Isolation, Characterization, Structural Elucidation and Anti-Bacterial Activities of Roots Extracts of *Cucumis Ficifolius*

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Abstract

Cucumis ficifolius (family Cucurbitaceae) is traditionally used for the treatment of diarrhea, teeth-ach, wound, burns, bleeding from external cuts and against different infectious diseases. With the absence of previous scientific report on the roots of the plant, this study was focused on isolation of chemical constituents and evaluation of their anti-bacterial activities of the roots of *C. ficifolius*. The n-hexane, ethyl acetate and methanol extracts of the roots of *C. ficifolius* were investigated for their phytochemical constituents and the results revealed that C. ficifolius is rich in many secondary metabolites such as saponins, terpenoids, glycosides, and steroids. But tannins and alkaloids were not observed. The ethyl acetate extract was subjected to column chromatography over silica gel and furnished three compounds namely **compound-1**, **compound- 2** and **compound-3**. The structures of these compounds were characterized using FTIR, 1H-NMR, 13C-NMR and DEPT-135. The crude extracts and the isolated compounds were tested against four bacteria strains (Gram negative, *E. coli* and *p. aeruginosa*; Gram positive*, S. aureus* and *B. subtilis*) using Agar well diffusion method, the results showed that the methanol extracts and **compound-2** were active against all the tested bacteria. Accordingly, 12.34, 18.25 and 23.75 mm zone of inhibition against *B. subtilis* were found at the concentration of 20, 30 and 40 µg/mL MeOH extract respectively. Similarly 9.5, 13.25 and 17.25 mm zone of inhibition were found for the same bacteria strains and concentrations of **compound-2**. These results showed the increase of inhibition zone with concentration. However, the n-hexane crude extract was found not to have anti-bacterial activity towards the tested bacteria strains.

Introduction

Plants have been used to treat a wide range of diseases throughout the history of human beings and this practice continues to date. This is mainly because most of these herbals are accessible, affordable and the extracted chemicals have little or no side effects as compared to drugs synthesized in the laboratory. Plants comprise the largest component of the diverse therapeutic elements of traditional health care practices both in humans and animals. The medicinal values of plants are due to the chemical substances that produce a definite physiological action on human body and are called phytochemicals. They are chemicals extracted from plants and the term is often used to describe the large number of secondary metabolic compounds found in plants [1, 2]. Naturally occurring compounds may be divided into two broad categories. The first class of compounds is known as primary metabolites. They occur in all cells and play a central role in the metabolism and reproduction of those cells. Primary metabolites include the nucleic acids, the common amino acids, sugars and the high molecular weight polymeric materials such as cellulose, lignins and proteins which form the cellular structures. Most primary metabolites exert their biological effect within the cell or organism that is responsible for their production. The second class of compounds is secondary metabolites. Such compounds are characteristic of a limited range of species and occur in plants in a high structural diversity. The major classes of secondary metabolites include tannins, glycosides, flavonoids, alkaloids, terpenoids, steroids, quinones and

saponins are among others and play significant role in drug discovery. Secondary metabolites have often attracted interest of researchers because of their biological effect on other organisms [3, 4].

The biologically active constituents of medicinal, commercial and poisonous plants have been studied throughout the development of organic chemistry. Many of these compounds are secondary metabolites. Natural products often have an ecological role in regulating the interactions between plants, micro-organisms, insects and animals. They can be defensive substances, anti-feedants, and attractants. Natural products from plants remain vital in drug discovery where they can be used directly as drugs or serve as leads to new drugs by providing chemical entities [5]. The currently accepted modern medicines have gradually developed over the years by scientific and observational efforts of scientists. However, the basis of their development remains rooted in traditional medicine and therapies. The approach to new drugs through natural products has proved to be the single most successful strategy for the discovery of new drugs [6]. Natural products therefore, continue to play a crucial role in drug development as they account for almost 50% of new chemical entities in drug discovery and hence providing a starting point for new synthetic drugs. The use of medicinal plants thus finds its natural expression and further development in primary healthcare where in many cases they bridge the gap between the availability and the demand for essential drugs [3].

Cucumis ficifolius belonging to the *genus Cucumis* and *family Cucurbitaceae* is known locally in Ethiopia as “Holotoo” in Afan Oromo and “Yemidir Embuay” in Amharic. The *Cucumis* are medium-sized, botanically highly specialized genus mainly climbing, hairy and both wild and cultivated plants. It is well represented in the moist and moderately dry tropics of the World, particularly in grass and bush land areas of Africa including Ethiopia [7]. Some species occur in semi-desert or even desert vegetation. Any part of *C. ficifolius* if tasted or chewed has a bitter taste. *Cucumis* are used actively as traditional herbal remedies as anti-inflammatory, antitumor, hepato protective, cardio vascular and immuno regulatory activities. *Cucumis* are reported to possess purgatives and anti-helminthic properties due to the secondary metabolite *cucurbitacin* content [8-10]. In most countries of the world, the plant *Cucumis ficifolius* has been used for many years as a traditional medicine, leaves infusion is used to treat dysentery and diarrheic diseases. In Niger, the populations in the prospected zones used the fruits in the treatment of measles. The plant is also widely distributed in different regions of Ethiopia and is traditionally used in the treatment of diarrhea, teeth-ach, wound, skin infections, cancer, coughing, rabies in dogs, bad breath, TB, eye disease and against different varieties of diseases [11]. It has also seen that different parts of the plant are used for the treatment of different conditions this includes treatments of skin cancer, pulverized roots made as pastes/ointments are applied directly onto affected areas [12]. Local peoples or traditional healers usually grind the roots of *C. ficifolius* and tie on the affected body of the patient to treat wound and give order orally to chew fresh roots for those who have teeth-ach and stomach-ach. To treat rabies, powder of roots eaten with „Teff kita/ Or Crushed fresh root with water fermented for three days and taken with honey early morning before breakfast orally until cure [13, 14]. An extract of the plant, if administered to a cow that has just calved, will help remove the placenta quickly. The plant, like endod (*Phytolacca dodecandra, Phytolaccaceae*), is used to cause abortion in women, Pregnant women take powdered roots of the plant with coffee to remove the embryo and they

use the same procedure to remove the placenta quickly during birth. This prostrate herb is often seen in pastures and has variously colored attractive fruits. These fruits are often picked by small boys to play with like a ball. Fruits, if eaten, are highly poisonous, perhaps due to a bitter principle [12, 15]. Peoples also use the matured fruits of this plant to treat wound on fingers (they cut fresh fruits into two or three parts and tie on the affected areas). However, to the best of our knowledge there is no published scientific report on the isolation and anti-bacterial activities of the roots extracts of this plant in Ethiopia. So, since such medicinal herbs are widely distributed in different regions of Ethiopia and are traditionally used in the treatment of different varieties of bacterial diseases [7], the researcher took a big interest in conducting this research for chemical and anti-bacterial investigation of the roots extracts of the plant which could be important to generate adequate knowledge to the societies. The rapid development of multi-drug resistant strains of bacteria increased the occurrence of bacterial infections that cannot be treated with conventional anti-microbial agents. Due to this reason, scientists are always searching for the new generation anti-biotic drugs. But the new generation anti-biotics are less available and expensive for resource poor communities. The increasing rate of resistance of disease causing micro-organisms to conventional anti-biotics and the insufficient number of health facilities, results for the continuous search in the affordable, safe and effective herbal medicine.

Traditionally, people in Ethiopia have been used medicinal plants to treat different diseases and this has great contribution in primary health care systems. *C. ficifolius* is one of those ethnomedicinal plants that have been commonly visited by traditional healers in most parts of Ethiopia. The fruits and roots parts of the plant have been widely used by the local people for the treatment of different alignments including skin infections, wound, fever, ear-ach, stomach-ach, diarrhea, sexual disorder, blood clotting, nose bleeding, and hormonal disorder. While the seeds and fruits of *C. ficifolius* have been studied, to the best of our knowledge, there is no published report on isolation and characterization of chemical constituents and evaluation of biological activities of the roots extracts of this plant in Ethiopia. Therefore, the present study was focused on the isolation and identification of compounds from the roots extracts of *C. ficifolius* and evaluation of anti-bacterial activities.

The *Cucurbitaceae family*

The *family Cucurbitaceae* includes a large group of plants which are medicinally valuable. It comprises of about 130 *genera* and about 800 *species*. The family is predominantly distributed around the tropical Africa (Mauritania to Eritrea and Somalia, south to Tanzania and southern Congo), where edible fruits are grown. The diversity of the *cucurbitacins* activities, especially cytotoxicity and anti-feedants are a good evidence for further investigations. Recently, they were exploited for their anti-tumor properties, differential cytotoxicity toward renal, brain tumor, and melanoma cell lines, inhibition of cell adhesion. Phytochemical analysis of the plants belongs to *Cucurbitaceae family* showed the presence of various phytochemicals like tannins, cardiac glycosides, terpenoides, carbohydrates, resins, saponins, Carotenoids and phytosterols [10]. It is also known to contain several bioactive compounds such as triterpens, sterols and alkaloids [16].

The *Genus Cucumis*

Cucumis is a genus of twining, tendril-bearing plant (producing annual stems up to 2.5 meters long from a perennial rootstock) in the *Cucurbitaceae family* [7]. The name „*Cucumis*' is the Latin word for the "*cucumber"* which was already cultivated in Ancient Egypt. It is a genus of more than 52 species, indigenous mainly to tropical Africa, also Asia, Australia and some islands in the Pacific. A lot of works that have been done by the researchers throughout the world on various plants of this family indicated a number of *cucurbitacins* (triterpens like compounds) were isolated from *genus Cucumis* [16, 17].

Ethynobotancal information of *Cucumis ficifolius*

Cucumis are medium-sized, botanically highly specialized genus mainly climbing, hairy plants and well represented in the moist and moderately dry tropics of the world, particularly in grass and bush land areas of Africa. Some species occur in semi-desert or even desert vegetation [7].

Use in Ethnomedicine

Results obtained in the survey conducted by different researchers indicated that *Cucumis* were used by the people of various regions and tribes in the world. The diseases for which these plants were used includes bed wetting in children, bleeding from external cuts and wounds, burns, cancer, cholera, diabetes, ear disorders, eye disorders, fever, stomach ache, gastro-intestinal, goiter, heart disorders, hepatic disorders, infections, infertility, inflammation, malaria, menstrual disorders, mumps, paralysis, pox, respiratory tract disorders, skin disorders, sexual disorders, sexually transmitted diseases, sun stroke, tetanus, tuberculosis, typhoid, and vomiting [17]. According to reported by researchers, the whole plant parts of Bitter gourd or bitter melon *Cucurbitaceae* family are used in the treatment of malaria in the south-western regions of Nigeria. The plant is reported to possess anti-fungal, anti-inflammatory, anti-parasitic and act as a digestive stimulant [18]. Different researchers were also reported on pharmacological evaluation of wound healing potential of *Cucumis sativus*. They concluded that aqueous extracts of *Cucumis sativus* have good effect on wound healing [19]. Reports from many researchers revealed that regular intake of cucumber fruit promote healthy hair growth. It is useful in skin problems, sunburn and also for curing swelling under the eye. Its juice is also efficient to soften the skin texture. Placing the two slice of cucumber on eyes for 10 minutes can decrease the inflammation and cure skin infection significantly. Its fruit is also considered traditionally for weight loss, intestinal worms and tapeworms. Leaves are boiled, mixed with cumin seeds, roasted, powdered and administrated in throat infections in the doses of 30 grams. Due to elevated content of potassium (50-80 mg/100g), cucumber can also significantly be helpful for blood pressure [20-23].

Biological Activities

According to reported from different researchers the anti-microbial activity of seeds extract of *Cucumis sativa (Cucumber), citrullus fistulosus (Tinda) and Cucurbita pepo* revealed that all the seeds extracts were active against *Escherichia coli, Streptococcus thermophilous, Fusariumoxy sporium, and*

Trichodermareesei [10]. Studies on the anti-fungal activities of the ethanol extract of *Cucumis sativus* showed that the ethanol extracts of *Cucumis sativus* were effective against six fungi after diameter of zone inhibition were compared with the activity of the standard drug [24].

The ethanolic extracts of leaf of *M. charantia, L. cylindrical and (Cucurbitaceae family)* also showed anti-microbial activity on the test human pathogenic micro-organisms used [25]. It was reported that fruit of *Cucumis melo* is useful in chronic eczema and medicinally used to promote skin hydration, to treat light burns and scrapes or wounds [21]. Dose dependent cytotoxic activities were exhibited by aqueous fruit extract of *Cucumis melo* in human prostate carcinoma cells. As the dose of the extract increased, the number of viable cells decreased. This shows the anti-cancer and cytotoxic potential of the fruit of *C.melo* [26]. The fruit is tonic, laxative, diuretic diaphoretic and galactagogue. The flowers are expectorant and induce vomiting. The seeds are used as cough suppressant, fever reducer, and a digestive aid. A seed powder is mixed with water and used as a vermifuge [21]. The fruit extract has a high Superoxide Dismutase Activity (SOD). The SOD activity is responsible for the in-vitro and anti-inflammatory properties of the extract [27]. It also reported that one of the compounds that isolated from *Cucumis melo* has therapeutic potential and a possible effect treatment for varieties of inflammation-mediated diseases due to the *cucurbitacin E* content. Therapeutic potential of *cucurbitacin E* could be limited by the bitter taste of this compound but special pharmaceutical formulation is highly recommended to overcome this issue. As different reports showed that the cytotoxicity of chloroform seeds extract and isolated compounds (cucurbitacin B) of *C. prophetarum*, towards human cancer cell lines, tested on mouse embryonic fibroblast and showed a positive result. *Cucurbitacin B* is most widely used for in-vitro studies on tumor inhibition [28].

Accumulated evidences have shown that *cucurbitacin B inhibits* the growth of numerous human cancer cell lines and tumor xenografts, including breast, prostate, lung, uterine cervix, liver, skin, and brain cancers. Combination therapy with multiple drugs is a common practice in the treatment of cancer to get an additive or synergistic effect and to reduce toxicity to the host [29, 30]. Anti-microbial activity tests on compounds reported from *C. maxima* (spinasterol and 24- ethyl-5α-cholesta-7, 22, 25-trien-3β-ol) indicated that it was slightly active against the fungi (*Aspergillus niger and Candida albicans*) and the bacteria (*Bacillus subtilis and Pseudomonas aeruginosa*). It was inactive against *Escherichia coli, Staphylococcus aureus, and Trichophyton mentagrophytes* [25]. *Cucurbitacins* and their derivatives are triterpenoids found in medicinal plants mostly in the *Cucurbitaceae family*, known for their diverse pharmacological and biological activities, including anti-cancer effects, throughout human history. Although initial attention *to cucurbitacin* as a potential anti-cancer drug withered for decades, recent discoveries showing that *cucurbitacin* is a strong cancer inhibitor and reclaimed the attention of the drug industry one more time. There is an increasing evidence showing that some *cucurbitacins* not only inhibiting disease causing pathogens but also affect other signaling pathways. Moreover, some reports have shown the synergistic effect of *cucurbitacins* with known chemotherapeutic agents, such as doxorubicin and gemcitabine [31]. Even though, the plant *C. ficifolius* has been used traditionally for the treatment of different alignments including bacterial infections, the phytochemical pertaining of this plant and biological activities have not been well addressed. Especially the roots part of the plant is not

yet in focus. Therefore, the present study was focused to identify secondary metabolites from roots of *C. ficifolius* of n-hexane, ethyl acetate and methanol crude extracts and characterized three compounds (*α-spinasterol, cucurbitacin B and cucurbitacin D*) from EtOAc extract and evaluated their anti-bacterial activities.

Phytochemistry

Cucumis sativus

C. sativus is a widely cultivated plant in the gourd genus, *Cucumis*. It is a creeping vine bearing cylindrical fruits that used as culinary vegetables. The yellow solid compound which was soluble in hot water and organic solvents but insoluble in cold water has been identified from flowers of *C. sativus* (5,7-di-O-methyl luteolin-6-C-(3□□-O-benzoyl)-β dxyloside) (**1**) [21].

Cucurbita maxima

C. maxima (Cucurbitaceae family) commonly known as squash is widely used as vegetable and a source of vitamin A, iron, phosphorus, and calcium. A recent study reported that biologically active compounds α-spinasterol (24-ethyl-5α-cholesta-7, trans, 22 -dien-3β-ol) (**2**) and 24-ethyl-

5α-cholesta-7, 22, 25-trien-3β-ol (**3**) were isolated from the flowers of *C. maxima* showed potential anti-carcinogenic and anti- genotoxic effect [25].

Cucumis prophetarum

Different reports showed that terpenoid like substances called Cucurbitacins are the major compounds of *Cucurbitaceae family* [16]. The phytochemical investigation of the fruits of C. prophetarum L., belongs to the *family Cucurbitaceae*, wild plant growing in the desert of Makah, 80 km from Jeddah Saudi Arabia, (Chloroform:Methanol 1:1) extract had been fractionated using different chromatographic techniques, led to purification of two cucurbitacin derivatives;

dihydocucurbitacin B (**4**) and cucurbitacin (**5**) [28].

Other cucurbitacin derivatives, Hexanorcucubitacin D (**6**) **,** cucurbitacin E (**7**), cucurbitacin J (**8**)**,** cucurbitacin K (**9**) , cucurbitacin A (**10**) , cucurbitacin C (**11**) , cucurbitacin I (**12**) and cucurbitacin L (**13**) were also reported from the fruits of *Cucumis melo* [32].

Materials And Methods

Apparatus and Instruments

The apparatus and instruments that were used for the study include column chromatography, UV chamber (254 and 365 nm), Rotary evaporator, and TLC plat (aluminum coated as stationary phase). FTIR was performed in KBr cubet, wave number (400-4000) measured in cm-1 and Intensity (%T), 1H-

NMR, 13C-NMR and DEPT-135 experiments were performed at laboratory of Chemistry, Chemistry Department, Addis Ababa University, Addis Ababa on a spectrometer machine operating at 400 MHz.

Chemicals and Reagents

The chemicals and reagents that were used in the study include: solvents n-hexane (99%), ethyl acetate ((97%) and methanol (97%) all with analytical grades), Anhydrous sodium sulphate, iodine vapor, $FeCl_3$ (British drug house ltd., England), DMSO, HCl, H_2SO_4 (98%), NaOH, NH_3,

Ethanol (97%) and acetic anhydride (Baker chemical co., USA), Silica gel (250-400 mesh, ASTM, Germany).For NMR measurement (recording), the samples were dissolved in deuterated chloroform ($CDCl_3$). Tetramethylsilane (TMS) was used as reference having chemical shift of ppm. Chemical shifts were given in ppm.

Collection and identification of the plant material

The plant material

The fresh root samples of *C. ficifolius* were collected from Hulul village, Robe Woreda, Arsi zone, Oromia, Ethiopia. The plant was authenticated by the botanist Shambel Alemu at Addis Ababa University, Addis Ababa, with voucher specimen number GW 006 and deposited in the National Herbarium, Department of Biology, Addis Ababa University, Addis Ababa, Ethiopia.

Extraction and Isolation

Extraction

Powdered roots of *C. ficifolius* (300 g) were successively extracted using maceration technique and solvents, n-hexane (1.5 L), EtOAc (1.5 L) and MeOH (1.5 L) at room temperature for 72hrs.

Each extract was filtered using Whattman No. 1 filter paper (180 mm) and concentrated under reduced pressure at $40^{o}C$ using rotary evaporator. The resulting solid extracts were stored in refrigerator at $4^{O}C$ until required for further use.

Preliminary Phytochemical Screening

The n-hexane, EtOAc and MeOH extracts were used for screening of phytochemicals such as tannins, saponins, alkaloids, terpenoids, flavonoids, glycosides, steroids, proteins, and phenols). Literature reported specific procedures were used to carry out specific tests for each class of secondary metabolites. i.e. saponins (Rosenthaler test, foam test), glycosides (Keller-Kiliani test), sterols and terpenes (Liebermann-Buchnard), alkaloids (Wagner, Meyer test), tannins (gelatin test), flavonoids (NaOH 10% test) and phenol compounds (ferric chloride test) [33-35].

Test for Tannins

About 0.5 g of the crude extracts were boiled in 20 mL of water in a test tube and then filtered. A few drops of 0.1% ferric chloride solution were added and observed for brown-green color.

Test for Saponins

Froth Test: Small amount of the sample were diluted with distilled water to 20 mL and shaken in a graduated cylinder for 15minutes, 1 cm layer of foam was observed.

Test for Alkaloids

0.5 g of the extracts were evaporated and the residue was heated on a boiling water bath with 2 N

HCl (5mL). After cooling, the mixture was filtered and the filtrate was divided into two equal portions. One portion was treated with a few drops of Mayer's reagent and the other with equal amounts of Wagner's reagent. The formation of precipitation was indicator for alkaloids.

Test for Terpinoids

5 mL of the dissolved extracts were added to 2 mL of $CHCl_3$ and 3 mL of conc. H_2SO_4 to form a monomer of red-brown color of the interface which indicates the presence of terpinoids.

Test for Flavonoids

5 mL of 10% dilute ammonia solution were added to a portion of the aqueous filtrate of the plant extract, followed by addition of conc. H_2SO_4 Formation of yellow color in the extract indicates the presence of flavonoids.

Test for Phenols

Ferric Chloride Test: Extracts were treated with 4 drops of ferric chloride solution. Formation of blue-black color indicates the presence of phenols.

Test for Glycosides

A small amount of the extracts were dissolved in 1 mL water and then aqueous Sodium hydroxide was added. Formation of a yellow color indicates the presence of glycosides.

Test for Cardiac Glycosides

Keller-Killani Test: To 2 mL of the extract, glacial acetic acid, one drop of 5% Ferric chloride and concentrated sulfuric acid was added. Appearance of red-brown color at the junction of the liquid layers indicates the presence of cardiac glycosides.

Test for Phytosterols

Liebermann Buchnard test: 0.5 mL of the extracts were treated with equal amount of CHCl3 and filtered. The filtrates were again treated with drops of acetic anhydride, boiled, cooled and

H_2SO_4 (conc.) was added to the mixture. The formation of brown ring at the junction indicates the presence of phytosterols.

Isolation and characterization of compounds

The EtOAc extract (5.24 g) of the roots of *C. ficifolius* was adsorbed on equal amounts of silica gel and subjected to silica gel (160 g) column chromatography. Elution was carried out with increasing polarities (5% increament as eluents) of n-hexane, EtOAc and methanol (100% nhexane, 95:5 n-Hexane: EtOAc, 90:10 n-Hexane: EtOAc, 85:15 n-Hexane: EtOAc and the experiment was stopped at 90:10 EtOAc:MeOH. A total of 96 fractions were collected and purity of each fraction was monitored using TLC. The spots on the TLC plate were visualized under

UV chamber (254 and 365 nm). TLC was developed in mobile phases containing n-Hexane and EtOAc by gradually increasing the polarity ratio (n-H:EtOAc) of the solvent system as (10:0, 9:1, 8:2, 7:3… up to 0:10) and or EtOAc:MeOH (100:0%, 90:10, 80:20, 70:30… up to 0:100%) to choose the appropriate solvent for the complete resolution of the spots. Spots those invisible under UV lamp were visualized with vanillin reagent (TLC plates were visualized by spraying with vanillin-H2SO4, then warming on a hot plate). The retention factor (Rf) values of all the spots were then determined in different solvent systems. Fractions of the same Rf values (fraction 25, 26 and 27) were mixed together, fraction 78 and 92 each has a single spots with different Rf values and finally, FTIR and one dimensional NMR (1H NMR, 13C NMR and DEPT-135) spectroscopic techniques were used to characterize the three isolated compounds.

Table 1. Summary of isolation of compounds from roots extracts of *C. ficifolius* using CC.

Crude Extract	Fraction	Eluent System	Under UV lamp	In Vanillin Reagent	Rf	Compound
EtOAc	25-27 (Combined)	n-H:EtOAc 85:15	Invisible	n-H:EtOAC 7:3, purple spot	0.45	1
„	78	n-H:EtOAc 6:4	Visible at 254 nm	n-H:EtOAc 2:8, Reddish spot	0.65	2
„	92	n-H:EtOAc 2:8	Visible at 254 nm	n-H:EtOAc 1:9, blue-black s	0.76	3

Anti-bacterial Activities

Anti-bacterial activities of the crude extracts and isolated compounds were investigated using Agar well diffusion method against four test bacteria strains (two gram-positive test bacteria; *S. aureus (ATCC 25923)* and *B. Subtilis* (ATCC 6633) and two gram-negative bacteria; *E. coli (ATCC 25922)* and *P. aeruginosa (ATCC 7553)*) obtained from laboratory of Biology Department, ASTU. The bacterial suspension was spread uniformly on the agar surface. Agar surface was perforated with 6 mm diameter holes, aseptically cut and filled with 100 µL in different concentrations of the extracts and isolated compounds. DMSO was used as a solvent and negative control while Chloroamphinicol was used as the positive control. The plates were incubated at 37^{O}C for 24hrs and then examined to verify inhibitions. A positive result was defined as inhibition zone of 9 mm or more around the holes [36]. Experiments were performed in triplicate (varying concentration of the samples 20, 30, and 40 µg/mL) and the developed inhibition zones were compared with those of reference.

Results And Discussion

Extract Yield

Powdered roots of *C. ficifolius* was extracted successively using solvents n-hexane, EtOAc and methanol and yielded a colorless crude extract (3.36 g, 1.12% w/w) of n-hexane, dark-reddish

crude extract(5.24 g, 1.75% w/w) of EtOAc and dark crude extract(12.79 g, 4.26% w/w) of methanol. From these results one can deduce that more polar compounds are found in the plant than non-polar ones as the percentage yields increase with polarity.

Phytochemical Screening

The phytochemical analysis of each crude extract of *C. ficifolius* revealed the presence of pharmacologically useful classes of secondary metabolites such as saponins, flavonoids, terpenoids, glycosides, steroids, phenols, and the absence of tannins, alkaloids reducing sugars and proteins (Table 2).

Table 2. Phytochemical constituents of the roots extracts of *C. ficifolius*.

Secondary metabolites	Crude extracts		
	n-hexane	EtOAc	Methanol
Tannins	–	–	–
Saponins	+	+	+
Alkaloids	–	–	–
Terpenoids	+	+	+
Flavonoids	+	+	+
Phenols	+	+	+
Glycosides	+	+	+
Cardiac glycosides	+	+	+
Phytosterols	+	+	+

Key (+) presences (-) absences

Characterization of Compounds

In the course of this work, three compounds were isolated from the EtOAc roots extracts of *Cucumis ficifolius*. Here in, is the detailed characterization of these compounds.

Compound-1

Compound-1 was isolated from the combined fractions (25-27). It was a white crystalline solid (30 mg) with a melting point and Rf value of 171-174oC and 0.45, respectively. It was soluble in chloroform, in the TLC a defined purple spot was revealed with vanillin reagent (7:3 n- Hexane:EtOAc).

In the FTIR spectrum of compound-1 (Appendix 1.1) the broad absorption band at 3432 cm^{-1} showed O-H stretching that indicates the presence of hydroxyl group. The strong absorption band at 2953 cm^{-1} showed the presence of the C-H stretching of alkanes. The weak absorption band at 1647 cm^{-1} showed C=C stretching in olefins. The medium absorption band at 1465 cm^{-1} could be attributed to the presence of –CH2 bending in alkanes. The medium absorption band at 1385 cm-1 is due to the -CH3 bending. The absorption bands at 1158 cm-1 and 1054 cm-1 showed the –C-O- stretch of alcohols. On the other hand, the medium absorption band at 963 cm-1 showed the presence of =CH bending in alkenes. So, from the FTIR data in the description, compound-**1** has hydroxyl, alkene and alkane functional groups. The 1H-NMR spectrum of Compound-1 (Table 3, Appendix 1.2) indicated for three olefinic protons at *δ*H 5.18 (1H, *m*), *δH* 5.08 (1H, *dd, J* = 4, 8 Hz), and at *δ*H 5.04 (1H, *dd, J =* 4, 8 Hz), a carbinyl proton at *δ*H 3.62 (1H, *m*,) and six methyl protons at *δ*H 1.06 (3H, *d*, *J* = 8 Hz), *δH* 0.57 (3H, *d, J* = 4 Hz), *δ*H 0.57 (3H, *d, J* = 4 Hz),

*δ*H 0.84 (3H, *t*, *J* = 4 Hz), *δH* 0.82 (3H, *s*) and *δH* 0.82 (3H, *s*). The 13C-NMRspectrum (Table 3, Appendix 1.3) of Compound-1 revealed four olefinic carbons at δC 117.5, 139.6, 138.2, 129.5, and a carbinyl carbon at δC of 71.1. The DEPT-135 spectral data also showed two quaternary carbons at the δC 34.3 and 43.3 the peak at a δC 139.6 is also quaternary carbon(not observed in DEPT) but was taken as olefinic carbon), nine methylene carbons (-CH_2 pointing down) at δC 37.18, 31.5, 38.0, 29.7, 21.6, 39.5, 23.1, 28.5 and 25.4. The results of the 1H-NMR, 13C-NMR and DEPT-135 of compound-1 were compared with the spectral data of α- spinasterol reported literature for the same compound and showed good agreement [25, 37]. Hence, from the comparison (Table 3), compound-1 was identified as the sterol compound, α-spinasterol.

Table 3. The spectral data of compound-1 along with literature reported for α-spinasterol [25]

Spectral data of Compound-1			The literature data of α-spinasterol	
Position	δC	δH, multiplicity, J(Hz)	δC	δH, multiplicity, J(Hz)
1	37.2	1.10, 2H, m	37.2	1.02, 2H
2	31.5	1.27, 2H, m	31.5	1.36, 2H
3	71.1	3.61, 1H, 1H, m,	71.1	3.52,1H, m
4	38.0	1.27, 2H, m	38.1	1.27, 1.70,2H
5	40.3	1.40, 1H, m	40.3	1.4, 1H
6	29.7	1.77, 2H	29.7	1.22, 1.74,1H
7	117.5	5.18, 1H, m	117.5	5.15, 1H,br. s
8	139.6	q	139.6	q
9	49.5	1.55 , 1H, dd, J = 4.0, 8,0	49.5	1.66, 1H
10	34.1	q	34.2	q
11	21.6	1.51 , 2H, m	21.6	1.48, 2H
12	39.5	0.96, 2H, dd, dd, J = 4.0, 8.0	39.5	1.23, 2H
13	43.3	q	43.3	q
14	55.2	1.79, 1H, t, J = 2.8	55.1	1.81, 1H, t
15	23.1	1.43, 2H, m	23.1	1.40, 1.52, 2H
16	28.5	1.27, 2H	28.5	1.25, 2H
17	55.9	1.58, 1H, m	55.9	1.5, 1H
18	12.1	0.82, 3H, s	12.1	0.55, 3H, s
19	13.1	0.82, 3H, s	13.1	0.8, 3H, s
20	40.8	2.04, 1H, m	40.8	2.05, 1H, m
21	21.4	1.06, 3H, d, J = 8.0	21.4	1.02, 3H
22	138.2	5.08, 1H, dd, J = 4.0, 8.0	138.2	5.17, 1H, dd
23	129.5	5.04, 1H, dd, J = 4.0, 8.0	129.5	5.09, 1H, dd
24	51.3	1.58,1H, m	51.3	1.55, 1H
25	31.9	1.40, 1H, m	31.9	1.55, 1H

26	21.1	0.57, 3H, d, J = 8.0	21.1	0.85, 3H, d
27	20.9	0.57, 3H, d, J = 8.0	20.9	0.84, 3H, d
28	25.4	1.13, 2H , m	25.4	1.18, 1.42, 2H
29	11.9	0.84, 3H, t	12.2	0.81, 3H, t

Reported biological activity of this compound showed that it was slightly active against the gram

+ve bacteria B. subtilis and gram -ve bacteria P. aeruginosa [25],which was also agreed with the anti-bacterial activity of this work (Table 6).

Compound-2

Compound-2 (25.5 mg) was isolated from fraction 78. It was a light-orange solid (Figure 12 a) with melting point of 184-186^{O}C. It displayed spot with n-Hexane:EtOAc (3:2) as well as UV active at 254 nm and Rf of 0.65. In the FTIR spectrum of compound-2 (Appendix 2.1)**,** the broad absorption band at 3453 cm^{-1} showed O-H stretching that indicated the presence of hydroxyl group. The sharp and strong absorption band at 2924 cm^{-1} showed the presence of the C-H stretching of alkanes. The strong band at 1725 cm^{-1} showed the conjugation of -C=O stretching for esters. The medium absorption band at 1710 cm^{-1} showed the presence of –C=O bond in ketones. The medium absorption band at 1628 cm-1 is due to the- C=C for alkenes. On the other hand, the sharp absorption band at 1258 cm^{-1} showed the presence of -C-O- stretching bonds of alcohols. Similarly, the medium absorption band at 1130 cm^{-1} indicates the presence of C-O bond in esters. So, from the FTIR data compound-2 has hydroxyl, ketones, esters, alkene and alkane functional groups. The 1HNMR spectrum of compound-2 (Table 4, Appendix 2.2) revealed three olefinic protons at δH 7.09 (1H, *d, J* =16.0), 6.51 (1H, *d, J =* 16.0) and 5.79 (1H, *t, J* = 4.0), two protons on oxygenated carbons at the δH 4.37 (1H, *m, J =* 8.0) and 4.23 (1H, *m, J* = 4). The 13C-NMR spectrum (Table 4, Appendix 2.3) deduced a carbonyl group appeared at δC 213.0, 212.2 and 202.5 are ketone and at δC 170.3 is ester. Four signals appeared at δC 140.4, 120.4, 120.5 and 151.9 showed the carbon carbon double bonds (olefins), three peaks appeared at δC 71.7, 71.29, 78.3 (Table 4) represents a carbon atom connected with hydroxyl group. The DEPT-135 spectrum (Appendix 2.4) analysis also showed eleven quaternary carbons at the δC 213.0, 50.3, 140.4, 48.5, 212.2, 50.7, 48.7, 78.3, 202.5, 79.4 and 170.3. The four peaks pointing down at δC 36.0, 23.9, 48.6 and 45.3 showed the presence of four CH_2 groups. Comparing the spectral data of compound-2 with reported data of cucurbitacin B indicated that compound-2 was identified as one of the cucurbitacin group, the cucurbitacin B [28].

Table 4. The spectral data of compound-2 and literature reported NMR data of Cucurbitacin B.

Spectral data of Compound-2			The literature data of Cucurbitacin B	
Position	δ*C*	δH, multiplicity, J(Hz)	δ*C*	δH, multiplicity, J(Hz)
1	36.0	1.99, 2H, ddd , J = 4.0	35.81	2.30, ddd, 13.0, 6.0, 3.0
2	71.7	4.37, 1H, m, J = 8.0,	71.5	4.42, dd, 13.0, 6.0
3	213.0	q	213.9	q
4	50.3	q	50.1	q
5	140.4	q	140.3	q
6	120.4	5.79, 1H, t, J = 4.0	120.3	5.79, t, 5.5
7	21.9	2.32, 2H, m J = 8.0	23.7	2.38, ddd, 15.5, 5.5, 2.5
8	42.4	2.32, 1H, m, J = 8.0	42.1	2.00, dd,7.5, 2.5
9	48.1	q	48.2	q
10	33.8	2.77, 1H, d, J = 16	33.5	2.75, d, 13.0
11	212.2	q	212.9	q
12	48.5	a)3.27, 1H, d, J = 12.0 b)2.71, 1H, d, J = 12.0	48.5	a)3.27, d, 14.5 b)2.68, d, 14.5
13	50.7	q	50.5	q
14	48.7	q	48.1	q
15	45.3	a)1.37, 1H, dd, J = 8.0, 13.0 b)1.29, 1H, dd, J = 8.0, 13.0	45.3	a)1.87, dd, 13.0, 8.0 b)1.49, dd, 13.0, 8.0
16	71.3	4.24, 1H, m, J = 4.0	70.8	4.35, q, 8.0
17	58.2	2.52, 1H, d, J = 8.0	57.6	2.57, d, 8
18	19.9	0.99, 3H, s	19.6	0.97, s
19	20.1	1.09, 3H, s	19.8	1.07, s
20	78.3	q	78.8	q
21	24.0	1.09, 3H, s	24.2	1.34, s
22	202.5	q	202.0	q
23	120.5	7.09, 1H, d, J = 16	120.2	7.05, d, 16
24	151.9	6.51, 1H, d, J = 16	151.8	6.48, d, 16
25	79.4	q	81.8	q

26	26.0	1.45, 3H, s	25.6	1.55, s
27	26.4	1.56, 3H, s	26.0	1.57, s
28	29.7	1.27, 3H, s	29.2	1.28, s
29	21.3	1.27, 3H, s	21.1	1.36, s
30	18.9	1.58, 3H, s	18.6	1.44, s
AC	170.3	q	170.3	q

Recently, these compounds were exploited for their differential cytotoxicity toward renal, brain tumor, inhibition of cell adhesion [28].

Compound-3

Compound-3 (26.5 mg) was obtained from fraction 92 as a colorless crystalline solid with a melting point and Rf of 190-193^{O}C and 0.76, respectively. In the FTIR spectrum of compound-3 (Appendix 3.1) the broad absorption band at 3437 cm^{-1} showed OH stretching that indicated the presence of hydroxyl group. The sharp and strong absorption band at 2929 cm^{-1} showed the presence of the C-H stretching of alkanes. The strong peak at 1701 cm^{-1} showed C=O stretching for ketones. The medium absorption band at 1710 cm^{-1} showed the presence of C=O bond of ketones. The medium absorption band at 1627 cm^{-1} is due to the C=C for alkenes. On the other hand, the medium absorption band at 1266 cm^{-1} showed the presence of C-OH stretching of alcohols. Similarly, the medium absorption band at 1093 cm^{-1} indicates the presence of C-H bending. From this, it is possible to presume compound-3 contains alcohols, ketones, alkenes and alkanes functional groups. The 1H-NMR spectrum of compound-3 (Table 5, Appendix 3.2) revealed three olefinic protons at δH 7.15 (1H, *d, J* = 16.0, H-23), 6.68 (1H, *d, J* = 16.0, H-24) and 5.80 (1H, *t*, H-6), two carbonyl protons at the δH 4.41 (1H, *m,* H-2) and 4.16 (1H, *m,* H-16). The 13C-NMR spectrum of compound-3 (Table 5, Appendix 3.3) revealed a carbonyl groups appeared at δC 213.0 (C-3), 212.8 (C-11), and 202.5 (C-22) are ketone functional groups. Four signals appeared at δC 140.5 (C-5), 119.1 (C-6), 120.3 (C-23) and 155.7 (C-24) showed the presence of carbon carbon double bonds (olefinic protons), three peaks appeared at δC 71.7 (C-2), 71.3 (C-16), 78.3 (C-20) represents a carbon atom connected with hydroxyl group. The DEPT-135 spectral analysis also showed the presence of eleven quaternary carbons at the δC 213.0 (C-3), 50.8 (C-4), 140.5 (C-5), 48.5 (C-9), 212.8 (C-11), 50.3 (C-13), 48.1 (C-14), 78.3 (C-20), 202.5 (C-22) and 79.4 (C-25). The four peaks pointing down at δC 36.0 (C-1), 23.9 (C-7), 48.7 (C-12) and 45.5 (C-15) showed the presence of four methylene (-CH2) groups in the structure. Comparing the results of the 1HNMR, 13C-NMR and DEPT-135 of compound-**3** with the spectral data of compound-2 (Table 5), results showed good agreement except that compound-**2** contain esters functional groups (Ac) at δC 170.3 (C-25). Hence, compound-**3** was identified as another cucurbitacin group of compound, the cucurbitacin D [28]

Table 5. Comparison of 1H-NMR, 13C-NMR data of Compound-3 with Compound-**2.**

Spectral data of Compound-3			Spectral data of Compound-2	
Position	δC	δH, multiplicity, J(Hz)	δC	δH, multiplicity, J(Hz)
1	36.0	2.32, 2H, m	36.0	1.99, 2H, ddd,
2	71.7	4.46, 1H, m, J = 8.0	71.7	4.46,1H, dd, J = 8,16
3	213.0	q	213.0	q
4	50.3	q	50.3	q
5	140.5	q	140.4	q
6	119.8	5.80, 1H, t, J = 4.0	120.5	5.80, 1H, t, J = 8.0
7	23.9	2.72, 2H, ddd, J = 2.8,12	23.9	2.68, 2H, ddd, J = 2,8,12
8	42.4	2.00, 1H, dd, J = 7.5,15.5	42.4	1.91,1H, dd, J = 7.5,13
9	48.4	q	48.5	q
10	33.8	2.97, 1H, m, J = 8	33.8	2.77,1H, d, J = 8
11	212.3	q	212.2	q
12	48.7	a)3.31, 1H, d, J = 12 b)2.58, 1H, d, J = 12	48.7	a)3.27,1H, d, J = 12 b)2.71,1H, d, J = 12
13	50.8	q	50.7	q
14	48.3	q	48.1	q
15	45.5	a)1.57, 1H, dd, J = 8,13 b)1.44, 1H, dd, J = 8,13	45.4	a)1.37,1H, dd, J = 8,13 b)1.29, 1H, dd, J = 8,13
16	71.1	4.16,1H,m, J = 8.0	71.3	4.23, 1H, q, J = 4
17	57.8	2.58, 1H, d, J = 8	58.2	2.52, 1H, d, J = 8
18	21.2	0.98, 3H, s	20.1	0.99, 3H, s
19	19.2	1.08, 3H, s	19.9	1.09, 3H, s
20	78.2	q	78.3	q
21	24.5	1.37, 3H, s	25.0	1.37, 3H, s
22	202.8	q	202.5	q
23	120.3	7.15, 1H, d, J = 16	120.5	7.09, 1H, d, J = 16
24	155.7	6.6, 1H, d, J = 16	151.9	6.51, 1H, d, J = 16
25	70.2	q	79.4	q

26	24.7	1.41, 3H, s	26.0	1.45, 3H, s
27	28.7	1.34, 3H, s	26.4	1.56, 3H, s
28	29.7	1.26, 3H, s	29.7	1.27, 3H, s
29	22.3	1.26, 3H, s	21.3	1.27, 3H, s
30	18.9	1.34, 3H, s	18.9	1.58, 3H, s

Based on the given evidences the structure of compounds-2 and 3 was determined to be cucurbitacin like triterpens.

Anti-bacterial Activities

The anti-bacterial activities of roots extracts of *C. ficifolius*, were investigated against *S. aureus* (ATCC 25923) and *B. subtilis (ATCC 6633)* (gram +ve) and *E. coli (ATCC 25922)* and *P. aeruginosa (ATCC 7553)* (gram -ve) by testing the three crude extracts (n-hexane, ethyl acetate and methanol), respectively, and the isolated compounds, compound-1, Compound-2, and Compound-3 using Agar-well diffusion methods. Both group of samples showed anti-bacterial inhibitory activities at different concentrations (20, 30 and 40 µg/mL), most prominent with the methanol crude extract and negligible inhibition diameter zone of the n- hexane extract. The negligible inhibition zone of anti-bacterial activities of n-Hexane extract may be due to its low polarity.

Table 6. Growth inhibition zone of the four bacteria strains using Agar- well diffusion method in the crude extracts and isolated compounds of *C. ficifolius.*

Samples	Conc.	Inhibition zone in mm (mean±SD)			
		Gram positive bacteria		Gram negative bacteria	
		B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*
EtOAc crude extract	20	8.0 ± 0.5	7.0 ± 0.71	_	_
	30	13.2 ± 0.02	10.0 ± 0.1	7.2 ± 0.01	11.5 ± 0.05
	40	15.1 ± 0.57	12.2 ± 0.35	11.1 ± 0.72	13.5 ± 0.56
MeOH crude extract	20	12.4 ± 0.75	15.5 ± 0.72	13.2 ± 0.72	7.9 ± 0.25
	30	18.5 ± 0.02	20.5 ± 0.04	16.1 ± 0.01	6.3 ± 0.07
	40	23.7 ± 0.72	23.5 ± 0.35	18.5 ± 0.71	10.1 ± 0.73
Compound-1	30	10.4 ± 0.03	7.2 ± 0.02	7.7 ± 0.02	8.2 ± 0.03
	40	13.1 ± 0.26	11.5 ± 0.73	12.7 ± 0.55	12.6 ± 0.77
Compound-2	20	9.6 ± 0.74	10.5 ± 0.36	9.7 ± 0.80	7.0 ± 0.45
	30	13.5 ± 0.01	13.2 ± 0.04	12.5 ± 0.05	13.5 ± 0.03
	40	17.2 ± 0.75	15.2 ± 0.50	12.9 ± 0.71	14.6 ± 0.82
Compound-3	20	9.5 ± 0.76	8.9 ± 0.35	_	_
	30	13.5 ± 0.05	12.5 ± 0.01	7.2 ± 0.03	10.4 ± 0.06
	40	14.5 ± 0.73	15.5 ± 0.72	10.3 ± 0.99	11.5 ± 0.7
Chloramphenicol	20	19.4 ± 0.3	18.7 ± 0.25	20.2 ± 0.32	19.6 ± 0.4

Key (-) not detected, the mean values at each concentration were obtained by measuring the inhibition zone three times in different directions around the hole.

Anti-bacterial inhibition zone of any sample can be expressed in terms of the diameter of the inhibition zone, less than 9 mm, inactive, 9-12 mm, partially active, 13-18 mm, active and greater than18 mm, very active [36]. Accordingly, the methanol crude extract displayed inhibition zone of 23.5 and 23.75 mm against S. *aureus* and *B. subtilis* at 40 µg/mL respectively and hence categorized as very active. On the other hand compound-2 was also active with inhibition zone of 15.12 and 17.25 mm respectively against the same bacterial strains and concentration. These result revealed that Gram-positive bacteria were more susceptible than Gram-negative bacteria because it is considered less resistance. This observation is consistent with previous reports [38]. The result also showed an agreement with the result of anti-bacterial activities of sterol compounds reported from *C. maxima* [25]. The anti-bacterial investigation of this work revealed the anti-bacterial activities of the crude extracts and the isolated compounds increase

with concentration. The anti-bacterial activities of crude extracts and isolated compounds from *C. ficifolius may* be due to the presence of cucurbitacins [28, 39]. Therefore, chemical constituents of *C. ficifolius* are valuable in synthetic anti-bacterial agents in the future. Studies should also be extended to evaluating the practical effectiveness of these compounds against the growth of different health harming bacteria. This study also suggests that the methanol extracts and compounds of *C. ficifolius* can be used in the control of bacterial transmitting diseases because their anti-bacterial activities were comparable with the reference. The following concentration versus inhibition zone graph shows how anti-bacterial activities increased with increasing concentration (C1, C2 and C3 are corresponding to concentrations at 20, 30 and 40 µg/mL of the samples respectively and B, S, E and P stands for the four tested bacteria, *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa* respectively) (Figure 8).

Conclusion

Qualitative phytochemical screening tests were done on the crude extracts of the roots of *C. ficifolius,* and the results showed the presence of saponins, terpenoids, sterols, glycosides and tannins. The EtOAc crude extract of the plant was subjected to column chromatography and revealed three compounds namely Compouns-1 Compound-2 and Compound-3 that were characterized by FTIR and 1D NMR spectroscopic techniques. The study showed the antibacterial activities of chemical constituents of *C. ficifolius* on the bacterial strains through *invitro* appeared interesting and promising against *S. aureus and B. subtilis* (gram positive). The study supports the traditional use of the plant.

Declarations

I declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

I wish to draw the attentions of the editors to the following facts which may be considered as

1. I wish to confirm that there are **no known computing interests** associated with this publication.
2. I wish to confirm that there has been **no significant financial support** for this work.
3. I wish to confirm that on **availability of data and materials**, the datasets used and/or analyzed during the correct study are available from the corresponding author on reasonable requests and all data generated or analyzed during this study are included in this published article.
4. I wish to confirm that on authors contribution, the role of second authors was editing, guiding and supporting the research and others activities was done by corresponding authors.

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Figures

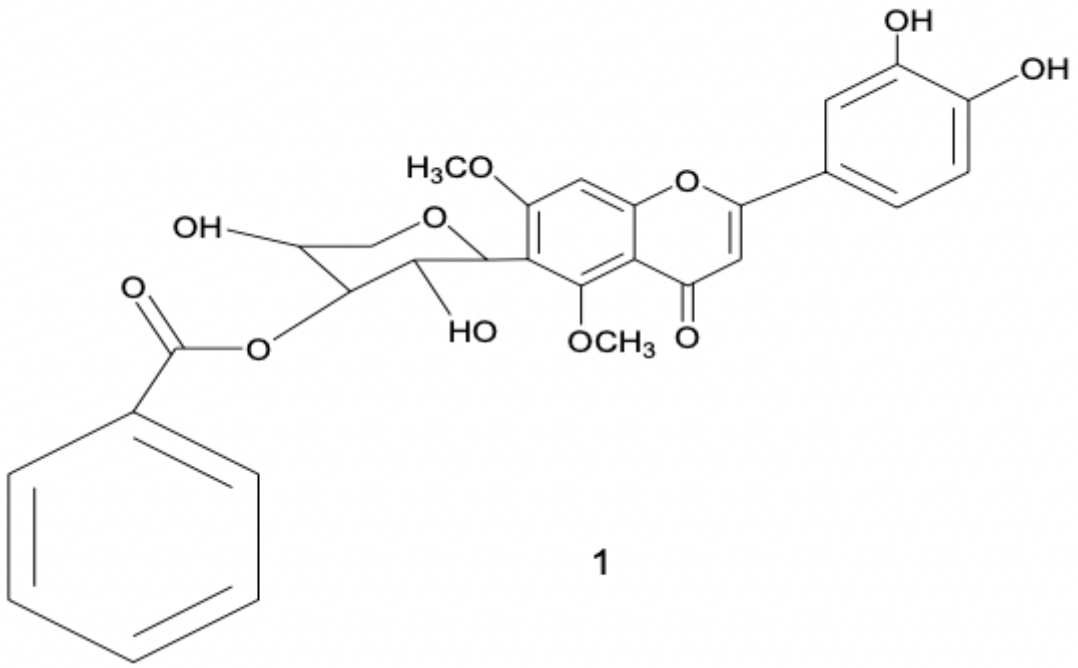

Figure 1

The Chemical structure reported from C. sativus flowers.

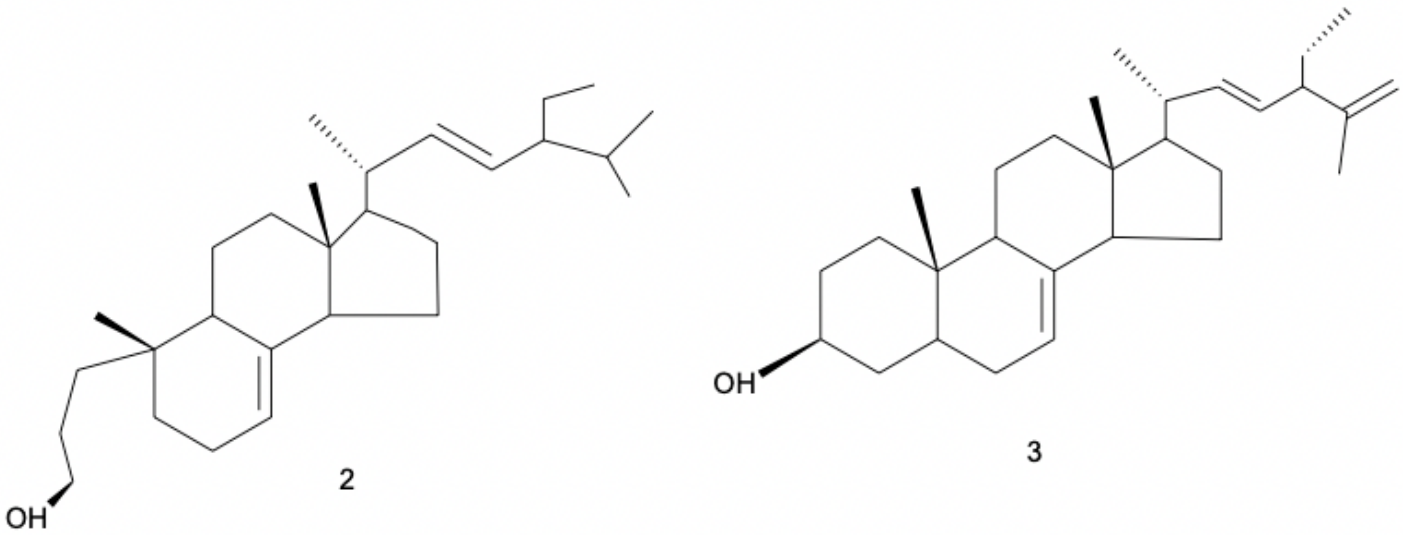

Figure 2

The Chemical structures of compounds reported from C. maxima.

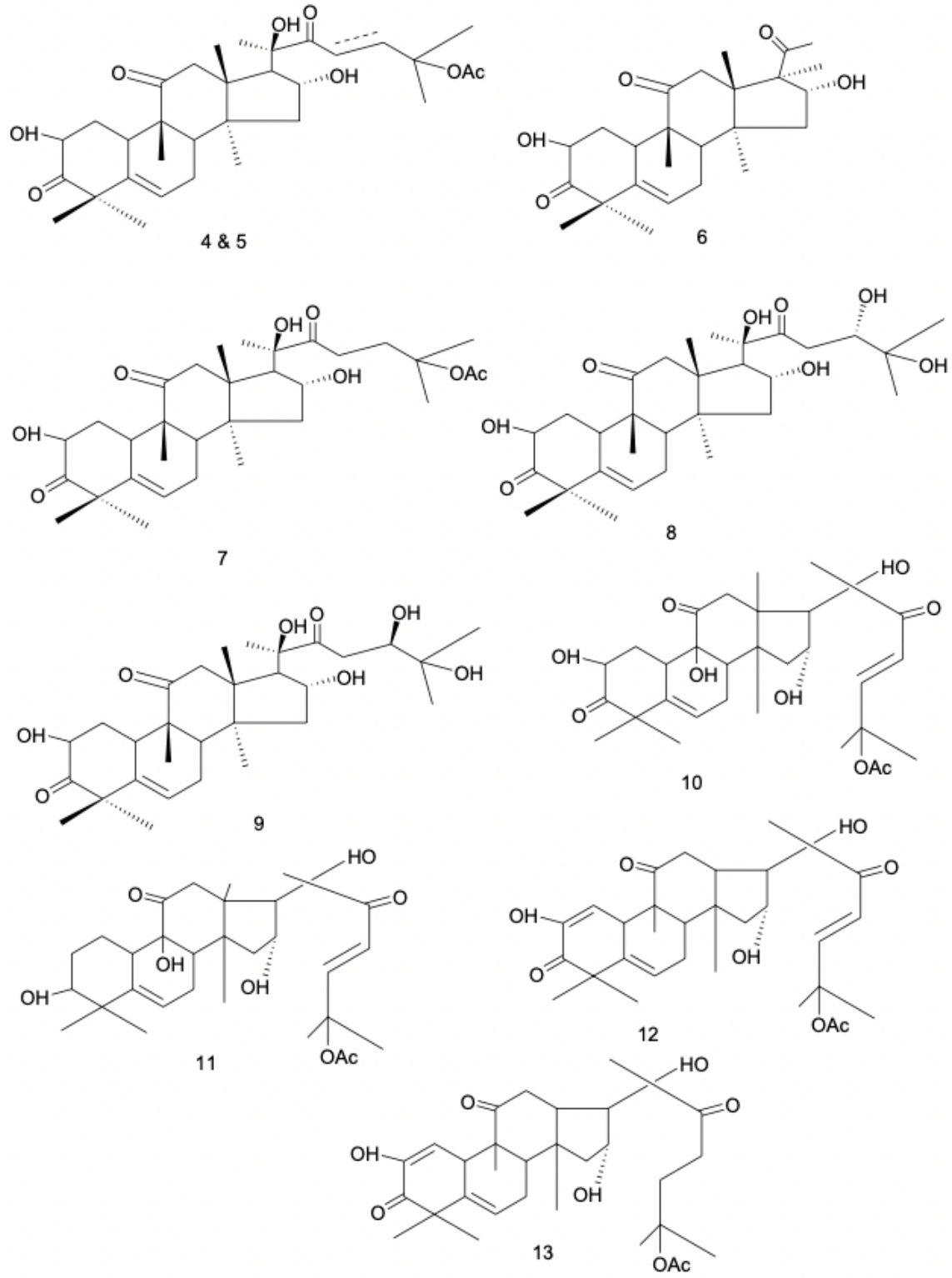

Figure 3

The Chemical structures of cucurbitacins reported from fruits of C. melo.

Figure 4

The areal and the roots of C. ficifolius

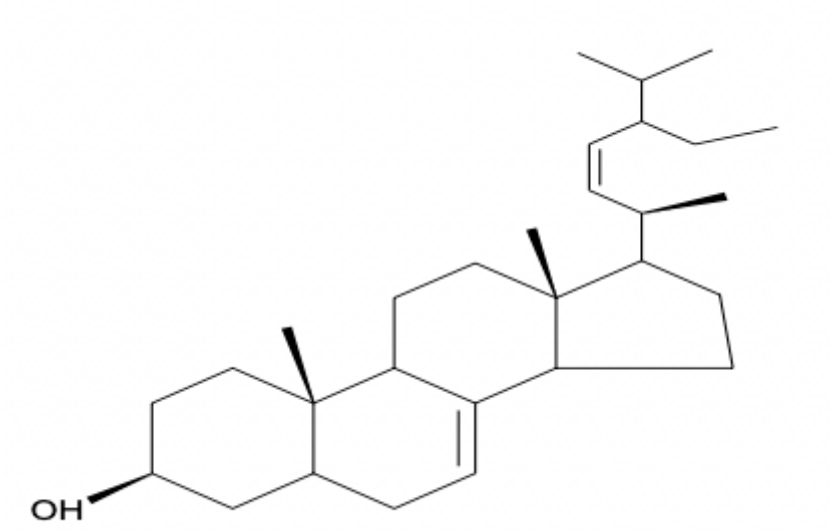

Figure 5

The proposed molecular structure of compound-1

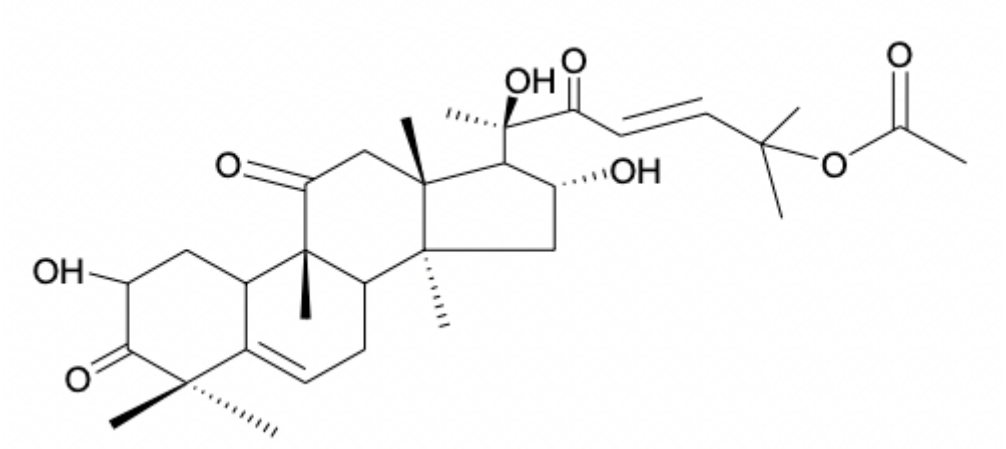

Figure 6

The proposed structure of compound-2.

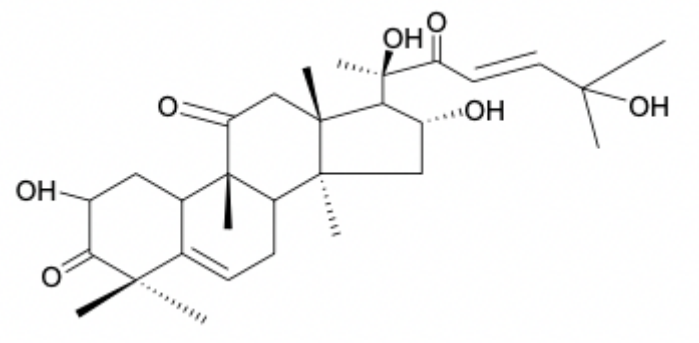

Figure 7

The proposed structure of compound-3.

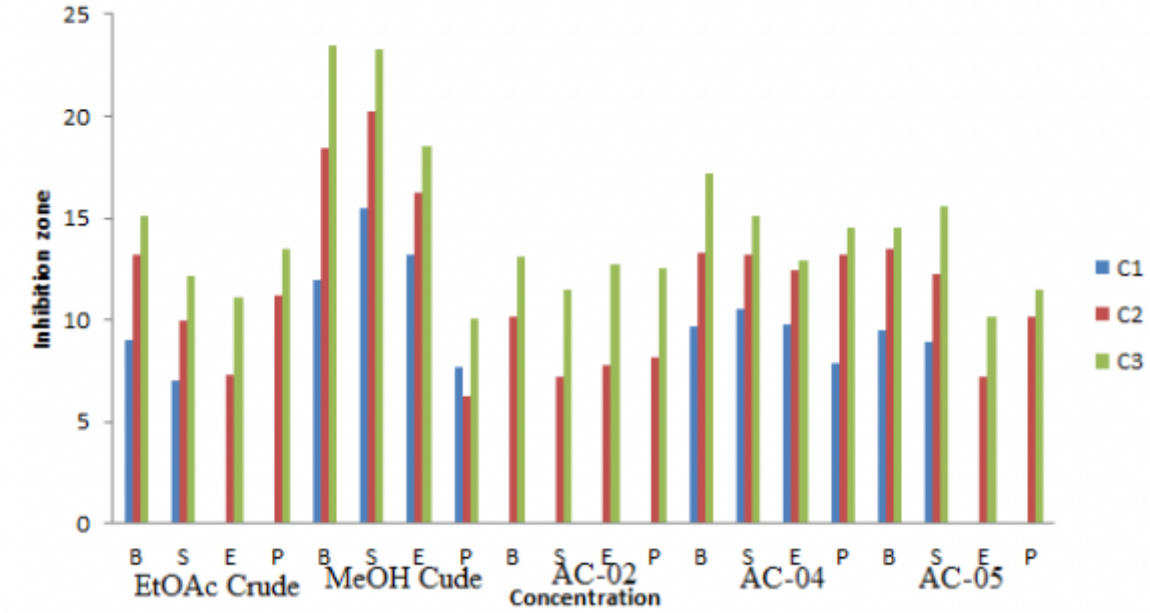

Figure 8

The graph of concentration versus inhibition zone of anti-bacterial activities of crude extract and the isolated compounds from C. ficifolius.