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In silico, in vitro: antioxidant and antihepatotoxic activity of gnetol from *Gnetum ula* Brongn

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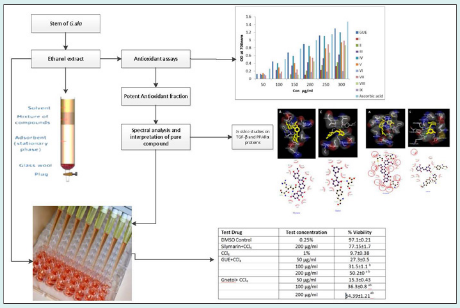
Abstract



Introduction: *Gnetum ula* is a notable medicinal plant used to cure various ailments. The stem part of the plant is used traditionally to treat jaundice and other disorders. The present work is to investigate the *in vitro* hepatoprotective and antioxidant activity of ethanol extract of stem of *G. ula* (GUE) and its isolated compound gnetol.
Methods: Column chromatography was carried out for GUE and various column fractions were obtained. DPPH and reducing power assays were performed for GUE and column fractions. The potent fraction was characterized, interpreted and tested for *in vitro* hepatoprotective activity on the BRL3A cell line. *In silico* docking studies of gnetol compound on the protein TGF-β (transforming growth factor – β) and Peroxisome proliferator-activated receptor α (PPARα) was carried out.
Results: DPPH scavenging and reducing power assay showed that the fourth column fraction has antioxidant potential than other fractions. The fourth column fraction was characterized to obtain gnetol compound. BRL3A cell line was used for the toxicity study of GUE and gnetol. Both, the extract and the isolated compound were found to be nontoxic with CTC_{50} value more than 1000 µg/mL. At the concentration of 200 µg/mL, GUE and gnetol offered cell protection of 50.2% and 54.3%, however, silymarin showed 77.15% protection at 200 µg/mL concentration against CCl_4 treated BRL3A cell line. The docking results of the ligand molecule TGF-β showed that gnetol has the binding affinity of -7.0 and standard silymarin being -6.8. TGF-β showed good hydrophobic interactions and formed two hydrogen bonds with the amino acids. For PPARα protein, gnetol showed the binding affinity of -8.4 and silymarin with -6.5. Hydrogen bonding and good hydrophobic interactions against the amino acid molecules in relation to the PPARα protein are shown.
Conclusion: *Gnetum ula* stem extract and its isolated compound are safe and offered significant hepatoprotection against CCl_4 induced toxicity. Isolated compound gnetol exhibited a potent antioxidant activity offering protection to liver damage. However, *in vivo* studies need to be carried out to validate the traditional use of *G. ula*.

Introduction

Liver is the largest organ in the human body performing major roles in various metabolic functions. Any sort of slight damage to the liver will lead to serious issues. Treating liver complications with plant-based medicine has become important in complementary and alternative medicine.[1] Human beings are using widely many plants for medicinal purposes. In spite of having a long history of usage, still, there are some plants whose impacts on liver disorders are not studied.[2] There is a tremendous usage of herbs for liver diseases, many are departed without proper investigation with respect to its traditional aspects.[3] Demand for a safe and efficacy hepatoprotective drug is a need for coping up with the liver disorder.

Medicinal plants contain various phytoconstituents responsible for antioxidant properties.[4] Thousands of

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secondary metabolites have been identified and known to possess antioxidant activity.[5] Considerably phenolic compounds exhibit more scavenging activities via their hydrogen or electron-donating groups.[6]

Several leads are obtained from the plant as hepatoprotective agents.[7] Some of them are silymarin, andrographolide, neoandrographolide, curcumin, picroside, kutkoside, phyllanthin, hypophyllanthin, and glycyrrhizin.[8] Nonetheless liver dysfunction remains as one of the serious problems without proper antihepatotoxic drugs in medical practice. However, plants with hepatoprotective properties, which are used traditionally, lack scientific assessment.

Gnetum ula belonging to the family Gnetaceae is a large woody climber. It is considered a sacred plant by Kodavas of Karnataka, India. Stem extracts are used in treating jaundice.[9-11] and leaf extracts are used in the treatment of liver enlargement.[12] Stem and roots are used as antiperiodic.[13] The stem is also given for penetrating wounds caused by horn thrust, also for treating piles, hemicranias.[14] Seed oil and roasted fruit is used in the treatment of rheumatism.[15,16] The fruits of *G. ula* are edible and seeds produce oil that can be used for medicinal purposes and for burnt wounds.[17,18]

Phenols are one of the important secondary metabolites of plants.[19] Polyphenols like stilbenes have been isolated in this genus *Gnetum*, which may contribute to their therapeutic values. Isolation of biergenin, 2-hydroxy-4-benzyloxy acetophenone and the related dimer of stilbenes was reported from the *G. ula.*[20] A stilbene called Gnetin from stem-wood of *G. ula* assigned has 3, 4-methylenedioxy-4´-methoxy-trans stilbene.[21] Phenolic compound gnetol was isolated from the stem of *G. ula* and characterized as 2, 6, 11, 13-tetrahydroxy-trans-stilbene.[22] Another stilbene from the wood part of *G. ula* was reported as gnetulin, a dimer of 3, 4, 5- trihydroxy -3- methoxystilbene.[23] Gnetifolin was isolated from the *Gnetum montanum* along with other compounds of resveratrol, 4, 5, 7-trihydroxy-3´-methoxyflavone, gnetol, daucisterol, β-sitosterol and, tetracosanoic acid.[24] Stilbene dimers were isolated from the dried bark of lianas of *Gnetum parvifolium* namely parvifolol A, B, C and D, 2b-hydroxyampelopsin F, gnetulin.[25] Trimeric stilbenes were isolated from the root of *Gnetum gnemon*, Gnemonol D, E and F.[26] Three phenolic compounds from the stem bark of *G. gnemon* namely 3, 4-dimethoxychlorogenic acid, resveratrol, and 3-methoxy resveratrol were reported.[27]

Based on the literature survey and traditional usage, the stem of *G. ula* has been selected to evaluate its antioxidant and hepatoprotective activity.

Material and Methods

Plant material

The stem of *G. ula* was collected in Biligirirangana Hills (B.R. Hills) of Chamarajanagar district; Karnataka State, India. Botanical identification of the plant was carried out and authenticated by Dr. Shiddamallayya. N, at National Ayurveda Dietetics Research Institute, Department of AYUSH, Govt. of India, Bangalore. The voucher specimen (No: RRCBI-MUS-0107) was deposited for future references.

Chemicals

AR grade solvents petroleum ether, ethanol, hexane, and chloroform were purchased from S D fine-chemicals limited (SDFCL), Mumbai. HPLC grade of Toluene, ethyl acetate, formic acid, and acetic acid were purchased from RANKEM Thane, Maharashtra. TLC silica gel 60 F254 aluminum sheets 20×20 cm was purchased from Merck Analytical chromatography, Germany. AR grade concentrated sulphuric acid (assay 98%) and glacial acetic acid were purchased from SDFCL, Mumbai. Vanillin powder was purchased from Sigma-Aldrich. Additional all the chemicals used were analytical grade obtained from Sigma-Aldrich (St. Louis, USA) and E-Merck (Mumbai, India).

Extraction

Shade dried powder of stem of *G. ula* (500 g) extracted successively with petroleum ether, chloroform, and ethanol using the soxhlet apparatus. Then, extracts were filtered and concentrated using a rotary evaporator (Make: BUCHI, Model: R-210), dried on a water bath and preserved in desiccator till use.

Isolation

Ethanol extract of *G. ula* (GUE) was macerated for 24 hr successively with hexane, chloroform and finally concentrated to get an ethanolic fraction of *G. ula*. The dried ethanolic fraction was subjected to the thin-layer chromatography (TLC) to fix the mobile phase for the separation of phytoconstituents; toluene: ethyl acetate: formic acid: acetic acid (7.5: 2.5: 1:1). The identification of bands was done under the UV after spraying vanillin sulphuric acid.

Column chromatography

Ethanolic fraction (5 g), was dissolved in 10 mL of methanol and 10 g of silica gel was added, air-dried to become as a free-flowing powder. Column chromatography was performed with Hexane and Ethyl acetate solvents. Initially, Hexane was eluted with 100%, subsequently with hexane:ethyl acetate ratios (98:2, 96:4, 94:6, 92:8, 90:10.88:12, 86:14, 80:20) and ethyl acetate (100%). All the column fractions were collected separately and concentrated by using a rotary evaporator under the vacuum. Further, concentrated fractions were subjected to *in vitro* antioxidant activity.

In vitro antioxidant activity

Radical scavenging activity and reducing power assay was assessed for GUE and its various column fractions obtained, and ascorbic acid was used as standard.

DPPH radical scavenging assay

The DPPH radical is purple in color and upon reaction with hydrogen donor changes to yellow color. It is a discoloration assay, which is evaluated by the addition of the extracts/fractions to a DPPH.[28] About 5.0 mL (0.2 mg) of DPPH solution in methanol was added to 50 µL of various concentrations (7.81 -250 µg) of GUE and column fractions. After 30 min of incubation period at room temperature, the absorbance was read at 517 nm.

Scavenging activity was expressed as the inhibition percentage (I) calculated by using the formula,

$$I\ (\%\ of\ Scavenging\ activity) = \frac{Absorbance\ of\ control - Absorbance\ of\ test}{Absorbance\ of\ Control} \times 100$$

Reducing power assay

The ability of the extracts to reduce iron III was assessed by the method of Oyaize M (1986). Different concentrations (50-300 µg/mL) of GUE and column fractions were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% aqueous potassium hexacyanoferrate solution was added. Incubated for 30 minutes at 50^{o}C, 2.5 mL of 10% TCA (trichloroacetic acid) was mixed and centrifuged at 3000 rpm for 10 minutes. 2.5 mL of supernatant was collected and mixed with 2.5 mL of water and 0.5 mL of 0.1% aqueous $FeCl_3$.The amount of iron ferricyanide complex was determined by measuring the formation of Perl's Prussian blue at 700 nm.[29] Higher the absorbance indicates high reducing power.

Potent Column fraction obtained from DPPH scavenging activity and reducing power assay were analyzed and TLC checked to find the phytoconstituent present. The further active fraction was purified and characterized by Mass spectroscopy and NMR data to predict the compound.

Hepatoprotective activity on BRL3A cell line

Hepatoprotective activity of ethanol extract of *G. ula* (GUE) and a potent isolated and characterized antioxidant compound were assessed for MTT assay.[30,31] Later safer or non-toxic doses of GUE and pure compound were tested for *in vitro* hepatoprotective activity on BRL 3A cell line.

Cell lines and culture medium

In the present study, the BRL3A cell line was used to assess the hepatoprotective function of GUE and isolated compounds. BRL3A was obtained from National Centre for Cell Sciences (NCCS), Pune, India. It was cultured in DMEM (Dulbecco's modified eagles medium), supplemented with 10% inactivated fetal bovine serum (FBS),100 IU/mL of penicillin,100 µg/mL of streptomycin and 5 µg/mL amphotericin in a humidified atmosphere of 5% CO_2 at 37^{o}C until confluent. Later the cells were dissociated with TPVG solution containing 0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS. Stock cultures were grown in 25 cm^2 culture flasks and the study was carried out in 96 microtitre plates.

Preparation of test solutions

A stock solution of 10 mg/mL concentration of the test sample was prepared by dissolving the sample in DMSO and then the volume was made up with DMEM, supplemented with 2% inactivated FBS. The stock was serially diluted to get lower concentrations.

Determination of cell viability by MTT assay

MTT assay was carried out for GUE and isolated compounds to assess its non-toxic doses. A monolayer cell culture was trypsinized and its count was adjusted to 1.0×10^5 cells/mL using DMEM containing 10% FBS. Approximately 10 000 cells (0.1 mL diluted suspension) were added to each 96 well microtitre plate. After 24 hours, the supernatant was flicked off to form a partial monolayer of cells and was washed with the medium. About 100 µL of different test concentrations (62.5-1000 µg/mL) was added to each well and then incubated at 37^{o}C for 3 days in 5% CO_2 atmosphere. Microscopic examination and observations were noted in 24 hours time interval. After 72 hours, test samples were discarded and 50 µL of MTT in PBS was added to each well. Again, incubated at 37^{o}C for 3 days in 5% CO_2 atmosphere, the supernatant was removed, 100 µL of propanol was added and the plates were gently shaken to solubilize the formazan formed. Microplate reader at a wavelength of 540 nm was used to read the absorbance and the percentage of growth inhibition was calculated using the formula. CTC_{50} (cell cytotoxicity), the concentration of test drug needed to inhibit the cell growth by 50% is generated from the dose-response curves for test samples.

% Growth Inhibition = 100 – (Mean OD of individual test group / Mean OD of the control group) × 100

Determination hepatoprotective activity

The protocol was followed as mentioned for the MTT assay. Along with 50 µL of different non-toxic test concentrations, 50 µL of DMEM and 1% CCl_4 toxicant was added. The absorbance was measured using a microplate reader at a wavelength of 540 nm.[31] The percentage of cell viability was determined, based on which the percentage protection offered by GUE, pure compound and standard drugs was calculated over the DMSO control.

% Viability = OD of the Test sample / OD of the Control × 100

In silico studies of Gnetol

Proteins selected for the present study are TGF-β (transforming growth factor-β), which plays a major role in liver fibrosis and peroxisome proliferator-activated receptor α (PPARα), is a ligand-activated transcription factor involved in liver homeostasis and other metabolic functions.

The crystal structure of the target was obtained (TGF-β1 and PPARα), from Protein Data Bank (PDB ID; 1VJY and 5HYK respectively. Structures of phytoconstituent, Gnetol were drawn and analyzed using ChemDraw Ultra 12.0. The 3D coordinates were obtained by using PRODRG online server.[32] Active pockets for proteins were obtained from the CASTp server.[33] ADT (AutoDock Tools), Graphical User Interface program was used for energy minimization, while protein and ligands preparation and grid box creation were completed using Graphical User Interface program AutoDock Tools (ADT). AutoDock saved the prepared file in PDBQT format. AutoDock/ Vina was employed for docking using protein and ligand information along with grid box properties in the configuration file. (Grid size for TGF-β1 was centre_ x =8.549, centre_ Y=63.166, centre_ Z=14.79, Size_ X=22.0, Size_ Y=20.0, Size_ Z=20.0. Grid size of PPARα was centre_ x =12.045, centre_ Y=27.605, centre_ Z=21.024, Size_ X=24.0, Size_ Y=30.0, Size_ Z=30.0). AutoDock/ Vina employs iterated local search global optimizer.[34,35] Throughout the docking procedure, both the protein and ligands were considered as rigid. The results of less than 1.0 Å in positional root-mean-square deviation (RMSD) were clustered together and represented by the result with the most favorable free energy of binding. The pose with the lowest energy of binding or binding affinity was extracted and aligned with receptor structure for further analysis.[36,37]

Results

Extraction and isolation of phytoconstituents from G. ula

Extraction of the stem of *G. ula* with petroleum ether, chloroform, and ethanol yielded 0.96%, 2.24%, and 4.45% respectively. Defatting was done with petroleum ether first and in order to concentrate polar compounds further extracted successively with chloroform and ethanol. Ethanol extract (GUE) was further macerated to get an ethanolic fraction of *G. ula*, studied for its active constituent present and hepatoprotective nature.

Column chromatography

Ethanolic fraction subjected to a column resulted in different column fractions from I to IX (Table 1), which were collected separately and concentrated by using a rotary evaporator under the vacuum. Further, concentrated fractions were tested for antioxidant activity.

Table 1. Column fractions of ethanol extract of *G. ula*

Elutions	No of fractions collected from each elution	Major fractions number
Hexane – 100%	5	I
Hexane: Ethyl acetate (98:2)	10	II
Hexane: Ethyl acetate (96:4)	8	III
Hexane: Ethyl acetate (94:6)	12	IV
Hexane: Ethyl acetate (92:8)	10	V
Hexane: Ethyl acetate (90:10)	14	VI
Hexane: Ethyl acetate (88:12)	12	VII
Hexane: Ethyl acetate (80:20)	19	VIII
Ethyl acetate (100%)	6	IX

In vitro antioxidant activity

Antioxidant potential of GUE and its column fraction of *G. ula* were determined by DPPH radical scavenging assay and reducing power assay. In DPPH radical scavenging assay, GUE and ascorbic acid exhibited antioxidant potential with the IC_{50} values of 16.28 µg/mL and 8.9 µg/ mL. Whereas, the fourth fraction of the column showed better scavenging activity with the IC_{50} of 17.15 µg/mL than all other remaining fractions (Table 2). Reducing the power of standard ascorbic acid and GUE increased with an increase in concentration, while the fourth fraction of the column has a good reducing power than other fractions (Fig. 1). Comparatively, the fourth fraction of the column showed better antioxidant potential, which was further analyzed and checked with TLC.

The fourth fraction was TLC checked with the same mobile phase (mentioned above), to find one major spot. Hence, the fourth fraction was subjected to the crystallization process to get a pure isolate. The fourth fraction of the column was considered for its antioxidant activity and characterized by NMR and Mass spectroscopy.

Characterization of IV fraction

Physical state – Yellow in color, m. p: 270°C.

IR -KBR 3242.23 cm^{-1}, 2986.70 cm^{-1}, 1604.69cm^{-1}, 1019.44cm^{-1} (Fig. 2), **MS m/z** =243(M^{-1}) (Fig. 3).

^{1}H NMR (Fig. 4) (400 MHz, CD_3 OD) δ - 1.14(d, J=0.45Hz, 1H), 6.12(t, J=2.45,2.45 Hz, 1H), 6.31(d, J=2.52 Hz, 2H), 6.45(d, J=2.58 Hz , 2H), 6.81(t, J=2.73,2.72 Hz, 1H), 7.41(d d, J=2.98, 2.95 Hz, 2H).

Table 2. IC_{50} values of GUE and column fractions for DPPH assay

Test samples	IC_{50} µg/mL
Ascorbic acid	8.9±0.12
GUE	16.28±0.24
I	Nil
II	<250
III	234.35±0.5
IV	17.15±0.09
V	218.85±0.67
VI	<250
VII	24.48±0.44
VIII	>250
IX	>250

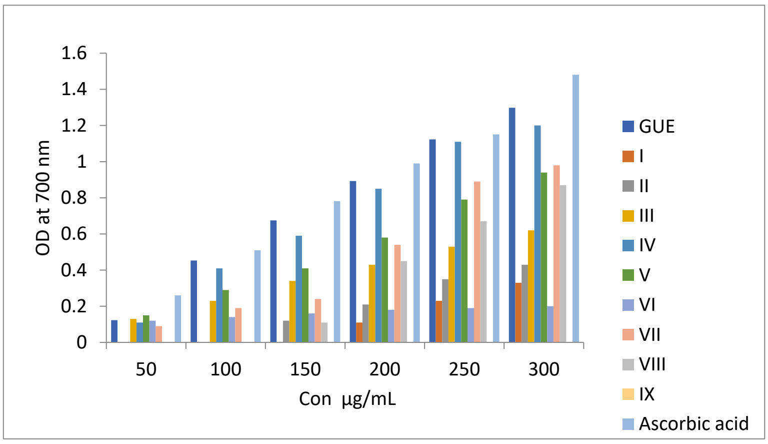

Fig. 1. Reducing power assay for GUE and Column fractions.

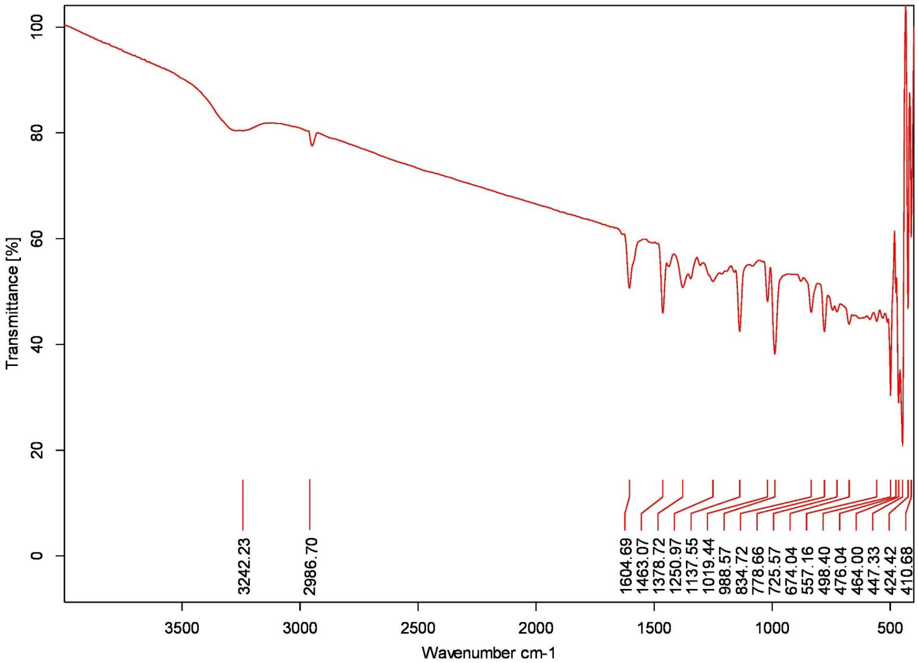

Fig. 2. IR studies of Gnetol compound.

[13]CNMR (Fig. 5) (100MHz, CD_3 OD)**;** δ =25.24, 102.29, 105.72, 107.94, 113.35, 121.71, 128.78, 132.10, 143.18, 158.03, 159.52.

[1]H NMR and **[13]C NMR** along the IR, mass studies, the compound was interpreted as gnetol with molecular formula- $C_{14}H_{12}O_4$. It is a polyphenol - 2,3', 5',6-tetrahydroxy-trans –stilbene (Fig. 6.).

Determination of cell viability by MTT assay

Cytotoxicity assay was performed for the GUE and its isolated compound gnetol on the BRL3A cell line. Concentration ranging from 62.5-1000 µg/mL was tested, which revealed that CTC_{50}% cytotoxic concentration was more than 1000 µg/mL. Table 3 shows the cytotoxic property of GUE and gnetol.

In vitro hepatoprotective activity of GUE and Gnetol on BRL3A cell line

The non-toxic dose of GUE and gnetol was tested for hepatoprotective function in CCl_4 induced BRL3A cell line. Standard silymarin at 200 µg/mL showed 77% protection, whereas GUE and gnetol at 200 µg/mL significantly offered the highest protection of 50.2% and 54.3% respectively against the toxicant. A lower dose of GUE and gnetol (50 µg/mL) also shielded the cell line from the toxic effects of CCl_4. Overall the test samples showed the protection, dose-dependently (Table 4).

In silico studies of Gnetol

After being tested on the cell line, the isolated compounds

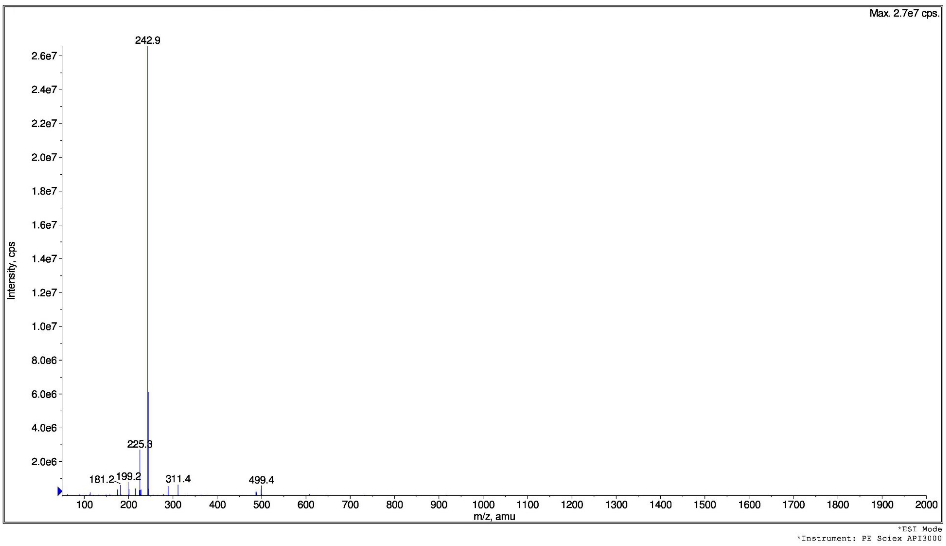

Fig. 3. Mass studies of Gnetol compound.

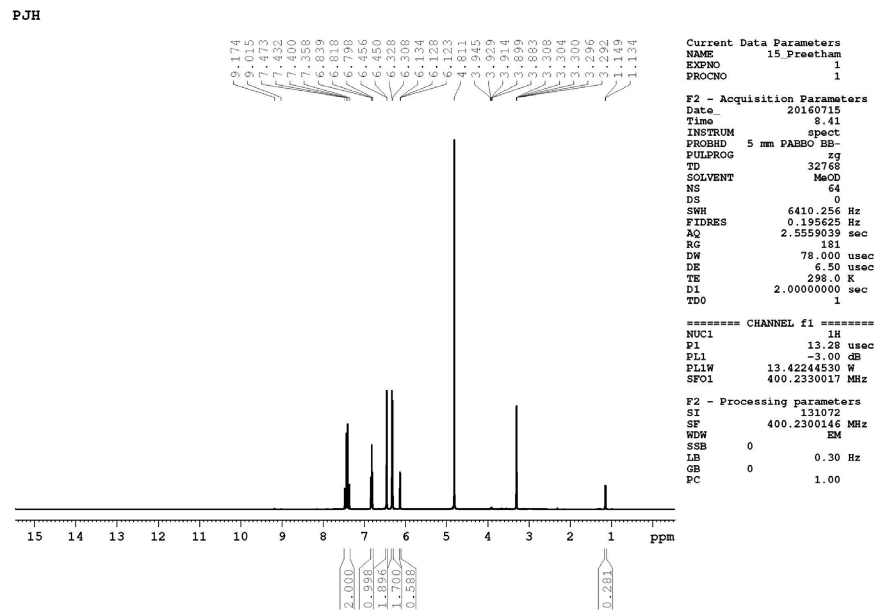

Fig. 4. ^{1}H NMR data of gnetol compound.

were further considered for *in silico* docking studies. The results of molecular docking assess the quality and energy binding of the structures with the molecules of bio targets. The results obtained were related to the standard, silymarin, and present in Table 5.

The docking results have proven that the ligand molecule, gnetol showed the binding affinity of -7.0 and standard silymarin being -6.8. Hydrogen bonding's of 2 and good hydrophobic interactions against the amino acid molecules in relation to the TGF -β protein.

In the case of PPARα protein, gnetol showed the binding affinity of -8.4 and silymarin with -6.5. Hydrogen bonding and good hydrophobic interactions against the amino acid molecules in relation to the PPARα protein are mentioned in Table 5.

Docking studies of TGF- β are presented in Fig. 7, which showed the Ligplot analysis and docking results showing the crystal structure of TGF-β with ligand Silymarin (A) and ligand gnetol (B). Hydrophobic interactions are found in the proteins active pocket (Glu247, Phe243, Ile367, Arg244, Gly353, Leu354, Val373, Phe216, Glu238, Arg237). TGF-β formed two hydrogen bonds with the amino acids Arg 240 and Ser 241. Ligplot analysis and docking results showed the crystal structure of PPARα with ligand Silymarin (A) and ligand gnetol (B) presented in Fig. 8. Silymarin was able to interact with Cys275, Ser280, Thr283 to form 3 hydrogen bonds. Gnetol with Tyr464, His440, Tyr314 formed 3 hydrogen bonds.

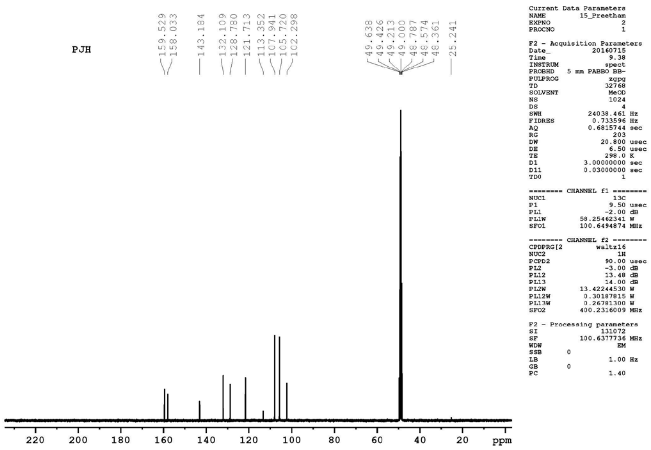

Fig. 5. ^{13}C NMR data of gnetol structure.

From this study, it is predicted that the inhibitory efficiency of the gnetol is more than the standard drug Silymarin. In the case of PPARα, activation efficacy of the ligand gnetol is more effective than the standard used. From the present study, it reveals that gnetol protects the liver in disease conditions.

Discussion

In the present study, *in vitro* antioxidant, cytotoxicity and hepatoprotective activity of ethanol extract and isolated compound gnetol of *G. ula* were evaluated. Reactive oxygen species (ROS) and free radicals are continuously generated with the exposure of endogenous and exogenous factors. They play a very important role in the pathogenesis of many disorders, wherein it also affects the normal functioning of the liver. Antioxidants from natural products detoxify the toxins, scavenges the free radicals, removes excess ROS and anti-lipid peroxidizes.[38] Many studies have been conducted on traditional medicine in order to develop new drugs as an antioxidant and to treat hepatic disease.[39]

Ethanol extract of *G. ula* has phytoconstituents viz phenols and flavonoids. Total phenol and total flavonoid content were found to be more in ethanol extract than other extracts.[40] In the present findings, *in vitro* antioxidant activity of GUE and different fractions could be credited to the presence of phenolic and flavonoids compounds, which were reported to have hepatoprotective activity.[41-44]

Bioactivity-guided isolation of a phytoconstituent gnetol from GUE has been evaluated for its antihepatotoxic activity. GUE and its isolate gnetol showed protective in function against the CCl_4 toxicant. Gnetol has been isolated in many species of *Gnetum* genus. Apart from *G. ula*,[22] it has also been isolated from *Gnetum gnemon*,[26] *Gnetum montanum*,[24] *Gnetum hainanense*[45] and *Gnetum klossii*.[46] It is a polyphenol compound belongs to the stilbenes family. Generally, phenolic structures have very good antioxidant potential as hydrogen donors, reacting with oxygen and nitrogen species, chelating metal ions. This group

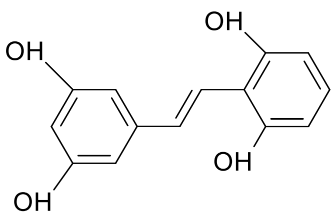

Fig. 6. Structure of Gnetol.

Table 3. Cytotoxic property of test drug GUE and Gnetol on BRL3A cell line by MTT assay

Con µg/mL	GUE (% of inhibition)	Gnetol (% of inhibition)
62.5	12.4±2.2	2.8±1.1
125	17.7±0.9	5.3±1.4
250	27.1±0.9	8.6±2.49
500	38.9±3.1	9.7±2.07
1000	48.4±1.3	14.4±1.9
CTC_{50}(µg/mL)	>1000	>1000

GUE, ethanol extract of *G. ula*

Table 4. Hepatoprotective effects of GUE and gnetol in CCl_4 induced BRL3A cell line

Test Drug	Test concentration	% Viability
DMSO Control	0.25%	97.1±0.21
Silymarin+CCl_4	200 µg/mL	77.15±1.7
CCl_4	1%	9.7±0.38
GUE+CCl_4	50 µg/mL	27.3±0.5
	100 µg/mL	31.5±1.1 [b]
	200 µg/mL	50.2±0 [a b]
Gnetol+ CCl_4	50 µg/mL	15.3±0.43
	100 µg/mL	36.3±0.8 [ab]
	200 µg/mL	54.39±1.21 [ab]

Values are expressed as mean ±SEM; n=3.
[a] Significance level: P <0.05, compared to DMSO control.
[b] Significance level: P<0.05, compared to CCl_4 intoxicated.

Table 5. Molecular docking interactions of Gnetol compound with Target protein TGF -β and PPARα

Ligands	Target protein	Affinity (kcal/ mol)	No. Hydrogen bonds	Hydrogen bond length (å)	Hydrogen bond with amino acids	Hydrophobic interactions with amino acids
Silymarin	TGF -β	- 6.8	2	3.17 3.18	Arg240 Ser241	Glu247, Phe243, Ile367, Arg244, Gly353, Leu354, Val373, Phe216, Glu238, Arg237
Gnetol	TGF -β	- 7.0	2	2.81 2.82	Tyr249 Asp351	Leu260, Leu278, Lys232, Val219, Leu340, Lys213, Gly212, Ala350, Glu245
Silymarin	PPARα	- 6.5	3	3.29 2.94 3.13	Cys275 Ser280 Thr283	Cys276, Met355, Phe318, Leu321, Met330, Ile317, Met220, Phe218, Glu286, Val324, Met320, Ala333, Val332, Thr279, Leu331
Gnetol	TGF -β	- 8.4	3	2.82 2.99 3.12	Tyr464 His440 Tyr314	Cys276, Leu321, Met330, Glu282, Tyr334, Thr283, Val324, met320, Ser323, Val332, Thr279

of compounds having the capacity to inhibit enzymes involved in radical generation such as cytochrome P_{450} isoforms, lipoxygenases, cyclooxygenases etc.[47,48] Gnetol is a positional isomer of oxyresveratrol hinting many pharmacological activities.[49-51] In this present study, the antioxidant activity of GUE and Gnetol on DPPH radical scavenging and reducing power may be attributed to the capacity in trapping free radicals by donating electrons or hydrogen atoms.

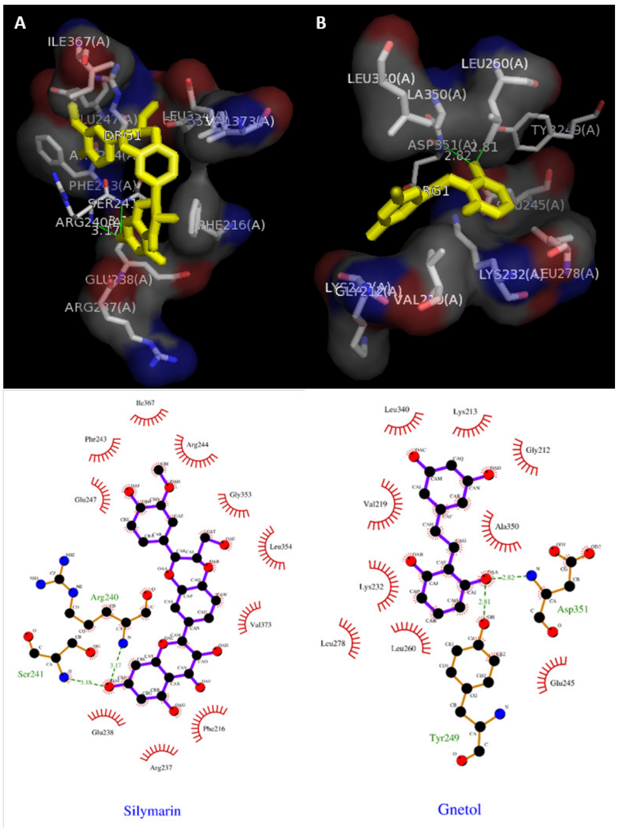

Fig. 7. Ligplot analysis and docking results. (A) The crystal structure of TGF-β with ligand Silymarin as the (standard drug). (B) The crystal structure of TGF-β with the ligand gnetol.

A study on the herbal drug becomes more important when it ameliorates any disease conditions.[43] *In vitro* cytotoxicity and hepatoprotective activity of traditionally used herbal plants have become important for primary level screening. BRL3A cell line was considered for this study which shows similar functioning to rat liver cells. MTT, tetrazolium dye is used widely to assess the cell viability and also used to determine non-toxic doses for GUE and gnetol for the hepatoprotective studies. The CTC_{50} values for GUE and gnetol found to be above 1000 µg/mL, so the concentration used for the study was found to be nontoxic for the BRL 3A cell line.

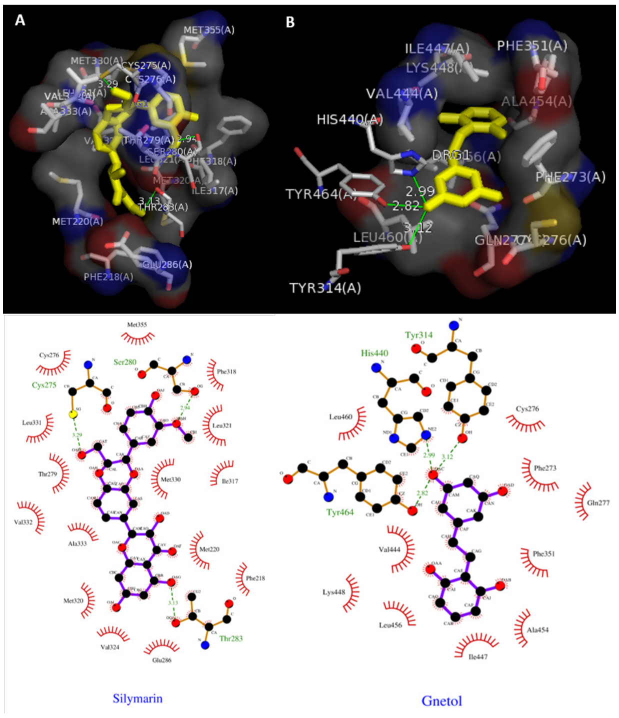

Fig. 8. Ligplot analysis and docking results. (A) The crystal structure of PPARα with ligand Silymarin as the standard drug. (B) The crystal structure of PPARα with the ligand gnetol.

Liver damage induced by CCl_4 is the best system of xenobiotic induced hepatotoxicity.[52-53] CCl_4 mediated hepatoxicity is a reliable studied model, variations associated with CCl_4 –induced liver injury is similar to that of acute viral hepatitis.[54] The CCl_4 gets accumulated in the parenchymal cells and metabolically activated by cytochrome P_{450}-dependent monooxygenases to form trichloromethyl radical CCl_3 and trichloromethyl peroxyl free radical ($CCl_3O_2^o$), further leading to increased liver damage. Lipid peroxidation of the cell membrane produces an MDA metabolite (malondialdehyde) used as an indicator of cell damage.[55] The level of MDA might have reduced by GUE and gnetol which suggests the curative activities against liver damage. GUE and gnetol at a concentration of 200 μg/mL were able to protect the cells, otherwise damaged by MDA. Both, GUE and gnetol showed their protective function of the liver in a dose-dependent manner. This may be due to the antioxidant potency of the GUE and Gnetol as antioxidants are the basis for inhibiting carbon tetrachloride-induced liver damage.

Researchers explore the herbal products and discovering the novel compounds for hepatoprotection. But only a few are targeted for hepatoprotective genes/proteins. Interaction studies of the drugs for activation of proteins or inhibition of the proteins are still a major lacuna, which essentially now an important criterion in the development of a new hepatoprotective drug.

In silico studies conducted for the isolated compound gnetol against an inhibitory protein TGF-β and activator protein, PPARα showed that gnetol is having a reliably good interaction with the proteins. TGF-β plays a significant function in chronic liver diseases, regulating in all stages of liver diseases.[56] Gnetol was able to inhibit the protein TGF-β and its interaction towards TGF-β was more than silymarin. Thus, targeting this protein in particular cell at the right time helps to achieve a therapeutic effect on liver problems. PPARα in the ligand-activated protein found in the liver helps in various metabolic issues.[57] Activation of PPARα is a benefit for treating metabolic disorders. Compare to silymarin, gnetol proved its efficacy through the interaction with PPARα.

In this postgenomic era, there are more prospects for active phytoconstituent from herbs, while traditional medicine helps to discover new drugs towards dreadful diseases. The success rate for the development of new synthetic drugs is one in ten thousand, whereas for the new medicinal phytoconstituent from traditionally used plants can be as high as one fourth or still more.[58] In this regard, we have justified the traditional usage of *G. ula* by using *in vitro* and *in silico* approaches to bring out a more reliable drug for hepatoprotection.

Conclusion

Our findings provide evidence that the ethanolic extract and its isolated compound gnetol from *G. ula* exhibit antioxidant and hepatoprotective activity. This study supports the usage of stem extract of *G. ula* by various tribes for the treatment of liver disorders. Detailed *in vivo* studies on liver protection activity are in progress to support the data obtained.

Research Highlights

What is the current knowledge?

√ The research on plants for treating liver diseases are continuously evaluated, where multiple herbs work synergistically in polyherbal formulations and active component responsible for liver treatment remains unknown.

What is new here?

√ In the present study, active compound Gnetol has been isolated and tested for antioxidant and hepatoprotective activity with supporting evidence of docking studies for two important proteins related liver diseased condition.

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Competing interests

No potential conflict of interests was reported by the authors.

Ethical Statement

There is none to be declared.

Authors' contributions

PJ: The data collection, contribution to data analysis and drafting the manuscript. SR and KSRR: Designing the experiment and data analysis, interpretation and critical revision. SGPS: Supporting for conduction of experiments, interpretation of data and revision. SS: supporting for computational studies and analysis.

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