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In-Vitro antioxidant, anti-lipid peroxidative activities and *In-Silico* study of *Terminalia chebula* bioactive compounds

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Abstract

Objective: To evaluate the antioxidant activities and to identify the bioactive compounds in hot water extracts of *Terminalia chebula* fruit.

Methods: The antioxidant activities were determined by DPPH assay, lipid peroxidation assay, iron chelation and total antioxidant assay. The phenolic composition was determined by HPLC-DAD. Human Rab8b Protein was used for the validation of compounds as anti-inflammation. String analysis for protein synergism was used.

Results: The analysis of *Terminalia chebula* Retzius (Combretaceae) phenolics showed anti-inflammatory effect. The specific phenolic compositions were determined by high performance liquid chromatography (HPLC) and resulted in the identification of rutin, catechin, caffeic acid, gallicacid, ellagic acid, epicatechin, and quercetin as antioxidant compounds. Human Rab8b protein is selected for protein docking and all compounds except rutin showed good results. ADMET properties were checked by using AdmetSar and all seven compounds showed validation for AMET properties. The synergisms of compounds were analyzed by STRING analysis and our ligands shows strong binding with human Rab8b proteins. The aqueous extract was capable of inhibiting the lipid peroxidation in egg yolk phospholipid homogenate. The extract scavenged the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) (IC_{50},71.5 ± 2.1 μg/ml). The extract displayed the high metal chelation activities and reducing abilities on the phosphomolybdenum assay.

Conclusions: It is concluded that extracts of *T. chebula* have good antioxidant and anti-inflammation activities and are rich in phenolics.

Keywords: *Terminalia chebula*, Protein docking, Lipid peroxidation, STRING analysis, Metal chelation, HPLC analysis

Introduction

Biomolecules such as DNA, lipids, proteins and RNA are damaged by oxidative stress, in human body that results in lipid peroxidation, injury to cells, impairment of tissues and gene mutation. Aging is due to free radicals and free radical cause different diseases like cardiovascular disorders, cancer, neurodegenerative diseases, inflammation [1]. In addition, lipid peroxidation caused by free radicals results in spoilage of food during processing and storage [2]. Currently interest in antioxidant compounds has increased because they play essential role in health and diseases and also have nutritive value.

Terminalia chebula Retzius (Combretaceae), is an important medicinal plant which exhibits many medicinal activities due to its rich phytochemical composition. The plant's crown is round and its branches are spread, its bark is dark brown in color and has cracks along its length. The leaves are elliptical and the petiole's top is occupied with two large

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glands at its tip. The fruit size is approximately 1–2 in. The fruit of the plant has five lines present on its skin and has shown protective effect against liver damage induced by CCl_4 and tert-butyl hydroperoxide [3]. The fruits also display cytoprotective [4], antidiabetic [5], antioxidant [6], antibacterial [7], anti-arthritic [8], hypo-cholesterolaemic [9] and anti-inflammatory activities [10]. Chebulanin, chebulinic acid, 1,6-di-O-galloyl-b-D-glucose and Casuarinin were isolated from *T. chebula* and showed significant antioxidant activity [11]. The fruits of *Terminalia chebula* are added to salads and are used in food preserves [12].

The search of literature has shown that there is less information on antioxidant and phytochemical analysis of hot water extracts of *Terminalia chebula*. Moreover, hot water extracts are traditionally used in preparation of teas from fruit of *Terminalia chebula*. The antioxidant activities of plants are different when different prooxidants are used. In this study, the iron and sodium nitroprusside are used to induce lipid peroxidation in phospholipid homogenate and the antioxidant effect of aqueous extract was studied. Hence, this study was aimed to determine the composition of phenolics by HPLC and in vitro antioxidant activities of aqueous extract using different assays.

Materials and methods

Chemicals

Standards used in HPLC analysis were purchased from Sigma Aldrich. Iron, sodium nitroprusside, DPPH, ammonium molybdate and 1,10-phenanthroline were purchased from Biochemicals (Lahore, Pakistan).

Preparation of fruit extract

The fruits of plant were locally purchased, identified by a botanist and a voucher specimen was deposited at the Herbarium of University of Poonch, Department of Botany (Ref. No. BOT/2017/51).

Finely grounded fruit material of the plant (25 g) was placed for 15 min in boiling water (500 ml) was cooled and filtered with filter paper No. 1 (Pore size, 11 μM). The solvent was evaporated by rotary evaporator (45 °C) producing 3 g (12% w/w) extract.

In vitro lipid peroxidation assay

The anti-lipid peroxidative properties of aqueous extracts were studied by a method [13]. In brief the egg yolk was weighed to 1 g and diluted to 100 ml with 100 mM Tris-HCl, pH 7.4 and used as homogenate. The homogenate was incubated with Fe (II) or sodium nitroprussside with or without the extract and colour reaction was carried out by adding 600 μl of TBA and 600 μl of acetic acid (pH 3.4) for 1 h. The tubes were cooled and 2 ml of n-butanol was finally added and centrifuged. The absorbance was read at spectrophotometer at 532 nm.

DPPH radical scavenging activity

The scavenging of the DPPH radical was reported by the method [14]. Briefly, a 0.25 mM solution of the DPPH radical (0.5 mL) was added to a sample solution in ethanol (1 mL) at different concentrations (25–400 μg/mL) of the aqueous extracts. The mixture was shaken vigorously and left to stand for 30 min in the dark, then the absorbance was measured at 517 nm. The capacity to scavenge the DPPH radical was calculated using the equation:

$$(\%)\ \text{scavenging} = [(\text{AoA1})/\text{Ao})] \times 100$$

Where, Ao is the absorbance of the control reaction and A1 is the absorbance of the sample.

Metal chelating activity

The iron chelating ability of the aqueous extract was studied by the method [15]. Briefly 150 μL of freshly formed 2 mM $FeSO_4{\cdot}7H_2O$ was added in a mixture which have 168 μL of the 0.1 M tris HCl (pH 7.4), (218 μL) of saline and (25–200 μL/ml) concentration of plant extracts. The mixture of sample was incubate for 5 min before addition of 13 μL of 0.25% 1,10-phenanthroline (w/v). Absorbance was checked at 510 nm in spectrophotometer.

Antioxidant potential assay

The reducing ability of the aqueous extract was followed by phosphomolybdenum method [16]. The

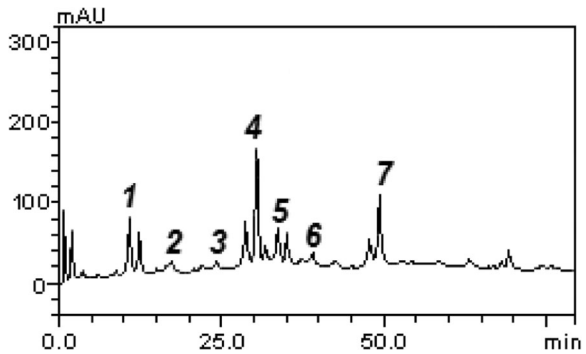

Fig. 1 Representative high performance liquid chromatography profile of *Terminalia chebula* fruit. Gallic acid (peak 1), catechin (peak 2), caffeic acid (peak 3), ellagic acid (peak 4), epicatechin (peak 5), rutin (peak 6) and quercetin (peak 7)

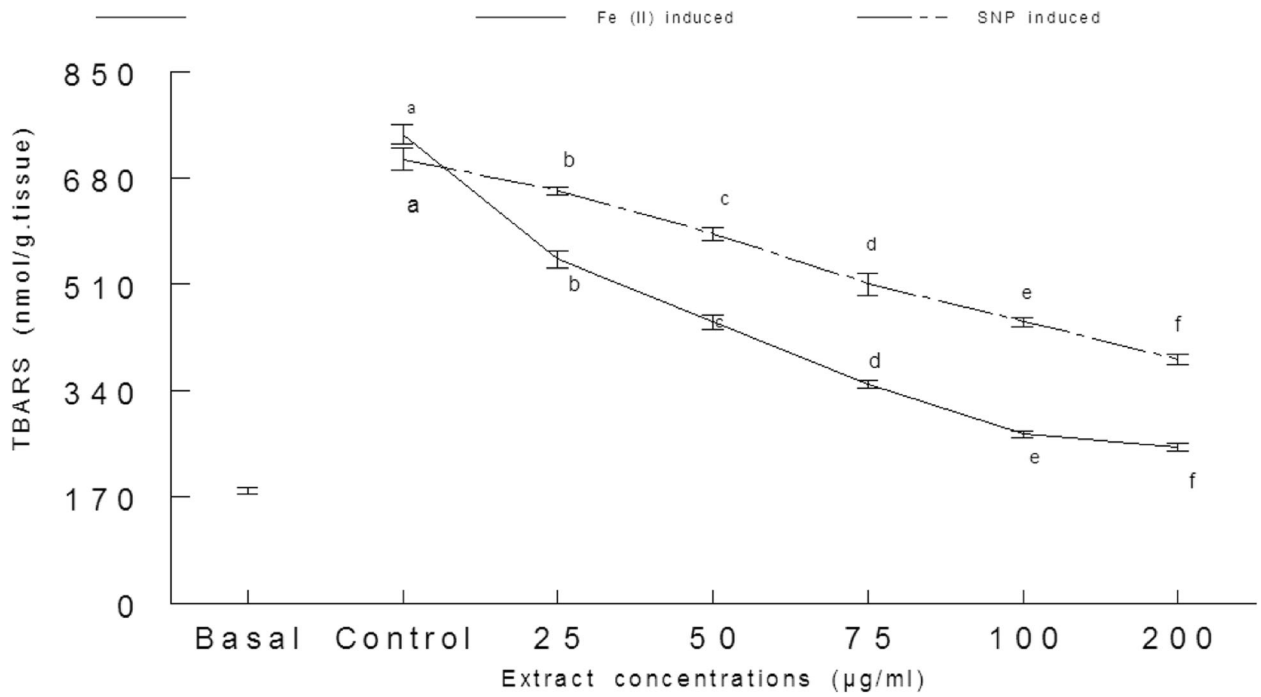

Fig. 2 Inhibitory effect of *Terminalia chebula* on lipid peroxidation induced by 10 μM Fe (II) and 5 μM sodium nitroprusside in egg yolk. Values represent the means of three separate experiments in duplicate ± SD. $p < 0.05$ is significantly different from control by DMRT. Values in figures which share different letters are significantly ($p < 0.05$) different from each other by DMRT

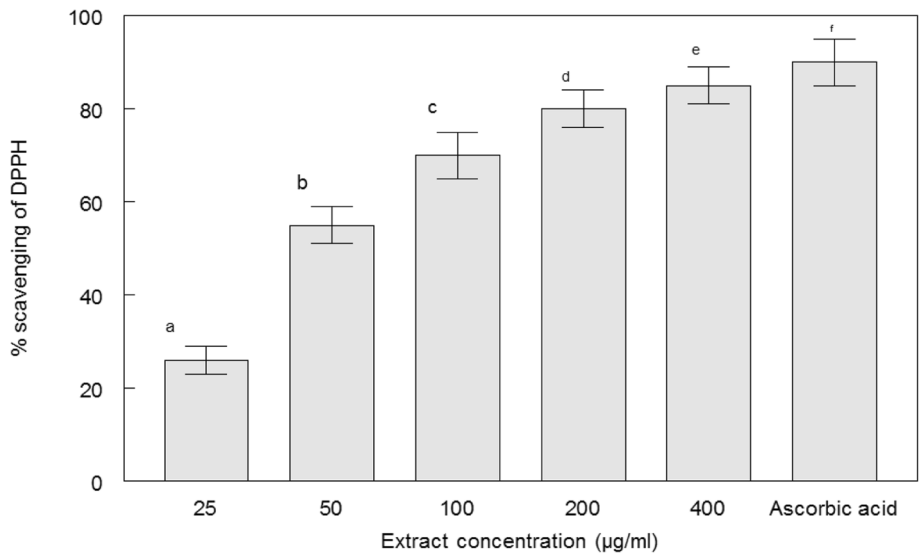

Fig. 3 DPPH radical scavenging activity of aqueous extract obtained from fruit of *Terminalia chebula*. Ascorbic acid at concentration of 100 μg/ml. Values are means±SD ($n = 3$). Values in figures which share different letters are significantly ($p < 0.05$) different from each other by DMRT

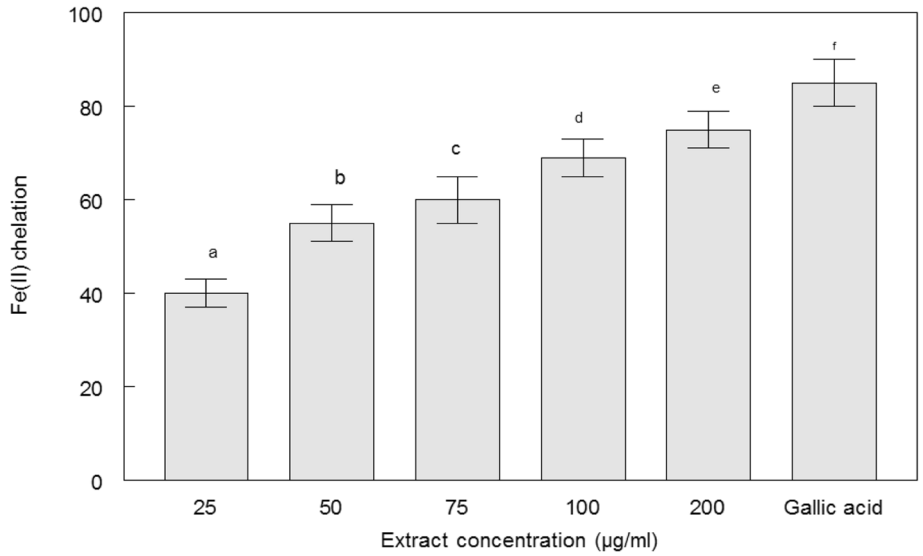

Fig. 4 Iron chelating abilities of *Terminalia chebula* fruit extract. Gallic acid at concentration of 50 µg/ml. Values are means±SD ($n = 3$). Values in figures which share different letters are significantly ($p < 0.05$) different from each other by DMRT

results were expressed as ascorbic acid equivalent. The assay was based on the reduction of molybdenum, Mo (VI)–Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acidic pH. The extract (0.1 mg/ml) was mixed with 3 ml of the reagent solution (0.6 M H2SO4, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated at 95 C for 90 min. The mixture was cooled to room temperature and the absorbance of the solution was measured at 695 nm.

HPLC analysis of phenolics and flavonoids

T. chebula aqueous extract (1 mg/mL) was dissolved in HPLC grade methanol filtered and subjected for analysis by Shimadzu HPLC system as reported by Khaliq et al., [13].

Molecular docking

Human Rab8b Protein was used for the validation of compounds as anti-inflammation. The 3D structures of protein were downloaded from RCSB database. For docking PyRxvina docks tool were used.

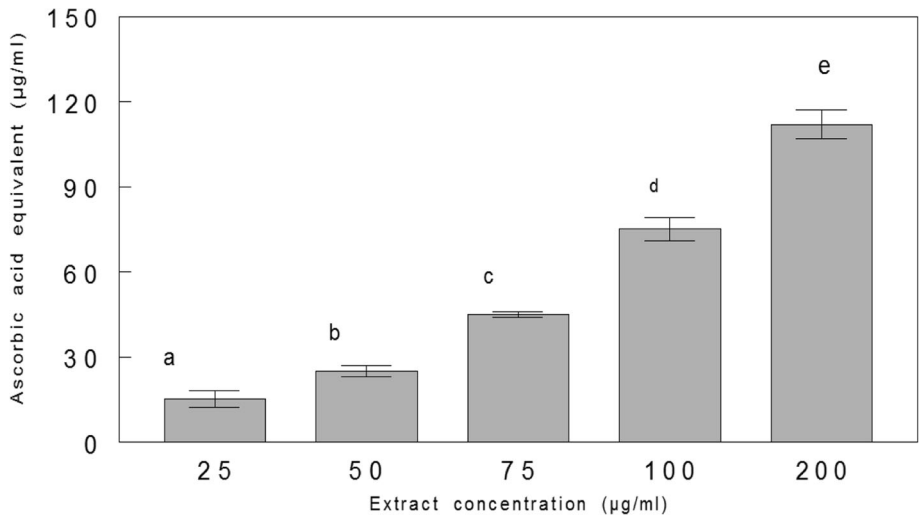

Fig. 5 Total antioxidant activity of *Terminalia chebula* fruit extract. Values are means±SD ($n = 3$). Values in figures which share different letters are significantly ($p < 0.05$) different from each other by DMRT

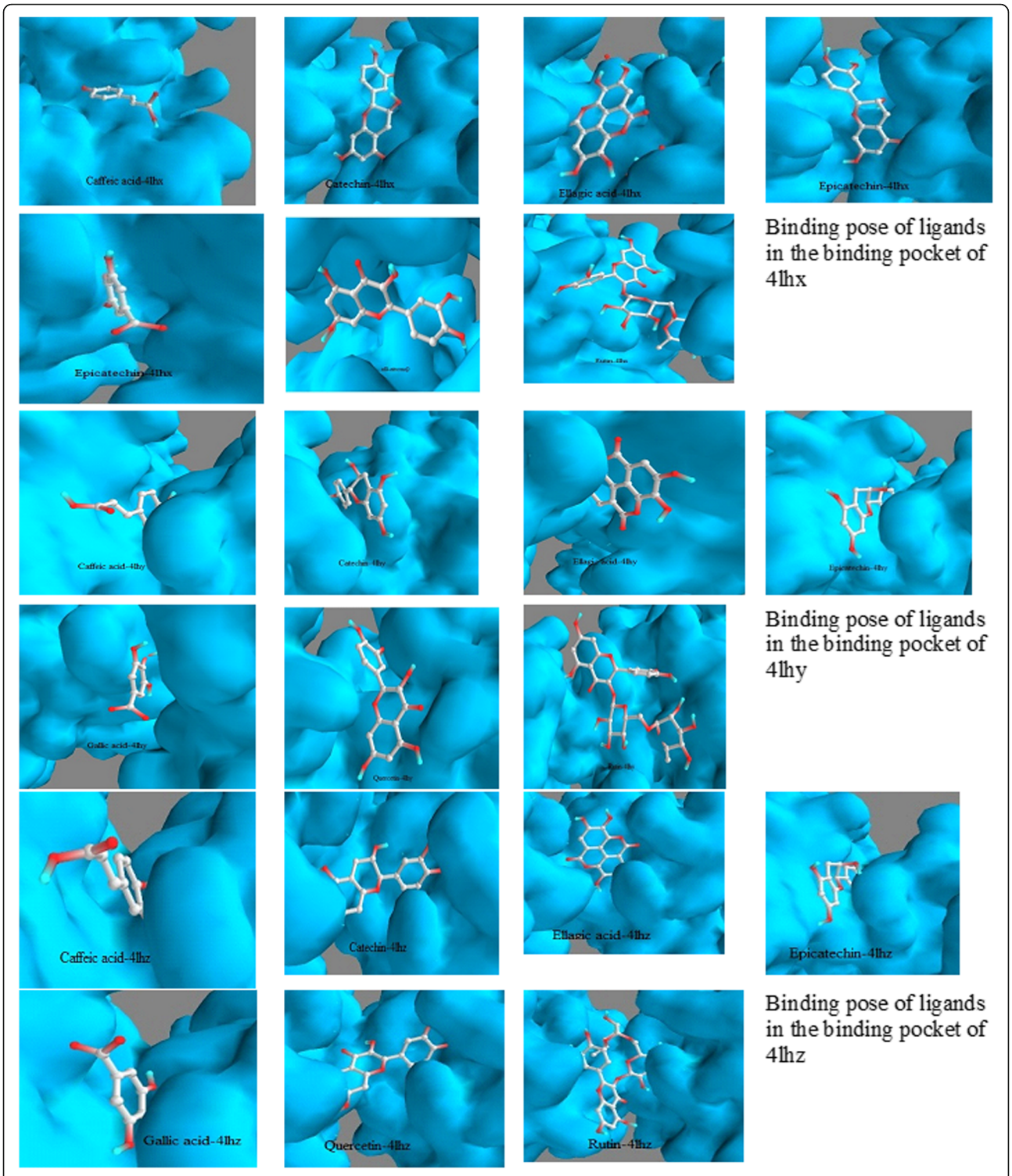

Fig. 6 Binding poses of the Ligands in the binding pockets of human Rab8b proteins with a surface view of the proteins

Table 1 Affinity score of ligand with Human Rab8b proteins

Ligand	4lhx	4lhy	4lhz
Caffic Acid	−5.8	− 5.7	−5.8
Catechin	−6.4	− 6.7	−6.9
Ellagic Acid	−7.2	−7.3	−7.6
Epicatechin	−6.6	−6.6	− 6.7
Gallic Acid	−5.5	− 5.5	−5.5
Quercetin	−6.6	−6.7	− 6.6
Rutin	−7.1	− 7.1	−8.1

Synergism of compounds

String is a free online software to analysis protein interactions. To check the synergism between compounds relevant protein of the compounds were analyzed.

Data analysis

The results were expressed as means± SD. The obtained data was analyzed by one way ANOVA and different group means were compared by Duncan Multiple Range Test (DMRT) wherever necessary; $p < 0.05$ was considered significant in all cases. Statistica (version 4.5; StatSoft Inc., Tulsa, OK, USA) was used as software package.

Results

In HPLC chromatogram of *Terminalia chebula* fruit extract the peak of gallic acid appeared at retention time of 10.18 min (peak 1), catechin at 16.53 min (peak 2), caffeic acid at 24.09 min (peak 3), ellagic acid at 30.15(peak 4), epicatechin at 33.78 min (peak 5), rutin at 39.04 min (peak 6) and quercetin at 49.56 min respectively (Fig. 1).

Lipid peroxidation in phopholipid homogenate was stimulated when iron and SNP were used as prooxidant. The Thiobarbituric acid reactive species (TBARS) production was enhanced to 79% in phospholipid homogenate compared to the basal or normal (Fig. 2). However, treatment with *Terminalia chebula* shunted the lipid peroxidation in control treatments compared to the control.

The DPPH activity of *T. chebula* is shown in Fig. 3. The activity was the highest at 200 μg/mL, with an IC_{50} value of 45.5 ± 2.1 μg/mL ($r^2 = 0.971$). The vitamin C showed an IC_{50} value 31 ± 1.1 μg/mL ($r^2 = 0.97$). The extract was effective in chelating iron from the mixture (Fig. 4). The chelating ability was increased when concentration was raised and comparable to the standard gallic acid. The phosphomolybenum assay is an indirect method which measures the total antioxidant activities. In the phosphomolybdenum assay, the extract showed their ability to donate electrons and showed an antioxidant activity of 111 ± 2.5 μg/ml as ascorbic acid equivalent at a maximal concentration (200 μg/mL) (Fig. 5). Molecular docking was done by using PyRx software and the given results were shown in Table 1 and pose of ligand on Human protein Rab8b were showed in (Fig. 6). All AMET characters were checked by using AdmetSar and results are showed in Tables 1, 2, 3. All compound showed good results for Lipinski rules (Table 3). The synergism of all compounds were analyzed by using STRING analysis (Fig. 7).

Discussion

Some important natural products such aschebulic acid, chebulagic acid, corilagin, mannitol, gallic acid, ellagic acid, tannic acid, ethyl gallate, and ascorbic acid were detected in the extracts of *T. chebula* [17]. *T. chebula* was rich in tannins (32%) [18]. Here the HPLC analysis has revealed the presence of gallic acid (4.97 ± 0.01 mg/g), catechin (0.83 ± 0.03 mg/g), caffeic acid (0.56 ± 0.04 mg/g), ellagic acid (9.15 ± 0.01 mg/g), epicatechin (2.74 ± 0.02 mg/g), rutin (0.80 ± 0.05 mg/g) and quercetin (6.03 ± 0.03 mg/g) in aqueous extract of *Terminalia chebula* fruit (Table 4). Plants are rich in phenolic compounds which inhibit the lipid peroxidation by neutralizing the free radical species [19, 20].

This study focused on the anti-lipid peroxidative properties of *T. chebula* in egg yolk phospholipid. Iron stimulated the lipid peroxidation as it can generate one electron transfer reaction and increases the

Table 2 Predicted Absorption of seven Ligands

	Caffeic acid	Catechin	Ellagic acid	Epicatechin	Gallic acid	Quercetin	Rutin
Blood-Brain Barrier	BBB-	BBB-	BBB+	BBB-	BBB-	BBB-	BBB-
Human Intestinal Absorption	HIA+	HIA+	HIA+	HIA+	HIA+	HIA+	HIA+
Caco-2 Permeability	Caco2+	Caco2-	Caco2-	Caco2-	Caco2-	Caco2-	Caco2-
P-glycoprotein Substrate	Non-substrate	Substrate	Substrate	Substrate	Non-substrate	Substrate	Substrate
P-glycoprotein Inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
Renal Organic Cation Transporter	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor

Table 3 Lipinski rule

	MW	AlogP	Hdon	Hacc	OB (%)	Caco-2	BBB	DL	FASA-	TPSA
Caffeic acid	180.17	1.37	3	4	54.97	0.27	0.11	0.05	0	77.76
Catechin	290.29	1.92	5	6	54.83	−0.03	−0.73	0.24	0	110.38
ellagic acid	302.2	1.48	4	8	43.06	−0.44	−1.41	0.43	0.43	141.34
epicatechin	290.29	1.92	5	6	48.96	0.02	−0.64	0.24	0.34	110.38
gallic acid	170.13	0.63	4	5	31.69	−0.09	−0.54	0.04	0.41	97.99
Quercetin	302.25	1.5	5	7	46.43	0.05	−0.77	0.28	0.38	131.36
Rutin	610.57	−1.45	10	16	3.2	−1.93	−2.75	0.68	0	269.43

production of reactive oxygen species. The overload of Iron results in the implication of different diseases such as cancer, hepatic, cardiac, brain disorder and neurodegenerative disorders [21]. It is evident from the results (Fig. 2) that the aqueous extract of *T. chebula* are capable of causing significant ($P < 0.05$) inhibition of lipid peroxidation which is partly due to its iron chelating abilities.

Increase in lipid peroxidation is strong indicator of tissues damage due to excess of iron and SNP. In biological system sodium nitroprusside (SNP) decompose to generate nitric oxide (NO^o) radical [22]. The released NO reacts with other reactive oxygen species (ROS) notably superoxide radical to form peroxynitrite radical [23]. *Terminalia chebula* has reduced the lipid peroxidation induced by SNP, as the water extractable phytochemicals of the plant scavenge the NO^o produced by the SNP, thus protecting the phospholipids against oxidative stress [23]. DPPH method is a fast and simple method which is in routine screens the antioxidant activities of plant extracts and synthetic compounds. The DPPH free radical being soluble in ethanol showed reduction on treatment with extract. *T. chebula* showed high percentage scavenging of the DPPH radical.

Table 4 Composition of *Terminalia chebula* fruit

Compounds	Extract	
	mg/g	%
Gallic acid	4.97 ± 0.01 a	0.49
Catechin	0.83 ± 0.03 b	0.08
Caffeic acid	0.56 ± 0.04 c	0.05
Ellagic acid	9.15 ± 0.01 d	0.91
Epicatechin	2.74 ± 0.02 e	0.27
Rutin	0.80 ± 0.05 b	0.08
Quercetin	6.03 ± 0.03 c	0.60

Results are expressed as mean ± standard deviations (SD) of three determinations
Averages followed by different letters differ by Turkey test at $p < 0.01$

Iron chelation assay is indirect method of evaluating the antioxidant activity. O-phenanthroline is a chemical which selectively chelates iron. Chelating agent reacts with O-phenanthroline and thus disrupts the complex formation and thus intensity of the color is decreased in the assay. *T. chebula* extract showed dose dependent scavenging of ferrous ions. Enhanced oxidative stress caused by ferrous ions leads to the many diseases like Alzheimer's syndrome which is a life threatening disease [24]. Naturally plants contain phytochemicals which are responsible for the metal chelation and thus reduce the lipid peroxidation [25].

In phosphomolybdenum method the Mo (VI) is reduced to its less common Mo (V) by the active compounds present in the extract. The absorbance of sample increases compared to the control which is indicative that the extract is capable of donating hydrogen atoms.

To check the strength of ligands (compounds) against inflammation, we used human Rab8b protein as a target for docking. Three proteins were selected i.e. 4lhx, 4lhy and 4lhz. In the molecular docking study, the X-ray structure of human Rab8b proteins were used to dock with our ligands. This regulation is particularly important in immune cells for mounting specialized immune defenses. By controlling the formation, transport and fusion of intracellular organelles, Rab8b serve as master regulators of membrane trafficking. As a result of cellular and molecular mechanisms Rab8b regulate immunity and inflammation.

The results of the PyRx docking showed a docking scores of − 5.5 to − 8.1 kcal/mol against the protein. Our ligands are capable of binding with human Rab8b proteins as well as show affinity toward the surrounding amino acids.

Conclusion

In conclusion, HPLC-DAD method was effectively utilized to determine the phenolic compounds in *T. chebula* fruit. Seven phenolic compounds were

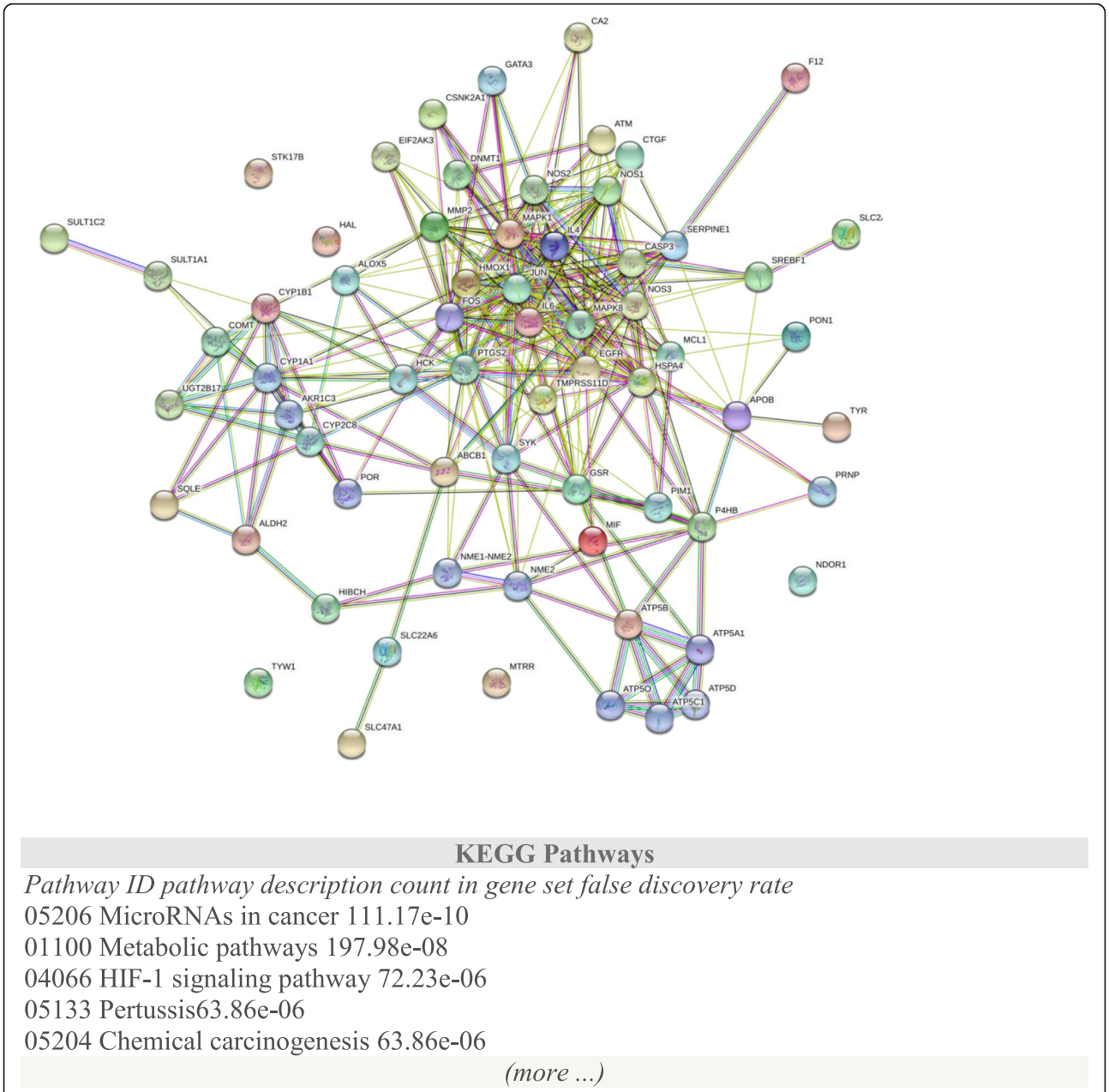

Fig. 7 Network and enrichment analysis showing results obtained upon entering a set of 61 proteins suspected to be involved in Amyotrophic Lateral Sclerosis (55). In the bottom inset, one enriched function has been selected, and the corresponding protein nodes in the network are automatically highlighted in color

identified in the fruit. Crude extracts of *T. chebula* possess different biological activities such as DPPH radical scavenging activity, reducing activities and anti-lipid peroxidative properties. Molecular docking of ligands was shown high affinity for anti-inflammation. All seven compounds showed validation for AMET properties. The synergism of all compounds is shown strength against inflammation. This justifies the use of the fruit in nutrition, industries and medicines. However, more systematic understandings of in vivo and in silico studies and safety evaluation are required.

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Authors' contributions
SM Sabir designed the study and wrote the manuscript. SR Abbas performed the In-silico studies. S Shahida is responsible for organization of the results and manuscript editing. MF Khan performed the statistical analysis. All the authors have read and approved the final manuscript.

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Competing interests
The authors declare that no competing interest involved in the study.

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