Understanding Tea and Vascular Function

A thesis submitted towards partial fulfillment of
BS-MS Dual Degree Programme

by

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Certificate

This is to certify that this thesis titled “Understanding Tea and Vascular Function” submitted towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research - Pune represents original research carried out by Shikari Sravani at Unilever R&D, Bangalore, under the supervision of Dr. Manoj K. Joshi, Discover Leader, Unilever R&D, Bangalore during the academic year 2013-2014.

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Dr. Manoj K. Joshi

Declaration

I hereby declare that the matter embodied in the report entitled “Understanding Tead and Vascular Function” are the results of the investigations carried out by me at Unilever R&D Bangalore, under the supervision of Dr. Manoj K. Joshi and the same has not been submitted elsewhere for any other degree.

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And my mom who has been my inspiration.

Sravani.

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Abstract:

Consumption of tea has been associated with reduced cardiovascular risk (CVD). Epidemiological and clinical studies have demonstrated that tea intervention may improve vascular function by enhancing nitric oxide bioavailability. Majority of the mechanistic studies indicate endothelium dependent nitric oxide (NO) pathway to contribute towards the observed benefit. However, recent evidence suggests that endothelium independent pathway may also contribute equally towards the benefit. In this study, we aimed to prioritize various biomarkers clinically influencing vascular function based on literature evidences and developed *in vitro* bioassays to determine their modulation in response to tea/tea ingredients. Nitric oxide enhancement, Angiotensin-converting enzyme inhibition, Endothelin-1inhibition and Reactive Oxygen Species were selected as potential biomarkers. We observed that gallated catechins majorly contribute towards Nitric oxide potentiating effects and Endothelin-1 inhibition in green tea, while in black tea it was majorly influenced by theaflavins. Additionally, we found for the first time that only theaflavins majorly contribute towards Angiotensin-converting enzyme inhibition, while the effect of catechins were less evident. Thus, targeting multiple pathways influencing vascular function could provide valuable clues for the prevention or treatment of cardiovascular diseases.

Chapter 1: Introduction

1.1 Cardiovascular disease (CVD)

1.1.1 Prevalence of cardiovascular disease:

Cardiovascular disease is a leading cause of mortality and morbidity across the globe. In 2008, 17.3 million deaths were reported due to CVD, which represents around 30% of total mortality (WHO, 2011). By 2030, it is estimated that this number would increase to 23.3 million, maintaining CVD as the leading cause of death. Over 80% of CVD-deaths have been reported to occur in low and middle income countries (WHO, 2011). In India, CVD contributes to about 25% of overall deaths. More alarmingly, the onset of CVD manifestation is observed a decade earlier compared to the global average (Engelgau et al., 2012). Improved early detection and a better understanding of the progression and pathogenesis of CVD may help in addressing the growing pandemic.

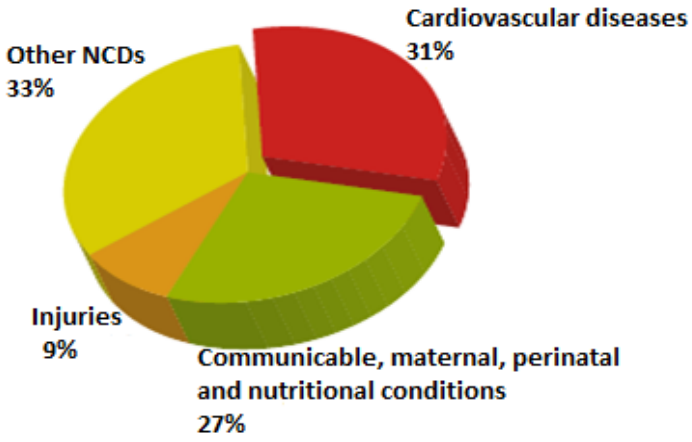


Figure 1: Global distribution of major causes of death. Adapted from WHO, 2011.

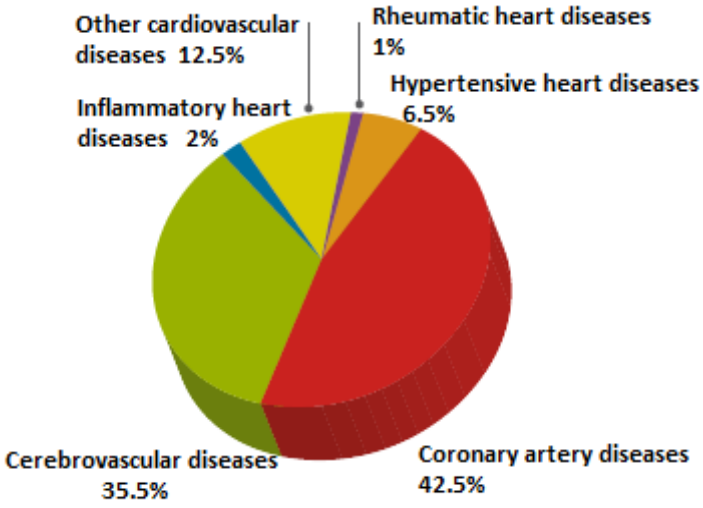


Figure 2: Global distribution of CVD deaths due to different types of cardiovascular diseases. Adapted from WHO, 2011.

1.1.2 Classification of cardiovascular disease:

Vascular disease majorly comprises of diseases associated with the arteries that supply blood to various organs, while the manifestation that occurs in arteries of the heart is termed as CVD. CVD includes coronary artery disease (CAD), cerebrovascular disease (stroke), congenital heart disease, peripheral artery disease (PAD) and rheumatic heart disease, out of which CAD and stroke contribute to the majority of deaths (WHO, 2011). Although the causative mechanism in all these vascular diseases (CAD and PAD) remains similar, the molecular pattern is best understood during the pathophysiology of atherosclerosis.

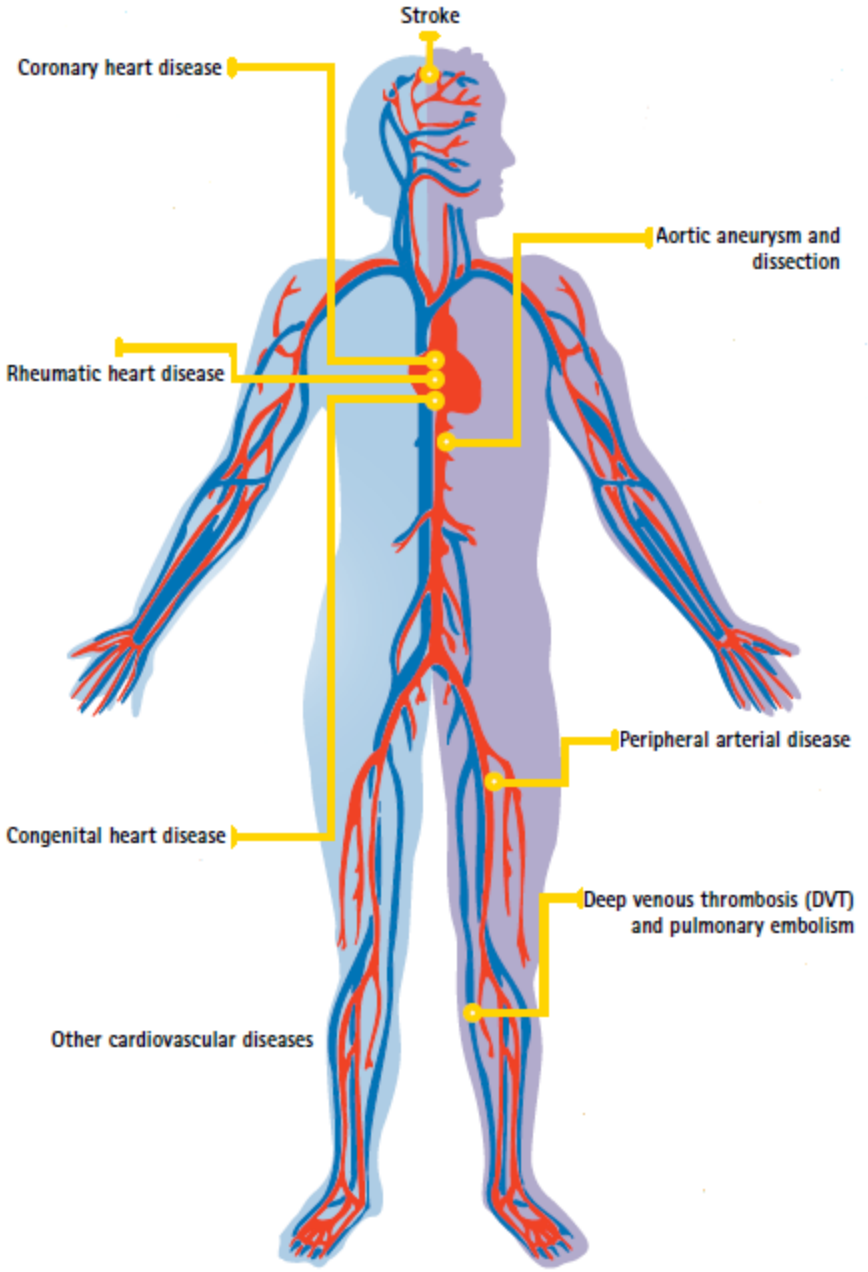


Figure 3: Types of cardiovascular disease. Adapted from WHO, 2011

1.2 Etiology associated with atherosclerosis

1.2.1 Atherosclerosis:

Atherosclerosis is a progressive disease initiated by reduced blood vessel flexibility, increased vascular adhesion and platelet aggregation which finally culminates into

inflamed and fibrotic vessels. In due course, these fibrotic lesions mature and lead to the formation of plaques. While plaques may cause a restriction of blood flow to cause clinical complications, the rupture of a plaque leads to severe clinical events such as hemorrhage. It may also expose the pro-thrombotic material in the plaque into the circulation, resulting in occlusion of the artery. Such a blockage in the arteries supplying to the heart leads to myorcardial infarction; whereas in the arteries which perfuse the brain causes ischemic stroke (Hansson and Libby, 2006).

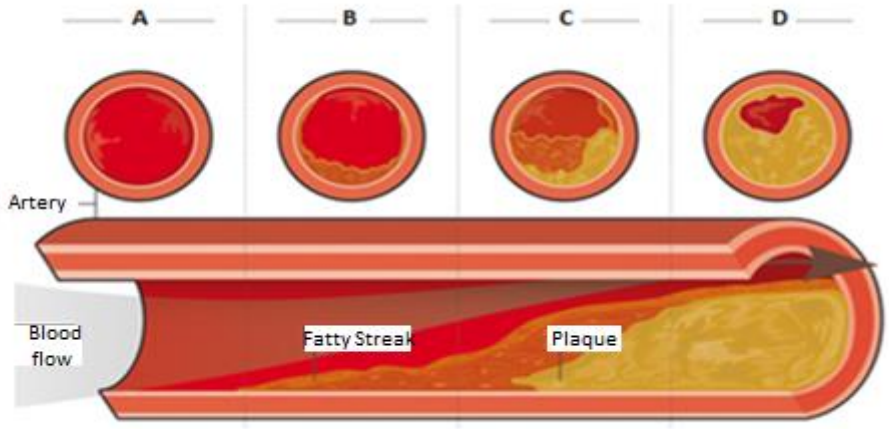


Figure 4: Onset and progression of atherosclerosis. A: Healthy arteries allow the blood to flow freely, without any blockage. B: Atherosclerosis starts with a "fatty streak" of material building up in the artery wall. C: Over time the material can form a plaque that might become a thrombosis or clot. D: A clot might cause a fatal blockage in the artery. Adapted from Cannon, 2013.

1.2.2 Development of atherosclerosis – an example of changing endothelial scenario:

Atherosclerosis is characterized by increased inflammation of the vessel wall coupled with endothelial dysfunction and an increase in intimal thickness (Hansson and Libby, 2006). Under homeostatic regulation, the vascular tone of the endothelium is guided by the shear stress exerted by blood flow. Endothelial cells release various dilator and constrictor substances to regulate vascular tone. Nitric oxide-mediated signaling is the

major characteristic of a healthy endothelium and contributes to about 80% of the observed vasodilatory benefit (Deanfield, 2007). However, such vascular benefits are compromised during the process of atherosclerosis. Compromised nitric oxide (NO) coupled with increased reactive oxygen species (ROS) leads to decreased bioavailability of NO. Such state of the endothelium is referred to as endothelial dysfunction (Kietadisorn, 2012). Increase in ROS levels promotes a pro-inflammatory, proliferative and pro-thrombotic endothelial milieu. Such a condition may have autocrine, paracrine and juxtacrine effects. Increased superoxide in the lumen of the vessel leads to increased oxidation of LDL. This biologically active lipid induces endothelial cells to produce adhesion molecules like VCAM1 and ICAM1. This allows monocytes and T-cells to adhere to the endothelial cells and respond to locally produced chemokines by migrating inside the arterial tissue. Subsequently, monocytes differentiate into macrophages which results in the uptake of ox-LDL and lead to formation of foam cells. These foam cells undergo necrosis to form thrombus (Hansson and Libby, 2006). An increase in the generation of ROS also reflects increase in intracellular oxidative stress. There are various endogenous mechanisms of ROS generation including mitochondrial respiration, enzymatic action of NADPH oxidase, xanthine oxidase and the phenomenon of eNOS uncoupling. The superoxide anion rapidly degrades nitric oxide by forming peroxynitrite leading to a decrease in NO bioavailability (Forstermann and Munzel, 2006).

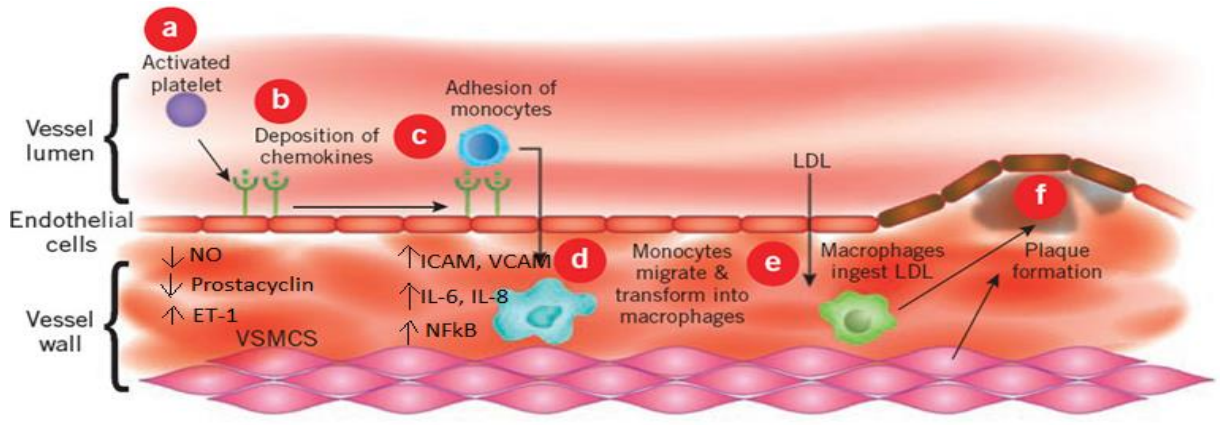


Figure 4: Pathogenesis of atherosclerosis: Increase in ROS levels leads to (a)- platelet activation, (b) - increase in inflammation and (c) – increase in adhesion

molecules which allow monocytes to adhere to endothelial cells and (d) – migrate inside the arterial tissue. (e) - Macrophages ingest ox-LDL and form foam cells which results in (f) – plaque formation. Adapted from Hansson and Libby, 2006

1.2.3 Circulating Biomarkers:

Due to change in molecular patterns, an alteration in the expression of various biomarkers has been observed. A plethora of biomarkers are associated with the progression of atherosclerosis. These include inflammatory markers (CRP, Interleukins, TNFα, NFκB, MIF, IFNY) (Smith et al., 2004), metabolic markers (Adiponectin, insulin, leptin), markers of oxidative stress (ROS, NOX, COX, XO, homocysteine), lipid markers (ox-LDL, LDL, HDL, apolipoprotein A1, lipoprotein (a)), coagulation markers (PAI-1, fibrinogen, tPA, von Willebrand factor) (Vaughan, 2005), adhesion markers (VCAM-1, ICAM-1, E-selectin, p-selectin) (Hwang et al., 1997) and markers of vascular function (nitric oxide, endothelin-1, angiotensin II, BH_4, ADMA, arginases, angiotensin converting enzyme (ACE)) (Boger et al., 2005). Out of these, markers reflecting vascular function and oxidative stress are of prime importance as they directly underscore endothelial dysfunction. Also, these markers serve as a link between atherosclerosis and endothelial function.

1.3 Vascular function

1.3.1 Vascular function and health:

The major function of blood vessels is to supply oxygenated blood, thereby maintaining the balance between tissue oxygen supply and metabolic demand. This is achieved by the endothelium via secretion of various vasoactive compounds regulating the vascular tone (Lerman and Bumett, 1992). Alteration in these vasoactive compounds disturbs vascular homeostasis resulting in the development of vascular complications. Alteration of markers like NO, endothelin-1, ACE, BH_4, ADMA, arginases and angiotensin II, reflect that the vascular function has been compromised and is indicative of early prognosis of various disease etiologies (Vita and Keaney, 2002). Decrease in NO bioavailability has been associated with pathological conditions like atherosclerosis,

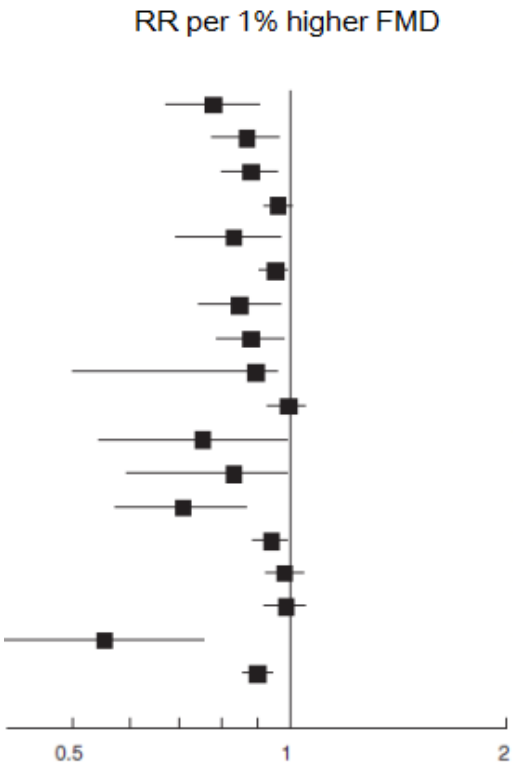
hypertension, hypercholesterolaemia, type 2 diabetes and septic shock (Li and Forstermann, 2000). Increase in endothelin-1 levels has been associated with hypertension, atherosclerosis, vascular remodeling, myocardial infarction, vascular hypertrophy and renal failure (Kitada et al., 2012). Increase in the levels of ACE is associated with hypertension, type 2 diabetes, arthritis and various cancers (Moskowitz and Johnson, 2004). Similarly, an increase in ADMA and a decrease in BH_4 levels have been observed in conditions like atherosclerosis and hypercholesterolaemia (Forstermann and Munzel, 2006).

1.3.2 Measuring vascular function:

Endothelial function can be measured by various invasive and non-invasive techniques. The gold standard for measuring vascular function is to evaluate the intracoronary/intrabrachial response to vasoactive agents like acetylcholine, bradykinin, serotonin, etc. However, its major disadvantage of being invasive has led to the development of new methods of measurement like flow-mediated dilataion (FMD), venous occlussion plethysmography, carotid intima-media thickness, pulse waveform analysis, peripheral arterial tonometry, etc.

Endothelial dysfunction results in vessel constriction and vascular remodeling. Structural remodeling leads to a dramatic change in the arterial waveform. Hence, pulse waveform analysis reflects arterial shape and stiffness. Measurement of carotid-intima media thickness using ultrasound gives arterial thickness. Venous occlusion plethysmography measures forearm blood flow while peripheral arterial tonometry gives blood flow upon a hyperemic stimulus. Flow-mediated dilatation is an extensively used reliable measure of endothelial function. This testing involves measuring the diameter of an artery before and after increase in shear stress, provided by reactive hyperemia, using ultrasound. The degree of dilatation reflects endothelium-dependent vasodilation mediated by the release of nitric oxide by the endothelium (Korkmaz and Onalan, 2008). Studies demonstrate that FMD is independently inversely associated with cardiovascular risk and has prognostic value for the prediction of long-term cardiovascular events. A recent meta-analysis, comprising of 23 prospective studies,

demonstrated a 10% reduction in cardiovascular risk per 1% higher FMD (Ras et al., 2013), while an earlier study indicates a 13% reduction (Brevetti et al., 2003).



Reference	*N*	*RR*	*95% CI*
Neunteufl, 2000	73	0.78	0.67; 0.90
Brevetti, 2003	131	0.87	0.78; 0.97
Gokce, 2003	199	0.88	0.80; 0.96
Fathi, 2004	444	0.96	0.92; 1.01
Katz, 2005	149	0.83	0.69; 0.97
Yeboah, 2007	2791	0.95	0.90; 0.99
Hu, 2008	279	0.85	0.75; 0.97
Mullesan, 2008	172	0.88	0.79; 0.98
Rossi, 2008	2264	0.89	0.50; 0.96
Suzuki, 2008	819	0.99	0.93; 1.05
Takase, 2008	103	0.75	0.54; 0.99
Shechter, 2009b	82	0.83	0.59; 0.99
Wang, 2009	101	0.71	0.57; 0.87
Yeboah, 2009	3025	0.94	0.88; 1.00
Anderson, 2011	1574	0.98	0.92; 1.04
Lind, 2001	912	0.99	0.92; 1.05
Yilmaz, 2011	304	0.55	0.40; 0.76
Overall	13422	**0.90**	0.86; 0.94

Figure 6: Forest plot showing studies with continuous risk estimates. Each square and line represents the relative risk (RR) of cardiovascular disease and accompanying 95% confidence interval. The risk estimate is expressed per 1% higher flow-mediated dilation (FMD). The pooled overall risk estimate for CVD is 0.90, reflecting a 10% decrease in CVD risk per 1% higher FMD. Adapted from Ras et al., 2013.

1.3.3 Dietary factors and CVD risk:

Evidence from epidemiological and intervention studies demonstrate a relationship between dietary patterns and CVD risk (Van Horn et al., 2008). A growing list of natural products has been shown to improve endothelial function by enhancing endothelial nitric oxide production/bioavailability. Identification of such compounds would help in formulating prevention strategies by including such components in daily diet. Also,

characterization of their cellular actions may help better our understanding of the regulation of endothelial function and could provide clues for prevention and treatment of cardiovascular diseases.

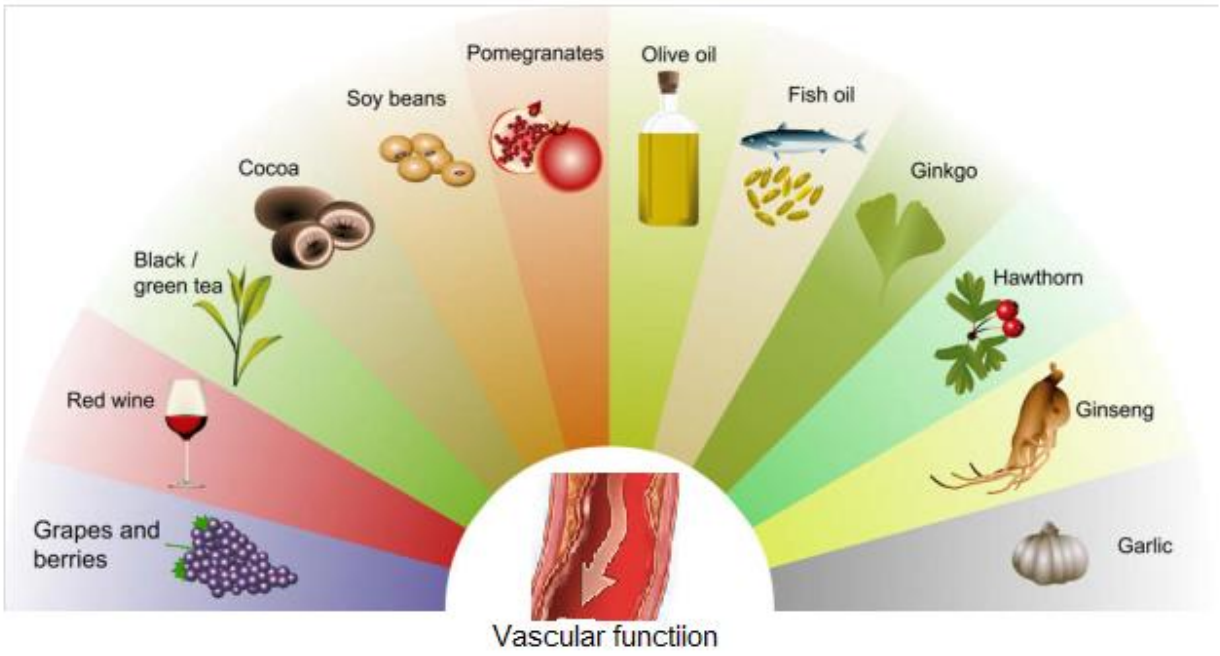


Figure 7: Prominent natural products shown to enhance vascular function.

Among the various neutraceuticals, evidence from epidemiological, interventional and *in vitro* studies demonstrate tea to improve endothelial function and reduce risk for various disease conditions. In addition, tea is the most widely consumed beverage worldwide which makes it an attractive contender. Hence, in this study, we aim to understand the modulation of identified biomarkers of endothelial function with selected tea ingredients.

1.4 Tea and Health:

1.4.1 Tea – its composition and health benefits:

The term “tea” refers to the plant *Camellia Sinensis*, its leaves and infusions derived from them. There are different types of tea, green, black, white, oolong and pu-erh tea, based on different modes of manufacture. Of these, black and green teas are the

varieties majorly consumed. Tea leaves contain specific polyphenols and the enzyme polyphenol oxidase. Black tea is produced by allowing the activity of polyphenol oxidase leading to the oxidation of polyphenols. This enzymatic oxidation is inhibited to produce green tea. Evidence from epidemiological studies highlights the health-promoting actions of tea. Tea consumption has been shown to be associated with a reduced risk of cardiovascular disease, type II diabetes, various cancers and impaired cognitive function (Peters et al., 2001; Yang et al., 2014;). Tea has been shown to improve vascular health via a multitude of effects including vasorelaxation, antioxidant, antiproliferative, antithrombotic, anti-inflammatory and anti-angiogenic properties due to its interference with various molecular targets (Stangl et al., 2007). .

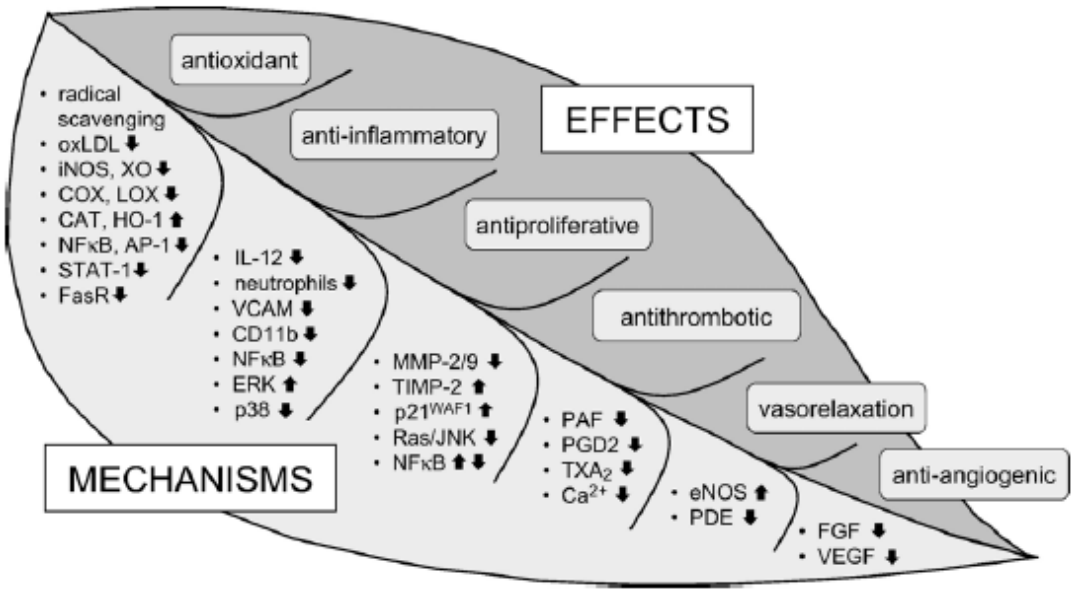


Figure 8: Effects and molecular mechanisms of tea polyphenols in the cardiovascular system. Underlying mechanisms for the beneficial effects of tea compound include antioxidative, anti-inflammatory, antiproliferative, and anti-angiogeneic properties, as well as vasorelaxation. Tea polyohenols interfere with a plethora of molecular targets, thus exerting beneficial cardiovascular effects. Adapted from Stangl et al., 2007

1.4.2 Evidence of tea on health:

In 2001, in a meta-analysis Peters et al., estimated that the incidence of myocardial infarction reduced by 11% with increase in tea consumption of 3 cups/day. A meta-analysis by Arab et al., showed a 21% reduced risk of stroke in people consuming 3 cups of tea per day. Similarly, a meta-analysis by Wang et al., showed that an increase in green tea consumption of 1 cup/day reduced CAD risk by 10% while no significant effect was seen with black tea consumption.

1.4.3 Evidence of tea on flow-mediated dilatation:

Hooper et al., showed that chronic ingestion of black tea led to an increase of 3.40% in FMD, while acute administration did not show any significant effect (1.70% increase in FMD). Subsequently, Widlansky et al., demonstrated that an acute ingestion of EGCG showed an increase in FMD by 1.5%. A recent meta-analysis by Ras et al., reported an increase of 2.6% in FMD upon median daily consumption of 500 mL (2-3 cups) of tea. The physiological effect of tea on acute and chronic ingestion is not well understood and must be investigated further.

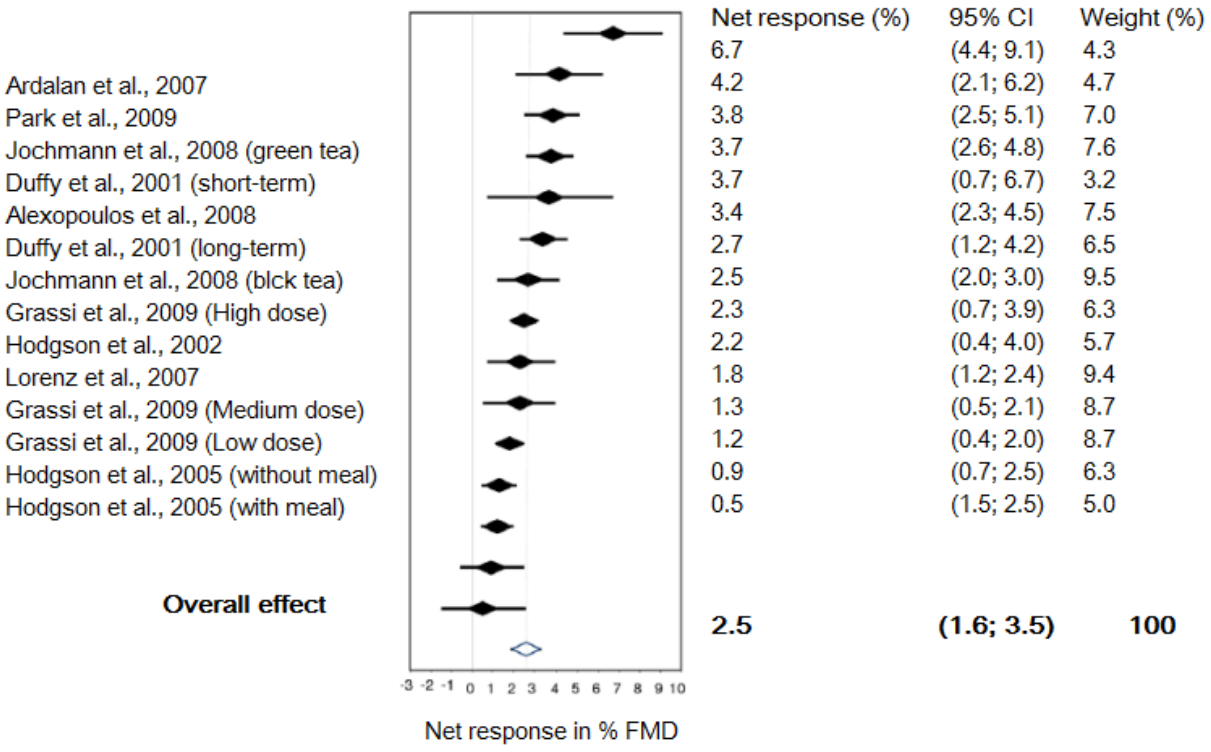


Figure 9: Forest plot showing the net flow-mediated dilation (FMD) responses and 95% confidence intervals of 15 study arms from 9 studies investigating the

effect of tea on FMD. The pooled overall effect of tea is an increase in FMD by 2.6%. Adapted from Ras et al., 2011.

1.4.3 Evidence of tea on blood pressure:

Acutely, green tea has been shown to increase blood pressure. However, subsequent studies have reported antihypertensive effects of tea. While a meta-analysis carried out by Taubert et al., demonstrated that tea consumption did not affect blood pressure, a recent meta-analysis by Onakpoya et al., showed a significant reduction in blood pressure upon green tea consumption. Though the mechanisms have not fully been explored, flavonoids found in tea have been shown to be responsible for the beneficial effects exerted by tea.

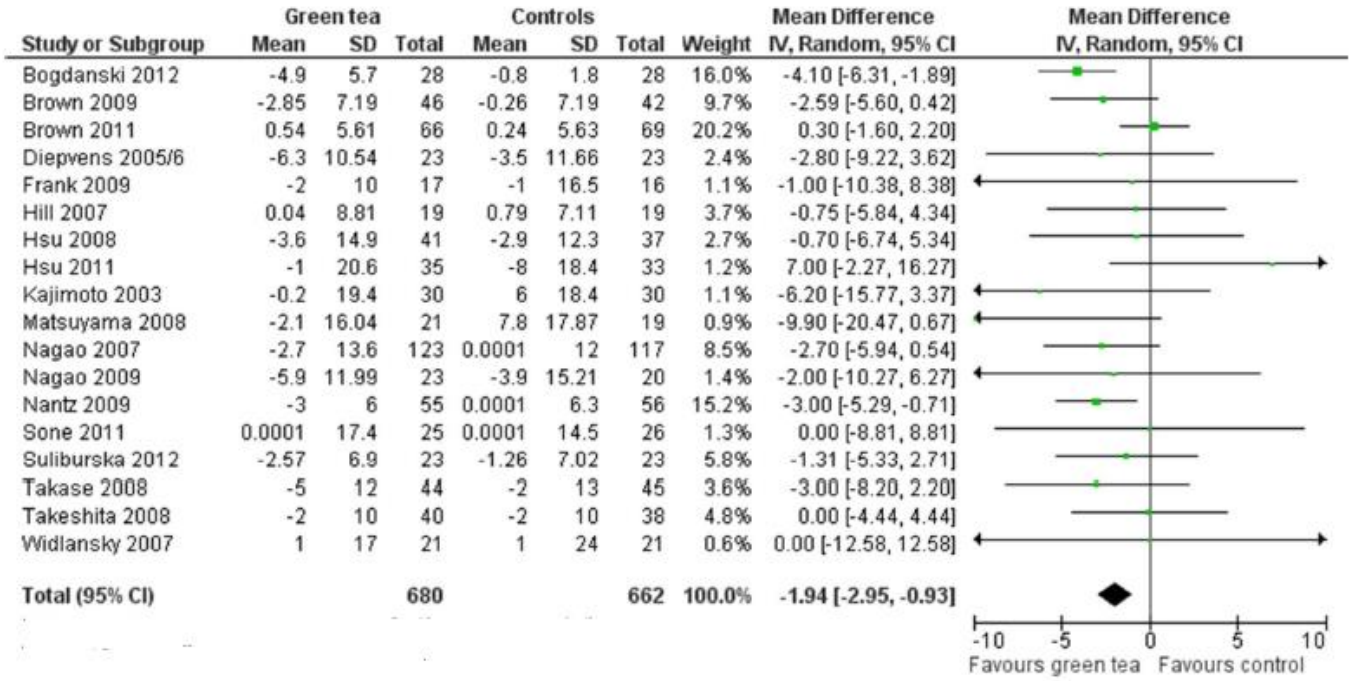


Figure 10: Green tea supplementation reduces systolic blood pressure. Adapted from Onakpoya et al., 2014.

1.4.4 Tea flavonoids and health:

Flavonoids are polyphenols which are the major constituents of tea. This class of compounds is classified into six subclasses: flavones, flavonols, flavan-3-ols, isoflavones, flavanones, and anthocyanidins. Out of these, tea majorly contains flavan-3-ols followed by minute amounts of flavonols.

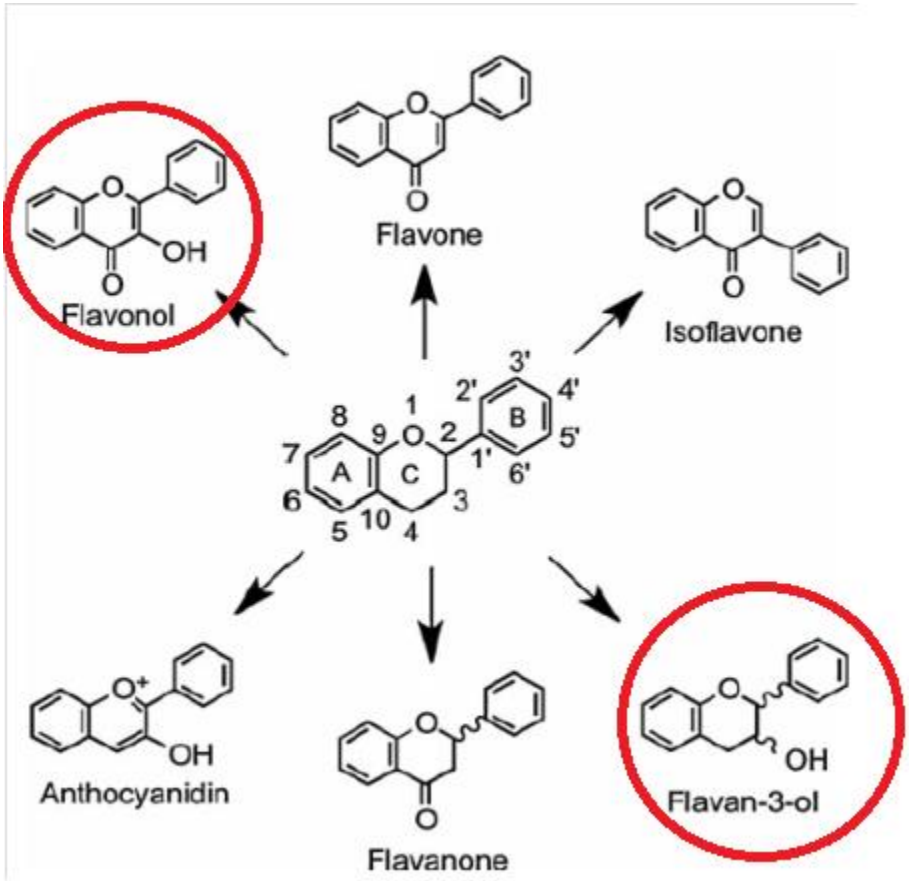


Figure 11: Classification of flavonoids into different subclasses. Flavan-3-ols and flavonols are present in tea.

Several human intervention studies have been carried out to investigate the association between flavonoid intake and incidence of various diseases. Liu et al, in a meta-analysis reported that an increase in the intake of 500mg/day of flavonoids causes a 5% reduction in the incidence of type II diabetes. Hooper et al reported a 1.34% increase in FMD upon chronic and 3.19% increase on acute ingestion of cocoa, which is rich in flavonoids. A meta-analysis by Huxley and Neil, in 2003, reported a reduction of 20% in

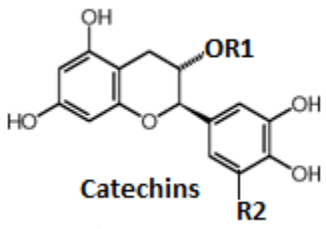
coronary heart disease mortality upon flavonol intake. Similarly, Hollman et al reported a 20% reduction in fatal and non-fatal stroke upon flavonol consumption. In an elaborate meta- analysis, McCullough et al. demonstrated the reduction in cardiovascular risk upon consumption of various subclasses of flavonoids in a dose-dependent manner. Flavan-3-ols were found to reduce CVD mortality by 17%, greater than that observed by other compounds.

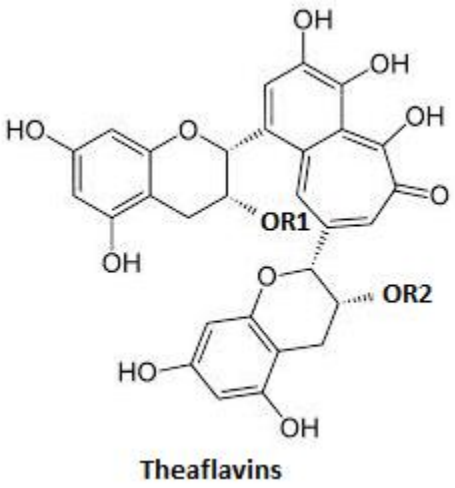
Hence, it can be conclusively derived that flavonoid consumption exerts health-promoting effects by reducing cardiovascular risk. Out of the different subclasses of flavonoids, flavan-3-ols have been demonstrated to provide maximum benefit.

The major tea flavan-3-ols are catechins - epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG), gallocatechin gallate (GCG) and epigallocatechin-3-gallate (EGCG) – of which EGCG is the most predominant in green tea. During the production of black tea the catechins are oxidized by polyphenol oxidase to high molecular weight theaflavins and thearubigins, predominant in black tea. The group of theaflavins includes theaflavin (TF1), theaflavin-3-monogallate (TF2A), theaflavin-3′-monogallate (TF2B), and theaflavin-3,3′-digallate (TF3). Thearubigins on the other hand, have not been well-characterized and are not included in this study. In this study, we aim to study the effect of tea catechins and theaflavins on vascular function.

		Men and women (n=98,469)		
Quintile	Median intake (range)	Deaths	Age-adjusted RR	Multivaiate-adjusted RR (95% CI)
Total flavonoids	mg/d			
1	94.5 (<121.5)	615	1.00 (-)	1.00 (-)
2	146.5 (121.5-172.3)	538	0.77 (0.69, 0.87)	0.84 (0.75, 0.95)
3	201.9 (172.4-238.0)	552	0.77 (0.68, 0.86)	0.87 (0.77, 0.98)
4	286.0 (238.1-359.6)	551	0.74 (0.66, 0.83)	0.86 (0.76, 0.96)
5	512.5 (>359.6)	515	0.72 (0.64, 0.80)	0.82 90.73, 0.92)
P-trend			<0.0001	0.01
Flavan-3-ols				
1	7.0 (<9.5)	621	1.00 (-)	1.00 (-)
2	11.8 (9.5-14.0)	534	0.74 (0.71, 0.89)	0.88 (0.78, 0.98)
3	16.8 (14.1-20.3)	548	0.77 (0.69, 0.86)	0.88 (0.79, 0.99)
4	26.3 (20.4-37.1)	548	0.76 (0.68, 0.85)	0.89 (0.79, 1.01)
5	63.7 (>37.1)	520	0.72 (0.64, 0.81)	0.83 (0.74, 0.93)
P-trend			<0.0001	0.02
Flavonols				
1	6.9 (,8.5)	657	1.00 (-)	1.00 (-)
2	9.9 (8.5-11.3)	542	0.76 (0.68, 0.86)	0.83 (0.74, 0.93)
3	13.0 (11.4-14.7)	555	0.77 (0.69, 0.87)	0.87 (0.78, 0.98)
4	17.2 (14.8-20.5)	516	0.72 (0.64, 0.81)	0.84 (0.74, 0.94)
5	27.2 (>20.5)	501	0.74 (0.66, 0.83)	0.84 (0.75, 0.94)
P-trend			<0.0001	0.03

Figure 12: Relative risks (RR) and 95% confidence intervals shown for cardiovascular disease mortality by quintile of energy-adjusted flavonoid intake (of various flavonoid subclasses) in men and women. Both age-adjusted and multivariate-adjusted RR values are shown. Adapted from McCullough et al., 2012.





	R1	R2
• Epicatechin (EC)	H,	H
• Epigallocatechin (EGC)	H,	OH
• Gallocatechin-3-gallate (GCG)	G,	OH
• Epicatechin-3-gallate (ECG)	G,	H
• Epigallocatechin-3-gallate (EGCG)	G,	OH
• Theaflavin (TF1)	H,	H
• Theaflavin-3-gallate (TF2)	H,	G
• Theaflavin-3'-gallate (TF3)	G,	H
• Theaflavin-3-3'-gallate (TF4)	G,	G

Figure 13: Chemical structures of catechins and theaflavins indicating the numbers and positions of the galloyl groups in the compounds.

Objective of the Study:

- To scope for relevant biomarkers influencing endothelial function.
- To establish and validate *in vitro* bioassays influencing endothelial function.
- To determine the role of tea flavan-3-ols in modulating markers of endothelial function using the validated bioassays.

Chapter 2: Material and methods

2.1 Cell Culture

EA.hy926 (human endothelial cells, obtained from ATCC, CRL-1548) were maintained in DMEM high glucose (Sigma) and 10% (v/v) fetal bovine serum (Gibco). Cells were sub-cultured regularly by trypsinization and incubated at 37°C in 95% humidified air and 5% CO_2. Cells were grown to confluence and seeded in 24, 48 well plates for 24 hours before treatment.

2.2 Treatment with tea actives

Working concentration of tea actives was prepared in DMEM without fetal bovine serum (FBS). Cells were given two washes with 1X PBS and treatment with tea actives (green and black tea, tea catechins, tea theaflavins) was given for specified time. The cells were incubated at 37ºC and 5% CO_2.

2.3 Quantification of polyphenol content in tea infusion using Folin-Ciocalteu method

The polyphenol content in green and black tea infusions was determined using Folin-Ciocalteu (FC) reagent. Briefly, 20 µl of 1:20 diluted tea infusion was mixed with 40 µl of Na_2CO_3 in a 96-well microtiter plate. 50 µl of FC reagent was added after 5 minutes and the plate was incubated in dark for 1 hour. Post incubation, measurements were taken using plate reader (Tecan). Gallic acid was used as calibration standard.

2.4 Quantification of Theaflavins (TFs) by HPLC

Theaflavins were quantified using HPLC analysis. HPLC conditions used are as follows:

Instrument: Shimadzu LC -10A HPLC

Column: Nova-Pak C-18, 60A, 4 µM, 3.9 x 150 mm

Mobile phases: A – 2% Acetic acid B - Acetonitrile

Flow rate: 1ml/min

Detector: PDA

Wavelength: 380 µm

Injection Volume: 20 µl

Column Temperature: 40ºC

Analysis time: 55 minutes

Using above conditions four theaflavin standards of different concentrations were run at 380 nm. Peak area of standards was used to generate calibration curve. Theaflavin concentration was determined as (% w/w).

2.5 MTT cell viability assay

The cytotoxic level of tea actives was determined via the MTT assay. In this assay, the yellow MTT (tetrazolium dye) gets reduced to purple formazan in the mitochondria of living cells and hence viability is quantified.

EA.hy926 cells were grown in 96-well plates at a density of 10^4 cells/well in DMEM (with FBS). After an incubation of 24 hours, the spent media was removed and tea actives prepared in DMEM (without FBS) at concentrations ranging from 3 μM or 1 μl/ml to 100 μM or 27 μl/ml were added to cells. The plate was incubated for 24 hours at 37°C and 5% CO_2. Post incubation, media containing tea actives was removed and washed twice with 1XPBS. Subsequently, a 5mg/ml of MTT reagent was prepared in 1XPBS (stock solution). 100 μl of working MTT reagent (1:10 dilution in 1XPBS) was added to the wells and incubated for 4 hours. After incubation, the reagent was discarded and 100 μl of DMSO was added to each well to lyse the cells. Absorbance was measured at 570nm using a plate reader (Tecan). Cell viability was expressed as % viability as compared to control.

2.6 Measurement of intracellular nitric oxide (NO) using flow cytometry

Intracellular nitric oxide production was quantified using flow cytometry. EA.hy926 cells were seeded at a density of $3x10^4$ cells/well in 48-well tissue culture plates. After adherence, cells were starved for 12-14 hours to reduce basal NO levels. The spent media was removed and a working concentration of DAF-FM DA dye, purchased from Invitrogen, (1:1000 silution) was prepared in DMEM without FBS. 300 μl of the dye was added to the wells and incubated for 30 minutes at 37°C and 5% CO_2. After incubation, the dye was removed and washed twice with 1XPBS. Subsequently, the cells were stimulated with different concentrations of tea actives (prepared in DMEM without FBS)

for 30 minutes at 37ºC and 5% CO_2. Post incubation, the media was removed and washed twice with 1XPBS. Cells were fixed with 4% paraformaldehyde solution after trypsinization with 0.25% trypsin 0.25 mM EDTA solution. Cell suspension was transferred to a 96-well microtiter plate. A population of 10,000 cells were gated and segregated based on their relative fluorescence intensities at 480 nm using Cell Quest Pro software by flow cytometer (BD FACS Calibur). The mean fluorescence yield was measured and compared to the population of untreated cells. The dye is non-fluorescent until it reacts with NO to form a fluorescent benzotriazole.

2.7 Determination of free radical scavenging property using DPPH assay

Radical scavenging capacity of tea actives was determined using the DPPH radical method. Briefly, in a 48-well plate 500 µl of DPPH (Sigma) solution (60 µM) prepared in ethanol was added to 500 µl of tea actives (prepared in ethanol). The mixture was shaken vigorously and the absorbance was measured at 517 nm after 15 and 30 minutes, after which the absorbance reached a steady state. α-tocopherol (Sigma) was used as a positive control. 500 µl of absolute ethanol added to DPPH reagent served as control. Inhibition was calculated as follows:

% Inhibition = ((OD of Control – OD of sample)/ OD of Control) x 100

2.8 Measurement of endothelin-1 secretion using ELISA

EA.hy926 cells were seeded in 24-well plates at a density of 10^5 cells/well. After 16 hours of stabilization, the spent media was removed and washed twice with 1 X PBS. Tea actives prepared in fetal bovine serum free DMEM were added and incubated for 6 hours at 37ºC and 5% CO_2. Post incubation, the supernatant from each well was collected and stored at -20ºC. ELISA kit procured from R&D systems (Quantikine SET100) was used for measuring endothelin-1 in the supernatant. Endothelin standards were prepared as directed to give the calibration plot. Working concentration of supernatant (250 µl) was prepared by diluting (1:10) with assay diluent. In the microplate strips provided, 150 µl of assay diluent was added to each well. To this, 75 µl

of sample, controls and standards were added. The plate was incubated for 1 hour at 25°C on a shaker. After incubation, the contents were removed and washed using the Wash Buffer. The process was repeated for four times blotting the plate against dry paper towels to ensure complete removal of liquid. Subsequently, 200 µl of Endothelin-1 Conjugate was added to each well and incubated for 3 hours on a shaker at 25°C. Post incubation, the contents were removed and washed (four times). 200 µl of Substrate Solution was added and incubated for 30 minutes, protecting from light. Post incubation, 50 µl of Stop Solution was added to each well and mixed properly. Measurements were taken using a microplate reader by subtracting reading at 450 nm from reading at 540 nm. Endothelin-1 was measured using the calibration plot and compared to untreated control cells.

2.9 Measurement of ACE activity using Rabbit Lung ACE

The solutions of ACE-inhibitor captopril (200 nM) and tea actives were prepared in 150mM tris-base buffer fresh before the experiment. 0.75 mU of ACE (Rabbit lung) enzyme procured from Sigma was prepared in 150 mM tris-base buffer (pH 8.3). In a 96-well white flat bottom plate, 25 µl of tea actives were added in each well. To this, 25 µl of enzyme was added. The assay mixture was incubated for 1 hour at 37°C and 5% CO_2. Subsequently, ACE substrate [Phe-Arg-Lys-(Dinitrophenyl) P-OH-o-Amino benzoic acid) procured from Sigma was prepared at a concentration of 40 µM in 150 mM tris-buffer (ph 8.3) and 1.125 M NaCl. To the reaction mixture, 50 µl of fluorescent substrate solution was added. The plate was tapped for uniform mixing and readings were taken at 30 minutes, 1 hour, 2 hours and 3 hours post addition. The fluorescence was measured at an excitation wavelength of 320 nm and emission wavelength of 430 nm using a plate reader (Tecan). The plate was kept in a shaker at 37°C, protected from light.

Chapter 3: Results and Discussion:

3.1 Selection of *in vitro* biomarkers showing association with CVD:

Various clinical biomarkers are associated with functionality of the endothelium. The biomarkers were prioritized based on the physiological effects exerted by them and its relevance in clinical end benefit. These are reported in Figure 14.

Reference	Biomarker	Clinical Measurement	Contribution to CVD
Ras et al., 2013	Nitric Oxide	Flow-mediated dilation (FMD)	10% decrease in CVD risk per 1% increase in FMD
Galie et al., 2009	Endothelin-1	Pulse wave velocity (PWV)	Intervention with Endothelin-1 receptor blockers (ERBs) showed a reduction in all-cause mortality by 40%
Ferari and Boersma, 2013	Angiotensin-Converting Enzyme	Carotid intimal-media thickness (cIMT)	Intervention with ACEIs decreased CVD mortality by 12%
Ye and Song, 2008	Reactive oxygen species	Forearm venous occlusion plethysmography post supplementation with antioxidants	30IU/day consumption of Vitamin E showed 4% decrease in CHD risk

Setoguchi et al., 2001	Tetrahydrobiopterin	Forearm venous occlusion plethysmography followed by folic acid/ BH_4 supplementation	BH_4 supplementation increased coronary blood flow by 143±40%
Kielstein et al., 2004b	Assymetric dimethylarginine	Carotid intimal-media thickness	Application of ADMA reduced cardiac output by 14% and increased vascular resistance by 11%
Shemyakin, et al., 2012	Arginase	Venous occlusion plethysmography after supplementation with arginase inhibitors	Significant increase in FBF post intervention with arginase inhibitors

Figure 14: Biomarkers, their clinical measurement and contribution in CVD risk reduction.

3.1.1 Nitric oxide:

In response to physiological shear stress, the endothelium produces nitric oxide (NO) by the action of the eNOS enzyme which utilizes L-arginine as substrate and calmodulin, heme, BH_4, NADPH, FAD and FMN as co-factors. The NO produced diffuses into vascular smooth muscle cells where it binds to the heme of soluble guanylyl cyclase (sGC) followed by activation of the enzyme to produce cGMP. The secondary messenger leads to activation of myosin light chain kinase (MLCK) resulting in vasodilatory response (Förstermann and Münzel, 2006).

Such provascular effects are compromised in disease states such as hypercholesterolaemia, hyperglycemia, hypertension, etc. Cellular investigations have

revealed that a decrease in NO via various mechanisms is associated with compromised vessel function. Such state of the blood vessel is referred to as endothelial dysfunction. Endothelial dysfunction reflects in decreased FMD and is an independent predictor of CVD risk (Kietadisorn et al., 2012). The molecular regulation is illustrated in Figure 15.

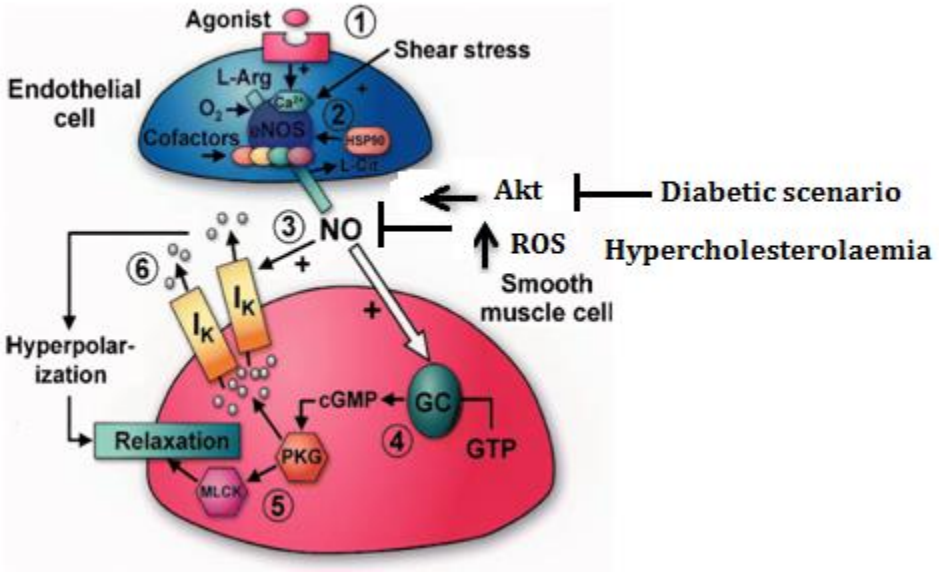


Figure 15: Nitric oxide synthesis in health and disease: Nitric oxide biosynthesis is triggered by the binding of agonists or by shear stress (1). eNOS enzyme converts the amino acid L-arginine into NO, with L-citrulline as byproduct (2). NO diffuses into adjacent smooth muscle cells (3) where it activates its effector enzyme guanylate cyclase (GC). GC converts GTP into cGMP, which activates protein kinase G (PKG), leading to modulation of myosin light chain kinase and smooth muscle relaxation (5). PKG also causes activation of potassium channels (I_K), thereby increasing cell membrane hyperpolarization and causing relaxation (6). In disease condition like diabetes, eNOS phosphorylation by Akt is inhibited which leads to decreased NO. In hypercholesterolaemia, increase in ROS decreases NO levels. Adapted from Khurana and Meyer, 2003

Understanding regulation of NO levels in the endothelium remains an ideal marker in several disease pathophysiology. Various methodologies have been implicated in

measurement of NO. It can be measured by its direct interaction with fluorescent dyes (DAF, DAR) (Kojima et al., 1998a; Kojima et al., 2001). It can also be measured indirectly as nitrates and nitrites by colorimetry (Griess) or fluorescent dyes (DAN) (Misko et al., 1993). Fluorescent dyes which are specific to NO have been investigated. Detection of intracellular NO remains more logical due to its quenching effect atleast for screening polyphenols. Currently, fluorescent dyes in their esterified forms, which can selectively permeate through living cells are used to detect NO intracellularly. The esterified form is less fluorescent but due to the presence of cellular esterases is cleaved to its non-ester form which can selectively bind to NO giving higher fluorescence (Itoh et al., 2000). Thus, such a fluorescent measurement can be used as an ideal *in vitro* tool to measure NO in endothelial cells. However, heterogeneity in endothelial cells is reported *in vitro* due to its differentiation pattern, eNOS expression and eNOS localization. This acts as a drawback for determining the exact levels of NO in endothelial population. Flow cytometry based segregation of responding and non-responding cells followed by estimation of NO levels in responding population would reflect the exact level of NO. Thus, flow cytometry was used in the current study.

3.1.2 Endothelin-1:

Blood vessel flexibility is mostly regulated by elasticity of vascular smooth muscle cells in response to physiological stimulus. This elasticity of blood vessel is compromised in conditions such as diabetes, hypertension, hypercholesterolaemia, and atherosclerosis. The molecular understanding of such a phenomenon is governed by the levels of endothelin-1 (ET-1) in the plasma (Pernow et al., 2012). The role of ET-1 on vascular function has been recently reported in a metaanalysis, wherein interventions with ET-1 receptor antagonists have been shown to give cardiovascular benefit (Pernow et al., 2012). However, the exact mechanism of how ET-1 compromises vascular function is not clear. A proposed mechanism is based on an increase in ROS which would dissipate NO and induce smooth muscle cell proliferation. ET-1 is synthesized from the precursor peptide big ET-1, which is cleaved into the 21-amino acid ET-1 by the action of the endothelin-converting enzymes (ECEs). While endothelial cells are the major source of ET-1, it is also found in vascular smooth muscle cells, central nervous system

and reproductive tissues. The functional ET-1 is reported to have diverse function based on its interaction with specific receptors. ET-1 signals via two receptor subtypes: ET_A and ET_B. ET_A receptors are found on vascular smooth muscle cells (VSMCs), while ET_B receptors are found both on endothelial cells and VSMCs. Both the receptors found on the VSMCs mediate vasoconstriction and cell proliferation while ET_B receptors on endothelial cells (sparsely expressed) mediate vasodilation (Unic et al., 2011). Although ET-1 binding to its receptor on smooth muscle cells regulates blood vessel flexibility, ET-1 release occurs in endothelial cells. Therefore, regulating ET-1 release rather than ET-1 function would be a relevant target. The molecular pattern has been illustrated in Figure 16.

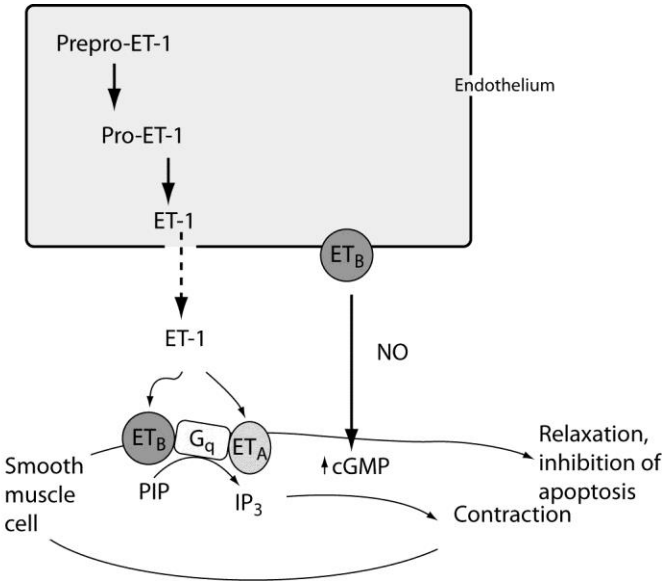


Figure 16: The product of ET1 transcription is prepro-ET-1, which is cleaved by a neutral endopeptidase to form the active precursor pro-ET-1 or big ET-1. Big ET-1 is converted to the mature peptide by the metalloproteinase endothelin-converting enzyme-1 (ECE-1). Two ET receptors have been identified in the vasculature: ET type-A receptors (ETA) reside in vascular smooth muscle cells and mediate vasoconstriction and cell proliferation, whereas ETB receptors reside on endothelial cells and are mainly vasodilatory through NO. ETB receptors on smooth muscle cells can elicit vessel contraction. ETA stimulation results in phospholipase C (PLC) activation generating 1,4,5-inositol triphosphate

(IP_3) and diacylglycerol (DAG) resulting in Ca^{+2} influx and phosphokinase c (PKC) activation leading to constriction. Adapted from Unic et al., 2011.

ET-1 is measured both *in vivo* and *in vitro* using ELISA. Since ET-1 is secreted by endothelial cells, ELISA serves as a sensitive and specific method for detecting ET-1

3.1.3 Angiotensin-converting enzyme (ACE):

Angiotensin II (Ang II), the principal effector of the Renin-Angiotensin-Aldosterone System (RAAS) hormonal cascade, which regulates blood pressure, also acts as a potent vasoconstrictor. AT_1 and AT_2 are the two receptor subtypes which mediate the effects of Ang II. While AT_1 is ubiquitously expressed in the cardiovascular system, AT_2 expression is lower. The vasocontrictory effects of Ang II are via AT_1 receptor while AT_2 has been demonstrated to exert vasodilation. Ang II is reported to increase ET-1 expression by inducing its transcription. It has also been reported to inhibit insulin-mediated signaling, thereby blunting the protective effects of nitric oxide. Angiotensin-converting enzyme (ACE) is the rate-limiting enzyme of the cascade which cleaves Ang I to give Ang II. Increase in plasma levels of Ang II have been associated with increased arterial stiffness, intimal media thickness and blood pressure resulting in hypertension and atherosclerosis (Ferario et al., 2006). Recently, ACE inhibitors have been evaluated for vascular benefits. A recent metaanalysis with ACEI intervention showed a 12% in CVD mortality (Ferari and Boersma, 2013). Another metaanalysis reported that ACEIs increased FMD by 1.26% (Shahin et al., 2011).ACEIs are reported to be more effective in improving FMD than other antihypertensive agents (Shahin et al., 2011). Hence, evaluating ACE activity is essential for assessing vascular benefit.

Tea is associated with reduced CVD risk and is reported to modulate above mentioned biomarkers. However, tea has a multitude of actives and an understanding of active-specific function for all these biomarkers remains elusive. Thus, in this study we investigated green tea and black tea and its known ingredients for its potential to modulate the biomarkers using various *in vitro* bioassays.

3.2 Effect of tea and tea ingredients on the viability of human endothelial cells

As the first step of investigation we determined the sub-toxic concentration of tea and tea ingredients using MTT assay. The results are shown in Figure 17 and 18.

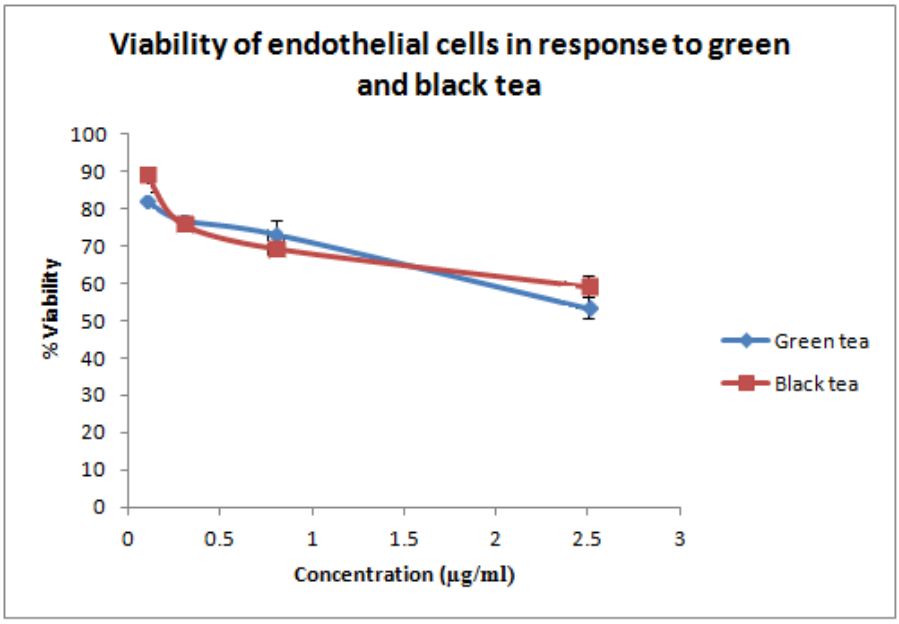


Figure 17: Dose-dependent effects of green tea and black tea (0.1µg/ml-2.5µg/ml) on viability of EA.hy926 cells. Results shown as mean ± SD.

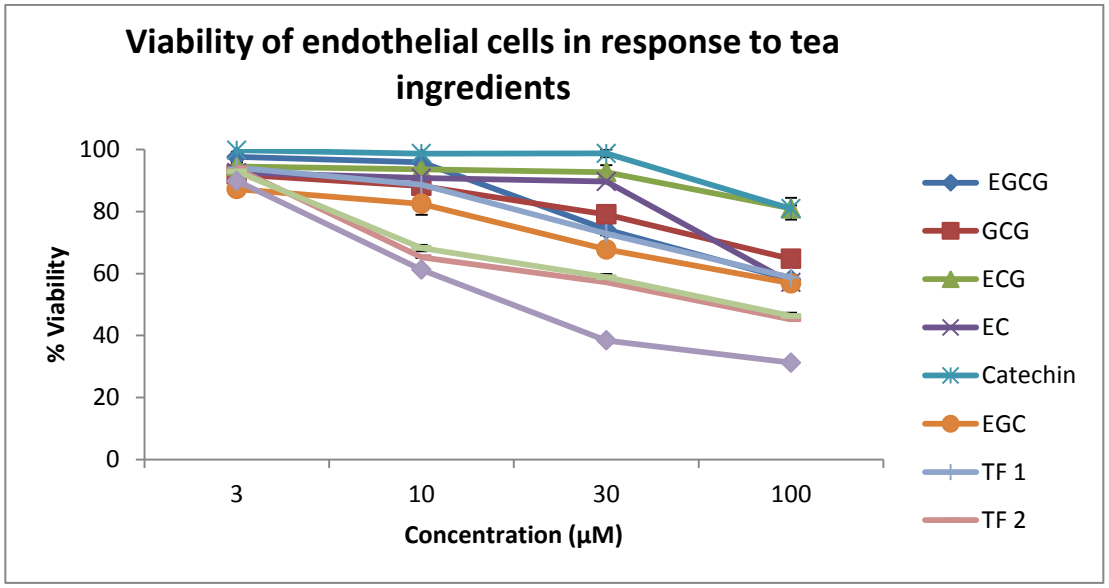


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Figure 18: Dose-dependent effects of tea catechins and theaflavins (3µM-100µM) on viability of EA.hy926 cells. Results shown as mean ± SD.

3.3 Modulation of in vitro biomarkers by tea and tea ingredients

As described earlier, tea may exert its beneficial actions on health through a multitude of ingredients. Here, we aim to focus on key intracellular, extracellular, and enzymatic targets and their modulation by tea.

3.3.1 Effect of tea and tea ingredients on nitric oxide production in endothelial cells

Tea has been shown to improve vascular function majorly by increase NO bioavailability. While epidemiological studies show that green tea consumption reduced CVD risk, studies using black tea either show positive or neutral effects. The positive effects are mainly attributed to flavonoids in tea. Though the flavonoid levels remain similar, green tea majorly consistes of catechins while black tea contains theaflavins and thearubigins. Although, both green tea and black tea have diverse flavonoid profile, the physiological benefits remain similar. Hence, we aim to compare the effects of green and black tea and its ingredients on nitric oxide production.

3.3.1.1 Effect of green and black tea on nitric oxide levels

In order to determine the effect of green and black tea on intracellular nitric oxide levels, EA.hy926 human endothelial cells were treated with DAF- FM. The cells were treated with green and black tea for 30 minutes. Subsequently, nitric oxide levels were measured using flow cytometry. The results are reported as fold change of mean intensity values with respect to the untreated control (Figure 19).

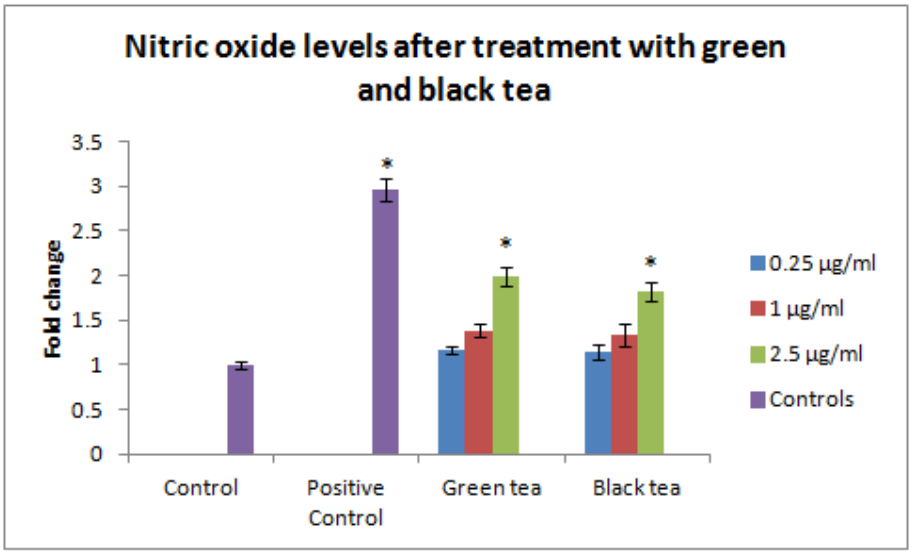


Figure 19: Fold change in the levels of nitric oxide after treatment with different concentrations of green and black tea (0.25µg/ml-2.5µg/ml) with respect to the control (no treatment) in endothelial cells. Cells were also treated with eNOS stimulator as positive control. Bars with * show significant increase with respect to control ($p<0.01$). Data has been reported as mean ± SD.

Treatment of endothelial cells with both green and black tea at a concentration range from 0.25µg/ml-2.5µg/ml showed significant increase in nitric oxide levels as compared to control cells. Both green and black tea showed similar increase in intracellular nitric oxide. These results are in agreement with a previous study which demonstrates that both green and black tea show similar increase in FMD and eNOS activity by increasing phosphorylation of the enzyme (Jochmann et al., 2008). Studies have demonstrated that tea flavonoids are responsible for the health-promoting effects observed upon tea consumption (Riemersma et al., 2001). Hence, we aim to evaluate the effect of known tea flavonoids.

3.3.1.2 Effect of tea catechins and theaflavins on nitric oxide levels

The effect of tea catechins (1µM-10µM) and theaflavins (1µM-10µM) on nitric oxide production was evaluated in endothelial cells. The results are reported in Figure 20.

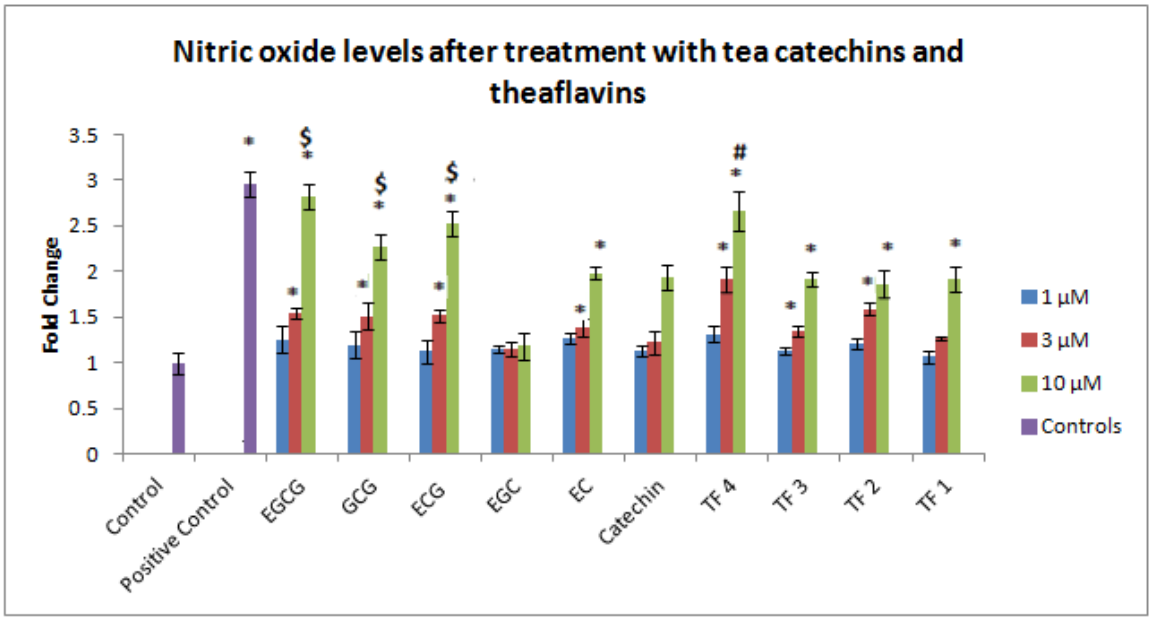


Figure 20: Fold change in the levels of nitric oxide after treatment with different concentrations of tea catechins and theaflavins (1µM-10µM) with respect to the control (no treatment) in endothelial cells. Cells were also treated with eNOS stimulator as positive control. Bars with * show significant increase with respect to control ($p<0.05$). Bars with $ show significant increase as compared to treatment with Epicatechin (10µM) ($p<0.01$). Bar with # show significant increase as compared to Theaflavin 1 (10µM) ($p<0.01$). Data has been reported as mean ± SD.

At a concentration of 10µM, all tea catechins and theaflavins show a significant increase in nitric oxide levels compared to control. The response was concentration-dependent (Supp. Fig. 4A) and followed the following pattern:

EGC<TF1≤TF2≤TF3<Catechin<EC<GCG<ECG<TF4≤EGCG.

Consistent with previous observations, gallated catechins (R^2=0.9) are more potent in increasing nitric oxide levels than non-gallated catechins (R^2=0.6) (Lorenz et al., 2009). The correlation graphs are shown in Supp. Fig. 4B, C.

The difference observed in nitric oxide levels upon treatment with gallated and non-gallated catechins has been attributed to the differential eNOS phosphorylation. EGCG has been reported to increase eNOS activity by increasing phosphorylation at Ser 1177 (Lorenz et al., 2004) while EC has been shown to increase eNOS activity by phosphorylating ser 1177 and 633 and dephosphorylating Thr 495 (Ramirez-Sanchez et al., 2010). However, complete illustration of molecular regulation of eNOS phosphorylation by different catechins is yet to be studied. This differential regulation of the enzyme may account for the difference in nitric oxide levels observed. Black tea polyphenols have been reported to increase eNOS activity by stimulating estrogen receptor and phosphorylating eNOS at Ser 1177 and dephosphorylating Thr 495 (Anter et al., 2005). The mechanism exerted by theaflavins to enhance nitric oxide has not been understood yet. Thearubigins in black tea are also not well characterized. Hence, further studies must be conducted to understand the mechanism employed by various tea flavonoids on modulation of eNOS enzyme.

3.3.2 Extracellular free radical scavenging by tea

Increase in ROS production has been shown to be crucial in the pathogegenesis of atherosclerosis. High levels of ROS have also been associated with various diseases like diabetes, cardiovascular and neurodegenerative diseases. Free radicals tend to react with available lipids and proteins, and alter their function. The best example being the oxidation of LDL to form ox-LDL, a critical biomarker of atherosclerosis. The health-promoting effects of tea have been attributed to the potent radical scavenging property of tea flavonoids. Here, we aim to measure the radical scavenging property of tea and tea ingredients.

3.3.2.1 Effect of green and black tea on free radical scavenging property

To measure the free radical scavenging property, both green and black tea (2.5 µg/ml, 7.5 µg/ml) were incubated with DPPH (60µM) for 30 minutes. Post incubation, the ability to prevent DPPH reduction was measured by reading the absorbance at 517nm. The results are represented as % inhibition as compared to untreated control (Figure 21).

Both green and black tea show significant radical scavenging with green tea exhibiting greater scavenging property than black tea. This is consistent with previous reports showing green tea to have higher antioxidant capacity than black tea (Lee et al., 2002). Radical scavenging property has been attributed to the flavonoids found in tea (Nanjo et al., 1996). The difference is proposed to be due to the different composition of the two teas.

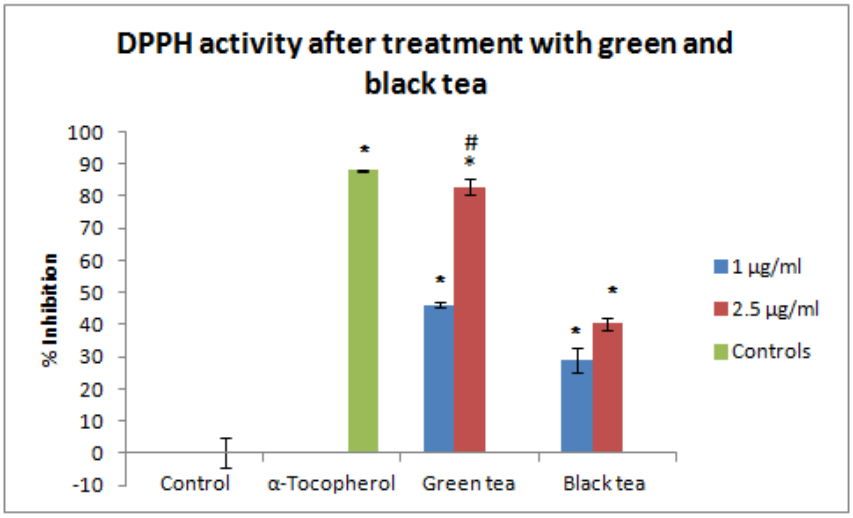


Figure 21: Percent inhibition in DPPH activity after treatment with different concentrations (1µg/ml, 2.5µg/ml) of green and black tea. α-Tocopherol was used as positive control (100nM). Bar with * shows significant decrease in DPPH activity as compared to control ($p<0.01$). Bar with # shows significant increase as compared to black tea (2.5 µg/ml) ($p<0.01$). Data has been reported as mean ± SD.

3.3.2.2 Effect of tea catechins and theaflavins on free radical scavenging property

To measure the free radical scavenging property, tea catechins and theaflavins (10µM, 30µM) were incubated with DPPH (60 µM) for 30 minutes. Results are shown in Figure. 22.

All the tea ingredients showed significant inhibition of DPPH radical activity at both the concentrations tested. The potency of radical scavenging observed here, at 30μM is: Catechin≤EGC<EC≤TF1<GCG≤TF2≤TF3≤EGCG<ECG<TF4.

Further, a dose-dependent response was shown by gallated catchins. This is consistent with previous observation which reports that theaflavins and catechins have similar antioxidant properties (Leung et al., 2001). However, gallated catechins (R^2=0.5) were shown to be more potent than non-gallated catechins (R^2=0.3) (Supp..Fig.7E-F), consistent with previous reports (Nanjo et al.,1996).

Catechins exert their antioxidant property by chelating metal ions such as copper (II) and iron (III) to form inactive complexes and preventing the generation of potentially damaging free radicals. Catechins also scavenge free radicals by forming stable semiquinone free radicals, thus, preventing the deaminating ability of free radicals (Sutherland et al., 2006). The galloyl moiety at 3 position in flavan-3-ols is essential for their radical scavenging property which accounts for the greater potency of gallated catechins and digallated TF4. Also, the presence of atleast an ortho-dihydroxyl group in the B ring is crucial for maintaining the effectiveness of the radical scavenging ability (Leung et al., 2001).

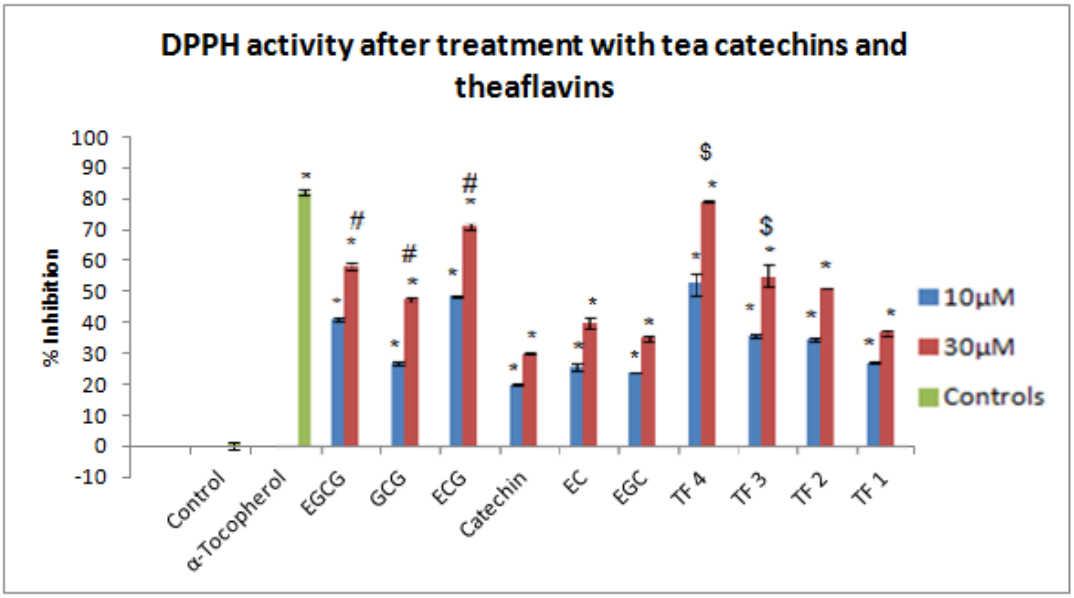


Figure 22: Percent inhibition in DPPH activity after treatment with different concentrations (10μM, 30μM) of tea catechins and theaflavins. α-Tocopherol (100nM) was used as positive control. Bars with * represent significant decrease in DPPH activity compared to control ($p<0.01$). Bars with # shows significant increase as compared to epicatechin at the same concentration ($p<0.01$). Bar with $ shows significant increase as compared to theaflavin 1 at the same concentration ($p<0.01$). Data has been reported as mean ± SD.

3.3.3 Extracellular endothelin-1 modulation by tea

As described, endothelin-1(ET-1) is a potent vasoconstrictor secreted by endothelial cells. Although ET-1 has been shown to play an important role in various pathological conditions, only a few studies have been conducted till date which investigate the effect of tea on ET-1 regulation. Hence, we aim to determine the effect of tea and its ingredients on ET-1 secretion by endothelial cells.

3.3.3.1 Effect of green and black tea on endothelin-1 secretion

To determine the effect of green and black tea on endothelin-1 secretion in endothelial cells, EA.hy926 cells were treated with various concentrations of green and black tea. After 6 hours of incubation, the supernatant was collected and ELISA was performed to measure the amount of endothelin-1 secreted by the endothelial cells. The results are shown as % reduction in ET-1 secretion as compared to untreated control (Figure 23).

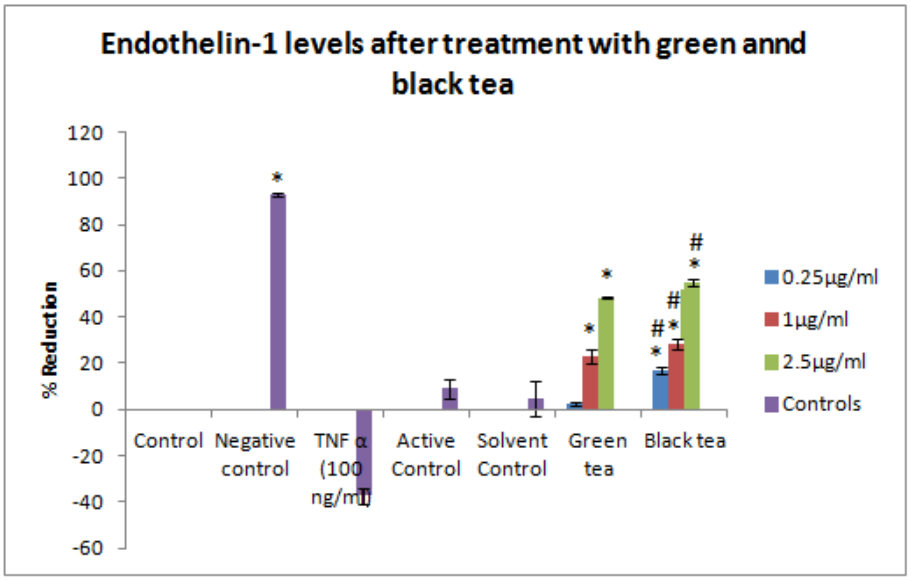


Figure 23: Percent reduction in the levels of endothelin-1 secretion after treatment with different concentrations of green and black tea (0.25µg/ml-2.5µg/ml) with respect to the control (no treatment) in endothelial cells. Cells were also treated with TNFα (100 ng/ml) as positive control and endothelin antagonist as negative control. Bars with * represent significant reduction in endothelin-1 secretion as compared to control (p<0.01). Bars with # show significant increase as compared to treatment with green tea of the same concentration (p<0.01). Data has been reported as mean ± SD.

Treatment of endothelial cells with both green and black tea showed a significant decrease in ET-1 secretion as compared to untreated control cells. These results are consistent with previous observations which suggest that ingestion of green tea extract by rats reduces ET-1 and prepro-ET-1 mRNA expression (Kim et al., 2006). Till date, the effect of black tea on ET-1 has not been investigated. A proposed mechanism would be that the flavonoids in green and black tea suppress ET-1 transcription by increasing NO bioavailability. Another hypothesis is that flavonoids may act as tyrosine kinase inhibitors thereby suppressing ET-1 (Larsen et al., 2010). It has been demonstrated that resveratrol, a phenolic component of red wine reduces ET-1 expression via interfering with the ERK1/2 pathway by attenuating ROS (Liu et al., 2003). Flavonoids may also modulate ET-1 via this pathway as they have been shown to efficiently reduce ROS

levels. However, direct effects of flavonoids on ET-1 transcription needs further investigation.

3.3.3.2 Effect of tea catechins and theaflavins on endothelin-1 secretion

In order to measure the effect of tea catechins on endothelin-1 secretion, human endothelial cells were treated with different concentrations of tea catechins (3µM-30µM). Results are shown in Fig. 24.

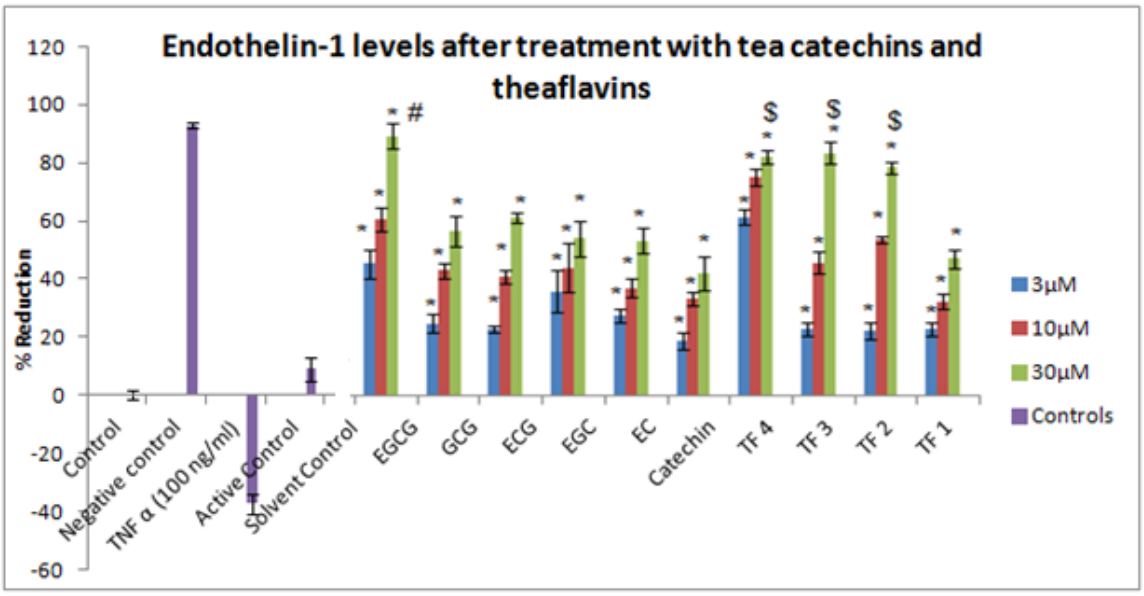


Figure 24: Percent reduction in the levels of endothelin-1 secretion after treatment with different concentrations of tea catechins and theaflavins (3µM-30µM) with respect to the control (no treatment) in endothelial cells. Cells were also treated with TNF α (100 ng/ml) as positive control and endothelin antagonist as negative control. Bars with * represent significant reduction in endothelin-1 secretion as compared to control (p<0.01). Bars with $ show significant increase as compared to treatment with Epicatechin (30 µM) (p<0.01). Bars with # show significant increase as compared to Theaflavin 1 (30 µM) (p<0.01). Data has been reported as mean ± SD.

Treatment of endothelial cells with all tea catechins showed significant reduction in endothelin-1 secretion. The potency observed here, at 30µM is:

Catechin<TF1≤EC≤EGC≤GCG<ECG<TF2≤TF3≤TF4<EGCG. Consistent with previous studies, EGCG is the most potent in suppressing ET-1 expression (Reiter et al., 2010). However, a clinical study also reported that EGCG consumption did not lower plasma ET-1 while EC consumption was found to reduce ET-1 (Loke et al., 2008). It must be noted, however, that this was an acute intervention. Previous studies have reported that EC lowers ET-1 secretion as observed in the current study (Loke et al., 2008; Loke et al., 2010). Till date, effect of the remaining catechins on ET-1 secretion has not been investigated. This study demonstrated that EGC, ECG and GCG are as potent as EC in ET-1 reduction. It also showed that non-gallated catechins show a dose-dependent response (Supp. Fig. 4I). However, further studies must be performed to validate these results. A previous study reported that theaflavin did not lower ET-1 levels (Loke et al., 2010). However, the study did not investigate the effect of TF2, TF3 and TF4 on ET-1. In the current study, it has been found that all theaflavins show significant reduction in ET-1 secretion, with TF4, TF3 and TF2 being more potent than EC. This may explain the greater potency of black tea (rich in theaflavins) in ET-1 reduction. However, this is the first study to have evaluated the effect of theaflavins on ET-1 secretion. Further studies must be performed to validate the results.

EGCG has been reported to cause phosphorylation of FOXO1 by Akt and AMPK-mediated phosphorylation, resulting in a reducing of ET-1 transcription (Reiter et al., 2010). The mechanism by which EC exerts its effect on ET-1 expression has not been elaborated yet. The efficient ROS scavenging capacity of theaflavins may be responsible for their ability to suppress ET-1. However, further studies need to be conducted to gain a better understanding.

3.3.4 Ezymatic modulation of angiotensin-converting enzyme (ACE) by tea

Angiotensin-converting enzyme (ACE) is the rate-limiting enzyme of the rennin-angiotensin-aldosterone system (RAAS) which has been shown to be modulated by tea. Here, aim to determine the effect of tea and its ingredients on ACE activity.

3.3.4.1 Effect of green and black tea on ACE activity

The enzyme was incubated with green and black tea at two concentrations (3µL/mL and 10µL/mL) for 1 hour. Post incubation, the substrate was added and measurements were taken using TECAN. The readings were taken at 30 minutes, 1 hour, 2 hours and 3 hours post substrate addition. Here, the data is represented as % inhibition in ACE activity as compared to untreated control cells after 3 hours (Figure 25).

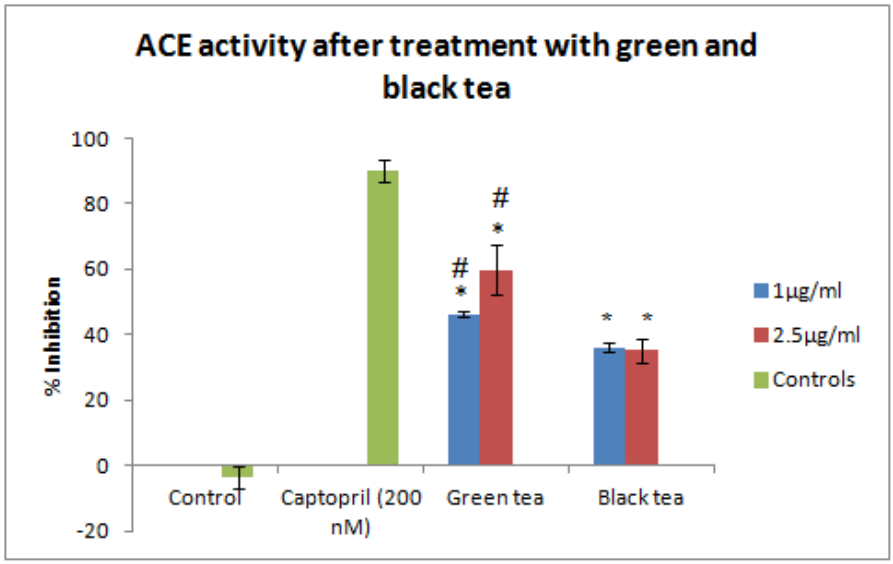


Figure 25: Percent inhibition in the activity of angiotensin-converting enzyme after treatment with different concentrations of green and black tea (1µg/ml and 2.5µg/ml) with respect to the control (no treatment) in endothelial cells. Captopril (200nM) was used as positive control. Bars with * represent significant inhibition in ACE activity as compared to control ($p<0.01$). Bars with # show significant increase as compared to treatment with black tea of the same concentration ($p<0.01$). Data has been reported as mean ± SD.

A significant reduction in ACE activity was observed upon treatment with both green and black tea, with green tea showing greater ACE inhibition. This is consistent with previous studies reporting a decrease in ACE activity upon treatment with both green and black tea with green tea having a lower IC_{50} compared to black tea (Persson et al., 2006; Dong et al., 2011). The study proposed that green tea inhibits ACE activity via

both irreversible inactivation of the enzyme as well as substrate-dependent mechanism while black tea acts by inactivating the enzyme alone.

3.3.4.2 Effect of tea catechins and theaflavins on ACE activity

The enzyme was incubated with tea catechins at two different concentrations (10µM and 30µM) for 1 hour. Subsequently, the substrate was added and measurements were taken using TECAN. Data is represented in Figure 26.

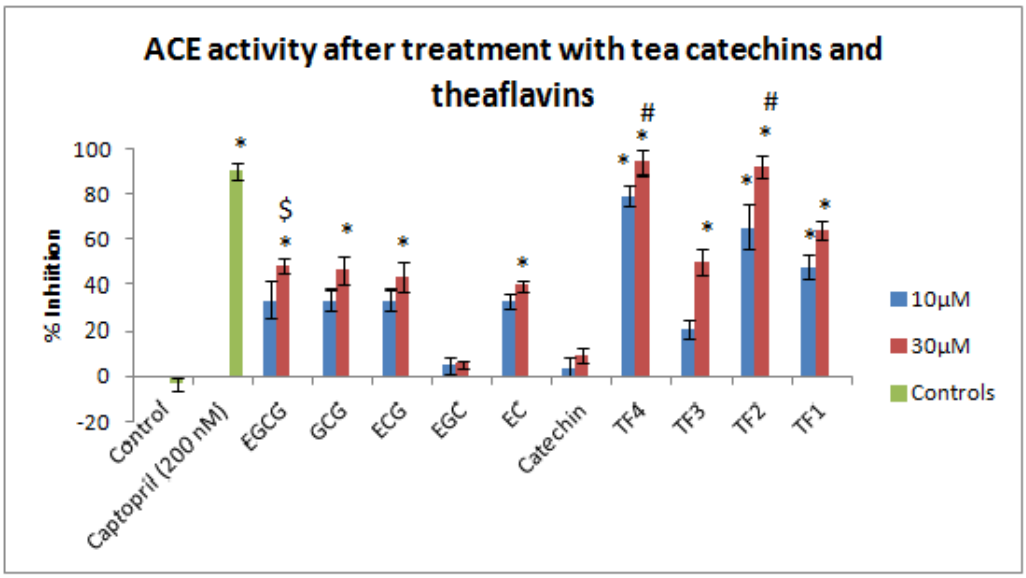


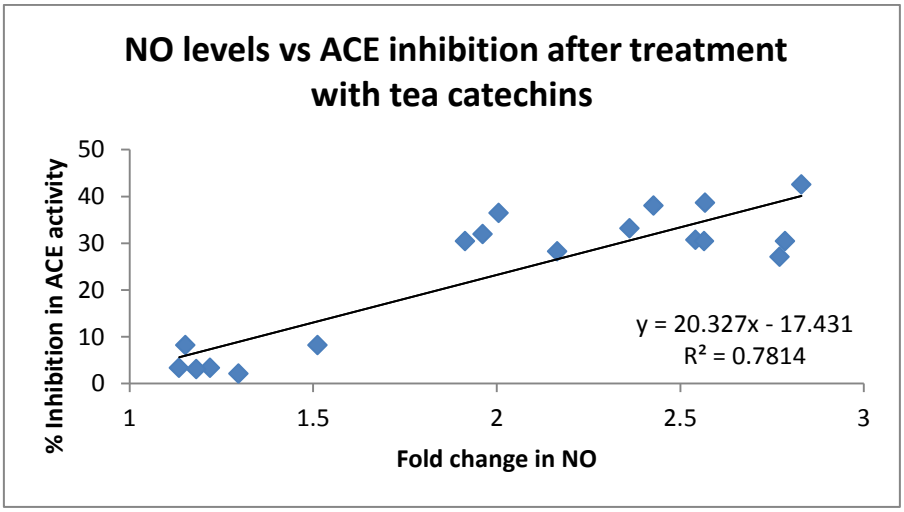
Figure 26: Percent inhibition in the activity of angiotensin-converting enzyme after treatment with different concentrations of green tea catechins and theaflavins (10µM and 30µM) with respect to the control (no treatment) in endothelial cells. Captopril (200nM) was used as positive control. Bars with * represent significant inhibition in ACE activity as compared to control ($p<0.01$). Bar with $ show significant increase as compared to treatment with Epicatechin (30µM) ($p<0.01$). Bars with # show significant increase as compared to Theaflavin 1 (30µM) ($p<0.01$). Data has been reported as mean ± SD.

At 30µM, all tea ingredients (except catechin) showed significant reduction in ACE activity. The potency of ACE inhibition observed here, at 30µM is: EGC<Catechin<EC≤ECG≤GCG≤EGCG≤TF3<TF1<TF2≤TF4.

These results are consistent with previous observations reported by using HUVEC cells (Persson et al., 2006). Till date, the effect of theaflavins on ACE inhibition has not been investigated. According to this study, theaflavins seem to be more potent in ACE inhibition than catechins. Lierature evidence also suggests significant reduction of blood pressure variability with black tea intervention, while green tea has no lowering effect. Catechins have been shown to chelate metal ions like zinc (II) which has been proposed as a mechanism for their ACE inhibiting property. An increased number of hydroxy groups and an addition of double-bond oxygen seem to increase ACE inhibition (Persson et al., 2006). Theaflavins have greater hydroxy groups than catechins which may increase their metal-chelating property. However, further studies need to be conducted to determine the effect of theaflavins on ACE activity and their mechanism of action.

3.4 Correlation between the selected biomarkers

The health benefits exerted by tea are proposed to be through the modulation of multiple molecular targets. Although the modulation of targets like NO, ET-1, ROS, etc by tea has been studied individually, the holistic effect of tea on different biomarkers has not been studied. Here, we aim to evaluate whether tea ingredients modulate various biomarkers together and if so, would it reflect in better vessel function. The following correlations were obtained (Figure 27): **A**.



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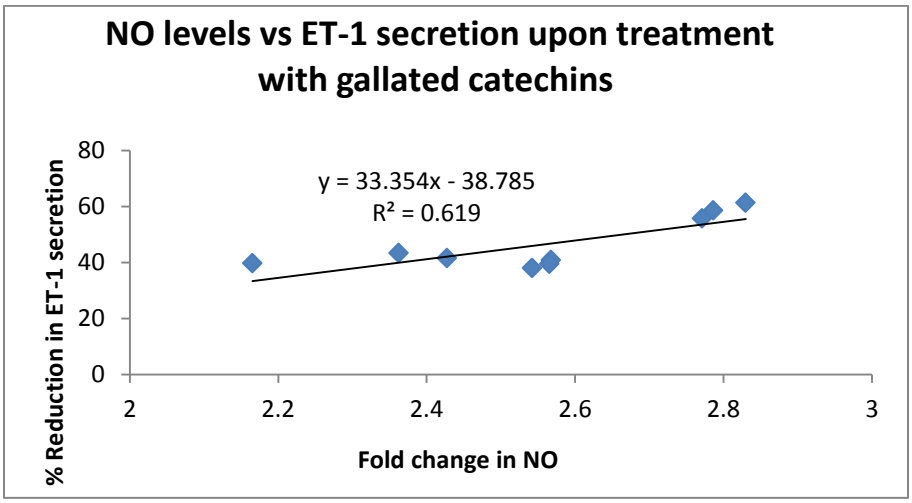
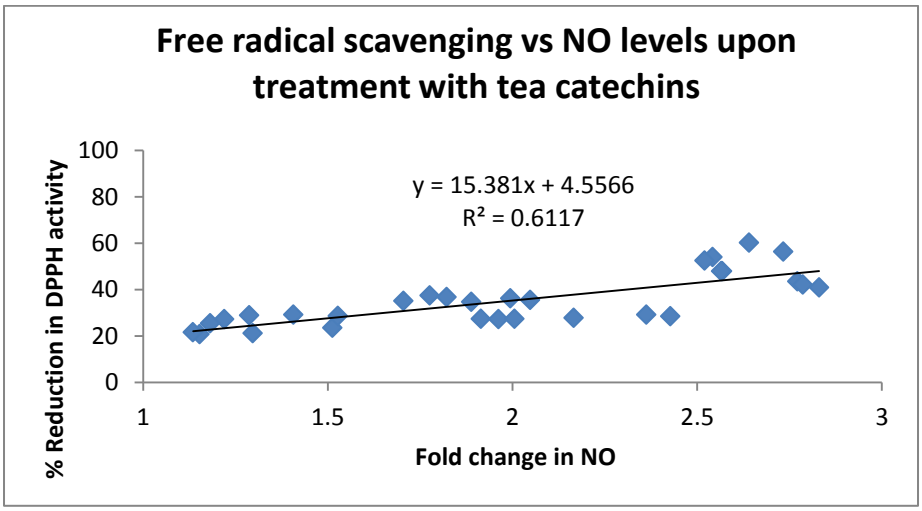
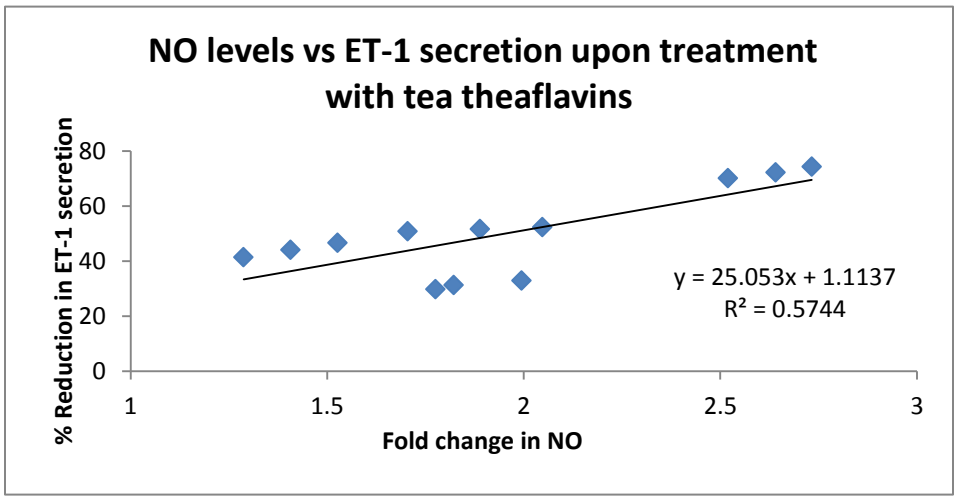
B.


NO levels vs ET-1 secretion upon treatment with gallated catechins
y = 33.354x - 38.785
R² = 0.619
% Reduction in ET-1 secretion
Fold change in NO
0
20
40
60
80
2
2.2
2.4
2.6
2.8
3

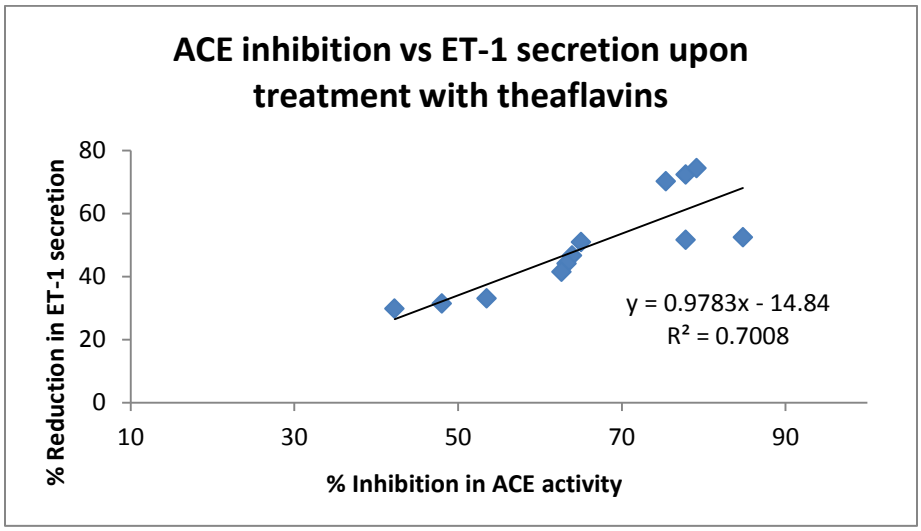
C.


Free radical scavenging vs NO levels upon treatment with tea catechins
y = 15.381x + 4.5566
R² = 0.6117
% Reduction in DPPH activity
Fold change in NO
0
20
40
60
80
100
1
1.5
2
2.5
3

D.


NO levels vs ET-1 secretion upon treatment with tea theaflavins
y = 25.053x + 1.1137
R² = 0.5744
% Reduction in ET-1 secretion
Fold change in NO
0
20
40
60
80
1
1.5
2
2.5
3

E.



F.

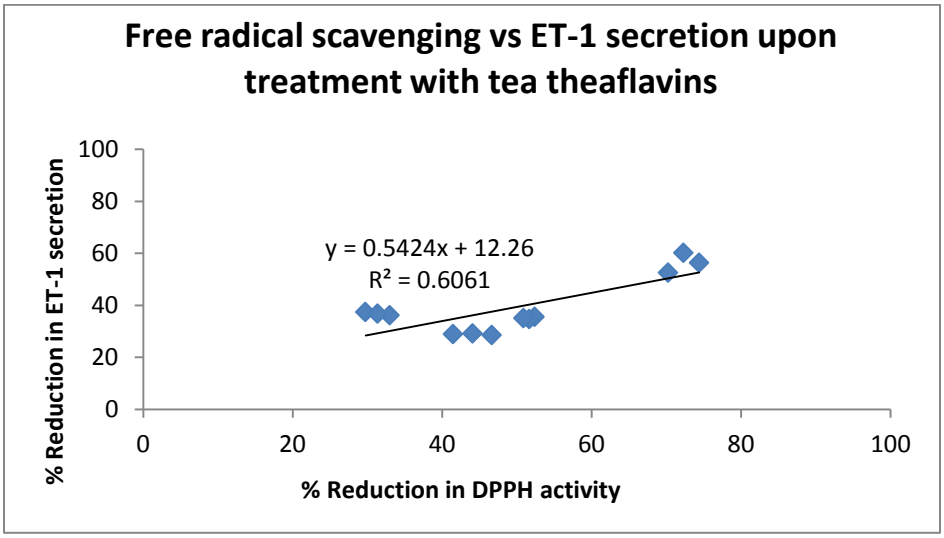


Figure 27: Correlation graphs- (A): correlation between intracellular nitric oxide levels and angiotensin-converting enzyme activity after treatment with tea catechins (10µM); (B): correlation between intracellular nitric oxide levels and endothelin-1 secretion after treatment with gallated tea catechins (10µM); (C): correlation between intracellular nitric oxide levels and free radical scavenging property after treatment with tea catechins (10µM); (D): correlation between intracellular nitric oxide levels and endothelin-1 secretion after treatment with tea theaflavins (10µM); (E): correlation between endothelin-1 secretion and

angiotensin-converting enzyme activity after treatment with tea theaflavins (10μM); (F): correlation between endothelin-1 secretion and free radical scavenging property after treatment with tea theaflavins (10μM).

The study indicated that molecules that showed higher NO activation potential also showed higher ET-1 inhibition (Fig. 28 B and D). This data is in agreement with the clinical evidence of black tea and green tea for decreased blood vessel occlusion (Peters et al., 2001). The association of ACE inhibition and NO production was also correlating with the clinical evidence on blood pressure lowering effect of tea (Onakpoya et al., 2014). Literature evidence on blood pressure lowering effects of tea suggests more benefit with black tea than green tea.

From the above correlation, it is important to note that upon treatment with tea catechins, especially gallated catechins, increasing intracellular nitric oxide levels corresponds to greater free radical scavenging property, and greater inhibition of ACE activity and ET-1 secretion. It is interesting that gallated catechins are the most potent in increasing nitric oxide bioavailability as shown in this study. A similar trend is observed upon treatment with theaflavins, which show maximum reduction in ET-1 secretion. Hence, it seems that the tea ingredients which are most potent in modulating a biomarker do so by exerting their effects on various targets. It can also be deduced that the biomarkers selected in this study are closely regulated by one another to maintain vessel function.

3.5 Way forward:

In this study, theaflavins exhibited greater potency in ET-1 reduction as well as inhibition of ACE activity. However, further studies need to be conducted to validate these results and to understand their mechanism of action. The mechanism by which catechins exert ET-1 reduction and ACE inhibition is not reported. Also, the modulation of eNOS enzyme by various catechins and theaflavins need to be studied. Further studies need to be conducted to understand the chronic effects of tea ingredients, their biotransformation and bioavailability. It must be noted that shear stress is a major regulator of vascular function in physiology. Therefore, the effect of shear stress must

be studied in health and disease conditions. Although a correlation has been observed in this study between various biomarkers, further studies must be conducted to understand the underlying mechanisms of these biomarkers and their effect on each other. Finally, a comprehensive model system must be developed to evaluate vascular benefit.

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Appendix:

1. Nitric oxide assay:

1.1 Response of EGCG at different concentrations

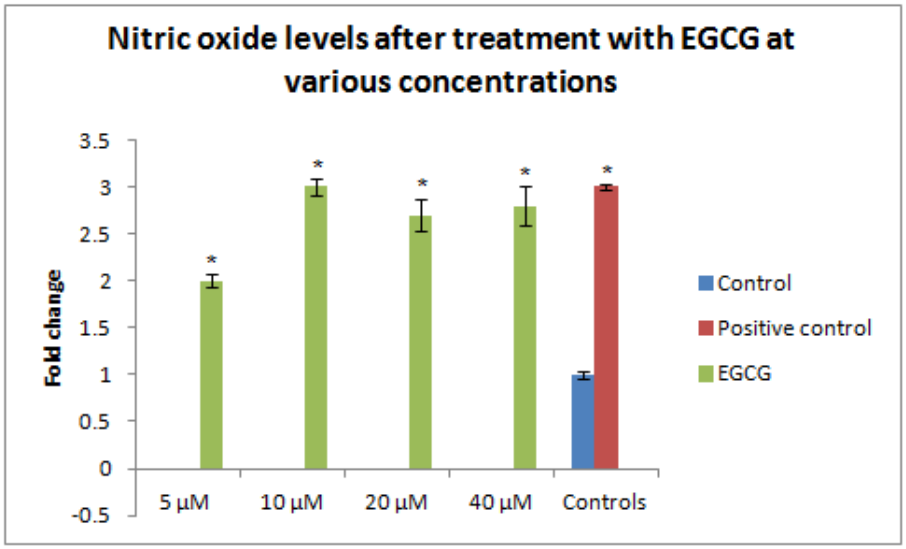


Figure 1: Fold change in nitric oxide levels after treatment with various concentrations of EGCG (5 µM-40 µM) as compared to control in endothelial cells. eNOS stimulator was used as positive control. Bars with * represent significant increase with respect to control (p<0.01).

Endothelial cells were treated with different concentrations of EGCG (5 µM-40 µM). The optimum response was observed at 10 µM with lower SD (Figure 1).

1.2 Response of EGCG with and without starvation

The endothelial cells were incubated with DMEM media with and without FBS for 16 hours. The starved endothelial cells had lower nitric oxide levels at baseline than cells incubated with FBS. The starved cells showed better respons upon treatment with EGCG (Figure 2).

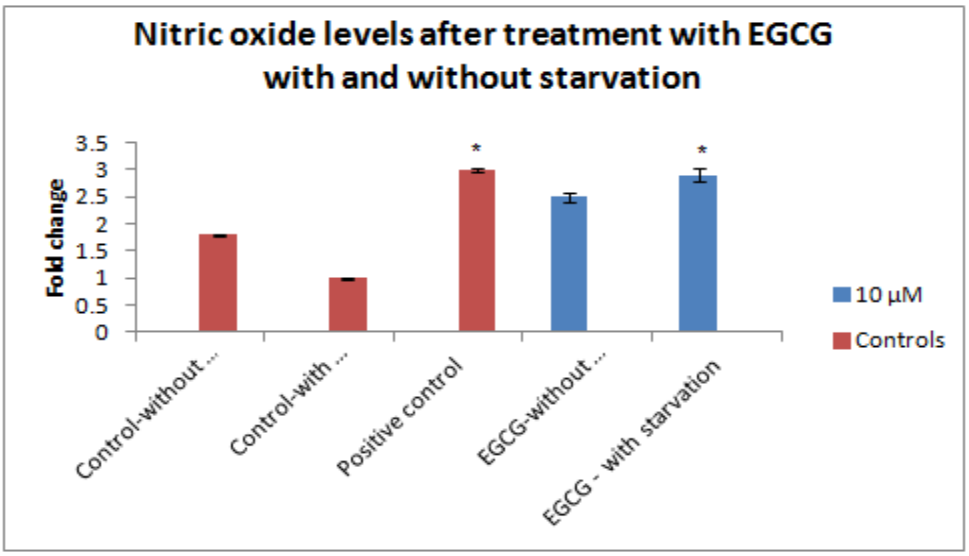
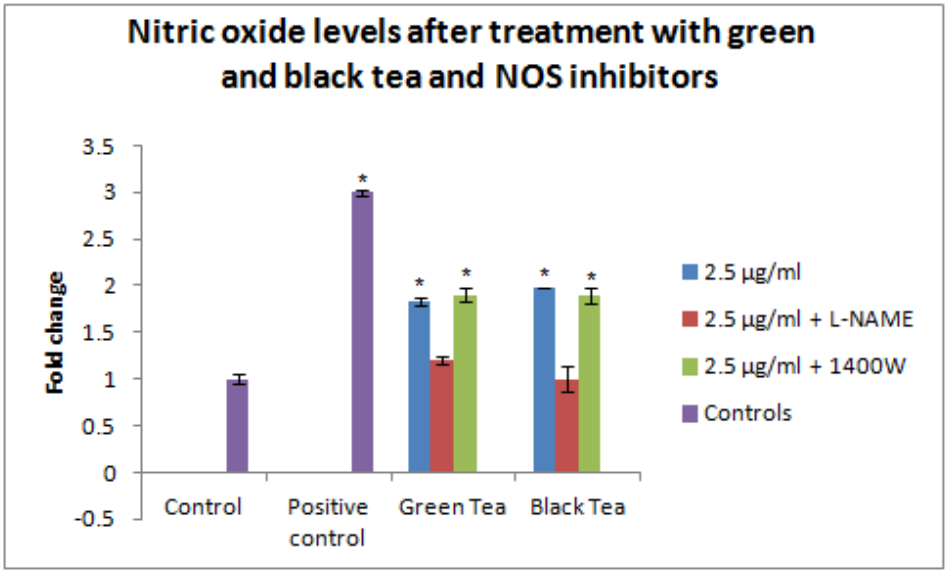


Figure 2: Fold change in nitric oxide levels after treatment with EGCG (10 µM) as compared to control in endothelial cells with and without starvation. eNOS stimulator was used as positive control. Bars with * represent significant increase with respect to control ($p<0.01$).

1.3 Response of tea/tea ingredients with NOS inhibitor (L-NAME) & iNOS inhibitor 1400 W

Endothelial cells were treated with NOS inhibitor L-NAME (N5-[imino(nitroamino)methyl]-L-ornithine, methyl ester, monohydrochloride) (Invitrogen) and inducible NOS (iNOS) inhibitor 1400W (N-(3-(aminomethyl)benzyl)acetamidine) (Invitrogen) for 24 hours. Subsequently, DAF-FM DA was added to the cells and incubated for 30 minutes. The cells were further treated with tea and tea ingredients. It was observed that all the tea ingredients showed significant increase in intracellular nitric oxide which was inhibited upon treatment with the general NOS inhibitor L-NAME while treatment with the iNOS inhibitor, 1400W did not show any reduction. This signifies that the observed nitric oxide is produced via the eNOS enzyme (Figure 3).

A.



B.

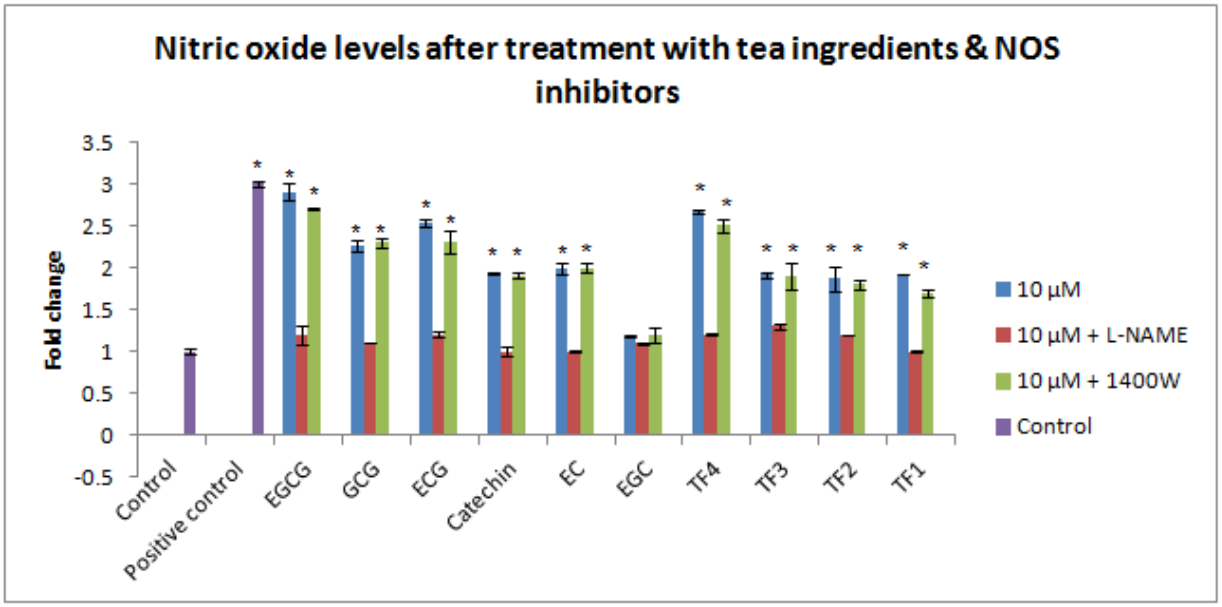


Figure 3: Fold change in nitric oxide levels after treatment with tea (A) and tea ingredients (B) (with and without NOS inhibitors, L-NAME and 1400W) as compared to control in endothelial cells. eNOS stimulator was used as positive control. Bars with * represent significant increase with respect to control ($p<0.01$)

2. Endothelin-1 assay

2.1 Reduction in endothelin-1 secretion after treatment with EGCG at various concentrations

Endothelial cells were incubated with EGCG at various concentrations (1 μM – 300 μM). The supernatant was collected and ELISA was performed. It was found that the optimum concentration which induced a significant reduction of ET1 secretion is 30 μM.

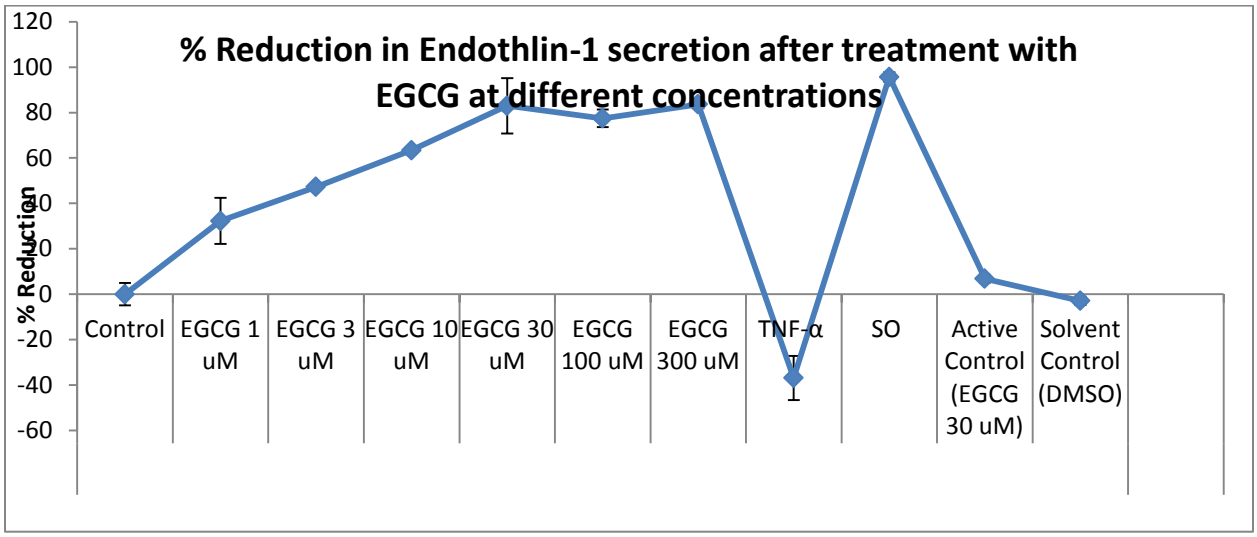


Figure 4: Percent reduction in the levels of endothelin-1 secretion after treatment with different concentrations of EGCG (1μM-300μM) with respect to the control (no treatment) in endothelial cells. Cells were also treated with TNF α (100 ng/ml) as positive control and endothelin antagonist as negative control.

2.2 Reduction in endothelin-1 secretion after treatment with EGCG at different time points

Endothelial cells were treated with EGCG (30 μM) and incubated for a range of time period (1 hour – 24 hours). The supernatant was collected and ELISA was performed. It was found that the maximum ET-1 reduction is seen upon incubation for 6 hours.

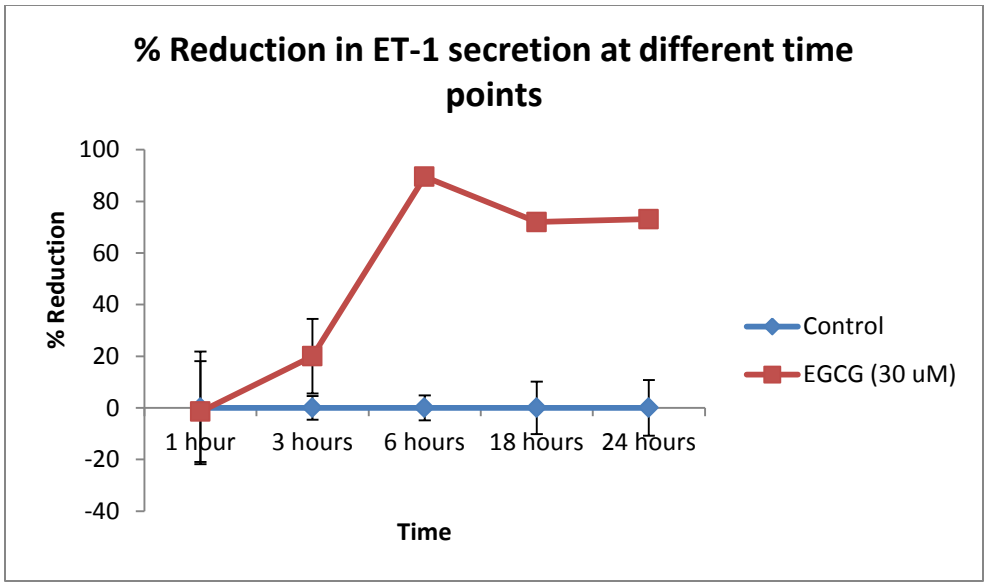
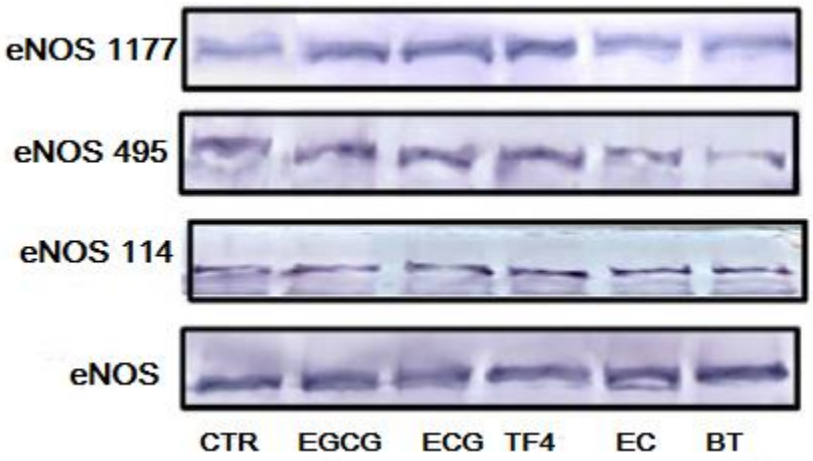


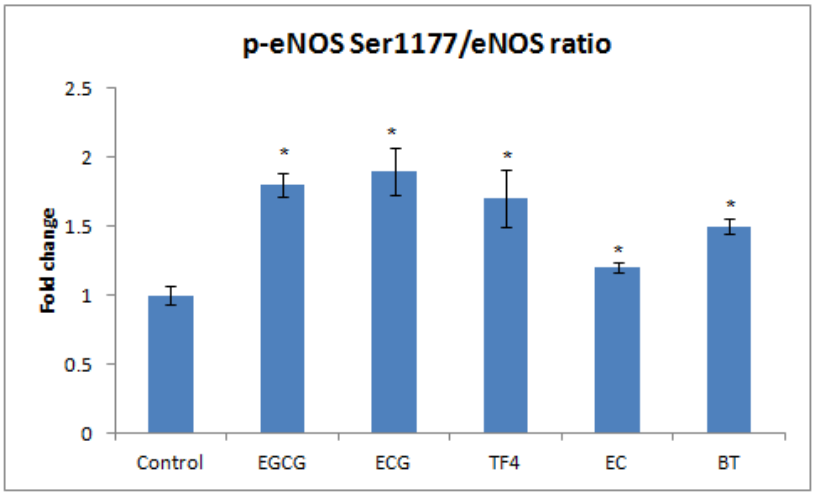
Figure 5: Percent reduction in the levels of endothelin-1 secretion after treatment with EGCG (1μM-300μM) with respect to the control (no treatment) at different time points (1 hour-6 hours) in endothelial cells. Cells were also treated with TNF α (100 ng/ml) as positive control and endothelin antagonist as negative control.

3. Differential phosphorylation by tea ingredients of eNOS enzyme

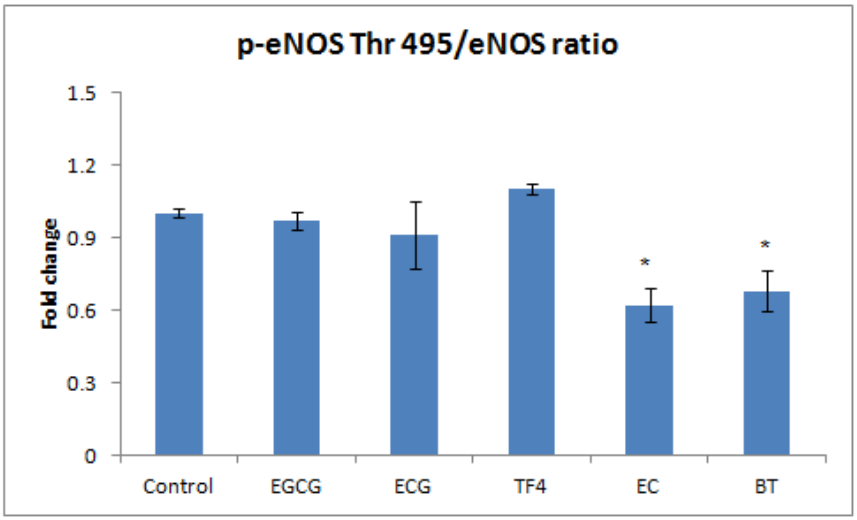
A.



B.


p-eNOS Ser1177/eNOS ratio
Fold change
2.5
2
1.5
1
0.5
0
Control
EGCG
ECG
TF4
EC
BT

C.


p-eNOS Thr 495/eNOS ratio
Fold change
1.5
1.2
0.9
0.6
0.3
0
Control
EGCG
ECG
TF4
EC
BT

D.

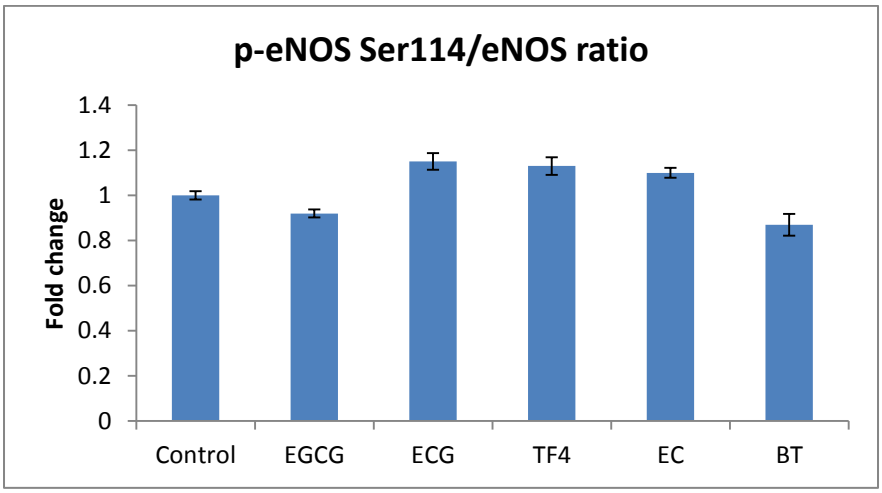
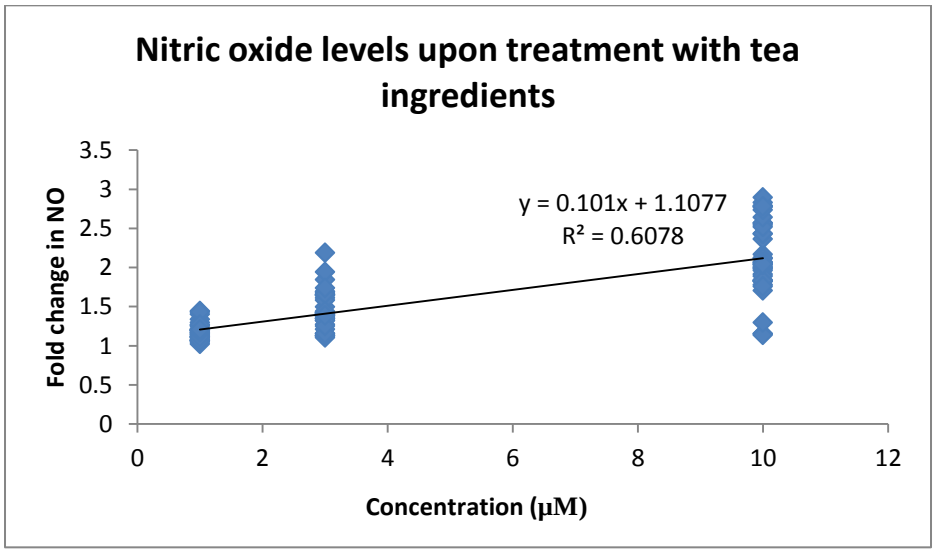


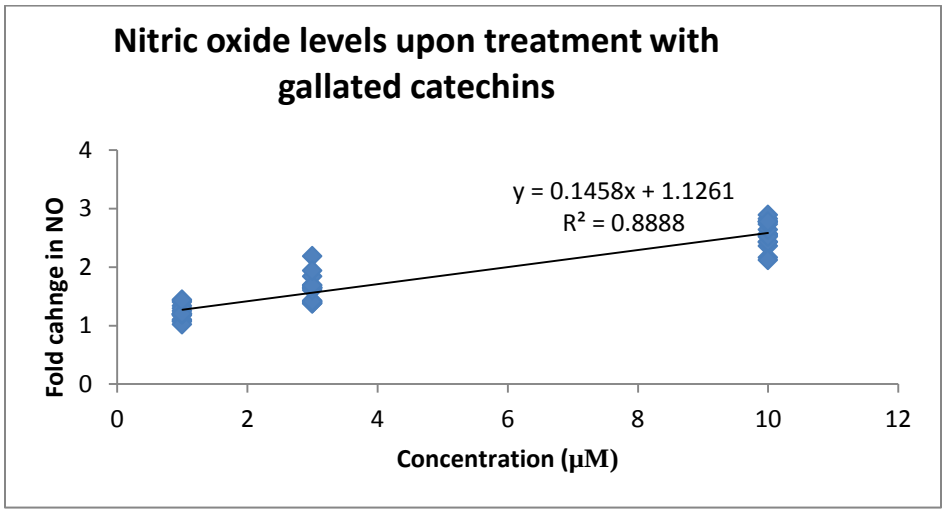
Figure 6: Western Blot analysis: (A) - western blot of eNOS phosphorylation at Ser1177, Thr495 and Ser114 by tea ingredients. (B, C, D) – ratio of phosphorylayed and non-phosphorylated eNOS forms. Bard with * represent significant change compared to control (p<0.01).

4. Correlation graphs:

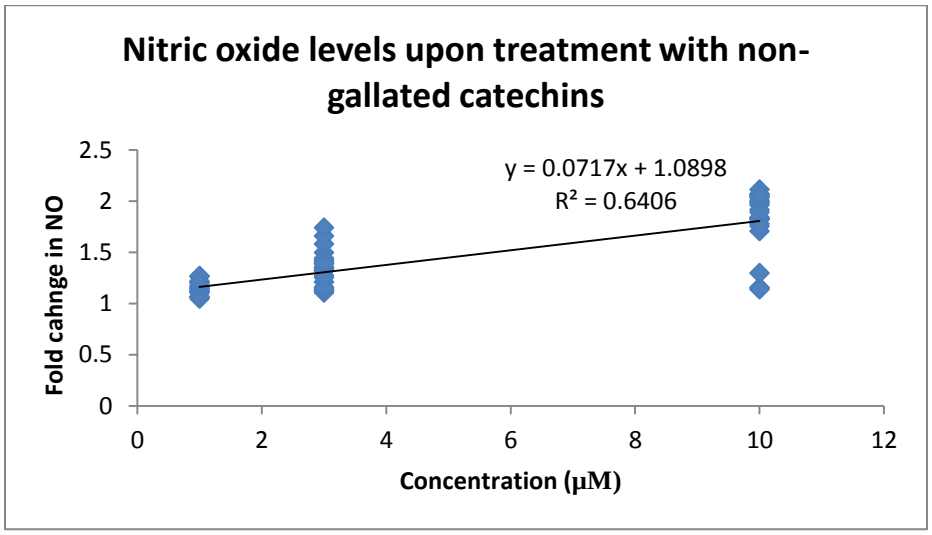
A.



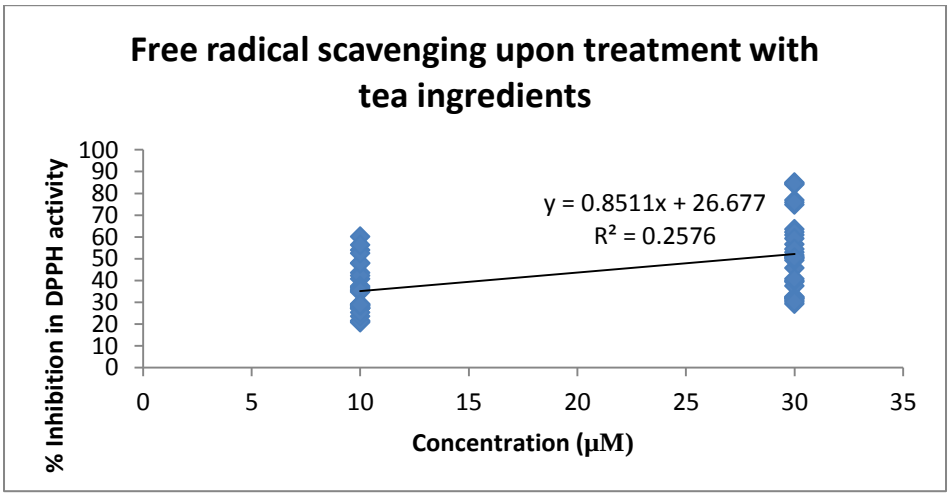
B.


Nitric oxide levels upon treatment with gallated catechins
y = 0.1458x + 1.1261
R² = 0.8888
Fold cahnge in NO
Concentration (µM)
0
1
2
3
4
0
2
4
6
8
10
12

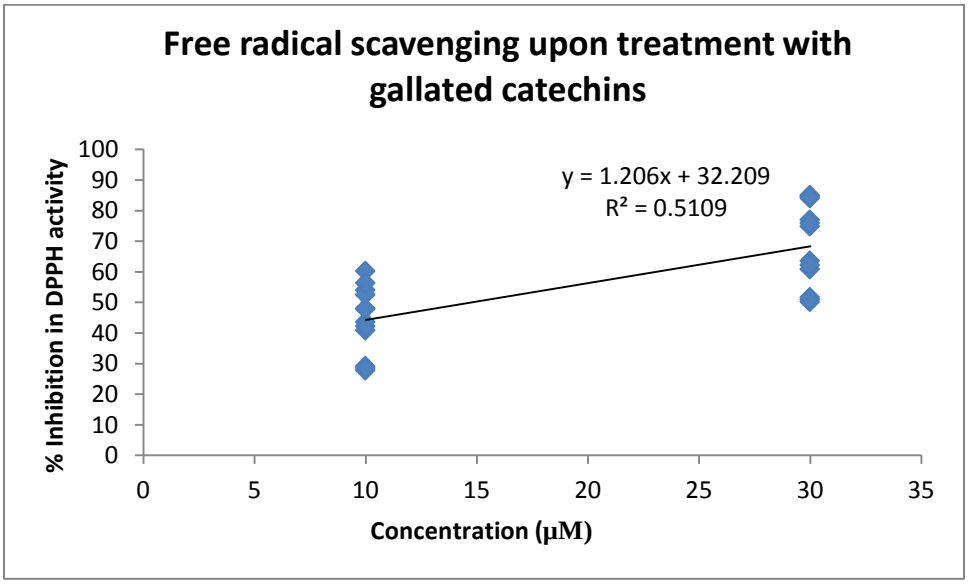
C.


Nitric oxide levels upon treatment with non-gallated catechins
y = 0.0717x + 1.0898
R² = 0.6406
Fold cahnge in NO
Concentration (µM)
0
0.5
1
1.5
2
2.5
0
2
4
6
8
10
12

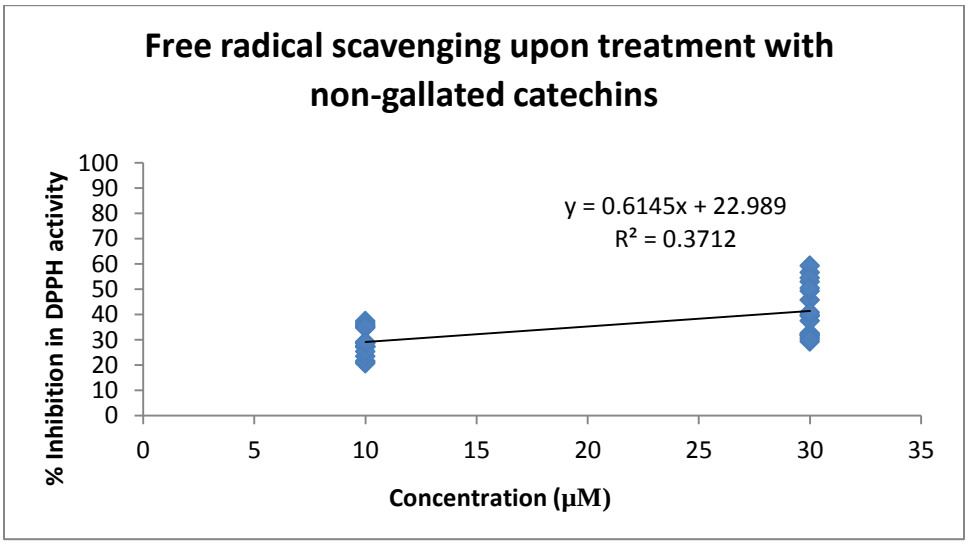
D.


Free radical scavenging upon treatment with tea ingredients
% Inhibition in DPPH activity
100
90
80
70
60
50
40
30
20
10
0
y = 0.8511x + 26.677
R² = 0.2576
0
5
10
15
20
25
30
35
Concentration (µM)

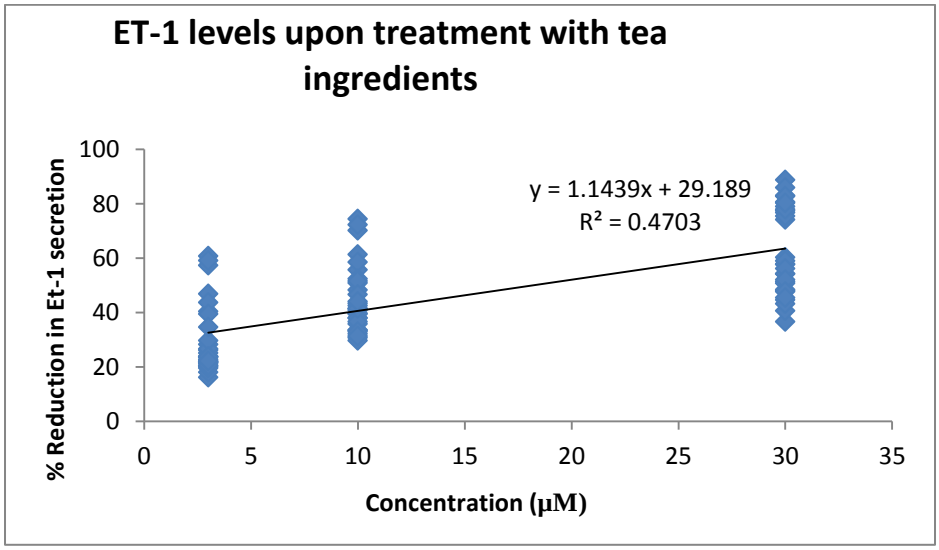
E.


Free radical scavenging upon treatment with gallated catechins
% Inhibition in DPPH activity
100
90
80
70
60
50
40
30
20
10
0
y = 1.206x + 32.209
R² = 0.5109
0
5
10
15
20
25
30
35
Concentration (µM)

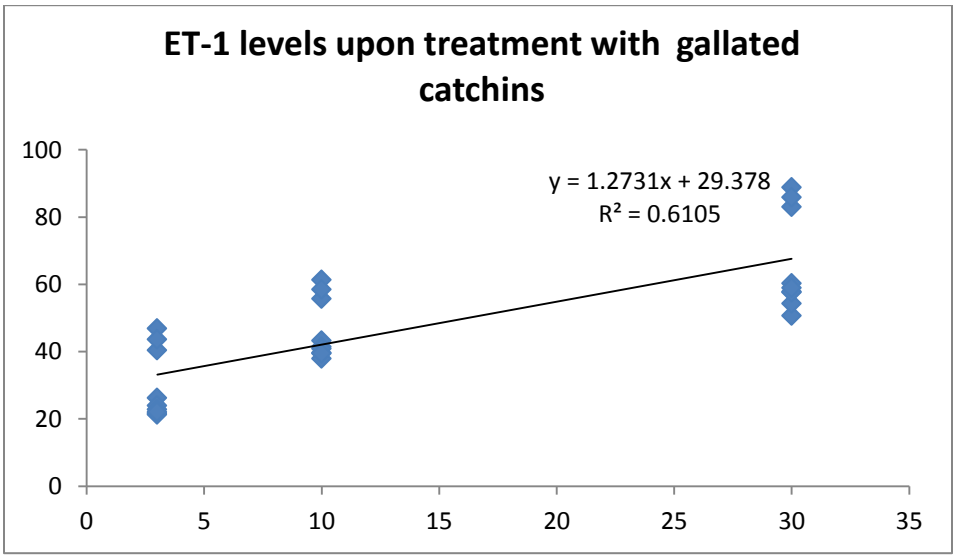
F.


Free radical scavenging upon treatment with non-gallated catechins
% Inhibition in DPPH activity
100
90
80
70
60
50
40
30
20
10
0
y = 0.6145x + 22.989
R² = 0.3712
0
5
10
15
20
25
30
35
Concentration (µM)

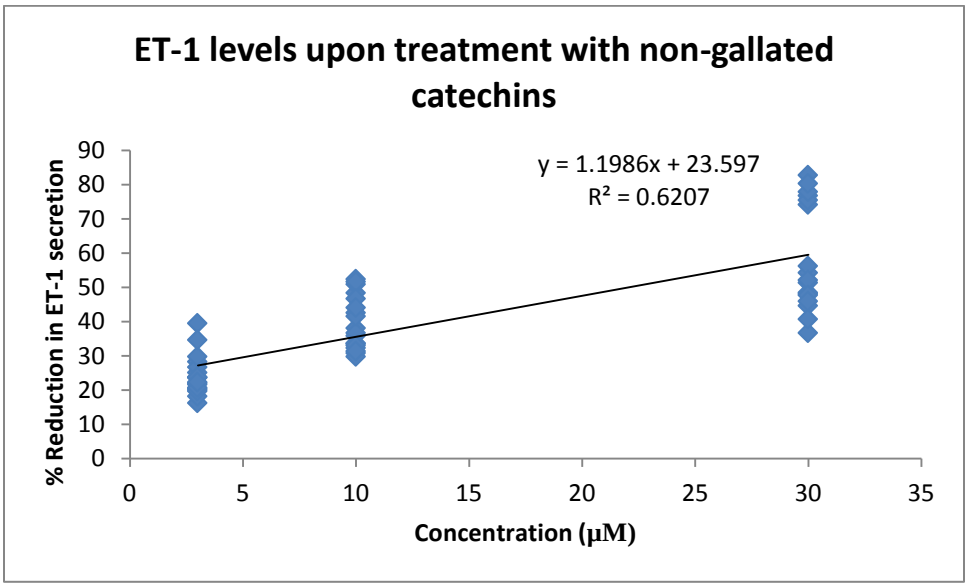
G.


ET-1 levels upon treatment with tea ingredients
% Reduction in Et-1 secretion
100
80
60
40
20
0
y = 1.1439x + 29.189
R² = 0.4703
0
5
10
15
20
25
30
35
Concentration (µM)

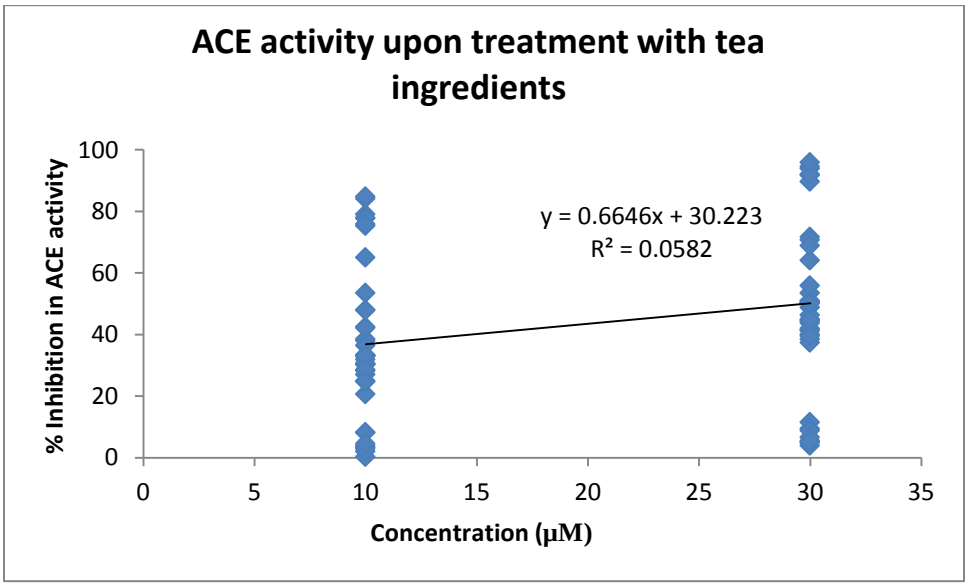
H.


ET-1 levels upon treatment with gallated catchins
y = 1.2731x + 29.378
R² = 0.6105
100
80
60
40
20
0
0
5
10
15
20
25
30
35

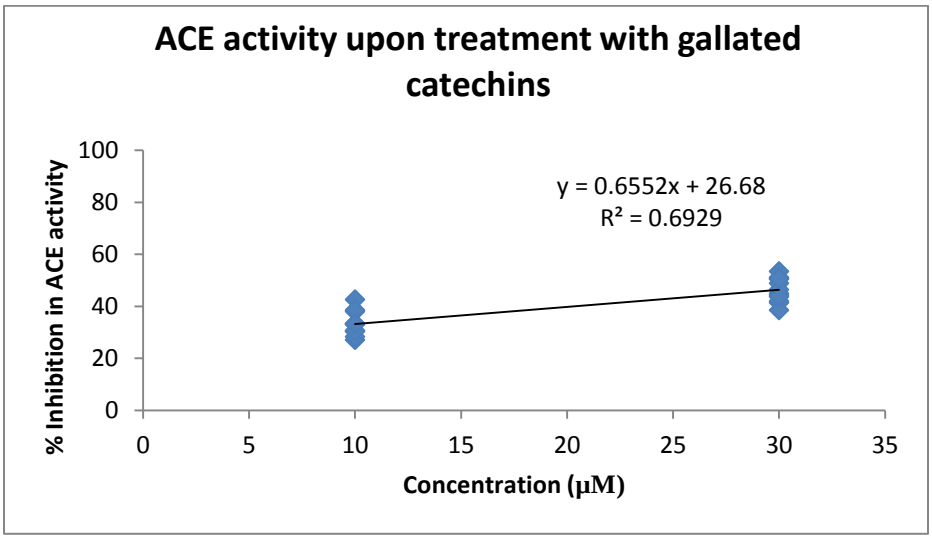
I.


ET-1 levels upon treatment with non-gallated catechins
y = 1.1986x + 23.597
R² = 0.6207
% Reduction in ET-1 secretion
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
Concentration (µM)

J.


ACE activity upon treatment with tea ingredients
% Inhibition in ACE activity
100
80
60
40
20
0
y = 0.6646x + 30.223
R² = 0.0582
0
5
10
15
20
25
30
35
Concentration (µM)

K.


ACE activity upon treatment with gallated catechins
% Inhibition in ACE activity
100
80
60
40
20
0
y = 0.6552x + 26.68
R² = 0.6929
0
5
10
15
20
25
30
35
Concentration (µM)

L.

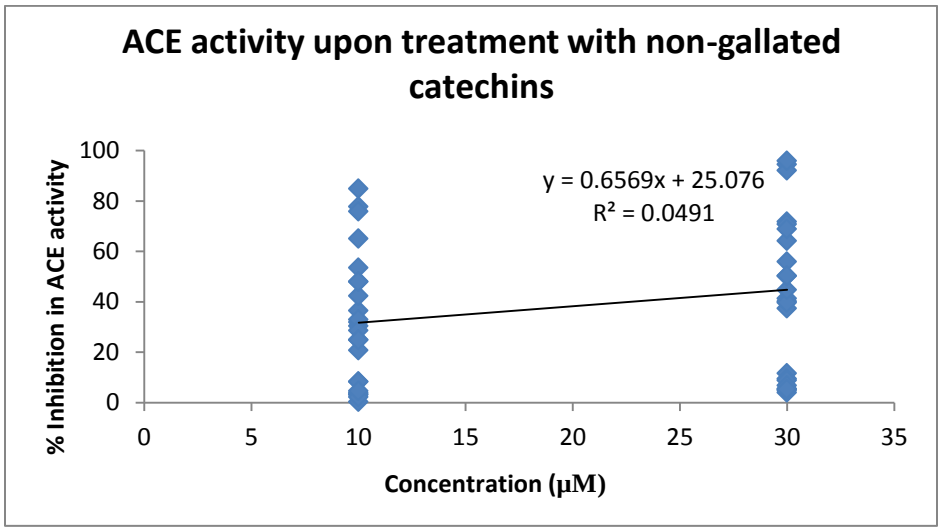


Figure 7: Correlation graphs: Intracellular nitric oxide levels and concentration of – (A)- tea ingredients, (B)- gallated catechins, (C)- non-gallated catechins. DPPH activity and concentration of – (D)- tea ingredients, (E)- gallated catechins and (F)- non-gallated catechins. Endothelin-1 secretion and concentration of (G)- tea ingredients, (H)- gallated catechins and (I)- non-gallated catechins. ACE activity and concentration of (J)- tea ingredients, (K)- gallated catechins and (L)- non-gallated catechins.