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Phytochemical and antimicrobial studies on extractives of *Calyptrochilum emarginatum* (SW) Schltr (Orchidaceae) growing in Nigeria

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Calyptrochilum emarginatum **is an epiphytic shrub belonging to the orchid family with numerous medicinal uses. Phytochemical investigation of the leaf extractives revealed the presence of tannins, flavonoids, carbohydrates, terpenes, sterols and saponins. Alkaloids and cardiac glycosides could not be detected. Antimicrobial studies of the extracts revealed that at a minimum inhibitory concentration (MIC) of 1.6 mg/ml, the hexane and methanol successive extracts exhibited bactericidal activities against *Staphylococcus aureus*. The straight run methanol extract and the successive ethyl acetate extract did not show any activity against all the microorganisms investigated namely, *S. aureus, Candida albicans, Klebsiella pneumonia, Escherichia coli* and Salmonella paratyphi at the same concentration.**

Key words: *Calyptrochilum emarginatum*, orchid, phytochemicals, antimicrobial, minimum inhibitory concentration (MIC).

INTRODUCTION

Calyptrochilum emarginatum (Afzel. ex Sw.) Schltr is an epiphytic shrub belonging to the orchid family. Orchid is an anglicized name given to every plant species belonging to the family Orchidaceae. Like other epiphytes, *C. emarginatum* grows on tree branches, unconnected to the ground and without being parasitic in any way. The leaves grow along a pendant stem up to 50 cm long with inflorescence appearing along the underside of the stem with 6 to 9 flowers. *C. emarginatum* is a shade-loving shrub with stems carrying distichous, ovate, leathery, unequally and obtusely bilobed (emarginated) apical leaves arising with an auxiliary. Flower inflorescence is characterized by strong scent, even nocturnal. The plant flowers approximately within 2 to 3 years. *C. emarginatum* can be found in Angola, Cameroon, Central African, Equatorial Guinea, Gabon, Ghana, Ivory Coast, Liberia, Nigeria, Sierra Leone and Zaire in tropical, evergreen and deciduous rainforest at elevation around sea level to 1000 meters (La Croix and La Croix, 1997).

There are only two recognized and acceptable species of *Calyptrochilum* and these are *C. chrystyanum* and *C. emarginatum.* Jayeola and Thorpe (2008) carried out electron micrograph of the genus *Calyptrochilum*. Kraenzl established taxonomical characteristics of the two species. *Chrystyanum* (Rchb.) Summerh was characterized by a network of horizontal grooves, dome shaped micro papillae and a mass of soft wax while the

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Emarginatum (Sw.) Schltr was distinguished by the presence of densely overlapping conical and globular type of micro papillae (Jayeola and Thorpe, 2008).

The medicinal orchids belong mainly to the genera: *Calanthe, Coelogyne, Cymbidium, Bulbophyllum, Cypipedium, Dendrobium, Ephemerantha, Eria, Galeola, Gastrodia, Gymnadenia, Habenaria, Ludisia, Luisia, Nevilia* and *Thunia* (Gutiérrez, 2010). Through the ages, several health-promoting benefits have been attributed to the use of orchid extracts such as anti-diuretics, anti-inflammatory, anti-carcinogenic, hypoglycemic activities, anti-microbial, anti-convulsive, neuroprotective and anti-viral activities (Gutierrez, 2010). For instance, the water soluble decoction of the whole plant of *Anoectochilus formosanus* was found to show potency for tumor inhibitory activities in experimental animals after subcutaneous transplantation of CT-26 murin colon cancer cells (Tseng et al., 2006). As a part of a screening study, methanol extract of the leaves of *Spiranthes mauritianum* (Orchidaceae) was found to possess anti-inflammatory activity and showed antibacterial activities against some Gram-positive organisms (Matu and Van Staden, 2003). In a study of herb extracts from Chinese medicinal plants, it was found that *Bletilla striata* (Orchidaceae) possessed antioxidant and anti-microbial properties (Luo et al., 2007). *Bletilla striata* tubers collected with a non-metal cutting tool, cleaned and dried were used to treat tuberculosis, hemoptysis, gastric and duodenal ulcers, as well as bleeding, and cracked skin on the feet and hands. Other uses in China, Mongolia, and Japan include the introduction of euphoria, purification of blood, strengthening and consolidation of lungs, as well as the treatment of pus, boils, abscesses, malignant swellings, ulcers, and breast cancer. Additional medical applications of the boiled or dried tubers of *B. striata* include treatment of flatulence, dispepsia, dysentery, fever, malignant ulcers, gastrointestinal disorders, hemorrhoids, anthrax, malaria, eye diseases, tinea, ringworm, tumors, and necrosis, silicosis, traumatic injuries, coughs, chest pain, tuberculosis, vomiting of blood, gastrorrhagia, enterorrhagia, internal bleeding, inflammation, and chopped skin. The powdered roots mixed with mineral oil have been used as an emollient for burns and skin diseases. Whole plant preparations are tonic and used as treatment against leucorrhea, hemoptysis and purulent coughs. Leaves collected in the autumn are reported to cure lung disease (Kong et al., 2003).

Two stilbenoids namely 3,3'-dihydroxy-2',6'-bis(p-hydroxybenzyl)-5-methoxybibenzyl and 3',5-dihydroxy-2-(p-hydroxybenzyl)-3-methoxybibenzyl were isolated from the methanolic extracts of tubers of *B. striata* which showed inhibitory effect of tubulin polymerization at IC_{50} of 10 µM. Also, 7,8-dihydro-5-hydroxy-12,13-methylene dioxy-11-methoxyldibenz[b*f*]oxepin, 7,8-dihydro-4-hydroxy-12,13-methylenedioxy-11-methoxyldibenz [b*f*]oxepin; and 7,8-dihydro-3-hydroxy-12, 13-methylenedioxy-11-methoxyldibenz [b*f*]oxepin, cumulatin, densiflorol A and plicatol B isolated from *Bulbophyllum kwangtungense* exhibited anti-tumor activities (Gutierrez et al., 2010).

The forkloric uses of *C. emarginatum* among the Takkad people of Southern Kaduna in Northern Nigeria have been reported to include treatment of infant convulsive fever (Mathias et al., 2007). In this work, we investigated the phytochemical and antimicrobial properties of the extractives of *C. emarginatum* found growing in Western Nigeria where people frequently use this plant in traditional medicine for the management of cough, tuberculosis and malaria.

MATERIALS AND METHODS

Chemicals and reagents

Reagents used were of Analar grade and unless otherwise stated were procured from Zayo-Sigma Abuja.

Plant

The plant material was collected in the month of June, 2012 in front of Conference Centre of the Obafemi Awolowo University, Ile-Ife, Osun State, South-West of Nigeria. The site of collection was characterized and geo-referenced N07 31.536, E004 31.536, with elevation 269 m, using a GARMIN GPS 60 Global Positioning System (GPS). The plant was identified and authenticated by Mr. Gabriel and Mr. Bennard at the herbarium of the Department of Botany, Obafemi Awolowo University, Ile-Ife, South-West of Nigeria.

Plant preparation and extraction

The fresh leaves were chopped into smaller bits and oven dried at a temperature of 40°C for a period of two weeks until constant weight. The dried plant sample was pulverized using mortar and pestle, and passed through sieve No. 22 of 710 µm nominal mesh aperture. Then 30 g of the powdered material was weighed and extracted with 300 ml of hexane in a stoppered container for 24 h at ambient temperature (28 to 30°C), with shaking during the first six hours at 120 rpm. The resultant mixture was vacuum filtered with Whatman No. 1 filter paper. The filtrate was concentrated to dryness using rotary evaporator at 40°C to yield the hexane extract of *C. emarginatum* (HECE). The air dried marc (29 g) was extracted successively with ethyl acetate and methanol for 24 h each, at ambient temperature (28 to 30°C). A separate straight run methanol extract was obtained using 30 g of the powdered sample and macerating with 300 ml of methanol for 24 h at ambient temperature (28 to 30°C), with shaking during the first six hours. The resultant mixture was vacuum filtered with Whatman No. 1 filter paper. The filtrate was concentrated to dryness using rotary evaporator at 40°C to yield *C. emarginatum* methanol extract straight-run (CEMEST). The dried successive ethyl acetate and methanol extracts of *C. emarginatum* were designated EECE and CEME, respectively. The yields of all the extractives were determined.

Phytochemical analyses

The phytochemical analyses of the powdered herb and extractives were performed using the methods described by Harborne (1998),

Evans (2002) and Sofowora (2008), with some modifications. The analyses were carried out in triplicate.

Test for alkaloids

One gram of the powdered herb was macerated with 10 ml of methanol at room temperature (28 to 30°C) for 3 h, with shaking at interval. The mixture was filtered with Whatman No. 1 filter paper. The filtrate was evaporated to dryness on a boiling water bath. Then the residue or 20 mg of each of the extractives were separately extracted with 10 ml of 1% HCl and filtered. The solution was divided equally into five test tubes, and two drops of the following reagents were added to the respective test tubes: Mayer's reagent (potassium mercuric iodide solution); Dragendorff's reagent (potassium bismuth iodide solution); Wagner's reagent (solution of iodine in potassium iodide); Hager's reagent (a saturated solution of picric acid); and 10% tannic acid solution. The formation of amorphous or crystalline precipitates or coloured precipitate in at least three or all of these tests indicate the presence of alkaloids.

Test for flavonoids

Lead acetate test for flavonoids

Two gram of the powdered herb or 10 mg of the extracts were wetted with acetone, and the acetone completely evaporated on a boiling water bath. The residue was extracted with 10 ml of warm distilled water and filtered. 3 ml of the filtrate was placed in a test tube and two drops of 10% (w/v) lead acetate solution added. The formation of a cream coloured precipitate indicates the presence of flavonoids (Evans, 2002).

Sodium hydroxide test for flavonoids

To 3 ml of the filtrate obtained earlier was added equal volume of 10% (w/v) sodium hydroxide solution. The formation of yellow coloured solution indicates the presence of flavonoids (Evans, 2002).

Test for terpenes and sterols

One gram of the powdered herb was extracted by maceration with 10 ml of chloroform. The extract was filtered and evaporated to dryness on water bath. The residue or 10 mg of extract was dissolved in 5 ml of anhydrous chloroform and filtered. The filtrate was divided into two equal portions and used for Lieberman-Burchard test for terpenes and Salkowski's test for sterols, according to (Sofowora, 2008) as indicated.

Lieberman-Burchard test for terpenes

To the first portion of the chloroform solution was added 1 ml of acetic anhydride and shaken. Then 1 ml of concentrated sulphuric acid was added down the wall of the test tube to form a layer underneath. The formation of a reddish-violet colour at the lower layer indicates the presence of terpenes (Sofowora, 2008).

Salkowski's test for sterols

Two mills of concentrated sulphuric acid was carefully added to the second portion of the chloroform solution, so that the sulphuric acid formed a lower layer. A reddish- brown colour at the interface indicates the presence of a steroidal ring.

Test for saponins

One-half gram of the powdered herb or 10 mg of the extract was added to 5 ml of 95% ethanol and boiled for 2 min. The mixture was filtered with Whatman No. 1 filter paper into a clean test tube. Then 10 ml of distilled water was added and shaken vigorously for about 30 s. The formation of a persisting honey comb indicates the presence of saponins (Evans, 2002).

Test for tannins

Ferric chloride test for tannins

10 ml of distilled water was added to 1 g of the powdered herb or 10 mg of the extract in a test tube and boiled for 3 min in a water bath. The mixture was allowed to cool and then filtered with Whatman No.1 filter paper. Then 1 ml of the filtrate was diluted with 4 ml of distilled water and two drops of 10% ferric chloride were added. Instant formation of blue-black or greenish coloured solution indicates the presence of tannins (Evans, 2002).

Test for anthraquinone derivatives (Borntrager's test)

About 0.5 g of the powdered herb or 10 mg of extract was placed in a dried test tube and 10 ml of chloroform added. The mixture was shaken for 5 min and filtered with Whatman No. 1 filter paper. To 3 ml of the filtrate equal volume of ammonia solution was added and shaken. Formation of a bright pink-red colour in the upper aqueous layer indicates the presence of free anthraquinones derivatives (Evans, 2002).

Test for carbohydrates (Molisch's test)

About 1 g of the powdered herb or 10 mg of the extract was boiled in 10 ml of distilled water on a water bath for 3 min. The mixture was filtered while hot. A few drops of Molisch's reagent was added to 2 ml of the cooled filtrate and shaken, and then a small quantity of concentrated sulphuric acid was added and allowed to form a lower layer. The formation of a purple ring at the interface indicated the presence of carbohydrates.

Keller-Kiliani's test for cardiac glycosides

About 0.5 g of the herb or 10 mg of the extract was placed in a test tube. Then 5 ml of water and 2 ml of glacial acetic acid containing one drop of 10% ferric chloride solution were added. The contents were thoroughly mixed and filtered. To 2 ml of the filtrate, 1 ml of concentrated sulfuric acid was added down the wall of the test tube to form a layer underneath. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides, indicative of cardiac glycosides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout the layer (Ayoola et al., 2008).

Antimicrobial activities

The four extracts were screened for antimicrobial activity against clinical isolates namely *Staphylococcus aureus, Candida albicans, Klebsiella pneumonia, Escherichia coli* and *Salmonella paratyphi*

Table 1. Colour and yields of dried extracts of *C. emarginatum*.

Extract	Colour	Yield (g)	Yield percentage (w/w)
HECE	Greenish	0.75	0.25
EECE	Greenish	0.51	0.17
CEME	Brown	0.46	0.15
CEMEST	Greenish brown	1.53	0.5

HECE = successive hexane extract of *C. emarginatum,* EECE = successive ethyl acetate extract, CEME = successive methanol extract, CEMEST = straight run methanol extract.

Table 2. Results of the phytochemical analyses of *Calyptrochilum emarginatum* herb and extracts.

Secondary metabolite	Herb	HECE	EECE	CEME	CEMEST
Alkaloids	-	-	-	-	-
Tannins	+++	-	-	+++	+++
Flavonoids	++	-	++	++	++
Terpenes	++	++	++	+	++
Steroids	++	+	++	+	++
Cardiac glycosides	-	-	-	-	-
Carbohydrates	++	-	-	-	-
Saponins	++	-	-	++	++
Anthraquinones	-	-	-	-	-

HECE = successive hexane extract of *C. emarginatum,* EECE = successive ethyl acetate extract of *C. emarginatum,* CEME = successive methanol extract of *C. emarginatum*, CEMEST = straight run methanol extract of *C. emarginatum,* + = slightly present, ++ = present, +++ = highly present, - = not detected.

obtained from the staff clinic of the National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria, using agar dilution streak technique (Mitscher et al., 1972). The test organisms were prepared by sub-culturing in freshly prepared nutrient broth at 37°C for 3 h, having approximately 1.25×10^6 to 1.25×10^7 colony forming units (cfu). Then 64 mg each of hexane and ethyl acetate extracts were dissolved in 2 ml dimethyl sulphur oxide (DMSO) to give a concentration of 32 mg/ml. Similarly, 64 mg each of the methanol extracts were dissolved in 2 ml of water. 1 ml of the prepared extracts each was introduced into 19 ml of molten nutrient agar placed in water bath at 45°C. These were mixed properly and poured into sterile petri dishes to give a final concentration of 1.6 mg/ml. The dishes which were prepared in duplicates were then allowed to gel and thereafter, the test organisms were inoculated by streaking onto the nutrient agar using a wire loop meant to deliver 0.002 ml containing approximately 2.5×10^3 to 2.5×10^4 cfu.

The organisms were also streaked on dishes containing only agar (organism viability control), dishes containing nutrient agar and DMSO and dishes containing nutrient agar and water, which also served as controls. The petri dishes were incubated for 24 h at 37°C after which they were observed for microbial growth inhibition. Both water and DMSO showed no inhibiting effect on the organisms.

Plates where growth inhibition of the organisms was observed were further incubated for 48 h and then a little of the inhibited organisms were sub-cultured in a freshly prepared nutrient broth and then incubated for 24 h at 37°C. Observation of clear broth indicates bactericidal activity while turbidity of the broth indicates bacteriostatic activity.

RESULTS

The phytochemical and antimicrobial properties of the extractives were determined. As shown in Table 1, the combined extractive value of HECE, EECE, and CEME obtained from successive extraction was higher than that from the straight run methanol extraction CEMEST. However, considering the individual extractive values, CEMEST had the highest extractive value. As shown in Table 2, the methanol extractives CEME and CEMEST contained majority of the phytochemicals detected namely tannins, flavonoids, terpenes, sterols and saponins. Antimicrobial studies of the extracts revealed that at a minimum inhibitory concentration (MIC) of 1.6 mg/ml, the hexane and methanol successive extracts exhibited bactericidal activities against *S. aureus*. The straight run methanol extract and the successive ethyl acetate extract did not show any activity against all the microorganisms investigated namely, *S. aureus, C. albicans, K. pneumonia, E. coli* and *S. paratyphi* at 1.6 mg/ml antimicrobial activities at the same concentration (Table 3).

Table 3. Antimicrobial activity of *C. emarginatum* extracts.

Extract	Concentration (mg/ml)	Microorganism	Biocidal activity	Biostatic activity
CEMEST	1.6	Sa	-	-
		Ca	-	-
		Kp	-	-
		Ec	-	-
		Sp	-	-
HECE	1.6	Sa	+	-
		Ca	-	-
		Kp	-	-
		Ec	-	-
		Sp	-	-
EECE	1.6	Sa	-	-
		Ca	-	-
		Kp	-	-
		Ec	-	-
		Sp	-	-
CEME	1.6	Sa	+	-
		Ca	-	-
		Kp	-	-
		Ec	-	-
		Sp	-	-

HECE = successive hexane extract of *C. emarginatum;* EECE = successive ethyl acetate extract of *C. emarginatum;* CEME = successive methanol extract of *C. emarginatum*; CEMEST = straight run methanol extract of *C. emarginatum;* Sa = *Staphylococcus aureus*; Kp = *Klebsiella pneumonia*; Sp = *Salmonella paratyphi*; Ca = *Candida albicans*; Ec = *Escherichia coli;* + = inhibition; - = no inhibition.

DISCUSSION

Phytochemical analyses

The colour and yields of the extracts studied are shown in Table 1. HECE and EECE were green in colour and had yields of 0.25 and 0.17% w/w, respectively. The successive methanol extract CEME was brown and the yield was 0.15%. On the other hand, CEMEST was greenish brown and the yield was 0.5% w/w. The results of the phytochemical tests of the herb and extracts of *C. emarginatum* presented in Table 2 showed that six major classes of secondary metabolites were detected namely, terpenes, sterols, saponins, tannins, flavonoids and carbohydrates. HECE contained terpenes and sterols. EECE contained terpenes, sterols and flavonoids. CEME and CEMEST contained terpenes, sterols, flavonoids, saponins and tannins. The methanol extracts CEME and CEMEST contained the majority of the secondary metabolites detected in which polyphenols are predominant. The phytochemical constituents of *C. emarginatum* from Northern Nigeria included flavonoids, saponins, steroids, terpenoids and tannins (Mathias et al., 2007), which is consistent with findings reported herein.

Antimicrobial studies

The four extracts HECE, EECE, CEME and CEMEST were screened against five microorganisms at 1.6 mg/ml concentration. As shown in Table 3, antimicrobial studies of the four extracts revealed that at 1.6 mg/ml concentration, the hexane extract (HECE) containing terpenes and steroidal compounds, and the methanol successive extracts (CEME) rich in saponins and polyphenols were active against *S. aureus* at 1.6 mg/ml. The straight run methanol extract (CEMEST) and the ethyl acetate successive extract (EECE) did not show any activity against all the microorganisms investigated namely, *S. aureus, C. albicans, K. pneumonia, E. coli* and *S. paratyphi*, at the same concentration of 1.6 mg/ml. HECE and the CEME inhibited the growth of *S. aureus*. On further observation, bactericidal activity was established.

Antimicrobial activity observed at the concentration of 1.6 mg/ml by any agent is indicative of potent activity (Mitscher et al., 1972). The antimicrobial activity result from this study supports the folkloric use of *C. emarginatum* for the treatment of respiratory tract and opportunistic infections.

Conclusion

C. emarginatum is rich in phytochemicals, some of which may be attributed to its ethnomedicinal uses for management of coughs, tuberculosis, and as antimalarial. The presence of some secondary metabolites like flavonoids, saponins, tannins, phenols, terpenes and sterols, all of which have been reported to exhibit physiological activities in man, animals and microorganisms, suggests that the plant may be used as a potent vegetable drug. Some phytochemicals are used in the pharmaceutical industry for the production of various drugs. Examples include the anticancer taxol as paclitaxel, artemisinin and its derivatives artesunate and artemether as antimalarials (Evans, 2002).

Flavonoids and saponins have been reported to possess antioxidants, hepatoprotective and anti-inflammatory activities, and are used as antimicrobial, anticancer and antiallergic remedies. Some tannins had been reported as anti-viral and anti-tumor agents as well as diuretics. Terpenes like the mono-, sesqui- and triterpenes, and sterols had been reported to exhibit various biological activities in animals and micro-organisms some of which include anti-inflammatory, anti-microbial and hormonal activities. Some steroidal compounds have been reported to exhibit anti-diabetic properties (Evans, 2002). The extracts from *C. emarginatum* namely HECE and CEME exhibited bactericidal activity against *S. aureus*, which is responsible for some respiratory tract and opportunistic infections. This finding corroborates the health-promoting benefits that have been attributed to the folkloric uses of *C. emarginatum*.

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