Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2020; 8(2): 424-430

Received: 16-01-2020
Accepted: 20-02-2020

Muzamil Hussain Dar
Lovely Professional University, Phagwara, Punjab, India

Amit Sehgal
Lovely Professional University, Phagwara, Punjab, India

Corresponding Author:
Muzamil Hussain Dar
Lovely Professional University, Phagwara, Punjab, India

Evaluation of antiangiogenic and antigenotoxic potential of green and black tea extracts by chicken chorioallantoic membrane assay

Muzamil Hussain Dar and Amit Sehgal

Abstract
Present work deals with analysis of the antiangiogenic and antigenotoxic properties of green tea and black tea extracts by chicken chorioallantoic membrane (CAM) assay *in vivo* and by radical scavenging assay. The extracts of green tea and black tea prepared in water were tested, administering at 48, 72 and 96 hrs. of incubation to observe angiogenesis of the CAM at 144 hrs. of development and antigenotoxic properties using radical scavenging assay. The extracts of green tea and black tea reduced neovascularization. Green tea extract showed highest inhibitory activity in angiogenic responses as compared to black tea. The quantitative analysis indicated inhibition in elongation and proliferation of both secondary and tertiary vessels. It seems to be the consequence of interference of extracts in signaling of angiogenic agents from epithelial cells or cellular apoptosis, which in its absence results in normal CAM angiogenesis. The result claims strong antiangiogenic and antigenotoxic properties of the green and black tea.

Keywords: Chicken chorioallantoic membrane, antiangiogenic, green tea, antigenotoxic, black tea

Introduction
Tea is the largest consumed beverage in the world just after water [1, 2]. The per capita consumption of tea is approximately 120 ml/day [3]. The main reasons known till date for higher consumption of tea is because of its low price, aroma, taste, cultural practices, having potential health benefits and its stimulating effect [4, 5]. Tea is mostly consumed for pleasure but its medicinal values have been deeply studied with references dating back to 5000 years ago [6]. Tea is prepared by boiling the leaves of the *Camelia sinensis* in the water. Tea is cultivated in more than 30 countries of the world; it is a member of family Theaceae [7]. Two main tea plant varieties are known; *C. sinensis var. sinensis* native to china is a bushy plant having small leaves also grown in South east Asian countries experiencing cold climate and *C. Sinensis var. assamica* native to Assam, India have larger leaves also grown in several other countries experiencing a semitropical climate [8]. Various different tea types are available depending on the basis of botanical varieties, geographical origin and level of fermentation [8]. Green tea is made by rolling leaves of tea and then steamed to decrease oxidation and deactivate polyphenol oxidase before drying [3]. Black tea is produced by rolling leaves of tea, then bringing phenolic compounds into contact with polyphenol oxidases, followed by oxidation of 90 -120 minutes. White tea is produced from very young tea leaves and buds, after the young leaves and buds are plucked they are steamed and dried immediately, to prevent oxidation, the tea has a light and delicate taste. Tea is particularly rich in proteins, polysaccharides, polyphenols, minerals, and methylxanthines (caffeine, theophylline and theobromine) which largely contribute to the health benefits of tea. The potential for the consumption of tea and tea polyphenols in order to prevent or mitigate chronic disease is currently the subject of extensive scientific studies [9].
Oxygen is important for life but its high concentration is toxic for the human health because of its reduction to reactive oxygen species (ROS) due to different metabolic pathways. These have an influential role in various human pathophysiological and physiological processes [10]. These are unstable and highly reactive molecules generated as a byproduct of cellular metabolism, particularly in mitochondria and include superoxide anion, hydroxyl radicals, perhydroxyl radical, nitric oxide, hydrogen peroxide, peroxynitrite, singlet oxygen and hypochlorus acid [11]. At low levels these are important for various physiologically important

processes like growth factor stimulation and control of inflammatory responses. ROS participate in the regulation of many cellular processes including growth, differentiation, proliferation, cytoskeleton regulation, apoptosis, contraction and migration. ROS causes oxidative damage to cells leading to a number of pathological conditions like hemorrhagic shock, cystic fibrosis, cardiovascular diseases, rheumatoid arthritis, neurodegenerative disease, metabolic disorders, gastrointestinal ulcerogenesis [12]. Some of the specific examples of ROS related diseases are Parkinson's disease, Alzheimer's disease, atherosclerosis, cancer [10].

Integrity of our DNA is crucial for our health but it is susceptible to the damage caused by the reactive oxygen species (ROS). Among the bases present in DNA guanine bases are more vulnerable [13]. It is estimated that about 10,000 oxidations hit the DNA of each cell on a single day within the human body and more than 35 different types of oxidized bases have been reported in the DNA in vitro [14]. However most of the damage done to the DNA is repaired by the DNA repair enzymes very effectively [15]. Some of the damaged DNA escapes repair thereby causing permanent damage and is thought to accumulate with age. The damaged DNA plays a central role in carcinogenesis [16] effects cell cycle, expression of genes and mitochondria/cytoplasm communication [12]. This damage done to the DNA is known as Genotoxicity.

Angiogenesis is defined as the formation of new blood vessels from already existing blood vessels. It plays a very important role in the pathological and physiological processes in living organisms such as wound healing, embryonic development, chronic inflammation, tumor growth and metastasis [17]. Angiogenesis occurs by a number of steps which include stimulation and activation of endothelial cells, breakdown of capillary basal lamina by activated endothelial cells, formation of new capillaries and their maturation [18]. These are regulated by a large number of factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (BFGF), interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) [19]. The searches for antiangiogenic drugs, dietary products and bioactive plants have been studied and various tests are done for their antiangiogenic properties [20].

The chick embryo chorioallantoic (CAM) assay is an extra embryonic membrane assay and has been most widely studied for demonstrating tumor angiogenesis and testing different agents for their antiangiogenic activities [21]. The CAM assay has the experimental advantages which include ease of preparation, low cost and the natural mature immune system is absent, but involves the complexity of the whole angiogenic process [22]. Chick embryo model commonly referred to as hens egg test (HET) and is used in different research fields from more than 100 years [23].

Materials and Methods

Preparation of tea

Tea extract was made by soaking 0.20 g of tea in 10ml of hot water maintained at 85 ± 5 ºC on heating mantle for 5 min. The aqueous extracts were filtered with whatmans filter paper, allowed to cool at room temperature and were used for various antiangionenic and antigenotoxic assays [24].

Hydroxyl radical scavenging assay

The formation of hydroxyl radicals (OH) from Fenton reagents was quantified using 2-deoxyribose oxidative degradation. Deoxyribose is degraded by hydroxyl radical generated by fenton systems and results in formation of malendialdehyde (MDA) which can be detected by its ability to react with thiobarbituric acid (TBA) to form a pink color. The reaction mixture contained deoxyribose (5 mM), $FeSO_4$ (6 mM), phosphate buffer (20 mM, pH 7.4), H_2O_2 (100 mM) and various concentrations (500-1000µg/ml) of the test compounds were added. Deoxyribose, tea extract and phosphate buffer were premixed before addition to the reaction mixture. After 15 minute incubation at room temperature, the reaction was stopped by addition of 0.75 mL of 4% phosphoric acid (v/v) and 0.75 mL of 1% aqueous solution of TBA was added to the sample; test tubes were heated at 95 ℃ for 15 min to develop the pink color. After cooling at room temperature, absorbance was measured by spectrophotometer at 532 nm against an appropriate blank [12].

$$\%\text{scavenging activity} = (Ac - At)/Ac \times 100$$

Where: At and Ac are the respective absorbance of test samples and control.

CAM assay

The fertilized eggs were brought and were cleaned with the help of paper towel from dirt, feathers, excreta etc.

A. Incubation of eggs: Fertilized eggs were incubated in an incubator kept at 37 ˚C for 72 hours and the upper side were the embryo resides was marked with the pencil. The eggs were rotated twice daily. After 48 hours the eggs were punctured on the big side of the egg and 10ml albumin was taken out, so that embryo sits well.

B. Ex Ovo Culture: After 72 hours eggs were removed from incubator. The window was made in the egg on the side which we have marked with pencil (hands sterilized with 70% ethanol).

C. Treatment: - 50µl of the tea extract was laid on the developing embryo through the window and the window was sealed with the help of cello tape [25].

d. Statistical analysis: - The statistical significance of the data has been determined using one way analysis of variance followed by post-hoc test of Tukey's. The results are represented as mean±s.d.

Results and Discussion

Present study was undertaken to assess and compare the antiangiogenic and antigenotoxic activity of green and black teas with the help of chick chorioallantoic membrane assay and hydroxyl radical scavenging assay.

Hydroxyl radical scavenging assay

The percentage of radical scavenging activity of green tea (GT) significantly increases with increasing concentration. The percentage scavenging activity was varying from 43.73% at 500µg/ml to 62.39% at 1000 µg/ml (Figure 1, Table 1).

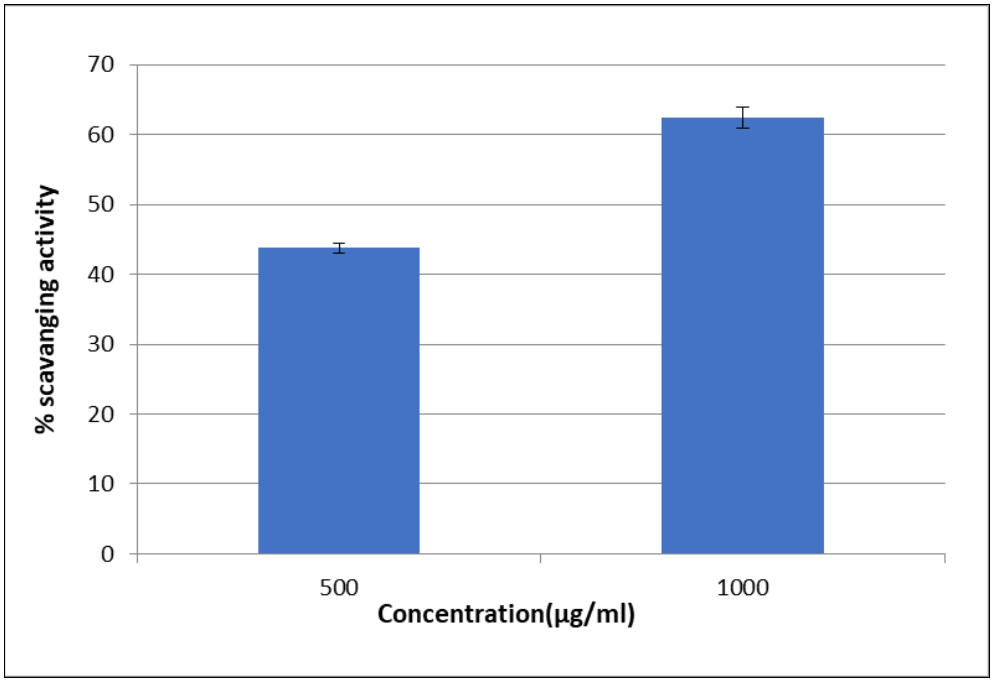


Fig 1: Hydroxyl radical scavenging activity of green tea (GT) (mean±sd, n=3).

Table 1: Hydroxyl radical scavenging activity of green tea (GT) (mean±sd, n=3).

Sample Concentration (µg/Ml)	% Scavanging Activity
500	43.73±0.65
1000	62.39±1.54

The percentage of radical scavenging activity of black tea (BT) significantly increases with increasing concentration. The percentage scavenging activity was varying from 32.05% at 500µg/ml to 50.0% at 1000 µg/ml (Figure 2, Table 2).

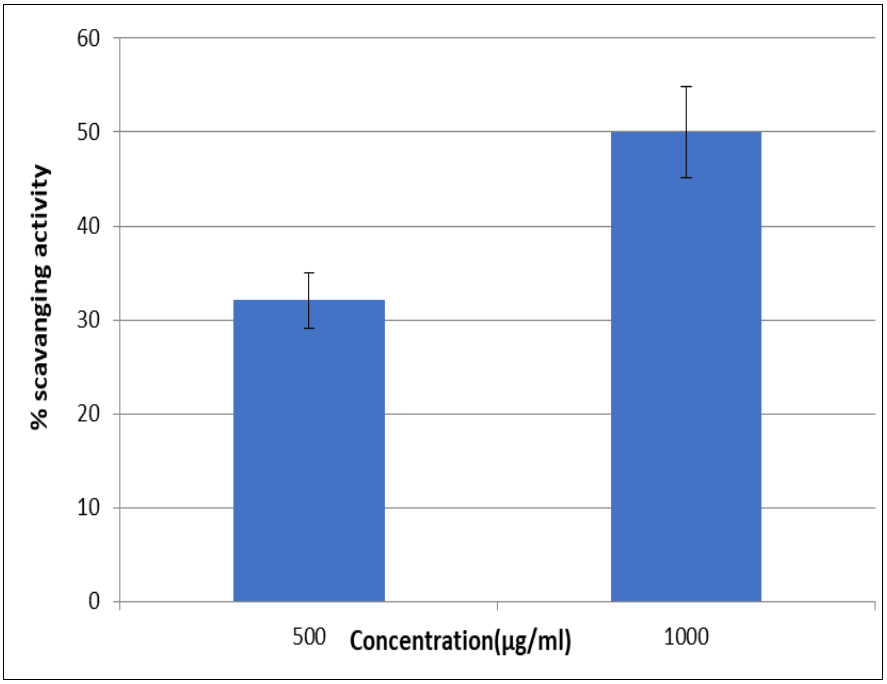


Fig 2: Hydroxyl radical scavenging activity of black tea (BT) (mean±sd, n=3).

Table 2: Hydroxyl radical scavenging activity of black tea (BT) (mean±sd, n=3).

Sample Concentration (µg/ml)	% Scavanging Activity
500	32.05±2.99
1000	50.0±4.83

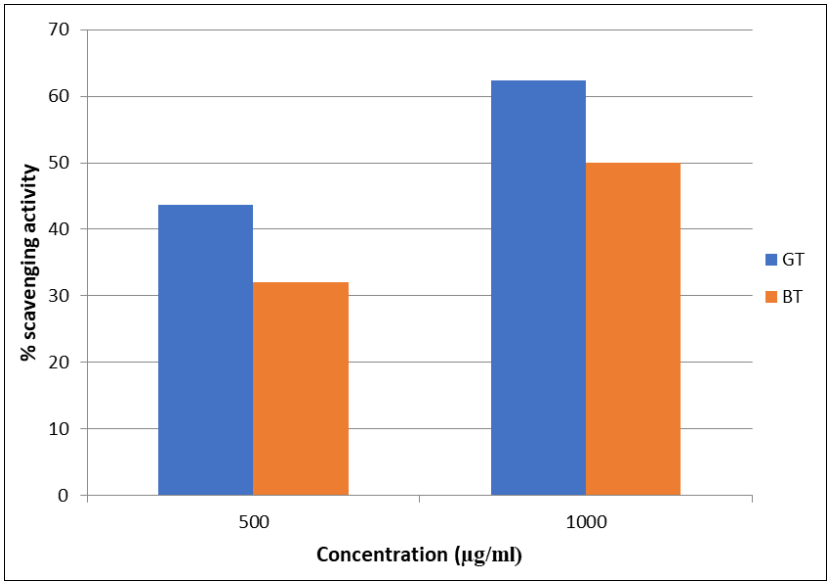


Fig 3: Comparison of hydroxyl radical scavenging activity of black tea (BT) and green tea (GT) (mean±sd, n=3).

GT showed more scavenging activity as compared to BT and the scavenging activity increased with increasing concentration in both tea types. A statistically significant difference was found between the scavenging activity of GT and BT (Table 3).

Table 3: Multiple comparison of hydroxyl radical scavenging activity of BT and GT at the concentration of 500µg/ml and 1000 µg/ml.

Multiple Comparisons

VAR00002
Tukey HSD

(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
GT 500	GT 1000	-18.6610*	2.42584	.000	-26.4294	-10.8926
	BT 500	11.6809*	2.42584	.006	3.9125	19.4493
	BT 1000	-6.2678	2.42584	.120	-14.0362	1.5006
GT 1000	GT 500	18.6610*	2.42584	.000	10.8926	26.4294
	BT 500	30.3419*	2.42584	.000	22.5735	38.1103
	BT 1000	12.3932*	2.42584	.004	4.6248	20.1616
BT 500	GT 500	-11.6809*	2.42584	.006	-19.4493	-3.9125
	GT 1000	-30.3419*	2.42584	.000	-38.1103	-22.5735
	BT 1000	-17.9487*	2.42584	.000	-25.7171	-10.1803
BT 1000	GT 500	6.2678	2.42584	.120	-1.5006	14.0362
	GT 1000	-12.3932*	2.42584	.004	-20.1616	-4.6248
	BT 500	17.9487*	2.42584	.000	10.1803	25.7171

Based on observed means.
The error term is Mean Square(Error) = 8.827.

*. The mean difference is significant at the 0.05 level.

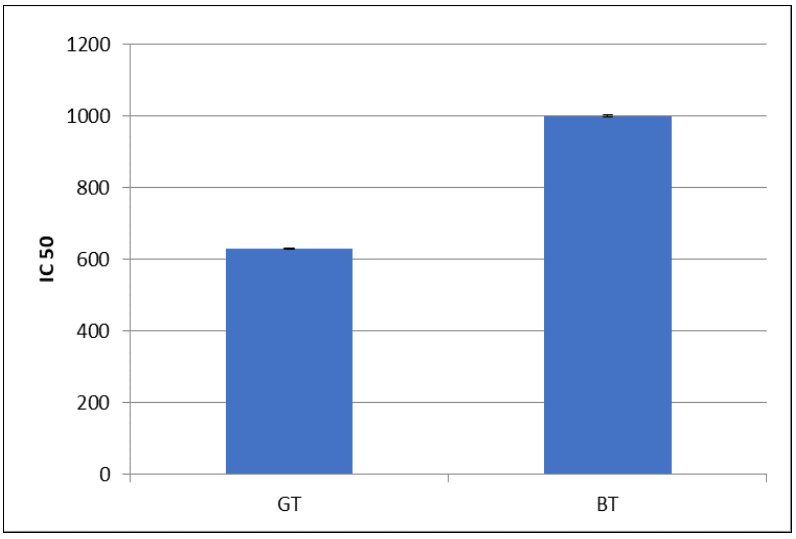


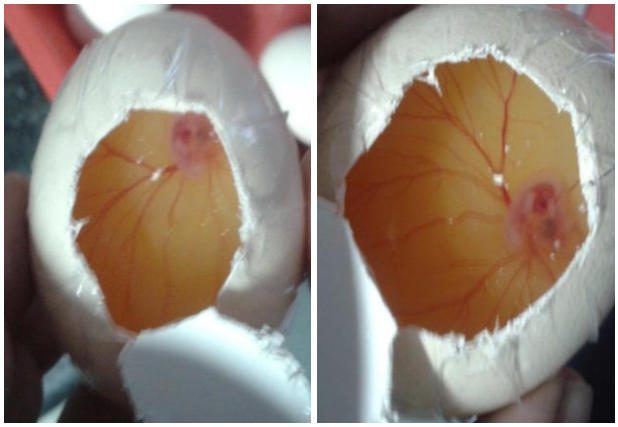
Fig 4: IC 50 values of GT and BT hydroxyl radical scavenging activity.

The effect of green tea and black tea on deoxyribose damage was assessed by using a sensitive technique known as the hydroxyl radical scavenging assay. The hydroxyl radical generated by fentons reaction induces severe damage to the biomolecules and can cause severe oxidative damage to the lipids, proteins and DNA. The hydroxyl radical along with transition metal (Fe^{2+}) causes degradation of deoxyribose into malanodialdehyde that gives a pink color with thiobarbituric acid with a maximum absorbance at 532 nm [26]. Hydroxyl radical scavenging activity of tea was quantified by measuring the inhibition of the degradation of Deoxyribose. The tea reduces the hydroxyl radical, which is unable to attack deoxyribose and hence less formation of malanodialdehyde [27]. In the present study green tea has the hydroxyl radical scavenging activity of 43.73% and 62.39%, and that of black tea 32.05% and 50% at concentrations of 500µg/ml and 1000µg/ml respectively. The green tea (IC50 value of 631.07±1.09µg/ml) was found to be more effective in quenching the hydroxyl radicals produced in the reaction mixture as compared to black tea (IC50 value of 1000±3.92. It is evident from our study that different tea types obtained from *Camellia sinensis*, possess an appreciable free radical scavenging ability. This is in agreement with previous studies which also showed similar observations. Yen and Chin [28] measured the electron proton resonance (EPR) signal intensity of DMPO-OH system, showed that different tea types have dose dependent inhibition of scavenging activity. The efficacy of different tea extract using hydroxyl scavenging assay was determined and it was depicted that the efficacy of green tea was more as compared to black tea [29].

Chorion allantois membrane (CAM) assay

CAM assays have been widely used to study angiogenesis, tumor cell invasion and metastasis. The CAM model has many advantages, such as the highly vascularized nature of the CAM greatly promotes the efficiency of tumor cell grafting, high reproducibility, simplicity and cost effectiveness, and finally as the CAM assay is a closed system, the half-life of many experimental molecules such as small peptides tends to be much longer in comparison to animal models, allowing experimental study of potential anti-metastatic compounds that are only available in small quantities.

The tea extract treatment was given to the eggs after 72 hours of incubation and then kept in the incubator for next 24 hours in order to see the effect of the green and the black tea in comparison to the control.

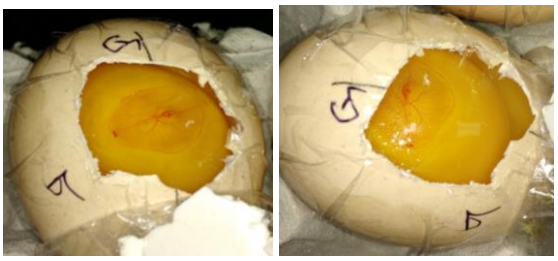


a. b.

Fig 5(a, b): Controlled CAM of 72 hours (no treatment).

a. b.

Fig 6(a, b): 72 hour CAM (to be treated with black tea extract).



a. b.

Fig 7(a, b): 72 hours CAM (to be treated with green tea extract).

a. b.

Fig 8(a, b): controlled CAM of 96 hours

a. b.

Fig 9(a, b): 96 hours CAM (24 hour treatment of black tea)

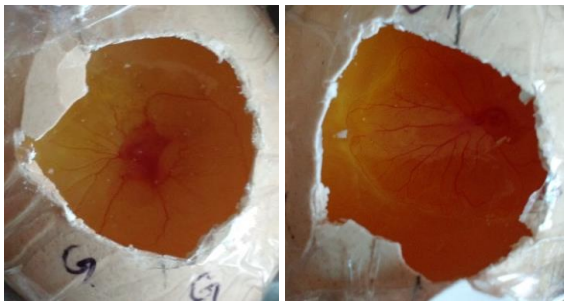


Fig 10(a, b): Green 96 hrs (24 hr treatment of green tea)

Using chick CAM model, the pharmacological effects of tea are confirmed to inhibit angiogenesis. Our results confirmed

that the tea extracts had a significant antiangiogenic activity. Out of the two tea extracts used the green tea showed the more antiangiogenic response as compared to black tea.

Treatment of green tea and black tea suppress the normal branching of blood vessels in the CAM development. Several growth factors are known to play a role in angiogenesis like VEGF, BFGF, IL-8, and TNF [30]. It is believed that the antiangiogenic action of tea may be due to inhibition of VEGF signaling. Thus through this tea seem to suppress the proliferation of secondary and tertiary blood vessels and inhibit the CAM angiogenesis.

The antiangiogenic property of tea may be attributed to the tea catechins particularly EGCG. Many plant compounds are shown to inhibit proliferation of angiogenesis of tumor cells *in vitro* which include polyphenols, flavonoids and terpenoids [31].

Conclusion

Green and black teas obtained from *Camelia sinensis* possess an appreciable free radical scavenging activity but upon comparison the green tea has more potency of radical scavenging as compared to the black tea. Both the tea types also inhibit the angiogenesis in the CAM model but green tea inhibits more as compared to black tea. Hence, regular consumption of tea may be effective in preventing the genotoxic damage and inhibition of angiogenesis in humans. Consequently, additional efforts are needed to understand the mechanisms, so that tea could be efficiently be used in different pharmaceutical formulations.

Acknowledgement

The authors are grateful to head department of zoology for providing necessary facilities.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

Funding

Funding was not available for the present research.

References

1. Vinson J. Black and green tea and heart disease: A review. Bio Factors. 2000; 13(1-4):127-132.
2. Cheng T. Will green tea be even better than black tea to increase coronary flow velocity reserve?. The American Journal of Cardiology. 2004; 94(9):1223.
3. McKay D, Blumberg J. The Role of Tea in Human Health: An Update. Journal of the American College of Nutrition. 2002; 21(1):1-13.
4. Baptista J, Tavares J, Carvalho R. Comparison of catechins and aromas among different green teas using HPLC/SPME-GC. Food Research International. 1998; 3(10):729-736.
5. Baptista J, Tavares J, Carvalho R. Comparative Study and Partial Characterization of Azorean Green Tea Polyphenols. Journal of Food Composition and Analysis. 1999; 12(4):273-287.
6. Wheeler D, Wheeler W. The medicinal chemistry of tea. Drug Development Research. 2004; 61(2):45-65.
7. Lapez V, Calvo M. White Tea (*Camellia sinensis* Kuntze) Exerts Neuroprotection against Hydrogen Peroxide-Induced Toxicity in PC12 Cells. Plant Foods Hum Nutrition. 2011; 66(1):22-26.
8. De Mejia E, Ramirez-Mares M, Puangpraphant S. Bioactive components of tea: Cancer, inflammation and behavior. Brain, Behavior, and Immunity. 2009; 23(6):721-731.
9. Rusak G, Komes D, Likia S, Horaia D, Kovaa M. Phenolic content and antioxidative capacity of green and white tea extracts depending on extraction conditions and the solvent used. Food Chemistry. 2008; 110(4):852-858.
10. Autreaux B, Tolendano MB. ROS as signaling molecules, mechanism that generate specificity in ROS homeostasis. Nature Reviews Molecular Cell Biology. 2007; 8(10):813-824.
11. Lau AT, Wang Y, Chiu JF. Reactive oxygen species; current knowledge and applications in cancer research and therapeutic. Journal of Cellular Biochemistry. 2008; 104:657-667
12. Halliwell B. Antioxidants in Human Health and Disease. Annual Review of Nutrition. 1966; 16(1):33-50.
13. Moller P, Loft S. Oxidative DNA damage in human white blood cells in dietary antioxidant intervention studies. American Journal of Clinical Nutrition. 2002; 76:303-310
14. Halliwell B. Why and how should we measure oxidative DNA damage in nutritional studies? How far have we come? American Journal of Clinical Nutrition. 2000; 5:1082-1087.
15. Jackson A, Loeb L. The contribution of endogenous sources of DNA damage to the multiple mutations in cancer. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2001; 477(1, 2):7-21.
16. Burdon RH. Genes and the environment. (Philadelphia, PA, London: Taylor and Francis), 1999
17. Folkman J, Yuen S. Angiogenesis. The Journal of Biological Chemistry. 1992; 267(16):10931-10934.
18. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nature Medicine. 1995; 1(1):27-30.
19. Ferrara N, Davis-Smyth T. The Biology of Vascular Endothelial Growth Factor. Endocrine Reviews. 1997; 18(1):4-25.
20. Loboda A, Cisowaski J, Zarebski A, Jazwa A, Rivera Nunez D, Kyprotakis Z *et al*. Effect of plant extracts on angiogenic activities of endothelial cells and keratinomycetes. Journal of Physiology and pharmacology. 2005; 1:125-137.
21. Quigley J, Armstrong P. Tumor Cell Intravasation Alu-cidated. Cell. 1998; 94(3):281-284.
22. Ribatti D, Nico B, Vacca A, Roncali L, Burri P, Djonov V. Chorioallantoic membrane capillary bed: A useful target for studying angiogenesis and anti-angiogenesis in vivo. The Anatomical Record. 2001; 264(4):317-324.
23. Lupke NP. The hen's egg test (HET) - an alternative toxicity test. Dermatatolgy. 1986; 115:133-135,
24. Santana-Rios G, Orner GA, Amantana A, Provost C, Wu S, Dashwood RH. Potent Antimutagenic Activity Of White Tea In Comparison With Green Tea In The Salmonella Assay. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 2001; 495(1):61-74.
25. Jadhav J, Mane A, Kanase A. Antiangiogenic properties of *Boerhaavia diffusa* extracts in chick Chorioallantoic Membrane (CAM). International Journal of Drug Development and Research. 2011; 3(4):307-317.
26. Cheng, Zhiyong, Li Y, Chang W. Kinetic deoxyribose

degradation assay and its application in assessing the antioxidant activities of phenolic compounds in a Fenton-type reaction system. Analytica Chimica Acta, 2003; 478:129-137.
27. Husain SR, Cillard J, Cillard P. Hydroxyl radical scavenging activity of flavonoids. Phytochemistry. 1987; 26:2489-2491.
28. Yen G, Chen H. Antioxidant Activity of Various Tea Extracts in Relation to Their Antimutagenicity. Journal of Agriculture and Food Chemistry. 1995; 43:27-32.
29. Erickson L. Rooibos Tea: Research into Antioxidant and Antimutagenic properties. Herbal Gram. 2003; 59:34-45.
30. Gu J, Makey K, Tucker K, Chinchar E, Mao X, Pei I, *et al*. a major green tea catechin suppresses breast tumor angiogenesis and growth via inhibiting the activation of HIF-1α± and NFκB, and VEGF expression. Vascular cell. 2013; 5(9):1-10.
31. Singh, Brahma N, Sharmila Shankar, Rakesh K. Srivastava. Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications. Biochemical pharmacology. 2011; 82(12):1807-1821.