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Antibacterial Activity of the Bioactive Fractions of *Cyathula uncinulata* (Amaranthaceae)

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Authors' contributions

This work was carried out in collaboration between all authors with joint design of the study by authors MABJ and CLO. Author MABJ managed the literature searches, wrote the protocol, performed laboratory study, the statistical analysis and wrote the first draft of the manuscript. Authors CLO, JNE, TH, YO and BBS managed the analyses and supervision of the study. All authors read and approved the final manuscript.

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ABSTRACT

Cyathula uncinulata (Schrad.) Schinz is used in ethnomedicine in various parts of the world. This study isolated and characterized a bioactive compound from *C. uncinulata* based on its antibacterial activities. Separation of the bioactive compound from the ethyl acetate fractions of the plant was by solvent-solvent fractionation followed by repeated column chromatography. The structure of the compound was elucidated by nuclear magnetic resonance and mass spectroscopic

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methods. The NMR spectra of the isolated compound showed that the compound with formula $C_{22}H_{38}O_7$ and molecular weight 414.5329 has a long aliphatic chain made up of sugar and fatty acyl moiety. The minimum inhibitory concentration (MIC) of fractions of extracts ranges from 0.39 to 2.5 mg/ml. The MIC of the final purified compound was 0.34 mg/ml compared to the MIC of 0.19 of kanamycin indicating a reasonable bioactivity. This study thus supports the traditional use of *C. uncinulata* in the treatment of bacterial infections.

Keywords: Cyathula uncinulata; bioactive compound; spectroscopy; minimum inhibitory concentration.

1. INTRODUCTION

Cyathula is a large genus of sub-shrubs of about 27 species distributed in Asia, Pacific Islands, Africa, and North and South America belonging to the family *Amaranthaceae.* The stems are erect or ascending and leaves are opposite, petiolate with entire margin. The flowers are clustered in cymose partial inflorescences, 1-3 in each cluster, hermaphroditic and partly accompanied by sterile ones. The flowers are bracts ovate, membranous and often spiny *Cyathula uncinulata* (Schrad.) Schinz is a weedy plant occurring widely all over Africa. The seeds are oblong or ellipsoid. In South Africa, *C. uncinulata* was described as one of the 1195 species of the Sneeuberg mountain complex (Eastern Cape), one of the botanically least known regions, where the plant is locally referred to as *Isinama* [1]. In terms of classification, a phylogenetic analysis found that *Cyathula*, some other members of the families *Amaranthaceae* and *Chenopodiaceae* constituted a monophyletic group and hence supported the call for *Chenopodiaceae* to be merged with *Amaranthaceae* [2].

Previous studies on *C. uncinulata* (CU) have reported some of its ethnobotanical uses [3]. It has different uses from country to country [4]. Various ethnic groups in parts of Democratic Republic of the Congo (DRC) use CU in the treatment of malaria [5,6]. In the Eastern part of former Zaire, CU is being used by men as a philter or medicine for love [7]. Furthermore, in Ethiopia, CU is regarded as one of the untapped herbaceous plants with high nutritive value used as animal feed and in the treatment of various ailments singly and in combination [8,9]. In a preliminary screening of some South African medicinal plants, *C. uncinulata* which was selected based on ethnobotanical survey of plants used in the treatment of HIV gave promising antibacterial activity [10]. In Lesotho CU is used as an emetic [11], whereas, a root-decoction of the plant is taken for urethral 'stricture', and the root-ash is used to make soap [12]. In Madagascar, the plant is considered to be anti-syphilitic [12]. In Rwanda, some of the therapeutic potentials of CU for human diseases include use as antimicrobial, antisterility, antibrucellosis, anti-prolapse, treatment of lactation deficiency [13,14], complement modulating activity, antifungal and anti HIV-1 [15]. According to a Rwandese study CU is used in the treatment of diarrheal diseases. Aqueous and ethanolic extracts had some activity against diarrheagenic bacteria: *Salmonella typhi, Shigella flexneri and Escherichia coli* [16].

Previous studies have reported bioactivity of some members of this genus. *Cyathula officinalis* Kuan (COK) contain polysaccharides which could increase mouse red cell immunity function by strengthening red cell immune adherence and cleanup of circulating immune complex [17], increase both cellular and humoral immune responses via up-regulating Dendritic cells maturation and suppressing the frequency of Treg cells [18,19]. COK whose root is a commonly-used traditional Chinese herbal medicine with a wide range of pharmacological activities [20,21,22] has also demonstrated anti-tumor activity. Components of COK were able to suppress the growth of mouse S180 tumor, H22 liver cancer cells [23]. A sulphated derivative of *Radix Cyathulae officinalis Kuan* polysaccharides (sRCPS) extracted from COK had antiviral activity comparable with the commonly used antiviral drug ACV (0.5 mg/ml) at concentrations of 2.0 mg/ml and 4.0 mg/ml [24]. Glycoside and oleoanic acids were isolated from COK and these compounds were tested for their inhibitory activities against MDA-MB-231 (a human breast cancer cell line) by Zhou and his co-worker [25]. Fructans which exist as a wide range of oligo- and polysaccharides in many species of bacteria, fungi, and plants were isolated from COK by Chen and Tian [26]. Bioassay showed that the graminans-type fructan that is comprised of a β-D-fructofuranosyl backbone could inhibit growth of Lewis pulmonary carcinoma implanted in mice [26]. A new heterocyclic compound, named 5,5′-diisobutoxy-2,2′-bifuran was isolated for the first

time from COK [27]. In their bid to reinvestigate phytoecdysteroids of the *C. oficinalis,* Okuzumi and co-investigators [28], isolated two cyasterone stereoisomers and a known cyasterone reported to possesses antitumor-promoting activity [29].

Several C_{29}-phytoecdysteroids such as cyasterone [30,31] capitasterone, [32] and makisterone C [33] which possesses potent insect molting hormone activity were isolated from *Cyathula capitata*. In a clinical experiment with a traditional Chinese medicine: concentrated herbal extracts of cooked *Cyathula* and concoction of 9 other ingredients restored ovarian function effectively and promptly in a patient with premature ovarian failure (POF) and 8-year secondary amenorrhea, thus the combination may offer another option for treating infertility in patients with POF [34]. *Cyathula* is a component of some Chinese formulations acclaimed to be therapeutic against endometriosis and have severe blood stasis effects [35].

C. prostrata was described as a potent source of anticancer agent in animal experiments [36,37]. The shoots of *C. prostrata* (L.) Bl. is used in India as ethnotherapy of dysentery, skin diseases and as an appetizer [38]. In Kenya, the leaves and roots of *C. schimperiana* non Moq are used as decoction (internal) for malaria, as antidiarrheal, against fungal infections while the root of *C. cylindrica* Moq roots is used as decoction (internal) for malaria, purgative and as emetic [39]. Among the native Ecuadorian, the Quichua people apply the flowers and shredded leaves of *C. achyranthoides* to dog bites [40] while the Shuar eat the raw young leaves to relieve headaches [41]. However, based on literature search, studies on the isolation of bioactive compounds from *Cyathula uncinulata* are very rare. This study explored the isolation of active compounds from *C. uncinulata* and determined their biological activity.

2. MATERIALS AND METHODS

2.1 Plant Collection

The leaves of *C. uncinulata* were collected in December 2009 from Flagstaff in the Eastern Cape province of South Africa. *C. uncinulata was* identified by the Kei Herbarium curator, Dr. Immelman of the Department of Botany, Walter Sisulu University, South Africa. The photographic images of CU are shown in Fig. 1.

2.2 Extraction of Plant Material

Powdered plant materials were exhaustively extracted with methanol (MeOH) as previously described by Oshima et al. [42]. Briefly, the supernatant was filtered through cotton wool and Whatman No. 1 filter paper using a Buchner funnel. The extract was concentrated at 45ºC using a Rotavapor (Eyela N-1100, Rikakikai, China). The reduced extract was transferred to a pre-weighed glass vial and sunction-dried at room temperature with a vacuum pump (ULVAC KIKO, Tokyo). The quantity extracted was then determined to be 2 g.

Fig. 1A. Leaves of *C. uncinulata* plant (Picture taken from source garden at Flagstaff, Eastern Cape, South Africa). B. *C. uncinulata* showing the plant flowering
Source: http://www.metafro.be/prelude/view_symptom?si=V(027)

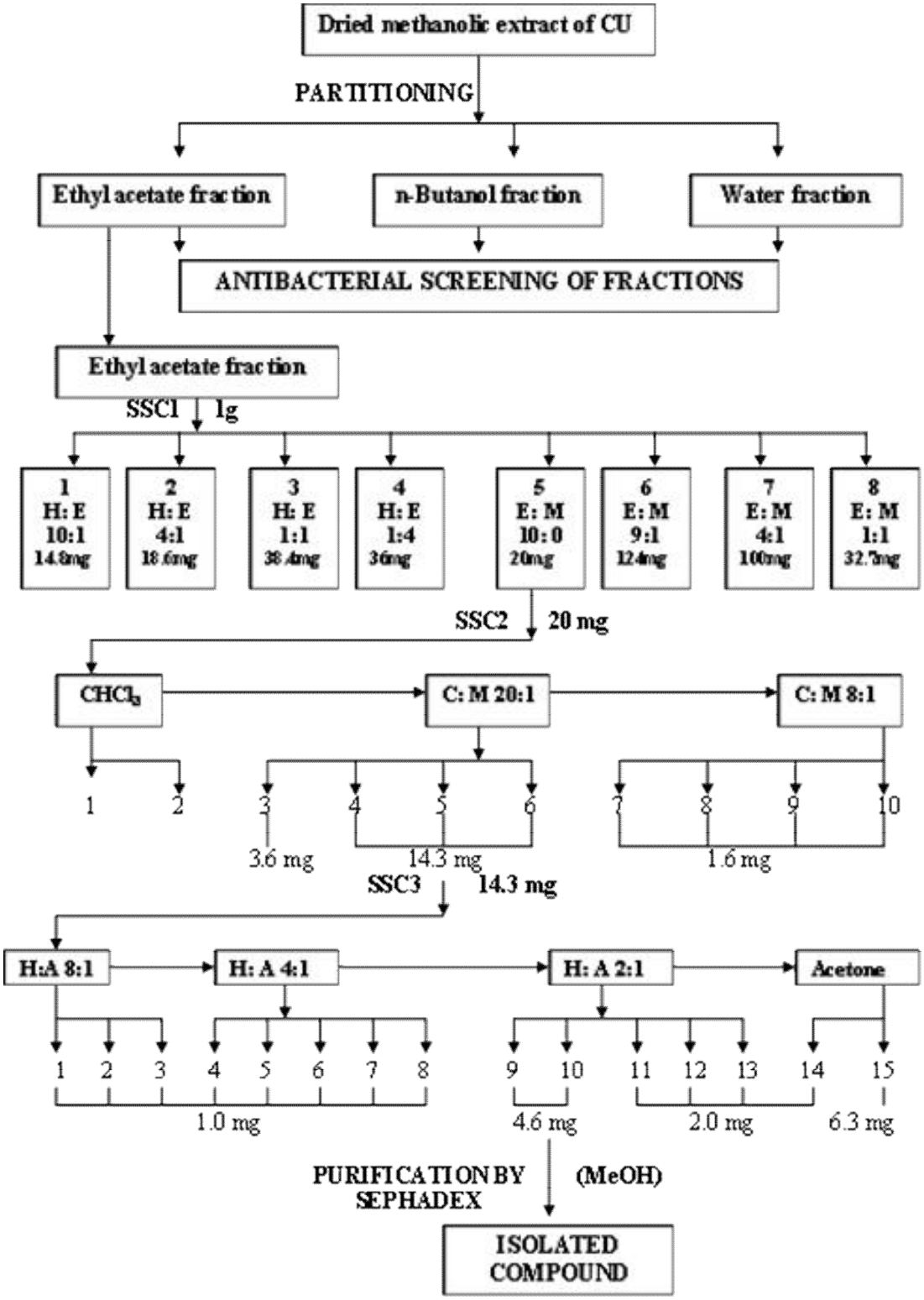


Fig. 2. Schematic diagram of solvent-solvent fractionation of *C. uncinulata*

2.2.1 Solvent-solvent fractionation of dried plant extract

The dried methanolic extract of *Cyathula* leaves was subjected to solvent-solvent fractionation as follows [42]: the MeOH extract was partitioned sequentially in ethyl acetate (EtOAc), n-butanol and water. The EtOAc fraction (1 g) was subjected to column chromatography over 100 g silica gel 60 (70- 230 mesh) eluting with *n*-hexane–ethyl acetate (10:1 → 4:1 → 1:1 → 1:4, v/v) and ethyl acetate–MeOH (10:0 → 9:1→ 4:1 → 1:1, v/v) to yield fractions designated as SSC1-1 to SSC1-8. Fraction SSC1-5 (20 mg) denoted SSC2 was further subjected to column chromatography over a lipophilic Sephadex (bead size 25-100 µℓ; Sigma-Aldrich) eluting sequentially with Chloroform and Chloroform-MeOH (20:1 → 9:1). The sub-fractions obtained were combined based on their TLC fingerprint pattern to yield 4 sub-fractions SSC2-1-2, SSC2-3 (3.6 mg); SSC2-4-6 (14.3 mg) and SSC2-7-10 (1.1 mg). A final column chromatography was performed with sub-fraction SSC2-4-6 now denoted as SSC3 using a finer silica gel 60 (40 – 50 µm Cica-Reagent, Kantochemical, Japan). The solvent flow of eluent was n-hexane-acetone (8:1 → 4:1 → 2:1) and acetone only. The fractions with similar TLC fingerprints were pooled together to yield four sub-fractions designated SSC3-1-8; SSC3-9-10; SSC3-11-14 and SSC3-15. A scheme of the steps involved in the fractionation of extract and fractions is shown in above Fig. 2.

2.2.2 TLC fingerprinting of fractions

The dried fractions were dissolved at a concentration of 10 mg/ml in methanol and TLC fingerprinting was done as described previously [43]. Several mobile systems were used. For the TLC of SSC1 fractions the mobile systems used were: H: E 4:1, H: E 1:1, C: M 20:1, C: M 8:1, C: M 2:1 and C: M: W: A 1:1:0.1:0.05 where H represents hexane, E - ethyl acetate, C - chloroform, M - methanol and W - water. For the TLC of SSC2, the mobile systems were H: A 2:1 and C: M 20:1. Finally for the TLC of SSC3, the mobile system was C: M 20:1. The detecting agent for chromatograms was *p*-anisaldehyde spray reagent.

2.2.3 Isolation of pure compounds

Based on TLC analysis and the result of step by step determining of antibacterial activities sub-fraction SSC3-9-10 (Fig. 2) was selected for purification and further characterization. SSC3-9-10 was purified by fractionation on a lipophilic Sephadex LH-20 (bead size 25-100 µℓ; Sigma-Aldrich) and eluted with MeOH as the mobile phase. The final pure compound was then subjected to NMR analysis.

2.3 Preparation of Pure Compound for NMR Analysis

The vacuum concentrated isolated pure compound was added to an NMR capillary tube and was dissolved by adding Chloroform-d for NMR (ACROS ORGANICS) and 99.8+% D, stab. with silver foil, 0.03 v/v % TMS (Merck) to a depth of 4 cm. The NMR capillary tube was then fitted in place for the ^{13}C and ^{1}H NMR analysis in a JEOL-AL 400 FT NMR system 400 MHz (JEOL FT NMR System AL400).

2.4 Antimicrobial Assay of Fractions

The microplate dilution assay as previously described [44] was used to determine the minimum inhibitory concentration (MIC) values of fractions of *C. uncinulata* extracts against *Escherichia coli* HB101 strain using kanamycin as positive control.

3. RESULTS

The MeOH extract of 2 g represented 2% of the starting material. The solvent-solvent fractionation yielded four fractions. The highest quantity (739.9 mg) of plant material was in the water fraction while the lowest quantity was in the ethyl acetate fraction (300.7 mg). In total, 1242.4 mg was extracted from the 2 g starting methanol extract of *C. uncinulata* giving a 60% yield.

The hexane-ethyl acetate combinations led to a better separation of compounds of the crude extract visualized with the *p*-anisaldehyde spray reagent than the chloroform-methanol combinations. The pooling together of fractions with the same R_f values led finally to the isolation of a pure compound with R_f of 0.38.

The MICs of the fractions of *C. uncinulata* against *E. coli* ranged from 0.39 to 2.5 mg/ml whereas the MIC of kanamycin, the positive control was 0.19 mg/ml (Table 1). The lowest MIC was observed in ethyl-acetate fraction while the highest MIC values were recorded in n-butanol and water fractions. The MIC of the

selected sub-fraction of the ethyl acetate (SSC 1) fractionation was 0.63 mg/ml while for the final purified compound MIC was 0.34 mg/ml.

Table 1. Average MIC values of the fractions of *Cyathula uncinulata* leaf extracts tested against *E. coli*

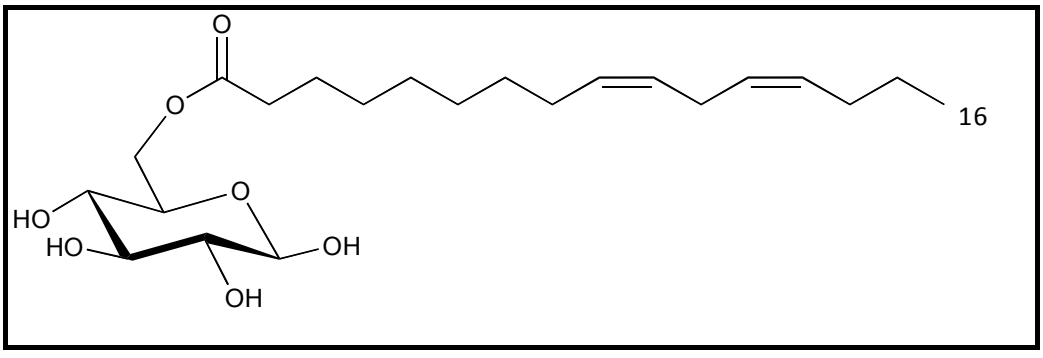
Fractions	MIC of fractions (mg/ml)
Methanol	1.093
Ethyl acetate	0.39
n-butanol	2.34
Water	2.5

The NMR spectra of the isolated compound showed that the compound has a long aliphatic chain. The proposed structure for the isolated compound as determined by 1H NMR spectrum and the elemental analysis is as shown in Fig. 3. The formula of the compound is $C_{22}H_{38}O_7$ while the molecular weight is 414.5329.

4. DISCUSSION

Solvent-solvent fractionation and column chromatography were employed to isolate the pure compound. The structure of this compound was elucidated by using nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy. NMR essentially provides a means of determining the structure of an organic compound by measuring the magnetic moments of its hydrogen atoms. The NMR proton spectra of the fraction yielding the pure compound are presented as supplementary materials A - C. The spectrum of the isolated compound shown in Fig. 3 is made up of glycosylated oleanolic acid: sugar and fatty acyl moiety composed of hydrocarbon tails. Glycosides of fatty acids or glycolipids are found in bacteria, yeasts and fungi but also in several plants. The functions and potential applications of this compound have been reviewed by Kitamoto and co-worker [45]. Sugar-based esters of fatty acids are nonionic surfactants of great potential applications. They are nontoxic, odourless, tasteless and biodegradable and because of their multi-functional properties and safety, this group of compounds has attracted attention from cosmetics, pharmaceutical, surfactant and nutrition scientists [45].

The aqueous fraction of the extract of *C. uncinulata* contained the highest quantity of plant components while the ethyl acetate fraction extracted the lowest. However, the least antibacterial activity was recorded in the aqueous fraction as opposed to the ethyl acetate fraction which had the highest activity with an MIC of 0.39 mg/ml (Table 1) hence the choice of ethyl acetate fraction for further fractionation and isolation of bioactive compound. There was a direct relationship between the polarity of the fractions and antimicrobial activity. The higher the polarity the lower the activity as also found by other authors [46,47]. The final compound had an average MIC of 0.34 mg/ml which is very close to 0.19 mg/ml of kanamycin the positive control, hence possessing a considerable antibacterial activity.



Chemical Formula: $C_{22}H_{38}O_7$
Exact Mass: 414.2618
Molecular Weight: 414.5329
m/z: 414.26 (100.0%), 415.27 (24.5%), 416.27 (4.3%)
Elemental Analysis: C, 63.74; H, 9.24; O, 27.02

Fig. 3. Structure of the isolated compound

Different compounds have been isolated from members of the genus *Cyathula*. Zhou et al. [48] isolated a new glycoside together with some oleonoic acids from the roots of *C. officinalis* Kuan. Four new compounds 4-[(1-ethoxy-2-hydroxy) ethyl] phenol (1), 2, 3-isopropylidene cyasterone (2), 24-hydroxycyasterone (3) and 2, 3-isopropylidene isocyasterone (4), together with fourteen known compounds were later isolated from the same species [25]. *C. officinalis* Kuan seems to be the most widely studied of the genus whereas report on isolation of bioactive compound from *C. uncinulata* is scarce. Other studies on the genus *Cyathula* reported mainly on biological activities and cytotoxicity effect of extracts of *C. achyranthoides* and *C. prostata* [49]. This study is a report on a bioactive compound from *C. uncinulata.*

5. CONCLUSION

This study isolated a compound from *C. uncinulata* (Schrad.) Schinz. The compound had reasonable antibacterial activity compared to the crude extract and the control antibiotic. This is an indication that this plant may be a candidate or serve as template for new antimicrobial. The main focus of several studies are the isolation and identification of bioactive compounds from plants, nonetheless, it is imperative to recognize the complexity of plants and that a single compound may not be responsible for the observed activity but rather a combination of compounds acting by synergism or as complements. This may further explain why bioactivity is lost in some cases in the course of isolation of active components.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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