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Isolation and Biological Investigation of Bioactive Compounds from *Croton grattissimus* (Burch) in Mthatha, Eastern Cape, South Africa

Morobe IC[1*], Obi LC[2], Oyedeji AO[3], Majinda RTT[1], Hattori T[4], Idiaghe JE[5] and Vasaikar SD[6]

[1]*Department of Biological Sciences, University of Botswana, Mabuto Drive, Gaborone, Botswana*

[2]*Division of Academic Affairs, University of Fort Hare, Alice, Fort Hare, South Africa*

[3]*Department of Chemistry, Walter Sisulu University, Nelson Mandela Drive, Eastern Cape, South Africa*

[4]*Laboratory Emerging Infectious Diseases, Internal Medicine, Graduate School of Medicine, Tohoku University Sendai, Japan*

[5]*Department of Primary Education Studies, College of Education, Agbor, Delta State, Nigeria*

[6]*Department of Medical Microbiology, Walter Sisulu University, Nelson Mandela Drive, Eastern Cape, South Africa*

[*]**Corresponding author:** Morobe IC, Department of Biological Sciences, University of Botswana, Mabuto Drive, Gaborone, Botswana, Tel: +26773130037; E-mail: morobe@mopipi.ub.bw

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Abstract

The therapeutic applications of aromatic plants used for antimicrobial purposes are well documented in ancient history. Extracts of *Croton grattisimus* (Burch) are used for anointing the sick, treating chest pains, coughs, fever and sexually transmitted diseases such as syphyllis. In an endeavour to establish the bioactive compounds of the plant, plant materials were collected, washed with sterile distilled water and left at room temperature for 7 days to dry. The dried material (50 g) was ground into a coarse powder using Macsalab mill. The leaves powder was then soaked in different solvents systems for 48 hr each with frequent shaking. The samples were then filtered, and the filtrate was concentrated to dryness under reduced pressure using a rotary evaporator, collected in 10 ml of the solvent, placed into small beakers and allowed to dry at room temperature. NMR spectroscopic methods were used for the structural elucidation of the active compounds. The percentage yield of *C. grattisimus* were, hexane (32.8 mg), dichloromethane (46.6 mg), ethylacetate (10.1 mg), methanol (61.2 mg) and water (10.6 mg). The R_f-values ranged from 0.64 to 0.70, with the most antimicrobial activity observed at 0.67 for *H. influenzae* and 0.64 for *S. pneumoniae*. The phytochemical investigations of methanolic extracts of *C. grattisimus* leaves led to the isolation of triterpenoids. This study reveals the characterization and structures of the non-volatile components of *C. grattisimus* extracts.

Keywords: *Croton grattisimus* (Burch); Aromatic plants; Sexually transmitted diseases; Antimicrobial activity; Triterpenoids

Introduction

Croton grattisimus (Burch) belongs to the family Euphobiaceae which consists of several species as trees, shrubs and herbs. *C. grattisimus* is a semi-deciduous tree species widespread in sub-Saharan Africa, occurring on rocky hillside and coastal areas throughout much of the warmer and drier regions, from North East South Africa to Cape Verde [1]. Van Vuuren reported that of the 750 species of *C. grattisimus* found in the tropics, only 10 species were native to the flora of Southern African region, including the Northern Eastern Cape and Zululand [2]. The leaves of this plant are aromatic in nature and are often used in folk medicine for the treatment of ailments such as respiratory and sexually transmitted diseases. The therapeutic applications of aromatic plants used for antimicrobial purposes are well documented in ancient history. The dried and powdered leaves of *C. grattisimus* and essential oils derived from this plant are used for anointing the sick, treating chest pains, coughs, fever and sexually transmitted diseases such as syphyllis [3].

Because the clinical efficacy of many existing antibiotics is threatened by the emergence of multidrug-resistant pathogens [4] the antimicrobial compounds derived from plants that can either inhibit the growth of pathogens or kill them and have no or least toxicity to host cells are considered candidates for developing new antimicrobial drugs. Antimicrobial agents inhibit the growth of microorganisms by interfering with specific physiological characters or metabolic functions of microorganisms [5]. It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug resistant microbial pathogens [5]. It was previously reported [6] that different races of people used *C. grattisimus* for different purposes. For instance, the Zulus and Xhosas used milk infusions of the bark as purgatives for stomach or intestinal disorders and uterine disorders. These remedies were applied by blowing the powdered bark into the womb. They further treated pleurisy by rubbing the powdered bark into chest skin incisions to act as a counter irritant [7]. In Venda, leaves were dried and smoked for the treatment of influenza, colds and fevers. It was reported [7] that Zimbabweans treat coughs with smoke from the leaves of the plant and take root infusions for abdominal pains. In Botswana, a decoction prepared with the leaves was taken for coughs [8]. Other countries use *C. grattisimus* bark in treating painful respiratory conditions, fevers and bleeding gums. *C. grattisimus* have many uses in southern Africa [9,10]. For example, the aromatic lavender like scent of the dried and powdered leaves of this plant have been used to treat chest pains, coughs, fever and sexually transmitted diseases such as syphilis. However, the bark was frequently used to treat bleeding gums, abdominal pains, skin inflammations and earache [11]. Generally essential oils derived from *C. grattisimus* have been widely used in the treatment of respiratory tract, urinary tract, gastrointestinal tract infections as well as skin infections. *C. grattisimus* contains organic compounds such aspumarane, kaurane, labdane, clerodane, cembrane, diterpenoides, isoquinoline, alkaloids and triterpenoides [12-17]. Secondary

metabolites with antiinfective activities have attracted attention as natural products that can be used as substitutes for antibiotics resistant to pathogenic bacteria, fungi and helminthes [5]. In this study we investigated the chemical and biological composition of non-volatile components of *C. grattisimus* and their antimicrobial activity against *H. influenzae* and *S. pneumoniae.*

Materials and Methods

Instrumentation and general experimental procedures

Plants were extracted using sequential solvent extraction methods. Vacuum Liquid Chromatography (VLC), Column Chromatography, Preparative Thin Layer Chromatography (PTLC) and Sephadex LH20 were used for isolation, sample clean-up and purification. Chloroform and Methanol (9:1) were used as solvent systems for isolation of active compounds. Active compounds were then identified using NMR (Proton 300 and 600 MHZ, DEPT-135, 2D (COSY), HMQC, HSQC, and HMBC. The spectra were acquired on Bruker Advance DPX 300 and 600 Spectrophotometer.

Plant Material

Collection of plant material: From June 2010 to December 2011, the leaves were collected from Lusikisiki in the Eastern Cape, South Africa, based on its ethnomedical application in the treatment of respiratory tract infections, guided by the information from traditional healers and others on the basis of its various uses. The plant was identified and authenticated by Taxonomist (Immelman K.L.) in the Department of Botany, Walter Sisulu University, South Africa. Voucher specimen MI008 was deposited at the University herbarium.

Preparation of plant crude extracts: Plant materials were washed with sterile distilled water and left to dry at room temperature for 7 days as previously described [3,4]. The dried material (50 g) was ground into a coarse powder using Macsalab mill (Model 200 LAB), Eriez, Bramley.

Solvent extractions: The leaves powder was then soaked in different solvent systems for 48 hr each with frequent shaking. The samples were then filtered through the funnel and Whatman No.1 filter paper and the filtrate were evaporated to dryness under reduced pressure using a rotary evaporator, collected in 10 ml of the solvent, placed in small beakers and allowed to dry at room temperature. After plant extracts had dried, the percentage yield of each extract (Hexane (32.8 g), Dichloromethane (46.6 g), Ethylacetate (10.1 g), and Methanol (61.2 g) [3,4].

Bioassay

Antimicrobial assay of plant extracts

Detection of zone of inhibition against microbial agents on TLC bioautography: The agar overlay or immersion bioautography was used. This is where chromatograph (TLC plates of 0.25 mm thickness) were placed into a beaker containing a known solvent system and the inoculum (agar seeded with *H. influenzae, S. pneumoniae* and *Candida albicans* was rapidly distributed over the TLC plate with a sterile Pasteur pipette. After solidification of the medium, TLC plates were incubated overnight at 37°C in a plastic-bowels lined with moist cotton wool. The bioautograms were sprayed with an aqueous solution (2.5 mg/ml) of tetrazolium salt (3-4,5-Dimethylthiazole-2-yl-2,5-diphenyl-tetrazolium bromide (Aldrich, Gillingham, England) and incubated at 37°C for 2 h. Active compounds appear as clear spots against a purple coloured background due to formation of formazen dye [2].

Culture media: Microorganisms are media sensitive thus the bacterial strains were maintained on tryptic soya agar slants. All the cultures were introduced aseptically (using a heat-sterilized wire loop) into 250 ml Elernmeyer flasks containing tryptic soya broth. To achieve a homogenous distribution of microorganism in the nutrients, the cultures were shaken during incubation at 37°C for 24 h using a LAB-LINE ORBIT shaker. All media was autoclaved at 121°C for 15 minutes prior to inoculation [9,13].

Inoculums for the Assay: Tryptic soya agar (TSA) was used as a solid media for the bioautographic overlays. The molten media were maintained in water bath at approximately 42°C and seeded with microorganisms from the tryptic soya broth; 10 ml of the broth culture was introduced into 100 ml of tryptic soya agar. The optical density at 540 nm (OD_{540}) of the agar was measured with Spectronic 21D (Milton Roy) UV/VIS spectrophotometer and OD_{540} equal to 1 corresponds to approximately 10^7 cells/ml [13].

Isolation of bioactive compounds using column chromatography: Isolation of bioactive compounds from *C. grattisimus* was done using column chromatography method. The column was pre-washed with 100% hexane and then the solvent mixtures were determined. The ratio of the crude extract to silica gel was 1:30. The solvent was added to the column and the slurry of the silica gel was poured into the column. The tap was slowly opened accompanied by a gentle tapping while the gel was packing. The level of the gel was marked on the column and the solvent was left to cover the top of the gel. Sand was added to separate the compound from silica gel and to protect the silica gel from breaking. The extract was slowly added accompanied by addition of the solvent and gently tapping the column to allow the extract to drop down. The column was run until the coloured gel approached the end of the column and then about 40 drops of the eluent was collected into pre-weighed tubes. The tubes were left to dry at room temperature. When the fraction was completely dry, then about 100µl of the solvent was added to the fraction, mixed and spotted on the TLC plate to determine the correct solvent system ratios and the similarities and differences between bands of bioactive compounds.

The column was eluted using a gradient solvent system starting with a mixture of % n-hexane, n-hexane/chloroform, % chloroform, chloroform/methanol and % methanol to give 146 fractions of 40ml each. With the guide of TLC observations, the concentrated fractions with similar TLC profiles were combined and labeled as Fa (% $CHCl_3$) [fraction 1-7], Fb ($CHCl_3$/MeoH; 9.5:0.5) [fr.8-15], Fc ($CHCl_3$/MeoH; 9:1) [fr.16-19], Fd ($CHCl_3$:MeoH; 9:1) [fr.20-24], Fe ($CHCl_3$/MeoH; 8:2) [fr.25-31], Ff ($CHCl_3$/MeoH; 7:3) [fr.32-46], Fg ($CHCl_3$/MeoH; 6:4) [fr.47-59], Fh($CHCl_3$/MeoH;1:1) [fr.60-101] and Fi (%MeoH; 102-146) [18].

TLC autographic assay of crude extracts and compounds: Crude extracts and compounds were developed in duplicate on separate TLC plates using an appropriate solvent system. One plate was sprayed with Vanillin-Sulphuric acid spray reagent and the other with the DPPH spray reagent. The plates were placed side by side and components showing antioxidant activity were identified, examined and recorded after 30 minutes. Yellow spots against a purple background when sprayed with DPPH showed a positive activity [18].

Results

Percentage yields of extraction solvents

The percentage yield of *C. grattisimus* extracts were, hexane (32.8 mg), dichloromethane (46.6 mg), ethylacetate (10.1 mg), methanol (61.2 mg) and water (10.6 mg). The R_f values ranged from 0.64 to 0.70, with the most antimicrobial activity observed at 0.67 for *H. influenzae* and 0.64 for *S. pneumoniae*. The phytochemical investigations of dichloromethane extracts of *C. grattisimus* leaves led to the isolation of non-volatile compounds.

Identification and isolation of bioactive compounds

Proton at 4.2 ppm (J9.4) correlated with 123.1 (3J) and 140.3 ppm (2J) and at 63.1 ppm correlated with carbon at 37.4 ppm (2J) and at carbon 25.8 (4J). Proton at 4.2 ppm (J=7.2 Hz, d, 1H) correlated with carbon at 123.1 ppm (12, 3J) and at 140.3 ppm (13, 2J) on HMBC. Proton at 3.69 ppm (t, J=6.6 Hz, 1H) correlated with carbon at 37.4 ppm (2J) and 25.8 ppm (4J) on HMBC. Compound 1 was isolated as a yellow powder of Melting point 241-245°C and responded positively to the Lieberman-Buchard test for triterpenes. The molecular formula was deduced as $C_{30}H_{46}O_2$ by Mass spectrophotometer (ESI-MS:437.2). The ^{1}H-NMR data showed singlet signals for seven methyl groups. The ^{13}C-NMR and DEPT spectra showed 30 signals for seven methyl, nine methylene, four methane, nine quaternary carbons, keto carbonyl and an β-unsaturated keto carbonyl. HMQC revealed ^{1}H-direct correlation between the protons of seven methyl groups and corresponding carbons. From this data an olean-type triterpene was isolated, as previously deduced [19]. This was confirmed by the HMQC and HMBC spectra, for the H-12 olefinic proton (δH 5.62) indicated ^{1}H-^{13}C long range correlations with the methane carbons C-9. Therefore compound 1 was found to be 12-oleanene-3,11-dione or 14b-icosahydropicen-3-ol. This compound was isolated [20]. In this study, the ^{1}H and ^{13}C-NMR assignments for compound four were carried out on the basis of ^{1}H-^{1}H, DEPT, HMQC, COSY (homonuclear correlation spectroscopy), HMBC and NOESY experiments.

Moronic acid is a phytochemical in the class of simple Triterpenoid keto acids, belonging to the group of new Terpenes extracted from *Rhus javanica* [20]. Health benefits of monoric acid include, HIV maturation inhibitor, Anti-Espstein-Barr virus [20]. It inhibits the capacity of Rta to activate a promoter that contains an Rta-response element, indicating that monoric acid interferes with the function of Rta [20], antifungal effects, antimicrobial activity against the Gram positive bacteria isolated from *Ozoroa mucronate* [20] and anti-herpes simplex virus. It was reported that monoric acid suppres virus yield in the brain more efficiently than those in the skin [21]. Therefore in view of this report Moronic acid may be expected to be beneficial in preventing central nervous system complications. Moronic acid, betulinic acid and their derivatives have been reported to exhibit anti-human immunodeficiency virus (HIV) activity as inhibitor of HIV-1 entry [20,22,23]. However, the mode of antiviral action of moronic acid against HSV may be different from that of betulinic acid against HIV. Thus, further study of moronic acid may clarify the mechanism of anti-HSV action *in vitro* and its toxicological and anti-HSV therapeutic effects at high doses *in vivo* for clinical application. Moronic acid was reported [21] to be less toxic than betulonic acid. Ursolic acid (3b)-3-hydroxyurs-12-en-28-oic acid) and oleanolic acid [(3β)-3-Hydroxyolean-12-en-28-oic] are triterpenoid compounds that exist widely in food and medicinal herbs with molecular weight of 456.68 and melting points of 300°C [24]. Oleanolic acid has been marketed in China as an oral drug for human liver disorders [24]. Ursolic acid is also known as urson, prunol, micromerol and malol compound which naturally occurs in large numbers of vegetarian foods, medicinal herbs and other plants including apples, bilberries, cranberries, antimicrobial and antifungal properties. It was reported that ursolic acid has medicinal action, both topically and internally. It has been used in many cosmetic preparations for its anti-inflammatory, antitumor and antimicrobial effects [24,25].

Tests have shown that rsolic acid inhibits the growth of several strains of Staphylococci, Candida albicans and Microsporium lenosum [26]. It was reported [27] that ursolic acid was also used as an ointment to treat burns, photoaged skin and prevent the appearance of wrinkles and age spots by restoring the skin's collagen bundle structures and its elasticity. Concentrations of ursolic acid ranging from 0.01 to 50mg have been reported [27] for inclusion in skin treatment preparations. Ursolic and oleanolic acids are non-toxic and have been used to enhance hair growth, prevent scalp irritation and have been recommended for skin cancer therapy in Japan [25]. Ursolic acid rarely occurs without its isomer oleanolic acid [(3β)-3-Hydroxyolean-12-en-28-oic] [25]. Triterpenoids are comprised of a triterpenoid aglycone linked to one or more sugar moieties and these compound structures are similar in pharmacological activity [27]. Several ursolic acid derivatives, ursane-type triterpenoid saponins are known to occur naturally as secondary metabolites [27].

The leaves of *C. grattisimus* were separated using repeated column chromatography over silica gel to yield compound 4, which was a colourless oil. NMR analysis showed that purified compounds are not stable on standing, but appear to be stable in the unpurified form [1]. The NMR spectra of compound 4 was similar to a compound previously isolated from the stem bark of *C. grattisimus* [1]. Y-Lactone are widely distributed hydrofurans in nature and represent around 10% of all-natural compounds that display a broad biological profile including strong antibiotic, antihelmentic, antifungal, antitumour, antiviral, anti-inflammatory and cytostatic properties which make them interesting lead structures for new drugs [28].

It has been reported that the α-methelene group in the lactone ring is potentially able to blind the nucleophilic sites of biomolecules by conjugate addition and manifests its own biological activity [28]. Y-lactones can also be synthesised from butenolides by catalytic hydrogenation method [1]. The latest methods of hydrolysing y-lactones include; y-lactones from ring transformation, y-lactones from C-C Bond Cyclization, y-lactones by C-O Bond Cyclization from a C4 block [1].

Bioassay

Antimicrobial assay of extracts: Table 1. Minimum Inhibiting quantities (MIQ) of the *C. grattisimus* crude extracts. The minimum inhibition quantity (MIQ) from the semiquantitative analysis showed antimicrobial activity in both organisms (Table 2). However, the activity between the two organisms was different for extracts and isolated compounds, for instance Table 1 Showed higher activity for *H. influenzae* than *S. pneumoniae*, whereas in Table 2 *S. pneumoniae* showed higher activity than *H. influenzae*. The antimicrobial activity bioautograms showed the highest activity in methanolic extracts and compounds followed by Hexane and Dichloromethane. Ethylacetate showed the least activity after 24 hrs. This was a reversal of the activity trend observed in the antimicrobial activity tests [29-31].

Microorganism and MIQ of the extract (µg)[A]		
Extract code	***H. influenzae***	***S. pneumoniae***
Hex	12	9
DCM	10	7
EtoAc	5	5
MeoH	15	11
Ampicillin	0.001	0.001
Chloramphenicol	0.001	0.001
Hex, DCM, EtoAc, MeoH: Hexane, Dichloromethane, Ethylacetate and Methanol respectively. A= Zone of inhibition (mm)		

Table 1: Minimum Inhibiting quantities (MIQ) of the *C. grattisimus* crude extracts.

Microorganism and MIQ of the extract (µg)[A]		
Compound code	***H. influenzae***	***S. pneumoniae***
C1	6	10
C2	5	8
C3	NA	3
C4	11	16
Ampicillin	0.001	0.001
Chloramphenicol	0.001	0.001
C1, C2, C3, C4: Compound 1, 2, 3 and 4 A= Zone of inhibition (mm).		

Table 2: Minimum inhibiting concentrations (MIQ) of isolated compounds.

Antioxidant activity of free radical scavenging of plant extracts and compounds from *C. grattisimus* stem bark DPPH Antioxidant Scavenging Autobiograms of *C. grattisimus* extracts and compounds intervals from 30 minutes to 2 hrs. Active extracts and compounds appeared as yellow spots against a purple background on a TLC plate and the observed chromatograms are as shown below Figures 1 and 2. The EC_{50} were determined from the plots similar to those shown Figure 3 using microsoft Excel program.

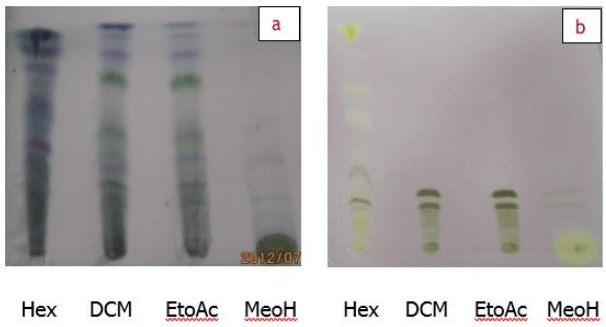

Figure 1: Vanillin sulphuric acid TLC bioautograms of *C. grattisimus* crude extracts and fractions.

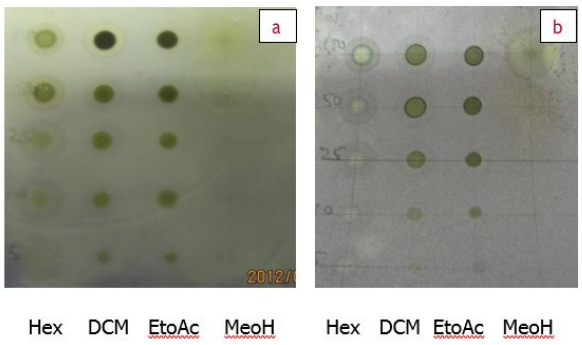

Figure 2: 3-(4,5 dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) TLC bioautograms showing *C. grattisimus* extracts antimicrobial activity against *S. pneumoniae* and *H. influenzae.*

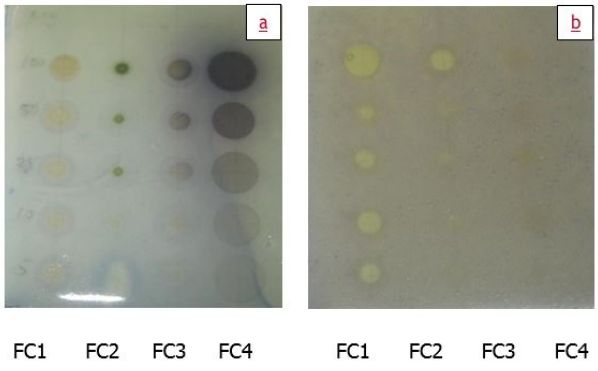

Figure 3: 3-(4,5 dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) TLC bioautograms showing antimicrobial activity of *C. grattisimus* compounds against *S. pneumoniae* and *H. influenzae*.

Discussion

Compound 1 was isolated as a white powder of melting point 240-245°C and the molecular formula was deduced as $C_3H_{46}O_2$ by ESI-MS. The chemical shift of compound 1 in the ^{13}CNMR spectrum were typical for triterpenes. Compound 1 was characterized as a 12-Oleanene triterpene with one carboxylic acid function in the upper rings as previously reported [32]. Compound one was identified as oleanolic acid by the close match of the ^{13}CNMR data (Table 3), similar to the one compiled [16].

The 'HNMR data (Table 3) showed singlets signals for seven angular methyl groups. The ^{13}CNMR and DEPT spectra showed 30 signals for seven methyl, nine methylene, four methine and nine quartenary carbons including a keto carbonyl and α, β-unsaturated keto carbonyl. HMQC experiments revealed 'H-^{13}C direct correlation between the protons of seven methyl groups and corresponding carbons were observed and Olean-type triterpene skeleton was deduced. The resonance assignments for seven methyl groups were at δ1.4, 2.0, 2.05, 2.35, 2.15, to H-23, 24, 25, 26, 27, 28, 29 and 30 respectively. The protons to two methyl groups (Δh 3.15, δC 25.8 and δH 1.63, δC 22.3 and two methylene groups (δH 2.0, δC 39.9 and δH 1.95, δC 37.4) had 'H-13C long range correlation with the carbon at δC 174.2, establishing the carbonyl to be C-3.

Position	δC	δH
1	29.4	1.62a
2	25.2	5.4a
3	77.2	5.4a
4	37.3	
5	39.9	2.0 m
5a	24.5	1.4 m
6	29.3	2.35 s
6a	32.9	2.35 m
7	39.4	5.4a
8	36.2	
9	123.1	2.05 m
9a	144.3	2.81 dd (5.6,12,5)
10	37.3	5.02b
11	28	6.98d
12	32	
13	29.7	2.15 m
13a	59.4	2.35 m
14	37.4	1.95 s
14a	29.7	1.5 s
15	25.8	1.62a
16	32.7	0.91d (6.6)
17	19.5	1.25 s
18	22.3	1.63 s
19	25.8	3.15 s
20	174.2	
OCH_3	50.4	

Table 3: ^{1}H (300 MHZ) and ^{13}C (600 MHZ) NMR data for compound 1 in Chloroform-d.

The hydrogen signal appearing at δ'H 5.4a (s), corresponding to the only present Olefenic hydrogen atom correlates to the carbon atom at δC 123.1 in the HMQC spectrum. The Olefinic C-13 atom was quaternary and appeared at 174.2 and at a keto C-atom appearing at 199.7 suggested compound 1 had an Olean-12-ene-11 keto skeleton [29]. These assumptions were confirmed by the HMQC and HMBC spectra, for the H-12 Olefinic proton (δH 5.4a) indicated 'H-^{13}C long range correlations with the methane carbons C-9 (δc 59.4), C-14 (δc 39.9) the H-18 proton (δH 2.35) with C-13 (δc 29.3) in the HMBC spectrum (Table 3). Therefore compound 1 was considered to be 12-Oleanene-3-11-dione. This compound was previously isolated [29]. The ^{13}C-NMR assignments for this compound were not reported until 1994 [30,31]. In this study, the 'H and ^{13}CNMR assignments for compound 1 were carried out on the basis of DEPT, HMQC, 'H-'H, COSY and HMBC experiments.

Total antioxidants capacity (Figures 4 and 5) indicated that all for *C. grattisimus* extracts and compounds possessed antioxidants activity. However, methanolic crude polysaccharide extracts exhibited stronger antioxidants. Similar results were reported [15]. It is well known that *C. grattisimus* possesses a wide variety of bioactive properties such as carotenoids, riboflavin, ascorbic acid, thiamine, monosaccharides vitamin antioxidants and 17 amino acids, which are the major constituents of hypoglycemic effect.

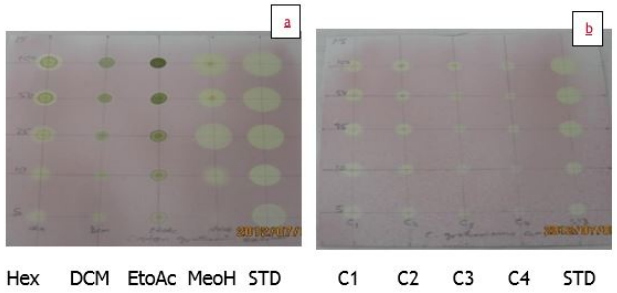

Figure 4: Chromatograms representing active extracts, compounds and standard (std) against DPPH observed after 30 minutes of reaction.

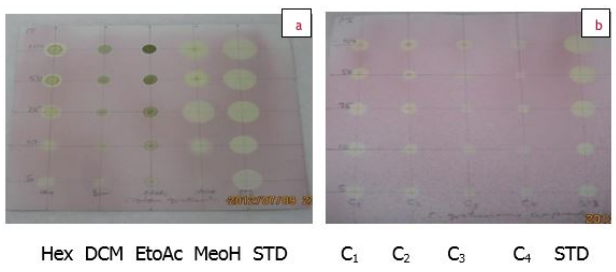

Figure 5: Chromatograms representing active extracts, compounds and standard (std) against DPPH observed after 2 hours of reaction.

DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The model of scavenging the stable DPPH radical is widely used for relatively rapid evaluation of antioxidant activities compared to other methods [11]. In the quantitative assay, the extract exhibited a noble dose dependent inhibition of the DPPH activity, with a 50% inhibition (IC_{50}) at a concentration of 0.01-0.3 mg/ml, while the IC_{50} value of the positive control (Gallic acid) was found to be 0.2 mg/ml. The results of this study suggest that the *C. grattisimus* crude extracts possess both strong free radical scavenging and antitumor activities. Therefore, further studies are needed to determine the efficacy *in vivo* or clinical usefulness.

Of the independent sample extracts, dichloromethane ethylacetate and methanolic extracts showed good antibacterial activity against both bacterial strains (Table 3). On the other hand, the good antibacterial activity was recorded in *C. grattisimus* against *S. pneumoniae*. *C. grattisimus* extracts in this study leads to the conclusion that active constituents in these crude extracts are among the lipophilic (non-polar) group of compounds. Although hexane extracts contain more non-polar compounds than DCM, the two solvents generally extract non-polar classes of compounds compared to methanol, which extracts mostly polar compounds. DCM extracts showed good antibacterial activity.

The results indicated that the efficacious interaction effect may be dependent on the precise concentrations of certain compounds derived from the extracts. In spite of the fact that most plant-derived extracts have shown weak potency against pathogenic bacteria compared to antibiotics, plants almost always fight infections successfully in their natural environment. The aspect of a synergistic mechanism becomes the apparent strategy employed by plants. Generally, good antibacterial activity shown by extract yields and extracts of DCM and methanol in almost all samples in this study suggest that non-polar compounds interact more than the polar compounds. The trend is, however, consistent with most of the findings in other non-interaction studies, in which non-polar extracts demonstrated better antimicrobial activity than polar ones [16]. Considering the scarcity of plant extracts with good antimicrobial activity against Gram-negative bacteria in most of the previous studies with different plant materials [2,16] the aspect of synergistic mechanism becomes the apparent microbial therapeutic tool.

In light of the global threat by the emerging antibiotic resistant bacterial strains which cause infectious diseases, results of this study provides an insight into the potential sources of such remedies and further confirm the efficacy of medicinal plant extracts. Such a scenario, however, highlights the complexity of the interaction effects of suitable chemical compounds found within a plant. Chemotherapy is often employed in clinical practice for the treatment of infectious diseases. As previously reported, the good antibacterial activity demonstrated by the phenolic rich extracts of *C. grattisimus* corresponds with the high phenolic content previously identified [3,11]. Garlic (*Allium sativum*) and tea tree (*Melaleuca alternifolia*) are reported as broad-spectrum antimicrobial agents [11]. The oil from *Lantana camara* is reported to pose insecticidal repellent activities towards bees, mosquitoes and cattle fly [17]. The oil derived from *L. camara* also exhibits ovipositional and antimicrobial activities [17]. However, chances are high that the good activity shown by these extracts in this study may be as a result of these phenolic compounds.

Conclusion

This study reveals the characterization and structures of the non-volatile components of *C. grattisimus* extracts. NMR analyses showed that purified bioactive compounds were unstable on standing but appear to be stable in the unpurified form. Researching the ^{13}C-NMR rule of the triterpenoids is helpful for deducing structures of unknown analogs. The results of this study suggest that the *C. grattisimus* extracts possess both strong free radicals scavenging, antimicrobial activities and further studies are needed to determine its efficacy *in vivo* or clinical usefulness.

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