International Journal of Current Biotechnology
ISSN: 2321 - 8371
Journal Homepage : http://ijcb.mainspringer.com

Extraction of Theaflavin from Black Tea and Its Bioconjugation with Chitin

Ganesan Vijaiyan Siva*, Gothandam Kirubananthan and Chinnaiah Alagarasan

Department of Biotechnology, University of Madras, Guindy Campus, Chennai-600025, India.

ARTICLE INFO

Article History:
Received 08 July 2017
Received in revised form 14 July 2017
Accepted 20 July 2017
Available online 30 July 2017

Key words:
Theaflavin, chitin, chitin–theaflavin bioconjugates, *Camellia sinensis.*

ABSTRACT

Fresh leaves of black tea are crushed and allowed to undergo oxidation induced by polyphenol oxidase resulting in the formation of poly phenolic dimer known as theaflavins. Tea and its derivatives have different polyphenol profiles, which are the bioactive chemical entities. This study was designed to confirm the presence of theaflavin in *Camellia sinensis* extract and the development of chitin–theaflavin bioconjugates. They were analysed by applying spectrometry, TLC, FT-IR, and GC-MS. The advantages of such a product could be effected on drug delivery in chronic disorders viz., diabetic, anti-inflammatory, protective agents against cardiovascular and cerebrovascular diseases, aging etc.

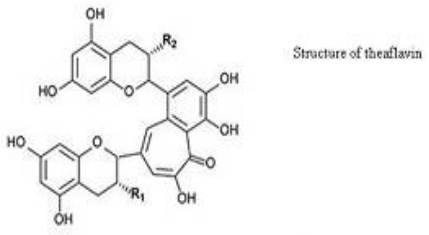
Introduction

Camellia sinensis (black tea) is one of the most popular beverages in the world and has been used as a daily beverage in Asia including India, China and Japan for thousands of years. It contains numerous compounds, such as polyphenols, theanine, theaflavins (TF), minerals, and purine alkaloids (Hayat *et al*., 2015; Mizukami *et al*., 2007; Tao *et al*., 2016). On the basis of manufacturing process, three different types of tea are produced, such as non-fermented green tea, semi-fermented oolong tea, and fully fermented black tea. Fermentation of tea leaves induces enzymatic oxidation (polyphenol oxidase) of flavan-3-ols that leads to the formation of black tea, TFs, and thearubigins (TRs) (Menet *et al*., 2004). Among them, TFs account for 2–20 g/kg of the dry weight of solids in brewed black tea (Sang *et al*., 2004). TFs are a type of polyphenol present in black tea comprises an aromatic ring with one or greater hydroxyl substituents. Phenolic materials commonly are water-soluble and are often blended with a sugar as glycosides and are commonly placed in the vacuole. TF is orange-red in colour and contains a seven-membered benzotropolone ring**.** TFs are mainly of four types, TF1, theaflavin-3-O-gallate (TF2A), theaflavin 32 -O-gallate (TF2B) and theaflavin-3, 32 -di-O-gallate (TF3), formed by the co-oxidation of selected pairs of green tea catechins (one di-hydroxy flavan-3-ol and one tri-hydroxy flavan-3-ol) during fermentation.

*Corresponding author.
Email address: gvsbio@gmail.com

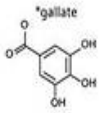
Several meta-analyses have also shown that consumption of black tea results in significant primary prevention of cardiovascular diseases as a consequence of decreased levels of plasma LDL cholesterol and blood pressure (Hartley *et al*., 2013 Santesso *et al*., 2014). TFs are major chemical constituents of black tea having anti-cancer (Gosslau, 2011), anti-inflammatory (Zu, 2012), cerebrovascular diseases, aging, caries formation effects (Kong *et al*., 2015; Kundu *et al*., 2006; Miller *et al*., 1996; Wu *et al*., 2016). Black tea has antioxidant, antimicrobial (Almajano, 2008; Friedman, 2007), and antiviral abilities, including bovine coronavirus, bovine rotavirus (Clark, 1998), HIV-1 (Liu, 2005), influenza (Zu, 2012) and HSV-1 (Cantatore, 2013). The flow-mediated dilatation (FMD) was increased significantly 2 h after ingestion of black tea, with this increase being similar to that observed in green tea (Jochmann, 2008) and ability of black tea flavanoids to increase FMD occurred in a dose-dependent manner (Grassi *et al*., 2009). The involvement of the sympathetic nervous system on hemodynamic changes induced by TF, evaluated using an adrenaline receptor blocker (Akiko *et al*., 2016).

Chitin $(C_8H_{13}O_5N)n$ is a biopolymer of an N-Acetyl glucosamine (GlcNAc / **A**-unit)with â-1,4 glycosidic linkages similar to cellulose found in abundance in marine arthropods such as crustaceans (e.g., crabs, lobsters and shrimps) and insects, fungi and other soft tissues of fish conjugated with proteins (Merzendorfer and Zimoch, 2003). It is a polysaccharide that contains nitrogen; it is synthesized from the units of N-acetyl-D-glucosamine (to be precise, 2-(acetyl amino)-2-deoxy-D-glucose). Therefore, chitin may be described as cellulose with one hydroxyl group on each monomer replaced with an acetyl

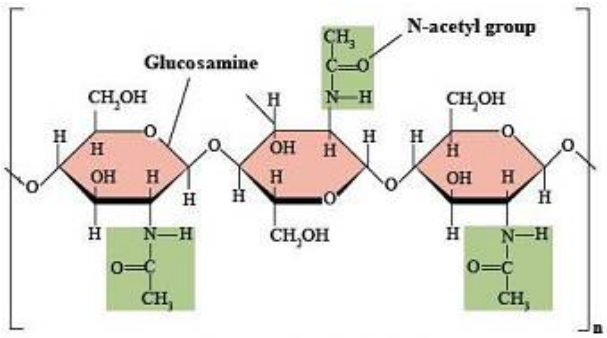


Structure of theaflavin

	R_1	R_2
Theaflavin	OH	OH
Theaflavin-3-gallate	*gallate	OH
Theaflavin-3'-gallate	OH	*gallate
Theaflavin-3,3'-digallate	*gallate	*gallate



Structure of Chitin



amine group. This allows for increased hydrogen bonding between adjacent polymers, giving the chitin–polymer matrix increased strength (Gilbert and Lawrence, 2009). Chitin derivatives such as Cationic chitosan in mixture with different herbal polymers has been shown to increase the drug encapsulation performance of liposomes via the layer-by-layer (L-b-L) self-assembly method (Haidar *et al*., 2008).

Materials and Methods

Chemicals

Standard TF and chitin were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All other chemicals such as acid, bases, solvents and salts used for the investigation were of analytical grade (AR) and were obtained from Glaxo Laboratories, SRL, Mumbai, India and Fisher Inorganics and Aromatics, Chennai.

Sample Collection

500 gm of commercially available tea powder was procured from the local market of Nilgiri hills region of Tamilnadu, India. Chitin was isolated from shells of *Portunus sanguinolentus* (Ganesan Vijaiyan Siva *et al*. 2017).

Preparation of Black Tea Extract

The powdered material (125 g) was packed in the filter paper and placed gently in the soxhlet apparatus and treated with both ethanol and hexane separately. Then extraction was carried out for 15 cycles at 50°C. The crude extract obtained was further concentrated by using rotatory evaporator at 250 rpm for 72 hr at room temperature and used for further studies.

Isolation of TF from *Camellia sinensis*

Ethanol extract of *Camellia sinensis* was subjected to column chromatography on silica gel (60–120 mesh size), which was eluted with mobile phase constituted of solvent A (0.1% formic acid) and solvent B acetonitrile) with gradient elution, i.e. solvent B was increased from 7 to 45% within 30 min and then dramatically decreased to 7% within 5 min. The flow rate was 1.4 ml/min and detection was made at 260–280 nm. The eluted fractions were analyzed by thin-layer chromatography (TLC).

TLC Chromatogram of TF

Using micropipette, about 10 µl of 1% w/v solution of ethanol black tea extract was loaded on the line drawn from the bottom of the pre-coated aluminium with silica gel 60 F 254 TLC plate (20 × 20, 0.5 mm thick) and placed in chloroform–methanol with a ratio of 2:3 (*v/v*) solvent system. The plates were developed by dipping them in iron (III) chloride-ethanol reagent resulted in the separation of TF by applying two-dimensional methods. They were examined under the UV-Vis lamp, and the spots were identified.

Gas Chromatography –Mass Spectrometry (GC-MS)

GC-MS analysis was carried out on an Agilent GC-MS (122-5532) system interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column DB-5MS (30 m x 250 µm x 0.25 µm), operating in electron mode at 70eV, helium as a carrier gas at a flow rate of 1 ml/min. 10 µl of each ethanol and hexane extract was injected in to the column and maintained oven temperature of 70°C with column and analyzed for 30 min. Interpretation of mass spectral detail was carried out by using Mass hunter library version NLST14.L software.

Preparation of Chitin–Theaflavin–Yeast Bioconjugate and Theaflavin–Yeast

250 gm of commercially available tea powder was boiled in 500 ml of water, cooled at room temperature and filtered. The filtrate measuring 250 ml was supplemented with 1% glucose and incubated with overnight grown cultures of yeast (*Saccharomyces cervisiea,* OD-1.8) and it was kept on a rotary shaker at 250 rpm for 72 hr. The phenolic content was estimated by the method suggested by Krishnan and Maru (2006), and also examined by FTIR and spectrometry. Equal amount of chitin and fermented filtered tea extract was further incubated for 48 hr at room temperature in a rotary shaker at 250 rpm. Chitin with theaflavin bioconjugate was obtained by centrifugation at 10000 rpm for 15 min. The bioconjugated chitin with theflavin was dried and analysed by FTIR and spectrometry.

Preparation of Chitin–Theflavin Bioconjugate

Chitin was isolated from shells of *Portunus sanguinolentus* (Ganesan Vijaiyan Siva *et al*. 2017). The crude ethanolic extract was further for examined. Equal amount of chitin and ethanolic crude extract blended and kept on a rotary shaker at 250 rpm for 72 hr to get chitin–theflavin bioconjugate.

Fourier Transform-Infra Red (FTIR) Spectrum Analysis

10 mg of each sample, extracted TF, TF–yeast, chitin–TF bioconjugate, chitin–TF–yeast bioconjugate, was mixed with 100 mg of liquid tetra hydro fluorine (THF) and compressed to prepare a salt disc. The disc was then read spectrophotometerically (Perkin Elmer Spectrum two model FT-IR c101375). The infrared frequencies of different components of extracted TF, TF–yeast, chitin–TF bioconjugate, chitin–TF–yeast bioconjugate were analyzed.

Results

Spectral Analysis of Standard Extracted Chitin and TF

Figure 1. UV visible light absorption spectral curve of (A) standard chitin and (B) biologically extracted chitin.

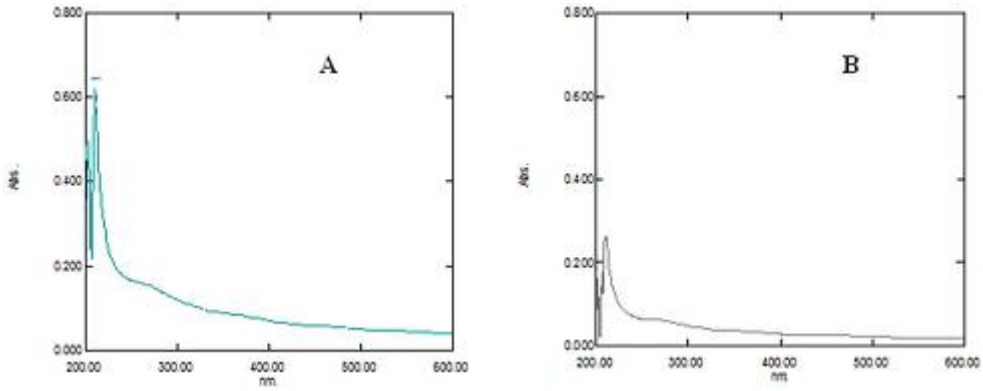


Figure 2. UV visible light absorption spectral curve of (A) standard TF and ethanolic extracted TF

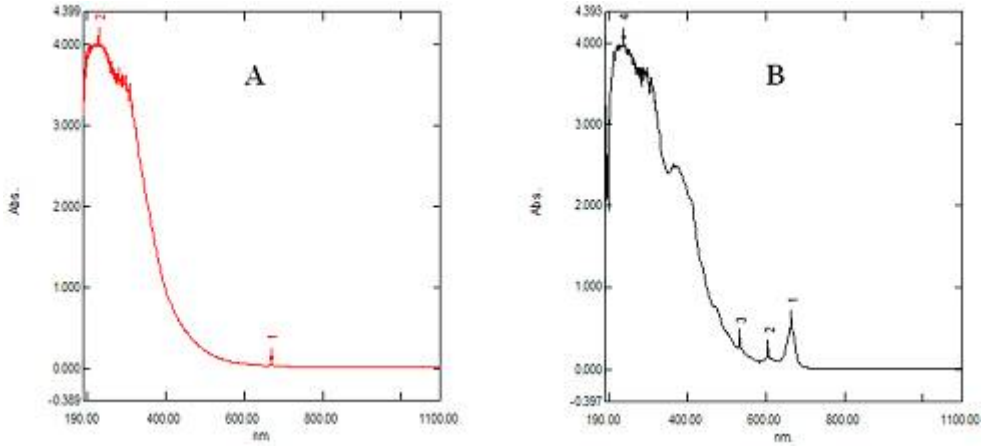


Table 1. UV visible light absorption and wavelength (ënm) of different combination of TF*

Contents	nm	absorbency
Theaflavin	245	4.000
Theaflavin +yeast	235	4.000

*All values are means of three replicates.

Figure 3. TLC chromatogram of standard and extracted theaflavin

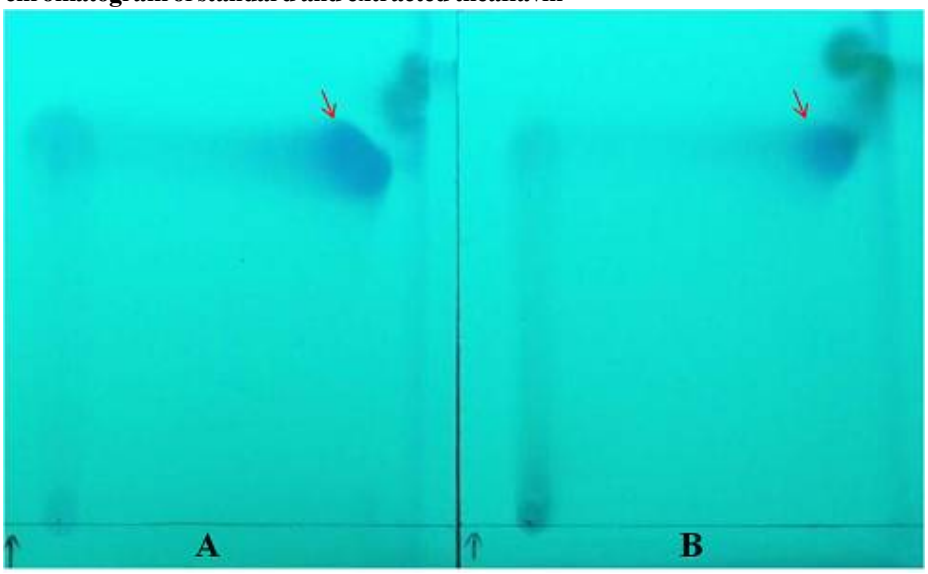


Figure 4. GC-MS image of extract theaflavin

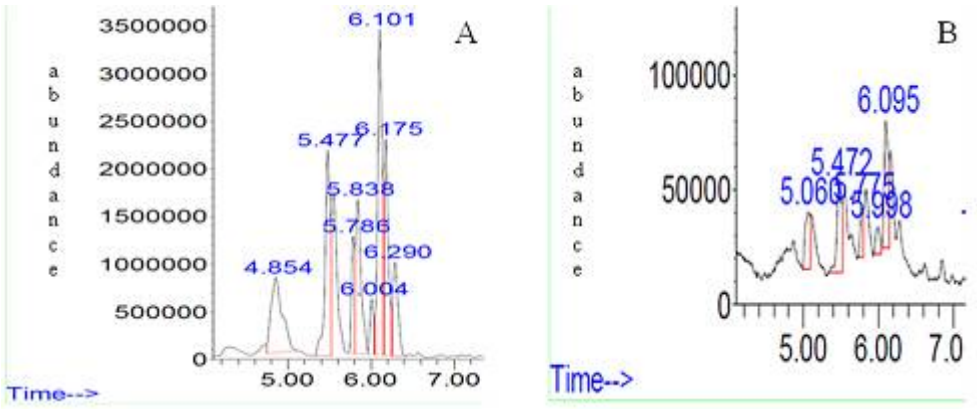
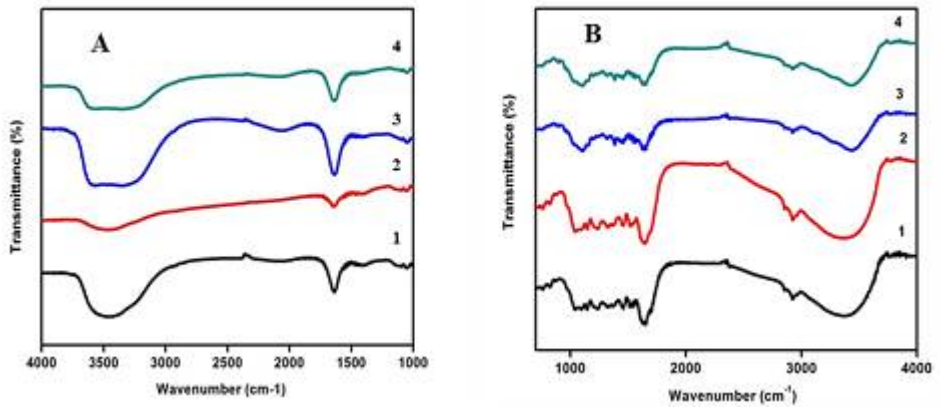


Table 2. Organic compounds present in both in ethanolic and hexane extract of black tea GC-MS

Peak no	RT	Compounds	Quality
Ethanolic extract of black tea			
1	4.848	Dodecane, 4,6-dimethyl-,	64
2	5.477	Octane, 6-ethyl-2-methyl-	59
3	5.786	1-Undecene, 7-methyl-	52
4	5.838	3-Heptene, 4-methyl-	43
5	6.004	Nonane, 1-iodo-	64
6	6.101	Decane, 3,6-dimethyl-	64
7	6.175	Decane, 3,6-dimethyl-	64
8	6.29	Undecane, 4-methyl-	64
Hexane extract of black tea			
1	5.060	2(1H)-Pyridinone, 6-hydroxy-	50
2	5.472	Dodecane, 2,6,10-trimethyl-	59
3	5.775	Cyclopentane, propyl-	46
4	5.998	1-Octadecanesulphonyl chloride	27
5	6.095	Carbonic acid, eicosyl vinyl ester	64
6	7.474	Methyl salicylate	50

Figure 5. The FT-IR spectrum of extracted TF, TF with yeast, TF with chitin and TF–chitin with yeast.



The absorbance values were measured at 210 nm and 245 nm in a UV-visible spectrophotometer (Shimadzu U-1800) for standard & extracted chitin and standard & exthanolic extract theoflavin, respectively. Fig 1 and 2.

TLC Chromatogram of TF

The standard theaflavin and ethanolic extract were analyzed for the presence of the TF. The individual sample was spotted on the TLC plates. The known sample was compared with the unknown sample and confirmed the presence of theaflavin. Fig 3.

Gas Chromatography –Mass Spectrometry (GC-MS)

Various organic compounds were present both in (A) ethanolic extract and (A) hexane extract of black tea (Table 2, Fig. 4).

Fourier Transform Infrared (FT-IR) Spectroscopy

The FT-IR spectrum of extracted TF, TF–yeast, chitin–TF bioconjugate, chitin–TF–yeast bioconjugate show stretches between 1000 and 4000 cm^{-1} for both liquid sample and dried sample (Fig. 4A and B). The results of theaflavin and yeast-treated theaflavin are same. chitin–TF bioconjugate and chitin–TF–yeast bioconjugate are similar. The results of liquid sample show single peak whereas dried sample shows multiple peaks.Fig.5.

Discussion

Extract of *Camellia sinensis* species belongs to Theaceae family is one of the most popular beverages consumed in many parts of the world. Black tea contains large amounts of TFs, formed by condensation and polymerization catechins during the fermentation process. In the GC-MS study Ethanolic black tea extracted may be have Dodecane, 4,6-dimethyl-, Nonane, 1-iodo-and Decane, 3,6-dimethyl- of various components nearest match to ethanolic black tea extracted sample quality. Methanol black tea extracted may be have Carbonic acid, eicosyl vinyl ester, Dodecane, 2,6,10-trimethyl-,2(1H)-Pyridinone, 6-hydroxy- and Methyl salicylate of various components nearest match to ethanolic black tea extracted sample quality.

In our sample, chloroform–methanol solvent system with a ratio of 2:3 (*v/v*) showed reasonably good results with two dimensional method. The plates were developed with iron (III) chloride–ethanol reagent resulted in the separation of TF by applying two-dimensional methods. Yang *et al***.** **(**2003**)** have reported that 400 mg of the crude TF mixture was dissolved in 2 ml of the aqueous phase **(**used as the mobile phase**)**, while using the Hexane, Ethanol, Methanol, Water at system 1**:**3**:**1**:**6 v/v. Wang *et al*. (2008) used a 30 mg sample of crude TF in 5 ml of the mobile phase while the solvent system used for HSCCC was hexane-ethylacetate-methanol water-acetic acid (1:5:1:5:0.25 v/v). Savitri Kumar *et al*., **(**2016**)** have reported three different compositions of the biphasic solvent system hexane–ethyl acetate-methanol-water (2:5:2:5 solvent systems A) 1:4:1:4 (solvent systems B) and 1:5:1:5 (solvent systems C) were studied. The aqueous phase was used as the mobile phase in the head to tail elution mode. Flow rates of 2.0 –2.8 mL min-1, revolution speeds of 800-1000 rpm and a 200 mg sample of TF in 5 mL of the aqueous phase with settling times 17–18 s (solvent systems B and C) gave the best separations. Ethanolic extract of black tea absorbance was noticed at 245 nm and extracted chitin was noticed at 210 nm.

Conclusion

Presence of theaflavin in black tea extract was confirmed by various analytical methods. This theaflavin can be used for various clinical applications such as diabetic, anti-inflammatory, protective agents against cardiovascular and cerebrovascular diseases, aging etc.

References

Akiko Saito, Risa Nakazato, Yoshitomo Suhara, Masahiro Shibata, Toshiki Fukui. *et al*. (2016) The impact of theaflavins on systemic-and microcirculation alterations: The murine and randomized feasibility trials. J Nutr Biochem. 32:107–114.

Almajano MP, Carbó, Rosa, Jimenez, JAL, Gordon MH. Antioxidant and antimicrobial activities of tea infusions. Food Chem 2008;108:55–63.

Cantatore A, Randall SD, Traum D, Adams SD Effect of black tea extract on herpes simplex virus-1 infection of cultured cells. BMC Complem Altern Med 2013;13:139.

Clark KJ, Grant PG, Sarr AB, Belakere JR, Swaggerty CL, Phillips TD, Woode GN. An in vitro study of theaflavins extracted from black tea to neutralize bovine rotavirus and bovine coronavirus infections. Vet Microbiol 1998;63:147–157.

Friedman M. Overview of antibacterial, antitoxin, antiviral, and antifungal activities of tea flavonoids and teas. Mol Nutr Food Res 2007;51:116–134.

Ganesan Vijaiyan Siva, Gothandam Kirubananthan, Chinnaiah Alagarasan and Rajan Deepika, Comparative study of Chitin and Chitosan extracted from *Portunus sanguinolentus*. International Journal of Current Biotechnology. 2017; 5: 1-12.

Gosslau A, En Jao DL, Huang MT, Ho CT, Evans D, Rawson NE, Chen KY. Effects of the black tea polyphenol theaflavin-2 on apoptotic and inflammatory pathways in vitro and in vivo. Mol Nutr Food Res 2011;55:198–208.

Grassi D, Mulder TP, Draijer R, Desideri G, Molhuizen HO, Ferri C. Black tea consumption dose-dependently improves flow-mediated dilation in healthy males. J Hypertens 2009;27:774–781.

Haidar ZS, Hamdy RC, Tabrizian M. Protein release kinetics for core-shell hybrid nanoparticles based on the layer-by-layer assembly of alginate and chitosan on liposomes. Biomaterials. (2008). 29: 1207–1215.

Hartley L, Flowers N, Holmes J, Clarke A, Stranges S, Hooper L, *et al*. Green and black tea for the primary prevention of cardiovascular disease. Cochrane Database Syst Rev 2013;6:CD009934.

Hayat K, Iqbal H, Malik U, Bilal U, Mushtaq S. Tea and its consumption: benefits and risks. Crit Rev Food Sci Nutr 2015; 55:939–954.

Jochmann N, Lorenz M, Krosigk Av, Martus P, Böhm V, Baumann G, Stangl K, Stangl V. The efficacy of black tea in ameliorating endothelial function is equivalent to that of green tea. Br J Nutr 2008;99:863–868.

Kong L, Qi X, Huang S, Chen S, Wu Y, Zhao L. Theaflavins inhibit pathogenic properties of *P. gingivalis* and MMPs production in *P. gingivalis*-stimulated human gingival fibroblasts. Arch Oral Biol 2015; 60:12–22.

Kundu T, Dey S, Roy M, Siddiqi M, Bhattacharya RK. Induction of apoptosis in human leukemia cells by black

tea and its polyphenol theaflavin. Cancer Lett 2006;230:111–121.

Liu S, Lu H, Zhao Q, He Y, Niu J, Asim KD, Wu S, Jiang S. Adult-type hypolactasia and regulation of lactase expression. Biochim Biophys Acta 2005;1723:270– 281.

Menet MC, Sang S, Yang CS, Ho CT, Rosen RT. Analysis of theaflavins and thearubigins from black tea extract by MALDI–TOF mass spectrometry. J Agric Food Chem 2004;52:2455–2461.

Miller NJ, Castelluccio C, Tijburg L, Rice-Evans C. The antioxidant properties of theaflavins and their gallate esters—radical scavengers or metal chelators? FEBS Lett 1996;392:40–44.

Mizukami Y, Yusuke Sawai A, Yamaguchi Y. Simultaneous analysis of catechins, gallic acid, strictinin, and purine alkaloids in green tea by using catechol as an internal standard. J Agric Food Chem 2007;55: 4957–4964.

Sang S, Lambert JD, Tian S, Hong J, Hou Z, Ryu JH, Stark RE, Rosen RT, Huang MT, Yang CS, Ho CT. Enzymatic synthesis of tea theaflavin derivatives and their anti-inflammatory and cytotoxic activities. Bioorg Med Chem 2004;12:459–467.

Santesso N, Manheimer E. A summary of a cochrane review: green and black tea for the primary prevention of cardiovascular disease. Glob Adv Health Med 2014;3:66–67.

Savitri Kumar N, Nimal Punyasiri PA, Wijekoon WAMB The hexane-ethyl acetate-methanol-water system for the separation of theaflavins from black tea (Camellia sinensis) using high-speed counter-current chromatography. Ceylon J Sci 2016;45(2):79–86.

Tao W, Zhou Z, Zhao B, Wei T. Simultaneous determination of eight catechins and four theaflavins in green, black and oolong tea using new HPLC–MS–MS method. J Pharm Biomed Anal 2016;131:140–145.

Wang K, Liu Z, Huang JA, Dong X, Song L, Pan Y. Preparative isolation and purification of theaflavins and catechins by high-speed countercurrent chromatography. J Chromatogr B 2008;86:282–286.

Wu Y, Jin F, Wang Y, Li F, Wang L, Wang Q, Ren Z, Wang Y. In vitro and in vivo anti-inflammatory effects of theaflavin-3,3'-digallate on lipopolysaccharide-induced inflammation. Eur J Pharmacol 2017;794:52–60.

Yang C, Li D, Wan X. Combination of HSCCC and Sephadex LH-20 methods. An approach to isolation and purification of the main individual theaflavins from black tea. J Chromatogr B, 2003;861:140–144.

Zu M, Yang F, Zhou W, Liu A, Du G, Zheng L. In vitro anti-influenza virus and anti-inflammatory activities of theaflavin derivatives. Antiviral Res 2012;94:217–224.