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Research Article

Evaluation of Antimicrobial Activity of Formulated Water-in-oil Cream Containing Extracts of *Cajanus cajan* (Pigeon Pea) L. Leaves

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Abstract

This work involved extraction of the active components of Cajanus cajan (Pigeon pea) leaves with various organic solvents in order of increasing polarity using n-hexane, ethyl acetate, ethanol and water. The extracts obtained were screened for phytochemical components. Extracts with antimicrobial susceptibility were formulated into water-in-oil creams in varying concentrations using reference standard. Formulated creams were evaluated using various parameters such as organoleptic property, homogeneity, pH, viscosity and irritability test on rabbit skin. Extracts were tested against the following typed cultured organisms *Staphyloccus aureus* NCTC 6571, *Pseudonomas aeruginosa* ATCC 27853, *Bacillus subtilis, Escherichia coli* NCTC 10418, *Salmonella typhi* and *Candida albican* using agar well diffusion method. Preliminary phytochemical screening of Cajanus cajan leaves showed presence of alkaloid, phenol, flavonoid, saponin, terpenoids. Ethanol and ethyl acetate extracts inhibited the growth of all bacteria and showed antimicrobial activity against fungi while the aqueous extract had no activity on the organism. N-hexane extract was only active on gram positive bacteria. Highest antimicrobial activity on gram negative bacteria was shown by ethyl acetate extract while ethanol extract showed highest activity on gram positive bacteria, *Bacillus subtilis*. The minimum inhibitory concentration of the extracts on the organism was in the range of 3.125 mg/mL – 50 mg/mL. Products showed the same antimicrobial activity with the extracts. The formulated creams have the same activity as the extracts with Cream containing 5% of the ethanol extract having the highest activity against Bacillus subtilis. Formulation C5 containing 1% ethyl acetate extract showed activity against all organisms. Differential Scanning Calorimetry (DSC) studies of the creams showed that the extracts and the excipients were compatible. Also, the products (C1-C6) were tested on the rabbit skin to check the irritability potential, it did not irritate rabbit skin, indicating the creams were safe for topical applications.

Keywords: *Cajanus cajan*; Antimicrobial Activities; Minimum Inhibitory Concentration; Phytochemicals; Topical Cream; Bacteria; Susceptibility

Introduction

Human beings from ancient times, have been faced with various health challenges which have hampered their economic, social, financial and personnel development. Among these health challenges that faces humans are predominantly caused by microorganisms. There are various topical synthetic products that are used to combat these infectious microorganisms, but their cost, unavailability, irrational use and abuse; and multi drug resistance has created the need to source for other alternative, safe and effective drugs from natural sources which are readily available and affordable [1]. Microbial contamination has increasingly become a health challenge in the global healthcare sector [2,3].

As reported in WHO 2020 "Policy to combat antimicrobial resistance", pathogenic micro-organisms has continued to pose as a risk to many medical and health procedures and increasing infections and hospitalization time has impacted adversely on the economy. In traditional folk medicine, majority of plant species like *Cajanus cajan* has shown antimicrobial activities and has been used in treatment of several diseases and ailment [4]. Though *Cajanus cajan* is mainly planted because of the seeds that is consumable, it has other inherent properties to be harnessed, therefore, it may be said to be a specie with numerous purposes and uses [5,6].

It has various medicinal uses such as the roots decoction used as an antihelminthic, the seeds as a sedative in traditional Chinese medicine [4]. The leaves are used to stimulate energy and manage hyperglycaemia in Garo people of the Bangladesh [7,8]. The leaves are used to manage food poisoning, manage colic and treat difficulty in stooling in Trinidad and Tobago [9]. It is utilized in stopping bleeding, used as pain relieve and to eradicate parasitism in folktale of Chinese people [7,8]. In some parts of India, the leaves, seeds and stems that are not matured are used to cure inflammation of the gums, anaemia, and stomatitis and can be used as a toothbrush [10]. Immature leaves are chewed in the treatment of boils on the tongue. A decoction is used for washing wounds that have opening. Also leaves when boiled can be used in sores and wounds to hasten its healing. The leaves decoction also used as an expectorant and in treatment of pulmonary conditions example cough, pneumonia and bronchitis in people affected and suffering such ailment in their area [8]. Freshly prepared leaf juice has a nullifying effect of alcohol intoxication and can be used as laxative in cases of constipation. The leaf decoction can be used to treat genital discomfort, urinary urgency while seeds that have not reached up to full age are helpful to treat kidney and liver issues [11]. The juicy side of the leaves is used for the eye in epileptic people in the Congo Republic by the Kongo, Dondo and Lari tribe [12].

The antimicrobial study of the Supercritical fluid extract (SFE-CO_2) of *Cajanus cajan* plant in the laboratory and in animal model were carried out in a work done by Yuan-gang., *et al.* (2010). A prominent susceptibility effect of the organisms *S. epidermidis*, *Staph. aureus* and *Bacillus subtilis* were shown using SFE extracts [16]. The microwave and hydro distillation extraction of essential oil that was collected from solvent free extraction have shown an excellent antimicrobial activity against both gram negative and gram positive bacteria, *B. subtilis* and *Proponibacterium acnes* [13,17]. Cajanuslactone, Pinostobin and cajaninstilbene acids have an increased activity against the bacteria *Staphylococcus aureus* [16]. In the work of Proma., *et al.* (2018), extracts of *Cajanus cajan* leaves has highlighted to be potent against some disease causing bacteria and results showed that the extract could inhibit successfully the growth of bacterial *Salmonella typhi.* The major component of the leaves extract stilbene and flavonoids could effectively inhibit growth of *Salmonella typhi* and *Escherichia coli* [14].

The various extracts were formulated into creams. Creams are viscous liquids or semi-solid emulsions of either oil-in-water or water-in-oil type. The water-in-oil cream was formulated using various solvent extract (n-hexane, ethyl acetate and ethanol) that shown microbial inhibitory activity [15].

Materials and Methods

N-hexane, ethyl acetate, ethanol were all analytical grade (Sigma Aldrich Germany), Muller Hinton agar, Nutrient broth, Sabroud dextrose broth (Titan Biotech LTD, Rajasthan, India), Distilled water, Dragendorff's reagent, alkaline reagent (NaOH) (JT Bayer, USA), ferric chloride, dimethyl sulphur oxide (DMSO) (JT Bayer, USA).

The microorganisms used were *Staphylococcus aureus* NCTC 6571, *Bacillus subtilis, Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi,* and *Candida albican. Escherichia coli* NCTC 10418. These microorganisms were typed cultured organisms obtained from Microbiology laboratory, Ahammadu Bello University (ABU) Zaria, Kaduna State.

Preparation of the plant material

Cajanus cajan (pigeon pea) was collected from a farm in Nnewi South Local Government of Anambra State in July 2021 when the leaves were all out. It was identified by a taxonomist in the faculty of agriculture and wild Life University of Port-Harcourt and a voucher specimen kept in the university's herbarium. Samples of the plant part (leaves) were separated into various parts, the leaves, stems, seeds and flowers by hands. The plant leaves were kept under a shade, dried in the open air at ambient temperature until it was properly dried. It was coarsely grinded in a milling machine, weighed and stored in an airtight container for further use.

Determination of Ash Profile of the plant material

Total ash

Dried leaves of the *Cajanus cajan* was incinerated to determine the ash content. The coarsely grinded leaves of *Cajanus cajan* 6 g was weighed and put into three nickel crucibles at 2 g each, the powder was incinerated in a furnace by gradually increasing the heat until the sample was free from carbon at about 450°C. It was cooled and kept in a desiccator. The formed ash was weighed, the percentage of total ash concerning the air-dried sample was calculated.

$$\frac{\text{Weight of ash (After incineration)}}{\text{Weight of original plant}} \times \frac{100}{1} \quad \text{.....Equation 1}$$

Acid insoluble ash

The total ash gotten was taken into dilute HCL 25 mL in a beaker, boiled in a water bath for five minutes until it boiled. It was filtered with ashless filter paper. The beaker and crucible were washed repeatedly using water until it is free from acid, it was passed through the filter paper. The filter paper was dried in the oven, folded into a narrow cone, inserted into a tarred nickel crucible and heated at 450°C in a furnace until it was completely turned to ash. The residual ash was heated and cooled in a desiccator after it was weighed.

Water soluble ash

A 2 g of the plant material was put in a crucible and heated in a furnace at 450°C until it's free from carbon. The crucible was cooled in a desiccator and weighed. Heating continued until a constant weight was achieved. The ash was transferred into a 25 mL beaker of water, heated for 5 minutes in a water bath. The mixture was filtered through ash less filter paper. Both were dried in the oven, compressed into a cone. Then transferred into a crucible and heated until the ash less paper was entirely eliminated. The ash were weighed and recorded.

Extraction of the plant material

A total of 600 g coarsely milled air dried leaves of *Cajanus cajan* was poured into a glass stoppered jar, then 2.5 L of n-hexane was poured into the glass jar containing the leaves, which gave a concentration of 0.24 g/mL, it was kept for 3days with mild shaking at 24 hours intervals. On the 3rd day, the n-hexane extract was filtered with the aid of a separating funnel. The filtrate was concentrated in a water bath, after concentration, it was transferred to appropriately labelled crucible where the residual solvent were allowed to evaporate to dryness.

The Marc obtained from N-hexane extraction (after collection of the filtrate) was air-dried and soaked in another 2.5 L of ethyl acetate solvent which gave approximately a concentration of 0.24 g/mL. It was kept for 3 days with mild shaking at 24 hours intervals. On the 3rd day, the ethyl acetate solvent extract was filtered using separating funnel. The same procedure was repeated for all the solvents ethanol and water. The different solvent extracts obtained were stored in a desiccator for further analysis [17].

Preliminary qualitative phytochemical screening

Phytochemical screening of the extract was carried out, for the presence of alkaloids, Dragendorff's test was employed. A 1 mL volume of extract was measured with a 1 mL pipette and emptied into a test tube. Then, 1 mL of potassium bismuth iodide solution (Dragendorff's reagent) was added into the test tube and the mixture shaken. An orange coloured precipitate was formed which showed that alkaloids were present.

For Flavonoids, alkaline reagent test was employed. A 1 mL volume of the extract was emptied into a few drops of NaOH solution in a glass tube for test, and mildly agitated, an obvious intense yellow mass which turns no colour when diluted acid was added was see. Presence of Saponin was detected by Frost Test. Diluting ethanolic extract with 20 mL distilled water and was shaken for 15 minutes in a graduated cylinder. A 1 cm formation of foam layer shows the presence of Saponin.

To test for phenol using Ferric Chloride Test. According to Pandey and Tripathi (2014), a bluish black colour was observed when a 1 mL volume of plant extract was added 1% gelatin solution which contains sodium chloride in a test tube. After shaking for a minute, the colour was observed which indicates Phenol presence [18].

Gelatin's Test was used for tannin. A I mL of ethanolic extract was collected and placed into a test tube. A 1% gelatin solution which contains sodium chloride was added and agitated, white precipitate was observed which showed that tannins were present.

About 1 g of the powdered drug with 5 mL of alcoholic KOH for 5 minutes mixed in a test tube, was transferred to an aqueous solution containing $FeSO_4$ and $FeCl_3$, which was maintained at 60-70°C for 10 minutes. The contents were transferred to 20% HCL. The uncovering of a Prussian blue colour which is distinct, confirmed phenol.

For Phytate, about 10 g of *Cajanus cajan* was extracted with HCL acid. It was reacted with phytase and phosphatase enzymes Megazyme K-PHYT, phosphorus was released, which indicated the presence of phytate.

Quantitative determination of the phytochemical constituents

Flavonoid determination

The method of Boham and Kocipai was used. A 10 g quantity sample was weighed and extracted using 80% methanol of 100 mL at 27°C continuously. Filtered with 125 mm filter paper No 42. The filtrate was taken to a pre-weighed crucible and evaporate until it dried. It was reweighed till the weight did not change. % Flavonoid = Weight of flavonoid /Total weight used X 100 ……..Equation 1

Alkaloids determination

The gravimetric method of Harbone (1973) was used for alkaloids. A 5 g quantity of sample introduced into solution of 10% acetic acid plus ethanol. It was left for 4 hours after agitation, then filtered. In a water bath, solvents were allowed to dry off. NH_4OH was introduced in drips which precipitated the alkaloids. It was cooled and filtered. The cloudy mass were dried at 60^0C in an oven and weighed [19].

The (%) of alkaloid was calculated using equation 2.

Alkaloid (%) = (weight of filter paper + alkaloid) – (weight of the filter paper) X 100/ Weight of plant material used (5 g)……Equation 2

Saponin determination

The method of Obadoni and Ochuko (2001) were used for Saponin. A 20 g quantity of the sample was weighed into a conical flask. A 100 ml of 20% aqueous ethanol was added. Heat was applied for 4 h in a water bath with stirring continuously at about 55^0C. The solution was filtered and the residue was re-extracted with another 200 ml of 20% ethanol. The extract was concentrated in the water bath to 40 ml at 90^0C. A 20 ml volume of diethyl ether was added and shaken vigorously to separate. The aqueous layer was recovered [20,29].

A 60 ml of n-butanol was added followed by washing with 10 ml 5% aqueous sodium chloride. The remaining solution was collected into a weighed petri dish (W1). The petri dish was dried in an oven at about 90°C. The petri dish was re-weighed and recorded as W2. The Saponin was calculated as in equation 3.

% Saponin = W2 – W1/ W0 X 100 …….. Equation 3

Tannin determination

Tannin was determined as described by Pearson (1976). A 1.0 g quantity of the test sample was weighed, 10 mL distilled water added and shaken at 5 min interval for 30 min. It was filtered to get the extract. A 2.5 mL volume of the supernatant was transferred into a test-tube also 2.5ml of standard tannic acid solution was also transferred into a 50ml flask. A 1.0 mL volume Folin-Denis reagent was added into the flask, followed by 2.5ml of saturated Na_2CO_3 solution. The solution was made up to the mark with distilled water [30].

Absorbance of the resultant solution was read after 90 min. incubation at room temperature. The percentage of tannin present was calculated using equation 4.

%Tannin=An/AsXC/WX100Xvf/va…….. Equation 4

Where:

An = absorbance of test sample

As = absorbance of standard solution

C = Conc of standard solution

W = weight of sample used

vf = total volume of extract

va = volume of extract analyzed.

Determination of phenol by spectrophotometric method

A 2 g quantity of the test sample were weighed into a flask, it was de-fatted using petroleum ether. The residues were allowed to stand for few minutes to air dry. The residue were boiled with 50 ml of diethyl ether for 15min. to extract the phenol. 5 mL was transferred into test tube.

A 2 ml volume of NH_4OH solution and 5 ml of amyl alcohol were introduced and kept for about 30 minutes for development of inference. The absorbance was read at 765 nm using UV-VIS spectrophotometry (Jenway, UK). Concentrations of Garlic acid standard were prepared. The absorbance was read. A calibration curve of

the absorbance against concentration was plotted. The absorbance of the sample was read by extrapolation and by tracing the concentration to get the sample concentration.

Phenol (mg/100g) =

Conc. obtained in mg/L/ sample weight X volume of sample

sample weight ……Equation 5

Determination of cardiac glycosides

Cardiac glycosides of the samples were quantitatively determined according to Solich., *et al.* 1992. A 1.0 g quantity of the sample was mixed with 10 mL freshly prepared Baljet's reagent (95 mL of 1% picric acid + 5 mL of 10% NaOH). Cyanogenic glycoside is obtained from the relation: After an hour, the mixture was diluted with 20 mL distilled water and the absorbance was measured at 495 nm in a spectrophotometer [21].

Determination of phytate

A 2 g of the extract were weighed into a 250 mL conical flask, 100 mL of 2% concentrated HCL was added and allowed to soak for 3 hour, was filtered. 50 mL of the filtrate was pipetted into a 250mL beaker. 107 mL of distilled water was added to improve acidity. 10 mL of 0.3% ammonium thiocynate solution was added as indicator. Titration was then carried out with standard iron III chloride ($Fecl_3$) solution which contains 0.00195g iron/ml. the end point was a brownish yellow colour which persisted for about 5 minutes. The phytate is calculated as follows in equation 6.

Phytate in (%) = 0.00195 X Average titre value / 2 X 100 …. ……..Equation 6

Antimicrobial assay

Preparation of microbial cultures

The microorganisms used were *Staphylococcus aureus* NCTC 6571, *Bacillus subtilis, Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi,* and *Candida albican. Escherichia coli* NCTC 10418. These microorganisms were typed cultured organisms obtained from Microbiology laboratory, Ahammadu Bello University (ABU) Zaria, Kaduna State.

Fresh bacterial cultures were prepared by sub-culturing stock typed bacterial cultures into freshly prepared Muller Hinton agar and incubating at 37°C for 24 hours while fresh fungal culture was prepared by sub culturing stock *Candida albican* culture into freshly prepared Sabroud dextrose agar and incubated at room temperature for 24 hours [22].

These 24 hours old bacterial and fungal cultures were transferred into freshly prepared nutrient broth and Sabroud dextrose broth in a test tube. 10 mL normal saline was added to dilute the cells and these diluted cells were compared with standard 0.5 Mcfarland turbidity to obtain the desired cell density of 1 X 10^8 (cells/mL) [22].

All safety protocols were ensured in handling the microorganism culture as it involves risk of infection. Aseptic techniques and thorough wearing of PPE such as hand gloves, facemask and laboratory coat at all times during the work. Sterilization of the work area with diluted hypochloride solution were done before and after each session during the analysis.

Agar well diffusion Assay

The Agar well diffusion media were prepared using Muller-Hinton agar and Sabroaund dextrose agar for bacteria and candida respectively. It was sterilized in the autoclave, poured into already labelled petri-dishes and allowed to solidify. A 0.5 µL volume of inoculum of each microorganism was seeded into warm molten Muller-Hinton agar for bacteria and Sabroaund dextrose agar for candida. A sterile cork borer of 8 mm width was made into the agar plates. A stock solution of 100 mg/mL of each of the extract (N-hexane, ethyl acetate, ethanol and water) were prepared individually by dissolving 100 mg of each extract in 1 mL of the solvent. A 100 µL volume of stock solution of each plant extract was pipetted onto the hole, 100 µL of each of the solvent was pipetted into the hole at centre of each petri dishes which served as the negative control. Streptomycin antibiotic disc was placed on the agar plate as positive control. The diameter of the zone of inhibition were measured in mm. These experiments were repeated twice and the average values were recorded [22,23].

Minimum inhibitory concentration

The stock solution containing 100mg/mL of the different solvent extract were subjected to a serial dilution which gave a concentration 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL and 3.125 mg/mL respectively in the five tubes and this same procedure was carried out for ethyl acetate and ethanol extracts.

Inoculum of microorganism was made in an appropriately labelled sterile plate containing Muller-Hinton agar for bacteria and Sabrouand dextrose agar for *candida spp* as previously stated. A sterile cork borer measuring 8 mm was used to make five holes

in each plate and each concentration of the plant solvent extract was pipetted into the appropriate hole, a control containing only the solvent was also made at the centre which served as a negative control. These were incubated at 37°C for 24 hours. The minimum zones of inhibition were measured using a meter rule, results determined and recorded.

Differential scanning calorimetry of extract

Two of the extracts ethyl acetate extract and ethanol extracts and their formulated creams were subjected to DSC that uses heat measurements to characterize substances and biomolecules. The molecules in the substance interacts with each other and these interaction could be favourable or unfavourable which the DSC analyse. It also analyse the stability of these extracts through their thermodynamic changes using NYSE: MTD DSC (Switzerland).

Formulation of water-in-oil cream

Six different formulations C1, C2, C3, C4, C5 to C6 were formulated two each with varying solvent extracts, base and other ingredients. The amount of extracts in the different formulations were varied based on the antimicrobial activity of the crude extract [24,25].

Preparation of cream

Preparation of *Cajanus cajan* topical antimicrobial cream were carried out using the formulae in table 1.

Ingredients	Quantity Required			Quantity used	
Formulation code		**C1**	**C2**	**C3**	**C4**
Solvent extract		N- Hexane		Ethanol	
Crude extract		2 g	1 g	1 g	0.5 g
% Strength/ conc.		10%	5%	5%	2.5%
Beeswax	10 g	4 g	4 g	4 g	4 g
Liquid Paraffin	30 g	12 g	12 g	12 g	12 g
Borax	0.5 g	0.2 g	0.2 g	0.2 g	0.2 g
Distilled water	9.5 mL	sq	sq	Sq	sq

Table 1: Ingredients for 20 g of batch C1 to C4.

Procedure

Beeswax 4 g was weighed and poured into a clean ceramic mortar, it was grated using pestle, and the grated beeswax was melted with 12 g of liquid paraffin at a temperature of about 70°C. A known amount of the extract was weighed and added into distilled water containing 0.2 g borax, the solution was heated to a temperature of 70°C. The solution was gradually added to the mixture of beeswax and paraffin oil. It was stirred mechanically until the preparation started to thicken. It was then transferred into the cream jar and closed tightly to prevent dehydration. This same process was carried out for formulation code C2 to C4 with varying amount of crude extract.

Preparation of oily cream

Oily cream was prepared according to [24]. It was prepared for Formulation batch C5 and C6. The ingredients here were varied to ascertain if it has significant difference on the antimicrobial activity of the extract.

A 20 g Oily cream for each of formulation code C5 and C6 was as follows.

Procedure

A 10 g of wool alcohol ointment was weighed and were melted in a water bath at 60°C, then 0.2 g of ethyl acetate extract was dissolved in it, it was added gradually with constant stirring, the purified water previously boiled and cooled to 60°C in a warmed mortar and pestle was added. It was vigorously mixed until a smooth cream was obtained, stirring continued until it was cold, then it was transferred into the cream jar, polished, labelled and kept for analysis.

Wool alcohol ointment P.C was prepared, then 10 g was used in preparation of the Oily cream P.C [24].

Ingredients	Quantity Required	Quantity used	
Formulation code		**C5**	**C6**
Solvent		**Ethyl acetate**	**Ethanol**
Crude extract		0.2 g	1 g
% strength/ conc.		1 %	5 %
Wool alcohol ointment	500 g	10 g	10 g
Distilled water	500 mL	10 mL	10 mL

Table 2: Ingredients for batch C5 and C6.

Formula code	Solvent extract present	Quantity of extract present	%Strength/Conc.
C 1	N-hexane	2g	10%
C 2	N-hexane	1g	5%
C 3	Ethanol	1g	5%
C 4	Ethanol	0.5g	2.5%
C 5	Ethyl acetate oily cream	0.2g	1%
C 6	Ethanol extract oily cream	1g	5%

Table 3: Percentage strength of extracts in 20 g each of batches C 1 to C 6.

Batch	Solvent extract					Excipients	
		Extract qty	Borax	White beeswax	Liquid paraffin	Wool alcohol ointment	Water
C1	N-Hexane	2g	0.2g	4g	12g		sq
C2	N-Hexane	1g	0.2g	4g	12g		sq
C3	Ethanol	1g	0.2g	4g	12g		sq
C4	Ethanol	0.5g	0.2g	4g	12g		sq
C5	Ethyl acetate Oily	0.2g				10g	10mL
C6	Ethanol Oily cream	1g				10g	10mL

Table 4: Composition of the various batches of *Cajanus cajan* leaf extract.

Sq: sufficient quantity.

Physical evaluation of the topical formulations

Organoleptic characteristics of all formulations were tested for physical appearance, colour, texture, state, homogeneity and odour were evaluated visually and by inhalation.

Homogeneity was carried out by taking a little amount of the cream using a spatula, the cream was felt in between the fingers of the index and thumb. The smoothness and consistency were felt. The texture and homogeneity was checked by presence of coarse particles in the products formed [26]. It was graded as the case may be: Excellent. Very good. Good and Poor. [26].

pH values

A 50 mL of distilled water was used to dissolve 0.5 g quantity of the formulated cream of various extract. The hand pH meter (Hanna, China) was used to measure the pH. The meter was marked using standard solutions buffered before use [27].

Odour

This was tested by bringing the formulated creams close to the olfactory sense organ for smell and the odour was noted.

Stability Studies of the formulated creams

Stability studies were carried out as stated by the International Council for Harmonization (ICH) guidelines using Intermediate stability parameter.

The formulated cream was kept at a temperature of 30° ± 2°C/65% RH ± 5% RH in a cupboard for 4 months and the cream maintained its consistency and homogeneity as observed macroscopically after the period [28].

Viscosity test

The viscosity test was carried out with Shanghai Nirum Rotation Viscometer using spindle 4 at different rotation per minute (6, 12, 30, 60 rpm) and the results were recorded.

Irritation test

Irritation testing of the formulated cream was carried out on the skin of rabbits. Three rabbits were tested, each was tested with two of the formulated creams. A circumference of the skin was scrapped then 2 mg of the cream was applied on the skin and left for one hour, it was washed off and observed using a hand lens, the procedure was repeated for 5 days and observed for swelling (edema) and redness (erythema) (ISO 10993-1:2018 (E) FDA).

Assay of the cream

Zone of inhibition

The antimicrobial inhibition diameter of the various extracts was used to assay the cream formulated to ascertain that the extract was not affected by the compositions of the cream. The cream which was formulated with varying amounts of extracts and varying bases, excipients to get batches C1, C2, C3, C4, C5 and C 6 were assayed with the typed cultured organism to determine antimicrobial activity and to determine the minimum inhibitory zone diameter. The weight of the formulated cream C1, C2, C3, C4, C5 and C6 is 20 g each. A 5 g quantity of each of the cream was dissolved in 10mL DMSO to get a stock solution of 500 mg/mL. Five sterile cream jars was set up to carry out a serial dilution of the cream. A 1 mL volume of the DMSO was measured into the five cream jar each, then 1 mL from the stock solution was added into the first jar, mixed and 1 mL collected, then put into the second jar to give a concentration of 250 mg/mL and 125 mg/mL respectively. This same procedure was carried out into the third to fifth cream jar to get a concentration of 62.5 mg/mL, 31.25 mg/mL and 15.625 mg/mL respectively for each of the formulation while the concentration of the extracts differs relative to the quantity in the cream. The dilutions relative to the concentration of the extracts are expressed in the Appendix Table 9. Muller-Hinton agar were prepared, sterilized in an autoclave and 10 mL was introduced into each of the sterile plates and allowed to cool and set. The plates were seeded with labelled organisms each by streaking with a loopful of organism taken from the broth. Using sterile 8 mm cork borer, five holes were made in each of the plates and properly marked and labelled. Using a micropipette, 100 µL each of the dilutions was introduced into the already marked holes and incubated at 37°C for 24 hours for the bacteria organisms and at room temperature 27°C for fungi species for 24 hours. The zones of inhibition were measured and the minimum inhibitory concentration determined. This procedure was carried out twice and the average diameter zone of inhibition calculated.

Statistical data analysis

Results are given as a mean ± standard deviation. A one- way analysis of data (ANOVA) was carried out using Fisher's least significant difference (LSD) post hoc to decide significance level.

Results and Discussion

Plate 1: Showed the seed and flower bearing plant of the Cajanus cajan. (Source https://tropical.theferns.info)

Ash profile, water soluble ash and acid insoluble ash of the leaves extracts of *Cajanus cajan* were 1.85% and 6.2% respectively.

Extracts are light green to dark green in colour, smooth with aromatic odour.

Irritation test

Results of plates 1, 2, 3 and 4 showing application of the cream, when it was washed off, viewed for redness, swelling or any irritation sign without hand lens and with the use of hand lens.

Table 5: Percentage yield of the different extract of *Cajanus cajan.*

Solvent used	Weight of Extract (g)	% Extract yield
N-hexane	32.5	5.42
Ethyl acetate	30.81	5.14
Ethanol	32.88	5.5
Water	27.52	4.59

Table 6: Qualitative and quantitative phytochemical constituents in the ethanolic leaves extract of *Cajanus cajan* (Pigeon pea).

S/N	Test	Quantitative %	Qualitative
1) Flavonoid	Alkaline test	5.0	+++
2) Saponin	Frothing test	2.0	++
3) Alkaloid	Dragendorff's	3.0	++
4) Phenol	Ferric chloride	15.91	+++
5) Cyanogenic glycoside		3.28	++
6) Tannin	Gelatin	1.215	+
7) Phytate	Megazyme K-PHYT	0.07	Trace

Key: + present.

Table 8: Minimum inhibitory concentration of *Cajanus cajan* solvent extracts.

Organism	Extract	Concentrations (mg/ml)				
		50	25	12.5	6.25	3.125
Staphylococcus spp	N-Hexane	2 mm	-	-	-	-
	Ethyl- acetate	8 mm	6 mm	4 mm	2 mm	-
	Streptomycin (Negative control)	-	-	-	-	-
Pseudomonas spp	Ethanol	8 mm	6 mm	4 mm	2 mm	
	Ethyl acetate	10 mm	8 mm	5 mm	3 mm	1 mm
	Streptomycin (Negative control)	-	-	-	-	-
Bacillus subtilis	Ethyl acetate	6 mm	3 mm	2 mm	-	-
	Ethanol	8 mm	6 mm	3 mm	1 mm	-
Salmonella typhi	Ethanol	6 mm	2 mm	-	-	-
	Ethyl acetate	9 mm	4 mm	3 mm	2 mm	-
Escherichia coli	Ethyl acetate	8 mm	7 mm	5 mm	2 mm	1 mm
	Ethanol	5 mm	2 mm	1 mm	-	-
	Streptomycin (Negative control)	-	-	-	-	-
Candida spp	Ethyl acetate	3 mm	2 mm	-	-	-
	Ethanol	4 mm	3 mm	1 mm	-	-
	Nystatin (Negative control)	-	-	-	-	-

Table 8: Minimum inhibitory concentration of *Cajanus cajan* solvent extracts.

Organism	Extract	Concentrations (mg/ml)				
		50	25	12.5	6.25	3.125
Staphylococcus spp	N-Hexane	2 mm	-	-	-	-
	Ethyl- acetate	8 mm	6 mm	4 mm	2 mm	-
	Streptomycin (Negative control)	-	-	-	-	-
Pseudomonas spp	Ethanol	8 mm	6 mm	4 mm	2 mm	
	Ethyl acetate	10 mm	8 mm	5 mm	3 mm	1 mm
	Streptomycin (Negative control)	-	-	-	-	-

Bacillus subtilis	Ethyl acetate	6 mm	3 mm	2 mm	-	-
	Ethanol	8 mm	6 mm	3 mm	1 mm	-
Salmonella typhi	Ethanol	6 mm	2 mm	-	-	-
	Ethyl acetate	9 mm	4 mm	3 mm	2 mm	-
Escherichia coli	Ethyl acetate	8 mm	7 mm	5 mm	2 mm	1 mm
	Ethanol	5 mm	2 mm	1 mm	-	-
	Streptomycin (Negative control)	-	-	-	-	-
Candida spp	Ethyl acetate	3 mm	2 mm	-	-	-
	Ethanol	4 mm	3 mm	1 mm	-	-
	Nystatin (Negative control)	-	-	-	-	-

Table 9: Organoleptic result of the cream batch C 1 to C6.

	C1	C2	C3	C4	C5	C6
State	Semi-solid	Semi-solid	Semi-solid	Semi-liquid	Semi-solid	Semi-solid
Colour	Dark green	Dark green	Greenish	Greenish	Light greenish	Light greenish
Odour	Aromatic	Aromatic	Aromatic	Aromatic	Aromatic	Aromatic
Texture	Smooth	Smooth	Smooth	Smooth	Very smooth	Smooth

Table 10: pH values of Cream batches C1 to C6.

S/No Formulation	Solvent extract /Amount	pH
C1	N-hexane 2g	6.1[a]
C2	N-hexane 1g	6.0[a]
C3	Ethanol 1g	5.3[b]
C4	Ethanol 0.5g	5.8[d]
C5	Ethyl acetate oily 0.2g	5.2[c]
C6	Ethanol oily cream 1g	5.4[c]

Formulation Code	Concentration w/w	Dilution in mg/mL	*P. aeruginosa*	*B. subtilis*	*Staph.auerus*	*E. coli*	*S. typhi*	*Candida albican*
C1	10 %	15.625	-	-	-	-	-	-
		31.25	1.5 ± 0.5	-	-	-	-	-
		62.5	2.0 ± 0.0	4.0 ± 0.0	6.0 ± 0.0	-	-	-
		125	3.0 ± 0.0	8.0 ± 0.0	6.5 ± 0.5	-	-	-
		250	4.5 ± 0.5	9.5 ± 0.5	9.5 ± 0.5	-	-	-
C2	5 %	15.625	-	-	-	-	-	-
		31.25	-	-	-	-	-	-
		62.5	-	-	-	-	-	-
		125	2.0 ± 0.0	-	1.0 ±	-	-	-
		250	4.0 ± 0.0	3.0 ± 2.0	5.0 ± 0.0	-	-	-

C3	5 %	15.625	2.0 ± 0.0	3.5 ± 0.5	-	-	-	-
		31.25	4.0 ± 0.0	5.5 ± 0.5	4.0 ± 2.0	-	4.0 ± 0.5	-
		62.5	6.0 ± 1.0	10.0 ± 0.0	6.5 ± 0.5	3.0 ± 0.0	5.0 ± 0.0	-
		125	8.0 ± 0.0	14.0 ± 1.0	7.5 ± 0.5	4.0 ± 0.0	6.0 ± 1.0	3.0 ± 0.0
		250	10.0 ± 0.0	17.5 ± 0.5	7.0 ± 0.5	3.5 ± 0.5	8.5 ± 0.5	3.0 ± 1.0
C4	2.5 %	15.625	-	-	-	-	-	-
		31.25	-	2.5 ± 0.5	-	-	-	-
		62.5	2.0 ± 0.0	3.0 ± 1.0	3.0 ± 0.0	-	3.0 ± 1.0	-
		125	5.0 ± 1.0	5.0 ± 0.0	5.5 ± 0.5	-	5.0 ± 0.0	-
		250	5.5 ± 0.5	8.5 ± 0.5	6.0 ± 0.0	-	6.0 ± 0.0	-
C5	1 %	15.625	-	-	-	-	-	-
		31.25	2.0 ± 0.0	4.0 ± 0.0	3.0 ± 0.0	-	-	-
		62.5	5.0 ± 0.0	4.0 ± 1.0	9.0 ± 0.0	2.0 ± 0.0	5.0 ± 0.0	-
		125	8.0 ± 1.0	5.0 ± 0.0	12.0 ± 1.0	2.5 ± 0.5	5.0 ± 0.0	1.0 ± 0.0
		250	9.0 ± 0.0	6.5 ± 0.5	14.0 ± 0.0	2.0 ± 0.00	7.5 ± 0.5	2.5 ± 0.5
C6	5 %	15.625	-	-	-	-	-	-
		31.25	-	-	0.5 ±	-	-	-
		62.5	-	2.0 ± 0.0	5.0 ± 0.0	1.5 ± 0.5	5.0 ± 0.0	-
		125	3.5 ± 0.5	4.5 ± 0.5	5.0 ± 0.0	2.0 ± 0.0	6.5 ± 0.5	1.0 ± 0.0
		250	4.0 ± 0.0	5.0 ± 0.0	6.0 ± 0.0	3.5 ± 0.5	7.0 ± 0.0	-

Table 11: Assay of the creams.

Formulation C1 to C6 Minimum antimicrobial inhibitory zones in mm.

Each value is expressed as mean ± S.D (n = 2) and significant at p > 0.5.

- No zone of inhibition.

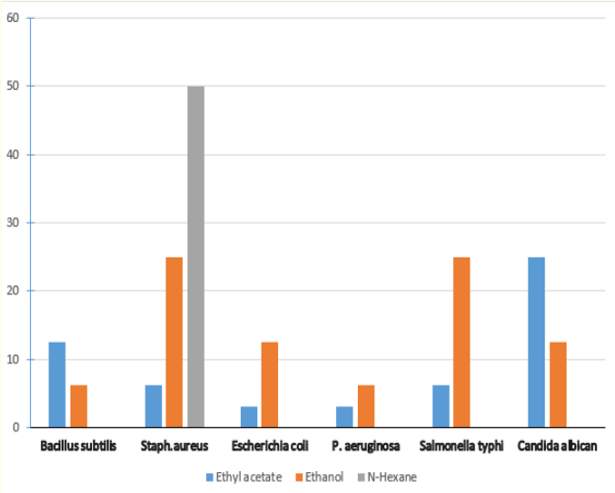

Figure 1: The Minimum Inhibitory concentration of the different solvent extracts of Cajanus cajan leaves in mg/mL.

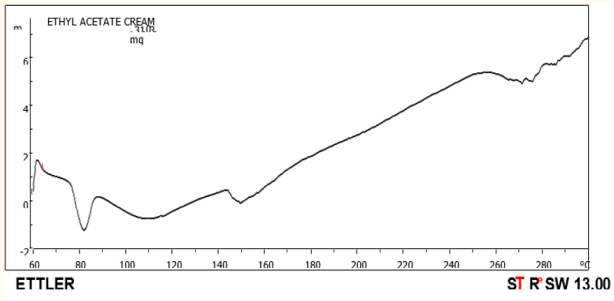

Figure 2: DSC of Ethyl Acetate cream.

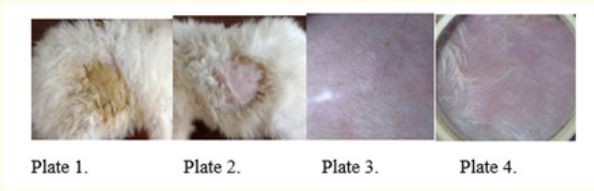

Figure 3

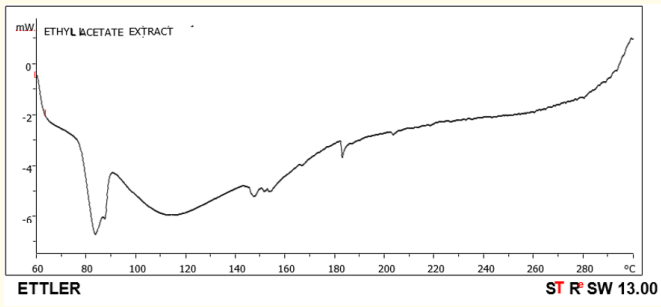

Figure 4: DSC of Ethyl Acetate extract.

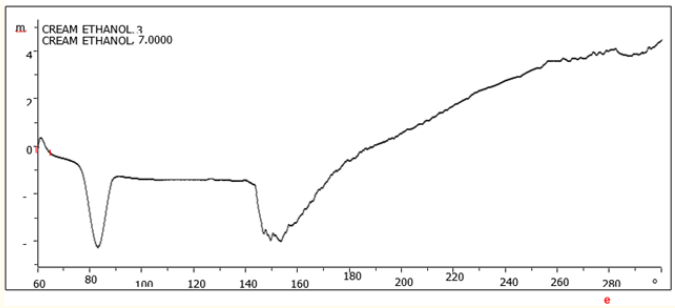

Figure 5: DSC of Ethanol Extract containing Cream.

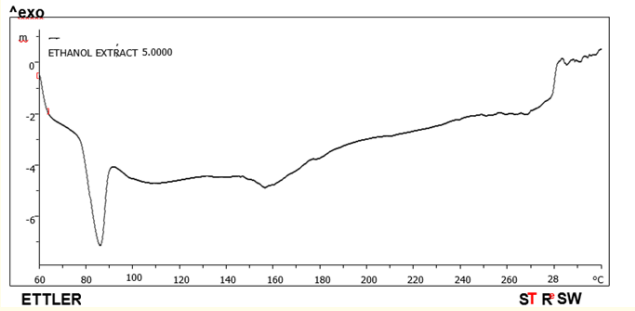

Figure 6: DSC of Ethanol Extract.

Discussion

A total ash value of 6.65%, water soluble ash 1.85% while acid insoluble ash were 6.2% respectively. The results showed that the ash value of the leaves extract are below the 14% maximum limit required of total ash in powdered medicinal plants [27]. Qualitative and quantitative phytochemical screening of the ethanolic extract of the leaves of *Cajanus cajan* are shown in Table 6. Flavonoids, Phenols, Saponins, alkaloids, Cyanogenicglycoside and Tanin were present in detectable amount while Phytate was present in trace amount. The ethyl acetate extract showed activity in all the organisms and was most active in *Pseudomonas* and S*almonella typhi spp.* The aqueous extract showed no activity while the control containing only the respective solvent, did not show any activity.

The serial dilution assay of the ethanolic extract of the *Cajanus cajan* showed MIC of 6.25 mg/mL for *Bacillus subtilis* and *Pseudomonas aeruginosa* and 12.5 mg/mL for *Escherichia coli* and *Candida albican*, while 25 mg/mL for *Salmonella typhi.*

The ethyl acetate extract had MIC of 3.125 mg/mL for *Pseudomonas aeruginosa* and *E.coli*, 6.25 mg/mL for *Staphlococcus aureus* and *Salmonella typhi,* 12.5 mg/mL and 25 mg/mL for *Bacillus subtilis* and *Candida albican* respectively. N-hexane extract showed MIC of 50 mg/mL for *Staphlococus aureus.* The results are represented in Table 8 and Figure 1.

Zone of inhibition of the various solvent extracts of the leaves of *Cajanus cajan* showed that the plant has antimicrobial activities as seen in the work done by [31]. All the extracts have varying antimicrobial activity except the water extract with no activity. The ethyl acetate and ethanol extract showed antimicrobial activity greater than N-hexane extract therefore, they were used in a lesser concentration in the formulations than that of N-hexane extract. The N-hexane extract showed activity only on *Bacillus spp* and *Staphylococcus spp.* The ethanol extract showed activity in all the organisms similar to result in [31] and had highest activity on *Bacillus spp.*

Organoleptic evaluation of all the cream formulations are semi-solid [32]. The colour of the creams ranged from dark green for C1 and C2 greenish for C3 and C4 and light greenish for C5 and C6 respectively. All creams had aromatic odour and C1, C2, C3, C4 and C6 are adjudged smooth while C5 was adjudged very smooth as showed in Table 9.

The result of the pH reading using standard buffer at a temperature of 33°C is shown in Table 10. Using Statistical Analysis of variance ANOVA and F-Fisher table at $p > 0.05$, there is no significant difference between the pH of formulation C1 and C2, formulation C5 and C6 which contains same ingredients in their formulation respectively. There is significant difference at $p > 0.05$ for the pH of C3 and C4 which has pH 5.3 and pH 5.8 respectively.

Viscosity of the various formulation were carried out with Rotation Viscometer using spindle 4 at 6.0, 12.0, 30.0 and 60.0 rotation per minute, which was further converted to rotation per second and Viscosity unit converted to $Pa.sec^{-1}$.

Stability studies shows that the formulated cream code C1, C2, C3, C4, C5 and C6 showed no observable change in the colour, odour and homogeneity after a weekly observation for four months kept at 30°C in a cupboard as per ICH guideline of 30°C ± 2/65% RH ± 5%RH.

Differential Scanning Calorimetry results graph at temperature of 85°C, peak was observed for ethanol extract and when compared with the formulated cream, the peaks was observed at almost at 85°C same with the extract. This indicates that there's compatibility of the excipients with that of the *Cajanus cajan* extract. It also shown the purity of the product. Also the graph depicts another peak between 145°C to 155°C of the ethanol cream which was not evident in the graph of the extract. This may be due to bond formed between the extract and the ingredient in the cream. Generally the ethanol extract is compatible and the ingredients of the cream did not interfere with the extract and stability of the product was also established.

For the DSC of the ethyl acetate extract, peaks were observed at temperatures of 82°C for both the extract and the cream formulation. The graph shown that the ingredients in the cream did not interfere with the extract and there's established purity of the products.

Conclusion

Ash profile is 6.5% ash value of the leave extracts which is below the 14% maximum limit required of total ash in powdered medicinal plants [2].

The ethanol extracts and ethyl acetate extracts of the *Cajanus cajan* leaves has shown a great potential of antibacterial activity against *Pseudomonas aeruginosa, Staphylococcus auerus, Bacillus subtilis, Esherichia coli, Salmonella typhi* and no cidal or static effect against *Candida albican* [33,34]. The extracts were produced into creams and the creams C1, C2, C3, C4, C5 and C6 were consistent in texture, homogenous in appearance, stable and the pH of the cream formulations were within the skin pH range. When tested for irritancy on rabbits skin, showed no irritation, which indicates it is safe to be used on a human skin.

Cream code C3, C5 and C6 showed antimicrobial activities against most of the typed organisms with MIC of 0.3125 mg/mL in C5 which contains ethyl acetate extract in an oil base. Stability testing showed that it was a good dosage form to deliver the extracts for topical activity as products showed no sign of instability through a period of four months. The antimicrobial assay shown that the excipients, varied bases do not hinder or limit the antimicrobial activities of the *Cajanus cajan* extracts. The Differential scanning calorimetry also further confirmed that the extracts were compatible with the ingredients used in the formulation of the cream, it did not hinder the antimicrobial activities of the extracts.

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