



The Anti-microbial Efficacy and Phytochemical Analysis of the Root Bark of *Uvaria chamaea*

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Abstract: Background: Medicinal plants show an important role in diseases prevention and treatment through the enhancement of anti-oxidant activity, inhibition of bacterial growth and modulation of genetic pathways. **Materials and Methods:** The anti-microbial efficacy of the root bark of *Uvaria chamaea* was evaluated using various solvents for extraction. The solvents used for extraction were hexane, methanol, petroleum ether, water and ethanol. The efficacy was tested on four different bacterial isolates: *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and a fungal isolate *Candida tropicalis*. Agar Well Diffusion (AWD) technique was used for the analysis. **Results:** It was observed that methanol extract at undiluted concentration (100%) and diluted concentrations (133.3mg/ml and 50mg/ml) was effective on *Pseudomonas aeruginosa*. The petroleum ether, water and methanol extracts at undiluted concentration (100 %) and diluted concentrations (133.3mg/ml and 50mg/ml) were not effective on *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli* and *Candida tropicalis*. Hexane at undiluted concentration (100%) was effective on *Klebsiella pneumoniae*. Also the hexane extract at undiluted concentration (100%) and diluted concentrations (133.3mg/ml and 100mg/ml) was effective on *Escherichia coli*. Only the ethanol extract at undiluted concentration (100%) and diluted concentrations (133.3mg/ml and 100mg/ml) was effective on *Candida tropicalis*. Flavonoids, alkaloids, tannins and terpenoids were detected in the *Uvaria chamaea* root bark. **Conclusion:** The hexane and methanol extracts of the root bark of *Uvaria chamaea* were effective on *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. The ethanol extract of the root bark was effective on *Candida tropicalis*. Hexane, methanol and ethanol seemed better solvents for the extraction of the anti-microbial compounds of the root bark of the plant. The root bark of *Uvaria chamaea* seemed not to possess broad spectrum activity but however active on *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Candida tropicalis*. In spite of the narrow spectrum activity of the root bark of *Uvaria chamaea*, the use of ethanol, hexane and methanol will be appropriate for the extraction of the anti-microbial constituents of the root bark of the plant during therapeutic development.

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Key words: *Uvaria chamaea*; root bark; anti-microbial; solvent extracts; pathogenic; bacteria; fungi; antibiotics

1. Introduction

Uvaria chamaea grows naturally in the savannah and rain forest regions of Africa and tropical areas of the world [1]. It is also commonly known as finger root or bush banana [2] and belongs to the family Annonaceae [3]. It has been reported to exhibit medicinal potentials [4].

The aim of this research study was to investigate the anti-microbial potentials of the root bark of *Uvaria chamaea* with view to proffering therapeutic alternative to synthetic drugs. The specific objectives of the study were to: determine the efficacy of solvent extracts of *Uvaria chamaea* on selected pathogenic isolates; compare the anti-microbial efficacy of the solvent

extracts of *Uvaria chamae* with specific conventional antibiotics; and phytochemically determine the bioactive constituents in the root bark of *Uvaria chamae*.

2. Materials and Methods

Collection of the Root bark of *Uvaria chamae*

The root bark of *Uvaria chamae* was collected at the Botanical Garden of the University of Ibadan, Ibadan, Nigeria. The root bark was authenticated at the Department of Botany, University of Ibadan, Ibadan, Nigeria.

Extracts and Microorganisms Used

The extracts used in this investigation were hexane, methanol, ethanol, petroleum ether and water. The microbial isolates (bacteria and fungus) used for the work were obtained from the Department of Microbiology and Parasitology, University College Hospital, Ibadan, Nigeria. The bacterial and fungal cultures used were preserved by sub-culturing the bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*) onto nutrient agar in plates and kept at 37°C [5]. The fungal isolate (*Candida tropicalis*) was sub-cultured onto potato dextrose agar plates and also kept at 37°C [5].

Culture Media Preparation

According to the manufacturer's instructions, the media used which were nutrient agar and potato dextrose agar were weighed separately using a Mettler weighing balance. Each medium was dissolved in 500 ml sterile distilled water in conical flasks. The media were properly mixed and placed into a homogenizer for thorough and proper solubility. The conical flasks were plugged with cotton wool wrapped with aluminium paper foil to avoid spilling/frothing. The media were autoclaved at 121°C for 15mins [6].

Preparation of *Uvaria chamae* Root Bark Extracts Using Solvents

The extraction method used was a modification of the methods of Adejuwon *et al.* [7, 8]. In line with traditional methods of preparation, shredded plant materials of the root bark of *Uvaria chamae* were put in sterile beakers containing any of sterile distilled water, hexane, ethanol, methanol or petroleum ether.

Sterile distilled water extract: Twenty (20) grams of milled root bark of *Uvaria chamae* was weighted into a beaker and dissolved with 100ml of the sterile distilled water. This was allowed to soak for 24hrs. The suspension was filtered first with muslin cloth to remove coarse particles and then through Whatman (No 1) filter paper.

Ethanol extract: Twenty (20) grams of milled bark of *Uvaria chamae* was weighted into a beaker and dissolved with 100ml of the ethanol. This was allowed to soak for 24hrs. The suspension was then

filtered first through muslin cloth to remove coarse particles and then through Whatman (No 1) filter paper.

Hexane extract: Twenty (20) grams of milled root bark of *Uvaria chamae* was weighted into a beaker and dissolved with 100ml of the hexane. This was allowed to soak for 24hrs. The suspension was then filtered first through muslin cloth to remove coarse particles and then through Whatman (No 1) filter paper.

Petroleum ether extract: Twenty (20) grams of milled root bark of *Uvaria chamae* was weighted into a beaker and dissolved with 100ml of the petroleum ether. This was allowed to soak for 24hrs. The suspension was then filtered first through muslin cloth to remove coarse particles and then through Whatman (No 1) filter paper.

Methanol: Twenty (20) grams of milled root bark of *Uvaria chamae* was weighted into a beaker and dissolved with 100ml of the methanol. This was allowed to soak for 24hrs. The suspensions were then filtered first through muslin cloth to remove coarse particles and then through Whatman (No 1) filter paper.

Inhibitory Tests for Bacterial Isolates

Using the modified method of Adejuwon *et al.* [7], each extract was tested for anti-microbial efficacy using Agar Well Diffusion technique. This method is dependent on the diffusion of the various extracts from a well cavity bored through the solidified agar layer in petri-dishes, such that growth of the inoculated microorganism is prevented entirely by the efficacy of the extract constituents thereby forming circular zone round the well containing the extract. This exhibited characteristic is as a result of the microorganisms' sensitivity to the extract.

Each bacterial isolate was streaked all over the surface of solidified nutrient agar plate using sterile swab stick. A sterile 8mm (diameter) cork-borer was used to make 5 uniform deep wells into the gel. Each well was filled with different concentrations of an extract. A well was perforated in the middle of the nutrient agar on plate. This was the control well which contained only the solvent (without extract). The dishes were allowed to stand for 45 minutes at room temperature to allow proper diffusion. The plates were later transferred into the incubator at 37°C for 24hrs. After the period of incubation, the diameter of zone of inhibition was measured using a meter rule. The zone of inhibition is regarded as being directly proportional to the potency of the extract.

Various concentrations for each of the extracts were serially prepared to get the final concentration.

Concentration of the undiluted

a) 20g powder of extract dissolve into 100mls solvent = $(20 \times 1000) = 20,000 \text{mg}/100 \text{ml}$

- b) $20,000/100 = 200\text{mg} = 200\text{mg/ml}$
 2) $10\text{ml} + 5\text{ml solvent} = 15\text{ml}$
 $200 \times 10\text{ml} / 15 = 133.3\text{mg/ml}$
 3) $10\text{ml} + 10\text{ml} = 20\text{ml}$
 $200 \times 10 / 20 = 100\text{mg/ml}$

Inhibitory Test for Fungal Isolate

Using the modified method of Adejwon *et al.* [7], Agar Well Diffusion technique used for bacterial isolates was also used for fungus. Instead of Nutrient agar (for bacteria), Potato dextrose agar was used here (for fungus). The potato dextrose agar was inoculated with the fungus on Petri plate at the centre using sterilized platinum wire loop. A well were bored into the inoculated potato dextrose agar on plates using a sterilized 8mm diameter size cork-borer. The well was filled a solvent extract of *Uvaria chamae*. The extract was allowed to diffuse into the gel for about an hour. The inoculated plate with solvent extract was incubated at 30°C for 48hrs after which the zone of inhibition was measured using a meter rule.

Antibiotic Sensitivity Test for Bacterial Isolates

The bacteria were tested for their susceptibility or resistance to conventional antibiotics. The antimicrobial efficacy and interpretation of sizes of zones of inhibition was in accordance with the Clinical and Laboratory Standards Institute for antimicrobial susceptibility tests [9]. Gram positive and Gram negative discs were used. The Gram positive discs used were: Cotrimoxazole (25 μg), Cloxacillin (5 μg), Erythromycin (5 μg), Gentamicin (10 μg), Augmentin (30 μg), Tetracycline (10 μg), Streptomycin (10 μg) and Chloramphenicol (10 μg).

The Gram negative discs used were: Augmentin (30 μg), Ofloxacin (5 μg), Gentamicin (10 μg), Nalidixic acid (30 μg), Nitrofurantoin (200 μg), Cotrimoxazole (25 μg), Amoxicillin (25 μg) and Tetracycline (25 μg).

Procedure:

Each bacterial isolate was streaked on solidified nutrient agar using sterile swab stick. Each sensitivity disc was carefully placed on the inoculated plate using sterile forceps. The plates were then placed in the incubator for 37°C for 24 hr. The zone of inhibition was measured using a meter rule.

Minimum Inhibitory Concentration (MIC)

The Minimum Inhibition Concentration (MIC) of the root bark of *Uvaria chamae* extracts from each solvent on isolates was determined. The mean value of triplicates was recorded. Individual solvent was used as control in each experiment. MIC is defined as the lowest concentration that will inhibit the visible growth of test organism by $>90\%$. This was represented by $<10\text{mm}$ diameter zone of clearance.

Minimum Bacteria Concentration (MBC)

The Minimum Bacteria Concentration (MBC), a measure of drug potency was also determined. This is

defined as the concentration where 99.9% or more of the initial inoculum is killed. This was represented by and significantly remarkable by $>10\text{mm}$ diameter zone of clearance.

Phytochemical Analysis of Root Bark of *Uvaria chamae*

The solvent extracts of *Uvaria chamae* was subjected to qualitative phytochemical screening to identify the presence of alkaloids, flavonoids, carbohydrates, saponins, steroids, tannins and terpenoids using the established methods as described by Sazada *et al.* [10] and Harborne [11]. Briefly, Alkaloids, flavonoids and tannins were respectively tested with Wagner reagent, concentrated HCl and 0.1% ferric chloride.

Tests Performed:

- Carbohydrates (Molisch's Test for CHO)**
Dissolve extract in water, add few drops of Naphtol to small portion follows by 1ml conc. H_2SO_4 run by the side of the test tube. Allow to stand for few minutes. A reddish or dull violent color at the inter-phase layer is formed to show the presence of carbohydrates.
- Alkaloids**
Quality test
Stir 1ml of extract with 5ml of 1% HCl in water bath
 - Filter, and heat 1ml of the filtrate with Mayer reagent
 - Turbid buff-coloured precipitate from Mayer reagent is an indication of presence of alkaloids
 For Mayer's reagent
Dilute a mixture of 1.36g of HgCl with 5g of KI in 100ml of water = creamy precipitate
- Tannins test**
 - Stir about 1g of plant extract with 10ml distilled water. Filter
 - Add 1% FeCl_3 to 2ml of filtrate
 - Appearance of blue-black, green ppt indicates positive
- Saponins test**
 - Boil 1ml of sample with 5ml of water. Filter and add 3ml water to the filtrate.
 - Shake vigorously for 5mins
 - Frothing which persist on warming, indicate presence of saponins
- Flavonoids test**
 - Dissolve about 1g of extract in ethanol.
 - Warm, filter and add 3 pieces of Mg chips to the filtrate, followed by few drops of Conc. HCl.
 - A pink, orange, red or purple coloration indicates the presence of flavonoids
- Anthraquinones test**
 - Shake about 0.2 g of plant extract with 10ml benzene

- Filter and add 10% $\text{NH}_3\text{SO}_{12}$, Shake and filter again.

- Appearance of pink or red or violent color indicate the presence of anthraquinones

7. Phlobatannins test

- Deposition of red ppt when an extract of each plant sample is boiled with 1% aqueous HCl is taken as evidence of presence of phlobatannins

8. Steroids test

- 2ml of acetic anhydride (acid) is added to 0.5g of plant extracts, then followed by 2ml Conc. H_2SO_4

- Color changes from violent to blue or green indicates the presence of steroids

9. Terpenoids

- 5ml of extract is mixed with 2ml of chloroform and 3ml Conc. H_2SO_4 carefully added to form a layer.

- A reddish brown coloration of the inter-phase indicates the presence of terpenoids.

3 Results

Table 1 shows the Minimum Inhibitory Concentration (MIC) of the solvent extracts of root bark of *Uvaria chamae* at various concentrations on selected pathogenic isolates. It was observed that the extracts: Petroleum ether (PE), Aqueous (AE) and Ethanol (EE) of the root bark of *Uvaria chamae* at undiluted concentration (100 %) and diluted concentrations (133.3mg/ml and 50mg/ml) were not effective on any of the Gram positive bacteria (*Staphylococcus aureus*) and Gram negative bacteria (*Klebsiella pneumonia*, *Escherichia coli* and *Pseudomonas aeruginosa*).

Methanol (ME) extract of the root bark of *Uvaria chamae* at undiluted concentration (100%) and diluted concentrations (133.3mg/ml and 50mg/ml) was effective on *Pseudomonas aeruginosa* but ineffective on *Staphylococcus aureus*, *Klebsiella pneumonia* and *Escherichia coli* (Table 1).

Hexane (HE) extract of the root bark of *Uvaria chamae* at a diluted concentration (100mg/ml) was effective on *Klebsiella pneumonia*. It was ineffective at a diluted concentration (133.3mg/ml) and undiluted concentration (100%) on *Klebsiella pneumonia*. Hexane extract of the root bark of *Uvaria chamae* (HE) at undiluted concentration (100%) and diluted concentrations (133.3mg/ml and 100mg/ml) was effective on *Escherichia coli* (Table 1).

Ethanol extract (EE) extract of the root bark of *Uvaria chamae* at undiluted concentration (100%) and diluted concentrations (133.3mg/ml and 100mg/ml) was effective on *Candida tropicalis*.

Petroleum extract (PE), hexane extract (HE), methanol extract (ME) and aqueous extract (AE) of the root bark of *Uvaria chamae* at undiluted concentration (100%) and diluted concentrations (133.3mg/ml and 100mg/ml) was not effective on *Candida tropicalis* (Table 1).

The Minimum Bacterial Concentrations of the solvent extracts of the root bark of *Uvaria chamae* on the selected pathogenic isolates are presented in Table 2.

All the pathogenic Gram positive bacterial isolates: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* used in this investigation were resistant to the Gram positive antibiotics: Cotrimoxazole, Cloxacillin, Erythromycin, Gentamicin, Augmentin, Streptomycin, Tetracycline and Chloramphenicol (Table 3).

Two of the pathogenic Gram negative bacterial isolates were sensitive to the Gram negative antibiotics. *Klebsiella pneumoniae* was susceptible to the actions of Augmentin. The zone of diameter of inhibition was 17mm. *Escherichia coli* was sensitive to the actions of Ofloxacin, Gentamicin and Nitrofurantoin with zones of diameter of inhibition 16mm, 12mm and 15mm respectively (Table 4). Flavonoids, alkaloids, tannins and terpenoids were detected in the solvent extracts of the root bark of *Uvaria chamae* (Table 5).

Table 1: The Minimum Inhibitory Concentration (MIC) of the solvent extracts of the root bark of *Uvaria chamae* on microbial isolates

Isolate	Solvent used for extraction	Undiluted extract	10ml+5ml solvent	10ml+10ml solvent	Control
		100mg/ml	133.3mg/ml	50mg/ml	
1 <i>Pseudomonas aeruginosa</i>	Ethanol	-	-	-	-
	Petroleum ether	-	-	-	-
	Methanol	25	10	20	-
	Hexane	-	-	-	-
	Aqueous	-	-	-	-
2 <i>Klebsiella pneumonia</i>	Ethanol	-	-	-	-
	Petroleum ether	-	-	-	-

		Methanol	-	-	-	-
		Hexane	-	-	18	-
		Aqueous	-	-	-	-
3	<i>Escherichia coli</i>	Ethanol	-	-	-	-
		Petroleum ether	-	-	33	-
		Methanol	-	-	-	-
		Hexane	10	16	31	-
		Aqueous	-	-	-	-
4	<i>Staphylococcus aureus</i>	Ethanol	-	-	-	-
		Petroleum ether	-	-	-	-
		Methanol	-	-	20	-
		Hexane	-	-	-	-
		Aqueous	-	-	-	-
5	<i>Candida tropicalis</i>	Ethanol	25	20	24	-
		Petroleum ether	-	-	-	-
		Methanol	-	-	-	-
		Hexane	-	-	-	-
		Aqueous	-	-	-	-

Table 2: The Minimum Bacterial Concentration (MBC) of the solvent extracts of the root bark of *Uvaria chamae* on microbial isolates

Isolate	Solvent used for extraction	Undiluted extract 10g into 100ml 200mg/ml	10ml+5ml solvent 133.3ml	10ml+10ml solvent 100mg/ml	Control
	Petroleum ether	-	-	-	-
	Methanol	25	20	10	-
	Hexane	-	-	-	-
	Aqueous	-	-	-	-
2	<i>Klebsiella pneumonia</i>	Ethanol	-	-	-
		Petroleum ether	-	-	-
		Methanol	-	-	-
		Hexane	18	-	-
		Aqueous	-	-	-
3	<i>Escherichia coli</i>	Ethanol	-	-	-
		Petroleum ether	-	-	-
		Methanol	-	-	-
		Hexane	31	16	10
		Aqueous	-	-	-
4	<i>Staphylococcus aureus</i>	Ethanol	-	-	-
		Petroleum ether	-	-	-
		Methanol	20	-	-
		Hexane	-	-	-
		Aqueous	-	-	-
5	<i>Candida tropicalis</i>	Ethanol	25	24	20
		Petroleum ether	-	-	-
		Methanol	-	-	-
		Hexane	-	-	-
		Aqueous	-	-	-

Table 3: Antibiotic sensitivity of microbial isolates using Gram positive disc

Antibiotic	Code	Concentration	<i>Escherichia coli</i>	<i>Klebsiella pneumoniae</i>	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>
Cotrimoxazole	Cot	25µg	R	R	R	R
Cloxacillin	Cxc	5 µg	R	R	R	R
Erythromycin	Ery	5 µg	R	R	R	R
Gentamicin	Gen	10 µg	R	R	R	R
Augmentin	Aug	30 µg	R	R	R	R
Streptomycin	Str	10 µg	R	R	R	R
Tetracycline	Tet	10 µg	R	R	R	R
Chloramphenicol	Chl	10 µg	R	R	R	R

Keys: Diameter of zones of inhibition/clearance was measured in millimeter; R = Resistance

Table 4: Antibiotic sensitivity of microbial isolates using Gram negative disc

Antibiotic	Code	Concentration	<i>Escherichia coli</i>	<i>Klebsiella pneumoniae</i>	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>
Augmentin	Aug	30 µg	R	17	R	R
Ofloxacin	Ofl	5 µg	16	R	R	R
Gentamicin	Gen	10 µg	12	R	R	R
Nalidixic Acid	Nal	30 µg	R	R	R	R
Nitrofurantoin	Nit	200 µg	15	R	R	R
Cotrimoxazole	Col	25 µg	R	R	R	R
Amoxycillin	Amx	25 µg	R	R	R	R
Tetracyclin	Tet	25 µg	R	R	R	R

Keys: Diameter of zones of inhibition/clearance was measured in millimeter; R = Resistance

Table 5: Phytochemical composition of the root bark of *Uvaria chamae*

Phytochemical	Root bark of <i>Uvaria chamae</i>
1 Molisch's Test for carbohydrate	Ab
2 Flavonoid	P
3 Phlobatannis	Ab
4 Alkaloids	P
5 Tannin	P
6 Saponin	Ab
7 Anthraquinones	Ab
8 Steroids	Ab
9 Terpenoids	P

Keys: Ab – Absent; P - Present

4. Discussion

The traditional medicine is the total sum of knowledge, resting rationally on theories, beliefs and experiences specific to one's own culture. It is used to maintain human beings in health so as to prevent, diagnose and to treat and heal physical and mental illnesses [12]. In Africa the therapeutic power of plants was known by our forebears and parents in an empiric way [12]. In a weak economic environment characterized by the high cost of the medicine, pharmacopeia and traditional medicine become a non-negligible alternative. Currently, more than 80% of the African population has resorted to drugs essentially made of plants found in their environments [13].

Solvent extracts of the root bark of *Uvaria chamae* used in this study showed antimicrobial efficacy on *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and *Escherichia col*. The phytochemical composition of the plant might play a significant role in its antimicrobial nature. In spite of the advent of modern generational antibiotics, in Africa generally especially Porto- Novo, the rate of frequentation of the traditional centers is superior to 80% [12]. Widespread antibiotic usage exerts a selective pressure that acts as a driving force in the development of antibiotic resistance [14, 15]. The association between increased rates of antimicrobial use and resistance has been documented for nosocomial infections as well as for resistant

community acquired infections [16, 17]. As resistance develops to "first-line" antibiotics, therapy with new, broader spectrum, more expensive antibiotics increases, but is followed by development of resistance to the new class of drugs [14]. The results of in vitro antibiotic susceptibility testing guide clinicians in the appropriate selection of initial empiric regimens and drugs used for individual patients in specific situations. The selection of an antibiotic panel for susceptibility testing is based on the commonly observed susceptibility patterns, and is revised periodically [9].

In this study, the test bacterial strains showed differences in their resistance pattern to different class of antibiotics. Sixteen antibiotics were used for screening of the five bacterial isolates. *Klebsiella pneumoniae* showed resistance to ofloxacin. *Escherichia coli* showed resistance to ofloxacin gentamicin and nitrofurantoin.

The results obtained in this study indicate that the hexane and methanol extracts of the root bark of *Uvaria chamae* inhibited the growth of a majority of the test bacterial isolates. This is an indication that the root bark of *Uvaria chamae* possesses active substances that can inhibit microbial growth. It was also observed that ethanol, aqueous and petroleum ether extracts of the root bark of *Uvaria chamae* were ineffective on the isolates. Methanol and hexane were better extraction solvents than water, ethanol and petroleum ether for the root bark of this plant.

Only the ethanol extract of the root bark of *Uvaria chamae* was effective on *Candida tropicalis*. The methanol, petroleum ether, hexane and aqueous extracts of the root bark were ineffective on this fungal isolate.

Phytochemical analysis which indicates the presence of flavonoids, alkaloids, tannins and terpenoids in the milled root bark of *Uvaria chamae* is evidence of the presence of naturally occurring bioactive compounds in the root bark of the plant.

Conclusion

The methanol and hexane extracts of the root bark of *Uvaria chamae* were effective on *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. The ethanol extract of the root bark was effective on *Candida tropicalis*. Hexane, ethanol and methanol seemed better solvents for the extraction of the anti-microbial compounds of the root bark of this plant. In conclusion, the root bark of *Uvaria chamae* seemed not to possess broad spectrum activity but however active on *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Candida tropicalis*.

Recommendation

In spite of the narrow spectrum of activity of the root bark of *Uvaria chamae*, the use of ethanol, hexane and methanol will be appropriate for the extraction of the antimicrobial constituents of the root bark of *Uvaria chamae* during therapeutic development.

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