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Original Article

CHEMICAL AND BIOLOGICAL ANALYSIS OF THE BIOCTIVE FRACTIONS OF THE LEAVES OF *SCAEVOLA TACCADA* (GAERTN.) ROXB

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ABSTRACT

Objective: *Scaevola taccada.* (Gaertn.) Roxb. is widely dispersed all along the coasts of Africa. It is used in folk medicine for diversity of ailments. This study aims to investigate the major phytoconstituents and biological activities of the leaves of *S. taccada* (Gaertn.) Roxb.

Methods: *In vitro* biological examination *viz.* antimicrobial, cytotoxic and antioxidant activities of the ethanol extract of the leaves (EE) and its fractions; (petroleum ether (PE), methylene chloride (MC), ethyl acetate (EA) and *n*-butanol(BuOH)) were carried out. Estimation of the phytochemicals of biologically active fractions was done.

Results: *n*-butanol fraction displayed remarkable antimycobacterium activity. Petroleum ether as well as *n*-butanol fractions evidenced a cytotoxic effect on breast carcinoma cell line (MCF7) and colon carcinoma cell line (HCT) with IC50 11.7 and 15.04 µg/ml respectively. Moreover, ethyl acetate fraction exhibits an antioxidant effect with EC50 476.7±0.57 µg/ml. *n*-tetradecane 1, α-amyrin palmitate 2, α-amyrin acetate 3, α-amyrin 4, stigmasterol 5*,* luteolin-7-*O*-*β*-glucoside 6, rutin 7 and alidyjosioside 8 were identified in *S. taccada* (Gaertn.) Roxb. leaves.

Conclusion: Petroleum ether fraction is a cytotoxic candidate, especially against (MCF-7). It exhibited a moderate antifungal and antibacterial against *certain* Gram-positive bacteria. Ethyl acetate showed an antioxidant effect along with moderate antifungal activity. *n*-butanol fraction exerted potential antimycobacterial, significant cytotoxic activity against (HCT), good antifungal and antibacterial against Gram-positive and Gram-negative bacteria. Stigmasterol**,** luteolin-7-*O*-*β*-glucoside**,** rutin and alidyjosioside were isolated for the first time from *S. taccada* (Gaertn.) Roxb. Leaves.

Keywords: *Scaevola taccada* (Gaertn.) Roxb., Iridoid glycosides, Antimicrobial, Cytotoxic

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INTRODUCTION

The plant kingdom includes many medicinally useful plants. Recently, the focus on plant study has increased all over the world and the importance of medicinal plants increased in various countries. Many plants are constantly screened for their possible biological activities, among which are those belonging to the family Goodeniaceae [1]. It is a family of flowering plants included within the Asterales and considered as a sister group to Asteraceae [2]. It comprises 11 genera and approximately 400 species. Most Goodeniaceae genera are indigenous to Australia, one genus; *Scaevola* is mainly dispersed throughout the pacific area [3]. Members of the family are either herbs or shrubs. The family is characterized by the presence of some important phytoconstituents: coumarins [4], iridoid glycosides [5], pentacyclic triterpenoids: myricadiol and taraxerol, betulin and betulinic acid [6] moreover, inulin [7]. Based on recent research, these compounds were found to exhibit various biological activities of medical importance to treat many diseases [8]. *Scaevola* is one of the widest genera of the family which is mainly distributed throughout Australia [9]. Several *Scaevola* species have been traditionally used as a remedy to treat various ailments, including mainly diabetes, inflammations, and infections. *Scaevola taccada* (Gaertn.) Roxb. is known as *S. frutescens* and *S. sericea* [10]. It is broadly spread down the coasts of Madagascar (Africa), other countries of Indian oceans, tropical Australia [9]. Traditionally, different parts of *S. taccada* (Gaertn.) Roxb. were used for the treatment of various diseases. Recently, leaves have been reported to act as an anti-diabetic, antipyretic, anti-inflammatory, anticoagulant, skeletal muscle relaxant as well as antimicrobial [11]. *S. taccada* (Gaertn.) Roxb. is reported to exert an antiviral effect aganist human immunodeficiency Virus Type-1(HIV1)[12]. Different extracts of *S. taccada* (Gaertn.) Roxb. were evaluated for their antibacterial and antifungal activities. *S. taccada* (Gaertn.) Roxb. is also reported to contain phenolics, terpenoid compounds, proteins, and carbohydrates, while alkaloids and saponins are completely absent [13]. Reviewing the current literature, no data was reported regarding the chemical and biological features of *S. taccada* (Gaertn.) Roxb. grown in Egypt. Therefore, the main objective was an *in vitro* biological exploration of the ethanol extract of the leaves and its four fractions. Furthermore, inspect the phytochemicals of the bioactive fractions.

MATERIALS AND METHODS

Plant material

Samples of *S. taccada* (Gaertn.) Roxb. leaves were collected during the years 2013-2014 from the private garden, Giza, Egypt. Herbarium specimen was kindly recognized by Eng. Therese Labib**,** Consultant of Plant Taxonomy at Ministry of Agriculture and the Former Director of Orman Botanical Garden, Giza, Egypt. A voucher specimen (28-6-2016) is kept at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University. Leaves were air-dried, pulverized and kept in the dark in firmly closed glass containers.

Preparation of extracts

A-Ethanol extract and fractions

Two kg of powdered leaves were extracted by cold maceration till exhaustion with 90% ethanol (6 x 850 ml). The solvent was evaporated to dryness under reduced pressure yielding 220 g of dry residue. Two hundred g residue was suspended in 500 ml distilled water, partitioned sequentially by petroleum ether (PE) (7 × 200 ml), methylene chloride (MC) (4 × 200 ml) followed by ethyl acetate (EA) (4 × 200 ml) and *n*-butanol (BuOH) saturated with water (6 × 200 ml). The solvents were evaporated under reduced pressure to give different fractions. The fractions were saved in dark tightly clogged glass containers and kept in desiccators for further studies.

B-Aqueous extract

Infusion in hot distilled water (4 x 500 ml) was prepared of air-dried pulverized leaves of *S. taccada* (Gaertn.) Roxb. (500 g). The solvent was evaporated under reduced pressure to give 45 g dry residue. Different dilutions of the extract were prepared in distilled water containing few drops of Tween 80 and saved for further biological study.

Material for biological study

a-Microorganisms

Bacterial strains: *Streptococcus pneumoniae* (RCMB 010010), *Bacillus subtilis* (RCMB 010067), *Vibrio cholerae* (RCMB 010041), *Escherichia coli* (RCMB010064), *Klebsiella pneumoniae* (RCMB 010073), *Shigella dysenteriae* (RCMB010098), *Salmonella typhimurium* (RCMB 010052), *Mycobacterium tuberculosis* (RCMB 010094-8). The strains of Fungus: *Aspergillus fumigatus* (RCMB 02568), *Fusarium solani* (RCMB 008023), *Fusarium oxysporum* (RCMB 008059), *Candida albicans* (RCMB 05031). All were maintained in the Regional Center for Mycology and Biotechnology (RCMB), Cairo, Egypt

b-Culture media

Media for antimicrobial activity

i-Middlebrook 7 H9 broth (Difco) for *mycobacterium tuberculosis*. ii-nutrient agar medium (Oxoid Laboratories, UK) was prepared foe bacteria. iii-Sabouraud dextrose agar (Oxoid Laboratories, UK) for fungi.

c-Chemicals, drugs and kits for biological activity

Gentamicin and Ampicillin (Sigma-Aldrich, Germany) as antibacterial. Amphotericin B (Sigma-Aldrich, Germany) as an antifungal. Isoniazid (Sigma-Aldrich, Germany) as antimycobacterial. Alamar Blue solution: Alamar Biosciences/Accumed, Westlake, Ohio for antimycobacterial activity. Doxorubicin (Sigma, St. Louis, MO, USA) was used as a cytotoxic standard. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH reagent): was obtained from Sigma-Aldrich, St. Louis, MO, USA and freshly dissolved in 99.9% methanol in a concentration of 0.004% for the determination of antioxidant activity. Ascorbic and gallic acids as reference antioxidants (Misr Company for Pharmaceutical Industry, Mataria, Cairo, Egypt).

Material for phytochemical study

Stationary phases: for vacuum liquid chromatography (VLC) Silica gel H 60, E. Merck, Germany. Silica gel 60 for column chromatography, Sigma-Aldrich Chemicals, Germany. Precoated TLC plates, silica gel 60 F254 (20× 20 cm), Sigma-Aldrich Chemicals, Germany. Sephadex LH 20, Pharmacia Fine Chemicals AB Uppsala, Sweden. Solvent Systems: S1: *n*-hexane (100%). S2: *n*-hexane: ethyl acetate (99:1 v/v). S3: *n*-hexane: ethyl acetate (97:3 v/v). S4: *n*-hexane: ethyl acetate (95:5 v/v). S5: *n*-hexane: ethyl acetate (90:10 v/v). S6: *n*-hexane: ethyl acetate (85:15 v/v). S7: ethyl acetate: methanol: water: formic acid (100: 16: 13: 0.2 v/v). S8: an upper layer of (butanol: acetic acid: water, 4: 1: 5). S9: isopropyl alcohol: water (3:1 v/v). Chromatographic Spray Reagents: *p*-Anisaldehyde-sulphuric acid spray reagent: (for sterols and triterpenes) [14]. Spray reagent for flavonoids 2% aluminum chloride [15]. Natural products/polyethylene glycol reagent (NP/PEG): (For phenolic compounds) [16]. Aniline phthalate spray reagent (for paper chromatography of sugars) [14]. Reagents and Chemicals for UV Spectroscopy: Shift reagents were prepared following the published measures [17] and chemicals used during the performance of the UV spectroscopic analysis of flavonoids were: 2.5 % aqueous sodium methoxide solution, 5% methanolic aluminum chloride solution. 0.5% aqueous hydrochloric acid solution, anhydrous sodium acetate powder and anhydrous boric acid powder. Sensitive analytical electric balance: Sartorius, Germany. Rotatory evaporator: Buchi, G. Switzerland. For localization of spots on chromatograms ultraviolet lamp Shimadzu; a product of Hanovia lamps: λ max = 254 and 330 nm. Bruker NMR-spectrophotometer: ^{1}H-NMR, 400 MHz, ^{13}C-NMR, 100 MHz, Japan. Using TMS as an internal standard, the NMR spectra were recorded in deuterated $CHCl_3$. Chemical shift values were recorded as δ ppm. NMR laboratory, Faculty of Pharmacy, Cairo University. Mass spectrometer: Thermo scientific ISQLT single quadrupole for recording mass spectra, Regional Center of Mycology and Biotechnology, Al-Azhar University. Schimadzu double beam spectrophotometer: (UV-1650), for determination of UV shifts of flavonoids, Pharmacognosy Department, Faculty of Pharmacy, Cairo University. Tecan infinite F50 absorbance microplate reader was for the determination of the antioxidant activity, Pharmacognosy Department, Faculty of Pharmacy, Cairo University.

Evaluation of antimicrobial action

The antimicrobial action was carried out using the agar well diffusion method as described by Holder and Boyce [18]. Results are recorded in table 1.

Evaluation of the antimycobacterial effect

Microplate Alamar blue assay was chosen for the evaluation of antimycobacterial activity due to rapid determination for the susceptibility of organisms to antimicrobials compared to the other methods, which require 3 w to get results [19]. Alamar blue is a soluble redox indicator, its oxidized form is blue and nonfluorescent; upon reduction by bacterial growth medium, it turns into pink and fluorescent; therefore, bacterial growth can be determined by a visual color change or fluorometrically [20]. Results were recorded at 24 h. post-reagent additions at 590 nm and presented in table 1.

Determination of the minimum inhibitory concentration (MIC)

The broth dilution method was used to evaluate Minimum Inhibitory Concentration (MIC) [21]. The MIC values of the tested samples and standards (ug/ml) are recorded in table 2. For antimycobacterial activity, visual MICs were distinct as the lowly concentration of drug that banned change in color. Inhibition Percentage was defined by Collins and Franzblau [19]. The results are recorded in table 2.

Evaluation of the cytotoxic activity

Latent cytotoxicity of the ethanolic extract (EE) of the leaves of S. taccada (Gaertn.) Roxb. was tested against HFB4 (normal melanocytes) tables 3 and 4. EE and its four fractions; PE, MC, EA and BuOH were tested against 2 human cancer cell lines; HCT (colon carcinoma) and MCF-7 (breast carcinoma) cell lines. Possible cytotoxicity was estimated by Sulphorhodamine B assay (SRB) [22] as compared to standard Doxorubicin. Results are recorded in tables 3 and 4.

Evaluation of the antioxidant activity

Antioxidant activity of EE of the leaves of *S. taccada* (Gaertn.) Roxb. and its four fractions were screened by adopting the 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) *in vitro* model. The examination was passed out as described by Romano *et al.*, [23] with a few modifications. The average EC_{50} of each extractive are presented in table 5 and those of the active samples are plotted against gallic acid and ascorbic acid (reference antioxidants).

Isolation and characterization of the phytochemicals

Commencing on the results of the *in vitro* cytotoxic examination on the different extractives of *S. taccada* (Gaertn.) Roxb. leaves, it was found that BuOH, as well as PE fractions, were the most active fractions against colon HCT, and breast MCF-7 carcinoma cell line, respectively. BuOH also exhibited promising antimicrobial activity against most of the tested micro-organisms. Though, the ethyl acetate fraction was the mainly active as DPPH radical scavenger. It was a promising finding that encouraged us to pressure more detailed investigation of the PE, EA and BuOH fractions in a trial to trace the bioactive constituents to which the defined actions may be attributed. This encouraged for investigation of bioactive constituents.

Isolation of the phytochemicals of the petroleum ether fraction

Twenty grams of PE portion was applied on VLC (silica gel H, 300 G, 7 cm L x 14 cm D). Gradient elution was performed starting with *n*-hexane, *n*-hexane/DCM and DCM/EtOAc mixtures. Fractions (200 ml, each) were collected, evaporated and residue monitored by TLC by S6 and the spots were then visualized after spraying with *p*-anisaldehyde/H_2SO_4 and heating at 110 ° C for 10 min. Similar fractions were pooled to give five main fractions elected as fractions I-V. Fraction I (0.5g) was rechromatographed on a silica gel 60

column (23 cm L x 2.5 cm D). Subfractions eluted with 100% *n*-hexane to yield about 25 mg of white wax (compound 1). Fraction II (2 g) was chromatographed on a silica gel 60 column (25 cm x 2.5 cm) as previously mentioned with fraction I to give two major subfractions. Subfraction II-a eluted with *n*-Hexane: EtOAc (99: 1 v/v) was collected and evaporated to yield 15 mg of white wax (compound 2). Subfraction II-b was further purified on a column of silica gel 60 (18 cm x 2 cm) eluted with a mixture of *n*-Hexane: EtOAc (97: 3 v/v) to yield 10 mg of white needle crystals (compound 3). Fraction III (2.5 g) was rechromatographed as fraction II. Subfraction eluted with *n*-hexane: EtOAc (96:4 v/v) was collected and evaporated to yield 45 mg of white needle crystals (compound 4). Fraction IV (3 g) was further chromatographed on a silica gel 60 column (30 cm x 2.5 cm). Subfractions eluted with 5% EtOAc in *n*-hexane to yield about 20 mg of white needle crystals (compound 5). Chromatographic and Spectral information of isolated compounds would be discussed later.

Isolation of the phytochemicals of the ethyl acetate fraction

Six grams of EA fraction were subjected to fractionation by VLC (silica gel H 60, 165 g, 11.5 cm L × 5.5 cm D). Gradient elution was performed using hexane, n-hexane/DCM mixtures, DCM/EtOAc mixtures and EtOAc/methanol mixtures. Fractions (200 ml, each) were collected and monitored by TLC using S7. Similar fractions were collected, yielding eight fractions designated as fractions I-VIII. The collective fractions were evaporated under reduced pressure, weighed and kept in desiccators for further analysis. Fraction VI (1g) was selected for further investigation; it was rechromatographed on a sephadex LH-20 column (22 cm L x 2.5 cm D) using methanol (50% v/v) for elution (isocratic elution). Fractions of (5 ml, each) were collected, monitored by TLC. Fraction containing the major spot was additional purified on another sephadex LH-20 column (20 cm L X 1.5 cm D) by methanol (50% v/v) for elution to yield 6 mg of a yellow powder (compound 6). Spectral data of compound 6 is recorded below.

Investigation of the constituents of the *n*-butanol fraction

Tweenty grams of BuOH fraction were applied on VLC (silica gel H 60, 280 g, 7 cm L × 14 cm D). Gradient elution was performed using DCM/EtOAc mixtures and EtOAc/methanol mixtures. Fractions with similar chromatographic pattern were pooled, yielding five fractions elected as fractions I-V. For isolation and purification of individual compounds, fractions IV and V were subjected to further fractionation. Fraction IV (1g) was rechromatographed on a sephadex LH-20 column (26 cm L X 3 cm D) using methanol: water (50:50 v/v) for elution (isocratic elution). Fractions of (5 ml, each) were collected and alike fractions were pooled together, yielding two subfractions. The subfraction was further purified on another sephadex LH-20 column (20 cm L X 1.5 cm D) using methanol: water (50:50 v/v) for elution to yield 12 mg of a yellow powder. Spectral data of compound 7 is recorded and discussed below. Fraction V (0.5 g) was chromatographed on a sephadex LH-20 column (25 cm L X 2.5 cm D) using butanol: isopropanol: water (40:10:50 v/v) (isocratic elution). Fractions of (10 ml, each) were collected together, yielding 15 mg of a white amorphous powder (compound 8), R_f =0.16 in S_7. Spectral data of 8 is presented and discussed with the other compounds.

Depiction of the isolated phytochemicals

Compound 1, white wax, soluble in *n*-hexane, gives blue color with ρ-anisaldehyde/H_2SO_4.^{1}H-NMR ($CDCl_3$, δ ppm) showed signals at δ_H 0.90 (6H, *t*, *J*=7 Hz) of two terminal methyl groups (CH_3-1, CH_3-14) and δ_H 1.2 (saturated alkyl chain (-CH2-) 12).

Compound 2 white wax, m. p 75-77 °C, soluble in *n*-hexane and methylene chloride and gives purple color with ρ-anisaldehyde/ H_2SO_4. ^{1}H-NMR: δ (400 MHz, CDCl3) showed signls at: 5.12 (1H, t, J=3.6 Hz, H-12), 4.51 (m, H-3), 2.28 (2H, m, H-2'), 1.25 (brs,-(CH2) n), 0.73 (3H, s, CH3-28), 0.70-0.81(overlapped) (12 H, br. s, CH3-23, 24,29 and 30), 0.91 (3H, s, CH3-26), 0.95 (3H, s, CH3-25), 1.00 (3H, s, CH3-27). ^{13}C-NMR: δ (100 MHz, CDCl3) showed signls at38.43 (C-1), 22.69 (C-2), 80.60 (C-3), 37.74(C-4), 55.27 (C-5), 18.20 (C-6), 32.87 (C-7), 40.02 (C-8), 47.63 (C-9), 36.79 (C-10), 23.24(C-11), 124.34 (C-12), 139.60 (C-13), 42.08(C-14),26.58 (C-15), 25.16 (C-16), 33.75 (C-17), 59.02 (C-18). 39.61 (C-19), 39.65 (C-20), 31.93 (C-21), 41.53 (C-22), 28.09 (C-23), 16.8 (C-24), 15.72 (C-25), 16.8 (C-26), 23.37 (C-27), 28.74 (C-28), 17.50 (C-29), 21.39 (C-30), 173.70(COO), 14.13 (CH3), 29.70 (CH2) n, 34.86 (C-2').

Compound 3 white needle crystals, soluble in n-hexane and methylene chloride, m. p. 223-227 °C and gives violet color with ρ-anisaldehyde/H_2SO_4. ^{1}H-NMR: δ (400 MHz, CDCl3) showed signals at: 5.13 (1H, t, J=3.4 Hz, H-12), 4.53(1H, m, H-3), 2.05 (3H, CH3-2'), 0.80(3H, s, CH3-28), 0.84 (overlapped) (12H, CH3-23, 24, 29, 30), 0.99 (3H, s, CH3-26), 1.02 (3H, s, CH3-25), 1.07 (3H, s, CH3-27). 13C-NMR: δ (100 MHz, CDCl3) showed signals at: 38.4 (C-1), 22.69 (C-2), 80.9 (C-3), 37.71 (C-4), 55.23 (C-5), 18.25 (C-6), 32.87 (C-7), 40.03 (C-8), 47.55 (C-9), 36.8(C-10), 23.22 (C-11), 124.34 (C-12), 139.63 (C-13), 42.07 (C-14),26.58 (C-15), 25.9 (C-16), 33.73 (C-17), 59.10 (C-18). 39.63 (C-19), 39.65 (C-20), 31.93(C-21), 41.53 (C-22), 28.03 (C-23), 16.8(C-24), 15.73 (C-25), 16.8 (C-26), 23.36(C-27), 28.74 (C-28), 17.49 (C-29), 21.31 (C-30), 171.02 (C-1', COO), 21.33 (C-2').

Compound 4: white needle crystals, soluble in n-hexane and methylene chloride, m. p. 185-188 °C and gives violet color with ρ-anisaldehyde/H2SO4. 1H-NMR: δ (400 MHz, CDCl3) showed signals at: 5.15 (1H, t, J= 3.56, H-12), 3.24 (1H, dd, J=10.48 Hz, J= 10.76 Hz, H-3), 0.82(3H, s, CH3-28), 1.28 (6H, s, CH3-24, CH3-27), 1.09 (3H, s, CH3-26), 0.93 (3H, s, CH3-23), 0.97 (3H, s, CH3-25), 0.82(3H, d, J= 6.2, CH3-29), 1.02 (d, J=4 Hz, CH3-30). 13C-NMR: δ (100 MHz, CDCl3) showed signals at: 36.87 (C-1), 26.62 (C-2), 79.04(C-3), 38.74 (C-4), 55.25 (C-5), 17.48 (C-6), 31.94 (C-7), 40(C-8), 47.7 (C-9), 36.9(C-10), 22.7 (C-11), 124.41 (C-12), 139.58 (C-13), 41.53 (C-14), 28.7 (C-15), 26.66 (C-16), 33.76 (C-17), 59.06(C-18), 39.69 (C-19), 39.62 (C-20), 31.26 (C-21), 41.55 (C-22), 28.12 (C-23), 15.68 (C-24), 15.63 (C-25), 16.87(C-26), 23.37 (C-27), 28.1 (C-28), 17.48(C-29), 21.4 (C-30).

Compound 5: white needle crystals, soluble in methylene chloride, m. p. 174-176 °C and gives blue color with ρ-anisaldehyde/H_2SO_4.^{1}H-NMR (400 MHz, CDCl3, δ ppm) exhibited signals at: 1.00 (3H, *d*, *J* = 7.28, H-21), 0.791 (3H, *t*, *J* =7.72, H-29), 0.84 (3H, *d*, *J* = 7.2, H-26), 0.688(3H, *s*, H-18), 0.77 (3H, *d*, *J* = 6.52, H-27), 1.2 (3H, *s*, H-19), 3.45 (1H, *m*, H-3), 5.34(1H, *d*, *J*= 5.2, H-6), 5.00 (1H, dd, H-22), 5.13 (1H, dd, H-23)., EI/MS m/z (70 eV) calculated for C29H48O with characteristic fragmentation at m/z: 412 (18.58%), 394 (2.49%), 369 (4.36%), 351(11.94%), 314 (6.70%), 301 (8.85%), 300(25.48%), 271 (48.59%), 255 (76.59%), 253(19.23%), 105 (53.02%), 55 (100%).

Compound 6 yellow powder, soluble in methanol, m. p.238-240 °C and gives a brown color in UV light, which changed to yellow ahead experience to ammonia vapor and gave an orange color on spraying by NP/PEG spray reagent.^{1}H-NMR spectra of compound **6** displayed signals 6.41(1H, *d*, *J* = 4 Hz, H-6), 6.71 (1H, *d*, *J* = 4 Hz, H-8), 6.51 (1H, *s*, H-3), 6.82 (1H, *d*, *J'*= 8 Hz, H-5'), 7.32 (2H, *dd*, H-2', H-6', overlapping). 4.98 (1H, d, J=8, H-1´´), 3.3-3.9 (sugar protons).

Compound 7 yellow powder, sparingly soluble in methanol and m. p.181-187 °C and gives a brown color in UV light = 365 nm which turned yellow on spraying with aluminium chloride and orange with NP/PEG spray. 1H-NMR ppm (400MHz, CD3OD) showed signals 7.68 (1H, d, *J*=1.2 Hz, H-2'), 7.65 (1 H, dd, *J*=(8.4,1.2) Hz, H-6'), 6.8 (1H, d, *J*=8 Hz, H-5'), 6.48 (1H, s, H-8), 6.23 (1H, s, H-6), 5.11 (1H, d, *J*=8Hz, glucose-H-1``), 4.53 (1H, br s, rhamnose-H-1```), 1.12 (3H, d, *J*=8Hz, rhamnose-H-6```). 13C-NMR ppm (100 MHz, CD3OD) showed signals at: 156.06 (C-2), 134.17 (C-3), 178.44 (C-4), 161.54 (C-5), 98.71 (C-6), 163.88 (C-7), 93.7 (C-8), 156.64 (C-9), 121.71(C-1`), 114.90 (C-2`), 144.36 (C-3`), 147.44 (C-4`), 116.48(C-5`), 122.46 (C-6`). Sugar carbons: 101.1 (C-1``),73.7(C-2``), 75.81 (C-3``),70.5 (C-4``), 74.50 (C-5``), 67.24 (C-6``),99.3 (C-1```), 70.5(C-2```), 70.05 (C-3```), 72.4 (C-4```), 68.24 (C-5```), 16.4(C-6```).

Compound 8 white amorphous powder, soluble in methanol, m. p. 93-96 °C and gives reddish violet color with *p*-anisaldehyde/sulphuric acid. ^{1}H-NMR (400 MHZ, MeOD δ ppm) showed signals at: 4.23(2H, br. s, H3), 2.68(1H, br. s, H4), 3.19(1H, br. s, H4a), 2.12.(2H, m, H5), 2.69(1H, br. s, H7), 5.25-5.36 (overlapped) (3H, br. s, H6, H8'), 2.7-2.82 (overlapped) (2H, m, H7a, H6'), 5.54(1H, br. s, H1'), 7.48(1H, s, H3'), 3.09(1H, br. s, H5'), 5.77(1H, br. s, H7'), 2.31(2H, m, H9'), 4.8(1H, br. s, H10'), 3.32(6H, s, Me-11', 12'), 1.16(1H, s, Me-8), 3.71(1H, br. s, Me10), 4.71(1H, br. s, H1'), 3.2-3.9 (sugar protons).^{13}C-NMR (400 MHZ, MeOD δ ppm) showed signals at174.35 (C-1), 67.9 (C-3), 44.03 (C-4), 38.8 (C-4a),

40.1 (C-5), 78.08 (C-6), 43.9 (C-7), 47 (C-7a), 13.06(C-8), 171.7 (C-9), 50.7(C-10), 167.34 (C-11), 96.4(C-1'), 152.28 (C-3'), 112 (C-4'), 31.66(C-5'), 44.03(C-6'), 134.5 (C-7'), 119.2(C-8'), 33.6 (C-9'), 103.87 (C-10'), 47.9 (C-11',C-12'). Sugar: 98.8 (C-1''), 73.2 (C-2''), 76.63 (C-3''), 70.17 (C-4''), 76.8 (C-5''), 62.9(C-6'').

RESULTS

Evaluation of the antimicrobial effect

Ethanol extract, PE, and BuOH exhibited a comparable activity against *Escherichia coli and Shigella dysenteriae.* EE showed potent activity against *Klebsiella pneumonia* with a high potency of 88 percent. Moreover, PE fractions exhibited an inferior activity against *Salmonella typhimurium,* and *Vibrio cholera*, with a potency of 71.78 and 70.04 percent, respectively, compared to standard Gentamicin.

From results presented (tables 1 and 2) BuOH fraction revealed the uppermost action alongside most of the tested organisms, where potency against Gram-positive bacteria; *Streptococcus pneumonia* and *Bacillus subtilis* were 93.69 and 74.69 percent, respectively, compared to the standard Ampicillin. Results obtained here are similar to those cited in the literature. BuOH was the most active antifungal against *Aspergillus fumigatus*, *Fusarium solani* and *Fusarium oxysporum* with potency 85.23, 83.10 and 81.18 percent, respectively, compared to Amphotericin (tables 1 and 2). EA fraction showed the lowest activity against the microbial strains, especially *B. subtilis and Klebsiella pneumonia.* The ethanolic extract, as well as the four fractions, exert no activity against *Candida albicans*. Regarding the antimycobacterial activity, BuOH is the most active one with a 55.70 % potency followed by EE with 26.9% potency while the other three fractions showed no activity (tables 1 and 2).

Table 1: Antibacterial, antifungal and mycobacterium tuberculosis activities of the ethanol extract of the leaves of *S. taccada* (Gaertn.) Roxb. and its four fractions

Sample tested bacteria	Diameter zone of inhibition (mm±SD) (potency % as compared to standard					
	EE	**PE**	**MC**	**EA**	***n*-BuOH**	**Positive control**
Gram-positive bacteria						Ampicillin
Streptococcus pneumonia	15.7±2. 1	20.2±1.2	17.8±0.7	16.3±0.5	22.3±2.1	23.8±0.63
(RCMB 010010)	65.96 %	84.87%	74.78%	68.48%	93.69%	100%
Bacillus subtilis	17.9±1.5	21.3±2.1%	19.3±0.63	18.3±1.2	24.2±1.5	32.4±1.2
(RCMB 010067)	55.24 %	65.74	59.56%	56.48%	74.69%	100%
Gram-negative bacteria						Gentamicin
Escherichia coli	18.3±0.58	16.4±0.5	15.2±1.5	14.3±0.63	18.3±0.63	22.3±1.2
(RCMB010064)	(82.06%)	(73.54%)	(68.16%)	(64.12%)	(82.06%)	100%
Salmonella typhi	20.6±0.58	18.3±0.63	NA	NA	21.6±1.2	25.6±0.18
(RCMB 10052)	(80.46%)	(71.48%)			(84.37%)	100%
Shigelladysenteriae	16.7±0.58	12.3±1.2	13.4±0.63	12.6±0.58	14.3±0.58	20.3±0.18
(RCMB010098)	(82.26%)	(60.59%)	(66.00%)	(62.06%)	(70.44%)	100%
Klebsiella pneumonia	23.3±0.22	19.3±0.72	16.2±1.5	15.6±1.5	22.4±1.5	26.4±1.5
(RCMB 010073	(88.2%)	(73.1%)	(61.36%)	(59.09%)	(84.84%)	100%
Vibrio cholera	15.8±0.58	13.8±0.44	11.3±1.2	NA	16.2±1.2	19.6±0.18
(RCMB 10041)	(80.61%)	(70.40%)	(57.65%)		(82.65%)	100%
Fungi (Amphotericin B)						
Aspergillusfumigatus	16.3±0.58	17.3±1.2	15.2±1.5	18.3±0.58	20.2±1. 2	23.7±0.1
(RCMB 02568)	(68.77%)	(73%)	(64%)	(77.2%)	(85.23%)	(100%)
Fusariumsolani	15.2±0.58	16.3±0.58	13.3±1.2	16.8±1.5	18.2±0.63	21.9±0.12
(RCMB 008023	(69.4%)	(74.42%)	(60.73%)	(76.71%)	(83.1%)	(100%)
Fusarium Oxysporum	18.9±0.22	18.4±0.63	15.4±0.63	19.3±0.72	23.3±0.58	28.7±0.22
(RCMB 008059	(65.85%)	(64.11%)	(53.65%)	(67.24%)	(81.18%)	(100%)
Candida albicans	NA	NA	NA	NA	NA	19.8±0.20
(RCMB 05031)						(100%)
Inhibitory % against *Mycobacterium tuberculosis*						Isoniazid
Mycobacterium tuberculosis (RCMB 010094-8)	22.34±1.5 26.85%	0	0	0	46.35±0.58 55.70%	83.2±2.1 100%

n-BuOH., Butanol; E. E., ethanol extract; EA, ethyl acetate; MC., methylene chloride; PE petroleum ether; NA.,no activity., Data are expressed as mean±SD.

Table 2: MIC (ug/ml) of the ethanolic extract of the leaves of *S. taccada* (Gaertn.) Roxb. and its four fractions on gram-positive, gram-negative bacteria, fungi and mycobacterium tuberculosis

Sample tested bacteria	EE	PE	MC	EA	BuOH	Positive control
Gram-positive bacteria						Ampicillin
Streptococcus pneumonia (RCMB 010010)	15.63	3.9.	7.81	15.63	0.98	0.98
Bacillus subtilis (RCMB 010067)	7.81	1.95	3.9	7.81	0.98	0.24
Gram-negative bacteria						Gentamicin
Escherichia coli (RCMB010064)	7.81	15.63	15.63	62.5	7.81	0.98
Salmonella typhi (RCMB 010052)	1.95	7.81	NA	NA	0.98	0.49
Shigelladysenteriae (RCMB010098)	15.63	125	62.5	125	62.5	3.9
Klebsiellapneumonia (RCMB 010073)	0.49	3.9	15.63	15.63	0.98	0.49
Vibrio cholera (RCMB 010041)	62.5	62.5	125	NA	15.63	3.9
Fungi						Amphotericin B
Aspergillusfumigatus (RCMB 02568)	31.25	15.63	62.5	7.81	3.9	0.98
Fusariumsolani (RCMB 008023	62.5	15.63	62.5	15.63	7.81	0.98
Fusarium Oxysporum (RCMB 008059	7.81	7.81	15.63	3.90	0.98	0.49
Candida albicans (RCMB 05031)	NA	NA	NA	NA	NA	3.9
Mycobacterium tuberculosis						Isoniazid
Mycobacterium tuberculosis (RCMB 010094-8)	62.5	0	0	0	15.63	1.95

n-BuOH., Butanol; E. E., ethanol extract; EA, ethyl acetate; MC., methylene chloride; PE petroleum ether; NA.,no activity.

Cytotoxic activity

EE showed cytotoxic activity on normal melanocytes (HFB4) with IC_{50} 19.4 µg/ml (tables 3 and 4). Tracing results of this study, it was found that PE fraction was the most potent cytotoxic sample on MCF-7 (breast carcinoma cell line) (IC_{50} = 11.7 µg/ml) followed by MC fraction and EE (IC_{50} = 24.2, and 25 µg/ml, respectively), while the other two fractions prevailed no cytotoxic action against breast carcinoma cell line (MCF-7). These results were compared to doxorubicin, which showed IC_{50} = 5 µg/ml. BuOH fraction was the most effective one against colon carcinoma cell line (HCT) (IC_{50} = 15.04 µg/ml), followed by EE, MC, EA, and PE fraction (IC_{50} = 16.4, 21.9, 21.9 and 22.7 µg/ml, respectively) compared to Doxorubicin which showed (IC_{50}= 4.19 µg/ml). Thus, EE and its four fractions showed non-selective activity. They affect both healthy and cancerous cell lines.

Table 3: Results of the cytotoxic activity of the ethanolic extract of the leaves of *S. taccada* (Gaertn.) Roxb. and its four fractions (Gaertn.) Roxb. on breast carcinoma cell line (MCF7), colon carcinoma cell line (HCT) and on normal melanocytes (HFB4)

Carcinoma cell line	Conc. (µg/ml)	Mean of the surviving fraction					
		EE	PE	MC	EA	Bu	Dox.
	0.0	1.000	1.000	1.000	1.000	1.000	1.000
MCF7	5.0	0.829	0.633	0.718	0.800	0.989	0.500
	12.5	0.688	0.485	0.632	0.755	0.890	0.545
	25.0	0.500	0.309	0.494	0.557	0.690	0.498
	50.0	0.394	0.358	0.451	0.622	0.698	0.513
	0.0	1.000	1.000	1.000	1.000	1.000	1.000
HCT	5.0	0.755	0.855	0.797	0.743	0.793	0.424
	12.5	0.573	0.700	0.677	0.526	0.515	0.379
	25.0	0.339	0.454	0.443	0.471	0.444	0.393
	0.0	1.000	-	-	-	-	-
HFB4	5.0	0.493	-	-	-	-	-
	12.5	0.260	-	-	-	-	-
	25	0.213	-	-	-	-	-
	50	0.173	-	-	-	-	-

Bu., Butanol; Dox., Doxorubicin; E. E., ethanol extract; EA, ethyl acetate; MC., methylene chloride; PE., petroleum ether

Table 4: IC_{50} of the ethanolic extract leaves *S. taccada*(Gaertn.) Roxb. and its four fractions of the on the tested cell lines

Extract	IC_{50} (µg/ml)		
	HFB4	MCF7	HCT
EE	19.4±0.35	25±0.30	16.4±0.13
PE	…….	11.7±0.21	22.7±1.5
MC	…….	24.2±2.5	21.9±2.2
EA	…….	…….	21.9±3.3
n-Bu	…….	…….	15.04±0.49
Dox.	4.2±0.20	5±0.42	4.19±0.54

Bu., *n*-butanol; ; Dox., Doxorubicin E. E., ethanol extract; EA, ethyl acetate; MC., methylene chloride; PE: Pet. ether.

Antioxidant effect

The EE of *S. taccada* (Gaertn.) Roxb. leaves (table 5) exhibited a weak DPPH scavenging activity with EC_{50} of 1134.43±6.26 µg/ml meanwhile, those of the reference antioxidants, gallic and ascorbic acids, were 18.85±0.59 and 19.75±0.77, respectively. Among the fractions, EA exhibited antioxidant activity at relatively low concentration followed by BuOH fraction with EC_{50} 476.7±0.57 and 643.8±5.43 µg/ml, respectively. Consequently, obtained results were comparable to those previously published.

Table 5: DPPH scavenging potency of the ethanolic extract of *S. taccada* leaves and its four fractions

Extract/Fraction	EC_{50}±SD µg/ml
EE	1134.43±6.26
PE	-----
MC	-----
EA	476.7±0.57
n-Bu	643.8±5.43
Gallic acid	18.85±0.59
Ascorbic acid	19.75±0.77

EC_{50}=effective concentration of the sample required to scavenge 50% of the DPPH free-radical as compared to the solvent control. AA., Ascorbic acid; Bu., *n*-butanol; E. E., ethanol extract; EA, ethyl acetate; GA., Gallic acid; MC., methylene chloride; PE: petroleum ether.

Characterization of the isolated phytochemicals

A total of eight compounds have been isolated from the leaves of *S. taccada* (Gaertn.) Roxb. leaves (Figure1). Five compounds have been isolated from PE. One compound from EA, while two compounds were identified in BuOH fraction. The identity of these compounds was based on their color reaction with different spraying reagents, melting points, spectral data and by comparison to the literature.

Compound 1 based on the previous data, is a hydrocarbon identified as *n*-tetradecane ($C_{14}H_{30}$). Compounds 2-4 are identified as alpha amyrin palmitate, alpha amyrin acetate and alpha amyrin based on their color reaction, melting points and spectral data and by the comparison to the published literature [24, 26], [25], [27, 28] for compounds **2, 3** and **4** respectively. Compound **5** exhibited a sterol behavior, where the mass scale showed a molecular ion peak at m/z 412 reliable with the molecular formula $C_{29}H_{48}O$, from the previous data together with its melting point, other spectral data and the literature [27, 30], it is identified as stigmasterol.

Compounds 6, 7 presented a flavonoid character after acid hydrolysis, compound **6** resulted in an aglycone moiety of flavone type**.** UV Spectral Data of compound 6 confirmed its flavone nature indicated by absorption of band I at 348 nm in methanol spectrum, which is luteolin. The absence of a free hydroxyl group at 7 positions was confirmed from the lack of bathochromic shift in band II with NaOAc reagent indicating that 7 position of flavone is substituted, so its flavone glycoside [17]. The sugar moiety was identified as glucose, which was confirmed by spotting versus authentic samples, so 6 was recognized as luteolin-7-*O*-*β*-glucoside. Compound **7** displayed a spectrum typical to that of a 3-OH substituted quercetin derivative [31] by comparison with published data [32] over and above co-chromatography with a genuine sample, 7 was identified as rutin.

Compound 8 was obtained as white amorphous powder soluble in methanol and gave reddish-violet color with *p*-anisaldehyde/H_2SO_4. Analysis using 1D and 2D-NMR spectral data allowed for the assignment of the compound. Some distinctive characteristics of compound 8 were concluded upon careful examination of its multiple spectra. ^{13}C-NMR revealed the presence of three carbons with downfield shifts at δ_c 174.35 (C-1), 171.7 (C-9) and 167.34 (C-11), indicating the presence of three carboxylic/ester groups. HMBC spectrum showed a correlation of the carbon at δ_c 167.34 (C-11) is conjugated to a double bond through its correlation to the olefinic proton at δ_H 7.4 (H-3'), which in turn showing a correlation to a quaternary olefinic carbon at δ_c 112 (C-4') and a further correlation to an oxi carbon at δ_c 96.4 (C-1'). The presence of terminal double bond could be deduced from the methylene signals in HSQC spectrum recorded at δ (5.3, 119.2) (HC-8'). The presence of three methoxy groups was confirmed by their resonance in HSQC spectrum, where two of them are identical resonating at 3.6 and 49.9 ppm (HC-11', 12') and the third one resonating at δ 3.96 and 52(HC-10). The signals in the region of 3.2-4.7 including one anomeric proton resonating at δ 4.7 (1H, br. s, H-1') supported the presence of a sugar unit. From the previous data, together with physical characters of the compound, it is concluded that compound 8 is a bis-iridoid glycoside (heterodimer) consisting of three parts, part A was assigned for secoiridoid moiety while part B was assigned for iridoid moiety and part C for the sugar unit, further confirmation of the structure was performed by a complete analysis of the different NMR spectra.

Part A was assigned for the secoiridoid moiety due to the presence of olefinic proton at δ_H 7.48 (H-3'), terminal double bond with two methylene protons resonating at δ_H 5.36 (2H-8') suggesting the presence of secoiridoid moiety, [35] this was confirmed by HSQC spectral data, where a signal at δc 152.28 which assigned for C-3' correlates to H-3' at δ_H 7.48 and a signal at δ_c 134.5 assigned for C-7' has a correlation to H-7' confirming the presence of terminal double bond of secoiridoid part. The presence of two methoxy groups (Me-11', 12') as a part of secoiridoid structure is confirmed from HSQC spectrum δ 3.6, 49.9 (HC-11', 12'). **Part B** was assigned for the iridoid moiety and characterized by the occurrence of two carbonyl groups at δc 171.7 (C-9) and 174.35 (C-1). Besides, the NMR data indicated the presence of iridoid-γ-lactone moiety, [36] the presence of the methyl group of the iridoid part was also confirmed by HSQC, which resonates at δ 1.16, 13.06 (HC-8). **Part C** the sugar part evidenced from the ^{13}CNMR and HSQC showed six signals assigned for the hexose sugar moiety. HSQC spectrum showed signals of hexose sugar H/C at *δ* (4.7, 98.8), (3.38, 73) and (3.39, 76.6), (3.31, 70.1), (3.33, 76.8) and (3.61, 62.8) which were assigned for HC-1''-HC-6'', respectively. Examination of HMBC spectra of **8** confirmed the site of glycosidation by H-1'', C-1' correlation at *δ* H/C (4.7, 96.4). As of the spectral information and by association with those previously available data [8]. The identity of sugar moiety was concluded to be *β*-glucose and **compound 8** could be identified as Alidyjosioside.

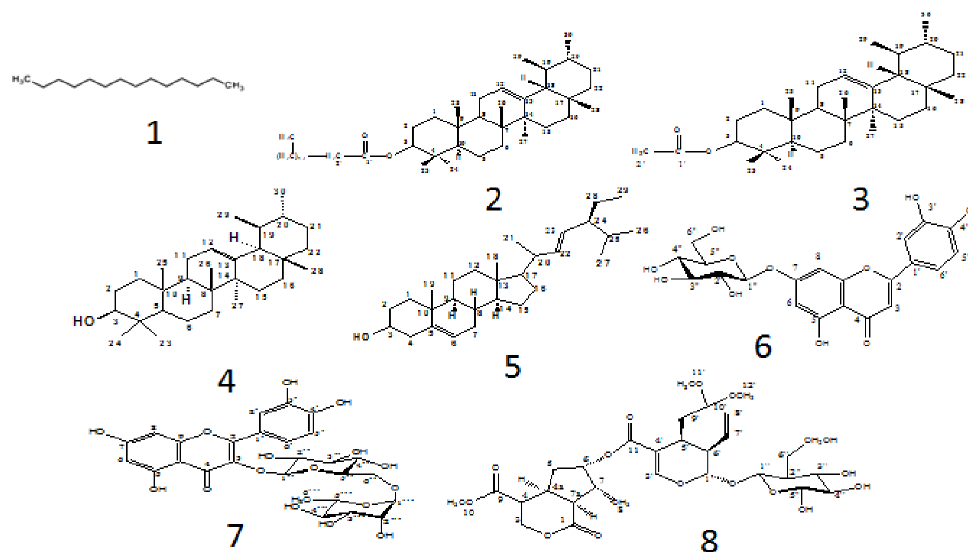

Fig. 1: Isolated compounds 1-8 from the ethanol extract of *S. taccada* leaves

DISCUSSION

There was a correlation between the inhibition zone width of the agar diffusion method and MIC values of the broth dilution method. The fractions that showed the highest zone of inhibition also had the minimum MIC values on all the tested microorganisms. Based on these findings, BuOH fraction showed the highest antimicrobial activity in addition to its antimycobacterial activity among the tested plant fraction followed by PE and finally EA.

By reviewing the available literature, it was found that the genus *Scaevola* exhibited cytotoxic activity against wide variety of cancer cell lines, such as the brain, skin, colon, pancreas, breast, lung, and ovary cell carcinoma [38]. Among the tested fractions, PE showed a cytotoxic against breast carcinoma cell line (MCF-7), while BuOH exhibited cytotoxic activity against colon carcinoma cell line (HCT). The cytotoxic effect of the PE fraction against MCF-7 due to its triterpene content (ursane type), while the activity of the BuOH against HCT cell line may be due to iridoid glucoside content [33].

Among the fractions, EA exhibited an antioxidant activity at relatively low concentrations followed by the *n*-butanol fraction. Consequently, obtained results were comparable to that previously reported [12].

Based on the spectral data, *n*-tetradecane ($C_{14}H_{30}$), α-amyrin palmitate, and α-amyrin acetate as much as the accessible literature alarmed; this is the first report on their isolation from the genus *Scaevola* [25]. *α*-amyrin was previously isolated from the leaves of *S. taccada* (Gaertn.) Roxb. leaves [29]. By reviewing the literature, stigmasterol is previously isolated from additional *Scaevola* kind, [31] but this is the first report on its isolation from *S. taccada* (Gaertn.) Roxb. leaves. Luteolin-7-*O*-*β*-glucoside, and (Quercetin-3-*O*-*α*-L-rhamnosyl-*β*-D-glucopyranoside) were identified in other *Scaevola* species, [8] but this is the first time to be isolated from *S. taccada* (Gaertn.) Roxb. leaves. Allidyjosioside was previously isolated from the genus, [8] but it is the first time for its isolation from *S. taccada* (Gaertn.) Roxb. leaves.

CONCLUSION

In conclusion, the *in vitro* biological activities of the locally cultivated *S. taccada* (Gaertn.) Roxb. leaves. assessed for the first time in this study, following those concerning the exotic plant. Previous biological studies were carried on crude alcohol extracts. The current fractionation procedure and evaluation of the bioactivities of the different extracts could help in the localization and isolation of the individual bioactive constituents. These compounds may be helpful for further chemo taxonomical studies.

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AUTHOR CONTRIBUTIONS

All authors were contributed to the idea, design the study, draft the article, review the data, and edit the article.

CONFLICT OF INTERESTS

Authors declare no conflict of interest

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