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Research Article

PHYTOCHEMICAL AND BIOLOGICAL STUDIES ON *PERSICARIA SALICIFOLIA* BROUSS. EX WILLD GROWING IN EGYPT

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ABSTRACT

The phytochemical investigation of *Persicaria salicifolia* Brouss. ex Willd herb resulted in isolation of trans phytol, *β*-sitosterol, *β*-sitosterol-3-O- -D-glucoside, luteolin, quercetin, quercitrin, hyperoside and rutin. The compounds were isolated using different chromatographic techniques. The isolated compounds were identified using different spectroscopic techniques including UV, IR, MS, ^{1}HNMR and ^{13}CNMR.The antitumor effect of total ethanolic extract of *Persicaria salicifolia* herb against solid Ehrlich carcinoma (SEC) was examined and the results revealed that the extract has strong antitumor activity. In vitro cytotoxic assay against human breast carcinoma (MCF-7) and prostate carcinoma (PC3) cell lines was carried out using different fractions of alcoholic extract. Methylene chloride and ethyl acetate fractions exhibited the highest activity against breast carcinoma, while petroleum ether extract exhibited the highest activity against prostate carcinoma cell line. Phytol, luteolin and quercitrin were isolated for the first time from *Persicaria salicifolia* Brouss. ex Willd.

Key words: Antitumor, Cytotoxic, Ehrlich carcinoma, MTT assay, *Persicaria salicifolia*.

INTRODUCTION

Family Polygonaceae includes many medicinally important species[1]. Six genera of this family are present in Egypt[2]; some species have been traditionally used for treatment of some diseases. For instance *Rumex dentatus* known as khallila, is used for treatment of pneumonia, cough, abscesses, stomachache and tumor[3,4].

Persicaria salicifolia is one of seven *Persicaria* species present in Egypt[2], and it grows in the Nile Delta as a helophyte and geophytes. It is a hydrophyte found on the river banks, shores of lakes, drains and canals [5]. Previous phytochemical studies focused on flavonoids and flavonoid glycosides[6-8]. Besides, only antioxidant activity was investigated[8]. Given these considerations, this study was undertaken to explore the antitumor and cytotoxic effects of *Persicaria salicifolia* total extract, different fractions and some isolated pure compounds.

MATERIALS AND METHODS

Plant materials

Aerial parts of *Persicaria salicifolia* Brouss. Ex Willd. were collected in October 2011 from the banks of fresh water canals in El Gharbia governorate, Egypt. The plant was kindly identified by Prof. Dr. Kamal Hussein Shaltout, Botany Department, Faculty of Science, Tanta University, Egypt. The aerial parts were washed, dried in shade and powdered.

Experimental animals

Adult Swiss female albino mice with an average body weight of 18-25 g were used. The study guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals were applied.

Cell lines

Ehrlich Ascites Carcinoma (EAC) cell line was supplied from Pharmacology and Experimental Oncology Unit of the National Cancer Institute (NCI), Egypt. Human Caucasian breast carcinoma (MCF-7) and human prostate carcinoma (PC3) were obtained from the American Type Culture Collection (ATCC).

Chromatographic materials and chemicals

Precoated TLC silica gel plates G F_{254}, silica gel for column chromatography (70-230 mesh) and reversed phase octadecylsilyl-silica gel (RP-C18) were obtained from E. Merck, Germany. Sephadex LH-20 was purchased from Sigma-Aldrich chemical Co.,USA. Diaion HP-20 was purchased from Mitsubishi Chemical, Japan. Polyamide was purchased from Fluka AG, Germany. Assay kits for analysis of biochemical parameters were purchased from Biodiagnostic, Egypt. Adriamycin® (Doxorubicin. HCl) IV 10 mg/5 mL, Pfizer, was used as a positive control.

Apparatus

Melting point determination was carried out using Gallenkamp melting point apparatus. IR spectra were recorded using KBr disc using Jasco FT/IR-6100 spectrophotometer. UV spectra were recorded using Jasco UV/Vis spectrophotometer, V-530, Japan. EI/MS spectra were recorded on Thermo Scientific ISQ Single Quadrupole MS, USA. ESI spectra were recorded by mass Spectrometer Thermo Scientific ISQ Quantum Access MAX triple Quadrupole, USA. ^{1}H and ^{13}CNMR spectra were measured on a Bruker Avance III 400 MHz Spectrometer,

Germany. Chemical shifts were given on a δ (ppm) scale with tetramethylsilane (TMS) as an internal standard. Tumor size was measured using Vernier caliber Tricle Brand, Shanghai, China. Spectrophotometer (Genesys 10-s, USA) was used for biochemical studies. Microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) was used to measure the absorbance in MTT assay.

Extraction and isolation

The dried powder of *Persicaria salicifolia* Brouss. ex Willd. (2 kg) was extracted with 95% ethanol till exhaustion using cold maceration method. The ethanolic extract was concentrated and evaporated to yield 76 g residue, which was suspended in 50% aqueous ethanol and successively fractionated with petroleum ether (40-60° C), methylene chloride, ethyl acetate and n-butanol.

Petroleum ether extract (2 g) was saponified by boiling under reflux with 30 mL 10% alcoholic KOH for 5 hours, the alcohol was distilled off and 20 mL water was added then extracted several times with ether. The combined ethereal extract was evaporated under reduced pressure to give unsaponifiable matter (1.8 g). The unsaponifiable matter was chromatographed on a silica gel column (3.5 × 34 cm, 95 g) by gradient elution using n-hexane-methylene chloride, then, methylene chloride. The collected fractions (25 mL each) were screened by TLC. Fractions 72-87 eluted with 50% and 60% methylene chloride were rechromatographed on silica gel column (1.0 × 20 cm, 11 g) by gradient elution using n-hexane-methylene chloride. Subfractions 24-34 (1mL each) eluted with n-hexane-methylene chloride (70:30) were combined and evaporated to yield compound **1** (30 mg) after recrystallization from methanol. The compound was obtained as white amorphous powder; R_f value 0.23 (solvent system: hexane-CH_2Cl_2; 3:7); IR (KBr) cm^{-1}: 3428, 2932, 2858, 1732, 1642, 1059 and 962; EI-MS [M^+] m/z 296; ^{1}HNMR [DMSO-d_6, 400 MHz] δ 5.25 (1H, *t*, H-2), 3.93 (2H, *t,* H-1), 1.92 (2H, *t*, H-4), 1.56 (3H, *s*, H-3a), 1.13-1.54 (*m*, CH_2 protons), 0.86 (3H*, br. s*, H-11a), 0.84 (6H, *br.s,* H-15a, 16), 0.82 (3H, *br. s*, H-7a); ^{13}CNMR [DMSO-d_6, 100 MHz] δ58 (C-1), 125.8 (C-2), 136.1 (C-3), 39.7 (C-4), 25 (C-5), 36.5 (C-6), 32.5 (C-7), 37.2 (C-8), 23.7 (C-9), 37.2 (C-10), 32.4 (C-11), 37.1 (C-12), 24.6 (C-13), 39.2 (C-14), 27.8 (C-15), 22.9 (C-16), 16.3 (C-3a), 19.9 (C-7a), 20.1 (C-11a). All assignments were substantiated by DEPT-135 and HSQC experiments.

Fractions 88-97 eluted with 70-90% methylene chloride were combined, concentrated, and recrystallized from methanol to yield compound **2** (75 mg).

The compound was obtained in the form of white needle crystals; m.p (137-139°C); R_f value 0.63 (solvent system: CH_2Cl_2- MeOH; 9.5:0.5); IR (KBr) cm^{-1}: 3428, 2957, 2866, 1645 and 961; EI-MS [M^+] at m/z 414; ^{1}HNMR [$CDCl_3$, 400 MHz] δ 5.34 (1H, *t*, H-6), 3.50 (1H, *m*, H-3), 0.99 (3H, *s*, H-19), 0.91 (3H, *s*, H-21), 0.84 (3H*, t*, H-29), 0.82 (6H, *d, J*=6 Hz, H 26,27), 0.67 (3H, *s*, H-18), 0.67-2.25 (*m*, H-1, 2, 4, 7-9, 11, 12, 14-17, 20, 22-25, 28); ^{13}CNMR [$CDCl_3$, 100 MHz] δ37.3 (C-1), 31.4 (C-2), 71.6 (C-3), 42.3 (C-4), 140.8 (C-5), 121.6 (C-6), 31.9 (C-7), 31.9 (C-8), 50.4 (C-9), 36.5 (C-10), 21.1 (C-11), 39.8 (C-12), 42.3 (C-13), 56.8 (C-14), 24.3 (C-15), 28.2 (C-16), 56.1 (C-17), 11.9 (C-18), 19.0 (C-19), 36.1 (C-20), 18.9 (C-21), 33.9 (C-22), 26.1 (C-23), 45.8 (C-24), 29.2 (C-25), 18.9 (C-26), 19.0 (C-27), 23.1 (C-28), 11.8 (C-29).

Methylene chloride extract (3.6 g) was chromatographed on silica gel column (4.5 × 25.5 cm, 117 g) using methylene chloride-methanol gradient. The collected fractions (50 mL each) were examined by TLC. Fractions 27-30 eluted with 92% methylene chloride in methanol were combined and evaporated to yield a residue (220 mg). The residue was chromatographed on sephadex LH 20 column (2 × 30 cm, 12g) using methanol as mobile phase. Subfractions 25-33 (1 mL each) were pooled, evaporated, and crystallized from methanol to give compound **3** (15 mg).

The compound was obtained as buff yellow powder, R_f value 0.27 (solvent system: CH_2Cl_2-MeOH-H_2O;9: 1: 0.1); UV λ_{max}MeOH: 255, 267, 290 (sh.), 348 nm; +NaOH: 271, 325 (sh.), 407; +$AlCl_3$: 273, 292 (sh.), 320 (sh.), 419; +$AlCl_3$/HCl: 267, 273, 297 (sh.),358, 384; +NaOAc: 271, 328 (sh.), 399; +NaOAc/Boric acid: 262, 290 (sh.), 373, 433 (sh.); ^{1}HNMR [DMSO-d_6, 400 MHz] δ 12.99 (1H, *s*, 5-OH), 7.42 (1H, *dd, J*=2, 8 Hz, H-6`), 7.4 (1H, *d, J*=2 Hz, H-2`), 6.88 (1H, *d, J*=8 Hz, H-5`), 6.66 (1H, *s*, H-3), 6.42 (1H, *d, J*=2 Hz, H-8), 6.18 (1H, *d, J*=2 Hz, H-6); $^{13}$CNMR [DMSO-$d_6$, 100 MHz] δ 164.4 (C-2), 103.2 (C-3), 182.1 (C-4), 161.9 (C-5), 99.4 (C-6), 164.9 (C-7), 94.3 (C-8), 157.8 (C-9), 104.0 (C-10), 121.8 (C-1`), 113.7 (C-2`), 146.3 (C-3`), 150.5 (C-4`), 116.5 (C-5`), 119.5 (C-6`).

Fractions 31-34 were combined and concentrated to give a white precipitate. The precipitate was dissolved in chloroform and recrystallized from methanol to give 30 mg of compound **4**. The compound was obtained as white amorphous powder; R_f value 0.33 (solvent system: CH_2Cl_2-MeOH; 9.5:0.5); IR (KBr) cm^{-1}: 3416, 2957, 2872, 1633, 1461 and 925; ESI-MS $[M+H]^+$ m/z at 577; ^{1}HNMR [DMSO-d_6, 400 MHz] δ 5.34 (1H, *t*, H-6), 4.22 (1H, *d, J*=8 Hz, H-1`), 3.63 (1H, *m*, H-3), 0.96 (3H, *s*, H-19), 0.90 (3H, *s*, H-21),0.84 (3H, *d, J*=6.8 Hz, H-26), 0.83 (3H, *t, J*= 8, H-29), 0.81 (3H, *d, J*=6.8 Hz, H 27), 0.67 (3H, *s*, H-18), 0.67-2.25 (*m*, H-1, 2, 4, 7-9, 11, 12, 14-17, 20, 22-25, 28), 2.93-4.43 (*m,* sugar protons);
^{13}CNMR [DMSO-d_6, 100 MHz] δ37.3 (C-1), 29.8 (C-2), 77.4 (C-3), 38.8 (C-4), 140.9 (C-5), 121.7 (C-6), 31.9 (C-7), 31.9 (C-8), 50.1 (C-9), 37.3 (C-10), 21.1 (C-11), 39.4 (C-12), 42.3 (C-13), 56.7 (C-14), 24.3 (C-15), 28.3 (C-16), 56.0 (C-17), 12.3 (C-18), 19.4 (C-19), 36.0 (C-20), 19.1 (C-21), 33.8 (C-22), 26.0 (C-23), 45.6 (C-24), 29.8 (C-25), 20.2 (C-26), 19.1 (C-27), 23.1 (C-28), 12.3 (C-29), 101.3 (C-1`), 74.0 (C-2`), 77.4 (C-3`), 70.6 (C-4`), 77.2 (C-5`), 62.2 (C-6`).

Ethyl acetate fraction (3.3 g) was chromatographed on silica gel column (4.5 × 23.5 cm, 110 g) by gradient elution using increasing polarity of methylene chloride-methanol mixtures. The collected fractions (25 mL each) were screened using TLC. Fractions 39-50 were pooled, evaporated and subjected to isocratic silica gel column chromatography (1 × 7 cm, 4.5 g) using methylene chloride-methanol-water (90:10:1). Subfractions 2-4 (2 mL each) were combined and concentrated to give white precipitate of compound **4** (8 mg).TLC examination of the supernatant revealed the presence of one major spot. The supernatant was concentrated and recrystallized to give compound **5** (5 mg).

Compound **5** was obtained as yellow amorphous powder; R_f value 0.32 (solvent system: CH_2Cl_2- MeOH-H_2O; 9:1:0.1); UV λ_{max}MeOH: 256, 371; +NaOH: 290, 426; +$AlCl_3$: 271, 455; +$AlCl_3$/HCl: 266, 302 (sh.), 361 (sh.), 427; +NaOAc: 271, 325 (sh.), 381; +NaOAc/Boric acid: 261, 389; ^{1}HNMR [DMSO-d_6, 400 MHz] δ 12.49 (1H, *s*, 5-OH), 7.68 (1H, *d, J*=2 Hz, H-2`), 7.55 (1H, *dd, J*= 2, 8 Hz, H-6`), 6.88 (1H, *d, J*=8 Hz,H-5`), 6.41 (1H, *d, J*=1.6 Hz, H-8), 6.19 (1H, *d, J*=1.6 Hz, H-6); $^{13}$CNMR [DMSO-$d_6$, 100 MHz] δ 148.1 (C-2), 136.1 (C-3), 176.3 (C-4), 161.1 (C-5), 98.7 (C-6), 164.3 (C-7), 93.9 (C-8), 156.6 (C-9), 103.4 (C-10), 122.4 (C-1`), 115.5 (C-2`), 145.5 (C-3`), 147.3 (C-4`), 116.0 (C-5`), 120.5 (C-6`).

Fractions (58-64) eluted with 85 % methylene chloride in methanol were combined and evaporated to yield 400 mg residue. The residue was rechromatographed on silica gel column (1.5 × 18 cm, 12 g) using a gradient elution of methylene chloride-methanol. Subfractions 49-12 (10 mL each) were pooled, evaporated, and further purified using sephadex LH 20 column (1 x 50 cm, 18 g) to give compound **6** (13 mg). The compound was obtained as a yellow amorphous powder; R_f value 0.44 (solvent system: CH_2Cl_2-MeOH-H_2O; 8:2:0.2); UV λ_{max}MeOH: 260, 267, 348; +NaOH: 270, 325 (sh.), 395; +$AlCl_3$: 269, 386; +$AlCl_3$/HCl: 268, 354, 402 (sh.); +NaOAc: 270, 325 (sh.), 370; +NaOAc/Boric acid: 263, 368; IR KBr cm^{-1}: 3416, 1655, 1359, 1303 and 1067; ESI-MS $[M+H]^+$ m/z at 449; ^{1}HNMR [CD_3OD, 400 MHz] δ7.23 (1H, *br.s,* H-2`), 7.2 (1H, *dd, J*=1.8, 8.2 Hz, H-6`), 6.81 (1H, *d, J*=8.2 Hz, H-5`), 6.27 (1H, *br.s,* H-8), 6.19 (1H, *d, J*=1.5 Hz, H-6), 5.25 (1H, *br.s,* H-1``), 3.17-4.12 (*m,* sugar protons), 0.84 (3H, *d, J*=6 Hz, H-6``); ^{13}CNMR [CD_3OD, 100 MHz] δ 157.9 (C-2), 134.8 (C-3), 178.3 (C-4), 161.8 (C-5), 98.5 (C-6), 164.8 (C-7), 93.3 (C-8), 157.1 (C-9), 104.8 (C-10), 121.6 (C-1`), 115.5 (C-2`), 145.7 (C-3`), 148.4 (C-4`), 115.0 (C-5`), 121.5 (C-6`), 102.1 (C-1``), 70.6 (C-2``), 70.7 (C-3``), 71.8 (C-4``), 70.5 (C-5``), 16.3 (C-6``). All assignments were supported by DEPT 135 and HSQC experiments.

Fractions eluted with 85-80 % methylene chloride in methanol (65-82) were pooled and evaporated to yield 940 mg residue. The residue was rechromatographed on silica gel column (2 × 34 cm, 50 g) using gradient elution of methylene chloride-methanol. Subfractions 33-48 (10 mL each) were pooled and rechromatographed on silica gel column (1.5 × 19.5 cm, 16 g) using 10% gradient of methylene chloride-ethyl acetate then ethyl acetate followed by gradient elution of ethyl acetate-methanol. Fractions 126-200 (5 mL each)were combined, evaporated, and rechromatographed on reversed phase C18 silica gel column (1.6 × 10 cm, 9 g) and eluted with gradient elution of methanol-water to yield compound **7** (15 mg). The compound was obtained as a bright yellow amorphous powder; R_f value 0.65 (solvent system: CH_2Cl_2-MeOH-H_2O; 7:3:0.3); UV λ_{max}MeOH 256, 355; +NaOH: 272, 323 (sh.), 369 (sh.), 405; + $AlCl_3$: 269, 420; +$AlCl_3$/HCl: 272, 325 (sh.), 379 (sh.), 393; + NaOAc 269, 352 (sh.), 399; +NaOAc/Boric: 259, 375; IR (KBr) cm^{-1}: 3384, 1655, 1359, 1302 and 1065; ESI-MS $[M+2H]^{+2}$ m/z at 466; ^{1}HNMR [CD_3OD, 400 MHz] δ 7.86 (1H, *d, J*=1.6 Hz, H-2`), 7.60 (1H, *dd, J*= 1.6, 7.2 Hz, H-6`), 6.91 (1H, *d, J*=7.2 Hz, H-5`), 6.42 (1H, *d, J*=1.6 Hz, H-8), 6.23 (1H, *br. s,* H-6), 5.17 (1H, *d, J*=7.6 Hz, H-1``), 3.42-3.88 (*m,* sugar protons); $^{13}$CNMR [$CD_3OD$, 100 MHz] δ 157.4 (C-2), 134.4 (C-3), 178.1 (C-4), 161.6 (C-5), 98.6 (C-6), 164.9 (C-7), 93.4 (C-8), 157.1 (C-9), 104.0 (C-10), 121.6 (C-1`), 116.4 (C-2`), 144.4 (C-3`), 148.6 (C-4`), 114.7 (C-5`), 121.5 (C-6`), 103 (C-1``), 71.8 (C-2``), 73.7 (C-3``), 68.6 (C-4``), 75.8 (C-5``), 60.5 (C-6``).

n-Butanol fraction (10 g) was suspended in deionized water and applied to Diaion HP-20 column (2.5 × 45 cm, 85 g). Elution was carried out using gradient of deionized water-methanol (25% increment). Fractions from each solvent were evaporated and freeze dried. Fractions eluted with 50 and 75% methanol were combined, evaporated (1.3 g) and rechromatographed on polyamide column (2.5 × 46 cm, 35g) using distilled water, followed by gradient elution of methanol-water. Fractions 24-44 (10 mL each) eluted with methanol (40-60%) were pooled, evaporated and lyophilized to yield a residue (140 mg) which rechromatographed on sephadex LH 20 column (1 × 50 cm, 18 g) using methanol for HPLC as eluting solvent. Subfractions 9-17 were combined and evaporated to yield compound **8** (15 mg). The compound was obtained as a bright yellow powder; R_f value 0.36 (solvent system: CH_2Cl_2-MeOH-H_2O; 7:3:0.5); UV λ_{max}MeOH:258, 269 (sh.), 309 (sh.), 357 nm; +NaOH:274, 324 (sh.), 413; +$AlCl_3$:271, 303 (sh.), 409; +$AlCl_3$/HCl: 269, 300 (sh.),362, 395; +NaOAc: 271, 309 (sh.), 408; +NaOAc/Boric acid: 266, 404; IR (KBr) cm^{-1}: 3385, 1651, 1362, 1293 and 1066; ESI-MS $[M+H]^+$ m/z at 611; ^{1}HNMR [CD_3OD, 400 MHz] δ 7.69 (1H, *br. s,* H-2`), 7.65 (1H, *d, J*= 8.4 Hz, H-6`), 6.89 (1H, *d, J*=8.4 Hz, H-5`), 6.42 (1H, *br. s,* H-8), 6.23 (1H, *br. s,* H-6), 5.12 (1H, *d, J*=7.6 Hz, H-1``), 4.88 (1H, *br. s,* H-1```), 3.33-3.84 (*m,* sugar protons), 1.14 (3H, *d, J*= 6.4 Hz, H-6```); ^{13}CNMR [CD_3OD, 100 MHz] δ 157.9 (C-2), 134.2 (C-3), 177.9 (C-4), 161.6 (C-5), 98.8 (C-6), 165.3 (C-7), 93.6 (C-8), 157.2 (C-9), 104.1 (C-10), 121.7 (C-1`), 114.7 (C-2`), 144.5 (C-3`), 148.5 (C-4`), 116.3 (C-5`), 122.1 (C-6`), 103.4 (C-1``), 74.3 (C-2``), 76.8 (C-3``), 70.8 (C-4``), 75.8 (C-5``), 67.2 (C-6``), 101.0 (C-1```), 70.7 (C-2```), 70.0 (C-3```), 72.5 (C-4```), 68.3 (C-5```), 16.47 (C-6```).

Acid hydrolysis of the isolated glycosides [9]

Complete acid hydrolysis was carried out by heating 5 mg of the glycoside with 10 mL of 5% H_2SO_4 in 90 % aqueous methanol at 100 ˚C for 2 hours under reflux. The hydrolysate was then extracted with ethyl acetate and evaporated to obtain the aglycone. The mother liquor of hydrolysate was neutralized with $BaCO_3$ and used to test the sugar moiety.

Biological activity

Antitumor activity against solid Ehrlich carcinoma (SEC)

Antitumor activity against solid Ehrlich carcinoma was carried out according to the method reported by Osman *et al*.[10].

Biochemical study

I- Determination of reduced glutathione (GSH)

Reduced glutathione was determined by the method of Moron *et al* (1979)[11]. The values are expressed as nmoles GSH/mL.

II- Determination of catalase activity (CAT)

Catalase activity was determined according to the procedures reported by Luck[12].

The activity of the enzyme is expressed in the terms of µmole of H_2O_2 consumed/min/mL serum.

III- Determination of superoxide dismutase (SOD)

Superoxide dismutase (SOD) was assayed according to the method reported by Kakkar *et al.* (1984)[13].

IV- Determination of nitric oxide scavenging activity (NO)

The procedures were carried out according to the method reported by Green *et al* (1982)[14]. Comparison between different groups was carried out by analysis of variance test (ANOVA) followed by Tukey test. The level of significance was set at $P \leq 0.05$. SPSS 11 computer software was used to carry out the statistical analysis.

Histopathological study

The excised tumors were immediately fixed in stoppered jars containing formol saline (10 % formalin in normal saline) routinely processed in ascending grades of alcohol, cleared in xylene, then embedded in paraffin. Paraffin embedded sections were cut on charged slides (3-5µm) and stained with hematoxlyin and eosin stain (H & E) to be studied by light microscope[15].

Determination of cytotoxic activity

Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan as described by Mosmann[16]. Different concentrations of extracts and pure compounds were used to give a final concentration of 100, 50, 25, 12.5, 6.25, 3.125 and 1.56 µg/mL in DMSO. Doxorubicin, a

natural cytotoxic agent, is used as a positive control in this study that gives 100% lethality at 100 µg/mL under the same conditions[17, 18]. A statistical significance was tested between samples and negative control (cells with vehicle) using independent t-test by SPSS 11 program. The percentage of change in viability was calculated. A probit analysis was carried for LC_{50} determination using SPSS 11 program.

Table 1: Effect of *P. salicifolia* extract on serum reduced glutathione, catalase activity, superoxide dismutase and nitric oxide in SEC bearing mice

Dose (mg/kg)	GSH nmol/mL	CAT µmol/min/mL	SOD U/L	NO µM
Saline (negative control)	3.82 ± 0.86	18.05 ± 4.94	14.59 ± 4.15	92.49 ± 10.23
P. salicifolia extract				
50	10.10^{a*} ± 1.07	43.42^{a*} ± 9.73	15.95^{d*} ± 2.72	58.70^{acd*} ± 5.44
75	10.81^{a*} ± 1.41	54.12^{a*} ± 11.32	23.09^{a*} ± 5.91	99.07^{b*} ± 8.64
100	11.92^{ab*} ± 0.61	73.61^{abc*} ± 8.70	32.32^{ab*} ± 6.39	98.94^{b*} ± 7.95
Doxorubicin 2.0	1.90^{abcd*} ± 0.41	36.91^{acd*} ± 5.54	20.79^{d*} ± 4.93	101.6^{b*} ± 15.27

Data represented as mean± S.D (n=8). F test (ANOVA, Significance between groups was done using Post Hoc Test (Tukey)
a: significant difference from saline group, b: significant difference from 50 mg/kg, c: significant difference from 75 mg/kg,
d: significant difference from 100 mg/kg, *: Statistically significant at $p \leq 0.05$

Table 2: Cytotoxic effect of methylene chloride and ethyl acetate fractions and phytol against MCF-7 cell line

Concentration (µg/mL)	% Inhibition			
	Methylene chloride	Ethyl acetate	Phytol	Doxorubicin
100	73.8 ± 0.75	79.8 ± 0.85	64.3 ± 0.93	100 ± 0.0
50	53.4 ± 0.34	56.1 ± 0.65	42.3 ± 0.46	100 ± 0.95
25	33.1 ± 0.59	27.7 ± 0.57	16.8 ± 0.28	49.3 ± 0.37
12.5	24.5 ± 0.99	11.9 ± 0.29	7.9 ± 0.19	7.65 ± 0.28
6.25	10.8 ± 0.82	4.3 ± 0.05	2.4 ± 0.1	5.37 ± 0.37
3.12	4.6 ± 0.73	1.9 ± 0.14	0.00	3.72 ± 0.17
1.57	1.3 ± 0.24	0.00	0.00	2.08 ± 0.15
LC_{50} µg/mL	60.1	58.9	74.7	26.1

*Data obtained from three determinations (n=3) and shown as mean ± S.E with confidence level 95%.
LC_{50} was calculated by probit analysis using SPSS 11 program.

Table 3: Cytotoxic effect of petroleum ether fraction, phytol and *β*-sitosterol-*3-O-β*-glucoside against PC3 cell line

Concentration (µg/mL)	% Inhibition			
	Petroleum ether	Phytol	β-sitosterol-*3-O-β*-glucoside	Doxorubicin
100	75.3 ± 1.15	100 ± 0.0	81.3 ± 1.37	100 ± 0.0
50	48.9 ± 0.95	100 ± 1.3	61.2 ± 0.98	100 ± 0.0
25	30.5 ± 0.95	44.5 ± 0.85	40.9 ± 0.84	100 ± 0.95
12.5	22.7 ± 0.79	19.5 ± 0.64	16.8 ± 0.35	43.6 ± 0.93
6.25	9.8 ± 0.57	6.4 ± 0.52	5.4 ± 0.27	7.84 ± 0.87
3.12	3.6 ± 0.23	2.1 ± 0.28	2.0 ± 0.05	6.52 ± 0.68
1.57	1.4 ± 0.18	0.0	0.0	5.37 ± 0.32
LC_{50} µg/mL	61.6	24.5	53.8	23.8

*Data obtained from three determinations (n=3) and shown as mean ± S.E with confidence level 95%.
LC_{50} was calculated by probit analysis using SPSS 11 program.

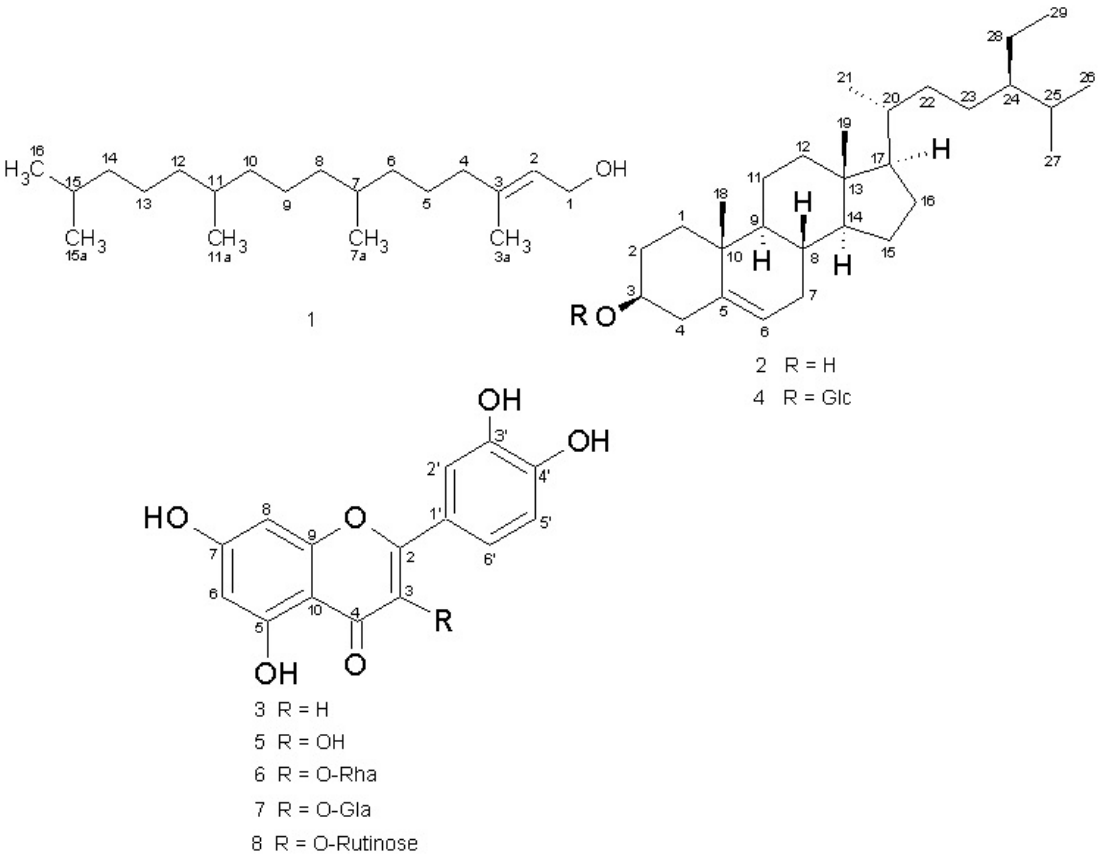

Figure 1: Compounds isolated from *P. salicifolia*

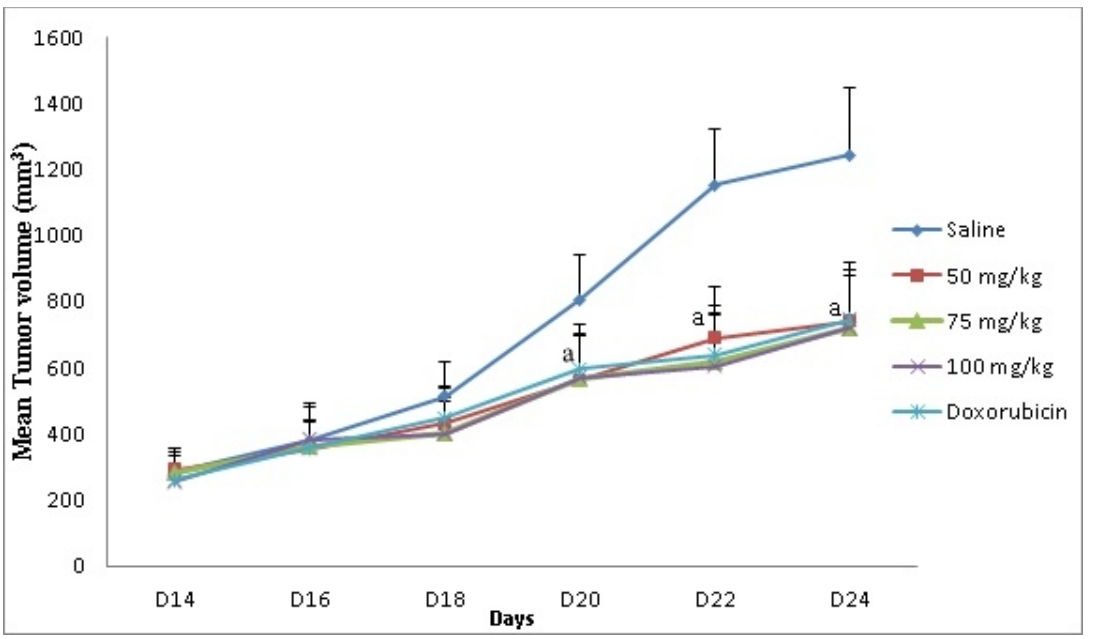

Figure 2: Time-course effects of different doses of *P. salicifolia* extract on the growth of solid Ehrlich carcinoma (SEC).
[a] Significant difference from the group injected with saline at $p<0.05$.

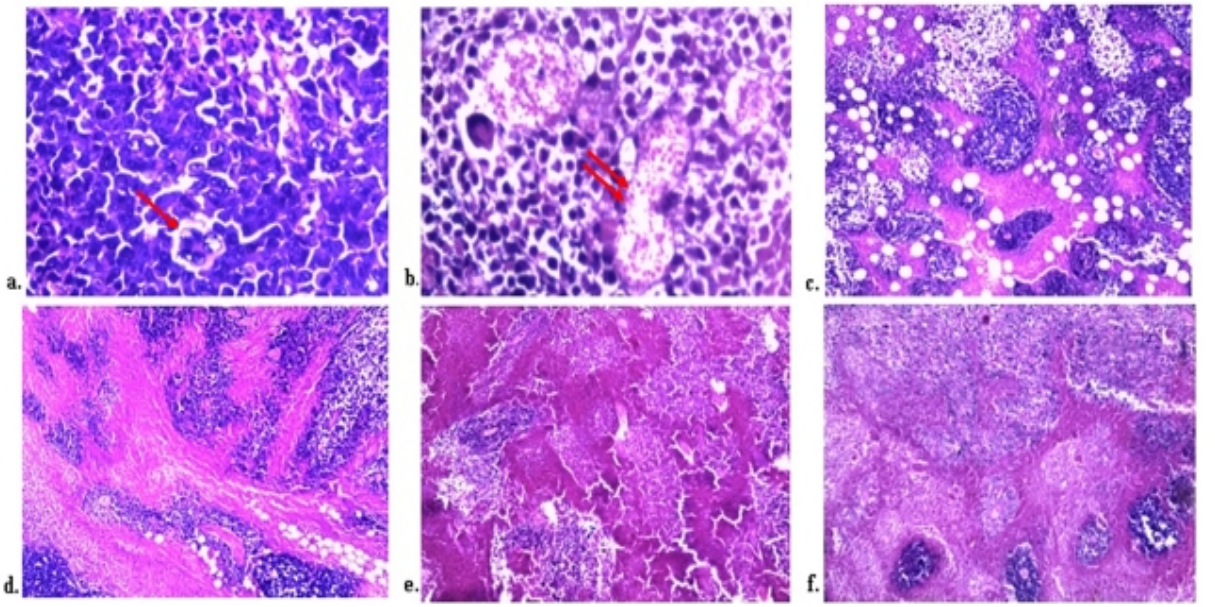

Figure 3: Light micrographs of haematoxylin and eosin staining showing:
(a) Ehrlich solid carcinoma in saline group (control group) with malignant tumor cells, malignant giant cell ↑ and minimal necrosis (X400);
(b) angiogenesis↑↑ in saline group (X400);
(c) *P. slaicifolia* 50 mg/ kg treated group with moderate necrosis and tissue oedema (X400);
(d) *P. slaicifolia* 75 mg/kg treated group with moderate necrosis tissue oedema (X400);
(e) *P. salicifolia* 100 mg/kg with severe coagulative necrosis and tissue debris (X100);
(f) doxorubicin (2 mg/kg) treated group showing extensive necrosis and tissue debris (X100).

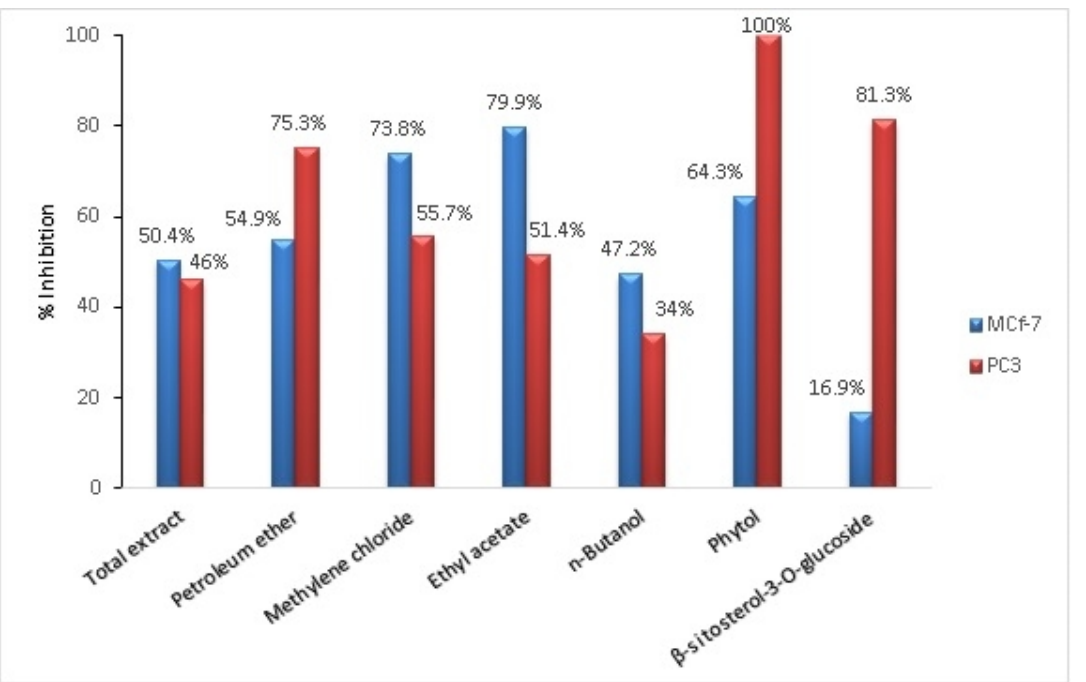

Figure 4: Single dose cytotoxic effect of *P. salicifolia* total extract, different fractions, phytol and *β*-sitosterol-3-*O*-*β*-D-glucoside against MCF-7 and PC3 cell lines at 100 µg/mL

RESULTS AND DISCUSSION

Phytochemical investigation

The phytochemical investigation of *Persicaria salicifolia* Brouss. ex Willd aerial parts extract lead to isolation of eight compounds (Figure 1). Structure elucidation was assisted by different spectroscopic techniques e.g. UV, IR, EI-MS, ESI, ^{1}HNMR and ^{13}CNMR.

Compound **1** was obtained as a white amorphous powder. It gave positive Salkowski's test and negative Leibermann's test. IR spectrum of compound **1** revealed the presence of alcoholic hydroxyl group at 3428 and 962 cm^{-1} for (C-O) stretching. The band at 1642 cm^{-1} indicated the presence of olefinic bond. EI-MS of the compound showed molecular ion peak $[M]^{+}$at m/z 296 which is consistent with molecular formula $C_{20}H_{40}O$. ^{1}HNMR spectrum of compound **1** exhibited a triplet signal at 5.25 ppm (1H, *J*=8 Hz) assigned to the olefinic proton H-2 and triplet signal at 3.93 (2H, *J*= 8 Hz) assigned to H-1 which indicated the presence of hydroxyl group at C-1. Moreover, the protons of methyl groups in positions 7a, 15a, 16 and 11a resonated at 0.82, 0.84, 0.84 and 0.86 ppm, respectively, while protons of the methyl group in position 3a appeared as singlet at 1.56 ppm. Furthermore, the presence of a triplet at 1.92 ppm (2H, *J*=8 Hz) assigned to H-4 confirmed the existence of an olefinic bond. Methine protons appeared as multiplets at 1.17-1.54 ppm. ^{13}C-NMR spectrum exhibited signals at 125.8, 136.1 ppm assigned to the olefinic carbons C-2 and C-3, respectively. In addition, the occurrence of a signal at 58.0 ppm confirmed the presence of hydroxyl group attached to C-1. Trans configuration of compound **1** was evident from the ^{1}H and ^{13}C shifts of C-3a and C-4 being consistent with those reported in literature[19]. DEPT-135 further confirmed these assumptions as the carbon resonating at 58.0 appeared as methylene, carbon resonating at 125.8 ppm appeared to be methine, and carbon resonating at 136.1 appeared to be quaternary. Also, cross peaks in HSQC spectrum lent further support to proton-carbon assignments. It showed proton resonating at 3.93 ppm is coupled with the carbon resonating at 58.0 ppm (C-1) and proton resonating at

5.25 ppm (C-2) is coupled with the carbon resonating at 125.8 ppm. The signals resonating at 16.34, 19.9, 20.1, 22.9 and 22.9 appearing as quartets in DEPT-135 spectrum, were assigned to C-3a, C-7a, C-11a, C-15a and C-16, respectively. Therefore, compound **1** was concluded to be trans 3a, 7a, 11a, 15a tetramethyl-2- hexadece-1-ol (trans phytol)[19].

Compounds **2** and **4** gave positive Liebermann's and Salkowski's tests, which suggested sterol nature of the compounds. Compound **2** was isolated as white needle crystals, m.p. 137-139 °C, while Compound **4** was obtained as a white amorphous powder. Based on IR, EI-MS, ^{1}HNMR and ^{13}CNMR data, m.p. and Co-TLC with authentic *β*-sitosterol and *β*-sitosterol-*3-O-β*-D-glucoside[21], it was concluded that compound **2** is 3-*β*-stigmast-5-en-3-ol (*β*-sitosterol) and compound **4** is *β*-sitosterol-*3-O-β*-D-glucoside. This was confirmed by comparing the obtained data to those published in literature[20-22].

Compound **3** was obtained as buff yellow powder. The UV spectral data revealed that compound **3** has λ_{max} at 348, 255 and 267 nm together with a shoulder at 290 nm indicating the flavone nature of this compound[9]. The free 4`–hydroxyl group is indicated by the large bathochromic shift in band I on adding NaOH, while free 5-OH group was proposed due to the large shift with $AlCl_3$ shift reagent, which was decreased upon addition of HCl supporting the presence of ortho-dihydroxyl system in ring B. On the other hand, the bathochromic shift in band II upon addition of NaOAc indicated the presence of free 7-OH group[9]. Examination of ^{1}HNMR data revealed the presence of five aromatic protons occurring as two doublets (*J*=2 Hz) at 6.18 and 6.42 ppm due to meta coupled protons at C-6, C-8 in ring A and three protons of ring B resonating at 6.88 (H-5`), 6.4 (H-2`) and 7.42 (H-6`). A singlet at δ 6.66 ppm is characteristic for H-3. $^{13}$CNMR revealed the presence of 15 signals, twelve of them are aromatic carbons. $^{1}$HNMR and $^{13}$CNMR data were consistent with those reported for luteolin (5,7,3`,4`tetrahydroxy flavone)[22, 23]. Luteolin is reported for the first time in *Persicaria salicifolia* Brouss. ex Willd.

Compound **5** showed UV pattern of a flavonol[9]. It showed the same ^{1}H and ^{13}CNMR data as quercetin[23,24]. It was further confirmed by Co-TLC with reference sample.

Compound **6** displayed UV, IR and ESI-MS spectral data consistent with a 3-O- flavonol glycosides.^{1}H and ^{13}CNMR lent further support to these assumptions as ^{1}HNMR showed *meta*-coupled doublets at 6.1 and 6.27 ppm (*J*=1.52Hz) each integrated for one proton assigned to H-6 and H-8, respectively. A doublet signal at 6.81 ppm assigned to H-5` (1H, *J*=8.2Hz). Sugar protons resonated at 3.17- 4.12 (*m*). ^{13}CNMR spectrum showed 21 carbons, 15 of them possessed the same chemical shift reported for quercetin. DEPT 135 experiment showed the presence of ten methine carbons, one methyl and ten quaternary carbons. Four methine groups were assigned to the flavonol moiety. HSQC spectrum exhibited cross peak at H_{δ} 7.23 ppm and C_{δ} 115.5 ppm assigned to C-2`, and $H_{\delta}$ 7.2 ppm and $C_{\delta}$121.5 assigned to C-6`.^{1}HNMR, ^{13}CNMR, DEPT 135 and HSQC data suggested the presence of one sugar moiety with one methyl group resonating at H_{δ} 0.84 ppm and C_{δ} 16.3 ppm which referred to rhamnose sugar moiety. Five of the methine groups were assigned to α-L-rhmanose moiety. The aglycone signals were consistent with quercetin and further confirmed by Co-TLC using authentic sample. Based on the obtained data, it was concluded that compound **6** is quercitrin (quercetin-*3-O-α*-L-rhmnopyranoside)[23,25]. This is the first report for isolation of quercitrin from *Persicaria salicifolia* Brouss. ex Willd.

The UV, IR and ESI-MS spectral data of compound **7** indicated the presence of 3 hydroxy flavone glycoside (flavonol glycoside). ^{1}HNMR showed meta-coupled doublets at 6.23 and 6.42 ppm (*J*=1.6 Hz) each integrated for one proton due to H-6 and H-8 respectively. A doublet signal at 6.9 ppm assigned to H-5` (1H, *J*=7.2 Hz). Sugar protons resonated at 3.42- 3.88 (*m*) in addition to anomeric protonat 5.17 ppm (1H, *d, J*=7.6*Hz*) which indicated *beta* configuration of the sugar moiety. ^{13}CNMR spectrum showed the presence of 21 carbons with anomeric carbon signal at 102.9 ppm. Carbons at 71.8, 73.7, 68.6, 75.8 and 60.5 ppm were assigned to sugar moiety and were consistent with galactose residue. Carbons at positions C-5 and C-7 were deshielded to 157.4 and 164.9 ppm respectively due to hydroxylation. Also, the downfield location of C-3` and C-4` at 144.4 and 148.6 ppm respectively, indicated the hydroxylation at C-3` and C-4`. These data suggested the aglycone to be quercetin. In addition, acid hydrolysis of the isolated glycoside and comparing to authentic sugar and aglycone samples, confirmed the sugar moiety to be galactose and the aglycone moiety to be quercetin. Based on the above data combined with ^{1}H and ^{13}CNMR data, compound **7** was identified as hyperoside (quercetin-*3-O-β*-D-galactoside)[26].

Compound **8** was isolated from n-butanol extract and it has pseudo molecular ion peak $[M+H]^{+}$ at m/z 611 pointed at flavonoid with two sugar moieties. The UV spectral data exhibited the hydroxylation pattern of 3-O-substituted flavonol. ^{1}HNMR spectrum exhibited five aromatic protons; two ortho coupled protons at 6.89 and 7.65 ppm with coupling constant 8.4

Hz assigned to H-5` and H-6` respectively; two meta coupled protons at 6.23 and 6.42 ppm assigned to H-6 and H-8; and a singlet aromatic proton at 7.69 ppm assigned to H-2`. Also, the presence of rhamnose and glucose moieties was evident with the rhamnose anomeric proton signal at H_δ 4.88 ppm (broad singlet) and glucose anomeric signal at δ 5.12 ppm (*d, J*=7.6 Hz) indicating *α* and *β* glycosidic linkage, respectively. A doublet of methyl group of rhamnose was observed upfield at 1.14 ppm integrated for 3 protons. The rest of protons in the sugar moiety resonated between 3.33 and 3.84 ppm. The ^{13}CNMR spectrum showed 27 carbon signals which indicated the presence of 15 carbon signals due to the flavonol skeleton. The characteristic signal of C-6``` of rhamnose at 16.5 ppm in addition to anomeric carbon C-1``` at 101.0 ppm confirmed the presence of rhamnose. Excluding the rhamnose signal from the aliphatic region left 5 carbons consistent with glucose carbons in addition to glucose anomeric carbon at 103.4 ppm. These data indicated the presence of rutinose sugar moiety. The obtained ^{1}H and ^{13}CNMR data were compared to the reported data and the compound was identified as rutin (quercetin-*3-O-α*-L-rhamnosyl (1```→ 6``) *β*-D-glucoside)[27].

Antitumor activity against solid Ehrlich carcinoma (SEC)

Ehrlich Ascites Carcinoma (EAC) cells are of mammary origin, where a spontaneous mouse breast carcinoma served as the original tumor[28]. Solid Ehrlich Carcinoma served as a good model for assessing the in-vivo anti-tumor effect of *P. salicifolia* extract. Tumor proliferation is usually based on the presence of free radicals, therefore, the present study focuses on assessing the direct effect of the extract on tumor size and proliferation and measuring serum antioxidant enzymes which antagonize the tumor growth. Intraperitoneal injection of different doses of *P. salicifolia* extract affected tumor size as shown in figure 2. Significant tumor size reduction was observed at 50, 75, and 100 mg/kg of *P. salicifolia* extract at $p<0.05$ compared to saline treated group. The reduction in the tumor size was not dose dependent as the different doses showed similar activities. The results were comparable to that obtained from doxorubicin treated group.

Biochemical study

The biochemical study carried out to assess the antioxidant effect of *P. salicifolia* extract involved measuring serum reduced glutathione, catalase activity, superoxide dismutase and nitric oxide scavenging activity. The results showed that treatment of Ehrlich bearing mice with 50, 75 and 100 mg/kg of *P. salicifolia* extract caused dose dependent increase in serum GSH, catalase activity and SOD. Nitric oxide was reduced at dose 50 mg/kg, while the higher doses almost maintained its serum level when compared to saline and doxorubicin groups (Table 1). These results indicate that *P. salicifolia* extract possesses antitumor effect through activating the endogenous antioxidant mechanisms[29, 30]. Increased serum NO may be attributed to macrophage-induced toxicity involved in the body defensive mechanism against the tumor[31].

Histopathological study

Histopathological investigation of the excised tumors lent further confirmation to the dose dependent antitumor effect of *P. salicifolia* extract as shown in Figure 3. Hematoxylin and eosin stained sections of saline group showed malignant tumor cells with multiple tumor giant cells (multinucleated) infiltrating muscles with minimal microscopic necrotic areas. Some areas showed multiple malformed widely dilated blood vessels (angiogenesis) in all members of the group. *P. salicifolia* extract treated groups showed moderate necrosis and tissue edema at doses 50 and 75 mg/kg which developed to severe coagulative necrosis and cellular debris at dose 100 mg/kg. This effect is comparable to that caused by treatment with doxorubicin. These results revealed that *P. salicifolia* ethanolic extract possessed strong in-vivo antitumor activity using solid Ehrlich carcinoma (SEC) bearing mice which suggests the extract to be a good alternative as a chemotherapeutic agent.

Cytotoxic activity

Evaluation of cytotoxic activity of the total extract and different fractions from *P. salicifolia* against MCF-7 and PC3 cell lines using single dose experiment was performed. The results revealed that both methylene chloride and ethyl acetate fractions exhibited highest inhibition against MCF-7, while petroleum ether fraction presented highest activity against PC3 cell line (Figure 4).The study was also performed on the two major isolated compounds phytol and *β*-sitosterol-3-*O*-*β*-D-glucoside. Trans phytol showed moderate activity against MCF-7 and strong activity against PC3 at concentration 100 µg/mL. On the other hand, *β*-sitosterol-3-*O*- *β*-D-glucoside exhibited low activity against MCF-7 and high activity against PC3. Multi-dose study to determine LC_{50} of fractions and compounds with highest activity was carried out. The results revealed that LC_{50} of methylene chloride is 60.1 µg/mL, ethyl acetate 58.9 µg/mL and phytol 74.7 µg/mL, compared to doxorubicin (LC_{50}= 26.1 µg/mL) against MCF 7 cell line as shown in Table 2. *β*-sitosterol-*3-O-β*-D-glucoside and petroleum ether fraction possessed LC_{50} 53.8 and 61.1 µg/mL, respectively against PC3 Cell line compared to LC_{50} 23.8 µg/mL of doxorubicin. Interestingly, phytol showed 100% inhibition against PC3 cell line at doses 50 and 100 µg/mL, with LC_{50} 24.5 µg/mL which is comparable to doxorubicin. This result suggests that phytol may be used as an alternative antitumor agent (Table 3).

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