Neutraceutical and antibacterial properties of methanol extract of *Plukenetia conophora* [Müll.-Arg. family Euphorbiaceae] leaves and physical properties of its cream formulations

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ABSTRACT

The neutraceutical and antibacterial properties of methanol extract of the leaves of *Plukenetia conophora* were studied as well as physical properties of creams formulated with the extracts. Phytochemical analyses of the contents of the extract were determined using standard methods. The antimicrobial assay of the extracts was investigated and the quality and stability of the creams formulated with the extracts were also studied. Proximate analysis for the extract revealed 6.86±0.07% moisture, 11.78±0.42% protein, 8.57±0.14% total ash, 20.12±0.74% crude fibre, 1.56±0.42% total fat and 51.85±0.08% total carbohydrate. The phytochemical groups identified include alkaloids, cardenolides, flavonoids, sugars, and tannins. Polyphenolic content analysis of the extract revealed:- total flavonoids (78.27mg/g), total proanthocyanidins (73.50mg/g) and total phenolic acids (110.71mg/g). Mineral content analysis showed that the extract had some vital minerals. The antibacterial assay of the extracts displayed activity against *Proteus mirabilis* and *Bacillus subtilis*. The formulated creams passed the physical and stability studies that were carried out.The phytochemicals present in the extracts indicate the wide range of physiological and medicinal activities of the extract and thus support the folk use of *Plukenetia conophora* as a nutraceutical and antimicrobial. Furthermore, the extract could be formulated as creams with desirable physical properties and stability.

Keywords: *Plukenetia conophora*, nutraceuticals, antibacterial, creams

INTRODUCTION

Plant materials have a huge importance as a source of new drugs and leads. The potential for developing phytomedicine into various health care products appears rewarding, both from the perspective of economy and safety. Various studies have suggested that many plant extracts are quite effective than the synthetic ones with minimal side effects although very little scientific research on their biological activity has been worked out (Friedman *et al*., 2007, Serafino *et al*., 2008). In the past decade, the incidence of resistance to antibiotics has increased considerably due to overuse of antibiotics, non-adherence to antibiotic therapy, undesirable side effects, high cost of treatment and sometimes prescribing and dispensing errors. This has driven mutation in microorganisms that bring about resistance (Gould and Bal, 2013, Ventola, 2015). Consequently, some antimicrobial agents are no longer useful in the treatment of infections hence, some infectious diseases become difficult to treat (Spellberg and Gilbert, 2014).

Plukenetia conophora formally referred to as *Tetracarpidum conophorum* belongs to the family Euphorbiaceae (Onawumi *et al*., 2013). It is an evergreen perennial tree often found growing in the moist forest zones of sub-Sahara Africa during the rainy season with no special soil requirement (Burkill, 1984). It is a climbing shrub 10-20 ft. long. It is known in the southern Nigeria as *ukpa* (Igbo), *awusa* or *asala* (Yoruba) and *okhue* or *okwe* in Edo state.

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The leaves, bark, root and fruit of *Plukenetia conophora* are considered to be medicinal. The leaves are used to treat venous insufficiency, hemorrhoids, hypoglycaemia, indigestion, constipation, diarrhoea, dysentery, syphilis, thrush, intestinal worm infection, asthma, prolonged and constant hiccups, eczema, pruritus, fungal and microbial infections, psoriasis and parasitic skin conditions mostly among children, the elderly and immunosuppressed (Payal *et al*., 2014, Urszula *et al*., 2014). Other uses include: as an antidote to snakebite, management of diabetes, tonification of kidneys, and strengthening of the back and knees (Nael *et al*., 2011). The bark is used in tea as laxative, chewed for toothache and to prevent and control high blood pressure while the root is used for healing of haemorrhoids, frost bite and varicose ulcers (Wolters 2009, Faramarz *et al*., 2013).

The antimicrobial potential of the extracts and fractions of *Plukenetia conophora* against wide spectrum of bacteria (including *Staphylocci, Clostridia, Esherichia* and *Pseudomonas*) and some fungi like *Aspergillus niger* and *Candida albicans* have been demonstrated (Ajaiyeoba and Fadare, 2006, Olabinrin *et al*., 2010). Enitan *et al*. (2014) demonstrated the antibacterial activity of the methanol extract of *Plukenetia conophora* leaf on selected urinary isolates using the agar punch-hole diffusion and agar broth dilution. The extract exhibited bactericidal activity against the uro-pathogens in varying degrees at different concentrations tested.

The methanol and ethanol-water extracts of the fresh and dried leaves extracts of *Plukenetia conophora* have been shown to exhibit good free radical scavenging activity. The broad range of antioxidant activity of these extract indicates the potential of the plant as a source of natural antioxidants or nutraceuticals with potential application to reduce oxidative stress and consequent health benefit (Amaeze *et al.*, 2011). Onawumi *et al*. (2013) also demonstrated that the crude samples of *Plukenetia conophora* leaf contained secondary metabolites, vitamins and mineral constituents and the result of proximate analysis showed that the leaf contained 29±0.71% moisture, 5.63±0.08% fat, 14.92±0.04% fibre, 16.62±0.30% protein, 12.89±0.02% ash and 20.94±0.01% carbohydrate. Hence they concluded that *Plukenetia conophora* leaf is a food and could be a potential source of useful drug formulation. The results of the studies on *Plukenetia conophora* have been shown that the part of the plant as well as the processing procedures of the extract affect their phytochemical, neutraceutical as well as antimicrobial properties

It is essential that the use of the extract of *Plukenetia conophora* leaf in the management of skin diseases needs to be explored as well as formulation of a stable topical preparation. This study was aimed at determining the neutraceutical and antimicrobial properties of methanol extract of the leaf of *Plukenetia conophora* (family Euphorbiaceae), and to develop a stable topical dosage form (cream) containing extracts of the leaves of *Plukenetia conophora* to be used for the treatment of skin infections and wound implicated by the susceptible microorganisms.

MATERIALS AND METHODS

Collection and identification of plant materials

Leaves of *Plukenetia conophora* were purchased from Ikire, Osun state, Nigeria in January, 2015. They were identified at the Herbarium of the Department of Botany, University of Lagos, Akoka, Lagos State, Nigeria, and a voucher specimen (LUH 6332) was prepared and deposited in the same herbarium. The leaves were cleaned and were allowed to dry at room temperature for 3 weeks and powdered to 40 mesh and stored in an airtight container at room temperature prior to the extraction.

Test microorganisms

The microorganisms used for the study were clinical isolates of *Staphylococcus aureus, Bacillus subtilis, Streptococcus faecalis, Escherichia coli, Proteus mirabilis*,and *Pseudomonas aeruginosa*obtained from Lagos University Teaching Hospital Lagos, Nigeria.

Extraction

The dried powdered leaf was extracted by maceration at 30°C in analar grade methanol for 72 hours. After extraction, filtration was done with the aid of sterile muslin cloth. The solvent was removed by rotary evaporator and further removal of the solvent was carried out by air drying to obtain the methanol extract. The dried methanol extract was stored in sterilized bottles at room temperature for further use.

Chemical and Phytochemical analysis

Proximate analysis of the extract, including the moisture, crude protein and total ash was carried out using Association of Official Analytical Chemists (AOAC) official methods of analysis (AOAC, 1990). Total carbohydrates, including fibre, were calculated by difference. Total fat was analysed by the Chloroform-Methanol method as

recommended by the Food and Agricultural Organisation (FAO, 1986).

For phytochemical investigation of the methanol extract of *Plukenetia conophora*, methods as recommended by Sofowora (1982) were adopted. The phytochemicals determined include alkaloids, anthraquinones (free and bound), cardiac glycosides (cardenolides and cardiac glycosides with steroidal ring), flavonoids, saponins, sugars (reducing sugars and monosaccharides), tannins (hydrolysable and proanthocyanidins), terpenoids and phlobatannins.

Quantitative analysis of polyphenolic contents were carried out. Total flavonoid was estimated using the method of Miliauskas *et al*., (2004). The total phenolic content of the extract was determined by the modified Folin-Ciocalteau method (Wolfe *et al*., 2003). The procedure reported by Sun *et al*. (1998) was used to determine the total proanthocyanidin.

Mineral content analysis was carried out by first digesting the extract with nitric acid and perchloric acid and then the aliquots were used for the determination of the mineral contents. Phosphorous was determined by UV spectrophotometer (Jenway USA 6405 uv–vis spectrophotometer) while sodium and potassium were determined by flame photometer (Jenway PFP7, Bibby Scientific Stone Staffordshire, UK) (Khalil and Mannan, 1990). Iron, copper, zinc, manganese, calcium and magnesium by atomic absorption spectrophotometer (Perkin Elmer, Walthman, MA, USA) (A.O.A.C., 1990).

Antibacterial assay

The *in vitro* antibacterial activity of the methanol extract of *Plukenetia conophora* was studied in different concentrations (100, 200 and 400 mg/ml) against six pathogenic bacteria, three Gram-positive (*Staphylococcus aureus, Bacillus subtilis, Streptococcus faecalis*) and three Gram-negative (*Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa*). The antibacterial study was carried out by agar plate diffusion technique (Alalor *et.al*., 2012). Levofloxacin was used as positive control. Antibacterial potential of the methanol extract well as the control were assessed in terms of zone of inhibition of bacterial growth.

The Minimum Inhibitory Concentration (MIC) of organisms susceptible to the extract was then determined by diluting the extract to various concentrations (0.25-128mg/ml). Various sets of agar plates containing varying volumes of growth medium (Mueller Hinton agar) were prepared. The extract at different concentration was mixed with the corresponding volume of growth medium in the agar plate, and allowed to set. A standardized inoculum of 0.5 McFarland turbidity standards which is equivalent to 1 x 10^6 cfu/ml (0.1 ml) was added to each plate. Incubation was done at 37 ºC for 72 h.

Formulations

The dried extract was used for the formulation of the creams. Two different formulations containing 1%w/w of the methanol extract (Table 1) were prepared, MF1 and MF2. For formulations MF1, the required quantities of bees wax, cetyl alcohol and stearic acid were weighed and transferred into a beaker and heated in a Uniscope SM801A laboratory water bath (Surgifriend Medicals, England) set at 70^{o}C until all the ingredients melted (oil phase). Then, the required quantities of water and triethanolamine were transferred into a larger beaker and heated in the water bath set at 70^{o}C. Then, the glycerine was added and the extract was incorporated. It was removed from the heat and the melted oil phase was slowly poured into the aqueous phase a little at a time, stirring constantly. Stirring was continued until a smooth, uniform paste was obtained. The resulting cream was then packaged into a plastic cream jar and stored at room temperature until further use. The same method was employed for formulation MF2 using the ingredients listed in Table 1.

Evaluation of the formulated creams

The prepared cream formulations were subjected to some quality control analysis. Evaluation of cream pH, cream homogeneity, cream appearance, after feel, ease of cream removal, irritancy test and accelerated stability studies were carried out according to previous work done by Ashish *et al*. (2013).

Statistical Analysis

Data obtained were expressed as mean ± SD (standard deviation). ANOVA was used to test if there were significant difference in the data obtained. p-values $\leq$ 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Proximate analysis of the leaf of *Plukenetia conophora* revealed the presence of moisture, crude protein, ash, crude fibre, carbohydrate and fat in the leaves (Table 2). The moisture content, crude protein, total ash, and total fat content obtained was much lower when compared to that obtained by Onawumi *et al*. (2013) however, a higher crude fibre and total carbohydrate content was obtained.

This could be due to differences in the time of collection of the leaves and the geographical location where the leaves were collected. The low moisture content of the leaves of *Plukenetia conophora* (6.86±0.07%) when compared to 29% obtained by Onawumi *et al*. (2013) implies that the leaves might have a longer shelf life and can be preserved for a longer period of time. Fibre has a physiological effect on the gastrointestinal function of promoting the reduction of tracolonic pressure which is beneficial in diverticular disease. Fibre also has a biochemical effect on the absorption and re-absorption of bile acids and consequently the absorption of dietary fats and cholesterol (Edeoga *et al*, 2006). As indicated by the higher fibre content of 20.12%, the leaves when incorporated in diets, will improve digestion and promote good gastrointestinal health. It will also help to improve the absorption of dietary fats.

Table 1: Percentage composition of the *Plukenetia conophora* methanol extract cream formulations MF1 and MF2

	INGREDIENTS	**FORMULATION (% w/w)**	
		MF1	**MF2**
	Methanol Extract	1.0	1.0
Water phase	Propylene glycol	-	5.0
	Tween 80	-	2.0
	Water	70.0	70.0
	Triethanolamine	2.0	2.0
	Glycerine	5.0	-
Oil phase	Cetyl alcohol	5.0	5.0
	Stearic acid	10.0	10.0
	Beeswax	7	-
	Arachis oil	-	5.0

- Indicates not present

Table 2. Proximate composition of the leaf of *Plukenetia conophora* on a dry weight basis

Dietary nutrient	**% composition in leaf of *P conophora***
Moisture content	6.86±0.07
Crude protein	11.78±0.42
Total ash	8.57±0.14
Crude fibre	20.12±0.74
Total fat	1.56±0.42
Total carbohydrate	51.85±0.08

Table 3. Polyphenolic content of the methanol extract of *Plukenetia conophora*

Polyphenols	**Concentration (mg/g)**
Total flavonoids (QE)	78.27 ± 2.01
Total phenolic acids (GAE)	110.71± 3.06
Total proanthocyanidins(CE)	73.50± 1.81

QE: Quercetin equivalent, GAE: Gallic acid equivalent, CE: Catechin equivalent.
Absorbances are expressed as mean ± standard deviation of triplicate determinations

Table 4. Mineral content (mg/g) of the methanol extract of *Plukenetia conophora*.

Composition	Methanol extract
Calcium	3.030
Potassium	0.986
Sodium	1.452
Magnesium	3.919
Zinc	1.028
Iron	3.621
Manganese	2.864
Copper	0.002
Chromium	0.031
Nickel	0.034
Cadmium	0.050

Table 5: *In vitro* antibacterial activity of the *Plukenetia conophora* methanol extract

Extract /Positive Control	Diameter of zone of inhibition (mm)					
	Bacillus subtilis	*Staph aureus*	*Streptococus faecalis*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Proteus mirabilis*
Extract (400mg/ml)	20.0±1.2	Nz	nz	nz	nz	**22.0±0.2**
Extract (200mg/ml)	17.0±1.5	Nz	nz	nz	nz	**17.00±1.8**
Extract (100mg/ml)	Nz	Nz	nz	nz	nz	**15.0±1.3**
Levofloxacin (20µg/ml)	34.0±0.8	29.0±0.6	22.0±0.3	18.0±0.5	26.0±1.4	nz
Levofloxacin (10µg/ml)	28.0±0.5	24.0±0.8	18.0±0.9	nz	23.0±0.8	nz
Levofloxacin (5µg/ml)	22.0±1.1	18.0±0.5	nz	nz	19.0±0.2	nz
Levofloxacin (2.5µg/ml)	18.0±0.5	15.0±1.2	nz	nz	17.0±0.8	nz

Values are the means of triplicate treatments with standard deviation; nz means No zone of inhibition

Preliminary phytochemical screening of the methanol extract for secondary metabolites, showed the presence of alkaloids, cardenolides, flavonoids, sugars (including reducing sugars), tannins and terpenoids. Alkaloids play an important role in the defence systems against pathogens and animals. Alkaloids belonging to beta-carboline group possess antimicrobial, anti-HIV and antiparasitic activities (Bouayad *et al*., 2011) The presence of tannins in the leaf of the *Plukenetia conophora* plant can support its strong use for healing of hemorrhoids, frost bite and varicose ulcers in herbal medicine (Onawumi *et al*, 2013).

The results of the quantitative analysis of polyphenolic contents are presented in Table 3. Plant phenolics are a major group of compounds acting as primary antioxidants or free radical scavengers (Kähkönen *et al*., 1999) Phenolic compounds contribute to quality and nutritional value in terms of modifying color, taste, aroma, and flavor and also in providing health-beneficial effects. They also serve in plant defense mechanisms to counteract reactive oxygen species in order to survive and prevent molecular damage and damage by microorganisms and insects (Esmaeili *et al*., 2012). The presence of the polyphenolic compounds indicates the extract could serve as good of nutraceuticals.

The result for the mineral analysis (Table 4) showed that the methanol extract of the dried leaves of *Plukenetia conophora* plant contained some vital minerals. Therefore, the methanol extract could be good potential source of vital minerals that are useful to the human body. This result become so important when the usefulness of such minerals like Ca, Mg, Na, K in the body is considered.

The result of the antimicrobial assay (Table 5) of the methanol extract showed that the methanol extract both displayed concentration-dependent antibacterial properties against *Bacillus subtilis* and *Proteus mirabilis* but showed no activity against *Staphylococcus aureus*, *Streptococcus faecalis*,

Pseudomonas aeruginosa and *Escherichia coli*. Interesting, at the minimum concentration used, 100 mg/ml, the methanol extract had a diameter of zone of inhibition of 15.00 mm against *Proteus mirabilis* while, the reference standard, levofloxacin used,, failed to inhibit the growth of *Proteus mirabilis* at the highest concentration used (20µg/ml). This implies that in the management of infections caused by the anaerobic bacteria *Proteus mirabilis*, treatment using the extract of *Plukenetia conophora* might be more effective than the use of antibiotics such as levofloxacin and similar drugs. The antimicrobial activity results of the same plant part tested most of the time varied from one study to another. The differences in the concentration of plant constituents of the same plant organ might be attributed to the age of the plant, differences in topographical factors, the nutrient concentrations of the soil as well as collection day and time, drying process, extraction procedures and other experimental procedure used for antimicrobial study. The relationship between chemical composition of plants and geographical location has been documented (Raoand Rout, 2003).

The Minimum Inhibitory Concentration (MIC) gave the lowest concentration of the extract that inhibited the growth of the test organisms. The MIC values obtained were 1.0mg/ml for *Proteus mirabilis* and 4.0mg/ml for *Bacillus subtilis*. In general, the lower the MIC values, the more sensitive the microorganisms to the antibacterial agent. The MIC values obtained for the extract of the plant further gives credence to the traditional use of *Plukenetia conophora*for the treatment of infections.

The prepared ointment showed no irritant effect when applied on the skin. The creams readily spread when rubbed gently on the skin. The pH of the formulations were between 6.8 -7.6 throughout the period of storage at 25 and 40^{O}C for 20 days. The pH of the formulations lies in the normal pH range of the human skin (6.8±1). Therefore, the cream is fit for application to the skin and no harsh effect will be produced on the skin. The absence of liquefaction throughout the 20 days of observation provided strong evidence for the stability of the formulations under investigation. Other physicochemical properties of the cream such as the homogeneity, appearance, after feel, type of smear and ease of removal did not change at elevated temperatures. All the formulations were safe in respect to skin irritation and allergic sensitization. However, the homogeneity of cream formulation, MF2, containing tween 80 was better. Tween 80 (Polysorbate 80) is a hydrophilic nonionic surfactant. The better homogeneity of the formulation containing tween 80 might be attributed to the surface active activity of tween 80. The lowering of the interfacial tension by tween 80 between the oil and aqueous phases used in the formulation facilitated a homogenous emulsion formation. For semi-solid dosage forms that are applied topically for therapeutic effect, it is desirable that the active substance should be released at the skin surface and should penetrate at a suitable rate in sufficient amounts to maintain an effective concentration at the site of action (Azubuike *et al*. 2015). Topical formulations present many challenges related to bioavailability and stability and hence therapeutic effectiveness. Control of critical material attributes such as particle size and viscosity are important to ensure formulation's target profile is achieved (Pharmaceutical Codex 1994). These as well as antibacterial activities of the formulated creams will be subject of further studies.

CONCLUSION

The nutraceutical and antimicrobial values of the methanol extract of *Plukenetia conophora* leaves have been established as evident in the results obtained from proximate, mineral content analysis and microbial assay of the extract and fraction of the leaves analysed. This discovery coupled with the presence of zinc and other phytochemicals further supports the formulation of extract and fraction of *Plukenetia conophora* leaf as a cream in the management of susceptible bacteria skin infection. Evaluation of the efficacy and stability of the formulated creams revealed their potential use in the treatment of susceptible bacteria skin infections.

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