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Phytochemical Extraction, QSAR and Molecular Docking Analysis of the Total Flavonoids from Green Tea as Aldose Reductase Inhibitors

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ABSTRACT

Tea is one of the most popular beverages consumed worldwide. It is consumed in different parts of the world as green (unfermented), black (fermented), or Oolong tea (Semi fermented). Amongst these, the most significant effects on human health have been observed with the consumption of green tea. The present study aims at identifying simple methods for performing qualitative and quantitative analysis of green tea leaves for the presence of flavonoids and molecular docking analysis of green tea flavonoids as aldose reductase inhibitors as a potential cure for diabetic retinopathy, neuropathy and nephropathy. Extraction was performed using tea bags and the wholesale leaves as samples and alcohol and water as solvents. Phytochemical analysis and identification was performed by employing Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC). A molecular docking study was performed to understand the interactions of aldose reductase enzyme (PDB ID: 3G5E) with the phytochemicals identified in green tea leaves. Quantitative Structure–Activity Relationship (QSAR) studies were carried out using Build QSAR and Molecular properties were calculated using MedChem designer. Docking and the QSAR studies indicate that the phytochemicals present in the green tea leaves extract could inhibit the activity of Aldose reductase enzyme due to their interactions with the binding pocket. The phytochemical studies enabled us to understand that green tea extract was indeed a rich source of flavonoids. Hence by consumption of these phytochemicals in green tea we could combat the complications of diabetes i.e., retinopathy, neuropathy and nephropathy.

Keywords: Diabetic retinopathy, Neuropathy, Nephropathy, Auto dock 4.0, Argus lab 4.0.1

INTRODUCTION

Tea is one of the most popular beverages consumed worldwide. Tea, from the plant *Camellia sinensis*, is consumed in different parts of the world as green, black or oolong tea. Among all of these, however, the most significant effects on human health have been observed with the consumption of green tea [1]. The health benefits of consuming green tea, includes the prevention of cancer, cardiovascular diseases, the anti-inflammatory, antiarthritic, antibacterial, antiangiogenic, antioxidative, antiviral, neuroprotective and cholesterol-lowering effects [2]. The majority of tea beverage is prepared from two types of manufactured tea-black and green [3]. Both green and black teas are rich dietary sources of flavonoids [4]. The health-promoting effects of green tea are mainly attributed to its polyphenol content, particularly flavones and flavonols, which represent 30% of fresh leaf dry weight [5]. The major flavonoids of green tea are various catechins which are found in greater amounts in green tea than in black or oolong tea [6]. There are four kinds of catechins mainly found in green tea: Epicatechin, epigallocatechin, epicatechin-3-gallate, and Epigallocatechin Gallate (EGCG) [7]. The preparation methods influence the catechins both quantitatively and qualitatively, the amount of catechins also varies in the original tea leaves due to differences in variety, origin, and growing conditions [8]. The preparation of fresh green tea cannot totally extract catechins from the leaves. Therefore, the concentration found differs from the absolute values determined through the complete extraction of leaves [9]. Our objective is to find a best method of extraction and identify the major flavonoids of green tea and to perform phytochemical analysis of green tea extract. To carry out *in silico* studies to assess the inhibitory activity with aldose reductase through computer simulations. Further this helps to throw some light on how to avoid the complications of diabetic retinopathy, neuropathy and nephropathy using natural compounds.

MATERIALS AND METHODS

Extraction

The tea bags and wholesale tea leaves were stored in their original containers in the dark at room temperature. As the efficiency of extraction is determined by several physical and chemical parameters such as temperature, solvent system, filtration, agitation, leaching as well as size of the particles, several alterations were made to the protocols to identify the optimal conditions [10].

Solvent system: Two systems, absolute ethanol and water were used to obtain tea extracts. Leaching Temperature: Leaching was carried out at two temperatures, room temperature and 4°C. According to Stoke-Einstein equation the efficiency of leaching should increase with rise in temperature. Leaching time: Leaching was carried out at different time intervals (0, 30, 90 and 120 min) and temperatures (0 and 4°C) to observe the effect of leaching time on the efficiency of extraction. Storage temperature: Tea extracts were stored at room temperature and 4°C. Agitation: Samples were subjected to leaching considering two conditions; one with agitation and one without agitation. Size of particles: The smaller the size of the particles the greater is the surface area that is available for the diffusion of flavonoids into the solvent (Fick's First Law).

Alcohol extraction

The tea extracts were prepared by following the micro wave-assisted extraction protocol described by Pan et al. with some variations. The dried tea leaves were pre-leached with 50% v/v ethanol for 90 min with a solvent to tea leaves ratio of 20:1 (ml/g) at room temperature. The samples were stirred for 5 min using magnetic stirrer. The extraction was then carried out using a microwave oven for 4 min by initially switching ON for 45 s consecutively switching OFF for 10 s and then switching ON for 3 s. The second step was repeated over the next 3 min. The extract was filtered initially with sieve and then with Whatmann filter paper No.1 and finally the samples were then stored at 4°C.

Water extraction

1 g of tea leaves was weighed and to it 100 ml of distilled water was added. The sample leaching was carried out at room temperature for 120 min with agitation. The mixture was then heated in the microwave oven for 2 min. The extract was then filtered initially with sieve and then with a Whatmann filter paper No.1. The samples were then stored at 4°C.

Confirmation tests for flavonoids

5 ml of dilute ammonia, 1 ml of Conc. H_2SO_4 was added to the extract. The presence of yellow colour that disappears on standing indicates the presence of flavonoids [11]. To 0.2 g of the tea powder few drops of dilute NaOH and HCl is added. The presence of yellow colour which further becomes colorless indicates the presence of the flavonoids [11].

Identification and estimation of flavonoids

The concentration of the flavonoids in the tea extracts are determined by aluminium chloride colorimetric assay [12]. The tea extracts were diluted up to 20 fold. An aliquot (2 ml) of the diluted sample is mixed with 0.2 ml of 5% sodium nitrite. After 5 min, 0.2 ml of 10% aluminium chloride is added to the mixture. After 6 min, 2 ml of 1 M sodium hydroxide were added to the mixture. The final volume of the reaction mixture was made up to 5 ml with 50% ethanol and water. Absorbance was measured at 510 nm against a blank. The total flavonoids content was determined using a standard curve of quercetin (0-50 mg/ml) and the results were expressed as Quercetin equivalents.

Thin layer chromatography (TLC)

100 ml of the mobile phase, a mixture of Acetone: Chloroform: Water (80:20:10) was first prepared. The stationary phase (TLC plate) was prepared by marking points 2 cm from the bottom of the plate and 1 cm apart. The samples along with the standards were then loaded onto the spots and then allowed to dry. The plate was placed in the chamber and the samples were allowed to run up to $3/4^{th}$ of the length of the TLC and the plate was then viewed under UV at 366 nm.

***In silico* studies**

Preparation of protein and ligands

The crystal structure of aldose reductase enzyme with PDB Id: 3G5E used in this study was retrieved from RCSB Protein Data Bank [13]. 25 compounds catechins and their derivatives were obtained from NCBI PubChem [14] and their structures were obtained using Mol-inspiration [15]. The ligands were prepared for docking using SPDBV [16].

Active site analysis

The active site analysis of 3G5E was performed using MetaPocket 2.0**.** It is an online server for automatic identification of residues given its 3D coordinates [17].

Calculation of physicochemical properties, QSAR studies and toxicity evaluation

The properties of the ligands such as the logP value, hydrogen bond acceptor, molecular weight, hydrogen bond donors and number of atoms were obtained using MedChem designer^{TM} 2.5.0.8 that also analyses the number of violations or deviations from Lipinski's rule of five [18]. QSAR Model was generated using Build QSAR [19]. Toxicity evaluation studies were carried out using Osiris Property Explorer [20].

Docking studies

Molecular docking analysis of aldose reductase enzyme with the ligands of green tea extract was carried out by Auto Dock4.0 [21] and Argus Lab4.0.1 [22] softwares. Docking was performed for 21 compounds which were considered for QSAR studies. The docking results were analysed using software Accelrys Discovery Studio 3.5 [23] to understand the protein ligand interactions.

RESULTS AND DISCUSSION

The extraction protocol was standardized based on the concentration of flavonoids obtained under each condition. The first condition that was considered was leaching time at room temperature and at 4°C. It was observed that the concentration of flavonoids decreased with increase in time of leaching at room temperature, whereas the concentration of flavonoids increased with increase in time of leaching at 4°C. The concentration values were obtained from the Quercetin standard curve. The results showed that the concentration of flavonoids was higher when leached at room temperature after 30 min than 4°C (Table 1).

Table 1: Concentration of flavonoids at 4°C and room temperature at different time intervals

Leaching time		OD at 510 nm	Concentration (µg/ml)		OD at 510 nm	Concentration (µg/ml)
30 min	**4°C**	0.27	21.673	**RT**	0.38	42.827
90 min		0.28	23.596		0.3	27.442
120 min		0.29	25.519		0.29	25.519

For further standardization of the extraction protocol we considered the particle size. Two types of samples were considered for this i.e., powder extract and leaf extract. The results showed that the concentration of flavonoids obtained using the powdered tea leaves was significantly more than that of tea leaves. Our result was hence in accordance with the Stoke-Einstein equation (Figure 1).

For further standardization of the extraction protocol we used water and alcohol as solvents. Based on the results it was observed that the alcohol extraction yielded a greater concentration of flavonoids when compared to that of water. It was observed that the samples that were subjected to agitation consistently yielded higher concentration of flavonoids. In order to confirm the presence of total flavonoids we further performed TLC analysis. On allowing the solvent to run $3/4^{th}$ of the TLC plate (9.5 cm from the line), three distinct bands as well as an intermediate region was observed. When observed under visible light the chromatogram consisted of a single green and two brown bands. When observed under UV, bands of three distinct colors (brown, pink, green) were observed (Figure 1). The bands and their corresponding R_f values are shown in Table 2.

Table 2: R_f values of the bands obtained on the plate

S. No.	Color	R_f
1	Brown	0.33
2	Pink	0.52
3	Green	0.69
4	Intermediate(brown-pink)	0.41

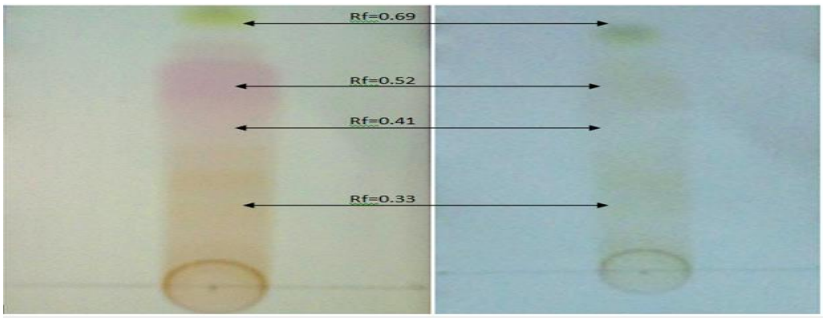
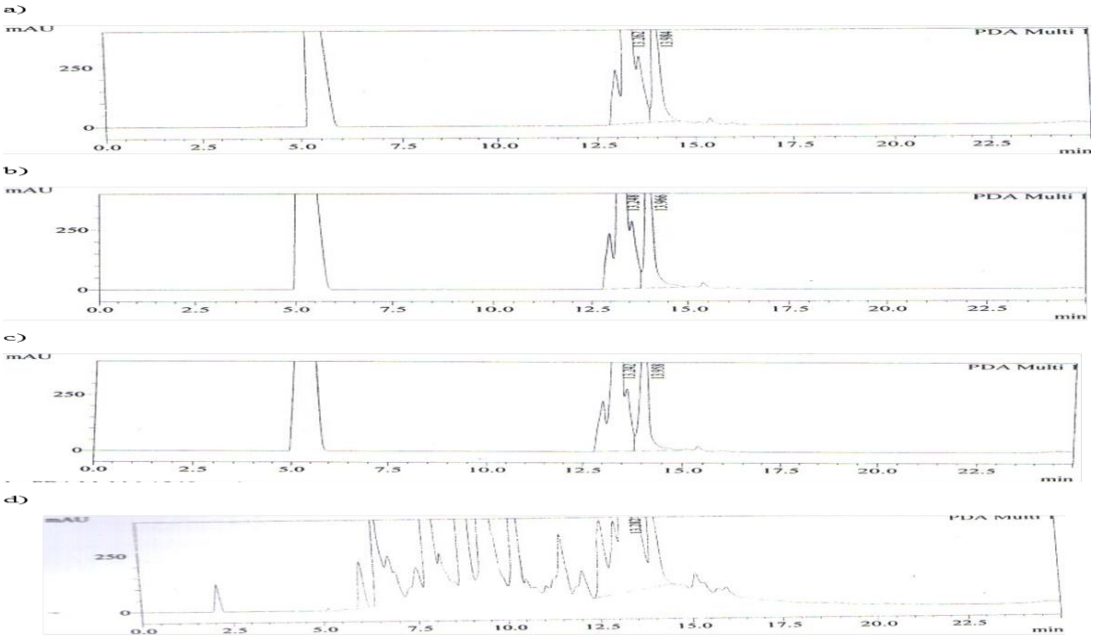


Figure 1: (a) TLC plate viewed under UV, (b) TLC Plate viewed under visible light

The 3 bands (brown, pink and green) were scraped and dissolved in the mobile phase methanol and subjected to further analysis using HPLC. HPLC analysis of the samples yielded peaks at different retention times ranging from 5-15 min. The nature of peaks observed in each of the samples were similar to those of the positive control, but had varied levels of disturbance. One peak, having a retention time of approximately 13 min, was consistently found in all of the samples s shown in Figure 2. This peak may be epicatechin. Further analysis could accurately identify and quantify specific flavonoids. Based on the phytochemical analysis (TLC, HPLC) of green tea extracts it was observed that the extracts predominantly contained compounds of the class catechins.



(a) Brown band of TLC plate; (b) Pink band of TLC plate; (c) Green band of TLC plate; (d) Positive control

Figure 2: HPLC chromatograms of the bands obtained from TLC and control

Calculation of physicochemical properties

The Physicochemical properties of the compounds are indicated in Table 3. Based on Lipinski Rule of five compounds namely 2,4,12 and 19 were eliminated for docking studies.

Table 3: Physicochemical properties of green tea extract ligands (Ligands with violations indicated in Bolt)

S. No.	CID	S+LogP	S+LogD	M Log P	M. Wt.	HBDH	M_NO	T_PSA	Rule of 5
1	44257125	1.204	1.184	1.007	304.302	4	6	99.38	0
2	72277	0.637	0.445	-0.002	306.274	6	7	130.61	1
3	14332899	1.076	1.061	1.007	304.302	4	6	99.38	0
4	107905	2.363	1.963	-0.489	442.382	7	10	177.14	1
5	2301	0.519	0.499	0.246	290.274	5	6	110.38	0
6	5280343	1.958	1.529	-0.235	302.242	5	7	131.36	0
7	21633047	1.687	1.681	1.25	318.329	3	6	88.38	0
8	9064	0.775	0.746	0.757	290.274	5	6	110.38	0
9	2302	1.529	1.186	-0.235	302.242	5	7	131.36	0
10	176920	0.666	0.634	0.248	320.301	5	7	119.61	0
11	2519	-0.153	-0.153	0.082	194.194	0	6	61.82	0
12	107905	2.17	1.574	-1.212	458.381	8	11	197.37	2
13	5280445	2.428	2.007	0.525	286.243	4	6	111.13	0
14	155660	4.264	4.253	2.389	388.464	4	6	99.38	0
15	2303	2.656	2.637	0.668	454.436	4	9	134.91	0
16	73160	0.775	0.746	0.757	290.274	5	6	110.38	0
17	5280443	2.858	2.53	1.296	270.243	3	5	90.9	0
18	21633048	2.207	2.204	1.488	332.356	2	6	77.38	0
19	5281672	1.723	0.979	-0.983	318.241	6	8	151.59	1
20	2304	2.682	2.502	0.457	440.409	5	9	145.91	0
21	9995184	1.162	1.15	1.007	304.302	4	6	99.38	0
22	44668576	3.092	3.059	1.948	360.41	4	6	99.38	0
23	5280863	2.243	1.848	0.525	286.243	4	6	111.13	0
24	21676357	1.274	1.245	0.316	428.398	5	9	153.75	0
25	72276	0.775	0.746	0.757	290.274	5	6	110.38	0

Docking and QSAR studies

Docking studies were performed for 21 compounds and the binding energy obtained from them was used as one of the variables for QSAR studies as indicated in Table 4. The binding energies of the green tea extract ligands were in the range of -6.25-12.67 kcal/mol. The final equation of QSAR studies was obtained by plotting binding energies of the compounds against its Log p as shown in Figure 3. Out of the 21 molecules selected to generate the QSAR model, 12 molecules showed correlation between their structure and activity in terms of binding energy and lipophilicity.

Table 4: Variables used for QSAR studies

S. No.	CID	Compound	S+LogP	Binding energy (kcal/mol)
1	44257125	Catechin-7 methyl ether	1.204	-11.0219
2	14332899	3-O-Methylcatechin	1.076	-9.81547
3	2301	Epigallocatechin modified	0.519	-10.657
4	5280343	Quercetin	1.958	-8.65
5	21633047	Catechin 7,4' dimethyl ether	1.687	-10.8212
6	9064	Catechin	0.775	-11.5197
7	2302	Myricetin modified	1.529	-6.25083
8	176920	Ourteacatechin	0.666	-9.48534
9	2519	Caffein	-0.153	-4.99424
10	5280445	Luteolin	2.428	-12.67
11	155660	Heptyl-3-catechin	4.264	-12.6142
12	2303	Epicatechn-3-gallate modified	2.656	-4.97623
13	73160	Catechol	0.775	-11.9148
14	5280443	Apigenin	2.858	-10.7
15	2163308	Catechin 5,7,4' trimethyl ether	2.207	-11.3284
16	2304	Epigallocatechin gallate 3 modified	2.682	-7.25501
17	9995184	4' O-Methylcatechin	1.162	-10.9771
18	5280863	Kampferol	2.243	-10.1
19	21676357	LMPK12020108	1.274	-7.04795
20	72276	Epicatechin	0.775	-10.19
21	44668576	Penta methyl catechin	3.092	-6.32

QSAR equation:

Binding energy = - 0.2656 (± 0.2319) Log P - 4.2459 (± 0.4218)

(n = 12 ; R = 0.628 ; s = 0.356 ; F = 6.509 ; p = 0.0288 ; Q2 = 0.198 ; SPress = 0.410 ; SDEP = 0.391)

Figure 3: QSAR model equation

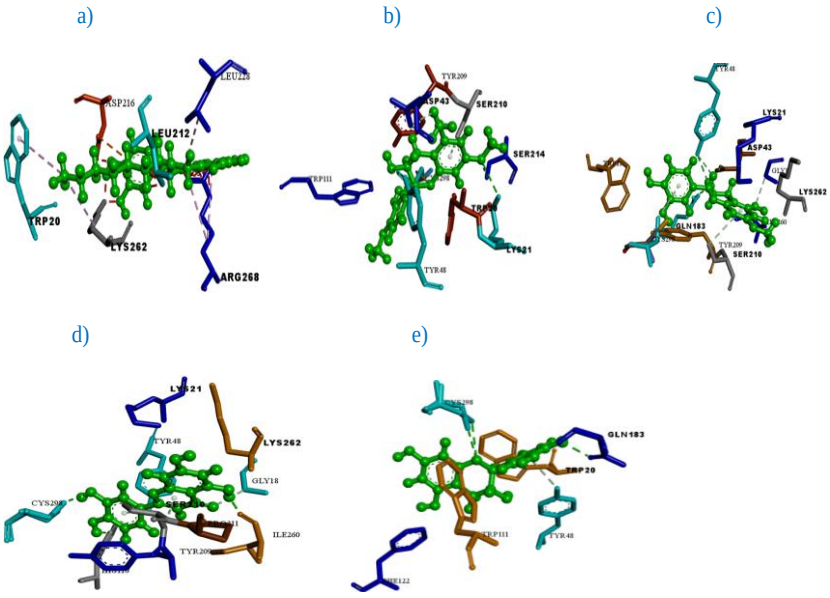
Toxicity evaluation

Of the 12 compounds that showed correlation between their activity and structure 5 compounds viz., 155660 (Heptyl 3 catechin), 21633048 (Catechin 5,7,4'-trimethyl ether), 44257125 (Catechin-7 methyl ether), 5280445 (Luteolin) and 73160 (Catechol) did not exhibit any mutagenic, carcinogenic, irritant and reproductive toxicity and also observed to have good drug score.

Binding interactions

The binding pocket of the aldose reductase enzyme identified using Meta Pocket contains the following residues GLY18, THR19, TRP20, LYS21, SER22, PRO23, PRO24, ASP43, HIS46, VAL47, TYR48, GLN49, ASN50, LYS77, TRP79, CYS80, LEU108, HIS110, TRP111, THR113, PHE115, PHE121, PHE122, SER159, ASN160, GLN183, ILE184, TYR209, SER210, PRO211, LEU212, GLY213, SER214, PRO215, ASP216, PRO218, TRP219, LEU228, GLU229, LYS234, THR243, THR244, ALA245, ILE260, PRO261, LYS262, SER263, VAL264, THR265, ARG268, GLU271, ASA272, VAL297, CYS298, ALA299, LEU300, LEU301, CYS303, THR304, TYR309, PRO310, PHE311. Out of the 21 compounds docked to determine their binding interactions and QSAR studies, 5 compounds exhibited better binding energies in the range of -10.8212 kcal/mol to -12.67 kcal/mol and interactions with the binding pocket.

Figure 4 depicts the interactions of five potent flavonoids of green tea i.e., 155660 (Heptyl 3 catechin), 21633048 (Catechin 5, 7, 4'-trimethyl ether), 44257125 (Catechin-7 methyl ether), 5280445 (Luteolin) and 73160 (Catechol) with the enzyme. Heptyl 3 catechin interacted with TRP20, LEU212, ASP216, LEU228, LYS262, and ARG268. Catechin 5, 7, 4'-trimethyl ether interacted with TRP20, LYS21, ASP43, TYR48, TRP111, TYR209, SER210, SER214 and CYS298. Catechin 7-methyl ether interacted with GLY18, LYS21, ASP43, TYR48, TRP111, GLN183, TYR209, SER210, ILE260, LYS262, and CYS298. Luteolin interacted with GLY18, LYS21, TYR48, HIS110, TYR209, SER210, PRO211, ILE260, LYS262, and CYS298. Catechol interacted with TRP20, TYR48, TRP111, PHE122, GLN183, and CYS298. The catechins mostly formed H-bonds with LYS262.



(a) Interaction of Heptyl-3 catechin; (b) Interaction with Catechin 5, 7, 4'-trimethyl ether; (c) Interaction of Catechin-7 methyl ether; (d) Interaction with Luteolin; (e) Interaction with Catechol (all ligands are in green colour).

Figure 4: Interaction of ligands with aldose reductase enzyme

CONCLUSION

The phytochemical studies enabled us to understand that green tea extract was indeed a rich source of flavonoids. Maximum amount of flavonoids can be extracted at room temperature, by continuous agitation from powdered samples using alcohol as the solvent. It is suggested that water extraction yields pure samples compared to alcohol extraction. Further purification and estimation methods must be carried out to quantify individual flavonoids. Docking studies were carried out for 21 compounds with aldose reductase, out of which 12 compounds showed correlation in their structure and activity on performing QSAR studies. The interactions with the binding pocket of aldose reductase enzyme indicate that Heptyl 3-catechin, catechin 5,7,4'-trimethyl ether, catechin-7 methyl ether, luteolin and catechol could be considered as the probable inhibitors of the enzyme. Hence these phytochemicals in green tea could be used to combat the complications of diabetes i.e. retinopathy, neuropathy and nephropathy.

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