Some Chemical Properties and Biological Activity of an Endemic Plant *Tripleurospermum callosum* as a Case Study

Aras, Abdülmelik*[+]

Department of Biochemistry, Faculty of Science and Arts, Iğdır University, Iğdır, TURKEY

ABSTRACT: *T. callosum an endemic plant, used in this study, efficiently inhibited enzymes, with IC50 values of 28.87 mg/mL, 15.75 mg/mL, 36.47 mg/mL, and 60.0 mg/mL, for AChE, BChE, α-Gly, and GST respectively. The antioxidant activities of the water and methanol extracts of T. callosum were investigated using four in vitro techniques. The antioxidant activity of water extract against ABTS radical was very strong as in the case of standards. Rosmarinic acid (2080.4 µg /g), quercetin-3-D-glycoside (853.8 µg /g), and shikimic acid (784.8 µg /g) were detected as the most intensive phenolic compound in T. Callosum by using the advanced LC-MS/MS technique. The computational screening of the studied ligands revealed the docking energies in the range of -4.217 to -9.027kcal/mol for used enzymes. Rosmarinic acid and quercetin 3-O-glucoside showed binding energies of < -8 kcal/mol with AchE and BChE respectively. In conclusion, the biological activities of the plant might be due to its rich chemical composition.*

KEYWORDS: *Tripleurospermum callosum; Phenolic compounds; Antioxidant; Enzyme inhibition; Docking.*

INTRODUCTION

Medicinal herbs contain chemical ingredients that can be used for therapeutic purposes [1, 2]. Green herbs, vegetables, and fruits are known as the primary antioxidant sources [3]. Several medicinal plants contain secondary metabolites including terpenoids, tannins, flavonoids, and alkaloids have a detoxification mechanism to alleviate the harmful effects caused by toxic metals [4]. *Tripleurospermum callosum* (Boiss.&Heldr.) E. Hossain is an endemic perennial plant, that belongs to the family Asteraceae (Compositae) and has medicinal properties [5], such as antiseptic, antifungal, antibacterial [6], anti-inflammatory, anti-ulcer, and antioxidant [7]. *Tripleurospermum* genera contains triterpenoids, volatile oils, flavonoids [8], coumarins, quinines, tannins [9], phenolic compounds, and carotenoids [10].

There is a balance between free radicals and antioxidants and this is necessary for the proper physiological functions of the body [11]. The defense system of the body is insufficient in need of exogenous antioxidants [12]. Flavonoids as exogenous antioxidants can protect the human body against oxidative diseases [13, 14] such as inflammation, Parkinson's disease, and Alzheimer's disease[15]. *T. callosum*, whose contents were examined by the advanced LC-MS method, was found to be rich in phenolic compounds

***** *To whom correspondence should be addressed.*
+ E-mail: abdulmelik.aras@igdir.edu.tr & abadi.49@hotmail.com
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. Some of the plant secondary metabolites are AChE inhibitors (AChEIs) [16] and are thought to have a therapeutic effect in the treatment of Alzheimer's Disease (AD) [17]. The AChE is responsible for the breakup of the acetylcholine in the synaptic of the brain cleft. Hence AD symptoms are prevented by keeping the acetylcholine in the synaptic cleft for neurotransmission [18]. Plants' natural phenolic compounds are acetylcholinesterase inhibitors (AChEi) and are believed to could help the treatment of AD [19]. GST, another enzyme we used in our study is responsible for detoxification metabolism. Prevents the formation of free radicals by discarding substances that are toxically entering the body [20]. However, some type of cancer cells uses GST to maintain their survival and obtain drug resistance. The finding that members of the GST enzyme are over-expressed in some types of cancer supports this [21]. α-glyph fourth enzyme we use has great importance in pharmaceutical investigations since these metabolic enzyme inhibitory properties play a role to perform lag glucose absorption and the separation of the glucose molecule from carbohydrates, resulting in decreased postprandial plasma blood glucose [22-24].

In this study, some chemical properties such as enzyme inhibition and antioxidant activity of *T. callosum* were described. The phenolic contents of the plant were analyzed using LC-MS/MS. Also, the molecular docking analysis of the most intensive phenolic compound of *T. callosum* was calculated.

EXPERIMENTAL SECTION

Chemicals

All chemicals were purchased from Sigma-Aldrich, Merc, and Fluka (AChE from Electric eel Electrophorus electricus, BChE from equine serum, and α-Glyfrom *saccharomyces cerevisiae*), Glutathione S-transferase from human placenta used as the enzyme. The standard compounds of LC-MS/MS were purchased from Sigma Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany). The reagents and chemicals for antioxidant methods were purchased from Sigma-Aldrich as well.

Plant sample

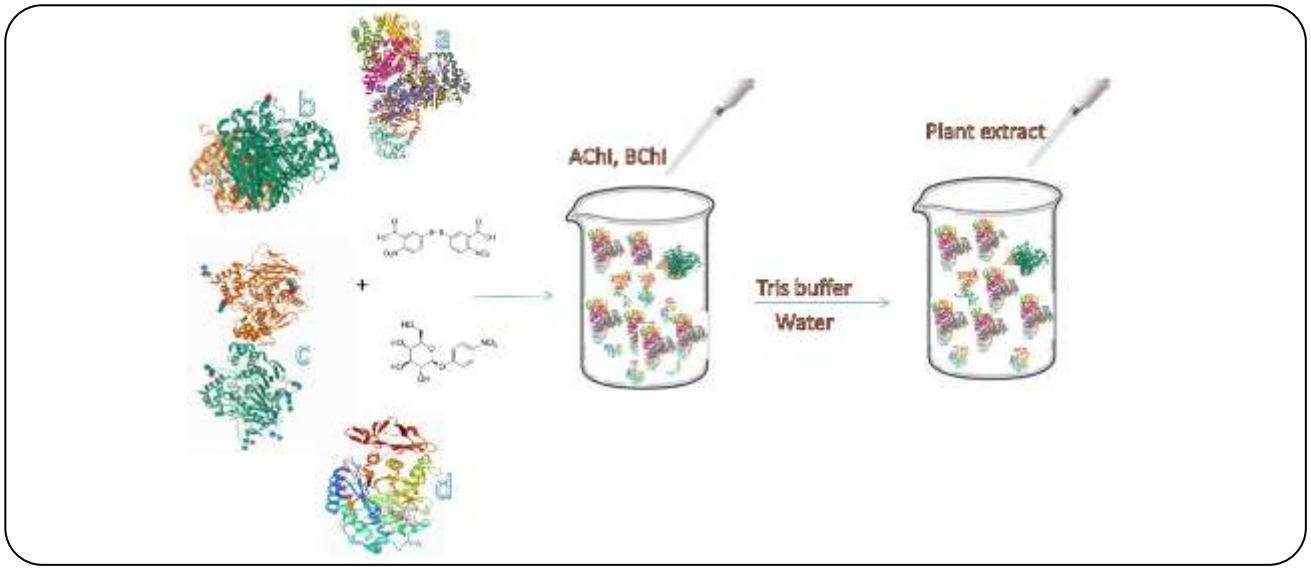
T. callosum plant was collected from Bingöl; Karlıova, vicinity of Göynük village, steppe, slopes, and damaged *Quercus* forest, 1850-2000m, 19.06.2017collected number 5197. The identification of the plant sample was made according to volume 5 of the Flora of Turkey (*Davis*, 1972).

Extract preparation

30 g of *T. callosum* leaves were dried in shadow levigated by a blender added to 300 mL of distilled water (1/10: w/v) and stirred at room temperature for about 24h. Then filtered by using filter paper. The same procedure was performed using methanol. The water sample was frozen in a lyophilizer (Labconco, Freezone 1 L) at 5 mm Hg at -50 °C and lyophilized. Stock solutions were prepared by dissolving the lyophilized extracts with distilled water at a rate of 1 mg/ml. It was stored at +4 °C for use in experiments. The filtered methanol sample was evaporated with an evaporator (Heidolph 94200, Bioblock Scientific).

Antioxidantmethods

The antioxidant properties of *T. callosum* were measured by the four in Vitro methods. The FRAP assay (ferric ion reducing antioxidant power) and the CUPRAC assay (cupric ion reducing antioxidant capacity) were used for reducing antioxidant potential. DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) cation radical scavenging technique method was used for radical scavenging potential. DPPH free radical solution (1 mM) was prepared in ethanol using a magnetic stirrer for 12 h at 25 ◦C. The absorbance of the control solution at 517 nm was adjusted to 1.5 ± 0.2 with ethanol. α-Tocopherol, ascorbic acid, BHA, and BHT were used as standard antioxidant compounds. Absorbances were measured using a UV spectrophotometer (Shimadzu, UV-1800, Japan). Extracts and standard antioxidants were prepared at (10-30 mg/mL) concentrations. 2,2-azino-bis (3-ethylbenzothiazloine-6-sulphonic acid) radical cation (ABTS·) [25], free radical scavenging on [1,1-diphenyl-2-picrylhydrazyl (DPPH·) radical[26], cupric ion reducing (CUPRAC) [27], ferric reducing antioxidant power (FRAP) [28], were used to determine the antioxidant activities of the *T. callosum* plant extract. The aliquot samples in different concentrations (10–30 μg/mL) were added to purpled color DPPH (1 mM) at room temperature. Absorbance was measured at 517 nm after 30 min incubation. Decreased absorbance demonstrated DPPH free radical scavenging capacity.



Schema 1: Enzyme study of T. callosum plant extract

Determination of phenolic by using LC-MS/MS

An Agilent Technologies 1260 Infinity II liquid chromatography System combined with a 6460 Triple Quad mass spectrometer was used for quantitative and qualitative analysis of 57 phytochemical compounds. Poroshell 120 EC-C18 (100 mm × 4.6 mm I.D., 2.7 μm) column was used for the chromatographic separation of the compounds. Mobile phase flow rate, column temperature conditions, and different mobile phases additives such as formic acid, ammonium acetate, and acetic acid were applied together with acetonitrile, purified water, and methanol mobile phases to achieve the most ideal separation and ionization of the compounds. Thus, in chromatographic separation, mobile phases of 0.1% formic acid and 5mM ammonium formate in water a mobile phase, and 0.1% formic acid and 5mM ammonium formate in methanol B mobile phase were used. Also using a flow rate of 0.4 mL/min, a gradient program of 15% for 1-12 min, 50% for 12-30 min, 90% for 30-32 min, and 10% for 32-35 min was applied in the B mobile phase, respectively. The column temperature was maintained at 40°C and the injection volume was 4.0 μL [29, 30].

An electrospray ionization (ESI) source operating in both negative and positive ionization modes was used to determine the mass-to-ion ratio (m/z) of the compounds. The ESI Source parameters were set at capillary voltage to 4000 V, nebulizing gas (N_2) flow to 11 L/min, nebulizer pressure to 15 psi, and gas temperature to 300 °C to ensure ideal ionization of all compounds and achieve the ideal peak intensity. The precursor and product ions, collision energies, and fragment or voltage of each compound were determined for quantitative measurement as Multiple Reaction Monitoring (MRM).

Precision and accuracy

Precision (intra-day and inter-day repeatability) and accuracy (recovery) studies for the developed method were evaluated by analyzing assay three replicates of samples three times within 1 week. Precision studies were evaluated using the RSD% (Relative Standard Deviation). Recovery was used to calculate by the following equation (Ellison and Williams 2012);

The recovery: recovery (%)=(detected concentration–original concentration)/spiked concentration×100.

Intra-day and inter-day repeatabilities were 2.16-13.10% and 2.25–13.85 % respectively and the calculated recovery value was found between 0.857-1.054 %.

Enzymes inhibitory activity studies.

Assessment of α-Glycosidase (α-Gly) inhibition

The inhibitory effect of *T. callosum on* α-Gly was determined by using p nitrophenyl-D-glucopyranoside (p-NPG) as the substrate [31]. Absorbance was measured at 405 nm.

Determination of GST inhibition

A series of experiments were applied to show the inhibitory effects of *T. callosum* extracts at 20-100 mM concentrations on GST enzyme activity [20].

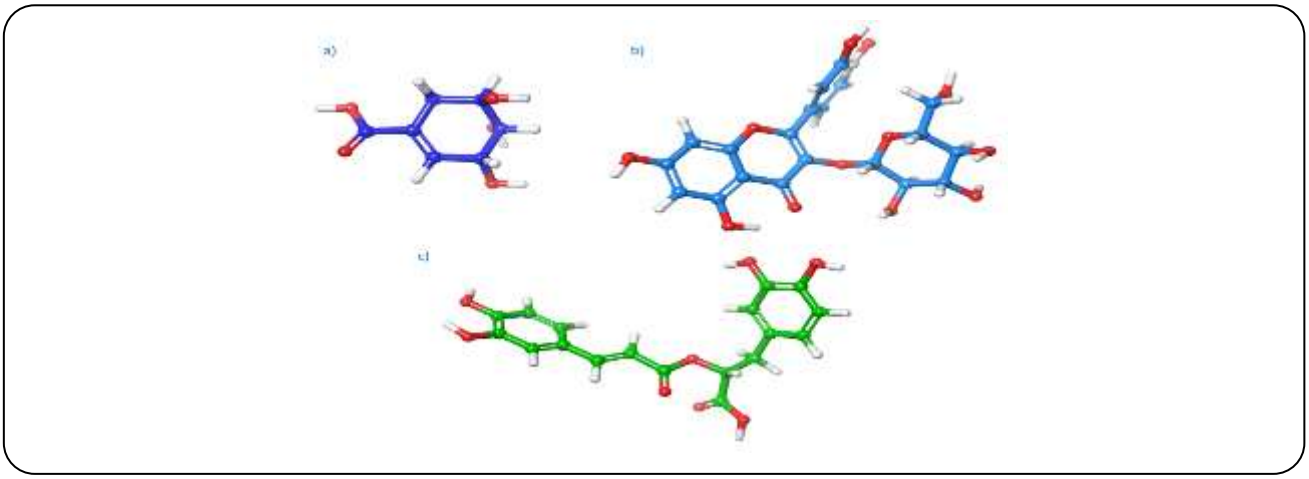


Fig. 1: Optimized 3D structures of the Phenolic compounds a) shikimic acid b) quercetin 3-O-glucoside c) Rosmarinic acid.

Evaluation of AChE/BChE inhibition activity studies

Acetylthiocholine iodide (AChI) and butyrylcholine iodide (BChI) as the substrates were used for the determination of AChE and BChE inhibitory activities of *T. callosum* extracts[32]. Absorbance was calculated at 412 nm.

Molecular modeling studies

In the molecular docking study, acetylcholinesterase (PDB ID: 4M0E), butyrylcholinesterase (PDB ID: 6SAM), glutathione s-transferase (PDB ID: 5JCU), and alpha-glucosidase (PDB ID: 3A4A) enzymes were used as receptors. Crystallographic structures of enzymes were obtained from the protein database (PDB) at the Research Collaboratory for Structural Bioinformatics (RCSB) (see http://www.rcsb.org/pdb). The Maestro Molecular Modeling platform (version 11.8) of the Schrödinger, LLC model was used as the docking program. The structures of the ligands were prepared with ChemBio3D as part of the ChemBioOffice 2019 Suite in SDF file format as 3D structures. (Fig. 1). Lig prep, Protein Preparation Wizard, and Receptor Grid Generation modules of Maestro software, at this stage all water molecules were removed and polar hydrogen atoms were added. A grid box was formed around the active site of the proteins containing natural ligands. These studies were carried out according to the methods used in the previous studies. The docking score and energy were analyzed for receptor binding affinity and the interactive nature of the Ligand - proteins. Molecular docking studies were performed with the Glide docking module under Maestro. The resulting receptor model, 2D and 3D interactions were visualized with Maestro and Discovery Studio 2017 version[33].

RESULTS AND DISCUSSION

LC-MS/MS analysis

Studies exhibited a strong relationship between antioxidant activity and phenolic compounds. Phenolic compounds are essential secondary metabolites that have antioxidant properties. These compounds are virtually found in all parts of the plants and are used for the human diet. Hydroxylated phenolic has many biological effects such as antiviral, antimutagenic, and antioxidant [34]. Due to their reducing properties as hydrogen or electron-donating agents, they have the potential to act as free radical scavengers (antioxidants) [35]. Medicinal plants contain vitamins, carotenoids, phenolic acid, and flavonoids are affecting the functioning of the organism by interacting with reactive oxygen species[34]. Antioxidants are a factor in preventing diseases such as cardiovascular, cancer, brain dysfunction, and cataracts [1, 36]. Method validation parameters such as accuracy (recovery), and precision (repeatability) were studied for standard uncertainties of each analyte according to EURACHEM Guide. Herein, phenolic compounds of the *T. callosum* methanol extract using the LC-MS/MS method were determined [37]. The results were given in Table 1.

The phenolic content of *T. Callosum* was identified and quantified using LC-MS/MS. All standards specified in Table 1 were detected in the plant extract. Rosmarinic acid (2080.4 µg/g), quercetin-3-D-glycoside (853.8µg/g), shikimic acid (784.8 µg/g), fisetin (724.5 µg/g), quercimeritrin (533.1 µg/g), scutellarin (437.0 µg/g), found to be the highest amount of *T. Callosum* phytochemicals, as reported in Table 1 and Fig. 2. Besides, 4-hydroxybenzaldehyde, 5-hydroxyflavone,

Table 1: Validation parameters of compounds by LC-MS/MS method and analysis of phenolic compounds in tripleusorperumcollasum(µg /g)

	Phenolics found in T. Collasum	Amount (µg /g)	RT	LOD (µg/L)	LOQ (µg/L)	Linearity Range (µg/L)	R2	Recovery
1	4-Hydroxybenzaldehyde	0.5305	5.77	8.78	26.7	62,5-2000	0.998	1.054
2	5-Hydroxyflavone	8.5436	23.69	7.81	23.76	62,5-2000	0.999	1.024
3	6,2,4-Trimetoxyflavone	4.3671	24.57	1.55	4.88	12,5-400	0.999	1.026
4	6-Hydroxyflavone	1.5778	19.60	1.97	6.15	12,5-200	0.996	0.952
5	Baicalein	33.3202	18.14	1.95	6.3	50-800	0.999	1.012
6	Baicalin	26.4582	12.53	0.39	2.21	6,25-800	0.999	0.979
7	Biochanin A	148.8448	20.59	2.45	7.81	62,5-2000	0.999	1.021
8	Catechin	47.7489	3.51	28.74	69.24	250-8000	0.999	0.962
9	Chlorogenic acid	85.7533	5.30	23.4	74.1	125-4000	0.998	0.976
10	Chrysin	343.6806	20.85	4.84	15.63	31,25-1000	0.999	1.034
11	Coumarin	19.8543	15.04	5.63	15.62	62,5-2000	0.999	1.016
12	Cynarin	24.3698	11.37	9.39	28.3	62,5-2000	0.994	0.977
13	Diosgenin	18.7531	34.51	3.13	8.19	25-800	0.999	1.013
14	Ellagic acid	21.6844	15.25	72.5	226.5	500-8000	0.992	1.021
15	Epicatechin	45.3013	6.83	8.45	19.69	62,5-4000	0.998	1.02
16	Fisetin	724.4637	13.53	20.8	68.5	125-4000	0.996	0.942
17	Flavone	14.8771	21.69	1.52	6.02	6,25-200	0.999	1.005
18	Gentisic acid	27.1214	3.15	9.45	32.5	125-2000	0.996	0.996
19	Hyperocide	124.5144	11.89	0.38	2.06	6,25-800	0.998	0.946
20	Isoquercitrin	41.8229	11.91	0.95	3.23	12,5-800	0.999	1.007
21	Kaempferol	144.9784	16.62	37.26	128.1	500-8000	0.998	0.998
22	Kaempferol-3-glucoside	174.5354	13.35	0.61	2.31	6,25-200	0.999	0.985
23	Morin	23.2236	15.97	3.19	12.6	62,5-2000	0.998	0.985
24	Naringenin	9.8671	15.53	2.8	7.81	31,25-4000	0.999	1.02
25	Naringin	286.6902	12.03	14.68	43.8	62,5-8000	0.998	0.989
26	Neohesperidin	2.6688	12.86	18.93	69.5	250-4000	0.998	0.962
27	Oleuropein	46.2110	13.84	9.68	36.2	125-4000	0.996	1.036
28	Protocatechuic acid	6.0813	2.77	4.62	14.77	31,25-1000	0.997	0.958
29	Quercetin	42.3638	15.03	4.54	12.6	15,625-1000	0.999	1.006
30	Quercetin-3-D-xyloside	853.8423	12.51	45.85	125.8	500-8000	0.999	0.938
31	Quercetin-3-glucoside	6.6037	11.91	1.04	3.12	12,5-800	0.999	1.008
32	Quercimeritrin	533.1198	10.38	3.13	10.21	31,25-2000	0.998	0.984
33	Resveratrol	136.3335	11.98	12.18	38.44	125-2000	0.998	1.017
34	Rosmarinic acid	2080.3735	12.17	22.72	69.78	125-4000	0.998	1.013
35	Rutin	210.0396	11.98	21.3	62.5	250-8000	0.999	0.994
36	Scutellarin	436.9988	11.20	2.3	6.2	12,5-800	0.997	0.992
37	Shikimic acid	784.7789	1.18	68.25	210.24	500-8000	0.991	0.924
38	Silibinin	25.0341	15.93	2.96	9.74	62,5-2000	0.999	0.996
39	Syringic acid	161.6072	6.34	26.98	83.2	250-8000	0.994	0.943
40	Tamarixetin	13.5494	16.20	4.73	15.68	31,25-8000	0.999	1.001
41	Trans-cinnamic acid	23.1226	14.35	60.35	190.1	500-8000	0.997	1.015
42	Trans-ferulic acid	15.8455	9.58	12.45	35.32	62,5-4000	0.997	0.956

R_t, retention time, LOD, and LOQ: limit of detection and limit of quantification, respectively.

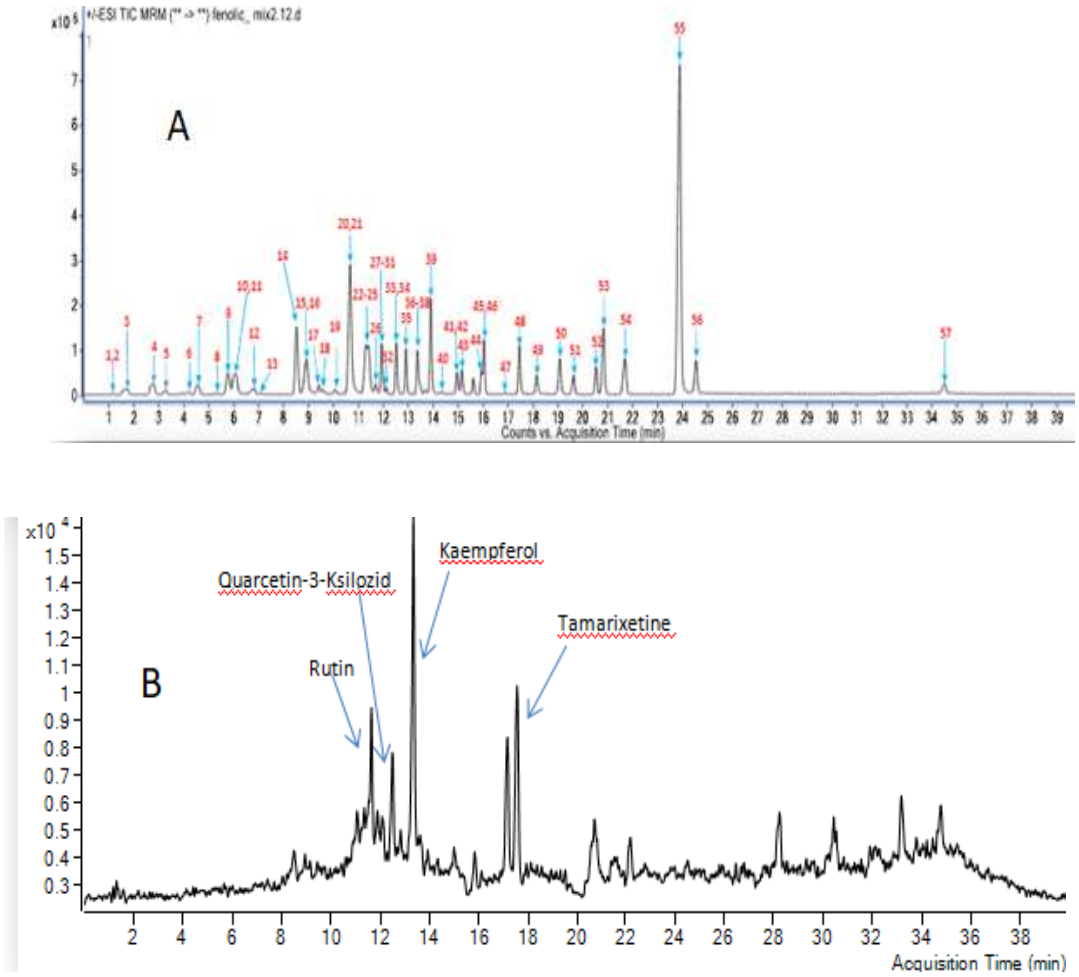


Fig. 2: LC-MS / MS chromatograms T. callosum and of 57 standards (1-2: Ascorbic acid, Shikimic acid, 3: Gallic acid, 4: Protocatechuic acid, 5: Genrretisic acid, 6: Catechin, 7: 4-Hydroxybenzoic acid, 8: Chlorogenic acid, 9: 4- Hydroxybenzaldehyde, 10- 11: Vanillic acid, caffeic acid, 12: Epicatechin, 13: Syringic acid, 14: p- coumaric acid, 15- 16: Salicylic acid, Taxifolin, 17: Polydatin, 18: t- ferulic acid, 19: Sinapic acid, 20-21: Queersmethrin, Coumarin, 22-25: Scutellarin, o-coumaricacid, Cynarine, Protocatechuic ethylester, 26: Hyperoside, 27-31: Quercetın, 3-Glycoside, Rutin, Iso quercetin Resveratrol, Naringin, 32: Rosmarinic acid, 33-34: Quercetın, -3- o-xyloside, Hesperidin, 35: Neohesperidin, 36-38: Kaempferol- 3-glycoside, Fisetin, Oleuropein, 39: Baicalin, 40: t-cinnamic acid, 41-42: Ellagic Acid, quercetin, 43: Naringenin, 44: Silibinin, 45-46: Hesperetin, Morin, 47: Campherol, 48: Tamarixetine, 49: Baikalein, 50: 7- Hydroxyflavone, 51: 6- Hydroxyflavone, 52: Biokanin A, 53: Chrysin, 54: Flavone, 55: 5- Hydroxyflavone, 56: 6'2'4 trimethoxyflavone, 57: Diosgenin) (B) compounds in the methanol extracts of T. callosum.

6,2,4-trimetoxy flavone, 6-hydroxy flavone, baicalein, baicalin, biochanin a, catechin, chlorogenic acid, chrysin, coumarin, cynarin, diosgenin, ellagic acid, epicatechin, flavone, gentisic acid, hyperocide, ısoquercitrin, kaempferol, kaempferol-3-glucoside, morin, naringenin, naringin, neohesperidin, oleuropein, protocatechuic acid, quercetin, quercetin-3-glucoside, resveratrol, rutin, silibinin, syringic acid, tamarixetin, trans-cinnamic acid, and trans-ferulic acid were other phytochemicals of *T. callosum* extract detected in different amounts. Rosmarinic acid was found the most abundant compound in *T. callosum*, is a very important molecule in the biosynthetic pathway of many aromatic compounds that exist in microorganisms and plants.

Antioxidant potential

Free radicals, such as Reactive Oxygen Species (ROS), and Reactive Nitrogen Species (RNS) are very reactive

molecules or atoms because of having unpaired electrons [38, 39] formed under pathological AD conditions [40]. The level of free radicals and antioxidants is balanced by the human body [41, 42]. Besides vegetables, fruit, and many plants are used as natural sources of chemical components with antioxidant activities [43]. The antioxidant potential of plants can be described as a level of inhibiting or limiting the oxidation of bioactive substances in the environment by the synergic effect of the antioxidant compound of those plants [44]. Presenting the antioxidant potentials of plants, the determination of the secondary metabolites that cause some biological activity such as antioxidant activity is important in terms of obtaining new and natural antioxidant compounds [45].

When the LC-MS / MS results were analyzed, the plant was found to be rich in phenolic compounds. Since it has been observed in previous studies that phenolic contents affect antioxidant activities, we applied four different *in vitro* methods to determine the antioxidant potential of *T. callosum*. CUPRAC (cupric ion reducing antioxidant capacity) and FRAP (ferric ion reducing antioxidant power) methods were used to measure the reducing power antioxidant activities. ABTS (2,2-azino-bis 3-ethylbenzothiazloine-6-sulphonic acid) and DPPH (1,1-diphenyl-2-picrylhydrazyl) methods were used to calculate the radical scavenging properties. DPPH and ABTS assay has extensively been used for the determination of radical scavenging. The radical scavenging level of a sample demonstrates its antioxidant potential that prevents oxidation chain initiation. DPPH radical scavenging capacity and ABTS cation radical inhibition of *T. callosum* samples and standard antioxidants (ascorbicacid,alpha-Tocopherol,BHA (butylated hydroxyanisole), BHT (Butylated hydroxytoluene) were compared. Decreasing absorption demonstrates the radical scavenging potential of substances. Compared to the standard antioxidants, water extracts have better antioxidant activity than some standards in the ABTS method. However, the Methanol extract of *T. callosum* sample demonstrated lower free radicals scavenging activity when compared to the water extract. Besides, the Methanol extract of *T. callosum* showed good antioxidant activity against the DPPH. However, the antioxidant activity of the water extract of *T. callosum* is very weak activity compared to the Methanol extract and standards. Fig. 3 shows an increased free radical scavenging potential by increasing the concentrations of standards and samples.

DPPH scavenging percentages of *T. callosum* extracts and standard antioxidants at the 30 µg/mL concentration and IC_{50} values were as follows respectively ascorbic acid (92.4±0.4%, 10.8±5.4), BHA (88.7±1.7%, 11.2±5.5), alpha-tocopherol (88.4±2.6%,11.2±5.3), BHT (76.8±6.4%,12.8±5.5), methanol extract (22.0±20.0%,73.2±51.5). Water extract demonstrated almost no activity. The lower IC_{50} values show strong scavenged DPPH and ABTS radicals. Both plant extracts and standard antioxidants decreased (Table 2).

ABTS cation radical inhibition percentages of *T. callosum* extracts and standards at 30 µg/mL concentration and IC_{50} values was as follows respectively: alpha-tocopherol (47.2±10.8%, 29.0±12.1), BHT (38.4±11.0%, 25.0±7.0), water extract (33.6±5.4%, 28.8±10.5), ascorbic acid (28.4±16.9%, 37.1±13.4), methanol extract (23.2±7.8%, 42.3±13.7), and BHA (18.2±6.6%, 53.7±15.9).

The increasing of the reducing power of a sample indicates it is antiradical potential. The antioxidant potential of samples of *T. callosum* was determined by FRAP and CUPRAC methods. Both the CUPRAC and FRAP method is a well-known methods to measure reducing the antioxidant potential of substances. Antioxidant substances cause the reduction of ferric (Fe^{3+}) ions to ferrous (Fe^{2+}) ions.

The cupric and ferric reducing potential of *T. callosum* samples were calculated and compared to the ascorbic acid, BHA, BHT, and α-Tocopherol. As seen in Fig. 3, reducing levels of the water extracts are higher than MeOH extracts in the FRAP method. At the 30 µg/mL concentration reducing antioxidant potential decreased as follows alpha-Tocopherol, BHT, BHA, ascorbic acid, *T. callosum* water extract, *T. callosum* methanol extract.

In the CUPRAC method, methanol and water extract of *T. callosum* demonstrated lower reducing capacities when compared to the standards. The water extract of the *T. callosum* sample showed remarkable antioxidant activity against cupric ions. At the 30 µg/mL concentration reducing capacities decreased as follows BHA, ascorbic acid, alpha-tocopherol, BHT, water extract, and methanol extract.

Determination of enzyme inhibition

Several plants reported having a large number of secondary metabolites with different biological activities [46]. In this study, AChE, BChE, α-Gly, and GST

Table 2: Docking score, enzyme inhibitory and antioxidant properties of the T. callosum plant extracts.

Methods	BHA	BHT	Ascorbic acid	α-Tocopherol	Methanol extract	Water extract	shikimic acid	quercetin 3-O-glucoside	Rosmarinic acid
Enzyme Inhibition Methods (IC_{50})							Docking Scores		
AChE	-	-	-	-	28.87 (R^2=0.905)	-	-6.401	-4.658	-9.027
BChE	-	-	-	-	15.75 (R^2=0.979)	-	-5.674	-8.192	-7.968
α-Gly	-	-	-	-	36.47 (R^2=0.965)	-	-6.246	-4.217	-6.589
GST	-	-	-	-	60.0 (R^2=0.980)	-	-5.413	-4.289	-7.347
DPPH	11.2±5.5	12.8±5.5	10.8±5.4	11.2±5.3	73.2±51.5	ND		-	-
ABTS	53.7±15.9	25.0±7.0	37.1±13.4	29.0±12.1	42.3±13.7	28.8±10.5		-	-
ND: Not detected									

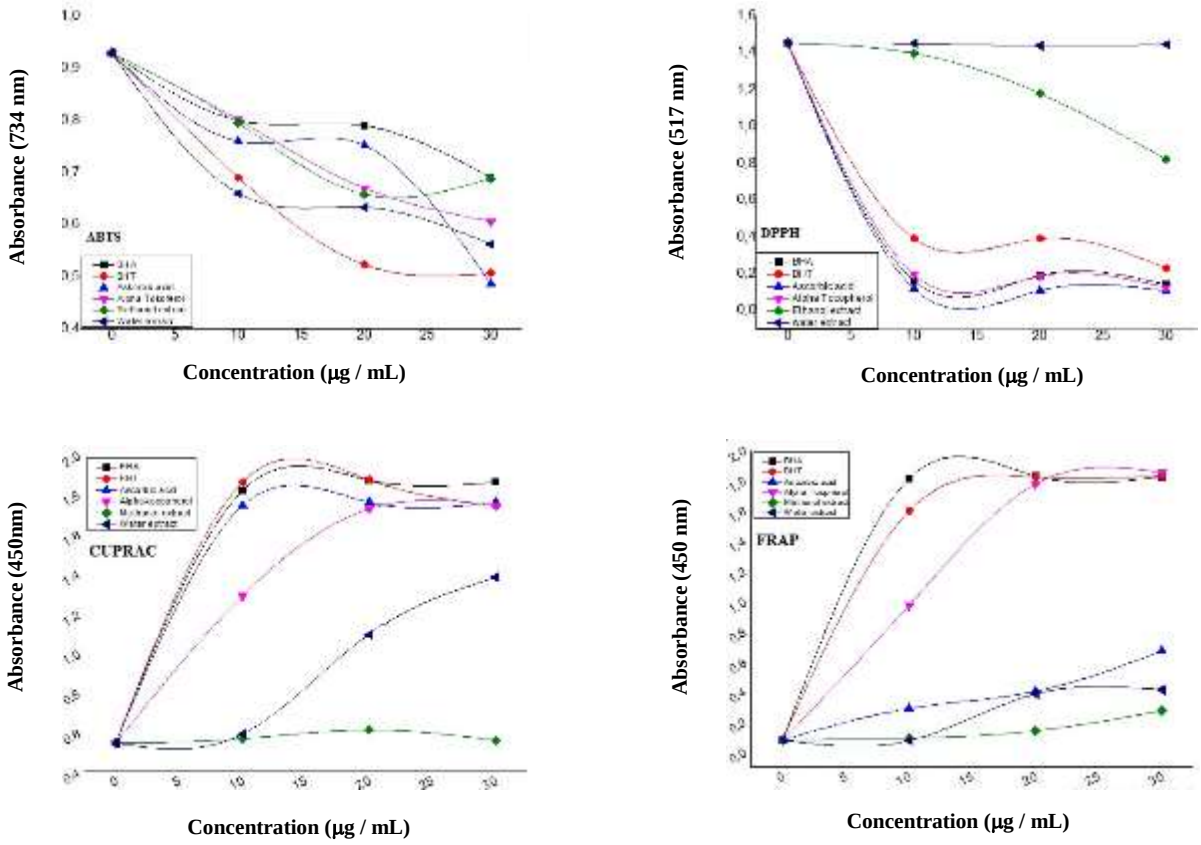


Fig. 3: Antioxidant activity of T. callosum and standards for ABTS, DPPH, CUPRAC, and FRAP methods.

were efficiently inhibited by *T. callosum* methanol extracts as shown in Fig. 4. TAC (9-amino-1,2,3,4-tetrahydroacridine) is a well-known reversible inhibitor of BChE and AChE and the first drug for the treatment of AD, therefore TAC can be used as a Standard to compare our obtained data. IC_{50} values for *T. callosum* methanol extract against the used metabolic enzymes were as follows: 60.0 mg/mL (r2: 0.98) for GST, 28.87 mg/mL (r2: 0.9046) forAChE, 15.75 mg/mL (r2: 0.9791) for BChE and 36.47 mg/mL (r2: 0.9654) for α-Gly. Studies have found significant

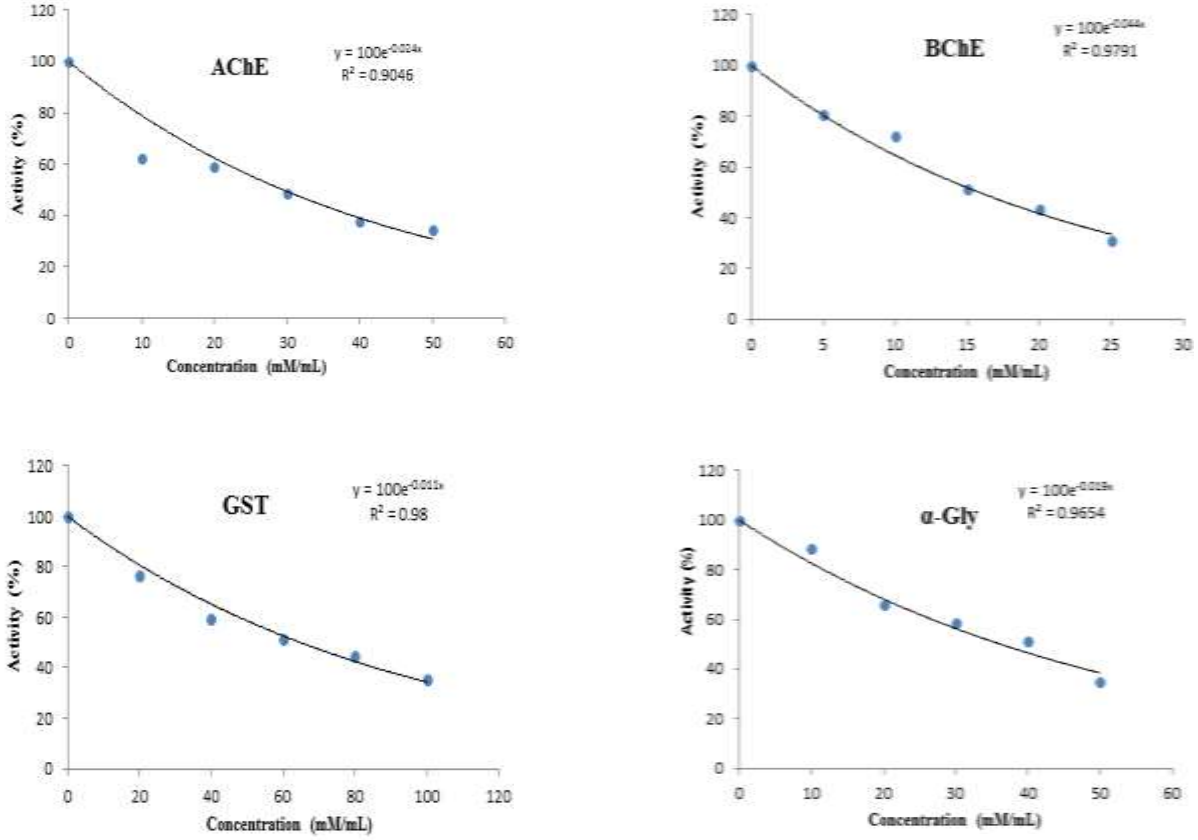


Fig. 4: The enzyme inhibition of T. callosum ethanol extract against AChE, BChE, GST, and α-glycosidase (C) enzymes.

results for TAC and acarbose at the micromolar level [47, 48]. All enzymes used were strongly inhibited by *T. callosum* methanol extracts. Especially, plant extract showed excellent activity against BChE (15.75 mg/mL). Results demonstrated that when compared to the tacrine used in studies the anti-AD potential of the methanol extract was relatively lower (Table 2).

Molecular Docking Studies

Molecular docking is useful for studying the binding mechanism between the ligand-receptor and understanding the interaction of possible binding modes at the molecular level. Here, molecular docking was performed to obtain the preferred binding sites of the ligands with the receptor and substantially confirm the experimental observations [49]. The study consists of 3 different compounds and 4 enzyme sets, 12 good docking results were obtained (Table 1). These ligands were placed in the catalytic active region of the enzyme and the docking results were analyzed on the basis of binding affinity and interaction mode. The halogenated structures at the meta position achieved a good binding score with all enzymes relative to the para position. However, as a molecular structure, the best binding affinity score was observed in AChE and BChE enzymes.

Molecular structural similarity to the natural ligand in protein structure increases this value. Fig. 5 shows the regions where proteins can interact with 3D crystal structures and small molecules (ligands). The dynamics of the protein play an important role in how proteins interact with Shiff base derivatives to form complexes, which can increase or inhibit its biological function[50]. Since AChE is in the binding inner regions of proteins, higher affinity was found compared to other enzymes [51]. Figs. (5, 6, and 7) show 3D crystal structures of the regions where proteins can interact with small molecules (ligands).

The residues on the enzyme achieved strong binding with PHE 295 (2.10), HIS 447 (2.80), TYR 124 (2.92) SER125 (2.42-1.64), GLY 121 2.10, TYR 337 (2.96), GLU 202 (1.61) strong conventional hydrogen bonds,

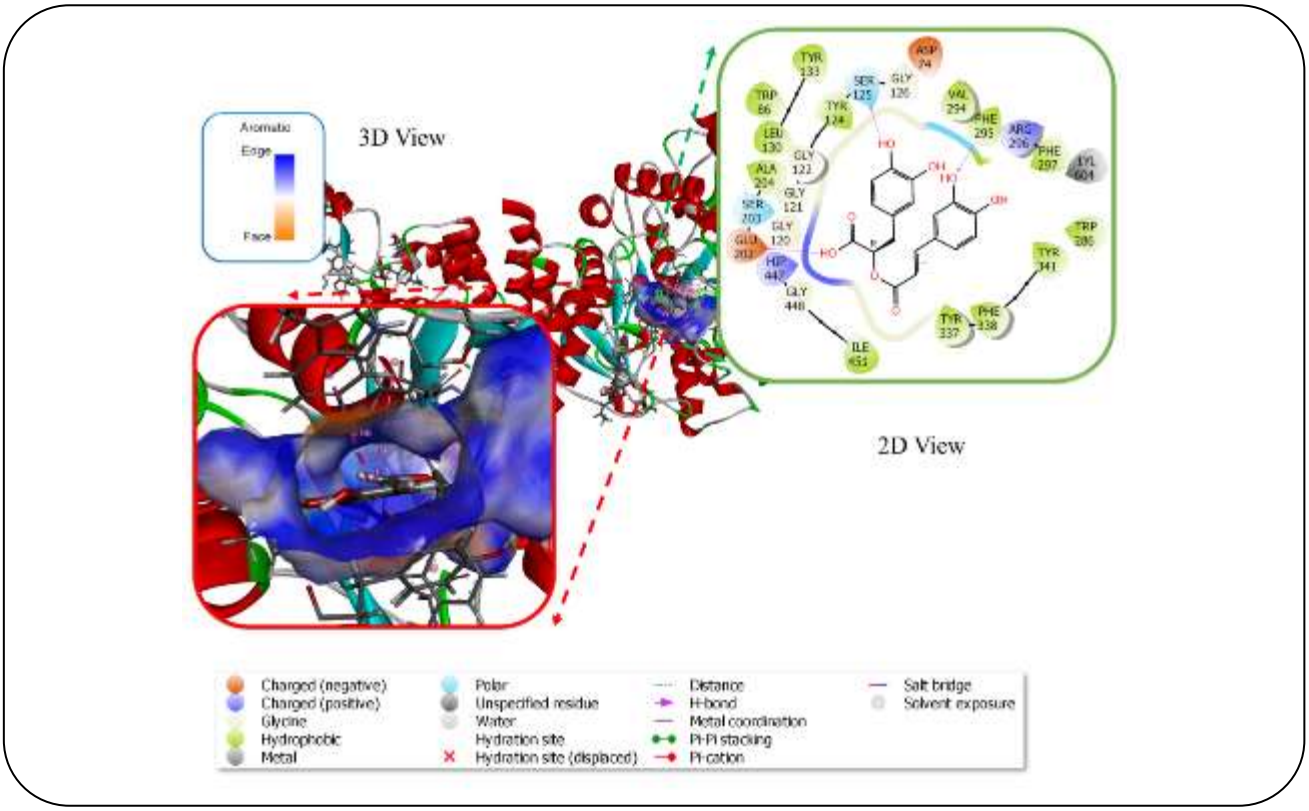


Fig. 5: 3D and 2D view of rosmarinic acid –AchE enzymeinteractions.

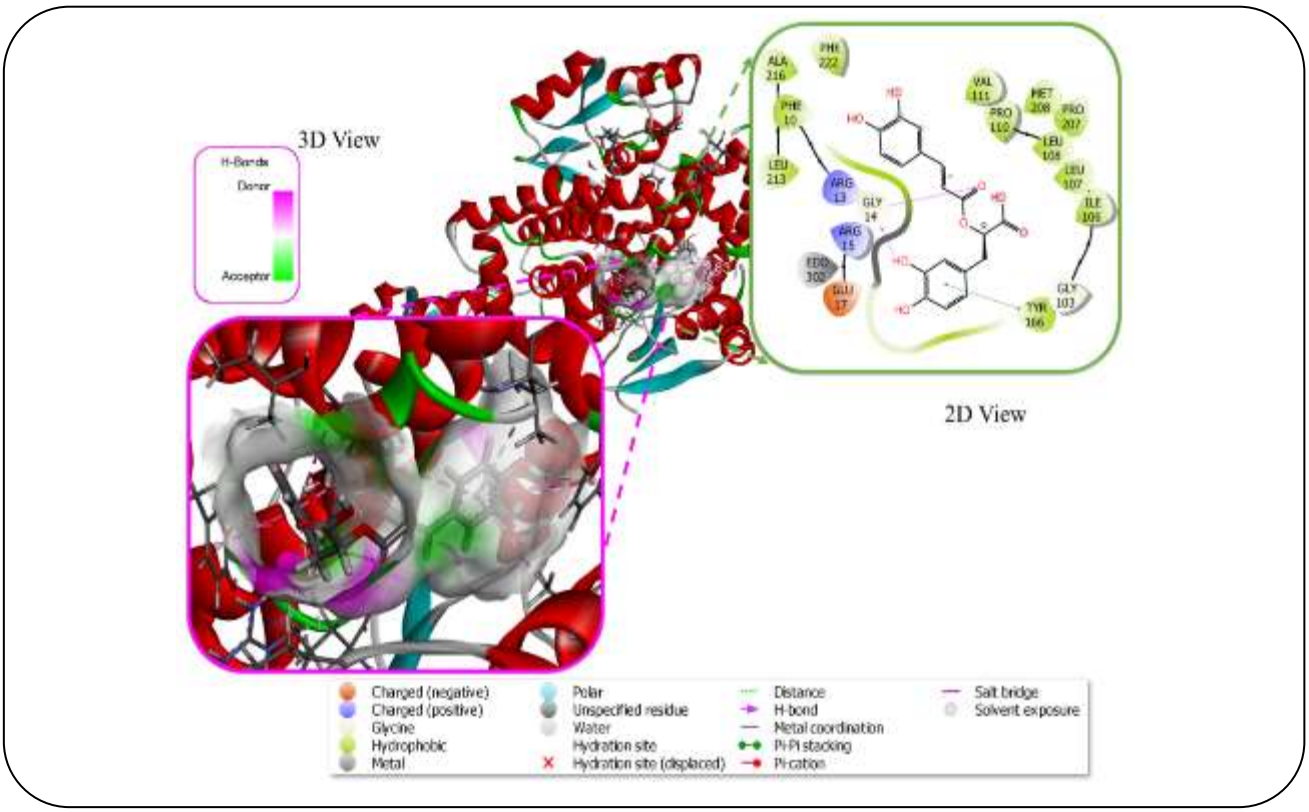


Fig. 6: The interaction mode between Rosmarinic acid - GST enzyme.

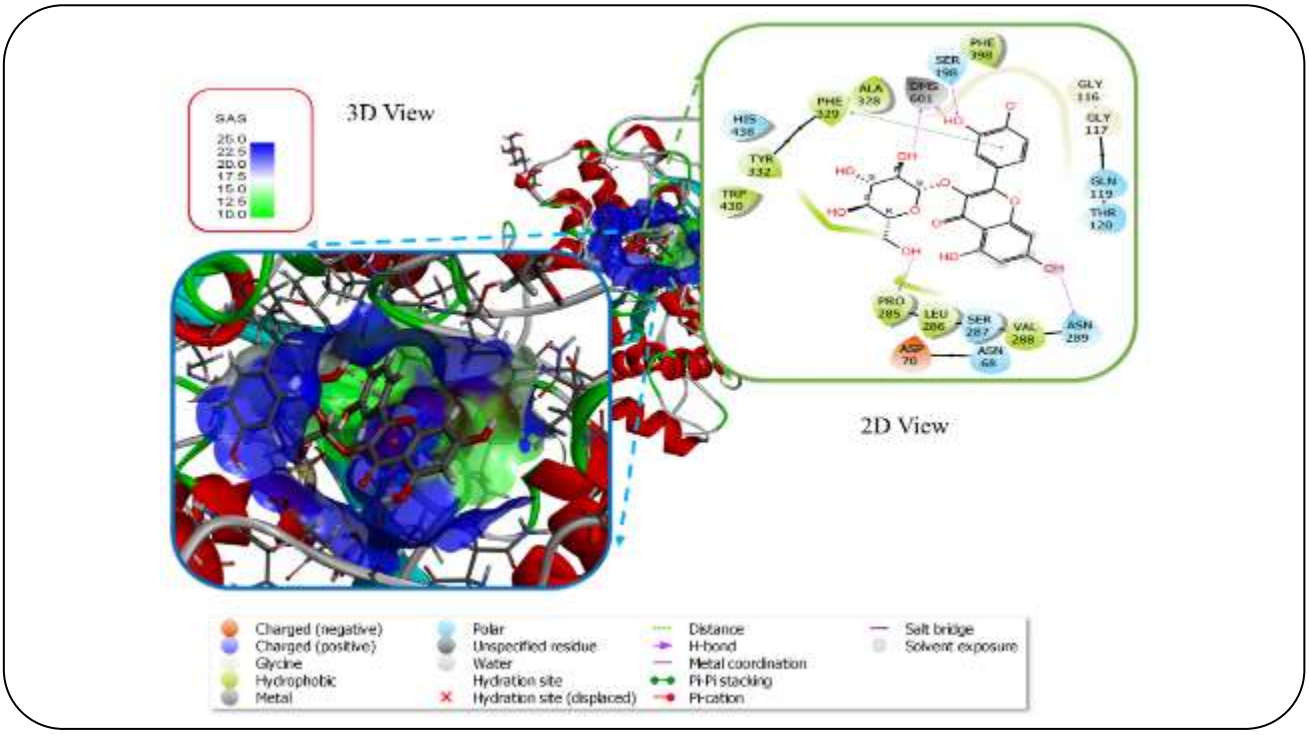


Fig. 7. 3D view of the hydrogen bonds donor/acceptor on the receptor d) 2D view of quercetin 3-O-glucoside -α-Gly enzyme interactions. Conventional hydrogen bonding GLY 14 1.76-2.41, Amide-pi Stacked TYR 166 4.09, ILE 106 5.10. pi-pi stacked ARG 13 5.49, LEU 107 4.72, pi-donor hydrogen bond PHE 222 5.66. PI-ALKYL VAL 111 5.26 AND ALA 216 5.08.

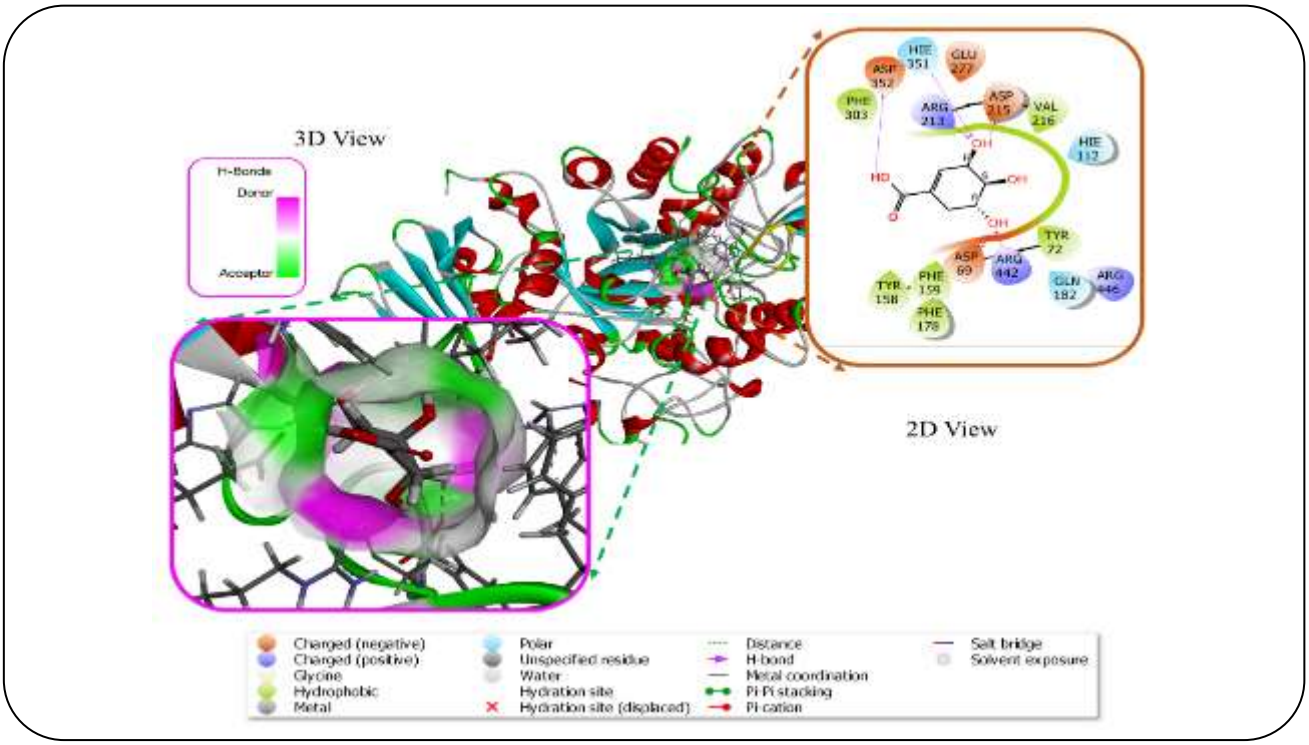


Fig. 8: The interaction mode between shikimic acid -BChE; 3D view of the hydrogen bonds donor/acceptor surface on the receptor and 2D view of shikimic acid -BChE enzyme interactions. ASP 69 1.87, ARG 442 1.85, ASP 1.70 ARG 213 2.70 HIS 351 2.53 ASP 352 1.96, carbon-hydrogen bonds with ASP 69 and 2.72, as well as VAL 216, TYR 72 and PHE 303 with Van der Waals bonds.

respectively. Pi-pi stacked TRP 286 5.66 and pi-pi t-shaped bond TYR 341 4.20. GLY 126 2.79 carbon-hydrogen bonds.

3D view of the hydrogen bonds donor/acceptor surface on the receptor and 2D view of ligand- enzyme interactions. Conventional hydrogen bonding GLY 14 1.76-2.41, Amide-pi Stacked TYR 166 4.09, ILE 106 5.10. pi-pi stacked ARG 13 5.49, LEU 107 4.72, pi-donor hydrogen bond PHE 222 5.66. PI-ALKYL VAL 111 5.26 AND ALA 216 5.08.

CONCLUSIONS

This study reported antioxidant properties, enzyme inhibition, and phenolic content of the *T. callosum* extracts. The results of four antioxidant methods demonstrated that *T. callosum* extracts have a significant radical scavenging ability. Moreover, the methanol plant extract was found to possess different inhibitory properties against the four studied enzymes. The best of these inhibition effects were achieved against BChE in vitro. The molecular docking studies of the three most intensive phenolic compounds of *T. callosum* with enzymes were performed using an induced-fit docking module. Considering the inhibitory activity of AChE, BChE, α-Gly, and GST enzymes plant bioactive content could be considered a novel candidate both in the food preservation industry as an additive of natural origin and the drug design processes of anti-Alzheimer and anti-diabetic. In conclusion, in vitro coupled with the in silico results stated in this study, indicated some basic important biological activities of *T. callosum*. However, more advanced investigations need for possible ingredients and potential activities of this plant.

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