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Short Communication

PROTEIN PRECIPITATION BY TEA POLYPHENOLS, *IN-SILICO* ANALYSIS OF ITS BIOAVAILABILITY, AND BINDING TO SARS-COV-2

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ABSTRACT

This study is undertaken to see precipitation of protein non-specifically by tannin in tea, a plant secondary metabolite. Tannins that are present as a part of the defense system of the plant get oxidized to its quinone/semiquinone state and readily react with protein to form a precipitate. In this study, we have estimated the polyphenol content of Assam tea which was decreased by 9.33% due to the precipitation reaction with BSA. We have visualized the precipitation of protein by tannin/polyphenol. In our studies, we have tried to remove highly reactive or protein precipitating polyphenols from ready-to-drink black tea with the help of charcoal beads. Bioavailability, including absorption, metabolism, distribution, and excretion of different types of tea polyphenols in the human body have been assessed using bioinformatics methods. Polyphenols are high molecular weight compounds and are less absorbable through the GI tract. We have also analyzed the binding affinity of 24 tea molecules to spike glycoprotein of SARS-CoV-2.

Keywords: Tea, Tannin, Polyphenol, Assam Tea, Charcoal Beads, SARS-CoV-2.

1. INTRODUCTION

Tea is the second most consumed beverage in the world and around two-third of the world population drink tea [1]. Tea is originated in South East Asia mainly in China, India, and Japan [1]. Tea has traces of proteins, sugars, amino acids, lipids, vitamins and minerals, polyphenols etc. [2]. Polyphenols account for the fragrance and color of tea [2]. Polyphenols are a group of plant secondary metabolites that are responsible for UV protection and defense against pathogen aggression in the plants [3, 4]. Tea polyphenols are categorised to catechins (20%-30%), flavonoids (50%-60%), thearubigins (10%), theaflavins (10%) and tannins (5.33%) [2, 5]. These phytochemicals present in tea are known to reduce heart disease, inflammation and cancer by controlling key cancer factors such as tumor necrosis factor-alpha, p53, cyclooxygenase 2 etc. [6]. However, it is important to note that many of these studies were performed *in-vitro* and the bio-availability or mechanism of action of many of these phytochemicals after absorption is not properly understood [6, 9]. In our study, we have estimated the polyphenol and the tannin content of ready to drink black tea (Assam Tea). We have also checked the bioavailability of these polyphenols by using swiss ADME.

Tea is categorized into different types depending on the process of manufacturing (CTC - crush, tear, curl) [2]. Green tea is made with minimal processing (unwilted and unoxidized), whereas black tea is made with the highest level of processing (Wilted and fully oxidized) [2]. During the manufacturing cycle, the polyphenols present in the tea are oxidized to highly reactive quinone and semiquinone forms [7]. Polyphenol oxidation is classified into two groups of reactions, namely enzymatic and non-enzymatic reactions [7]. The enzymatic reaction is catalyzed by the enzyme polyphenol oxidases (catecholase/laccases, intracellular enzymes of the plant) and oxygen [7]. Polyphenols are converted non-enzymatically to their quinone and semiquinone forms by auto-oxidation in presence of alkaline medium, metal ions or by acid-catalyzed solvolysis [7]. Low pH triggers the formation of more reactive polyphenols and tannins [7].

The highly reactive quinone form of polyphenol/tannin easily binds (non-specific) covalently or noncovalently to protein [7, 8]. The binding is due to the formation of a covalent bond or H-bond between the amine (-NH) group of protein and the oxygen (O) of the quinone component of polyphenol [8]. Amino conjugates are also formed by the reactions of amino groups and polyphenols [7]. Tannins in quinone/semiquinone form react with salivary proteins (mucin) in the buccal cavity to create astringency [9]. Tannins interact with the proteins in the

stomach to reduce the bioavailability of the digestive enzymes [9]. Mehanso *et al*. showed that tannin can cause liver damage by binding to the epithelial proteins and penetrating to the superficial cells [10]. Tannin is also reported as a tentative carcinogen by occupational safety and health administration (OSHA) of the USA [10]. Tannin used in leather industries in the process of tanning is described as a water pollutant as it affects aquatic biodiversity [11]. A study conducted in the Bogor district of Indonesia reported that regular intake of tea with tannin contributes to a decrease in iron and ferritin in the blood of pregnant women [12]. So, Rooibos tea with low tannin is usually favored in countries like South Africa [13]. Also, it is interesting to note that tannase treated green tea improved the chemopreventive potential of green tea [14]. We have tried to visualize this precipitation of protein (Bovine Serum Albumin -BSA) by tea extract. We have also estimated the change in polyphenol content of the tea extract after reaction with protein.

Tannin is removed from wastewater by using natural polymers, organic polymers, and inorganic polymers but there is very little research done to selectively remove the highly reactive tannin or protein precipitating components from tea [15]. Natural polymers like collagen fibers and macro reticular resins were used to remove polyphenols with galloyl groups [16]. Inorganic polymers like attapulgite clay (at pH 6), Carbon Nano Cage (CNC), polyacrylamide gel was made to separate components like tannic acid, catechin, etc [15, 17, 18]. Collagen fibers did not remove the highly polymerized polyphenols [16]. CNC is not very cost-effective and polyacrylamide with its potential to form acrylamide might be harmful [18-20].

Charcoal selectively absorbs tannic acid at a higher temperature and is used to treat drinking water [21, 22]. We have made charcoal beads and estimated the change in polyphenol content after treating the tea with charcoal beads, which might help to remove the highly reactive polyphenols and tannin from tea. This method could help us to make tea a better beverage in the future by removing its harmful or protein precipitating effect.

The present pandemic of COVID-19 is caused by the novel coronavirus sars-cov-2 discovered in Wuhan, China [23]. The virus infects the cells of lower respiratory airways by binding to cell receptors (angiotensin-converting enzyme-ACE2) of alveolar epithelial cells [24]. The ACE2 of human alveoli cells binds to the receptor-binding domain (RBD) of spike glycoprotein of the virus [24]. We have docked the different tea polyphenols to the RBD of spike glycoprotein to see the binding affinity of tea polyphenols.

2. MATERIAL AND METHODS

The tea leaves were bought from the local market of brand Nameri Gold Assam Tea (NGAT) with Batch No. NTBC/19. The 1st extract (Extract-1) was prepared by boiling a hundred milligrams of tea in 10mL distilled water for 10 min. The tea extract was collected using a steel strainer and the cycle mentioned above was repeated twice with fresh distilled water. The total volume of tea extract collected was 7.2 mL. The 2ndtea extract (Extract-2) prepared by heating 1g of tea in 25mL distilled water at 100°C for 10 minutes [25]. Both the extracts were centrifuged at 7500 rpm for 10 minutes in a mini centrifuge by Tarsons and the supernatant was stored in the LG refrigerator at 4°C. Extract-1 was diluted 10 times and the polyphenol concentrations of both the extracts were estimated using Folin's reagent (2N) according to the method of Zhang et al [26]. Gallic acid (Himedia A.R) was used as a standard in the polyphenol estimation.

The radial diffusion assay was also performed to see the protein precipitation by tannins in tea extract (Extract-2) [27]. One Petri plate without BSA is kept as a control and another plate has BSA in it (0.2 $mgmL^{-1}$). Tea extract-2 of 100µL was poured to the wells in both the Petri plates and stored at 37°C for 5 hours in an equitron incubator. Also, the tannin content in extract-2 was estimated using tannic acid (Loba chemicals- A.R.) as standard [28]. The extract-2 was diluted 3-times before estimating tannin content using folin ciocalteau reagent of commercially available 2N concentration (Himedia A.R). The charcoal beads were prepared by adding 4 grams of charcoal powder (Himedia) to 10 ml of 3% Na-alginate solution [29]. The beads are produced when the solution mixed with charcoal dropped from a height of about 20 cm with a syringe to 100 mL of 0.2 M $CaCl_2$ solution at room temperature (temp). The beads were stored at room temperature [29].

ADME (Absorption, Distribution, Metabolism, and Excretion), gastrointestinal (GI) absorption of 33 different molecules in tea extract were analyzed using swiss ADME webtool in order to check the bioavailability of the molecules [30, 31]. The 33 molecules analyzed are Afzelechin, Apigenin, Assamician-A, Caffeic Acid, Quinic Acid, Catechin, Chlorogenic Acid, Coumaroyl-quinic Acid, EGCG, Epitheaflavic acid 3' gallate, Eriocitrin, Gallic acid, Kaemferol, Leucocynidin,

Myercitin, Pentagalloyl Glucose, Proanthocyanidin, Quercetin, Theaflagallin, Theaflavinin, Theaflavin 3 gallate, Theaflavin 3' gallate, Theaflavin3,3' digallate, Theaflavin, Theogallin, Camelliatannin-a, Pheophorbide-a, Pheophorbide-b, Theasinensin-a, Theasinensin-f, Theobromine, Theanine, Spinasterol [30]. The docking of 24 molecules found in tea was performed against RBD of spike glycoprotein of SARS-CoV-2 by using Auto Dock Vina [32]. The 24 ligands for docking were downloaded on the basis of available 3D structure in pubchem database. The conformations with the highest binding energy and RMSD (root mean standard deviation) as zero were chosen for analysis. Pymol and discovery studio visualizer was used to visualize and analyze the binding site and polar interactions of ligand and receptor [33, 34].

3. RESULTS

The total polyphenol of NGAT estimated in tea extract-1 is 0.647 $mgmL^{-1}$ (GAE). The standard error in mean calculation (SE) in the estimation of total polyphenol is 0.049. The total polyphenol content in NGAT is estimated as 43.2mg/g of dry tea weight.

The precipitation of protein by tea extract-2 was visualized in agar plate diffusion assay. No precipitation ring was formed in the control petri-plate suggesting that precipitation ring is formed only in the presence of BSA i.e. protein (Fig.1). The precipitation ring of 2mm is formed in the plate with BSA and the diffusion of 6mm is observed in the petri-plates.

Fig. 1: Agar petri-plates with and without BSA respectively. It shows the formation of a precipitation ring in the presence of BSA.

It was seen a 9.33% decrease in the polyphenol content in the tea extract due to the formation of the precipitate with BSA (Fig.2). The polyphenol concentration changed from 0.762 mgmL-1 (GAE) to 0.691 mgmL-1(GAE) in a freshly prepared extract-1 due to the precipitation reaction with protein (BSA-0.2 $mgmL^{-1}$). SE in polyphenol estimation for freshly prepared extract-1 before precipitation reaction is 0.0303 and after the reaction is 0.0311.

The tannin content in the tea extract-2 was estimated as 0.563 $mgmL^{-1}$ which decreased to 0.5072 $mgmL^{-1}$ due to precipitation by BSA. There was a 9.91% decrease in the tannin content of the tea extract due to the formation of tea-BSA precipitate.

Charcoal Beads were used to absorb highly reactive polyphenols. The polyphenol concentrations were estimated at different time intervals while stirring the charcoal beads in extract-2. We found that the crude extract-2 contains 1.529 $mgmL^{-1}$ (GAE) polyphenol which decreased to 1.410 $mgmL^{-1}$ at 5 minutes and 1.219 mgmL-1 at 20 minutes after adding the charcoal beads (Fig.3). Two grams of charcoal beads decreased the total polyphenol content by 7.78% and 20.24% of the extract at 5 minutes and 20 minutes treatments respectively.

From ADME analysis of the 33 bioactive tea molecules we have found only 11 molecules to have high gastrointestinal (GI) absorption. The molecules are afzelechin, apigenin, caffeic acid, catechin, coumaroyl-quinic acid, gallic acid, kaempferol, leucocyanidin, quercetin, theobromine, l-theanine. Besides GI absorption they also have high drug likeliness. The lipophilicity value of 33 molecules suggests that spinasterol has the highest lipophilicity value followed by pheophorbide-A and apigenin. Overall, 17 molecules (of 33) show high lipophilicity with Consensus Logp>1. Among the molecules, with high GI absorption Afzelechin, Apigenin, kaempferol, quercetin has good lipophilicity also. However, none of the tea molecules have shown blood-brain barrier permeability. Most of the molecules have shown good solubility with log S>-5.0. The highest solubility is shown by theobromine.

Docking was performed between 24 bioactive molecules in tea and the ACE2 binding domain of spike glycoprotein (PDB-ID: 6W41). Blind docking has given us the binding affinities of different ligands to specific target protein sites. The highest binding energy was shown by theaflavin, eriocitrin, myricetin, proantho-cyanidin, quercetin, theaflagallin (Table 1). In Pymol we visualized the docking sites and bonds and interaction.

The interactions of tea molecules with different residues in spike glycoprotein RBD indicate that tea molecules do not imitate the polar binding of ACE2 residues to RBD.

Catechin and ACE2 use the same Gln474 residue in RBD binding. Tea molecules have a high binding affinity towards the spike glycoprotein of SARS-CoV-2.

An extended loop of the RBD interacts with the alpha-1 helix of ACE2 [35]. Alpha-2 helix of the ACE2 interacts with the beta 3/4 strands of RBD [35]. In the polar interactions Gln498, Thr500, and Asn501, Lys417, Tyr453, Gln474 and Phe486 of the RBD forms bonds with Tyr41, Gln42, Lys353, Arg357, Asp30, His34, Gln24, Met82 respectively of ACE2 [35]. The visualization of the polar interactions of the spike glycoprotein to tea polyphenols shows that tea polyphenols don't interact with the same residues as ACE2.

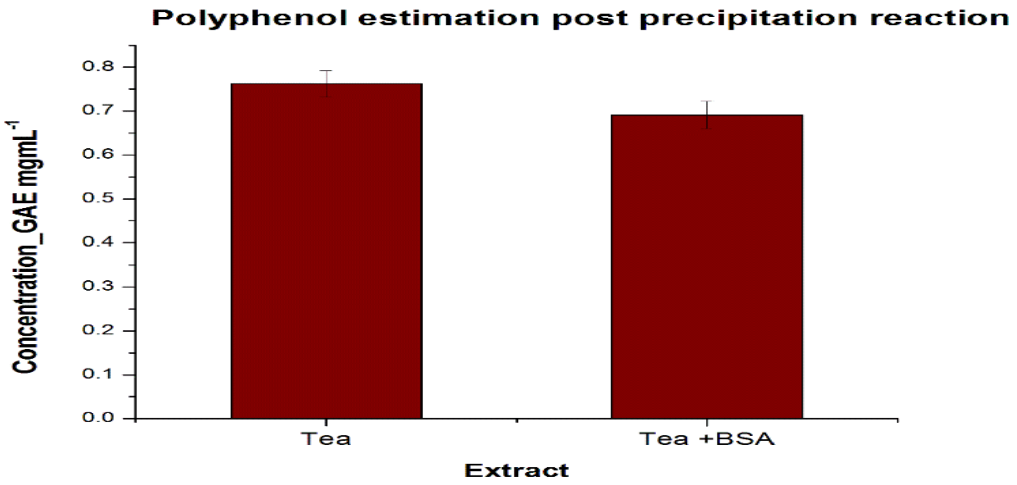

Fig. 2: Polyphenol concentration before and after precipitation reaction

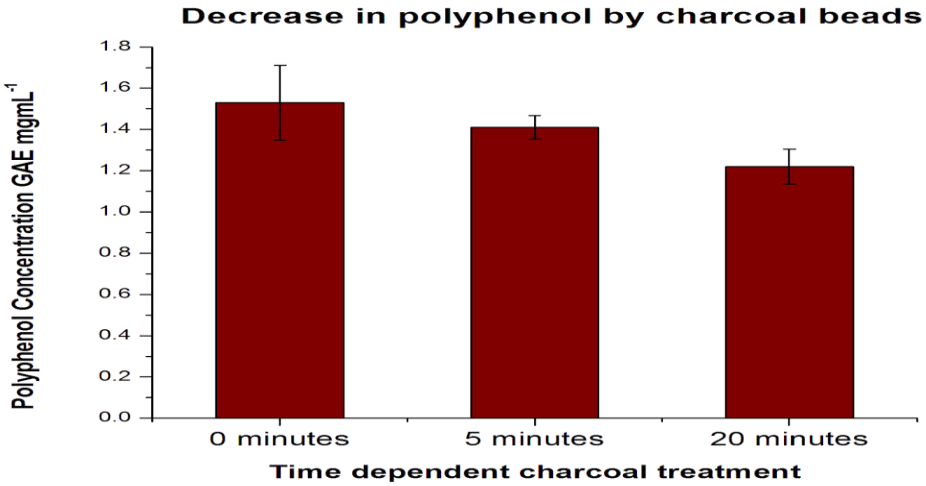

Fig. 3: Time-dependent decrease in total polyphenol content by the charcoal beads.

Table 1: Binding energies of tea molecules to spike glycoprotein of SARS-CoV-2

Ligand (Tea Molecules)	Binding Energy (Kcal/mol)
Theaflavin	-8
Eriocitrin	-8
Myricetin	-7.6
Proanthocyanidin	-7.6
Quercetin	-7.4
Theaflagallin	-7.4

4. DISCUSSION

The polyphenol concentration of NGAT is found to be 43.2 GAE mg/g which is slightly higher than that of Darjeeling tea [35].The polyphenol content of Darjeeling tea is 36 GAE mg/g [36]. Our results show

slightly higher polyphenol content because tea polyphenol content is also dependent on the extraction procedure as well as the tea shoot quality [37-39]. We have used water to extract the polyphenol as we wanted to mimic the tea making process in the households. The polyphenol content of the tea leaves bud is highest, which decreases towards the inter-node of the tea leaves [39]. The total amount of polyphenols decreased by 9.33 percent when interacting with BSA. BSA is taken as standard protein as tannin binds non-specifically to protein. Approximately 9.93 percent decrease in tannin content after precipitation reactions with BSA is observed. The binding of proteins is also visualized by an agar plate diffusion assay as the plate that contains BSA forms a precipitation ring with protein. It shows the binding of proteins to tannins/ polyphenols as a whole. It is interesting to note that people take both milk tea and black tea and it is expected that milk tea would help to protect the body proteins as milk proteins might form a precipitate and protect our body protein from the precipitation effect. But research indicates that there is no change in the blood plasma level of polyphenols due to the consumption of black tea and milk tea [40]. The highly reactive polyphenols in black tea might form a precipitate with body proteins like mucin before entering the body which might be the reason that we get the same level of polyphenols entering our plasma.

We have used charcoal beads to remove the highly reactive tannins/polyphenols. The 20.24% decrease in total polyphenol content in tea due to charcoal beads suggests that it can be used to remove harmful polyphenols or highly reactive polyphenols from tea extract. Even Though charcoal is highly effective in removing polyphenols at higher temperatures, charcoal calcium alginate beads are not stable at high temperatures. Tea is taken at a high temperature usually in Indian households so it might pose a challenge.

Most polyphenols are high molecular weight compounds that have the least absorption through the gastrointestinal tract. The high molecular weight polyphenols might be excreted out as precipitate with protein. Due to lockdown caused by covid-19 we were not able to carry out further wet-lab experiments. We have tried to perform *in silico* analysis of the possible inhibitory effect of tea molecules against covid-19.

The spike glycoprotein of SARS-CoV-2 interacts with ACE2 receptors of host cells and mediates membrane fusion for entry to the host cells, so the spike glycoprotein could be possible drug targets for blocking the virus entry to cells [41]. The inhibition of virus entry to the cell will also be effective in reducing the cytokine /inflammatory storm caused by covid-19 [42]. The highest binding energy shown by theaflavin, eriocitrin, myricetin, proanthocyanidin, quercetin, theaflagallin reveals its potential against covid-19. The flavanols like eriocitrin is also found to be effective diseases like hepatic steatosis [43]. The binding affinity (>7.4 kcal/mol) shown by the tea molecules is considered as a high binding affinity of flavanols against SARS-CoV-2 [41]. The binding energy of tea molecules to RBD of spike glycoprotein is higher than that of hydroxychloroquine. The binding affinity of hydroxylchloroquine spike glycoprotein is -5.6 kcal/mol [44]. Quercetin found in tea has high binding energy (7.4kcal/mol) as well as GI absorption. The interactions between ACE2 and RBD are mainly polar [35]. The docking of tea polyphenols especially theaflavin to spike glycoprotein of sars-cov-2 showed the high binding affinity but they don't inhibit directly. Inspite, the polyphenol binding residues might play inhibitory role by some other properties like steric hinderance. This suggests that tea can be effective in reducing the binding of virus to human body cells. There is no evidence till now that tea molecules directly inhibit sars-cov-2. The binding energy of theaflavin is -8 Kcal/mol and that of catechin is -7.1kcal/mol. The binding energy of catechin to Mpro (Covid-19 main protease) as -7.4 kcal/mol (Inhibition constant= 4.95μM) [45]. Drinking tea might inhibit the virus entry to the cells to a great extent. However, more research is needed to establish the effect of polyphenols on the covid-19 and health.

5. CONCLUSION

Polyphenols with their ability to precipitate proteins can cause adverse health problems even though a lot of health benefits are associated with tea as described above. Tea polyphenols with its low gastrointestinal absorption may precipitate body proteins in the GI tract even before any health benefits might occur. In this way, harmful components could play their function well before any gain could arise. We have tried to eliminate protein precipitation components from tea and we have seen a decrease in the amount of polyphenol in tea due to charcoal beads. This may be an innovative way to remove protein precipitating tannin from tea. But further work could lead to an easily available household product that extracts tannin from tea. It might lead us to make tea a better drink in the future. Some of the tea

molecules with its high binding affinity towards SARS-CoV-2 has shown promising results for the treatment against covid-19.

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Conflict of interest

Authors have no conflict of interest.

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