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Therapeutic profiling of *Thespesia populnea* seed acetone extract: A mangrove-associated source of antioxidant, antimicrobial and cytotoxic activities

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The seeds of *Thespesia populnea*, a mangrove-associated plant, remain understudied despite their potential bioactivity. Among various extracts, the acetone extract showed the highest polyphenol (186.13 ± 0.06 mg GAE/g) and flavonoid (475.71 ± 8.08 mg QE/g) contents. Antioxidant assays demonstrated a significant free radical scavenging activity. It exhibited strong antimicrobial effects, with inhibition zones of 16–18 mm against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and MIC values of 28 μg/mL (*Candida albicans*) and 51 μg/mL (*Aspergillus niger*). The MTT assay revealed selective cytotoxicity against MCF-7 breast cancer cells (IC_{50} = 76.8 ± 2.1 μg/mL) over L929 normal fibroblasts (IC_{50} = 286.34 ± 1.8 μg/mL). GC–MS and FTIR analyses confirmed the presence of bioactive phytochemicals responsible for these effects. This is the first comprehensive report highlighting the antioxidant, antimicrobial and anticancer potential of *T. populnea* seed extract, supporting its relevance as a promising natural candidate for biomedical applications.

Keywords: *Thespesia populnea*, antioxidant, antibacterial, anti-tumour, MCF-7

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INTRODUCTION

Thespesia populnea (L.) Soland. ex Corrêa, commonly known as the Portia tree, is a fast-growing, salt-tolerant mangrove associate species of the Malvaceae family widely distributed in tropical and subtropical coastal regions. It has garnered a considerable attention in traditional medicine systems, including Ayurveda, Unani and Siddha, where various parts of the plant have been used to treat ailments such as skin infections, inflammation, ulcers, jaundice and liver disorders. Traditionally, the bark and leaves are the most commonly utilised plant parts and a number of studies have since validated their antioxidant, anti-inflammatory and hepatoprotective effects [1, 2]. In spite of its ethnomedicinal value, it remains scientifically underexplored, thereby qualifying as a neglected and underutilised species (NUS) [3].

Mangroves and mangrove associates are plants that grow in unique environments characterised by a high salinity, moisture levels, tidal variations and strong winds [4, 5]. These challenging conditions have prompted adaptations in mangroves and their associates, potentially leading to the accumulation of biologically active phytochemicals that protect them from their harsh environmental surroundings [6]. These plants produce numerous secondary metabolites, such as polyphenols, flavonoids, alkaloids, terpenoids, etc. which play a pivotal role in defence mechanisms and are responsible for a wide array of bioactivities that form the foundation of many modern therapeutic agents [7, 8].

Considering the medicinal importance and phytochemical richness of *Thespesia populnea* (*T. populnea*), it is important to explore its potential in managing conditions linked to cellular damage. One of the major causes of such damage is oxidative stress, which occurs when the body produces more reactive oxygen species (ROS) and reactive nitrogen species (RNS) than it can neutralise. Elevated levels of these reactive species can harm cells and tissues and are known to play a key role in the development of various physiological ailments [9]. Research indicates that these plant-derived compounds play a vital role in reducing oxidative stress by either deactivating or neutralising free radicals [10]. The significance of plant metabolites is particularly important in the context of rising antimicrobial resistance and the global burden of cancer, where nature-derived compounds continue to offer structurally novel and biologically potent alternatives to synthetic drugs [11].

Despite the ethnopharmacological use of *Thespesia populnea*, there exists a noticeable research bias towards specific plant parts – particularly the bark, leaves and flowers [12–14]. In contrast, the seeds remain significantly underexplored, despite the emerging evidence supporting their rich phytochemical composition and comparable therapeutic potential [15]. This oversight represents a notable gap in the comprehensive phytopharmacological profiling of the species. The underutilisation of these seeds also has implications for sustainability, as they are typically discarded as agricultural or processing waste. Moreover, seeds offer advantages in terms of storage, transport and preservation, possessing a longer shelf life than most other plant parts [16].

The emergence of multidrug-resistant pathogens and chemoresistant cancers demand for multifunctional therapeutic agents capable of targeting diverse biological pathways. Plants that exhibit such multifunctionality are particularly valuable in the context of ethnobotanical drug discovery. The lack of selectivity profiling also presents a critical limitation in the existing literature. For a plant extract to be considered a viable candidate for therapeutic development, it must demonstrate a selective toxicity – preferentially targeting cancerous cells while sparing normal, healthy cells. However, few studies on *T. populnea* have evaluated this aspect. The present study addresses this gap by including the L929 mouse fibroblast cell line as a non-cancerous counterpart to the MCF-7 breast cancer cell line, thereby assessing the cytotoxic selectivity of the seed extract. In doing so, this work contributes novel insights to the phytomedicinal profile of *T. populnea* and establishes it as a multifunctional bioactive agent with potential applications in managing oxidative stress, microbial infections and cancer. By integrating pharmacological efficacy with ecological and therapeutic relevance, this study positions *T. populnea* seeds as a promising candidate in the biodiversity-to-biomedicine pipeline. The evidence-based valorisation not only reinforces the scientific foundation of traditional knowledge but also promotes sustainable natural product development while highlighting the need for wetland conservation.

MATERIALS AND METHODS

Materials

The seeds of *T. populnea* were collected from Thrissur-Kole wetlands (UNESCO recognised Ramsar Site), Kerala, India, on May 2023. The plant was identified in the field by Dr. Pramod Kumar, Assistant Professor and taxonomist, Department of Botany, NSS College, Ottapalam, Palakkad, Kerala, India, and it was deposited in the Regional Herbarium of Kerala, Department of Botany, St. Berchmans College, Changanacherry, under the accession number RHK7662. For the present study, analytical grade chemicals were purchased from Sigma Aldrich and used as received without further purification. Aqueous solutions were made in Milli-Q water. The HPLC grade of hexane, acetone, ethanol and methanol were used. For the antimicrobial studies, cultures of *Escherichia coli* (MTCC 723), *Klebsiella pneumoniae* (MTCC 109), *Pseudomonas aeruginosa* (MTCC 424), *Staphylococcus aureus* (MTCC 96), *Candida albicans* and *Aspergillus niger* were collected from the School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala, India. For cytotoxicity studies, DLA (T-cell lymphoma), MCF-7 (breast cancer) and L929 (mouse fibroblast) cells were sourced from the National Centre for Cell Sciences (NCCS) located in Pune, India.

Phytochemical analysis of *T. populnea*

Extract preparation

The seeds of *T. populnea* were thoroughly washed with distilled water, air-dried under sunshade for three days and subsequently ground into a fine powder. For extraction, 20 g of the powdered seed material was loaded into a cellulose extraction thimble and placed in an automated Soxhlet apparatus (SoxTron SOX 3, Tulin Equipments, India) equipped with a microprocessor-controlled time–temperature programming unit. Soxhlet extraction was carried out for 8 h using four solvents – hexane, acetone, ethanol and water – each applied separately at a sample-to-solvent ratio of 1:10 (w/v). The extraction temperatures were set according to the boiling points of the respective solvents: 68°C for hexane, 56°C for acetone, 78°C for ethanol and 100°C for water. After extraction, the solvents were completely removed under reduced pressure at 45°C using a rotary evaporator to obtain the dried crude extracts. The resulting hexane, acetone, ethanolic and aqueous extracts were stored at –20°C until further analysis [17].

The extraction yield is calculated by the following equation,

$$\text{Extraction yield (\%)} = \frac{W_{ext} \times 100}{W_p},$$

where W_p and W_{ext} are the mass of dry plant and the mass of crude extract, respectively.

Column chromatographic fractionation of acetone extract

The acetone extract of *T. populnea* seeds was subjected to silica gel column chromatography for the isolation of bioactive subfractions. A vertical borosilicate glass column (200 mm length × 15 mm internal diameter) was used for the separation process. Prior to packing, the column was thoroughly rinsed with acetone and dried completely. A piece of glass wool was placed at the bottom of the column to support the stationary phase. Silica gel (60–120 mesh) was employed as the stationary phase and a slurry was prepared using hexane. The silica slurry was carefully poured into the column, allowing a simultaneous drainage of solvent to ensure a uniform and compact packing of the silica bed, which occupied approximately two-thirds of the column length. A total of 39 mg of the acetone extract was dissolved in a minimal volume of hexane and carefully loaded along the inner wall of the column. The column was subsequently rinsed with an additional solvent to ensure the complete transfer of the sample. Fractionation was performed using a gradient elution technique, beginning with non-polar solvents and progressing to more polar solvents (i.e. hexane to methanol) in increasing polarity ratios. Collected subfractions were concentrated under reduced pressure using a rotary evaporator to remove residual organic solvents and were analysed for the DPPH activity and total antioxidant capacity.

Determination of total phenolic content (TPC)

The determination of total phenolic content in the extracts was conducted using the Folin–Ciocalteu method [18], employing gallic acid as the standard solution. The assay involved mixing 500 µL of the sample with 2 mL of 10% Folin reagent, followed by neutralisation with 4 mL of 7.5% sodium

carbonate solution. The prepared mixture was incubated at ambient temperature for 30 min with an occasional shaking. Following the incubation, its absorbance maximum was recorded at 765 nm. A calibration curve was generated using gallic acid as the standard ($y = 0.01122x$; $R^2 = 0.9991$, Fig. 1a, supporting information) and the results were expressed as milligrams of gallic acid equivalents (GAE) per gram of the dry sample.

Determination of total flavonoid content (TFC)

The total flavonoid content in the extracts was determined using the aluminium chloride method, employing quercetin as the standard solution [19]. To perform the assay, 500 µL of extract was mixed with 1.5 mL of 95% ethanol. Subsequently, 100 µL of 10% aluminium chloride and 100 µL of potassium acetate were added to the solution and the total volume was adjusted to 5 mL with distilled water. The resulting mixture was then incubated at room temperature for 30 min, after which the developed yellow colour was measured at 415 nm using a UV–vis spectrophotometer. The blank solution, used for calibration, contained the same volume of milli-Q-water in place of the 10% aluminium chloride solution. A standard curve was constructed using quercetin ($y = 0.0042x$; $R^2 = 0.9978$, Fig. 1b, supporting information), and the final results were reported as milligrams of quercetin equivalents (QE) per gm of the dry sample.

Antioxidant assays

The antioxidant potential of the extracts was systematically evaluated using a set of standard assays: DPPH, $ABTS^{+\bullet}$, SOD, FRAP and TAC.

(i) DPPH scavenging assay

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was evaluated following the procedure outlined by Aquino et al. [20]. Initially, 3 mg of DPPH was dissolved in 25 mL of methanol. Subsequently, various concentrations of the sample aliquots (0–100 µg/mL) were added to 187 µL of the freshly prepared DPPH solution. The total volume of the solution was adjusted to 1 mL with methanol. After an incubation period of 30 min, the absorbance values for different concentrations of the extract were measured spectrophotometrically at 515 nm. Each concentration was analysed in triplicate. The radical scavenging activity of the sample was quantified in terms of percentage inhibition. The IC_{50} value (half maximal inhibitory concentration) for each extract was determined using a linear regression method. Ascorbic acid was used as the positive control (Fig. 2a, supporting information).

$$\text{Inhibition on DPPH radical (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

(ii) $ABTS^{+\bullet}$ assay

The $ABTS^{+\bullet}$ (2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid) radical scavenging assay was conducted following the procedure outlined by Barton et al. [21]. Initially, the $ABTS^{+\bullet}$ radical cation was generated by the reaction between ABTS and $Na_2S_2O_8$. Subsequently, the 2 mL solution of $ABTS^{+\bullet}$ was mixed with different concentrations of the sample aliquots (0–100 µg/mL). After an incubation period of 6 min, the absorbance of the solution was measured at a wavelength of 734 nm. All measurements were performed in triplicate. The IC_{50} value for each extract was determined using a linear regression method keeping trolox as the positive control (Fig. 2b, supporting information).

$$\text{Inhibition of ABTS radical (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

(iii) SOD assay

The detection of superoxide (SOD) radicals ($O_2^{-\bullet}$) generated from the photo reduction of riboflavin was achieved using the NBT (nitroblue tetrazolium) reduction method of Mc Cord and Fridovich [22]. The reaction mixture comprised of 0.2 mL EDTA (6 mM) containing 3 µg NaCN, 0.05 mL riboflavin (2 µM), 0.1 mL NBT (50 µM), 2.63 mL KH_2PO_4-Na_2HPO_4 buffer (67 mM, pH 7.8) and 0.02 mL various concentrations of the extract (0–300 µg/mL), all in a final volume of 3 mL, was used. The reaction tubes were subjected to light exposure using an incandescent lamp for a duration of 15 min. Absorbance at 560 nm was taken before and after illumination. The percentage inhibition of superoxide generation was assessed by comparing the absorbance between the untreated control and the treated samples. The IC_{50} value of extracts

were calculated keeping ascorbic acid as the positive control (Fig. 2c, supporting information).

$$\text{Inhibition of superoxide radical (\%)} = \frac{\Delta \text{ Absorbance of control} - \Delta \text{ Absorbance of sample}}{\Delta \text{ Absorbance of control}} \times 100$$

Δ Absorbance = Absorbance after illumination – Absorbance before illumination

(iv) FRAP assay

In the ferric reducing ability of plasma (FRAP) assay, the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions facilitates the formation of a deeply blue-coloured complex between ferrous ions and tripyridyl-s-triazine (TPTZ), indicating a successful reduction and complexation reaction, with its absorbance peak at 595 nm. The FRAP reagent consisted of 25 mL of acetate buffer, 2.5 mL of ferric chloride and 2.5 mL of 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ). Different concentrations of the sample (0–50 μg/mL) were adjusted to 1 mL with the freshly prepared FRAP reagent. The reagent formulation was then incubated for 15 min at 37°C and subsequently measured against distilled water at 595 nm. The EC_{50} (effective concentration 50%) values of the extracts were calculated in reference to vitamin E as the positive control (Fig. 2d, supporting information). The amount of Fe^{2+} generated from the reduction of Fe^{3+} by the extract was determined using a standard curve prepared with a ferrous sulphate solution ($y = 0.00314x + 0.00153$, $R^2 = 0.9947$, where y is the absorbance at 593 nm and x is the concentration of ferrous sulphate solution (Fig. 3, supporting information) and the FRAP values were expressed as mM Fe^{2+} per μg of extract [23].

$$\text{Inhibition in FRAP radical (\%)} = \frac{\text{Absorbance of sample} - \text{Absorbance of control}}{\text{Absorbance of control}} \times 100$$

(v) Total antioxidant capacity (TAC)

This assay relies on the reduction of Mo(VI) to Mo(V) by the sample, resulting in the formation of a green-coloured phosphomolybdenum complex under acidic pH conditions. 50 mL reagent solution was prepared by combining 0.247 g of ammonium molybdate and 0.168 g of sodium dihydrogen phosphate with 1.6 mL of 0.6 M sulfuric acid. Subsequently, 50 μL of the extract was added to 1 mL of the reagent solution and incubated at 95°C for 90 min. Following the incubation, the mixture was cooled to room temperature and absorbance was measured at 695 nm against the blank. The reagent mixture without an extract served as the blank reference. Trolox was employed as the standard. The total antioxidant capacity (TAC) of extracts was determined by using a linear regression equation of the calibration curve for trolox $y = 0.0152x$, $R^2 = 0.9846$, where y is the absorbance at 695 nm and x is the concentration of Trolox in μg/mL (Fig. 4, supporting information), and was expressed as milligrams of trolox equivalents per gram (mg/g) of the sample [24].

Antimicrobial assays

The antimicrobial activity of the extracts was determined using the broth microdilution method and the agar well diffusion method.

(i) Broth microdilution method

The antimicrobial activity of the seed extracts was assessed against four bacterial strains – *E. coli* (MTCC 723), *K. pneumoniae* (MTCC 109), *P. aeruginosa* (MTCC 424) and *S. aureus* (MTCC 96), and two fungal species – *C. albicans* and *A. niger* using the broth microdilution method. All microbial strains were subcultured in nutrient broth and incubated aerobically at 37°C for 12 h. The resulting cultures were adjusted to a turbidity equivalent to the 0.5 McFarland standard (~1.5 × 108 CFU/mL) to ensure the uniform inoculum density. For microdilution, 100 μL of the standardised microbial suspension was added to the wells of a sterile 96-well microtiter plate containing 100 μL of two-fold serially diluted seed extracts in the appropriate growth medium, resulting in a final volume of 200 μL per well [25]. To correct for the inherent absorbance of the plant extract, a blank control containing the extract and broth without a microbial inoculum was included. Negative controls containing a microbial suspension and broth (without extract) and positive controls containing standard antibiotics or antifungal agents were also maintained. Nutrient broth was used as the diluent and cultivation medium for all bacterial strains, while Mueller–Hinton broth (MHB) was used for wells containing gentamicin as the positive antibiotic control. For

antifungal testing involving *C. albicans*, *A. niger* and amphotericin B, the RPMI 1640 medium buffered with 0.165 M MOPS (pH 7.0) was employed. The plates were gently shaken at 300 rpm for 20 min to ensure a uniform mixing and then incubated at for 24 h. Microbial growth was quantified by measuring absorbance at 600 nm using a MultiSkan FC Microplate Reader (Thermo Scientific, USA). The minimum inhibitory concentration (MIC) of each extract was determined as the lowest concentration that demonstrated no growth.

(ii) Agar well diffusion method

The antibacterial assessment of *T. populnea* extracts was also conducted using the agar well diffusion method [26]. In this procedure, nutrient agar plates were first inoculated with subcultures of *E. coli* (MTCC 723), *K. pneumoniae* (MTCC 109), *P. aeruginosa* (MTCC 424) and *S. aureus* (MTCC 96). Subsequently, five wells of 6 mm in diameter were created in the agar plates. 1 mg of all extracts (hexane, acetone, ethanolic and water), along with 10% DMSO (serving as the negative control), were added to wells 1 to 5, respectively. Following a 24-hour incubation period, the zones of inhibition were measured. Analysis was conducted in triplicate to ensure the robustness and reliability of the results.

Antitumour assays

Antitumour assays were performed on the extracts by in vitro cytotoxicity studies using the trypan blue exclusion method on DLA cell lines and MTT assay on MCF-7 cell lines.

(i) Trypan blue exclusion method on DLA cell lines

In vitro cytotoxicity activities of seed extracts from *T. populnea* were evaluated using Daltons lymphoma ascites cells (DLA) [27]. Tumour cells were extracted from the peritoneal cavity of tumour-bearing mice and were washed thrice with a normal saline solution to remove cellular debris and contaminants. Cell viability was determined by the trypan blue exclusion assay, to distinguish viable cells from non-viable ones. Viable cell suspensions (1×10^6 cells in 0.1 mL) were added to tubes containing various concentrations of the samples (0–200 μg/mL), with the volume adjusted to 1 mL using phosphate-buffered saline (PBS). Control tubes contained only cell suspensions. These assay mixtures were then incubated for 3 h at 37°C. Subsequently, the cell suspensions were mixed with 0.1 mL of a 1% trypan blue solution and were left for 2–3 min. Dead cells absorbed the blue colour of trypan blue, while live cells remained unstained. The numbers of stained (dead) and unstained (live) cells were counted using a hemocytometer separately to determine cytotoxic effects. The results were compared with the IC_{50} values of a standard cytotoxic drug 5 – fluorouracil and curcumin [28, 29].

$$\text{Cytotoxicity (\%)} = \frac{\text{No. of dead cells}}{\text{No. of live cell + No. of dead cell}} \times 100$$

(ii) Anti-proliferative assay of acetone extract using the MTT method

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method was employed to assess the anti-proliferative activity of acetone extract on the MCF-7 breast cancer cell line and on the L929 mouse fibroblast cell line. The cell lines were cultured in 25 cm^2 tissue culture flasks using DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS, L-glutamine, sodium bicarbonate and an antibiotic solution containing penicillin (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (2.5 μg/mL). Cultured cell lines were maintained at 37°C in a humidified 5% CO_2 incubator (NBS Eppendörf, Germany).

After reaching confluence, the cells were trypsinised, suspended in the 10% growth medium and seeded in 96-well tissue culture plates at a density of 5×10^4 cells/well. Samples were prepared by adding 1 mg of the sample to 1 mL of DMEM, which was then completely dissolved using a cyclomixer. The solution was subsequently filtered through a 0.22 μm Millipore syringe filter to ensure sterility.

Upon achieving a sufficient cell growth, the growth medium was removed and the freshly prepared samples were serially diluted five times in 5% DMEM using two-fold microdilution (100, 50, 25, 12.5 and 6.25 μg in 100 μL of 5% DMEM). Each concentration was added in triplicate to the respective wells and incubated at 37°C in a humidified 5% CO_2 incubator.

A solution of 15 mg of MTT was reconstituted in 3 mL PBS until completely dissolved and sterilised by

filter sterilisation. After a 24-hour incubation period, the sample content in wells was removed and 30 µL of reconstituted MTT solution was added to all test and cell control wells. The plate was gently shaken and then incubated at 37°C in a humidified 5% CO_2 incubator for 4 h. Following the incubation period, the supernatant was removed and 100 µL of the MTT solubilisation solution (DMSO) was added to solubilise the formazan crystals. The absorbance values were measured using a microplate reader at a wavelength of 570 nm [30]. A non-linear regression analysis was done to calculate the IC_{50} of the extract. To assess the extract's efficacy against MCF-7 cells and fibroblast cells, its IC_{50} values were compared with those of the standard anticancer drug taxol (Paclitaxel) [31].

The percentage of cell viability was calculated using the formula:

$$\text{Cell viability (\%)} = \frac{\text{Mean OD of samples} \times 100}{\text{Mean OD of control group}}$$

The experiment was repeated three times for validation. Following a 24-hour incubation period, cytotoxicity was directly observed using an inverted phase-contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera), allowing for detailed microscopic observations at 10X magnification. Images of the observed cytotoxic effects were recorded for documentation and analysis.

Characterisation techniques

The dried extracts were used for FTIR analysis. The IR spectra were recorded using a Shimadzu IR Prestige-21 FTIR spectrometer equipped with a ZnSe ATR crystal. Spectral acquisition was carried out over a scan range of 600–4000 cm^{-1}, with 30 scans per sample and a resolution of 4 cm^{-1} [32]. Qualitative and quantitative analysis of the extract using GC MS was conducted using a Thermo Fisher Scientific TRACE 1300 GC system coupled with a TSQ 8000 EVO mass spectrometer and a non-polar 5% phenyl capillary column (Thermo-17MS; 30 m × 0.25 mm, 0.15 µm film). Electron ionization at 70 eV was employed, with helium (99.99%) as the carrier gas at a flow rate of 1 mL/min. A 1 µL sample was injected in the pulsed splitless mode (25 psi for 1 min), with the inlet temperature of 280°C and the ion source temperature of 250°C. The oven program started at 70°C (2 min hold), ramped to 150°C at 25°C/min (2 min hold), then to 200°C at 3°C/min (2 min hold) and finally to 280°C. Mass spectral data were analysed using the Xcalibur software (Thermo Fisher Scientific Inc., Waltham, MA, USA) with reference to the NIST 17 electronic mass spectral library, NISA MS Search Program V.2.3 and the NIST MS Interpreter (NIST, USA) [33].

A Multi Skan FC microplate reader with the SkanIt software, Thermo Scientific, USA, was used for measuring the absorbance of samples in antibacterial and anti-proliferative studies. An inverted phase contrast tissue culture microscope, Olympus CkX41 with OptiKa Pro5 CCD camera, with 10X magnification was used for recording images of the MTT assay.

Data analysis

The IC_{50} and MIC values for each extract in all the assays, were calculated using the linear regression analysis of concentration–response curves. The data were presented as the mean of the sample size $n = 3$ and standard deviation (SD). Linear regression analyses were carried out using the OriginPro 8.5 software. The Pearson correlation coefficients were calculated to evaluate the relationships among TPC, TFC and antioxidant activities evaluated by DPPH, $ABTS^{+\bullet}$, SOD and FRAP assays. Student's t-test (significant at $p < 0.01$) was used to compare the antioxidant and antimicrobial activity of extracts. To assess the cytotoxic selectivity of the acetone extract between cancerous (MCF-7) and non-cancerous (L929) cell lines, a two-way ANOVA was performed followed by the Tukey's post hoc test.

RESULTS AND DISCUSSION

Extraction yield, total phenolic and total flavonoid content of extracts

In this study, among the four solvents tested, acetone yielded the highest extractable content, indicating its superior ability to solubilise a broad spectrum of bioactive compounds from *T. populnea* seeds. It was reported that the type of solvent used significantly influenced the extraction yield, as the efficiency of phytochemical solubilisation is largely dependent on the solvent's molecular

structure and polarity [34]. After 8 h of soxhlet extraction, the percentage extraction yields followed the descending order: acetone extract (14.4 ± 0.1%) > hexane extract (10.8 ± 0.2%) > ethanolic extract (9.7 ± 0.3%) > aqueous extract (8.7 ± 0.2%). These results suggest that acetone, due to its intermediate polarity, effectively extracts both polar and non-polar phytoconstituents, thereby enhancing overall recovery. In contrast, water, despite being highly polar, resulted in the lowest yield, possibly due to its limited capacity to solubilise hydrophobic or moderately polar secondary metabolites.

Phenolic compounds are widely distributed in medicinal plants and are recognised for their antioxidant potential and other health-promoting properties. In this study, the total phenolic content and total flavonoid content of four different solvent extracts of *T. populnea* seeds were quantified and the results are presented in Fig. 1. Among the extracts, the acetone extract showed the highest levels of both phenolics and flavonoids. The TPC and TFC of the acetone extract were found to be 186.13 ± 0.06 mg GAE/g dry weight and 475.71 ± 8.08 mg QE/g dry weight, respectively.

These results are consistent with previous studies that reported the suitability of acetone, a solvent of intermediate polarity, for the efficient extraction of flavonoids, which are primarily present in plants as glycosidic derivatives [35, 36]. The elevated flavonoid content observed in the acetone extract may also be attributed to the presence of multiple hydroxyl groups in flavonoid structures, which enhance solubility in moderately polar solvents. Similar observations were made by Dirar et al. in studies on *G. senegalensis*, *B. linariifolia* and *M. pseudopetalosa* [37].

Moreover, as a mangrove-associated plant, *T. populnea* naturally inhabits stress-prone environments such as a high salinity and an intermittent flooding. These abiotic stress conditions are known to influence the secondary metabolite production as part of the plant's adaptive defense mechanisms [38]. The biosynthesis and accumulation of phenolic compounds are often enhanced under such conditions, driven by enzyme-regulated metabolic pathways. Previous reports have confirmed the presence of phenolics and flavonoids in various parts of *T. populnea*, including the bark, leaves, roots and flowers [39–41].

Analysis of acetone extract using GC–MS and FTIR

Since the acetone extract exhibited the highest content of polyphenols, it was selected for phytochemical screening using GC–MS and FTIR. GC–MS general profiling of the acetone extract of *T. populnea* revealed the presence of several bioactive compounds, including β-sitosterol (25.14%), cholesta-5,24-dien-3β-ol (11%), (20(22)E)-cholesta-5,20(22)-dien-3β-ol (9.2%), oleic acid (6.2%), γ-tocopherol (6%), stigmasterol (4.9%), δ-cadinene (4%), α-muurolene (3%), 9Z,12Z-octadecadienoic acid (2.5%) and muurolol (1%) (Fig. 2).

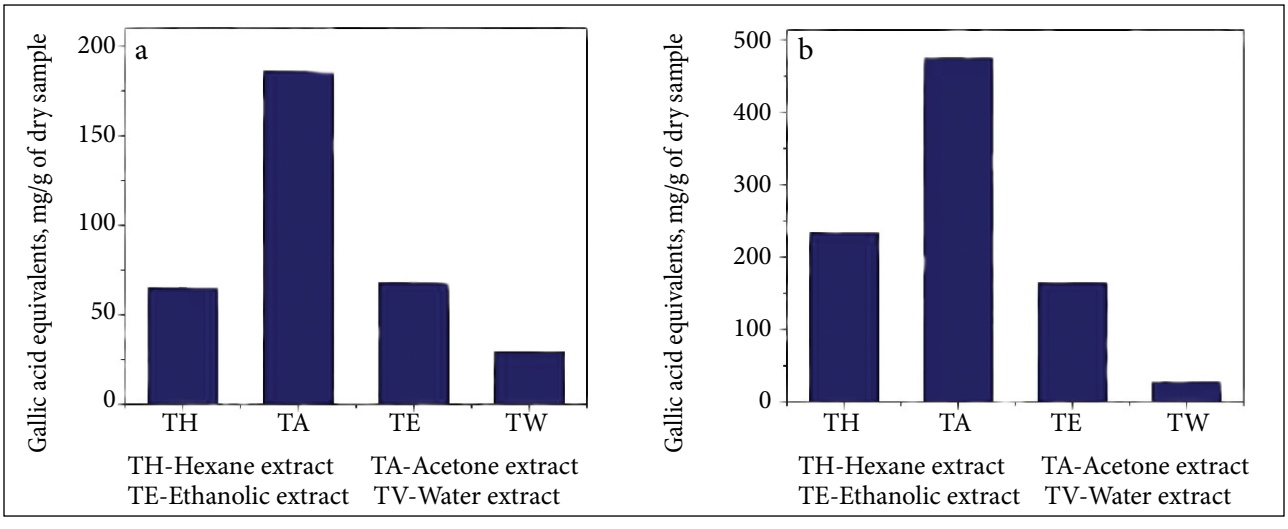

Fig. 1. (a) Total phenolic content and (b) total flavonoid content present in various extracts of *T. populnea* (Data are expressed as mean ± SD of triplicate experiments and the error bars are not visually distinguishable in the graph due to the very low SD associated with each measurement)

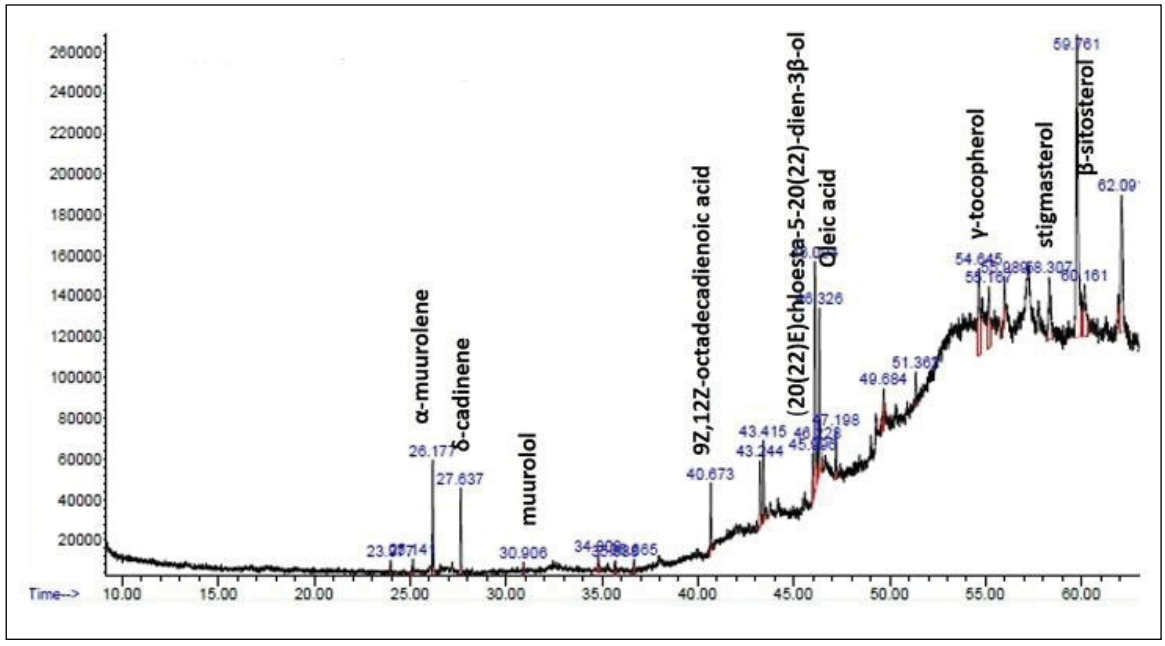

Fig. 2. GC-MS profile of the acetone extract of *T. populnea*

δ-cadinene, murolene and muurolol are sesquiterpenes having antioxidant [42], antifungal [43] and anticancer properties [44]. Tocopherol, stigmasterol and sitosterols identified from the acetone extract also exhibit many medicinal properties. Tocopherol, also known as Vitamin E, is a potent antioxidant that helps to protect cells from damage caused by free radicals, thereby reducing the risk of chronic diseases like heart disease and cancer [45]. It has also been reported to play a vital role in supporting the immune function and maintaining skin health [46]. Stigmasterol and sitosterols are plant sterols similar to cholesterol. They are used as a natural remedy for managing high cholesterol levels [47]. Sitosterols also possess anti-inflammatory properties and are important for prostate health [48].

The FTIR peak of the acetone extract of *T. populnea* is shown in Fig. 3. A broad intense peak at 3400–3200 cm^{-1} is the characteristic peak of the hydroxyl (-OH) group in alcohols or phenolic compounds. The acetone extract contains a high amount of phenolic compounds, which might be the reason for the appearance of this intense peak at 3306 cm^{-1} [49]. Strong peaks comprised in the domain 2860–2930 cm^{-1} correspond to the C–H stretching vibrations, particular to $–CH_3$ and $–CH_2$ in lipids and to C–H in aldehydes [50]. Bands observed at 1520–1650 cm^{-1} might be due to the C=C stretching vibrations from the aromatic rings in the phytosterols. A sharp and strong peak at 1392 cm^{-1} might correspond to the C–H bending of alkanes [51]. A strong peak at 1040 cm^{-1} can be attributed to C–O–C stretching vibrations, likely arising from glycosylated or polyoxygenated derivatives of β-sitosterol, stigmasterol, γ-tocopherol or fatty acid compounds [52, 53]. Thus, the results from GC–MS and FTIR further confirmed the presence of polyphenols in the acetone extract.

Antioxidant activities of extracts

The antioxidant activities of the seed extracts were comprehensively evaluated using multiple in vi-

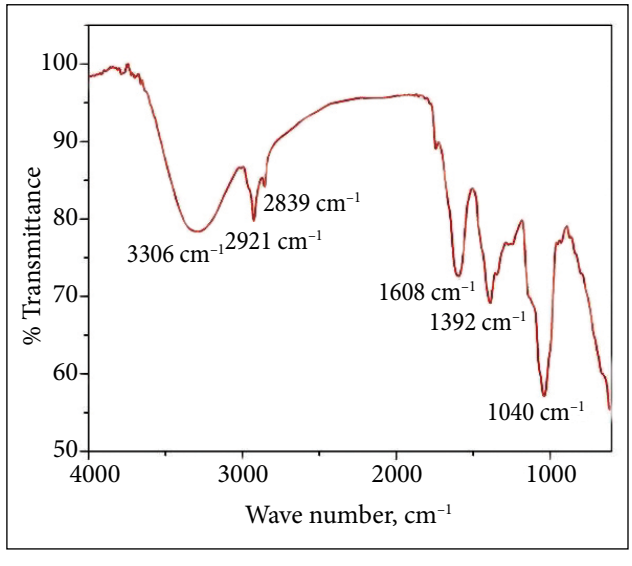

Fig. 3. FTIR spectra of the acetone extract of *T. populnea*

tro assays [54]. Across all assays, the acetone extracts consistently exhibited the highest antioxidant potential, followed by the ethanolic, hexane, and aqueous extracts [55] (Table 1). In the DPPH scavenging assay, the acetone extract showed a high free radical neutralisation capacity, with an inhibitory effect comparable to the positive control, ascorbic acid. Similarly, its ability to scavenge the $ABTS^{+\bullet}$ radical was significant, confirming its strong electron-donating properties. Similarly, in the superoxide radical (SOD) assay, the acetone extract showed activity (IC_{50} = 75.07 μg/mL) that was almost identical to that of ascorbic acid (IC_{50} = 75.91 μg/mL). This striking similarity indicates that the extract possesses radical-quenching efficiency comparable to well-established standard antioxidants (Fig. 4a–c).

The FRAP assay further confirmed the potent reducing power of the acetone extract, while its total antioxidant capacity (TAC) was approximately threefold higher than that of ethanolic and hexane extracts. The percentage of antioxidant activity by FRAP assay decreased in the order: vitamin E > acetone extract > ethanolic extract > hexane extract > water extract. (Fig. 5a). The reducing power of the extracts was compared with the standard ferrous ion solution. The calibration curve of ferrous sulphate was found to be linear with the equation, $y = 0.00314x + 0.00153$, $R^2 = 0.9947$. Among the extracts, the acetone extract of *T. populnea* exhibited the highest FRAP value of 10.86 ± 0.17 mM of Fe^{2+}equivalents/μg of the extract (Fig. 5b). Together, these findings establish the acetone extract as the most bioactive fraction of *T. populnea* seeds. When compared with the other extracts using Student's t-test, the differences were statistically significant ($p < 0.01$), confirming that the acetone extract exhibited the most potent antioxidant activity (Table 1, supporting information). The presence of sterols and tocopherols identified by GC–MS and FTIR analyses suggests that synergistic interactions among different classes of bioactive compounds in the acetone extract contribute to this comparable performance to standard antioxidants. This highlights the therapeutic potential of the acetone extract as a natural antioxidant source.

The Pearson correlation analysis was conducted to quantify the relationships between the phytochemical content and antioxidant activities (Table 2, supporting information). This statistical approach revealed a strong and highly significant positive correlation between the total phenolic content and total flavonoid content, suggesting that these two classes of compounds contribute synergistically to the overall phytochemical profile. Furthermore, both TPC and TFC exhibited significant inverse correlations with the IC_{50} values of the DPPH, $ABTS^{+\bullet}$ and SOD assays, as well as the EC_{50} value of the FRAP assay. This inverse relationship is a critical finding, as it signifies that higher levels of TPC and TFC are directly associated with a more potent antioxidant activity (indicated by lower IC_{50} and EC_{50} values). This statistical evidence strongly confirms that the phenolic and flavonoid compounds are the primary contributors to the extract's observed free radical scavenging, reducing, and superoxide inhibition activities.

When compared to literature, the present results are noteworthy. This aligns with findings

Table 1. **Antioxidant activities of seed extracts of *T. populnea***

Sample	DPPH (IC_{50} in μg/mL)	ABTS+• (IC_{50} in μg/mL)	SOD (IC_{50} in μg/mL)	FRAP (EC_{50} in μg/mL)	TAC (mg/g TE*)
Hexane	32.93 ± 0.07	27.17 ± 0.02	115.82 ± 0.19	7.08 ± 0.25	322.36 ± 7.96
Acetone	**11.42 ± 0.09**	**10.33 ± 0.004**	**75.07 ± 0.22**	**1.56 ± 0.10**	**1256.57 ± 8.59**
Ethanolic	16.45 ± 0.15	17.66 ± 0.17	207.29 ± 0.56	4.77 ± 0.52	388.81 ± 4.31
Water	165.56 ± 2.01	51.98 ± 0.002	>1000	9.75 ± 1.09	148 ± 5.23
Ascorbic acid	2.52 ± 0.03		75.91 ± 0.23		
Vitamin E				0.62 ± 0.06	
Trolox		8.86 ± 0.02			

TE* is Trolox equivalents, means are presented ± standard error of triplicate measurements, statistical significance was tested by student's t-test: highly significant ($p < 0.01$), significant ($0.01 \leq p < 0.05$), ns = not significant.

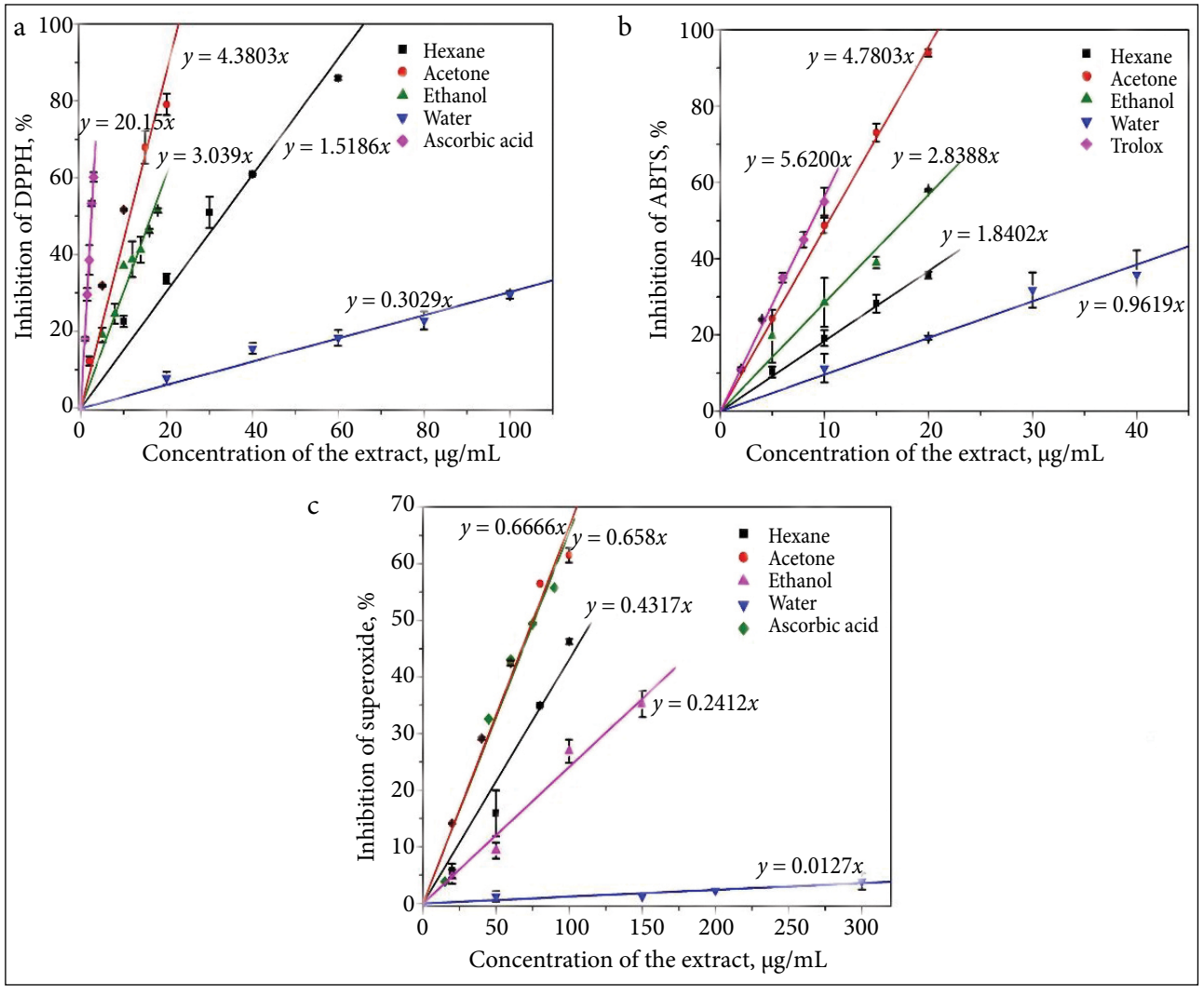

Fig. 4. (a) DPPH, (b) $ABTS^{+\cdot}$ and (c) SOD antioxidant activities of extracts of *T. populnea* (Error bars represent the standard deviation of triplicate measurements)

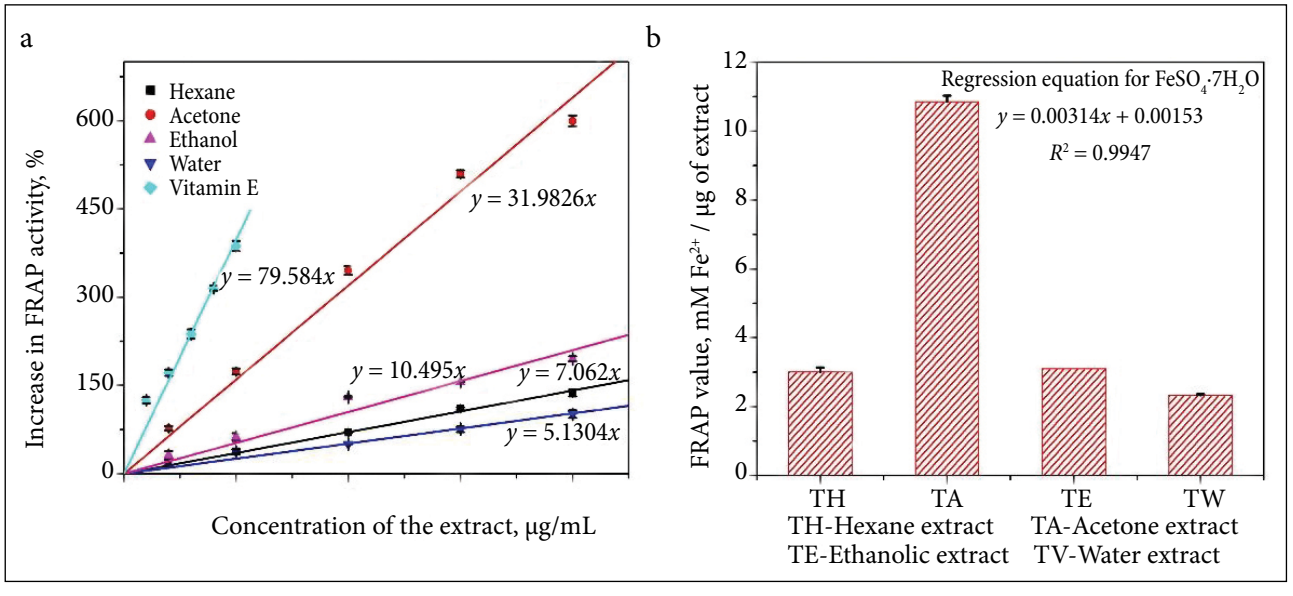

Fig. 5. (a) FRAP activity and (b) mean FRAP values of extracts of *T. populnea* (Error bars represent the standard deviation of triplicate measurements)

Table 2. **Antimicrobial activities of extracts of *T. populnea* by broth dilution method**

Sample	MIC value, µg/mL					
	E. coli	***P. aeruginosa***	***K. pneumoniae***	***S. aureus***	***C. albicans***	***A. niger***
Hexane	58 ± 2.06	66 ± 2.69	100 ± 5.69	85 ± 2.9	48 ± 2.56	55 ± 4.53
Acetone	**48 ± 3.05**	**55 ± 1.58**	**95 ± 3.03**	**65 ± 4.0**	**28 ± 1.36**	**51 ± 2.04**
Ethanolic	69 ± 5.02	57 ± 7.15	120 ± 4.26	86 ± 1.23	65 ± 1.36	76 ± 2.75
Water	71 ± 3.06	75 ± 0.52	200 ± 12.9	95 ± 1.59	48 ± 5.3	59 ± 1.0
Gentamycin	1 ± 0.25	10 ± 0.80	16 ± 0.93	0.5		
Amphotericin B					1 ± 0.03	1.5 ± 0.08

Each value is the mean of three replicates.

from prior research, which have also reported a positive correlation between the antioxidant activity and phenolic content [56]. Bettaieb Rebey et al. found that the acetone extract of cumin seeds possessed the highest levels of phenolics and flavonoids, which correlated with its superior antioxidant activity, as evidenced by a low DPPH IC_{50} value of 6.24 µg/mL [57]. In another study conducted by Do et al., the ethanolic extract of *Limnophila aromatica* showed a positive correlation between its total phenolic and flavonoid contents and antioxidant activity, as reflected by a low IC_{50} value of 70.06 ± 1.0 µg/mL in the DPPH assay [58]. Various studies have shown that the free radical scavenging activity of plant extracts is largely due to the presence of phenolic compounds rich in hydroxyl groups [59]. Additionally, the antioxidant activity of these phenolic compounds has been attributed to the degree of hydroxylation and position of the hydroxyl group in the structure of polyphenols within the extract [60]. Thus, the observed antioxidant effects of the acetone extract of *T. populnea* could potentially be elucidated by the presence of plant sterols, including β-sitosterol, stigmasterol and γ-tocopherol. It is plausible that the synergistic action of these phenolic compounds contributes to the overall antioxidant potential exhibited by the extract. In summary, those findings highlighted the potent antioxidant properties of the acetone extract of *T. populnea*, suggesting its potential for the isolation and development of effective antioxidant compounds.

Since the acetone extract of *T. populnea* exhibited a notably high antioxidant activity in preliminary assays, it was further subjected to silica gel column chromatography to isolate bioactive subfractions. A total of seven subfractions (TA-F1 to TA-F7) were obtained through gradient elution using solvents of increasing polarity. The antioxidant potential of each subfraction was assessed using the DPPH radical scavenging assay and TAC assay. Among the subfractions tested, TA-F2, eluted using 20% ethyl acetate in hexane (1:4, v/v), demonstrated the most potent antioxidant activity. TA-F2 was isolated in a yield of 6.28 ± 0.2% relative to the total acetone extract. As shown in Fig. 6, TA-F2 exhibited the highest DPPH radical scavenging activity, with an IC_{50} value of 25.27 ± 1.25 µg/mL, indicating a strong free radical neutralisation capacity. The linear regression slope further highlights its superior antioxidant response compared to the other fractions. In line with the DPPH results, TA-F2 also recorded the highest reducing power in the TAC assay, with a value of 457.23 ± 3.45 mg/g Trolox equivalents (TE). These findings collectively

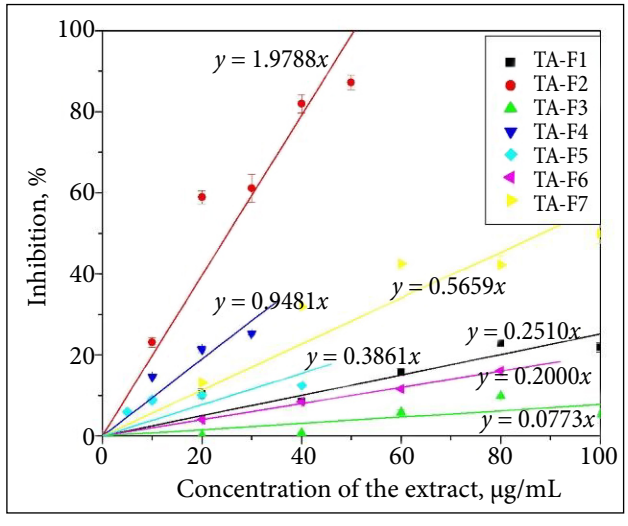

Fig. 6. DPPH radical scavenging potential of subfractions of the acetone extract of *T. populnea* (Error bars represent the standard deviation of triplicate measurements)

suggest that TA-F2 is the most bioactive antioxidant component within the acetone extract and warrants further investigation for potential pharmacological applications.

Antimicrobial activities of the extract

In vitro antimicrobial studies were conducted using the extracts of *T. populnea* and the results were compared with the standard antibiotic, gentamycin and antifungal drug, amphotericin B using the broth microdilution method (Table 2). All extracts were found to have a significant antimicrobial activity against tested bacterial and fungal species. The statistical comparison of the antimicrobial activities of different solvent extracts of *T. populnea* revealed that the acetone extract exhibited significantly higher inhibitory effects across most of the tested pathogens. As shown in Table 3, supporting information, the Student's t-test results demonstrated that the antimicrobial activity of the acetone extract was significantly greater than those of other extracts against *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *S. aureus*, *C. albicans* and *A. niger*. A marginal significance was observed in the case of *A. niger* when compared to the hexane extract, while the difference was not significant for *P. aeruginosa* vs ethanolic extract. Both gram positive and gram-negative bacteria were susceptible to the tested samples. The results showed that the acetone extract was found to be highly effective against the tested bacteria and fungi. In a similar study conducted by Famuyide et al., the antibacterial activities of acetone leaf extracts from nine *Eugenia* and *Syzygium* species were evaluated against *E. coli*, *P. aeruginosa* and *S. aureus*, revealing that all extracts exhibited significant antibacterial effects, with MIC values ranging from 80 to 310 μg/mL [61]. The acetone extract demonstrated its highest antibacterial efficacy against *E. coli*, exhibiting a MIC of 48 μg/mL. The acetone extract of *T. populnea* showed a high antifungal activity against *C. albicans* (MIC = 28 μg/mL) than *A. niger* (MIC = 51 μg/mL). 20% DMSO (negative control) showed no activity against any of the strains tested. In a study conducted by Ramadwa et al., the crude acetone extract of *Ptaeroxylon obliquum* and its isolated compounds demonstrated a significant antifungal activity, against *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*, with MIC values ranging from 2 to 16 μg/mL [62]. These findings supported the conclusion that the acetone extract of *T. populnea* possesses potent and broad-spectrum antimicrobial properties.

From agar well diffusion studies, the acetone extract exhibited antibacterial activity against both gram-negative and gram-positive bacteria (Fig. 7). The zone of inhibition was measured and the results are shown in Table 3. In our study, 1 mg acetone extract showed a significant antibacterial activity against *E. coli*, *P. aeruginosa* and *S. aureus* with the zone of inhibition of 16 ± 0.1, 16.5 ± 0.5 and 18 ± 0.1 mm, respectively. Thus, the significant antibacterial activity of acetone extract was further reinforced by the results from the agar well diffusion method. Consistent with this, Al Ansari et al. reported that the acetone extract of *Pongamia pinnata* leaves exhibited the most pronounced antibacterial activity among the tested extracts. Notably, when compared to their results, the acetone extract of *T. populnea* demonstrated a greater antibacterial efficacy, displaying larger zones of inhibition (at 10 mg/mL) against *P. aeruginosa* (16.5 vs 10 mm) and *S. aureus* (18 vs 3 mm), thereby highlighting its potent and broad-spectrum antibacterial

Table 3. **Antibacterial studies of extracts of *T. populnea* using the agar well diffusion method**

Tested bacterial species	Zone of inhibition, mm					
	Hexane extract, 1 mg	Acetone extract, 1 mg	Ethanolic extract, 1 mg	Water extract, 1 mg	Gentamicin, 100 μg	Streptomycin, 100 μg
E. coli	11 ± 0.3	16 ± 0.1	13.5 ± 0.2	ND	11 ± 1.3	30 ± 1.2
P. aeruginosa	ND	16.5 ± 0.5	ND	ND	20 ± 0.6	32 ± 1.7
K. pneumonia	no clear zone	no clear zone	no clear zone	no clear zone	ND	30 ± 1.1
S. aureus	11 ± 0.2	18 ± 0.1	14 ± 0.3	ND	15 ±0.5	35 ± 0.8

Means are presented ± standard error of triplicate measurements.

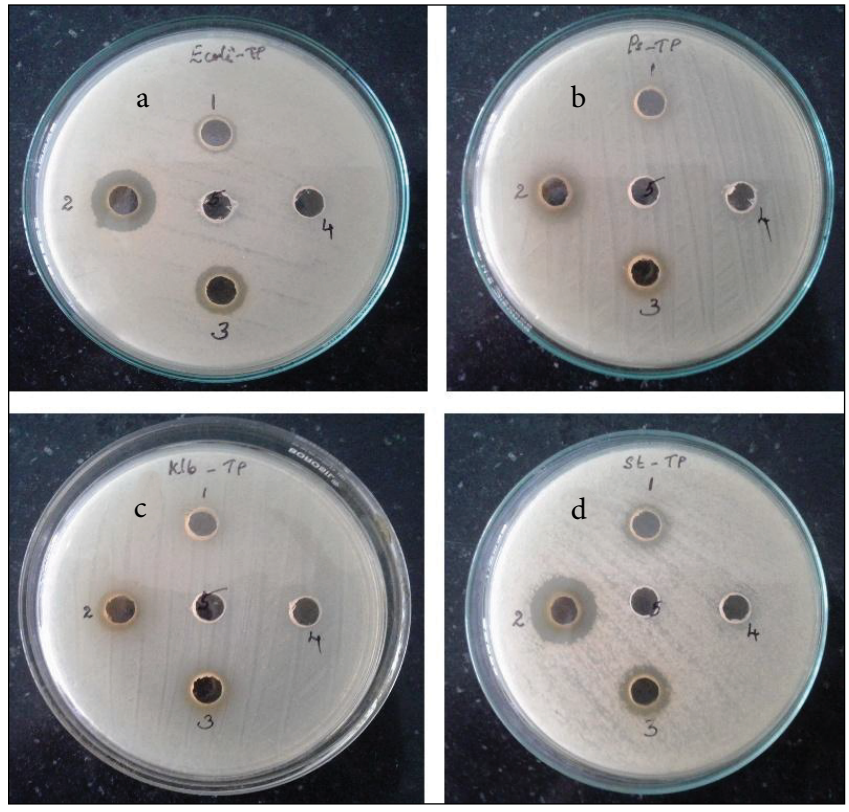

Fig. 7. Photographs of the antibacterial plates of (a) *Escheríchia coli*, (b) *Pseudomonas aerugínosa*, (c) *Klebsiella pneumoniae* and (d) *Staphylocȯccus aureus* after the agar well diffusion method; 1, 2, 3, 4 and 5 contain hexane, acetone, ethanolic, water extracts and 10% DMSO as the control, respectively

properties [63]. In a study conducted by Krishamoorthy et al., the chloroform leaf extract of *T. populnea* showed a 14.8 mm zone of inhibition against *S. aureus*, which is lower than the 18.0 ± 0.1 mm observed for the acetone seed extract in our study, indicating a superior efficacy of the seed extract [64]. The results were compared with standard antibiotics, gentamicin and streptomycin (Fig. 5, supporting information). The results revealed that 1 mg of acetone extract of *T. populnea* exhibited a comparable or even superior antibacterial activity to 100 μg of gentamicin, suggesting its potential as a promising candidate for future antimicrobial drug development. Also, from the results obtained, it was observed that all extracts of *T. populnea*, at the tested concentration, did not show any zone of inhibition against *Klebsiella pneumonia*, indicating a little antibacterial activity. The lack of activity of *T. populnea* extracts against *K. pneumoniae* may be attributed to the absence or insufficient concentration of phytochemicals capable of targeting key structural or metabolic components unique to this pathogen. Unlike gram-positive organisms, *K. pneumoniae* possesses robust defence mechanisms, including a protective outer membrane and resistance enzymes, that may render it less susceptible to the general phytochemical profile of *T. populnea* [65].

The GC–MS analysis of the acetone extract identified several natural compounds possessing antimicrobial properties, including β-sitosterol, octadecadienoic acid, oleic acid, δ-cadinene, α-muurolene and muurolol [39, 63, 64]. δ-cadinene, α-muurolene and muurolol are sesquiterpenoids that were reported to possess antibacterial activities against pathogenic bacterial strains viz. *S. aureus*, *K. pneumonia*, *E. coli*, *P. aeruginosa* and *Salmonella typhimurium* [68]. The antimicrobial activity of the acetone extract could be attributed to the presence of polyphenolic compounds, fatty acids, sesquiterpenoids and flavonoids within it. Sompong Boonsri et al. reported on the antibacterial potential of sesquiterpenoids derived from *T. populnea* heartwood [69]. Aly Soliman Derbalah et al. investigated the antifungal activity of methanolic leaf extracts from *T. populnea*

against *Sclerotium rolfsii*, identifying the biologically active components responsible for its antifungal effects [70]. β-sitosterol, a phytosterol, was noted for its efficacy in shielding against cell lysis induced by pneumolysin, a 53 kDa hemolytic protein toxin [71].

So, it could be concluded that though microbes differ in their respective cell wall structures, the acetone extract could actively destroy their activity. Mechanistically, the plant extract impedes bacterial growth by disrupting the peptidoglycan layer of the cell wall, consequently diminishing its mechanical integrity. Furthermore, phytochemical constituents can penetrate microbial cells, interfering with critical processes such as DNA replication and compromising the structural integrity of proteins and enzymes, which are essential virulence factors for pathogenicity [72]. Our findings collectively highlighted the potential utility of *T. populnea* seeds as an antimicrobial source, suggesting further research.

Antitumour assays

Given the promising antioxidant properties of the *T. populnea* seed extracts, we proceeded to evaluate their potential as a source of novel anticancer agents. To this end, the cytotoxic effects were assessed against Dalton's Lymphoma Ascites (DLA) cells, an established and widely used model for in vitro anticancer screening [73] (Table 4).

Table 4. **Antitumour studies of *T. populnea* extracts using the trypan blue dye exclusion method on DLA cell lines**

Sample	IC_{50} value, μg/mL
Hexane	100.6 ± 2.3
Acetone	45.6 ± 1.2
Ethanolic	80.4 ± 1.7
Water	>1000

Each value is a mean of three replicates.

The standard cytotoxic agents, 5-fluorouracil and curcumin, have reported IC_{50} values of 2.3 and 14.75 μg/mL, respectively, against DLA cell lines [28, 29]. The acetone extract of *T. populnea* exhibited the highest cytotoxicity with an IC_{50} value of 45.6 μg/mL. A similar result was obtained for the methanolic extract of *Kingiodendron pinnatum* with an IC_{50} value of 50.09 μg/mL against DLA cells [74]. The cytotoxic effect of acetone extract might be due to the presence of its high phenolic and flavonoid content. Hexane and ethanolic extracts also showed a significant cytotoxicity. Since the acetone extract demonstrated the strongest cytotoxic activity, it was chosen for further evaluation of anti-proliferative activity by MTT assay on MCF-7 human breast cancer cells.

MCF-7 cells were treated with the acetone extract varying from 6.25 to 100 μg/mL. From the non-linear regression analysis, the acetone extract exhibited a moderate anti-proliferative activity with a half minimal concentration of 76.8 ± 2.1 μg/mL. To evaluate the selectivity of MCF-7 cells by acetone extract, the MTT assay was performed on the non-cancerous L929 mouse fibroblast cell line. The results demonstrated that the acetone extract exhibited a promising anticancer activity on MCF-7 breast cancer cells, while showing a significantly lower toxicity on non-cancerous L929 fibroblast cells (IC_{50} = 286.34 ± 1.8 μg/mL) (Fig. 8). In contrast, the standard drug taxol (paclitaxel), although highly potent against MCF-7 cells (IC_{50} = 0.99 μg/mL) [31], also displayed a considerable toxicity towards normal cells (IC_{50} = 0.21 ± 0.12 μg/mL) with serious side effects such as neurotoxicity, myelosuppression and hypersensitivity reactions [75, 76]. This comparison highlighted the *T. populnea* extract's favourable safety profile and a selective action on cancer cells, making it a potentially safer alternative to conventional chemotherapeutics.

The anti-proliferative efficacy of acetone extract was further substantiated through a direct microscopic examination, wherein images depicting morphological alterations in MCF-7 (Fig. 9) and L929 cell lines (Fig. 6, supporting information) were presented.

The images clearly depict progressive alterations in cell morphology with increasing concentrations of the acetone extract. Untreated MCF-7 cells exhibited a typical cobblestone morphology, characterised by closely packed, polygonal, adherent cells with intact membranes and normal cell–cell contacts, reflecting a healthy proliferative growth. At low concentrations (12.5 and 25 μg/mL), early cytotoxic changes were evident, including a mild cell shrinkage and a reduced confluency. Some cells begin to round off, suggesting early apoptotic events. At 50 μg/mL, the marked cytoplasmic condensation, detachment from the substratum and cell rounding became prominent. The number of

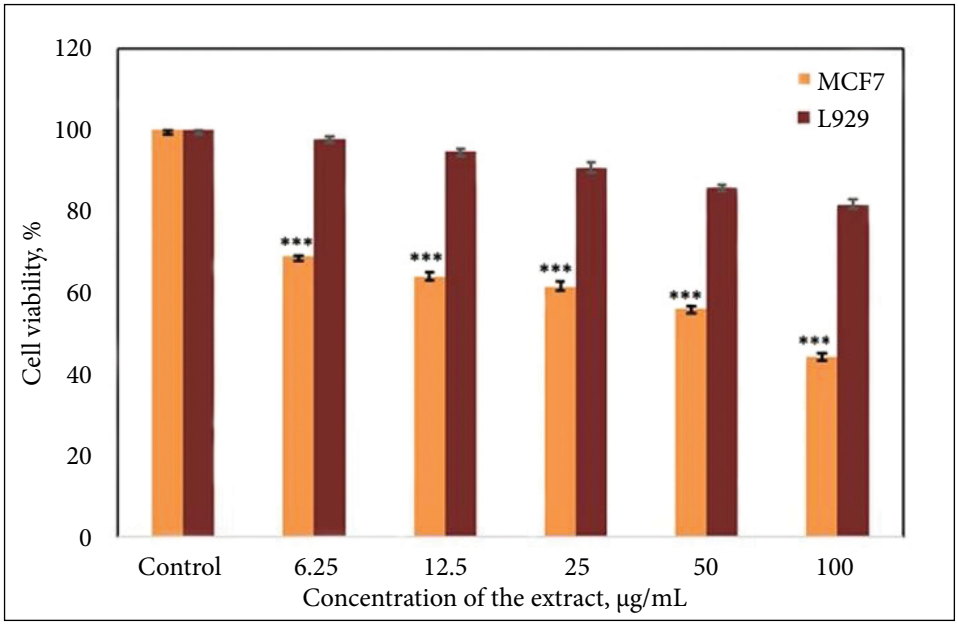

Fig. 8. MTT assay of the acetone extract of *T. populnea* on MCF-7 and L929 cell lines (Error bars represent the standard deviation of triplicate measurements. Two-way ANOVA was performed followed by Tukey's post hoc test, *** highly significant ($p < 0.001$))

viable, attached cells decreased significantly. At 100 µg/mL, an extensive cytotoxicity was observed, with most cells having lost adherence and showing a pronounced membrane blebbing and an apoptotic debris formation. Only a few intact cells remained, confirming a strong dose-dependent inhibitory effect on cell proliferation [77]. These progressive morphological changes from (a) through (e) confirm a clear dose-dependent cytotoxic effect of the acetone extract on MCF-7 cells. The reduction in viable, adherent cells directly corresponds to the decreased formazan crystal formation

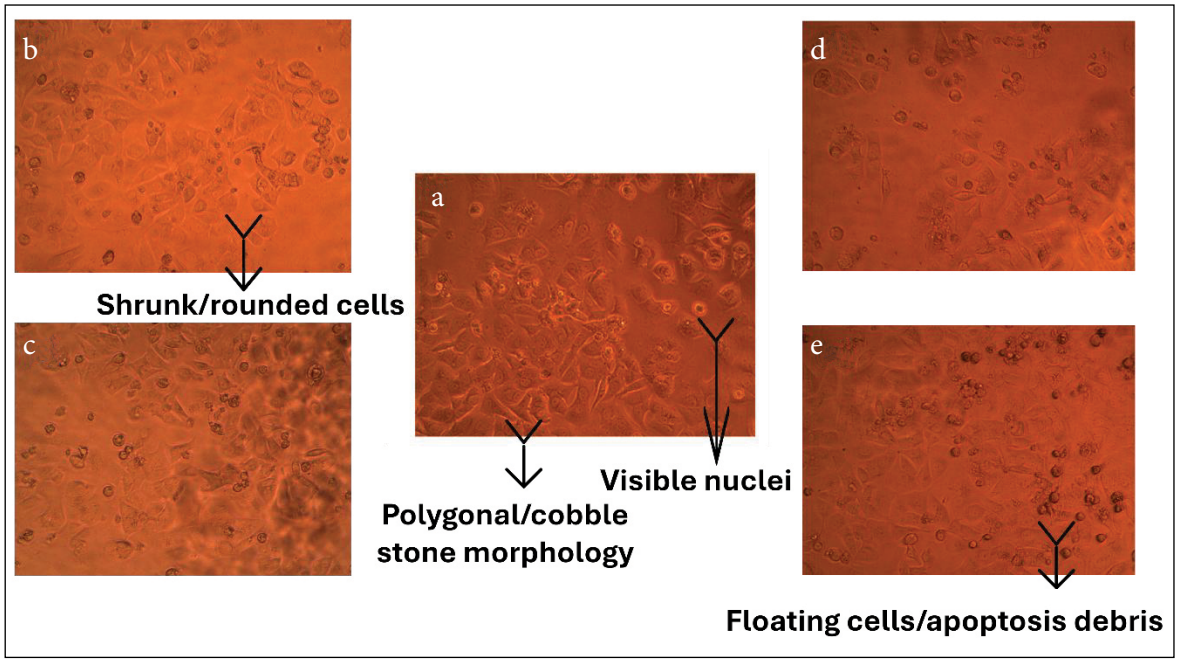

Fig. 9. Morphological changes occurred on treated MCF-7 cell lines by acetone extract of *T. populnea* (a) control, (b) 12.5 µg/mL, (c) 25 µg/mL, (d) 50 µg/mL and (e) 100 µg/mL

measured in the MTT assay, thereby validating the extract's strong antiproliferative activity.

To evaluate the cytotoxic selectivity of the acetone extract between cancerous (MCF-7) and non-cancerous (L929) cell lines, the two-way ANOVA was performed followed by Tukey's post hoc test. The two-way ANOVA revealed that both cell lines and extract concentration significantly influenced cell viability, with a notable interaction effect, indicating that the extract affects the two cell lines differently across concentrations (Table 4, supporting information). The Tukey's post-hoc test confirmed that MCF-7 cells exhibited a significantly reduced viability compared to L929 cells at all tested concentrations (6.25–100 μg/mL), highlighting the selective cytotoxicity of the acetone extract. Within MCF-7, viability decreased significantly in a dose-dependent manner (Table 5, supporting information). For L929, while there was a statistically significant decline in viability with an increasing concentration, the changes were relatively mild, maintaining above 80% viability even at the highest dose.

A primary mechanism for this selective toxicity involves the induction of oxidative stress. Malignant cells typically generate higher basal levels of reactive oxygen species (ROS) due to their rapid metabolism, rendering them a more vulnerable to additional oxidative stress induced by phytochemicals rich in polyphenols and flavonoids [78]. In contrast, the more robust antioxidant defense systems of normal L929 fibroblasts allow them to withstand this oxidative challenge without a significant harm. Furthermore, certain sterols and tocopherols identified in the extract are known to induce apoptosis selectively in cancer cells by modulating mitochondrial pathways and arresting cell cycle progression, while exerting minimal effects on normal cells. Furthermore, the selective action may be linked to a differential signalling pathway modulation. Numerous studies have shown that plant extracts can selectively induce apoptosis in cancer cells by interfering with key molecular pathways that are often dysregulated in malignancy. These mechanisms can include the activation of pro-apoptotic proteins, the inhibition of anti-apoptotic proteins (such as Bcl-2) and the induction of cell cycle arrest [79]. Sitosterol was reported for its anticancer activity by inhibiting cellular growth, inducing cell cycle arrest, and promoting apoptosis in cancer cells. Specifically, this compound induced arrest at the G2/M phase of the cell cycle, consequently downregulating the expression of c-Myc [80]. Dube et al. reported that stigmasterol, derived from the mutant plant *Typhonium flagelliforme*, exhibited an IC_{50} value of 0.1623 μM against the MCF-7 human breast cancer cell line. This potency surpassed that of cisplatin, as evidenced by its IC_{50} value of 13.2 μM [81]. Stigmasterol also reported to have the capability to provoke oxidative stress within MCF-7 cells, thereby triggering apoptotic pathways [82].

Table 5. **Summary of plants studied and their IC_{50} values for anti-proliferative activities on MCF- 7 breast cancer cell lines**

No.	Plant	Plant part	IC50, μg/mL	References
1	*Acacia nilotica*	leaves	493.3	[80]
2	*Aristolochia baetica*	root	216.06	[85]
3	*Origanum compactum*	aerial	279.5	[85]
4	*Graviola fruit*	dry fruit	4.75	[86]
5	*Punica granatum*	fruit	49.08	[87]
6	*Mangifera indica* L.	fruit kernel	15	[88]
7	*Christia vespertilionis*	leaves	24	[89]
8	*Annona muricata*	leaves	2.86	[77]
9	*Populus nigra*	buds	66.26	[90]
10	*Aristolochia foetida*	leaves	47.3	[91]
11	*Aristolochia foetida*	stem	45.9	[91]
12	*Saraca asoca*	bark	50.80	[92]
13	***T. populnea***	**seed (acetone)**	**76.8 ± 2.1**	**Present work**

Previous studies on plant extracts also support this selective pattern. Alkahatani et al. (2022) reported that the extract of *Barleria hochstetteri* induced apoptosis in HeLa cervical cancer cells while showed a limited toxicity to L929 fibroblasts which was mediated by the caspase-3 activation, a key enzyme in the apoptotic cascade [79]. Similarly, Mier-Giraldo et al. demonstrated that extracts from *Physalis peruviana* fruit caused a significant cytotoxicity in HeLa cells but with minimal effects on L929 fibroblasts [83]. More recently, Shahrokh Mojarradgandoukmolla et al. evaluated extracts from *Trigonella* species on MCF-7, L929 and HeLa cell lines and observed a selective cytotoxicity towards cancerous cells and a minimal toxicity to L929 cells [84]. The differential response observed in this study highlights the therapeutic potential of *T. populnea* as a source of compounds with specific anticancer properties.

A comparative summary of previously reported plant extracts and their IC_{50} values against MCF-7 breast cancer cells is presented in Table 5. The values vary widely, reflecting differences in phytochemical composition and extraction methods. Among those, extracts such as *Annona muricata* (2.86 μg/mL) and *Mangifera indica* (15 μg/mL) displayed a strong antiproliferative activity, while others like *Acacia nilotica* (493.3 μg/mL) and *Origanum compactum* (279.5 μg/mL) were considerably less potent. In this context, the acetone extract of *T. populnea* seeds, with an IC_{50} value of 76.8 ± 2.1 μg/mL, may be categorised as moderately active. Although its potency is lower compared to certain highly active extracts, the novelty of this work lies in being the first report on the antiproliferative potential of *T. populnea* seeds, a plant part that has remained largely unstudied compared to bark, leaves and flowers. This contribution expands the phytopharmacological profile of *T. populnea* seeds as a promising but underexplored source of bioactive compounds with the anticancer potential.

Boonsri et al. investigated the cytotoxic effects of nine compounds isolated from *T. populnea* on selected cancer cell lines, namely MCF-7 (breast cancer), HeLa (cervical cancer), HT-29 (colorectal adenocarcinoma) and KB (nasopharyngeal carcinoma) cell lines. Among these compounds, (+)-gossypol demonstrated a significant cytotoxic activity against HeLa and KB cell lines, with IC_{50} values of 0.08 and 0.04 μg/mL, respectively. Mansonone E exhibited a notable activity against all four cancer cell lines, particularly MCF-7 cells with an IC_{50} value of 0.05 μg/mL. Populene D and mansonone D displayed pronounced inhibitory effects on HeLa and MCF-7 cells, respectively, while populene C demonstrated a moderate inhibitory activity across all four cell lines [93]. Studies have reported that gossypol, a major compound isolated from *T. populnea*, exhibited insecticidal, antimicrobial, antiviral, antifertility, antiparasitic, anti-inflammatory and antioxidant properties in addition to anticancer activity [94]. Additionally, Breyer et al. reported the anti-proliferative activity of (-)-Thespesone, a highly oxo-functionalised sesquiterpene of the cadinane type, on MCF-7 breast cancer cell lines with an IC_{50} of 11 μg/mL [95]. Thus, it could be concluded that the synergistic effect of these polyphenols, flavonoids and sesquiterpenes contribute to the anti-proliferative effect of the acetone extract.

CONCLUSIONS

This study presents a comprehensive evaluation of the antioxidant, antimicrobial and antitumour properties of *T. populnea* extracts, emphasising its potential as a source of bioactive compounds for biomedical applications. The acetone extract consistently demonstrated a superior antioxidant activity, as evidenced by a high total phenolic content and a strong free radical scavenging capacity. The Pearson correlation analysis revealed significant associations between phenolic content and antioxidant assays, underscoring the functional relevance of polyphenolic constituents. The antimicrobial screening indicated a broad-spectrum activity of acetone extract, particularly against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*, while no inhibition was observed against *Klebsiella pneumoniae*. The cytotoxic screening via trypan blue and MTT assays confirmed the selective antitumour activity of the acetone extract. The MTT assay yielded an IC_{50} value of 76.8 ± 2.1 μg/mL against MCF-7 breast cancer cells, while IC_{50} against L929 normal fibroblasts was 286.34 ± 1.8 μg/mL, indicating a selective cytotoxicity and biological safety. The two-way ANOVA and Tukey's post hoc analysis validated the significant differences in cell viability across concentrations and cell lines.

Overall, this study demonstrated the novel multifunctional potential of *T. populnea* seeds, particularly its acetone extract, reinforcing its candidacy for future development in the biodiversity-to-biomedicine pipeline targeting antioxidant, antimicrobial and antitumour applications. Future research should aim to isolate and characterise the individual bioactive constituents to better understand their synergistic effects and therapeutic viability.

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HESPESIA POPULNEA SĖKLŲ ACETONO EKSTRAKTO TERAPINIS PROFILIAVIMAS: SU MANGROVĖMIS SUSIJĘS ANTIOKSIDACINIO, ANTIMIKROBINIO IR CITOTOKSINIO AKTYVUMO ŠALTINIS

Graphical Abstract