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ORIGINAL ARTICLE

Production of bioactive compounds from callus of *Pueraria thomsonii* Benth with promising cytotoxic and antibacterial activities

Yu Li [a], Pachaiyappan Saravana Kumar [a,*], Jiao qiu [b], Yalan Ran [c], Xuhui Tan [a], Renjun Zhao [a], Lunqiang Ai [a], Mingyuan Yuan [a,d], Ji Zhu [a], Meijun He [a,*]

[a] *Institute of Chinese Herbal Medicines, Hubei Academy of Agricultural Sciences, Enshi 445000, China*
[b] *Department of Central Hospital of Tujia and Miao Autonomous Prefecture, Hubei University of Medicine, Shiyan 442000, China*
[c] *Agricultural and Rural Bureau of Enshi Tujia and Miao Autonomous Prefecture, Enshi 445000, China*
[d] *College of Biological Science and Technology, Hubei Minzu University, Enshi 445000, China*

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Abstract For thousands of years *Pueraria thomsonii* Benth has been used to treat a number of diseases in traditional Chinese pharmacopeia. Despite these uses, there is still insufficient information on its biological activity and chemical composition. In this respect, the in vitro callus culture of *P. thomsonii* was subjected to identify anticancer and antibacterial compounds. Based on significant preliminary cytotoxicity and antibacterial activities; the chemical investigation led to the isolation of isoflavonoids, coumaric acid derivative and dihydroxyflavanone-type of compounds viz., daidzin **(1)**, puerarin **(2)**, biochanin A **(3)**, daidzein **(4)**, p-coumaric acid ethyl ester **(5)** and liquiritigenin **(6)**, respectively. These compounds were tested for their cytotoxicity and antibacterial activities. Among them, p-coumaric acid ethyl ester **(5)** exhibited significant cytotoxicity with GI_{50} values of 14.73, 15.64 and 20.88 µM/mL against 4T1, NC1-H1975 and A549, respectively; the other isoflavones and aflavonoid showed moderate to weak activities. Moreover, p-coumaric acid ethyl ester **(5)** inhibited the growth of *K. pneumonia*, MRSE and MRSA at very low MIC values of 6.01, 12.01 µg/mL 24.02, respectively. On the other hand compounds biochanin A **(3)** and liquiritigenin **(6)** showed moderate antibacterial activity. Because of the potential anticancer and antibacterial

Abbreviations: NAA, 1-Naphthaleneacetic acid; 6-BA, 6-Benzylaminopurine; µM, Micromole; mL, Milliliter; µg, Microgram; L, Liter; °C, degree Celsius; *P. thomsonii* calli extract, EtOH-PT; g, Gram; mg, Milligram

* Corresponding authors.
E-mail addresses: savanah.kumar@gmail.com, saravanakumar12deri01@loyolacollege.edu (P. Saravana Kumar), 840940513@qq.com (M. He).

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activities of bioactive compounds from *P. thomsonii*, they can be used to treat various cancer and emerging bacterial infections.

1. Introduction

Cancer has been a most common, devastating and life threatening disease worldwide in the past few decades (Siegel et al., 2019). Recently, diagnosis of new cancer cases in all ages and from both genders with high mortality has drastically increased globally because of aging, lifestyle choices, smoking, lower physical activity, unhealthy diets, obesity and high population rate (Arun et al., 2013, Mathew et al., 2019). Ten million cancer deaths are estimated to have occurred worldwide in 2020; it is a great threat to human health and safety (Mani et al., 2019, Sung et al., 2021). In this context, World Health Organization (WHO) reported that most common cancer deaths in 2020 were due to lung (1.80 million deaths) followed by colon and rectum (935,000 deaths), liver (830,000 deaths), stomach (769,000 deaths) and breast (685,000 deaths) accounting for almost 60% of all cancers diagnosed. Currently, the common treatment for cancer includes chemotherapy, immunotherapy, radiotherapy and surgery. However, all treatment modalities are associated with adverse effects and known to induce chemo-resistance after some period of time; they are not successful even with combination therapeutic strategy (Chen et al., 2020, Shantabi et al., 2020). Therefore, there is an urgent need to find out new anticancer drugs with efficiency to target various factors in cancer with minimal side effects.

Likewise, serious infections caused by bacteria and their resistance to antibiotics are some of the biggest healthcare threats to pharmaceutical and medical community; infections kill around 700,000 people each year worldwide (Tesfaye et al., 2020). While development of resistance to antibiotic is a natural evolutionary phenomenon, it has been exacerbated by systematic misuse and overuse of antibiotics in human medicine or to prevent diseases in healthy individuals. In view of this serious global threat, WHO has warned recently that antibiotic resistance has reached an alarming level in several parts of the world and that a continued increase in resistance could swell to 10 million people dying per year by 2050 if no action is taken.

To date many plant compounds have shown promising therapeutic anticancer and antibacterial activities alone or in combination due to their selective toxicity against the tumor cells rather than normal cells and in addition to their broad spectrum antibacterial activities with less or negligible side effects (Morales and Haza 2012, Tripathi et al., 2019, Huang et al., 2021, Ma et al., 2021). *P. thomsonii* is a plant variety of *Pueraria lobata* belonging to the family Leguminosae, distributed in East Asian countries, such as China, India, Thailand and Japan (Chen et al., 2014). It is an important edible Chinese medicinal plant used to treat influenza, cervical spondylosis, alcoholism, chest stuffiness, hemiplegia, vertigo, headache and other illnesses (Chen et al., 2017). *P. thomsonii* is reported to be a rich source of chemical constituents such as puerarin, tectorigenin, daidzin, daidzein, and genistein which are the predominant bioactive compounds traditionally shown to possess diverse biological activities (Lin et al., 2010, Niiho et al., 2010, Wang et al., 2020). Such reports place this plant's compounds in high demand to meet the needs of medicinal and pharmaceutical industries. Therefore, the callus culture could be a realistic source of bioactive secondary metabolites production with no time and space limitation and the technique has been widely used as an alternative for the production of plant secondary metabolites (Kamarul Zaman et al., 2020). Recently, Su et al., (2013) have studied the biosynthesis of puerarin, daidzein and total isoflavones in *P. thomsonii* callus culture suspensions under low light stress. However, the anticancer and antibacterial activities of these secondary metabolites and the chemical fingerprinting of the callus culture have not been scientifically proved yet. Therefore, induction of callus culture from *P. thomsonii* and identification of anticancer and antibacterial compounds in callus are of great significance. Hence, in this study we explored the anticancer and antibacterial potential of the callus culture extract and the compounds against bacterial pathogens and human lung adenocarcinoma cancer cell line (A549), human non-small lung cancer cell line (NCI-H1975) and mouse breast cancer cell line (4T1).

2. Materials and methods

2.1. General experimental procedures and chemicals

HRESIMS experiments were performed using a maXis II Q-TOF mass instrument (Bruker, Germany). NMR spectra were recorded on a Bruker NMR 600 spectrometer (Bruker, Germany) operated at 400 MHz for ^{1}H and 150 MHz for ^{13}C with tetramethylsilane (TMS) as internal reference. The chemical shifts were expressed in δ (ppm) referenced to the residual solvent signals. Silica gel 100–200 and 200–300 mesh (Merck), were used for gravity column chromatography (CC). Thin Layer Chromatography (TLC) was carried out on 0.2 mm thick silica gel plates (HS-GF254). The organic solvents used for extraction and purification were procured from Beijing chemical factory (Beijing, China). Analysis and preparation of compounds were performed using an Agilent 11260 Infinity II Prime with an ODS column (YMC, 250 × 10 mm, 5 μm) and the solvents used were of HPLC grade.

2.2. Plant material and callus induction

The young shoots of *P. thomsonii* were collected from Central China Medicinal Botanical Garden (Enshi, China, E109°45′24″; N30°10′51″). The collected plant material was identified by Dr Meijun He, Hubei Academy of Agricultural Sciences. Sterile solid Murashige and Skoog (MS) culture medium containing 3% sucrose, PGRs-free and 0.8% agar adjusted to pH-5.8 before sterilization (121 °C, 15 psi, for 15 min) was used. The young shoots of *P. thomsonii* were surface sterilized by following the methods of Su et al. and Li et al. (Li et al., 2021, Su et al., 2021) (Fig. S1a). Briefly, the young shoots were washed 5 times with sterile water and then soaked in 75% ethanol for 45 s followed by intermittent soaking in 0.1% $HgCl_2$ for 20 min. The shoots were washed with sterile water 3 times and then were cut into small pieces. Initially, four explants were transferred into jars containing 40 mL of MS medium with 1-Naphthaleneacetic acid (NAA, (0.25 mg/L) and 6-Benzylaminopurine (6-BA, 1.5 mg/L). The explants were incubated at 25 ± 2 °C in a growth cabinet under photoperiod of 16 h with white fluorescent light (50 μmol $m^{-2}s^{-1}$) for 30 days. After incubation the uninfected and well grown calli were harvested for extraction (Fig. S1b).

2.3. Extraction and isolation of bioactive compounds from callus culture

After incubation the fully grown calli were collected from MS culture medium and used for extraction of secondary metabolites. Briefly, the harvested calli were dried in an oven at 45 °C. The dried biomass of *P. thomsonii* calli weighing 500.25 g was extracted three times with 5 L of ethanol (EtOH). The solvent was evaporated under reduced pressure to obtain 5.36 g yellowish green gum (EtOH-PT). The extract was fractionated over silica gel chromatographic column and eluted with solvents of increasing polarity of $CHCl_3$/MeOH. A total of 8 fractions (Fr.A1- Fr.A8) were collected and similar fractions were combined based on their TLC profiles. Fr.A6 (423.12 mg) was purified over ODS column to obtain compound **5** (21.6 mg) and compound **6** (7.6 mg). Fr.A5 was subjected to silica gel column chromatography to yield compound **2** (18.3 mg). Based on TLC profile the Fr.A7 and Fr.A8 (128.48 mg) were combined together and fractionated using a silica gel column chromatography to obtain 11 fractions (Fr.B1-Fr.B11). Fr. B3-B5 was purified by semi-preparative HPLC with an ODS column to give compound **3** (12.5 mg) and compound **1** (10 mg). Fr.B6 was further purified by semi-preparative HPLC to give compound **4** (7.2 mg).

2.4. Biological activities

2.4.1. Cell lines and culture conditions

Human lung adenocarcinoma cells (A549), human non-small cell lung cancer (NCI-H1975) and mouse breast cancer cells (4T1) were grown in RPMI-1640 medium supplemented with 10% FBS (v/v), 1% antibiotic and antimycotic solution (1000 U/mL penicillin and 10 mg/mL streptomycin sulphate). The cells were grown and maintained in 5% CO_2 and 95% air with 90% relative humidity at 37 °C in a CO_2 incubator.

2.4.2. In vitro cytotoxic activity

The extract and compounds were evaluated for their cytotoxic activity against a panel of aforementioned human and mouse breast cancer cell lines. The in vitro cytotoxic activity was determined using MTT assay described previously by Su et al. (Su et al., 2021). Briefly, various concentrations of the extract (20–200 μg/mL) and compounds (20–100 μM/mL) were added to the 5×10^4 cells/well that were seeded onto the 96-well microtiter plate and incubated for 48 h. After treatment 10 μL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT 5 mg/mL) was added to each well and incubated at 37 °C in the dark for 4 h followed by addition of 100 μL of DMSO and allowed to stand for 30 min to dissolve the formazan crystals. The viability was measured at 544 and 590 nm in ELX800 Universal Microplate Reader. The percentage of growth inhibition (GI) was calculated using the formula: Growth Inhibition (%) = B/A × 100 (A- absorbance of control cells and B- absorbance of treated cells).

2.4.3. Microbes

The Gram positive methicillin resistant strains such as *Staphylococcus aureus* ATCC 43300 (MRSA), Methicillin resistant *Staphylococcus epidermidis* ATCC 12228 (MRSE) and Gram negative bacterium *Klebsiella pneumonia* ATCC 13883 were procured from the Key Laboratory of Tropical Marine Biological Resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences. Prior to the experiment, fresh cultures of bacterial inoculums were prepared by growing cells in sterile Mueller-Hinton broth (MHB, OXOID, USA) for 24 h at 37 °C and diluted with sterile MHB in order to attain McFarland standards 0.5 corresponding to $1 \times 10^{4\text{-}6}$ CFU/mL.

2.4.4. Disc diffusion assay

The antibacterial activity of the EtOH-PT against the aforementioned bacterial pathogens was assayed using paper disc method with slight modification (Kumar et al., 2020). Briefly, the sterile filter paper discs were impregnated with 20 μL for crude extract and 10 μL for compounds and dried under sterile conditions at room temperature. Further, the dried discs were placed on pre-coated bacterial plates (50 μL of freshly prepared test cultures, $1 \times 10^{4\text{-}6}$ CFU/mL) of Mueller-Hinton agar (MHA). DMSO (20 μL/disc) and Kanamycin (50 μg/disc) were used as solvent and positive controls. The plates were first incubated at room temperature for 30 min to allow proper diffusion of the extract into the medium and then re-incubated overnight at 37 °C. After incubation period, the inhibition zones were measured and expressed in millimeter (mm). An average inhibition zone was calculated for 5 replicates.

2.4.5. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC values of extract and compounds were determined by broth microdilution assay in sterile 96-well microtiter plate according to Kumar et al. (Kumar et al., 2017). Briefly, 25 μL of the extract and purified compounds were serially diluted to obtain the following concentrations ranging from 0 to 200 μg/mL for extract and 0–100 μg/mL for compound; 75 μL of MHB and 5 μL of freshly grown bacterial cultures were added to each well. DMSO and kanamycin were included as solvent and positive controls. The treated microtiter plates were then kept at 37 °C for 24 h followed by addition of 40 μL of iodonitrotetrazolium chloride (200 μg/mL) into each well and re-incubated at 3 °C for 30 min. The color changed from yellow to purple indicating the microbial growth in the well. The MIC values of the extract, isolated compounds and kanamycin which showed antibacterial activity at concentrations below10 μg/mL were grouped into significant activity; those which showed activity between 10 and 100 μg/mL were grouped into moderate activity and compounds which showed activity above 100 μg/mL were grouped into weak activities (Kuete 2010). The MBC of compounds were assayed by following the method of Rozman et al. (2017) (Rozman et al., 2017). To check the viability of the tested bacterial pathogens, the mixture in each well was streaked on MHA and incubated overnight at 37 °C.

2.5. Statistical analysis

All statistical analyses were performed using the SPSS software 24.0 (Systat Software, Systat, Evanston, IL, USA) and the data were presented as mean ± SEM. The significance among all groups was evaluated using one-way ANOVA. $P < 0.05$ was considered statistically significant. The Anova analyses

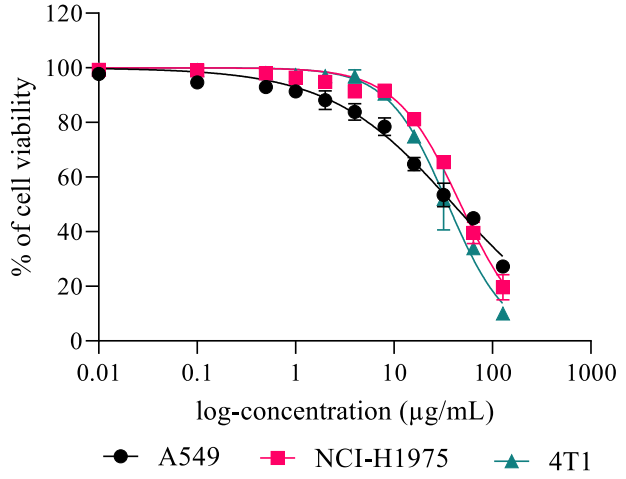

Fig. 1 (a) Graph showing cytotoxic effects of callus extracts from *P. thomsonii* on human lung adenocarcinoma A549 cells (A549), human non-small cell lung cancer NCI-H1975 (NCI-H1975) and 4T1 mouse breast cancer cells (4T1), **(b)** A comparative analysis of heatmap showing cytotoxic effects of callus extract from *P. thomsonii* on A549 cells, NCI-H1975 and 4T1.The color codes of rows and columns represent the concentration of crude extract and the cell lines tested; similarly, the color codes for % of cell viability of A549, NCI-H1975 and 4T1 are depicted on the left side of the heatmap

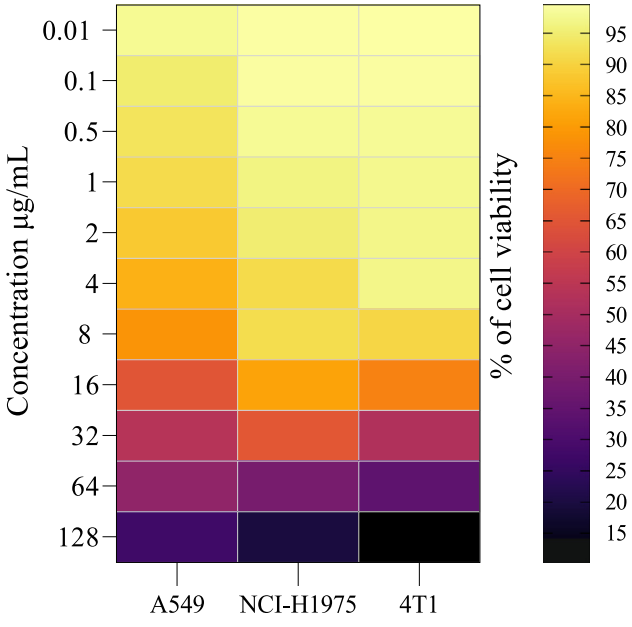

Fig. 1 (*continued*)

were carried out using Graphpad version 8.4.3 for Windows version 7.

3. Results

3.1. Cytotoxic effect of EtOH-PT

After 24 h of treatment, the EtOH-PT was highly effective in inhibiting cell proliferation in 4T1, NCI-H1975 and A549 cell lines in a concentration-dependent manner with the GI_{50} values of 59.45, 68.23 and 68.26 µg/mL, respectively. The results were comparable to the standard drug doxorubicin with the GI_{50} values of 6.94, 11.42 and 5.17 µM/L against 4T1, NCI-H1975 and A549 cell lines, respectively (Figs. 1a-b, S2a-f). Though considerable cytotoxicity was witnessed alongside all cancer cell lines, the higher inhibition percentages of 10.09 ± 2.21–99.48 ± 0.44%, 19.64 ± 4.56–99.27 ± 0.22% and 27.2 8 ± 1.45–97.74 ± 1.96% were seen against 4T1, NCI-H1975 and A549 cell lines, respectively.

3.2. Antibacterial activity of EtOH-PT

EtOH-PT exhibited significant antibacterial activity against MRSA with the zone of inhibition of 18.15 mm; it was followed by 14.19 ± 0.96 mm against *K. pneumonia*. The lowest zone of inhibition of 9.51 ± 0.36 mm was recorded against MRSE (Fig. 2a, S3a-c). The antibacterial activity was further validated by determining its minimum inhibitory concentration (MIC) and minimum bacteriostatic concentration (MBC) using broth dilution assay. EtOH-PT exhibited activities depending on bacterial strains with the MIC and MBC values in the range from 18.26 to 73.04 µg/mL and from 73.04 to 146.08 µg/mL, respectively (Fig. 2b). Among the strains tested, EtOH-PT showed remarkable activity against MRSA with MIC and MBC values of 18.26 and 73.04 µg/mL.

3.3. Isolation and identification of isosflavones from P. thomsonii calli

The remarkable cytotoxicity and antibacterial activities further encouraged us to investigate the bioactive constituents through silica gel column chromatography and semi-preparative HPLC using different solvent systems. This led to the isolation of six compounds (Fig. 3). The structures were elucidated based on physiochemical and detailed spectroscopic analyses including HRESIMS and NMR. Daidzin **(1)** was obtained as a white powder and the molecular formula was established as $C_{21}H_{20}O_9$ by positive HRESIMS *m/z* 417.1186 $[M + H]^+$

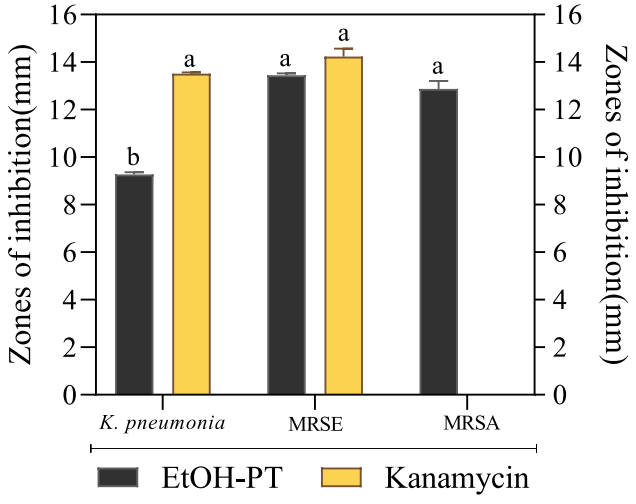

Fig. 2 (a) In vitro antibacterial activity of *P. thomsonii* callus extracts (EtOH-PT); (b) MIC and MBC of *P. thomsonii* callus extracts (EtOH-PT)

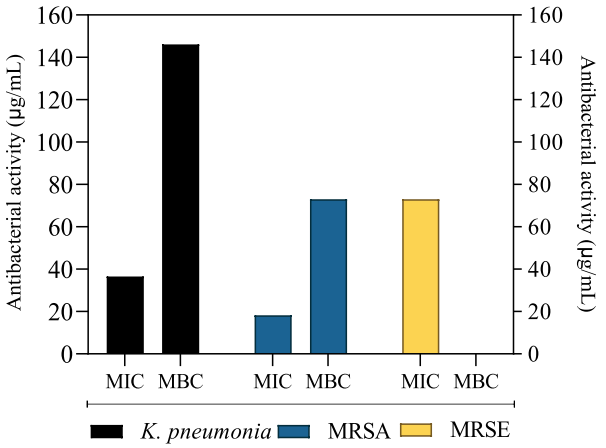

Fig. 2b (*continued*)

(calcd. for $C_{21}H_{20}O_9$) (Fig. S4).^{1}H NMR (400 Hz, DMSO d_6) δ ppm: 8.38 (1H, *s*, H-2), 8.05 (1H, *d*, *J* = 8.9 Hz, H-5), 7.40 (2H, *d*, *J* = 8.6 Hz, H-2′, 6′), 7.22 (1H, *d*, *J* = 2.3 Hz, H-8), 7.13 (1H, *dd*, *J* = 8.9 Hz, 2.3 Hz, H-6), 6.80 (2H, *d*, *J* = 8.8 Hz, H-3′,5′), 5.45 (1H, *d*, *J* = 4.5 Hz, OH-3′′), 5.16 (1H, *d*, *J* = 4.2 Hz, OH-2′′), 5.11 (1H, *d*, *J* = 7.6 Hz, H-1′′), 5.10 (1H, *d*, *J* = 5.2, 4′′–OH), 4.62 (1H, *t*, *J* = 5.5 Hz, OH-6′′), 3.66 ~ 3.74 (1H, *m*, H-6α′′), 3.42 ~ 3.50 (2H, *m*, H-5′′, H-6β′′), 3.26 ~ 3.32 (2H, *m*, H-2′′, H-3′′), 3.17 (1H, *m*, H-4′′) (Fig. S5). ^{13}C NMR (150 MHz, DMSO d_6) δ ppm: 175.22 (C-4), 161.87 (C-7), 157.73 (C-9), 157.50 (C-4′), 153.81 (C-2), 130.56 (C-2′, 6′), 127.43 (C-5), 124.17 (C-1′), 122.78 (C-3), 118.93 (C-10), 116.06 (C-6), 115.45 (C-3′, 5′), 103.85 (C-8), 100.44 (C-1′′), 77.68 (C-5′′), 76.94 (C-3′′), 73.59 (C-2′′), 70.09 (C-4′′), 61.10 (C-6′′) (Fig. S6-S7). Further, the identity of the compound was also confirmed by comparison with the spectroscopic data with those reported in the literature (Fu et al., 2015) (Fig. 3a). Puerarin **(2)** was obtained as a white powder and the molecular formula was established as $C_{21}H_{20}O_9$ by positive HRESIMS *m*/*z* 417.1179 $[M + H]^+$ and 439.0996 $[M + Na]^+$ (calcd. for $C_{21}H_{20}O_9$) (Fig. S8).^{1}H NMR (400 Hz, DMSO- d_6) δ ppm: 8.34 (1H, *s*, H-2), 7.93 (1H, *d*, *J* = 8.8 Hz, H-5), 7.38 (2H, *d*, *J* = 8.6 Hz, H-2′, 6′), 6.98 (1H, *d*, *J* = 8.8 Hz, H-6), 6.79 (2H, *d*, *J* = 8.6 Hz, H-3′, H-5′), 4.80 (1H, *d*, *J* = 9.4 Hz, H-1′′), 4.01 (1H, *t*, *J* = 8.7 Hz, H-2′′), 3.70 (1H, *J* = 11.3 Hz, H-6α′′), 3.16 ~ 3.28 (4H, *m*, H-3′, 4′, 5′) (Fig. S9).^{13}C NMR (150 MHz, DMSO- d_6) δ ppm: 175.38 (C-4), 161.56 (C-7), 157.61 (C-4′), 153.13 (C-2), 130.50 (C-2′, 6′), 126.71 (C-5), 123.53 (C-3), 122.99 (C-1′), 115.43 (C-6, 3′, 5′), 113.11 (C-8), 82.32 (C-5′′), 79.22 (C-1′′), 73.88 (C-2′′), 71.23 (C-3′′), 70.95 (C-4′′), 61.89 (C-6′′) (Fig. S10-S11). The identity of the compound was confirmed by comparing the spectroscopic data reported by Kim et al. (Kim et al., 2006) (Fig. 3b). Biochanin A **(3)** was obtained as a yellow powder and the molecular formula was established as $C_{16}H_{12}O_5$ by positive HRESIMS *m*/*z* 285.0759 $[M + H]^+$ (calcd. for $C_{16}H_{12}O_5$) (Fig. S12). ^{1}H NMR (400 Hz, DMSO- d_6) δ ppm:12.91 (1H, *s*, 5-OH), 10.90 (1H, *s*, 7-OH), 8.34 (1H, *s*, H-2), 7.48 (2H, *d*, *J* = 8.8 Hz, H-2′, 6′), 6.98 (2H, *d*, *J* = 8.8 Hz, H-3′, 5′), 6.86 (1H, *d*, *J* = 2.1 Hz, H-8), 6.22 (1H, *d*, *J* = 2.1 Hz, H-6), 3.77 (3H, *s*, MeO-4′) (Fig. S13). ^{13}C NMR (150 MHz, DMSO- d_6) δ ppm: 180.57 (C-4), 164.78 (C-7), 162.46 (C-5), 159.62 (C-4′), 158.05 (C-9), 154.75 (C-2), 130.63 (C-2′, 6′), 123.38 (C-3), 122.42 (C-1′), 114.16 (C-3′, 5′), 104.92 (C-10), 99.48 (C-6), 94.17 (C-8), 55.62 (MeO-4′) (Fig. S14-15). The structure of the compound was further confirmed by comparing the spectroscopic data reported by Formisano et al. (Formisano et al., 2012) (Fig. 3c). Daidzein **(4)** was obtained as yellow needles and the molecular formula was established as $C_{15}H_{10}O_4$ by positive HRESIMS *m*/*z* 255.0651 $[M + H]^+$ (calcd. for $C_{15}H_{10}O_4$) (Fig. S16). ^{1}H NMR (400 Hz, DMSO d_6) δ ppm: 8.27 (1H, *s*, H-2), 7.95 (1H, *d*, *J* = 8.8 Hz, H-5), 7.36 (2H, d, *J* = 8.6 Hz, H-2′, 6′), 6.92 (1H, dd, *J* = 8.8 Hz, 2.2 Hz, H-6), 6.85 (1H, d, *J* = 2.2 Hz, H-8), 6.82 (2H, *dd*, *J* = 8.6 Hz, H-3′, 5′) (Fig. S17). ^{13}C NMR (150 MHz, DMSO d_6) δ ppm: 175.17 (C-4), 162.99 (C-7), 157.89 (C-9), 157.64

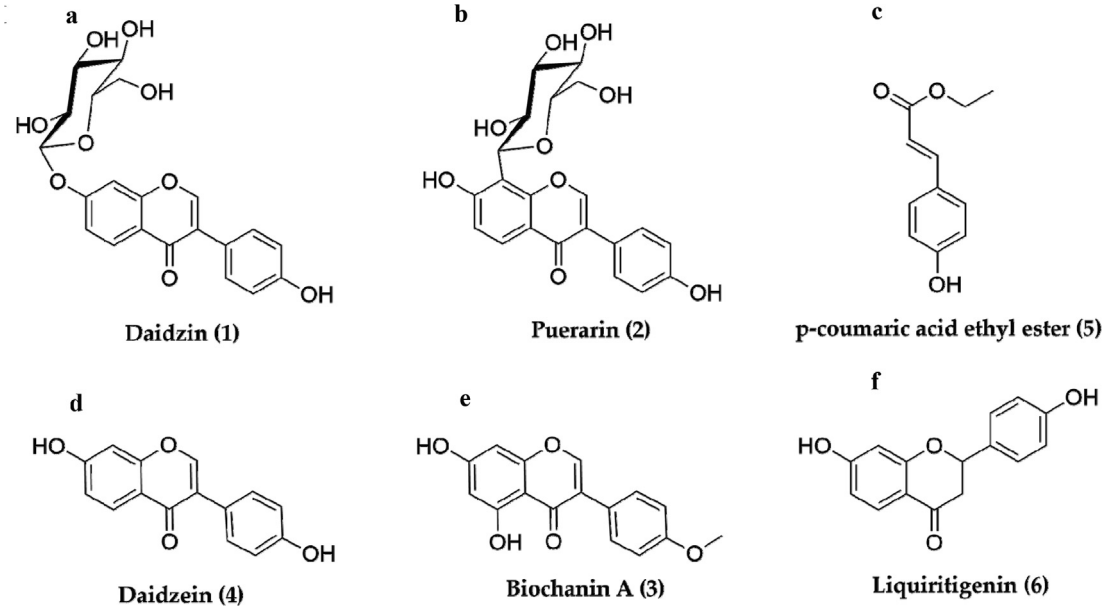

Fig. 3 Chemical structures of the compounds **(1–6)** isolated from *P. thomsonii* Callus

(C-4′), 153.28 (C-2), 130.55 (C-2′, 6′), 127.76 (C-5), 123.95 (C-3), 123.01 (C-1′), 117.09 (C-10), 115.60 (C-6), 115.41 (C-3′, 5′), 102.56 (C-8) (Fig. S18-19). The identity of the compound was confirmed by comparison of reported data of Lee et al. (Lee and Cho, 2012) (Fig. 3d). p-coumaric acid ethyl ester **(5)** was obtained as colorless crystals and the molecular formula was established as $C_{11}H_{12}O_3$ by positive HRESIMS *m/z* 193.0863 $[M + H]^+$ (calcd. for $C_{11}H_{12}O_3$) (Fig. S20). ^{1}H NMR (400 Hz, CD_3OD) δ ppm: 7.56 (1H, *d, J* = 15.9 Hz, H-7),7.39 (2H, *d, J* = 8.5 Hz, H-2, 6), 6.80 (2H, *d, J* = 8.5 Hz, H-3, 5), 6.26 (1H, *d, J* = 15.9 Hz, H-8), 4.17 (2H, *q, J* = 7.1 Hz, H-10), 1.26 (3H, *t, J* = 7.1 Hz, H-11) (Fig. S21). ^{13}C NMR (150 MHz, CD_3OD) δ ppm: 169.34 (C-9), 161.12 (C-4), 146.33 (C-7), 131.20 (C-2, 6), 127.16 (C-1), 116.87 (C-3, 5), 115.38 (C-8), 61.49 (C-10), 14.75 (C-11) (Fig. S22-23). The spectral data obtained were in agreement with the previous report of Peng et al. (Peng et al., 2011) (Fig. 3e). Liquiritigenin **(6)** was obtained as a faint yellow amorphous powder and the molecular formula was established as $C_{15}H_{12}O_4$ by positive HRESIMS *m/z* 257.0809 $[M + H]^+$ (calcd. for $C_{15}H_{12}O_4$) (Fig. S24). ^{1}H NMR (400 Hz, CD_3OD) δ ppm: 7.72 (1H, *d, J* = 8.7 Hz, H-5),7.32 (2H, *d, J* = 8.5 Hz, H-2′, 6′), 6.82 (2H, *d, J* = 8.6 Hz, H-3′, 5′), 6.49 (1H, *dd, J* = 8.7 Hz, 2.2 Hz, H-6), 6.35 (1H, *d, J* = 2.2 Hz, H-8), 5.36 (1H, *dd, J* = 13.1 Hz, 2.8 Hz, H-2), 3.04 (1H, *dd, J* = 16.9 Hz, 13.1 Hz, H-3α), 2.68 (1H, *dd, J* = 16.9 Hz, 2.9 Hz, H-3β) (Fig. S25). ^{13}C NMR (150 MHz, CD_3OD) δ ppm: 193.69 (C-4), 167.10 (C-7), 165.72 (C-9), 159.09 (C-4′), 131.49 (C-1′), 130.00 (C-5), 129.16 (C-2′, 6′), 116.45 (C-3′, 5′), 115.04 (C-10), 111.96 (C-6), 103.98 (C-8), 81.16 (C-2), 45.08 (C-3) (Fig. S26-27). The spectral data obtained were in good agreement with those of Zhang et al. (Zhang et al., 2018) (Fig. 3f).

3.4. Cytotoxic effects of compounds

To verify the in vitro cellular toxicity of daidzin **(1)**, puerarin **(2)**, biochanin A **(3)**, daidzein **(4)**, p-coumaric acid ethyl ester **(5)** and liquiritigenin **(6)** against adenocarcinoma epithelial cells, mouse breast cancer cells and human non-small cell lung carcinoma cells were studied at different concentrations to determine the growth inhibition (GI) GI_{50} values. Interestingly, p-coumaric acid ethyl ester **(5)** induced 6.181 ± 0.50–98.52 ± 1.88% of cell death followed by puerarin **(2)** 65.36 ± 0.68–97.01 ± 5.04%, liquiritigenin **(6)** 67.42 ± 2.09–99.65 ± 0.24%, biochanin A **(3)** 69.65 ± 0.54–99.80 ± 1.22%, daidzin **(1)** 71.18 ± 1.05–99.44 ± 1.06% and daidzein **(4)** 80.66 ± 0.75–100.33 ± 1.12% from 40 to 0.5 μM/mL with the GI_{50} values of 14.73, 57.86, 63.13, 57.43, 66.94 and 100.19 μM/mL against 4T1 cells, respectively (Fig. 4a). Similarly, p-coumaric acid ethyl ester **(5)** induced 6.00 ± 0.16–98.80 ± 0.97% of cell death followed by puerarin **(2)** 40.38 ± 0.71–99.38 ± 1.02%, biochanin A **(3)** 51.97 ± 0.72–100.42 ± 2.28%, liquiritigenin **(6)** 67.35 ± 2.14–100.96 ± 0.63%, daidzein **(4)** 68.84 ± 1.40–98.94 ± 2.32%, and daidzin **(1)** 77.88 ± 0.80–96.84 ± 2.89% from 40 to 0.5 μM/mL with the GI_{50} values of 15.64, 37.76, 52.20, 61.65, 96.52 and 98.82 μM/mL against NCI-H1975 cells, respectively (Fig. 4b). On the other hand p-coumaric acid ethyl ester **(5)** exhibited significant cytotoxicity against A549 cells with the GI_{50}value of 20.88 μM/mL followed by biochanin A **(3)** with the GI_{50} value of 58.93 μM/mL and liquiritigenin with the GI_{50} value of 97.02 μM/mL with 4.09 ± 0.05–100.08 ± 0.17, 53.21 ± 0.62–99.703 ± 0.52 and 68.093 ± 0.29–99.36 ± 0.88% of cell deaths, respectively (Fig. 4c). Contrarily daidzin **(1)**, puerarin **(2)** and daidzein **(4)** did not show any activity against A549 and the results were compared with that of well-known anticancer standard drug doxorubicin (Fig. 4d).

3.5. Antibacterial activities of compounds

As presented in Tables 1 and 2, all the six compounds displayed variable antibacterial activity against the pathogens tested viz., *K. pneumonia,* MRSE and MRSA. Among them p-coumaric acid ethyl ester **(5)** exhibited significant antibacterial activity against *K. pneumonia* with the zone of inhibition of 20.62 ± 0.11 mm followed by MRSE 13.01 ± 0.13 mm and MRSA10.15 ± 0.20 mm. The results were comparable with kanamycin on MRSA; no activity was observed on MRSA. At the same time compounds biochanin A **(3)** and liquiritigenin **(6)** displayed moderate activity against *K. pneumonia,* MRSE and MRSA with the zones of inhibition of 14.74 ± 1.38 mm, 11.85 ± 1.34 mm and 13.00 ± 0.85 mm, respectively. Based on significant antibacterial activity the compounds were tested on the bacterial pathogens to determine their MIC and MBC values. As expected, p-coumaric acid ethyl ester **(5)** efficiently inhibited the growth of *K. pneumonia* followed by MRSE and MRSA at very low MIC values of 6.01, 12.01 and 24.02 μg/mL and MBC values of 12.01, 24.02 and 48.04 μg/mL against *K. pneumonia*, MRSE and MRSA respectively. On the other hand, liquiritigenin **(6)** exhibited moderate activity against *K. pneumonia* and MRSE with the MIC value of 8.01 μg/mL and MBC value of 32.04 μg/mL, respectively (Fig. S31a-c).

4. Discussion

According to WHO the projected loss of life was 9.6 million in 2018; this will rise to over 13.1 million deaths in 2030 from cancer which is the most common and second leading cause of death worldwide (Fouad and Aanei, 2017). Currently, chemotherapies, radiotherapies, cytoredcutive surgery, endocrine and molecular therapies are commonly used strategies for cancer treatments (Geay, 2013). However, tumor heterogeneity, stage variations, drug resistance and toxicity seem to be critical obstacles that limit the therapeutic efficacy. Hence, search for rationalized and potential patient specific safe chemotherapeutic agents is continued globally as a priority area of research for exploring new lead molecules which can help minimize the side effects and assist in effective treatments.

Over the decades, the search for novel antibiotics continues to be of utmost importance in the fight against the challenge of antibiotic resistance (Falagas et al., 2010). However, in recent years, due to the extensive use and abuse of antibiotics, bacterial pathogens have developed drug resistance to commercial antibiotics. Consequently, the investigation for novel antibacterial compounds with unique structures for these bacteria is urgent (Aksoy and Unal, 2008, Dahiya and Purkayastha 2012, Park et al., 2015).

From ancient to modern medicines, plants represent vital resources and are considered to be easily accessible, cost-effective and safe to use in the treatment and management of various diseases, illnesses and infections (Aljubiri et al.,

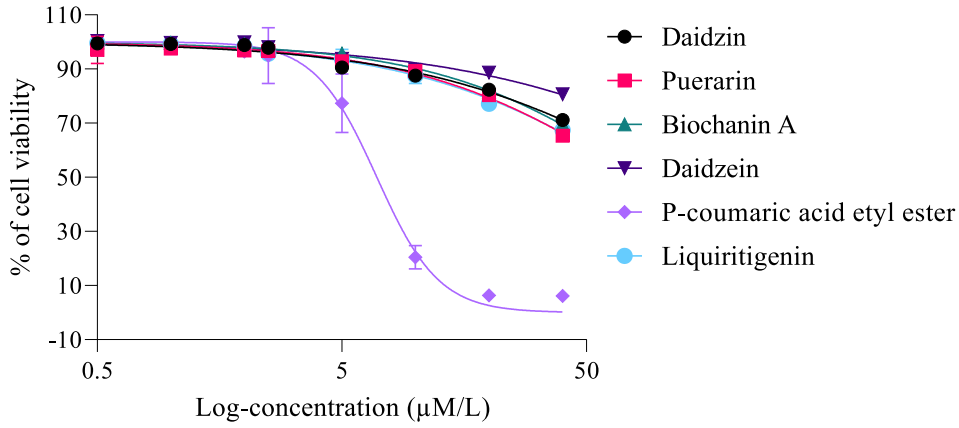

Fig. 4a Cytotoxicity of isolated compounds **(1–6)** against 4T1 mouse breast cancer cells

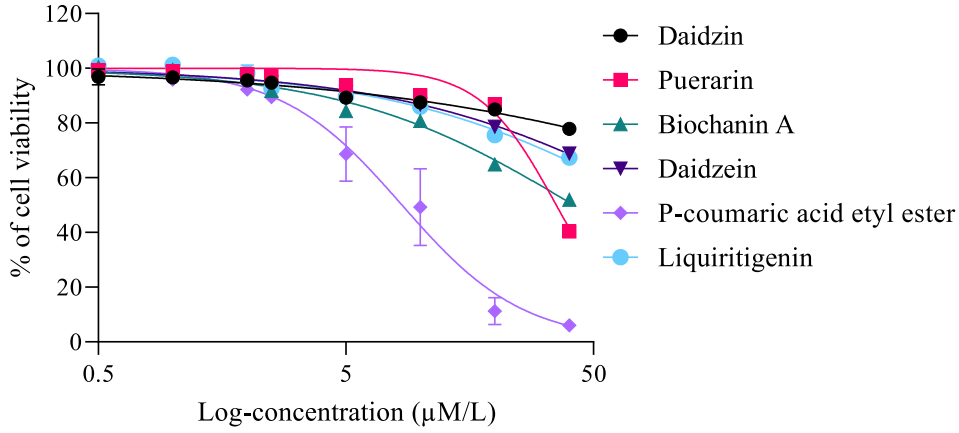

Fig. 4b Cytotoxicity of isolated compounds **(1–6)** against human non-small cell lung cancer NCI-H1975 cells

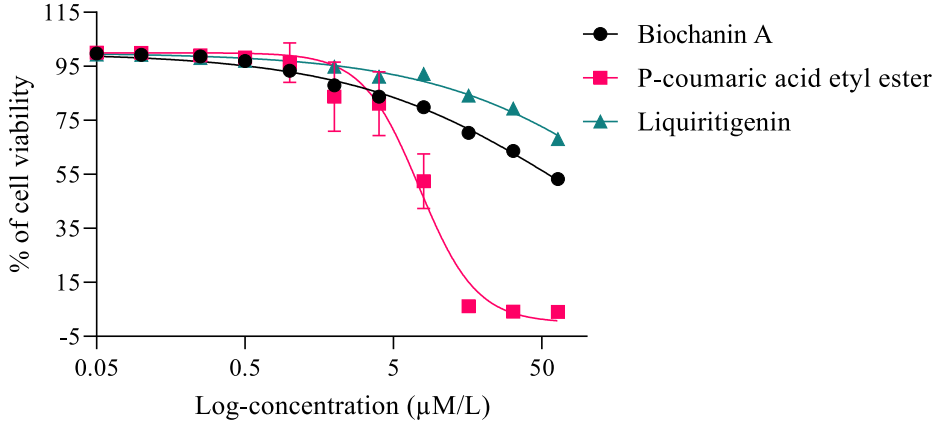

Fig. 4c Cytotoxicity of isolated compounds **(3, 5 and 6)** against human lung adenocarcinoma A549 cells

2021). In the past years, several lines of evidence showed that herbal extract and phytocompounds from various traditional medicinal plants had played a major role in treating various cancers and bacterial infections, thereby signifying the use of phytochemicals against cancer and bacterial pathogens as efficient anticancer agents and antibacterials for novel drug development (Wang et al., 2012, Fernández et al., 2016, Manickam and Preetha 2016). Furthermore, several medicinal plants are cultivated worldwide to be used in pharmacy due to therapeutic activities of their active phytoconstituents (Jones et al., 2006). Nowadays, compounds obtained from medicinal plants can be used in modern medicine with 80% correlation between their traditional uses and modern therapeutic activities (Sarkar et al., 2015).

As part of our continuous search for bioactive compounds from medicinal plants, *P. thomsonii* was subjected to isolation

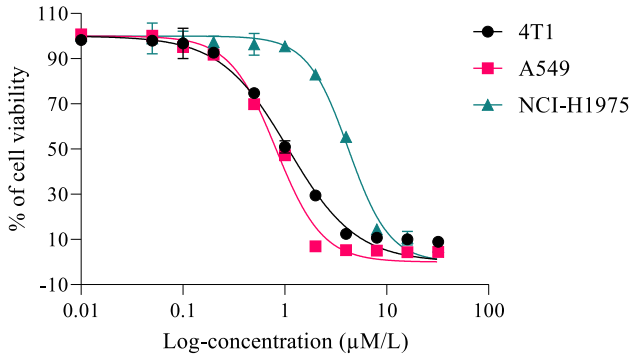

Fig. 4d Cytotoxicity of standard drug doxorubicin against human lung adenocarcinoma A549 cells (A549), human non-small cell lung cancer NCI-H1975 (NCI-H1975) and 4T1 mouse breast cancer cells (4T1)

Table 1 Antibacterial activity using disc diffusion assay

Compound	*K. pneumonia*	MRSA	MRSE
	Inhibition zones (mm)		
3	11.85 ± 1.34	–	–
5	20.62 ± 0.11	10.15 ± 0.20	13.01 ± 0.13
6	14.74 ± 1.38	–	13.00 ± 0.85
kanamycin	13.51 ± 0.06	–	14.23 ± 0.33

Table 2 Minimal inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the isolated compounds

Compound (μg/mL)	*K. pneumonia*		MRSA		MRSE	
	MIC	MBC	MIC	MBC	MIC	MBC
3	17.77	74.08	–	–	–	–
5	6.01	12.01	24.02	48.04	12.01	24.02
6	8.01	32.04	–	–	8.01	32.04
kanamycin	0.02	3.13	–	–	0.02	6.25

of bioactive molecules through in vitro micropropagation. *Pueraria* are perennial vine plants widely distributed in China (Liu et al., 2021). Among the nine species and two variants of *Pueraria* in China *P. thomsonii* was used often as medicine especially in traditional Chinese pharmacopeia (Wang et al., 2018). For more than thousands of years it has been widely used for the treatment of acute dysentery, obesity, cardiovascular diseases, diarrhea, diabetes, fever and flu, enhancing detoxification processes, improving liver function and relieving hangover (Wong et al., 2011). Over the past decades, abundant studies have been made on pharmacological activities of phytoconstituents isolated from *P. thomsonii*. Such reports cause this plant's bioactive compounds to be in high demand and the production of these active chemical constituents need to be enhanced using in vitro elicitation method in order to meet the needs of medicinal and pharmaceutical industries. Therefore, the present study focused on elicitation, isolation and characterization of potential compounds from the shoot tip derived calli of *P. thomsonii*. Interestingly, the callus culture conditions established in the present study induced the formation of fast growing friable calli from the explants. Our result is in agreement with the previous report of Thiem (2003) (Thiem 2003) in which *Pueraria lobata* was micropropagated from shoot tips for callus induction on MS medium. Moreover, this result also corroborated with the idea of other authors who showed that callus cultures were the realistic material to produce various secondary metabolites (Gomes-Copeland et al., 2018, Cabanas-Garcia et al., 2021).

The callus extract (EtOH-PT) was subjected to cytotoxicity and antibacterial studies against A549, 4T1 and NCH-1974 cell lines and bacterial pathogens Viz., *K. pneumoniae*, MRSE and MRSA. In the preliminary screening, EtOH-PT showed potent cytotoxic effects against 4T1 cells when compared to other tested cells which were considered as one of the useful parameters of anticancer effect. On the other hand, EtOH-PT exhibited remarkable antibacterial activity against *K. pneumoniae*, MRSE and MRSA with MIC and MBC values in the range of 18.26–73.04 μg/mL and 73.04–146.08 μg/mL. From the literature, it is evident that crude extracts from medicinal plants have proved to possess anticancer and antibacterial activities with lesser side effects. The remarkable anticancer and antibacterial activities of callus extract further encouraged us to study its chemical constituents responsible for significant cytotoxic and antibacterial activities. Further, the chemical exploration led to the isolation of four isoflavones along with a dihydroxyflavanone and a cinnamic acid-type of compound from the active callus extract and as far as the authors are concerned, this is the first time occurrence of isoflavones, dihydroxyflavanone and coumaric acid derivative in the callus culture of *P. thomsonii*. Briefly the compounds **1, 2, 3, 4, 5** and **6** were obtained as white powders, white amorphous powder, faint yellow amorphous powder, yellow needles and colorless crystals, respectively. The structures of compounds **1–6** were elucidated as daidzin, puerarin, biochanin A, daidzein, p-coumaric acid ethyl ester and liquiritigenin based on their comprehensive spectroscopic data and by comparison of their NMR data with those reported before (Kim et al., 2006, Peng et al., 2011, Formisano et al., 2012, Lee and Cho 2012, Fu et al., 2015, Zhang et al., 2018). Isoflavonoids, aflavonoid and cinnamic acid derivatives are a group of natural occurring compounds predominantly found in medicinal plants and also in plant products. Recently, they have gained substantial importance due to the diverse biological activities such as antimicrobial, antioxidative, cytotoxicity, contraceptive, insecticidal, oestrogenic and piscicidal properties (Devi et al., 2009). During our ongoing search for potent compounds from aforementioned plant, the compounds **1–6** were evaluated for their cytotoxic and antibacterial activities. As a result, p-coumaric acid ethyl ester **(5)** showed significant cytotoxicity against the tested cancer cell lines 4T1, NCI-H1975 and A549. The compounds daidzin **(1),** puerarin **(2),** biochanin A **(3),** daidzein **(4),** and liquiritigenin **(6)** showed moderate to weak cytotoxicity against 4T1, NCI-H1975. These results are supported by the earlier reports of Yu and Li (Yu and Li, 2006) in which puerarin **(2)** was proved to induce apoptosis in colon cancer HT-29 cells. Another study reported by Sarfraz et al. (Sarfraz et al., 2020) showed that biochanin A **(3),** a novel multifunctional bioactive isoflavone combated cancer development by inducing apoptosis, inhibition of metastasis and arresting cell cycle via targeting several deregulated signaling pathways of cancer. Similarly, Hua et al. (Hua et al., 2018) reported that daidzein **(4)** exerted anticancer activity towards SKOV3 human ovarian cancer cells by inducing apoptosis and cell

cycle arrest, and by inhibiting Raf/MEK/ERK cascade (Huang et al., 2021). For instance, it was reported that liquiritigenin **(6)** inhibited migration of A549 cells via downregulation of ProMMP-2 and PI3K/Akt signaling pathway (Wang et al. 2012). On the other hand daidzin **(1)**, puerarin **(2)** and daidzein **(4)** showed no activity against A549 cells. Most of the natural products with anticancer activities have been reported to exert a multistep process of carcinogenesis by cancer cells. Regarding anticancer potential, cytotoxicity is the most important criterion to be assessed to find out the selectivity toward cancer cells. In this context, the isolated compounds were further validated by comparing the cytotoxic capacity on normal cells. In accordance with Zubair et al. (2021) daidzin had no cytotoxic effect at the concentration of 100 μg/mL on Vero cells (normal kidney epithelial cells originated from *Cercopithecus aethiops*). Similarly, another study conducted by Zhou et al. (2020) reported that puerarin showed no cytotoxic effect on normal liver cell line (L02 cells) at the concentration of 50 mM. More recently, Hsu et al. (2018) and Wang et al. (2020) reported that biochanin A was less toxic to human gastric normal cell line (GES-1) and normal peripheral blood mononuclear cell (PBMC). On the other hand, Park et al. (2013) reported that daidzein showed no cytotoxic effect on normal human hepatocytes. Correlating the previous reports obtained for the normal cells which showed that the isolated compounds were found to be selective towards cancer cells over non-tumoral ones, this study also confirmed their selective anticancer activity. Therefore, the selective cytotoxicity by these compounds in the study was regarded as an important step in developing potent anticancer regimes. In addition, the isolated compounds showed strong to weak antibacterial activity against Gram negative bacterial pathogen *K. pneumonia* and Gram positive bacterial pathogens MRSE and MRSA at very low MIC and MBC values. Our results are in agreement with the previous report of Kumarihamy et al. who showed that a novel compound showed significant activity against the tested bacterial pathogens (Kumarihamy et al., 2021).

Further, to understand these differential effects of biological activities, a structure–activity relationship (SAR) study was performed by analyzing the chemical structure patterns of the most active compound. Based on significant cytotoxic and antibacterial activities, the SAR of the most active compound p-coumaric acid ethyl ester can be explained as follows. As shown in Figs. 4a-c, the naturally occurring cinnamic acid ethyl ester derivatives showed higher cytotoxicity against the tested cancer cell lines. These results were supported by the earlier reports of Menezes et al. 2017 and Pei et al. (2016) in which esterification significantly increased the partition coefficient and lipophilicity which improved p-coumaric acid's ability in crossing the membranes and led to higher pharmacological effectiveness of the ethyl ester derivative. This hypothesis was also further confirmed by similar study conducted by Menezes et al. in which the presence of single 4-OH functional group was the most favorable substitution pattern on the aromatic ring which influenced the cytotoxic activity of the test compound suggesting that this analogue with free hydroxyl group was generally more potent than other tested compounds (Menezes et al. 2017). This hypothesis was also further confirmed by another report of Alves et al. where hydroxyl (OH) group of the benzene ring position seemed to play an important role in the studied aflavonoid and phenolic compounds anti-MRSA activity. Correlating the previous reports, OH group seems to be essential for the antibacterial activity, supporting its SAR (Alves et al., 2013). However, the ethyl ester moiety may increase the interaction with the bacterial phospholipids cell membrane which triggers cell death by injuring plasma membrane and cytoplasmic distortion which justifies the remarkable antibacterial activities against the tested bacterial pathogens. Therefore, we hypothesize that the cytotoxic and antibacterial activities of the isolated compounds have similar mode of activities against tested cell lines and bacterial pathogens.

5. Conclusion

In conclusion, the present study demonstrated the in vitro callus induction through axillary shoot in *P. thomsonii* was the best in vitro condition for inducing the synthesis of anticancer and antibacterial compounds. Intriguingly, our results also showed that micropropagated callus tissue maintained the ability of high level of bioactive compounds' production which resulted in the isolation and identification of four isoflavones, aflavonoid and an ethyl ester of the coumaric acid as alternative source. In this line, all the isolated compounds **(1–6)** were tested for cytotoxic properties against 4T1, NCI-H1975 and A549 cells. Of these, p-coumaric acid ethyl ester **(5)** showed significant inhibitory activities against 4T1, NCI-H1975, and A549 cells with GI_{50} values of 14.73, 15.64 and 20.88 μM/mL, respectively. On the other hand compounds biochanin A < liquiritigenin < daidzein < daidzin < puerarin exhibited moderate to moderate activity against NCI-H1975. Similarly, puerarin < biochanin A < liquiritigenin < daidzin < daidzein induced appreciable cytotoxicity against 4T1 cells; biochanin A and liquiritigenin showed moderate cytotoxicity against A549 cells. Compounds (**1–6**) were also tested for their antibacterial activity against *K. pneumonia,* MRSE and MRSA. Among them p-coumaric acid ethyl ester **(5)** showed potent activity at very low MIC and MBC concentrations of 6.01, 12.01 and 24.02 μg/mL and 12.01, 24.02 and 48.04 μg/mL against *K. pneumonia*, MRSE and MRSA, respectively. In addition, it has also been hypothesized that the biological activities and their mode of action based on SAR have similar mechanism of action against cancer cell and bacterial pathogens. Finally, our results support the idea of callus culture for future scale-up optimization of active compounds production. Further, they can be used as natural anticancer and antibacterials in integrated cancer and bacterial infections management programs.

6. Contributors' statement

Yu Li- YL, Pachaiyappan Saravana Kumar- PSK, Xuhui Tan- XHT, Jiao qiu- JQ, Mingyuan Yuan- MYY, Renjun Zhao- RJZ, Lunqiang Ai- LQA, Ji Zhua- JZ, Yalan Ran- YLR, Meijun He- MJH. Conceptualization- YL, MJH and PSK; Methodology- JQ and XHT; Software and Validation, YLR, MYY and JZ; Formal analysis, investigation and resources- RJZ and PSK; Data curation- MJH; Writing – original draft preparation and writing-review and editing- YL and PSK; Visualization LQA and YLR; Supervision, RJZ; Project administration and funding acquisition- M.J.H and YL.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.arabjc.2022.103854.

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