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ORIGINAL ARTICLE

In vitro Antioxidant, Anti-inflammatory, Antitumor, and bactericidal properties of *Glycyrrhiza glabra* root extract

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ABSTRACT
Medicinal plants have been integral to medical management regimens since antiquity. This study aimed to assess the medicinal properties of methanolic root extract of Glycyrrhiza glabra viz antioxidant, anti-inflammatory, antitumor, and bactericidal activities. The antioxidant activity was evaluated by DPPH, ABTS$^{\bullet+}$, Nitric oxide, Hydroxyl radical, FRAP, and Phosphomolybdenum assay. The anti-inflammatory effect was determined using the heat-induced hemolysis method while the anticancer activity was studied on Hela cancer cells using MTT assay. The agar well diffusion method was used for assessing the antibacterial activity against six bacterial strains. Results indicate that the G. glabra root extract strongly scavenged free radicals in the order of ABTS$^{\bullet+}$ >Nitric oxide >Hydroxyl radical >DPPH radical, and the reducing power increased with increased concentration. The root extract inhibited hemolysis dose-dependently with an IC_{50} value of 82.91µg/mL. A concentration-dependent cell proliferation inhibition was observed in HeLa cells. G. glabra root extract had a stronger antibacterial effect against gram-positive bacteria. GCMS analysis showed the presence of isopropyl stearate, hexadecanoic acid, oleic acid, coumarin, phenolic compounds, and pyrazole. The findings indicate that G. glabra root extract contains different bioactive compounds of various polarities that can be used as natural therapeutic agents for addressing different disease conditions.
Keywords: Medicinal plants, *Glycyrrhiza glabra*, root extract, therapeutic properties, GCMS

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INTRODUCTION

Numerous studies have consistently emphasized the effectiveness of medicinal plants in treating acute and chronic disease conditions including cancer, diabetes mellitus, heart diseases, hypertension, and respiratory disorders with minimal or lesser side effects [1]. Secondary metabolites such as alkaloids, glycosides, phenolics, steroids, and terpenes, produced by the plants in response to biotic and abiotic stress conditions contribute to several medicinal properties [2]. Globally, India stands out as one of the 12 biodiversity hotspots and has about 8,000 species of medicinal plants, thereby reflecting the nation's profound biodiversity in herbal resources [3].

Glycyrrhiza, a perennial plant from the Fabaceae family, is cultivated in various countries such as China, Russia, Spain, Persia, and India. The Glycyrrhiza genus has around 30 species, including *G. aspera*, *G. eurycarpa G. glabra*, *G. inflata*, *G. korshinskyi*, *G. uralensis*, and. *G. glabra* is the most common species. The wide spectrum of medicinal properties associated with different parts of *G. glabra* include antimicrobial, antioxidant, anticancer, antidiabetic, antiulcer, anti-inflammatory, antipyretic, and antihyperlipidemic properties [4]. Studies indicate that the root extract of *G. glabra* has more medicinal properties than other aerial parts [5]. The root extract is commonly used as a natural sweetener and flavoring agent. Roots are an essential part of plants as they provide water and nutrients to all other parts of the plant. Roots of

many traditional medicinal plants have been used in treating many diseases and drug development. The objective of this study was to assess the medicinal properties of *G. glabra* root extract to optimize its use as a nutraceutical agent.

MATERIAL AND METHODS

Preparation of extract: *G.glabra* roots were obtained from Anna University campus, Chennai Tamil Nadu. The roots were cleaned, air-dried in a shaded area, and ground into fine powder. For extraction, 20 g of the powder was soaked with 200 mL of methanol for 48 h. The mixture was filtered to remove and concentrated to obtain crude extract.

DPPH radical scavenging assay: *G.glabra* RE (different concentrations) was mixed with 500 µL of 0.2 mM DPPH solution in MeOH and incubated in the dark at room temperature for 30 min. The OD was then measured at 517 nm, and the results are reported as % inhibition of DPPH radical [6].

% inhibition of DPPH radical: [(Control OD – Sample OD)/Control OD] x 100

$ABTS^+$ radical scavenging assay: $ABTS^+$ was obtained by mixing 7 mM ABTS and 2.45 mM $K_2S_2O_8$. The resulting solution was incubated for 16 h in a dark place at room temperature. The resulting $ABTS^+$ solution was diluted till an OD of 0.70 was obtained at 734 nm with 5 mM phosphate-buffered saline (pH 7.4). *G.glabra* RE (different concentrations) was mixed with 1 mL of diluted $ABTS^+$ solution and incubated for 10-15 min. The OD was measured at 734 nm and results are reported as % inhibition of $ABTS^+$ radical [7].

% inhibition of $ABTS^+$ radical: [(Control OD – Sample OD)/Control OD] x 100

Hydroxyl radical (OH^-) scavenging assay: *G.glabra* RE (different concentrations) was mixed with 1mL of reaction solution containing $C_5H_{10}O_4$ (2.8 mM), KH_2PO_4-NaOH buffer, pH 7.4 (0.05 M), $FeCl_3$ (0.1 mM), EDTA (0.1 mM), and H_2O_2 (1 mM). The final volume was made to 2 mL using de-ionized water and incubated for 30 min at 37°C. Later, 2mL of 2.8% (w/v) TCA and thiobarbituric acid were added, kept in a water bath for 30 min, and cooled. The OD was measured at 532 nm. Results are expressed as % inhibition of hydroxyl radical [8].

% inhibition of hydroxyl radical: [(Control OD – Sample OD)/Control OD] x 100

Nitric oxide radical (NO^- scavenging assay: *G.glabra* RE (different concentrations) was mixed with 2mL of 5mM sodium nitroprusside solution. The solution was incubated for 180 min at 25ºC followed by adding 4 mL of Griess reagent. The OD was measured at 546 nm after incubating the reaction solution for 10 min. Results are reported as % inhibition of nitric oxide radical [9].

% inhibition of nitric oxide radical: [(Control OD – Sample OD)/Control OD] x 100

Fe^{3+} reducing power assay: *G.glabra* RE (different concentrations) was mixed with 1000 µL of 0.2 M phosphate buffer solution (PBS) (pH 6.6) and 1000 µL of 1 % (w/v) $K_3[Fe(CN)_6]$, followed by incubation at 50 °C for 25 min. After cooling, 1 mL of 10% (w/v) TCA and 0.5 mL of 0.1% (w/v) freshly prepared $FeCl_3$ were added. The OD was measured at 700 nm and the results are reported as %reduction of Fe^{3+} to Fe^{2+} ions [10].

% reduction of Fe^{3+} to Fe^{2+} ions = [(Sample OD - Control OD)/Sample OD] x100

Phosphomolybdenum reducing assay: *G.glabra* RE (different concentrations) was mixed with 1000 µL of freshly prepared reagent solution [0.6 M H_2SO_4, 28 mM Na_3PO_4, and 4 mM $(NH_4)_2MoO_4$] followed by incubation for 90 min at 95°C. After cooling, the oOD was measured at 695 nm and results are reported as % reduction Mo (VI) to Mo (V) complex [11].

% reduction of Mo (VI) to Mo (V) complex = [(Sample OD - Control OD)/Sample OD] x100

For all antioxidant assays, ascorbic acid was used as the standard reference.

Anti-inflammatory assay: *G.glabra* RE (different concentrations) was blended with 0.2 mL of a freshly prepared 10% red blood cell (RBC). The solutions were then incubated at 56ºC for 30 min, then cooled and centrifuged at 2500 rpm for 10 min. The OD was measured at 560 nm. Diclofenac served as the standard. Results are reported as % inhibition of hemolysis [12].

% inhibition of haemolysis = [(Control OD – Sample OD)/Control OD] x 100

Cytotoxicity assay: The cytotoxic effect of *G.glabra* RE was studied against the cervical cancer (HeLa) cell line. The cells were incubated in a 5% CO_2 incubator at 37°C. The cells were grown in Dulbecco's modified Eagle's medium containing 10% FBS, supplemented with penicillin (120 IU/mL), streptomycin (75 µg/mL), gentamycin (160 µg/mL), and amphotericin B (3 µg/mL). The cells were sub-cultured after reaching 80% confluence. Cell viability was determined using an MTT assay. The cells were plated in a 96-well plate, (5000 cells/well), and varying concentrations of the extract were added. The cells were incubated at 37 °C for 48 h. After incubation, 0.05mL of MTT reagent was added and incubated for 2 h. The OD was measured at 490 nm after adding 0.05 mL of DMSO. Results are expressed as % inhibition of cell death [13].

% inhibition of cell death = [(Control OD – Sample OD)/Control OD] x 100

Antibacterial assay: The antibacterial activity of *G.glabra* RE was tested against gram-positive bacterial strains (*Bacillus subtilis, Staphylococcus aureus*, and *Micrococcus luteus*) and gram-negative bacterial strains (*Escherichia coli*, *Shigella flexneri*, and *Proteus vulgaris*) using the agar well diffusion method. Freshly cultured bacterial suspension was spread evenly on separate petri plate surfaces containing Mueller Hinton agar using a cotton swab. Wells (8mm) were made in each petri plate using a corrosion-resistant steel cork borer, and varying concentrations of the extract (250, 375, and 500 µg/mL) were added into the wells and incubated for 24 h at 37ºC. The standard used was tetracycline. The antibacterial activity was determined by measuring the diameter of the inhibitory zone formed in mm [14].

Gas Chromatography-Mass Spectrometry (GC-MS): Components present in *G.glabra* RE were analyzed using GC-MS. The analysis was performed with a GC-MS system equipped with an Agilent 6890N JEOL GC Mate II and an HP-5 column (30 m x 0.25 mm ID with 0.25 µm film thickness). The analysis was performed with helium as the carrier gas at a flow rate of 1 mL/min, an ionization voltage of 70 eV, and an ion source and interface temperature of 250°C. The column oven was kept at temperatures between 50°C and 250°C, increasing by 10°C per minute. National Institute Standard and Technology (NIST) database comprising more than 62,000 patterns was used for interpretation [15].

Statistical analysis: Results are presented as mean and standard deviation of three independent observations. IC_{50} value was calculated using Graph Pad Prism software.

RESULTS AND DISCUSSION

Table 1: Antioxidant potential of *G. glabra* root extract

% Inhibition of DPPH radical			Conc. µg/mL	% Inhibition of ABTS radical		% Inhibition of hydroxyl radical		% Inhibition of nitric oxide radical	
Conc. µg/mL	*G. glabra*	Ascorbic acid		*G. glabra*	Ascorbic acid	*G. glabra*	Ascorbic acid	*G. glabra*	Ascorbic acid
20	14.60 ± 0.13	51.66 ± 0.12	10	28.13 ± 0.37	56.90 ± 0.17	22.62 ± 0.33	41.42 ± 0.13	8.97 ± 0.40	19.28 ± 0.36
40	22.77 ± 0.25	57.06 ± 0.38	20	40.63 ± 0.11	65.43 ±0 .24	28.57 ± 0.29	44.39 ± 0.18	11.03 ± 0.42	33.17 ± 0.42
60	36.14 ± 0.36	62.78 ± 0.17	30	46.88 ± 0.29	70.94 ± 0.41	32.14 ± 0.24	56.89 ± 0.44	26.21 ± 0.14	39.15 ± 0.31
80	58.91 ± 0.10	71.89 ± 0.32	40	53.13 ± 0.33	77.82 ± 0.28	36.90 ± 0.18	65.92 ± 0.39	33.79 ± 0.16	56.47 ± 0.29
100	74.01 ± 0.46	78.16 ± 0.28	50	62.50 ± 0.14	81.11 ± 0.15	48.81 ± 0.11	69.50 ± 0.26	43.45 ± 0.34	58.03 ± 0.12
120	77.72 ± 0.28	84.89 ± 0.39	60	83.75 ± 0.21	86.12 ± 0.32	63.10 ± 0.30	73.88 ± 0.10	53.79 ± 0.29	64.77 ± 0.40
IC_{50} value (µg/mL)	64.02	19.23		35.93	5.87	49.23	20.59	55.97	32.42

Values are the mean of three independent observations

Table 2: Reducing power potential of *G. glabra* root extract

Concentration µg/mL	FRAP assay		Phosphomolybdenum assay	
	G. glabra	Ascorbic acid	*G. glabra*	Ascorbic acid
10	19.45 ± 0.18	32.19 ± 0.26	28.11 ± 0.35	42.17 ± 0.23
20	28.04 ± 0.25	45.11 ± 0.17	45.72 ± 0.38	49.04 ± 0.37
30	46.94 ± 0.36	54.83 ± 0.46	49.23 ± 0.17	56.19 ± 0.20
40	53.11 ± 0.11	67.11 ± 0.12	55.64 ± 0.22	61.11 ± 0.47
50	64.69 ± 0.15	73.77 ± 0.30	59.08 ± 0.10	64.23 ± 0.18
60	69.13 ± 0.42	78.26 ± 0.28	63.46 ± 0.24	69.36 ± 0.33
IC_{50} value (µg/mL)	35.69	26.29	36.95	27.36

Values are the mean of three independent observations

The increasing prominence of plant-based therapies, particularly those entrenched in traditional medicine, reflects a societal shift toward natural and holistic health approaches [16]. With a well-documented history of effectiveness and safety, traditional medicinal plants have captivated researchers to identify bioactive compounds with distinct medicinal properties from these botanical sources. The

prime objective of the present study was to evaluate the antioxidant, anti-inflammatory, anticancer, and antibacterial activities of *G.glabra* RE.

Free radicals are constantly produced in the body through diverse biological metabolic pathways. Oxidative stress occurs when the rate of production of free radicals exceeds the body's antioxidant defense mechanism. Oxidative stress induced by free radicals poses detrimental effects on human health. Conversely, antioxidants mitigate the adverse consequences of oxidative stress [17]. The antioxidant activity of *G.glabra* RE is presented in Table 1 and 2. The ability to scavenge free radicals increased linearly and can be ranked in the order of $ABTS^+$ >Nitric oxide >Hydroxyl >DPPH radical based on the IC_{50} value. Singh and Kumar [18] evaluated the antioxidant potential of petroleum ether, ethyl acetate, methanol, and aqueous extracts of *G.glabra* RE using DPPH, nitric oxide, and FRAP assays. Among the extracts tested, ethyl acetate had the highest antioxidant activity. Free radicals such as hydrogen peroxide, hydroxyl peroxide, nitric oxide, and superoxides are produced in the body due to mitochondrial dysfunction and activation of pro-oxidant enzymes [19]. Table 1 shows that *G.glabra* RE scavenged hydroxyl (63.10 at 120 µL) and nitric oxide radicals (53.79 at 120 µL) with an IC_{50} value of 49.23 and 55.97 µL. Ugbeni *et al*. [20] reported that methanolic and ethanolic extracts of *G.glabra* RE scavenged 77.51% and 78.92% of hydroxyl radicals with IC_{50} values of 0.42 mg/mL and 0.4 mg/mL respectively. Reducing power assays measure the ability of a substance to neutralize the harmful effects of free radicals by transferring electrons to them. The reducing potential of *G.glabra* RE was evaluated based on its ability to reduce molybdate complex (VI) to (V) and Fe^{3+} ions to Fe^{2+} ions. Table 2 shows that G.glabra RE could convert molybdate (VI) to (V) and Fe^{3+} ions to Fe^{2+} ions through electron transfer reaction. Tohma *et al*. [21] evaluated the radical scavenging potential and reducing power of lyophilized aerial parts and root extracts of *G.glabra* using FRAP and Cuprac assays. The authors concluded that the root extract has better antioxidant and reducing power than the aerial parts.

Table 3: Anti-inflammatory activity of *G. glabra* root extract

Concentration µg/mL	*G. glabra*	Diclofenac
20	16.35 ± 0.21	29.56 ± 0.10
40	22.25 ± 0.16	34.18 ± 0.36
60	33.42 ± 0.38	45.88 ± 0.22
80	37.99 ± 0.46	54.04 ± 0.13
100	57.86 ± 0.40	67.91 ± 0.34
120	71.30 ± 0.29	79.59 ± 0.18
IC_{50} value (µg/mL)	82.91	72.03

Values are the mean of three independent observations

Inflammation is the body's adaptive response to injury or any harmful stimuli. Stabilization of the liposomal membrane is essential to prevent inflammation reaction triggered by tissue damage caused by the release of lysosomal constituents, and bactericidal enzymes [22]. The anti-inflammatory activity of *G. glabra* RE shows a linear increase in hemolysis inhibition with a maximum activity of 71.30% at 120 µg/mL. However, diclofenac showed superior inhibition activity with an IC_{50} value of 72.03 µg/mL (Table 3). These findings suggest that *G. glabra* RE can effectively inhibit hypotonicity-induced lysis of the RBC membrane.

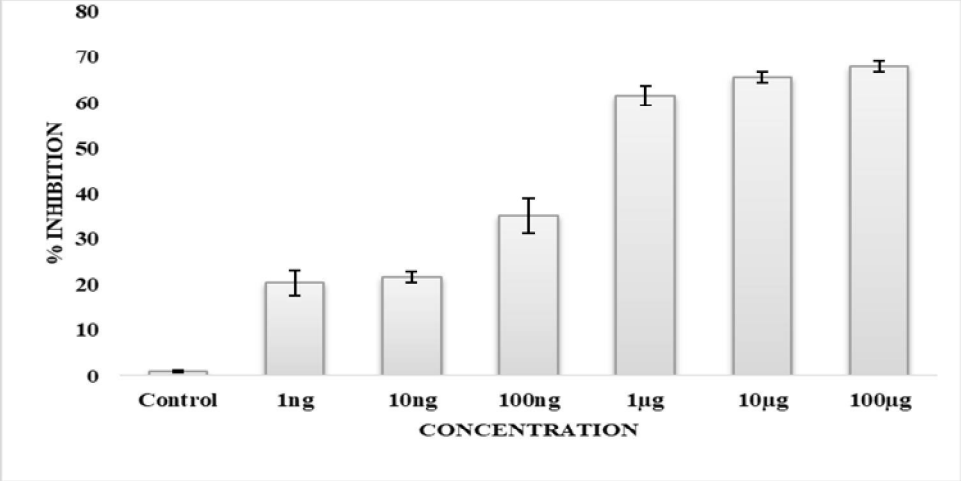
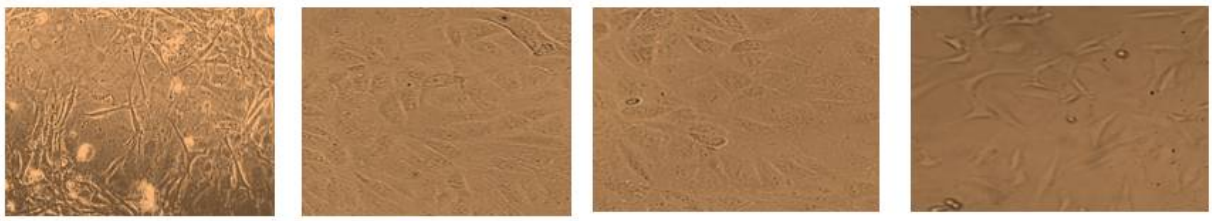


Figure 1: Cytotoxicity of *G. glabra* root extract against HeLa cell

Figure 2: Microscopic images of Hela cells treated with different concentrations of *G. glabra* RE



Control 1 µg/mL 10 µg/mL 100 µg/mL

Epigenetic changes in macromolecules such as DNA, nucleic acids, and proteins caused by chronic inflammation and oxidative stress become carcinogenic by initiating tumor formation, followed by tumor development, progression, and treatment resistance [23]. The anticancer activity of *G. glabra* was investigated using Hela cell lines. Results indicate that the anticancer effect was directly proportional to the concentrations of the extract. The IC_{50} value was found to be 152.56 ng/mL. Microscopic images of Hela cells treated with different concentrations of *G. glabra* RE showed alternations in cell morphology, particularly an increase in loss of adhesiveness, and cellular shrinkage that increased in a concentration-dependent manner. (Figure 2). Goel *et al*. [24] investigated the anticancer effect of hydroalcoholic RE of *G. glabra* against C6 glioma cells and reported the IC_{50} value to be 32 µg/mL. Similarly, Pandian and Chidambaram [25], demonstrated the anticancer activity of aqueous and silver nanoparticles derived from *G. glabra* on Hela cell lines with the nanoparticles exhibiting significant anticancer activity and showed an IC_{50} value of 125 µg/mL and 62.5 µg/mL respectively. Caroline *et al*. [26] investigated the anticancer effect of the ethanolic root extract of *Plectranthus amboinicus* and *G. glabra* on oral cancer (KB) cell lines. The authors observed a linear decrease in cell viability percentage. Comparing the two extracts, *G. glabra* demonstrated the highest anticancer activity with an IC_{50} value of 43.6 µg/mL. Results of this study along with previous studies, highlight the significant anticancer potential of *G. glabra* RE.

Table 4: Antibacterial activity of *G. glabra* root extract

Bacterial strains	Standard Tetracycline	Concentration		
		250 µg/mL	375 µg/mL	500 µg/mL
Bacillus subtilis	16.01 ± 0.17	16 ± 0.33	16 ± 0.47	18 ± 0.11
Micrococcus luteus	20.23 ± 0.36	17 ± 0.12	18 ± 0.18	18 ± 0.41
Staphylococcus aureus	15.10 ± 0.42	15 ± 0.46	17 ± 0.23	17 ± 0.25
Escherichia coli	23.12 ± 0.10	16 ± 0.26	16 ± 0.31	18 ± 0.43
Shigella flexneri	19.02 ± 0.13	16 ± 0.12	16 ± 0.29	17 ± 0.10
Proteus vulgaris	23.08 ± 0.22	20 ± 0.14	20 ± 0.40	21 ± 0.32

Values are the mean of three independent observations

The antibacterial activity of *G.glabra* RE was studied using three different concentrations (250, 375, and 500 µg/mL) against gram-positive and gram-negative bacterial strains. Table 3 shows a consistent increase in the antibacterial activity with an increase in concentration. Notably, the highest activity was observed against *Proteus vulgaris* with a zone of inhibition of 21 mm, followed by *Micrococcus luteus* (18mm), and *E.coli* (18mm) at a concentration of 500 µg/mL. The findings of the present study are in agreement with previous reports. Sultan *et al*. [27] showed that *G.glabra* RE was effective in inhibiting the growth of gram-positive (*S. aureus*, *Bacillus megaterium*, and *B. subtilis*) and gram-negative pathogenic bacterial strains (*E. coli*, *Pseudomonas aeruginosa*, and *Salmonella paratyphi*). In another study, Bhusal and Sharma [28] reported that the methanolic, ethanolic, and chloroform root extracts of *G. glabra* showed antibacterial activity against *B. subtilis* and *S. aureus.* Sedighinia *et al*. [29] tested the antibacterial activity of *G.glabra* RE against *Streptococcus mutans*, *Streptococcus sanguis*, *Actinomyces viscosus*, *Enterococcus faecalis*, *E.coli*, and *S. aureus*. The highest activity was observed in *S. mutans, A. viscosus*, and *E. faecalis* with a zone of inhibition of 27.3, 26.1, and 23.8 respectively at 100mg/mL.

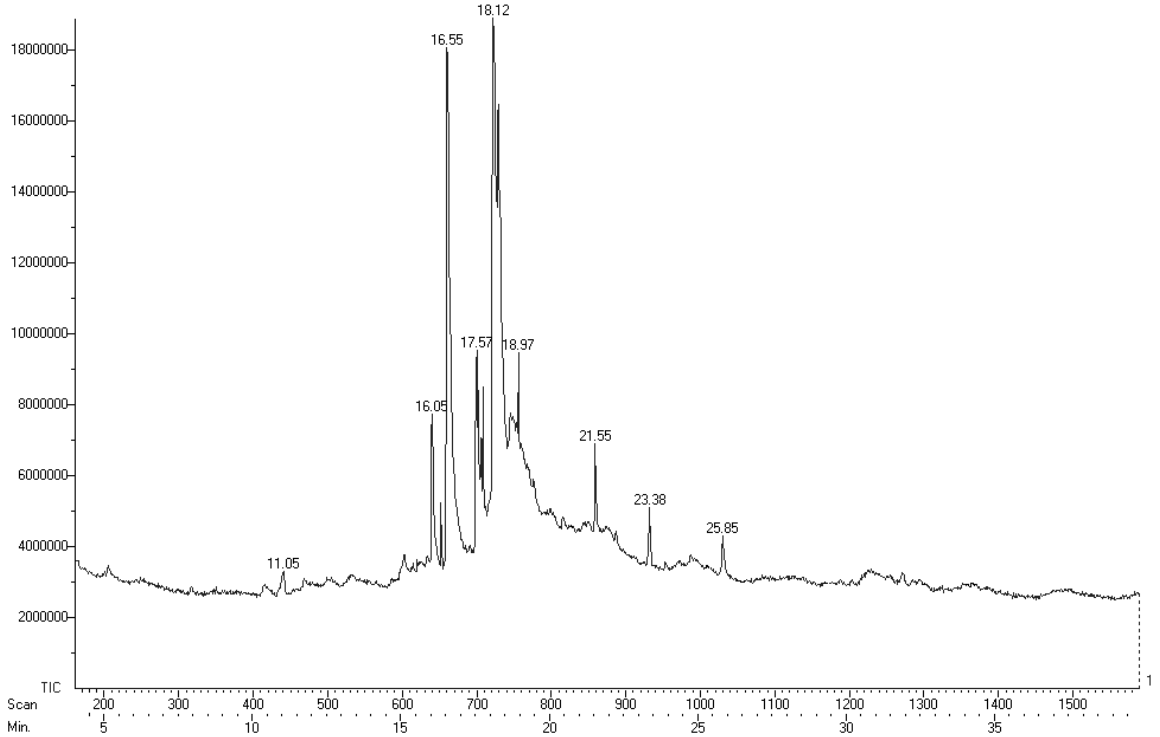


Figure 3: GCMS chromatogram of *G. glabra* root extract

Accurate certification and studies of phytochemicals are necessary, as these compounds serve as repositories for numerous potent drugs. This study identified the different bioactive compounds in G. glabra RE using GCMS analysis (Figure 3). The identified compounds include isopropyl stearate, elemene, n-hexadecanoic acid, 10-octadecenoic acid, methyl ester, oleic acid, coumarin, 3-(Benz oxazolyl)-8-methoxy, 1H-Pyrazole, 4,5 – dihydro – 1 – phenyl, pyrazole -3-carboxylic acid, 5[4-methoxyphenyl]-4-phenyl azo, methyl ester, and phenol 2,6-bis (1,1 dimethyl ethyl)-4-(4-hydroxy -3,5-dimethyl phenyl). Pyrazole-containing molecules exhibit anti-inflammatory, anticancer, antimicrobial, anticonvulsant, and antidepressant properties [30]. Research studies have shown that coumarins and their derivatives are effectively used as anticancer, anti-inflammatory, anti-HIV, and anticoagulant agents [31]. Polyphenols, which are secondary metabolites produced by plants contribute significantly to antioxidant and antimicrobial activity.

CONCLUSION

The findings of this study underscore notable medicinal properties of the root extract of *Glycyrrhiza glabra* as demonstrated through *in vitro* assays indicating its potential for treating chronic therapeutic conditions. However, further research studies focussing on the isolation, characterization, and purification of the active compounds are required to evaluate their therapeutic properties and the development of new drugs.

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