro and Ashorobi, 2008 had earlier reported rat erythrocyte membrane stabilizing property of *A. melegueta* extract. Since the erythrocyte membrane has been reported to be similar to the lysosomal membrane in structure and composition (Chowdhury *et al*., 2014), protection against hypotonicity induced lysis of bovine erythrocyte membrane confirms the antiinflammatory activity of *A. melegueta* extract. The membrane stabilizing potential of the extract at 5.0 mg/ml, was comparable to that of ibuprofen which displayed 88.9% inhibition at 10.0 mg/ml. Both drugs, ibuprofen and indomethacin, are non-steroidal antiinflammatory drugs (NSAIDs), but indomethacin exhibited a reduced ability to stabilize erythrocyte membrane. *In vitro* study by Chayen and Britensky, 1971, also revealed that many NSAIDs such as salicylates, indomethacin, flufenemic acid and colchicines do not act by stabilizing the lysosomal membrane.

CONCLUSION

Our findings therefore validate the antiinflammatory activity of *A. melegueta* seed extract reported by other researchers in other experimentally induced inflammation models. Stabilization of the lysosomal membrane could be one of the mechanisms by which this extract produces its action, and phytosterols present in the extract may be responsible for the observed biological effects.

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