



RESEARCH ARTICLES

HR-LCMS Profiling of phytochemical constituents and evaluation of antioxidant, antibacterial, anti-cancerous and anti-inflammatory potentials, plasma biocompatibility and cytotoxicity of *Grewia orbiculata* Rottler

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Abstract

Infectious diseases are one of the main reasons that are causing a greater number of deaths in the world owing to their strong resistance development and evolution. There is an immediate urgency for the discovery of drugs with a new class or new mode of action to combat these resistant bugs. In the past few decades, we have not been able to find new antibiotics, which are effective on resistant bugs. Instead of searching for synthetic molecules, if we divert our search for alternative sources that are abundant in nature, we can easily find new molecules. Plants are the best as they are known to possess complex molecules that are strong in their potency while being relatively safe for the host and tough on pathogens. With this rationale, the study was conducted to assess the phytochemical constituents of different parts of plant *Grewia orbiculata* Rottler using different solvents and to elucidate the biological activities. From qualitative analysis of all extracts, Methanolic Extract of Bark (MEB) and Ethyl acetate Extract of Leaf (EEL) were found to be rich in total phenolics and total flavonoids. Major phytochemicals found in MEB were Catechin, Epicatechin, and Carnitine and in EEL were Quinin acid, Gallic acid, Catechol, Isoquinoline, Coumaric acid, Kaempferol, and Quercetin of *G. orbiculata*. Upon testing the biopotentials of these extracts, it was found that among the different solvent extracts of leaves, twigs, buds, and bark, MEB showed the highest biological potential and therapeutic value. The antioxidant property of MEB assessed through DPPH and ABTS assays resulted in an IC₅₀ value of 50 µg/mL and 36 µg/mL, respectively. The metal chelating property of MEB gave a FRAP value of 24 ± 0.093 mmol/g equivalent to that of Tannic acid. Further, MEB was found to possess very good antibacterial activity against human pathogens such as *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Enterococcus faecium*, *Streptococcus epidermidis*, and *Mycobacterium smegmatis*. In addition, MEB also showed good anti-cancerous property against A549 cells, having IC₅₀ value of 98.73 µg/mL. The anti-inflammatory assay with MEB showed protection of BSA denaturation up to a concentration of 1000 µg/mL. Finally, the biocompatibility assay with blood showed no significant agglutination of RBCs up to a concentration of 200 µg/mL and cytotoxicity of MEB resulted in less than 50% inhibition of HTE cell proliferation at the highest concentration of 320 µg/mL, proving its non-toxic nature towards normal cells. Our study is the first to report and evaluate the therapeutic value of the plant *G. orbiculata*. MEB was found to possess very good therapeutic potential and can be used as potent antimicrobial agent to treat deadly human infections.

Keywords *Grewia orbiculata* · Antibacterial activity · Antioxidant property · Anti-cancerous activity · Catechin · Gallic acid · Quercetin

Abbreviations

MEB	Methanolic extract of bark
EEL	Ethyl acetate extract of leaf
A549	Human lung carcinoma epithelial cells
ABTS	2, 2'-Azino-Bis-3-ethylbenzothiazoline-6-sulfonic acid
ATCC	American type culture collection

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CFU	Colony forming unit
CLSI	Clinical & laboratory standards institute
DPPH	2,2-Diphenylpicrylhydrazyl
FRAP	Ferric reducing antioxidant power assay
HR-LCMS	High resolution liquid chromatograph mass spectrometer
HTE	Human tracheal epithelial cells
IC	Inhibitory concentration
IIT	Indian institute of technology
MIC	Minimum inhibitory concentration
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
SAIF	Sophisticated analytical instrument facility
TFC	Total flavonoid content
TPC	Total phenolic content

Introduction

The entire globe has just experienced the devastating and catastrophic impact of the pandemic, and we are still not free from the clutches of this lethal virus and its mutations even after three years. Many diseases caused by viruses, bacteria, and protozoans have taught us a lesson by showing how powerless and vulnerable we are! These infectious diseases are among the leading causes of mortality worldwide (Cinti et al. 2008). According to the CDC's (Centre for Disease Control) "AR Threats Report, 2019," many diseases are caused by a group of microorganisms known as ESKAPE pathogens. These are Gram-positive and Gram-negative bacteria that cause community and hospital-based diseases such as pneumonia, gut infections, and urinary tract infections. The World Health Organization (WHO) publications, since 2019, state that the primary issue with these pathogens is their propensity to acquire drug resistance quickly. According to CDC report of January 2022, antimicrobial resistance is the biggest issue as it is predominantly responsible for most of the worldwide mortality in developing nations. These resistant strains cause several dangerous illnesses that have no cure or effective treatments, which eventually increases mortality. Infections from resistant bugs, such as Carbapenem-resistant *Acinetobacter* have increased alarmingly at a rate of 78% on average in just one year, while infections from other 'Urgent threat' pathogens such as MRSA have increased by 15%, Carbapenem-resistant *Enterobacteriales* have increased by 35%, Vancomycin-resistant *Enterococcus* have increased by 14%, and Multidrug-Resistant *Pseudomonas aeruginosa* have increased by 32%.

According to recent reports, *E. coli*, *S. aureus*, *K. pneumoniae*, *S. pneumoniae*, *A. baumannii*, and *P. aeruginosa* are the pathogens that are linked to resistance. In 2019, they collectively caused 3.57 million (2.62–4.78) deaths that are linked to AMR (Murray et al. 2022) indicating an immediate

urgency to discover new antibiotics. Till date only six antibiotics under clinical development were designated as new, according to WHO reports of the year 2019. They also have cautioned that medicines would become less effective because of simple drug-resistance development, mainly caused by patient's misuse of antibiotics. These results make it clear that there is a need to discover new antibiotics with novel modes of action such that the pathogen can't rapidly acquire resistance (Fair and Tor 2014). As reported by NHS (National Health Service), the use of synthetic antibiotics has created severe negative effects and have lost their allure owing to resistance. To circumvent this limitation, we can rely on complex chemicals originating from plants or bacteria, which might be quite successful.

In this context, plant-derived biomolecules are known to work together to assault bacteria in a variety of ways, making it difficult for the pathogen to acquire resistance (Berdry 2012). They have the power to battle infections and also act as antioxidant, anti-inflammatory, and anti-cancerous agents, providing extra benefits to the body without causing negative effects. According to the 'American Cancer Society's Cancer Facts & Figures Report- 2022', this year around 609,360 deaths are projected due to cancer, equivalent to 1670 deaths per day. Lung cancer is the leading type of cancer in the world causing nearly 350 deaths per day (Siegel et al. 2022). Cancer is often treated with a variety of therapies such as chemotherapy, radiation, and chemical medications; Further these treatments are not only costly but also are linked with numerous side effects. Plant-based compounds are being researched as alternatives to synthetic medications for these reasons (Qamar et al. 2022). Natural sources account for more than 60% of anticancer medications, including Vinblastine, Vincristine, Camptothecin, Podophyllotoxin, and Combretastatin. Polyphenols such as Epigallocatechin-3-gallate, Catechins, Terpenoids, Paclitaxel, and Flavonoids such as Flavopiridol, have been reported to be particularly efficient against cancer and serve as antimicrobials as well (Parham et al. 2020a, b; Greenwell and Rehman 2015). Search for new sources in this direction has been going on aggressively as studies with some plant extracts have given promising results.

Encouraged by this, we set out to look for biomolecules in the hitherto unknown plant *Grewia orbiculata* Rottler to study and evaluate the biological potentials. This plant belongs to the family *Malvaceae* and the genus *Grewia*. There are several species in this genus that have been reported to possess antioxidant, anti-cancerous, and anti-bacterial properties. *G. asiatica*, *G. tiliaefolia* and *G. tenax* are some of the very well-studied species in this genus (Goyal 2012). β -sitosterol and daucosterol, phytosterols found in *G. tiliaefolia* were reported to possess anti-cancer activity against A549 lung cancer cells (Rajavel et al. 2017). Methanolic extracts of *G. asiatica* have shown potent

anti-cancerous ability against NCI-H522 lung cancer cells (Qamar et al. 2022). Folklore has several traditional applications for these plants and its components such as pods, leaves, bark, fruits etc. Bark concoctions are used to treat upper respiratory ailments like pneumonia and bronchitis (Suguna and Umesh 2022b). Locals also utilise various plant components to cure skin ailments such as abrasions, wound infections, and rashes etc. (Suguna and Umesh 2022b). We chose this genus *Grewia* based on extensive study and reports published on *G. asiatica* (Goyal 2012), *G. optiva* (Waliullah et al. 2011), *G. tenax* (Aadesariya et al. 2017a, b), and the therapeutic potentials they possess. The plant *Grewia orbiculata* Rottler, has remained unexplored scientifically till date but is well known to folklore (Suguna and Umesh, 2022a). This plant is commonly known in folklore as ‘Javane,’ ‘Karijavne,’ or ‘Karijaana’ in Kannada, the local dialect. People consume the bitter-tasting pods of this plant as fruits (Suguna and Umesh 2022a). We have evaluated the medicinal potential of different components of this plant such as leaves, buds, twigs, and bark, using different solvents such as chloroform, ethyl acetate, methanol, and water.

Materials and methods

Cell lines and bacterial strains

All bacteria, listed in Table 1 and cell lines listed in Table 2 were procured and cultured in recommended selective and propagation media as per the revival procedure provided by American Type Culture Collection (ATCC).

Collection and preparation of plant material

Different parts of plant *G. orbiculata* were collected in sufficient quantity and thoroughly washed with clean water, followed by 5% sodium hypochlorite solution wash to eliminate dirt and organic contaminants, and then rinsed with distilled water. Plant components were dried in the shade at room temperature, powdered into coarse powder, and kept in airtight containers. The voucher specimen number of the herbarium is Suguna, M. 123,449.

To extract phytochemicals from the plant *G. orbiculata* and its parts, different solvents were utilised; chloroform, ethyl acetate, methanol, and water were flashed consecutively using the Soxhlet apparatus to extract plant active compounds in leaves, bark, buds, and twigs for 6 h in each solvent (flow chart of extraction process is given in supplementary data Figure S1a). The solvents were chosen since they span a wide range of polar to non-polar solvents. Following extraction, the solvent was evaporated, and the weight of the extract was recorded to compute the extractive value (given in supplementary data Table S1b), which was

used to select extracts for further investigation. All extracts were kept at a temperature of 2–4 °C until further usage.

Qualitative phytochemical analysis of *G. orbiculata*

Qualitative phytochemical analysis was carried out by following the standard procedures (Harborne 1984; Sofowora 1993; Trease and Evans 1989).

Quantitative phytochemical analysis of *G. orbiculata*

Total flavonoids content

Total Flavonoid Content (TFC) of all the extracts of different parts of the plant were screened and quantified using Aluminium Chloride colorimetric method (Chatatikun and Chiabchalard 2013) (Procedure-supplementary data).

Total phenolic content and total tannin content

Total Phenolic Content (TPC)/total tannin content of all the extracts of different parts of the plant were evaluated using the method described in literature (Herald and Gadgil 2012). (Procedure-supplementary data).

Evaluation of antioxidant properties

DPPH and ABTS assay

Antioxidant activity of different solvent (chloroform, ethyl acetate, methanol, and water) extracts of different parts of the plant were analysed to assess the reducing power of DPPH and to determine the IC₅₀ value as published in the literature (De Torre et al. 2019). Percentage inhibition and IC₅₀ values of each extract were calculated. (Procedure-supplementary data).

Ferric reducing antioxidant power assay (FRAP) assay

Reducing power or metal chelating property of different extracts of different parts of the plant were determined according to the method described by Khatua et al. (2017). MEB which showed highest FRAP value at 100 µg was considered further to find the FRAP value of Tannic acid equivalent concentration (Procedure-supplementary data).

Metabolic profiling using HR-LCMS (Orbitrap)

Phytochemicals present in EEL and MEB were assessed using High Resolution- Liquid Chromatography Mass Spectroscopy (HR-LCMS)-Orbitrap (facility: SAIF, IIT Bombay)

to identify the phytochemicals. Refer supplementary data for LC and MS conditions used.

Evaluation of antibacterial property by micro broth method

Antibacterial activity of different solvents extract of different parts of the plant were evaluated following the method described by Shariati et al. 2020, as per CLSI guidelines. (Procedure-supplementary data). Ciprofloxacin (in $\mu\text{g/mL}$) was used as reference standard.

Biocompatibility with plasma assay

MEB was selected for biocompatibility test and was evaluated by the method recommended in literature (Afsar et al. 2016) (Procedure-supplementary data). Gallic acid of concentration up to 1000 μg was used as standard.

BSA denaturation assay

Protein denaturation assay with MEB was performed based on procedure as described in literature (Altundag et al. 2020) (Procedure-supplementary data).

Evaluation of cytotoxicity and in vitro anti-cancerous activity by MTT assay

Cytotoxicity and anticancer properties of MEB were assessed by conducting MTT assay as described in literature (Nordin et al. 2019). Two cell lines such as Primary HTE (Human Tracheal Epithelial cells- ATCC PCS-300-010) and A549- human lung carcinoma epithelial cells (ATCC CRM-CCL-185) were selected for MTT assay (Procedure-supplementary data).

Results

Qualitative and quantitative phytochemical analysis of *G. orbiculata*

Each extract of *G. orbiculata* was tested qualitatively for the presence of several phytochemicals (data in supplementary Figure S1b). Methanolic and water extracts of leaves and bark generated the most phytochemicals, including polyphenols, tannins, flavonoids, proteins, saponins and carbohydrates. Furthermore, each extract was statistically analysed for key elements based on qualitative analysis. TPC, TFC, tannins, proteins, and carbohydrates were found abundantly in MEB and EEL, as shown in Fig. 1. The standard quantification graphs are shown in supplementary data Figures S2a, S3a, S4a, S4b, and S4c. Protein and carbohydrate quantification can be found in S2b and S3b. Flavonoids were found to be prevalent in EEL and MEB. MEB was also found to be high in TPC, proteins, carbohydrates, and tannins.

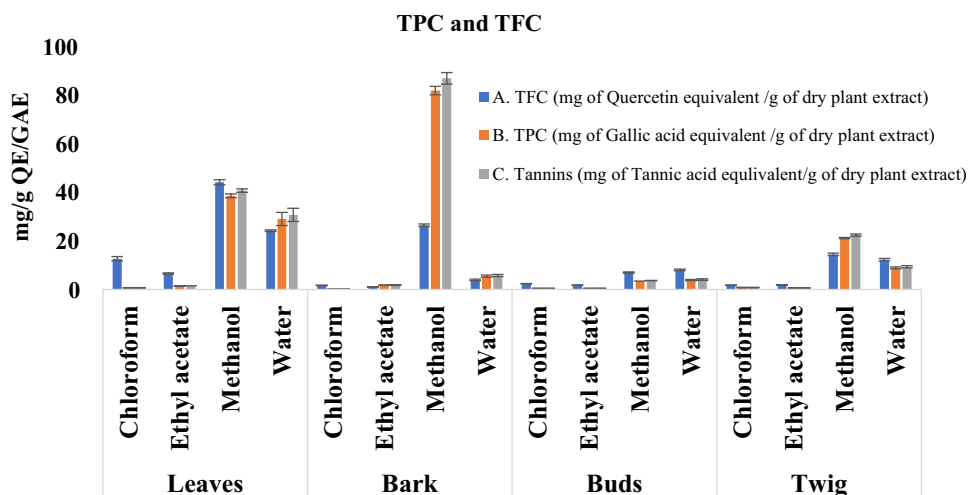
Evaluation of antioxidant properties

Antioxidant properties of all extracts were assessed by most widely used assays, DPPH and ABTS. Out of 16 extracts 6 showed prominent antioxidant property in DPPH and 3 showed good activity in ABTS assays.

DPPH radical scavenging assay

The DPPH test was initially performed on all extracts of *G. orbiculata* at a concentration of 100 μg to assess the activity possessed by these extracts, equivalent to the activity achieved using standard reference Ascorbic acid, as shown in the Fig. 2a. Methanolic and water extracts of the leaf, bark, and twig demonstrated significant antioxidant activity.

Fig. 1 Quantification of various phytochemicals in different parts of the plant *G. orbiculata*. The specific phytochemical contained in each extract is expressed as mg equivalent of concentration of reference standard per g dry weight of the explant. **A** TFC was tested against the reference standard Quercetin. **B** TPC was analysed using the reference standard Gallic acid. **C** Tannic acid was used as a reference standard for Tannin estimation



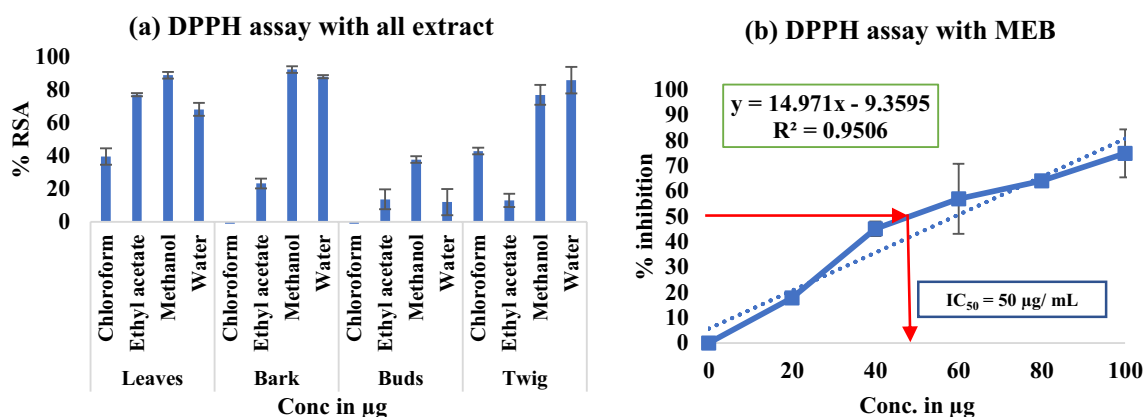


Fig. 2 **a** DPPH assay of leaves, bark, buds, and twig of plant *G. orbiculata*. **b** DPPH assay with MEB of *G. orbiculata*

These extracts were then tested through the DPPH assay at various concentrations to find the IC_{50} value and to compare their activity with ascorbic acid (reference standard). Supplementary Figures S5a and S5b show DPPH assay performed with reference standard and other extracts. Out of the 6 extracts, MEB showed prominent activity, therefore assay was conducted with MEB at different concentrations to find the IC_{50} value is as shown in Fig. 2b

ABTS radical scavenging assay

The ABTS test was carried out on all extracts of *G. orbiculata* at a concentration of 100 μg to determine the radical scavenging activity, as shown in Fig. 3a. Methanolic extracts of the leaf, bark, and twig exhibited significant radical scavenging action. These three extracts were then tested at different concentrations to find the IC_{50} value. MEB outperformed the other two extracts in terms of ABTS scavenging ability as shown in Fig. 3b. Supplementary Figures S6a and S6b show the standard graph and ABTS test of other extracts.

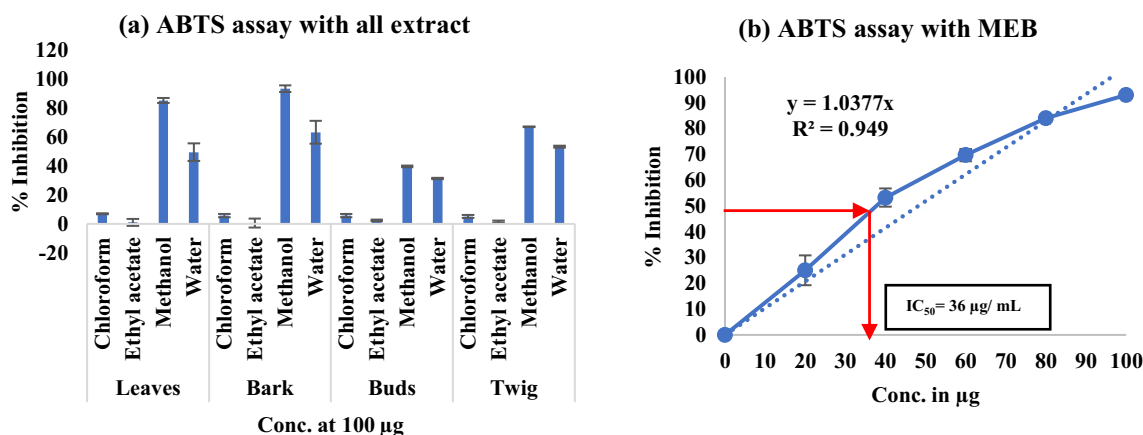


Fig. 3 **a** ABTS scavenging activity showed by different extracts of *G. orbiculata* **b** ABTS assay with MEB of *G. orbiculata*

Ferric ion reducing antioxidant property

The antioxidant properties of several extracts of *G. orbiculata* were evaluated through the FRAP test, as shown in supplementary Figure S7a. Out of the 16 extracts tested, MEB demonstrated a significant ability of metal chelating ability with magnitude of $24 \pm 0.093 \text{ mmol/g}$ compared to standard reference-Tannic acid, as shown in supplementary Figure S7b. Supplementary Figure S7c depicts the standard graph used for Tannic acid.

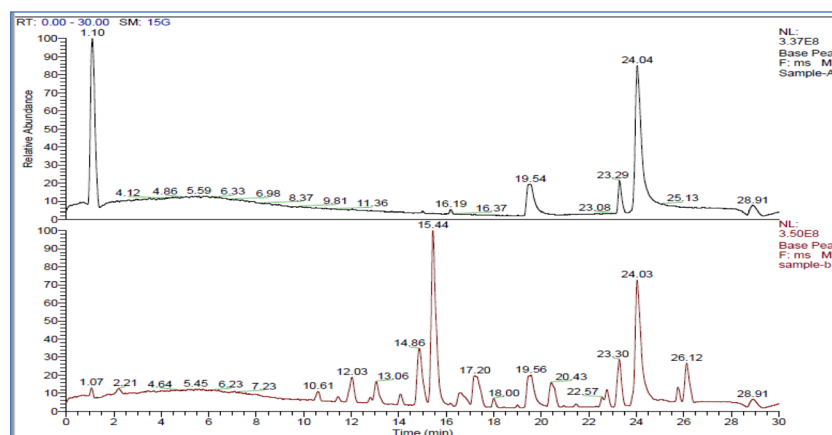
Metabolic profiling of *G. orbiculata* using HR-LCMS (Orbitrap)

Extracts were chosen for metabolic profiling based on the qualitative analysis of phytochemicals present in them. Quantitative analysis yielded abundance of total flavonoid, total polyphenols, and tannins in EEL and MEB. Phytoconstituent content profiling of EEL and MEB yielded various phytochemicals that act and contribute

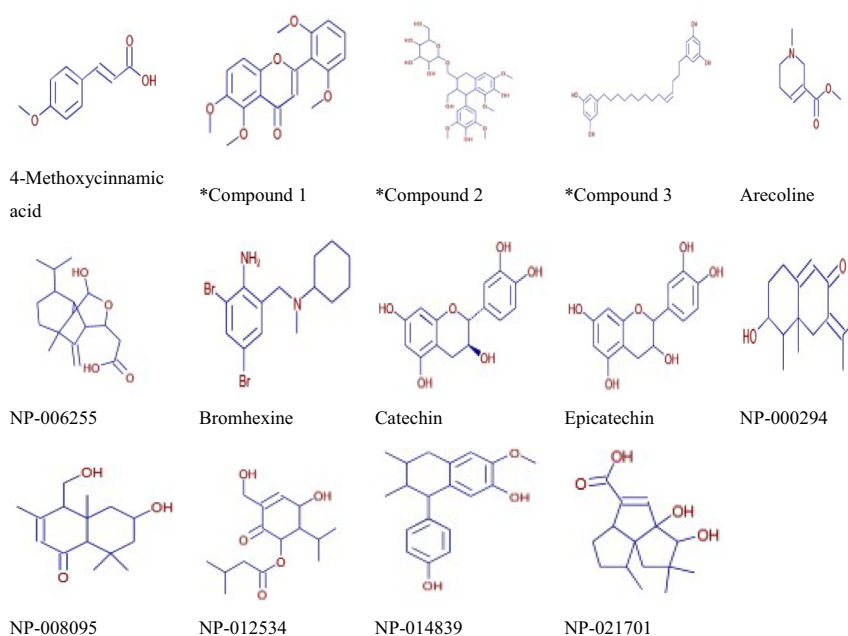
to the plant's antibacterial and antioxidant activities. Figure 4a depicts a chromatogram with multiple prominent peaks from two extracts. From the chromatogram, it is evident that there are many prominent phytochemicals present in the sample. The phytoconstituents are more in leaves compared to bark, but still the activities like antioxidant, metal chelating and primary Antibacterial Susceptibility test (AST) screening on *S. aureus*, indicated high therapeutic index to be owned by bark than leaves. This may be due to the low concentration of these chemicals present in leaves or in the solvent used for

extraction, therefore further purification of crude extract is planned to quantify these phytochemicals. Supplementary Table S3a, S3b contains list of phytochemicals with their specific formula, molecular weight, and retention time. Figures 4b, c represent the key phytochemicals with therapeutic index found in MEB and EEL, respectively. The major phytochemicals present in MEB were Catechin, Epicatechin, Bromhexine, 2-[[7-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-3-(hydroxymethyl)-6,8-dimethoxy-1,2,3,4-tetrahydronaphthalen-2-yl] methoxy]—6-(hydroxymethyl) oxane-3,4,5-triol etc., shown in Fig. 4a,

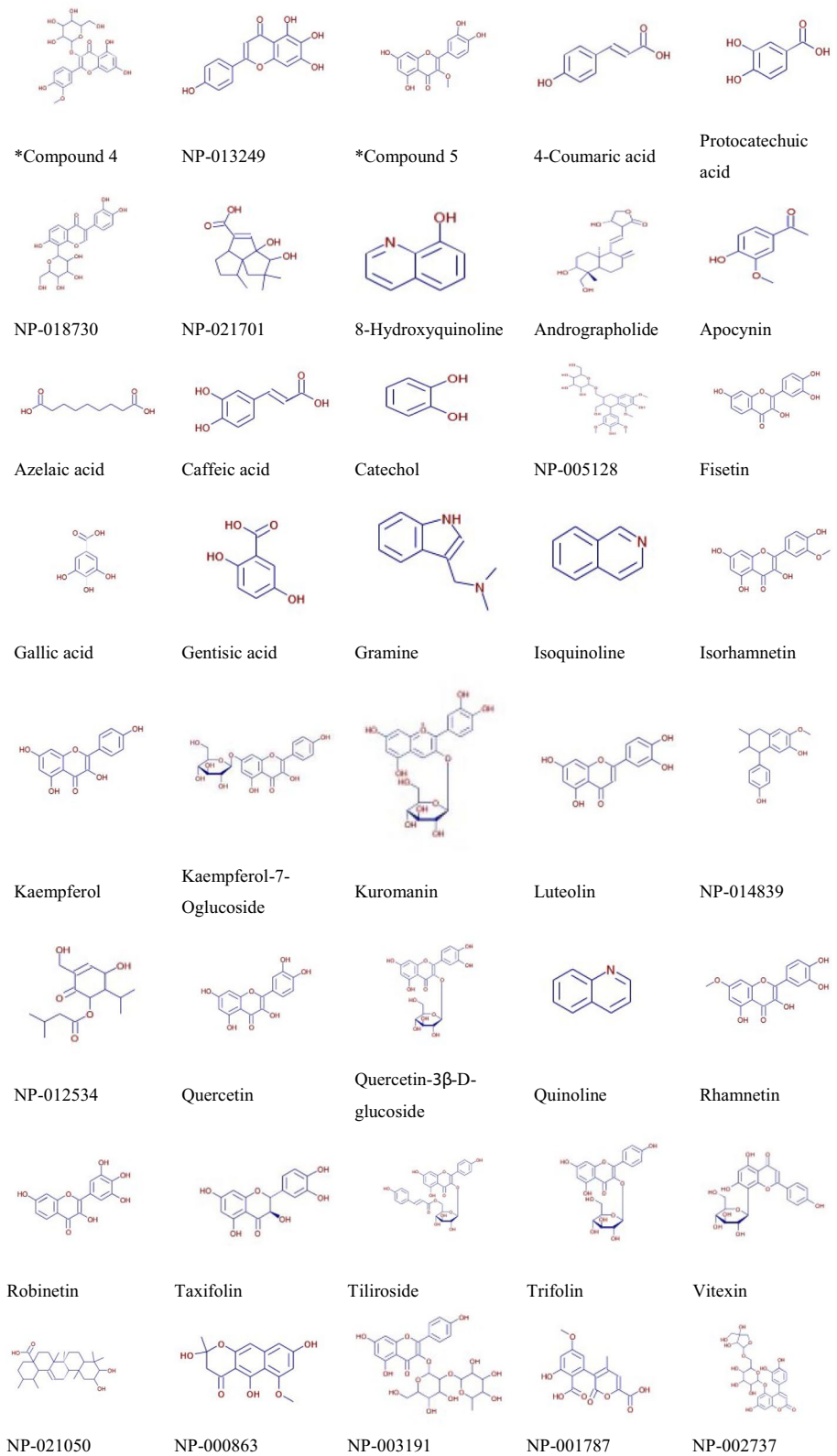
Fig. 4 **a** Chromatogram of MEB (sample A) and EEL (sample B). **b** List of major secondary metabolites present in MEB of *G. orbiculata*. Compound 1: 2-[[7-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-3-(hydroxymethyl)-6,8-dimethoxy-1,2,3,4-tetrahydronaphthalen-2-yl] methoxy]—6-(hydroxymethyl) oxane-3,4,5-triol, Compound 2: 2-(2,6-dimethoxyphenyl)-5,6-dimethoxy-4Hchromen-4-one, Compound 3: 5-[(10Z)-14-(3,5-dihydroxyphenyl) tetradec-10-en-1-yl]-benzene-1,3-diol.



(a) Chromatogram of MEB (sample A) and EEL (sample B).



(b) List of major secondary metabolites present in MEB of *G. orbiculata*. Compound 1: 2-[[7-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-3-(hydroxymethyl)-6,8-dimethoxy-1,2,3,4-tetrahydronaphthalen-2-yl] methoxy]—6-(hydroxymethyl) oxane-3,4,5-triol, Compound 2: 2-(2,6-dimethoxyphenyl)-5,6-dimethoxy-4Hchromen-4-one, Compound 3: 5-[(10Z)-14-(3,5-dihydroxyphenyl) tetradec-10-en-1-yl]-benzene-1,3-diol.

Fig. 4 (continued)

(c) List of major secondary metabolites present in EEL of *G. orbiculata*. Compound 4: 5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-3-([3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy)-4H-chromen-4-one, Compound 5: 3-Methoxy-5,7,3',4'-tetrahydroxyflavone.

are known to have antibacterial, anti-cancerous and anti-oxidant properties against many pathogens. Therefore, further purification of crude MEB is planned to quantify and to identify the phytochemicals present and their concentrations, to assess the major contribution of these components towards the therapeutic activity obtained. In EEL, we obtained vast variety of phytochemicals which are known to be potent antimicrobial, anti-cancerous agents such as Quercetin, Gallic acid, Coumaric acid, Kaempferol, Vitexin etc. as shown in Fig. 4b.

Evaluation of antibacterial property by Microplate broth method

Various pathogen species were grown in both differential/special and propagation media to ensure strain authenticity and minimise cross contamination. The supplementary data Table S1a contains information on several bacterial pathogens streaked on various nutritional medium. The antibacterial properties of all extracts were evaluated through microplate broth technique using 96 well plates. Initially, 16 extracts were tested on two bacteria, *E. coli* and *S. aureus* (Supplementary Table S4). Only methanolic extracts of bark, twigs, and leaves showed antibacterial activity among all the extracts tested. The pathogens were selected based on reports of CDC, in which, it is cautioned to be aware of these pathogens and their new variants with resistance that are causing major human diseases. Table 1 shows the MIC of methanolic extract of different plant parts such as leaves, bark, buds, and twig, against 10 bacterial pathogens. Among the four extracts, MEB showed significant efficacy against most of the pathogens examined, as shown in the Table 1. MEB is found to be potent against Gram- positive strains compared to Gram-negative.

Table 1 MIC of methanolic extract of leaves, bark, buds, and twig against human pathogens

Methanolic Extract/Standard	ATCC number	MIC (mg/mL)				
		Leaves	Bark	Bud	Twig	Ciprofloxacin
<i>MRSA</i>	43300	5	0.6	20	2.5	0.12
<i>Staphylococcus aureus</i>	25923	2.5	1.3	20	5	0.12
<i>Staphylococcus epidermidis</i>	4990	10	0.3	10	2.5	0.06
<i>Enterococcus faecium</i>	700221	20	1.3	20	5	1.00
<i>Enterococcus faecalis</i>	29212	2.5	1.3	10	5	0.25
<i>Escherichia coli</i>	25922	> 20	> 20	> 20	> 20	0.01
<i>Pseudomonas aeruginosa</i>	27853	20	2.5	20	> 20	0.06
<i>Klebsiella pneumoniae</i>	700603	> 20	> 20	> 20	> 20	0.06
<i>Acinetobacter baumannii</i>	17904	10	2.5	20	20	0.25
<i>Mycobacterium smegmatis</i>	607	> 20	2.5	10	10	0.01

MIC was determined by microplate broth method. Each plate had positive control (culture), standard antibiotic control (Ciprofloxacin) and negative control (Only media)

Biocompatibility assay

Plasma or erythrocytes serve an important function in the body by being the primary transport system. These erythrocytes are kept in the finest possible shape to ensure that all other components of the body function properly. As a result, it is critical to understand the potential response or interaction of any medicine or substance used in therapy with plasma. To ensure normal haematological functioning, it is necessary to assess the non-interference of these molecules with plasma. The interaction between plasma and plant component might result in either blood clotting or plasma disintegration. Based on various biological assays conducted, MEB was found to be potent among all the other extracts. Therefore, the interaction of MEB with plasma was studied to look for these signs, and the results of our study as depicted in Fig. 5a which reveals that neither they cause any significant haemolysis nor bring any changes in the morphology of blood cells.

In vitro anti-inflammatory activity

BSA denaturation assay study was done to assess the in vitro anti- inflammatory activity of MEB. The activity of MEB was evaluated by comparing the activity of diclofenac sodium of concentration range 10 to 100 µg/mL. As shown in the Fig. 5b, the highest concentration of MEB showed no significant denaturation of BSA and shows compatibility with plasma.

In vitro anti-cancerous property

The in vitro anti-cancerous property of MEB of *G. orbiculata* was assessed by conducting MTT assay. Result shown in the Table 2, reveals that the MEB taken at standard concentrations 10–320 µg/mL, exhibited prominent inhibition of

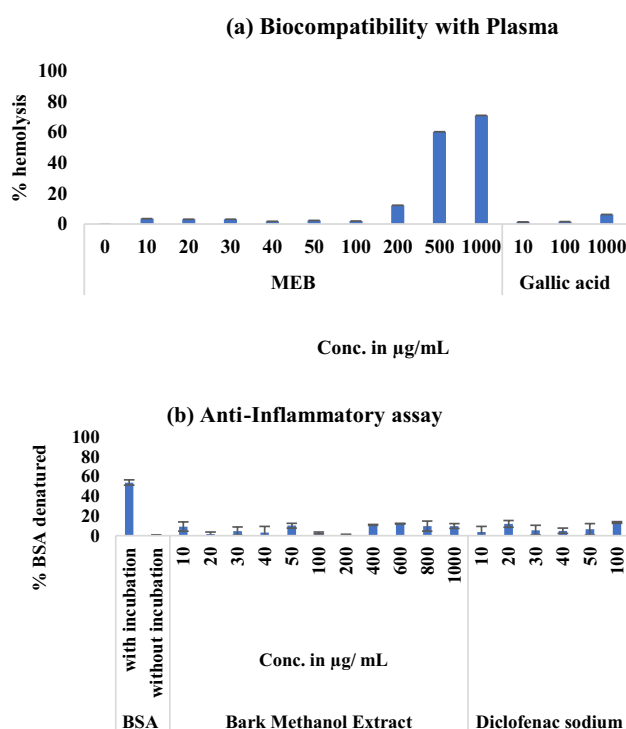


Fig. 5 Biocompatibility and in vitro anti-inflammatory activity. **a** Biocompatibility of MEB with plasma. **b** in vitro anti-inflammatory activity of MEB

A549 cell proliferation and there is a trend of dose dependent inhibition of A549 cells. Data obtained for reference standard Doxorubicin is shown in supplementary Table S5. The picture of the healthy cells in control and its inhibition in MEB treated plate is depicted in supplementary Figure S8.

Cytotoxicity

The cell line Primary Human Bronchial/Tracheal Epithelial Cells (HTE) lines were used to assess cytotoxicity of MEB at standard concentration of 10–320 µg/mL as shown in the Table 2 below. In case of HTE cells, highest conc. used

shows percentage of inhibition below 50 and therefore IC_{50} was not calculated. Data obtained for reference standard Doxorubicin is shown in supplementary Table S6. The healthy cell pictures of HTE cells in control and MEB treated plates are depicted in the supplementary Figure S9.

Discussion

Investigation of the phytoconstituents and therapeutic effects of the plant *G. orbiculata* are reported in this article. We have explored the therapeutic qualities of different plant parts such as leaves, buds, twigs, and bark. Initially, enough quantities of different plant parts were collected, and phytochemicals were extracted using various solvents through the sequential Soxhlet extraction. Extractive values were computed, and it was found that all methanolic extracts of different parts of the plant yielded a high yield, followed by water, ethyl acetate, and chloroform.

The qualitative and quantitative analysis of all extracts revealed the presence in significant quantity of TPC, TFC, Total protein, carbohydrate, and many more phytochemicals in EEL and MEB. As a result, more in-depth investigation was done on these two extracts. The metabolic profile of *G. orbiculata* utilising HR-LCMS (orbitrap) showed numerous compounds with well-known medicinal effects in both EEL and MEB. Column purification was performed on crude EEL and MEB to further purify the extracts. Antibacterial activity and DPPH assays were performed to identify the active fractions for which metabolic profiling was performed.

Many notable phytochemicals were found in EEL, including Quercetin, Quercetin-3-D-glucoside, Quinin acid, Gallic acid, Catechol, Isoquinoline, Coumaric acid, Vitexin, Isorhamnetin, and Kaempferol. Some of the phytochemicals found in *G. orbiculata* are also known to be present in other species of the same genus, such as *G. asiatica* and *G. optiva* (Goyal 2012; Kumar et al. 2022). The presence of these compounds distinguishes this plant since they have already been found to have excellent antioxidant property as well as

Table 2 MTT assay to access cytotoxicity on A549 cell lines and HTE cells lines

Compound Name	A549 cell lines				Primary HTEC lines			
	Conc. µg/ml	OD at 590 nm	% Inhibition	IC_{50} µg/ml	Conc. µg/ml	OD at 590 nm	% Inhibition	IC_{50} µg/ml
MEB of <i>G. orbiculata</i>	10	0.685	5.52	98.73	10	0.521	0.95	IC_{50} was not calculated due to inhibition less than 50%
	20	0.644	11.17		20	0.498	5.32	
	40	0.571	21.24		40	0.432	17.87	
	80	0.471	35.03		80	0.401	23.76	
	160	0.316	56.41		160	0.357	32.13	
	320	0.264	63.59		320	0.311	40.87	

MTT assay with A549 cell lines and HTE cells lines. Percentage inhibition obtained for different conc. of MEB used against A549 cells and HTE Cells

efficacy against various diseases. Pathogens are known to be destroyed by the action of some of the phytochemicals such as Quercetin, Coumaric acid, and Kaempferol with mechanism of action such as breaking up the cell wall, increasing cell permeability, and influencing protein synthesis and expression, influencing enzyme activity, and suppressing nucleic acid synthesis (Yang et al. 2020). Another phytochemical present in EEL, i.e. Gallic acid works by modifying the characteristics of membranes and making them irreversible like altering the charge, permeability, and physicochemical properties, and changing the membrane's hydrophobicity. It is also known to cause pore formation, resulting in the leaking of critical cell elements, and to reduce the negative surface charge (Borges et al. 2013).

MEB has yielded Catechin, Epicatechin, Docosanamide, and Carnitine, along with other components. Catechins are also found in the fruit of *G. asiatica*, which is widely studied and consumed (Qamar et al. 2022). Catechin in bark may be responsible for the high efficacy against a wide range of pathogens chosen which cause serious infections. We report that since, polyphenols were found to be abundant in bark, and these phenols are responsible for interference with bacterial quorum sensing, which is ultimately causing the inhibition of these pathogens and thereby imparting potent antibacterial activity to MEB. The major polyphenols present, Catechins and Epicatechin, are known to be exceptionally strong antibacterial agents, with a mechanism of action of hydrogen peroxide generation. These Catechins are also known to cause cytoplasmic membrane damage, and it has also been reported that it binds to the ATP site of DNA gyrase subunit b, limiting the activity of gyrase enzymes. It is also well recognised that these Catechins disrupt biofilms by destroying cell membranes and dissolving exopolysaccharides (Gopal et al. 2016).

The presence of phytochemicals such as Carnitine and Docosanamide imparts numerous other medicinal qualities such as anti-inflammatory and anti-cancer actions to MEB. Mechanism of action of L-carnitine has been investigated and it has been illustrated that L-carnitine contributes to the reduction of oxygen free radical production through its regulatory effects on membrane phospholipids and energy metabolism. L-carnitine was reported to limit NO generation and *iNOS* gene expression at the transcriptional level in Lipopolysaccharide-stimulated macrophages, with effects mediated through NF- κ B inhibition (Koc et al. 2011). So, the presence of these phytochemicals might have impacted positively, in combating the harmful effect of reducing agents which has resulted in MEB having very good anti-inflammatory action. There are also numerous complex compounds contained in both extracts that are less well-known names but have broad applications in natural treatments and cosmetics. The presence of these phytochemicals indicates the powerful antibacterial action found in both leaves and

bark. Further purification and analysis are planned to validate the key phytochemicals responsible for the activity, and to quantify them, that may disclose the plant's true mode of action in treating the infection.

Antioxidant capabilities measured by DPPH and ABTS have provided a good score with methanolic and water extracts of leaf, twig, buds, and bark. Among all the extracts examined, MEB gave the best results, with IC₅₀ values of 50 μ g/mL and 36 μ g/mL in the DPPH and ABTS tests, respectively. This result is in co-ordinance with the related literature, where it was reported that the methanolic extract of fruit of *G. asiatica*, a thoroughly investigated species in this genus, had an IC₅₀ value of 29 μ g/mL in DPPH assay (Qamar et al. 2022). Another study has shown that the methanolic extract of *G. tiliaefolia* leaves have a high antioxidant activity, with an IC₅₀ value of 71.5 μ g/mL. (Kumar et al. 2022). Furthermore, FRAP assays on all extracts revealed that MEB has substantial metal chelating activities with a FRAP value of 24 ± 0.093 mmol/g comparable to that of Tannic acid, whereas methanolic extract of *G. asiatica* fruit had a FRAP value of 46 mmol/g (Kumar et al. 2022).

Our initial assessment of antibacterial activity of all extracts against two main pathogens, *E. coli* and *S. aureus*, revealed that only methanolic extracts of leaves, bark, and twig were active, with bark being the most effective. The activity of MEB against *S. epidermidis* was found to be best, with a MIC of 0.3 mg/mL. It was also found to be effective against MRSA with a MIC of 0.6 mg/mL, followed by *S. aureus*, *E. faecium*, and *E. faecalis* with MIC of 1.3 mg/mL. It has a broad-spectrum range since it displays antimicrobial action against two prominent Gram-negative bacteria, *P. aeruginosa* and *A. baumannii*, with MIC of 2.5 mg/mL. Leaves were shown to be second best in antibacterial activity, with MIC ranging from 2.5–10 mg/mL on Gram-positive bacteria, whereas on *A. baumannii* it had MIC of 10 mg/mL and on *P. aeruginosa* it had MIC of 20 mg/mL. Interestingly, none of the extracts, even at the highest conc. of 20 mg/mL, were able to kill *E. coli* and *K. pneumoniae*. It will be very interesting to see if these extracts can kill these Gram-negative bacteria beyond the highest conc. used. Even passivity against *E. coli* and *K. pneumoniae* demonstrates that the activity obtained for MEB is not an artefact, since it is discovered to be quite effective against other pathogens, providing authenticity. It also demonstrates that the action is not random, rather it is pathogen specific. This piques our interest in investigating the molecules that are involved in bringing about this behaviour and whether these molecules are operating synergistically.

The antibacterial property found with MEB of *G. orbiculata*, is validated following similar results as reported on different species of this genus in literature. It has been previously reported that an acetone extract of *G. orientalis* leaves have strong action against important pathogens

such as *E. coli*, *B. subtilis*, *S. aureus*, *P. aeruginosa*, and *K. pneumonia*, with MIC ranging from 50 to 250 µg/mL. Acetone extracts of *G. flavescens* DC leaves and roots have shown antibacterial action, with MIC values of 0.03 mg/mL and 0.07 mg/mL against *S. aureus* and *E. coli* respectively (Kumar et al. 2022). Another study has found that different solvent extracts of *G. tenax* have varying degrees of antibacterial activity against *E. coli*, *S. marcescens*, *K. pneumoniae*, *S. typhi*, *P. aeruginosa*, *P. vulgaris*, *S. aureus*, *E. faecalis*, *S. epidermidis*, *S. pneumoniae*, and *B. subtilis* at concentrations of 1 mg/mL (Aadesariya et al. 2017a, b).

There is a great deal of opportunity to investigate in depth on biomolecules of this plant *G. orbiculata* and to determine their activities. It is also interesting to know if these phytochemicals function synergistically by interacting and co-operating with one another, or whether the therapeutic activity is brought about by the combined impact of each of these molecules acting separately. This can be accomplished by isolating the complex compounds and investigating their individual and combined activities at various concentrations and proportions. It will also be fascinating to explore these new compounds, which may have unique mechanisms of action, that may help to address the problem of drug resistance and to search for novel classes of antibiotics with potency comparable to synthetic antibiotics.

Antibiotics that are now in use have several negative effects with pathogenic resistance emerging. In our current investigation, we have found that the MEB is compatible with human plasma, and even at high concentrations, it does not denature the protein, indicating that it is likely to be non-reactive to host molecules. MEB biocompatibility with plasma was up to about 200 µg/mL, while the BSA denaturation experiment resulted in greater than 80% protection of BSA at 1000 µg/mL. The cytotoxicity test results of MEB also show that it is safe to normal host cells. As shown in the MTT experiment, MEB does not kill HTE cells completely even at 320 µg/mL, maximum concentration used. In the literature, it is reported that methanolic extracts of *G. asiatica* leaves demonstrated platelet aggregation inhibition efficacy, with 50% inhibition at conc. 5 mg/mL and 93% inhibition at 10 mg/mL. (Kumar et al. 2022).

It is also observed that *G. orbiculata* possesses anti-cancer properties against prominent and most widely studied lung carcinoma cell line such as A549. MTT assay at different concentrations of MEB of *G. orbiculata* has exhibited significant inhibition of A549 cell lines with an IC₅₀ value of 98.73 µg/mL, whereas other species of this genus, such as benzene leaf extract and methanolic bark extract of *G. tiliaefolia* have IC₅₀ values of 192 µg/mL and > 80 µg/mL respectively on lung carcinoma cell lines (Rajavel et al. 2017; Selvam et al. 2010). Overall, in the light of our study, we feel that there is lot of scope for further in-depth studies like antifungal, antiviral, and anti-diabetic activities to be

evaluated for this plant. This research might greatly aid in the development of plant-based antibiotics for the treatment of fatal illnesses.

Conclusion

In conclusion, our investigation has revealed the presence of many phytochemicals in *G. orbiculata*, that has given the plant the excellent medicinal properties. The evaluation of the therapeutic efficacy of this plant, particularly its bark, has given promising and fruitful outcomes. Due to the abundance of Catechin and Epicatechin in the MEB, it has obtained very potent antibacterial action against major pathogens. Our data imply that the likely mechanism of action against the pathogen studied may be hydrogen peroxide production, pathogen cytoplasmic membrane disruption, biofilm suppression, and polysaccharide breakdown which needs to be investigated further (Qamar et al. 2022; Gopal et al. 2016; Koc et al. 2011).

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s42535-022-00530-z>.

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Author contributions SM: Conceptualization, Methodology, Software, Formal analysis, Investigation, Resources, Data Curation and editing, Writing-Original draft, Project administration, Funding acquisition. US: Validation, Review, Visualization, Supervision.

Data availability The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that no conflicts of interest exist related to the manuscript.

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