PHYTOCONSTITUENTS AND BIOLOGICAL EVALUATION OF *JUSTICIA SPICIGERA* CULTIVATED IN EGYPT

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Abstract

Four compounds were isolated from the aerial parts of *Justicia spicigera* Schltdl. (Acanthaceae) and were identified as uracil (1), uridine (2), megastigmane vomifoliol-9-O-*β*-D-Glucopyranoside (3) and *β*-sitosterol (4). The structures of these compounds were characterized by spectroscopic methods (IR, ^{1}H-NMR, APT, 2D NMR and EI/MS). The different crude extracts showed selective inhibitory activity against *Mycobacterium tuberculosis* (TB), remarkable cytotoxic activities against Human breast cancer (MCF-7) cell lines and Human lung carcinoma (A-549) cell lines and weak antimicrobial activities. This is the first report concerning the isolation of compounds (1-3) from genus *Justicia*.

Key words: *Justicia spicigera*, phytoconstituents, Anti-TB and Cytotoxicity

Introduction

Acanthaceae is a large family comprising, 4300 species in 346 genera (*Wasshausen* & Wood, *2004*). Members of this family are found mostly in the tropics, but occur also in the temperate region, mainly in the Mediterranean *(Mabberley* & *David, 1993*). *Justicia* is the largest genus of Acanthaceae, with approximately 600 species that are found in pantropical and tropical regions (*Durkee, 1986*). The species of *Justicia* are described as erect perennial herbs or subshrubs (*Deng et al., 2016*). Leaves present cystoliths and are petiolate with a leaf margin that is usually entire. Species of *Justicia* are widely used in folk medicine to treatment of respiratory, gastrointestinal, and heart diseases, some of them are used for treatment of tuberculosis, diabetes, cancer, inflammation, rheumatism, and arthritis, also exhibiting antimalarial, antibacterial, antiviral, anti-HIV, and anthelminthic properties (*Correa & Antonio, 2012; Gomez-Verjan et al.,2012*). Compounds of different chemical classes have been isolated from *Justicia* species, such as coumarins, flavonoids, alkaloids, iridoids, diterpenes, and triterpenes. In addition, different lignan compounds are isolated from genus *Justicia* (*Correa & Antonio, 2012*; *Awan et al., 2014; Wen-Kun al., 2014*). *Justicia spicigera* plant is a shrub that grows in Mexico and known as muicle. It is used as immunostimulatory (*Alonso-Castro et al., 2012*). In traditional medicine it is mainly used against dysentery either from microbes or parasites and has an effect on intestinal motility. *Justicia spicigera* contains various bioactive compounds such as carbohydrates, pectins, glycosides, pigments, essential oils and minerals. Phenolic compounds, flavonoids such as kaempferitrin, and kaempferol trirhamnoside have been isolated from leaves and flowers (*Baqueiro-Pena et al., 2014*).

Material and methods:

Experimental:

General experimental procedures: EI/MS spectra were measured using El/MS 502 mass spectrometer having a direct inlet system and operating at 70eV. IR spectra were carried out on a Nicolet 205 FT IR spectrometer connected to a Hewlett-Packard Color Pro. Plotte. NMR spectra were recorded on a Varian Mercury 400 MHz spectrometer at 400 (^{1}H) and 100 MHz (APT) in DMSO-*d*, $CDCl_3$ and CD_3OD solution and chemical shifts were expressed in δ (ppm) with reference to TMS and coupling constant (*J*) in Hertz. Column chromatographic separation was performed on silica gel 60 (Si gel 60, Merck), sephadex LH-20 (Pharmacia) and C-18 Column (reversed SPE) (Phenomenex). TLC was performed on precoated TLC plates with silica gel 60 F254 (0.2mm, Merck). Developed chromatograms were visualized by spraying with 1% vanillin-H_2SO_4, followed by heating at 100 ^{0}C for 5 min.

Plant material:

Justicia spicigera aerial parts were collected in March 2015 from Giza Zoo garden and were kindly identified by staff members of Faculty of Science, Ain-shams University and Teriza Labib, Botanist of Orman Botanical Garden. A voucher herbarium specimen had been deposited in the Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, Nasr city, Cairo, Egypt.

Extraction and isolation:

The aerial parts of ***Justicia spicigera*** (3Kg) were air-dried, ground to fine powder and exhaustively extracted with 70% methanol three times to yield 330g of a dark solid extract, which was then suspended in water (500ml) and successively partitioned with *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol.

The *n*-butanol extract (30gm) was chromatographed on silica gel column (VLC) and eluted with dichloromethane-methanol (100:0–50:50) to obtain five fractions of A, B, C, D and E. Fraction B (1.13g) was rechromatographed on series of silica gel columns and eluted with dichloromethane-methanol (100:0-80:20 then 93:7-75-25) followed by final purification on Sephadex LH-20 columns eluting with 100% MeOH to afford compound 1 (20mg). Fraction C (1.2g) was rechromatographed on silica gel column and eluted with dichloromethane-methanol (100:0-75-25) followed by final purification on Sephadex LH-20 column eluting with 100% MeOH and C-18 column water-methanol (100:0-70:30) to afford compounds 2 (7mg) and compound 3 (10mg).

The ethyl acetate extract was chromatographed on silica gel column (VLC) and eluted with *n*-hexane–ethyl acetate gradient (100:0–50:50) to obtain four fractions of A, B, C, and D. Fraction A of ethyl acetate was further rechromatographed on silica gel column and eluted with *n*-hexane–ethyl acetate gradient (100:0–50:50) followed by final purification on sephadex LH-20 column eluting with 100% MeOH to give compound 4 (16mg).

Compound [1]: White amorphous powder; ^{1}H-NMR (400 MHz, DMSO-d_6), δ11 (brs, NH-1), 5.4 (1H,d ,J= 7.6Hz, H-5), 7.4 (1H,d, J=7.6Hz, H-6); APT-NMR (100MHz, DMSO-d_6), δ 164.35 (C-2), 151.52 (C-4) , 100.23 (C-5), 142.20 (C-6); EI /MS at m/z 112 $[M]^+$ (calc. for $C_4H_4N_2O_2$), 113 $[M+1]^+$.

Compound [2]: Yellowish brown amorphous powder; ^{1}H-NMR (400 MHz, CD_3OD), δ 5.7 (1H , d, J = 8Hz, H-5), 8.0 (1H, d, J = 8Hz, H-6), 5.9 (1H, d, J = 4.8Hz, H-1'), 3.5 (1H, m, H-2'), 4.0 (1H, m, H-3'), 4.17 (1H, m, H-4'), 3.7 (1H , dd , J= 12, 2.8Hz, H-5'a), 3.8 (1H , dd, J = 12 , 2.8Hz, H-5'b); APT-NMR (100 MHz, *CH_3OD*) δ 166.22 (C-2), 152.48 (C-4), 102.71 (C-5), 142.71 (C-6), 90.65 (C-1'), 75.66 (C-2'), 71.26 (C-3'), 86.31 (C-4'), 62.23 (C-5').; EI/MS at m/z 244 $[M]^+$ (calc. for $C_9H_{12}N_2O_6$, 245 $[M+H]^+$, and 140 $[C_5H_4N_2O_3]^+$.

Compound [3]: Yellowish brown crystals, ^{1}H-NMR (400 MHz, DMSO-d_6), δ 2.02 (1H, d , J =17Hz, H-2a), 2.5 (1H, d, J =17Hz, H-2b), 5.76 (1H, brs, H-4), 5.9 (1H,d , J= 15.6Hz, H-7), 5.6 (1H, dd, J= 6, 15.6Hz, H-8), 4.4 (1H, m, H-9), 1.2 (3H,d, J= 604Hz, H-10), 0.93 (3H, S, H-11), 0.91 (3H, S, H-12), 1.8 (3H.S, H-13), **glucose moiety;** δ 4.01 (1H, d, J=7.6Hz, H-1'), 2.9 (1H, m, H-2'), 3.5 (1H, m, H-3'), 3.02 (1H, m, H-4'), 3.8 (1H, m, H-5'), 3.7 (2H, m, H-6'); APT-NMR (100 MHz, DMSO-d_6) δ 49.82 (C-1), 41.42 (C-2), 197-87 (C-3), 126.01 (C-4), 164.27 (C-5), 78.39 (C-6), 132.10 (C-7), 131.92 (C-8), 77.62 (C-9), 22.5 (C-10), 24.5 (C-11), 23.5 (C-12), 19.1 (C-13); **glucose moiety;** δ 100.4 (C-1'), 72.5 (C-2'), 73.75 (C-3'), 70.5 (C-4'), 77.4 (C-5'), 61.5 (C-6'); EI-MS at m/z 386 $[M]^+$(calc. for $C_{19}H_{30}O_8$), 387 $[M+H]^+$, and 149 $[C_{10}H_{13}O]^+$.

Compound [4]: White crystals, ^{1}H-NMR (400 MHz, DMSO-d_6), 3.6 (1H, m, H-3), 5.4 (1H, m, H-6), 0.63 (3H, S, H-18), 0.95 (3H, S, H-19), 0.83 (3H,d, J= 6.5Hz, H-21), 0.80 (3H, d, J=6.5Hz, H-26), 0.87 (3H, d, J=6.5Hz, H-27), 0.92 (3H, t, J=7Hz, H-29); APT-NMR (100 MHz, DMSO-d_6) δ 33.86 (C-1), 31.79 (C-2), 71.95 (C-3), 39.92 (C-4), 140.91 (C-5), 121.86 (C-6), 31.6 (C-7), 31.79 (C-8), 50.28 (C-9), 36.65 (C-10), 21.22 (C-11), 37.40 (C-12), 42.43 (C-13), 56.91 (C-14), 23.21 (C-15), 28.39 (C-16), 56.20 (C-17), 12.0 (C-18), 19.54 (C-19), 36.29 (C-20), 19.17 (C-21), 34.09 (C-22), 24.44 (C-23), 45.98 (C-24), 29.29 (C-25), 19.96 (C-26), 18.92 (C-27), 23.21 (C-28), 12.12 (C-29); EI/MS m/z 414 $[M]^+$ (calc. for $C_{29}H_{50}O$).

Evaluation of antimicrobial activity

Agar well diffusion method:

The antimicrobial activity of different extracts of ***Justicia spicigera*** aerial parts was tested against a range of micro-organisms including two Gram-positive bacteria, two Gram-negative bacteria and two fungal species using the agar well diffusion assay method (*Hindler et al., 1994*) and the results were shown in (Table 1). All extracts were tested in concentration 5mg/ml and the inhibition zone was measure around each well after 24h.

Evaluation of antituberculosis activity

Microplate Alamar Blue Assay:

The antituberculosis activity of different crude extracts of ***Justicia spicigera*** was evaluated using the microplate alamar blue assay (*Franzblau et al., 1998*) which was performed in black, clear-bottomed, 96 well microplates. Outer perimeter wells were filled with sterile water to prevent dehydration in experimental wells. Extracts dilutions

were prepared in dimethyl sulfoxide and subsequent two-fold dilutions were performed in microplates 0.1 ml of 105 CFU/ml *Mycobacterium tuberculosis* inoculum was added to wells, additional control wells consisted of bacteria only as control. The different extracts and Isoniazide (standard drug) were tested at concentration of 100 µg. Starting at day 4 of incubation, 20 µl of alamar blue solution and 12.5 µl of 20% tween 80 were added to the entire plate. Plates were incubated at 37^0C, and results recorded at 24 h post reagent addition at 590 nm. Percent inhibition was defined as (mean of test well/mean of B wells) × 100.

Evaluation of cytotoxic activity:

Cell culture:

Human breast cancer (MCF-7) and Human lung cancer (A-549) cell lines were obtained from the National Cancer Institute (Cairo, Egypt) and maintained in Roswell Park Memorial Institute medium (RPMI1640) supplemented with 100 mg/ml of streptomycin, 100 units/ml of penicillin and 10% of heat-inactivated fetal bovine serum in a humidified 5% (v/v) CO2 atmosphere at 37°C.

Cytotoxic assay:

The cytotoxic activity of total extract, *n*-hexane, dichloromethane, ethyl acetate, *n*- butanol and water extract of ***Justicia spicigera*** aerial parts (serial dilutions 0-500 µg/ml) were evaluated against Human breast cancer (MCF-7) and Human lung cancer (A-549) cell lines using viability assay (*Mosmann & Tim, 1983*). After the end of the incubation period, media were aspirated and the crystal violet solution (1%) was added to each well for at least 30 minutes. The stain was removed and the plates were rinsed using tap water until all excess stain is removed. Glacial acetic acid (30%) was then added to all wells and mixed thoroughly, and then the absorbance of the plates were measured after gently shaken on microplate reader using a test wavelength of 490 nm. Treated samples were compared with the cell control in the absence of the tested compounds. All experiments were carried out in triplicate. The cell cytotoxic effect of each tested extract was calculated. The optical density was measured with the microplate reader to determine the number of viable cells and the percentage of viability was calculated as [1- (ODt/ODc)]x100 where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The relation between surviving cells and drug concentration is plotted to get the survival curve of each tumor cell line after treatment with the specified compound. The 50% inhibitory concentration (**IC50**), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose response curve for each concentration using Graphpad Prism software.

Results and discussion

Dried aerial parts of ***Justicia spicigera*** were extracted with 70% methanol and then fractionated with *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol. From these extracts and by using combined chromatographic separations, four compounds were isolated (Fig. 1). Their structures were elucidated using spectroscopic methods. The isolated compounds were identified as:

Uracil [**1**], Rf = 0.8, TLC solvent system (chloroform-methanol-water 80:20:2), obtained as white powder and showed a molecular ion peak at m/z 112 [M]+ in EI/MS.

In the ^{1}H-NMR spectrum of compound 1, two doublets of typical olefinic proton signals at δ 7.4 (J = 7.6Hz) and 5.4 (J= 7.6Hz) were observed. In APT-NMR, C-6 & C-5 were confirmed by presence of signals at δ_C 142.20 & 100.23 ppm respectively. Accordingly, the structure of compound 1 was elucidated as uracil by comparison of the spectral data, as described in the literature *(Huo et al., 2005*).

Uridine [**2**], R_f = 0.31, TLC solvent system (chloroform-methanol-water 80:20:2), obtained as yellowish brown powder and displayed a molecular ion peak at m/z 244 [M]+ in EI/MS. In the ^{1}H-NMR spectrum of compound 2, two doublets of typical olefinic proton signals at δ 8.0 (J = 8Hz) and 5.7 (J =8Hz) were observed which also confirmed by presence of signals at δ_C 142.71 & 102.71 ppm in APT-NMR. The presence of signal at δ_H 5.9 (1H, d, J= 4.8Hz) assigned for anomeric proton at C-1' which also confirmed by presence of signal at δ_C 90.65 in APT-NMR spectrum. The ^{1}H and APT-NMR spectra were in close agreement with those of a previous report (*Huo et al.,2005*).

Vomifoliol-9-O-*β*-D-Glucopyranoside [**3**], Rf = 0.6, TLC solvent System (chloroform-methanol 80:20), obtained as yellowish brown crystals and displayed a molecular ion peak at m/z 386 [M]+. The ^{1}H-NMR spectrum showed signal at δ_H 5.76 ppm (1H, S) indicates olefenic proton at C-4 which also confirmed by signal at δ_c 126.01 ppm in APT-NMR spectrum. Signal at δ_H 5.9 ppm (1H, d, J= 15.6Hz) assigned for H-7 and at δ_H 5.6 ppm (1H, dd, J=15.6 & 6Hz) assigned for H-8, which also confirmed by signals at δ_c 132.1 and 131.92 ppm in APT spectrum assigned for C-7 and C-8 respectively. H-9 was identified by presence of signal at δ_H 4.4 ppm (1H, m) which also confirmed by signal at δ_c 77.62 ppm assigned for C-9, while the two diasteriotopic protons at C-2 identified by signals δ_H 2.02 ppm (1H, d, J=17Hz) & 2.5 ppm (1H,d, J=17Hz) assigned for H-2a and H-2b and also confirmed by signal at δ_c 41.42 ppm while signals at δ_c 197.87, 164.27 & 78.39 ppm assigned for quaternary carbons C-3, C-5, and C-6 respectively. Singlet peaks at δ_H 0.93 (3H,S), 0.91 (3H,S) , 1.8 (3H, S) assigned for H-11, H-12 and H-13 respectively, which also confirmed by signals at δ_c 24.5, 23.5 and 19.1 ppm assigned for C-11, C-12 & C-13 in APT spectrum respectively, while the peak at δ_H 1.2 (3H, d, J= 6.4Hz) assigned for H-10 which also confirmed by signal at δ_c 22.5 ppm . Glucose moiety was identified by the presence of signal at δ_H 4.01 ppm (1H,d, J= 7.6 Hz) suggested an anomeric proton with *β*-configuration which also was confirmed by signal at δ_c 100.4 ppm assigned for C-1' (anomeric carbon). The compound 3 is suggested to be vomifoliol-9-O-*β*-D-Glucopyranoside by comparison it's spectral data with those in literature *(Herderich et al*., *1992*).

β-sitosterol [**4**], Rf = 0.5, TLC solvent systems (*n*-hexane-ethyl acetate 80:20), obtained as white crystals and displayed a molecular ion peak at m/z 414 $[M]^+$. Signals in the ^{1}H-NMR spectrum were observed mainly in the upfield region. The spectra exhibited two signals with high chemical shifts values; the first one resonated in the olefinic region and the other one was observed a little up field region. The olefinic signal at δ 5.3 (1H, m) appeared to be characteristic of the sterols, and it was assigned to H-6 proton in the *β*–sitosterol (4) chemical skeleton. The ^{1}H-NMR spectra of compound 4 also exhibited a signal corresponding to the proton connected to C-3 hydroxyl group which appeared as a multiplet at δ 3.52 (1H, m). The APT- NMR spectra exhibited 29 carbon signals, characteristic of phytosterols. Signals appeared at δ 33.86 (C-1), 31.79 (C-2), 71.95 (C-3), 39.92 (C-4), 140.91 (C-5), 121.86 (C-6), 31.6 (C-7), 31.79 (C-8), 50.28 (C-9), 36.65

(C-10), 21.22 (C-11), 37.40 (C-12), 42.43 (C-13), 56.91 (C-14), 23.21 (C-15), 28.39 (C-16), 56.20 (C-17), 12.0 (C-18), 19.54 (C-19), 36.29 (C-20), 19.17 (C-21), 34.09 (C-22), 24.44 (C-23), 45.98 (C-24), 29.29 (C-25), 19.96 (C-26), 18.92 (C-27), 23.21 (C-28), 12.12 (C-29). These data were in agreement with published literature for *β*-sitosterol and by comparison with authentic sample (*Pierre and Moses, 2015; Patra et al. 2010*).

To our best knowledge, compounds [**1-3**] suggested to be first reported in genus *Justicia*.

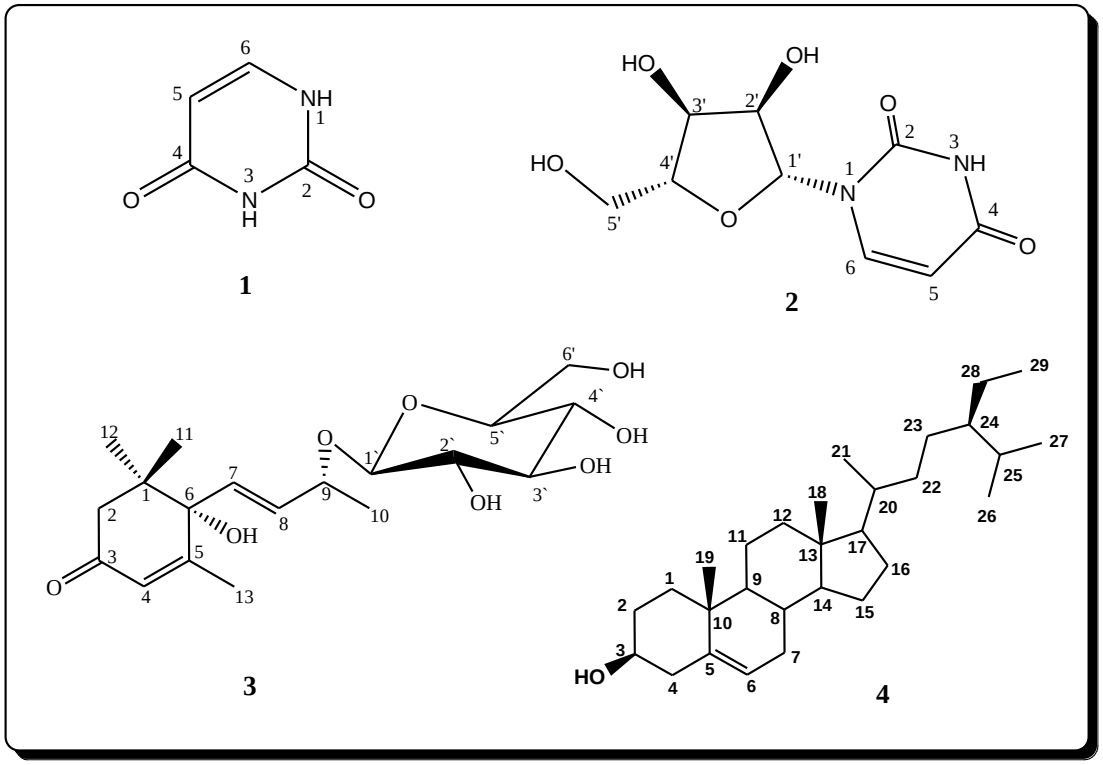

Figure 1. Compounds 1-4.

Antimicrobial activity of different extracts of *Justicia spicigera*

The current study provides an evidence that all extracts of ***Justicia spicigera*** showed no activity against *Aspergillus flavus and candida albicans* while the total extract and dichloromethane extract showed moderate activity against *Bacillus subtilis* (11mm and 18mm) respectively in comparison with ciprofloxacin (24mm) and the ethyl acetate extract showed moderate activity against *staphylococcus aureus* (14mm). The ethyl acetate extract was the only one showed remarkable activity against *Salmonella typhimurium* (16mm) in comparison with Gentamycin (17mm) and weak activity against *Escherichia coli* (16mm) in comparison with Gentamycin (30mm).

Table 1: Antimicrobial activity of *Justicia spicigera* extracts (zone of inhibition in mm):

	Control	**Total extract**	***n*-hexane**	**DCM**	**EtoAc**	***n*-BUOH**	**H_2o**
Fungi	Ketoconazole						
spergillus flavus	16	NA	NA	NA	NA	NA	NA
Candida albicans	20	NA	NA	NA	NA	NA	NA
Gram +ve Bacteria	Ciproflox acin						
Staphylococcus aureus	24	NA	NA	NA	**14**	NA	NA
Bacillus subtilis	26	**11**	NA	**18**	NA	NA	NA
Gram -ve Bacteria	Gentamycin						
Salmonella typhimurium	17	NA	NA	NA	**16**	NA	NA
Escherichia coli	30	NA	NA	NA	**15**	NA	NA

DCM=dicloromethane *n*-BUOH= *n*-butanol EtoAc= ethyl acetate
NA= no activity

Antituberculosis activity of different extracts of

The *n*-butanol extract of *Justicia spicigera* showed remarkable antibacterial activity against *Mycobacterium tuberculosis* (TB) with inhibitory percentage 61.48% in comparison with isoniazid 93.24% while the ethyl acetate and dichloromethane extracts showed inhibitory percentage 52.31% and 56.34% respectively as shown in (Table 2).

Sample	**Inhibitory % against Mycobacterium tuberculosis**
Standard (Isoniazid)	93.24 ± 2.1
Total extract	39.81 ± 0.72
n-Hexane fraction	NA
DCM fraction	56.34 ± 2.1
EtOAC fraction	52.31 ± 0.63
n-BUOH fraction	61.48 ± 1.5
H_2O fraction	NA

Table 2: Antituberculosis activity of *Justicia spicigera* extracts:

DCM=dicloromethane *n*-BUOH= *n*-butanol EtoAc= ethyl acetate NA= no activity

Cytotoxic activity of different extracts of *Justicia spicigera*

The *n*-hexane, ethyl acetate and *n*-butanol extracts showed remarkable cytotoxic activities against Human breast cancer (MCF-7) cell line with IC_{50} values 26.6, 49.3 and 43.4 µg/ml respectively and Human lung carcinoma (A-549) cell lines with IC_{50} values 23.7, 19.5 and 30.1 µg/ml respectively whereas weak activities were detected with the dichloromethane extract as shown in (Table 4)

Sample	**IC_{50} µg/ml (MCF-7)**	**IC_{50} µg/ml (A-549)**
Total extract	271	186
n-Hexane fraction	26.6	23.7
DCM fraction	116	101
EtOAC fraction	49.3	19.5
n-BUOH fraction	43.4	30.1
H_2O fraction	77.9	220

Table 3: Cytotoxic activity (IC_{50}) of *Justicia spicigera* extracts:

IC_{50}: the concentration required to cause toxic effect in 50% of intact cells.

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الملخص العربي

"التقييم البيولوجي والمواد الفعالة لنبات البستشيا الحمراء المنزرع في مصر"

للسادة الدكاترة

أحمد عثمان ، وائل محمدي عفيفي ، محمد عبدالرؤف ، لطفي دياب إسماعيل

مـــــــن

قسم العقاقير – كلية الصيدلة – جامعة الأزهر – القاهرة – مصر.

تم فصل أربعة مركبات من نبات البستشيا الحمراء بالطرق الكروماتوجرافية المختلفة وتم التعرف على المركبات المفصولة باستخدام الطرق الطيفية المختلفة وهي عبارة عن مركبين من مشتقات أحماض النوكليوتيدات يوراسيل (١) ، يوريدين (٢) ومركب ميجاستيجمان فوميفوليول-٩- بيتا جلوكوبيرانوسيد (٣) ومركب بيتا ستيروبد بيتاسيتوستيرول (٤).

أثبتت الدراسة الحيوية أن لخلاصة الخل الإيثيلي والميثلين كلوريد والبيوتانول تأثيرا قويا ضد ميكروب السل الرئوي بنسب تثبيط عالية ٥٢,٣١ ٪ و٥٦,٣ ٪ و٦١,٤٨ ٪ على الترتيب كما أظهرت الدراسة أيضا أن لخلاصة الهيكسان تأثيرا قويا ضد خلايا سرطان الثدي والرئة وتأثيرا قويا لخلاصة الخل الإيثيلي ضد سرطان الرئة.