Research Article

Comparative Antioxidant & Anti-inflammatory Studies of Quercetin, Hesperidin and Gallic Acid on Human Red Blood Cells (hRBC) as Compared to Vitamin E (Tocopherol)

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ABSTRACT

The aim of the study is to compare the antioxidant and anti-inflammatory activities of three different plant derived molecules (Quercetin, Hesperidin and Gallic Acid) on human RBC by using Vitamin E as standard. Here we had generated oxidative stress by using heat as well as chemicals like Hydrogen peroxide and the protective activity of the these three phytomolecules were observed in respect to vitamin E. The biochemical mechanism of action was investigated in terms of scavenging of free radicals. Hesperidin, Quercetin and Gallic Acid showed (10–200 µg/ml) decrease in cell damage in H_2O_2 induced damage & Hypo-saline model in a concentration dependent manner in *ex vivo* models. IC_{50} Value of Hesperidin, Quercetin, Gallic Acid in H_2O_2 models were 41.93 ± 0.34 µg/ml, 65.68 ± 0.72 µg/ml, 93.15 ± 0.53 µg/ml respectively whereas value of Vitamin E was 22.08 ± 0.32 µg/ml and IC_{50} Value of Hesperidin, Quercetin, Gallic Acid in Hypo-saline model were 43.19 ± 0.34 µg/ml, 75.91 ± 0.57 µg/ml, 105 ± 0.44 µg/ml respectively whereas value of Vitamin E was 17.79 ± 0.33 µg/ml. The antioxidant activity was compared on the basis of generation of oxidative stress in the hRBC cells & inhibition of oxidative stress by the phytomolecules. The study had established that Hesperidin mediates more antioxidant & anti-inflammatory activity than Quercetin and Gallic Acid in hRBC via its free radical scavenging activity.

Keywords: Gallic Acid, hRBC, H_2O_2 Induced Lysis, Hyposaline Induced lysis, Hesperidin, Quercetin, Vitamin E.

INTRODUCTION

Oxidative stress is the common pathological problem in inflammation, neurodegenerative disorder, neoplasm, Alzheimers' disease and ageing. Oxidative stress is the disturbance between production of reactive oxygen and nitrogen species (ROS, RNS) and anti-oxidant defense.[1] Oxidative stress is responsible for production of free radicals – which can damage the components of cells like protein, DNA, mitochondria and ultimately leads to the death of the cells. Protective mechanisms against oxidation include prevention of formation of reactive oxygen species (ROS), scavenging of various forms of ROS, and repair of oxidized cellular contents. In general any partial defect in any of these systems can cause damage to the cells and promote senescence. Cells contain a number of antioxidant defenses to decrease the production of ROS, but production of ROS in certain cases exceeds the cell's antioxidant capacity and as a result oxidative stress is generated. Host survival depends upon the ability of cells and tissues to adapt to or resist the stress, and repair or remove damaged molecules or cells. A number of stress response mechanisms have been evolved for these purposes, and they are rapidly activated in response to oxidative insults. Some of the pathways are preferentially linked to enhanced survival, while others are more frequently associated with cell death.[2-3] Recently it has been found that, inborn defects of the antioxidant system interfere with the various protective mechanisms of the RBC cells and effects of several pure flavonoids has good membrane protective activity against damage of the cells.[4] Antioxidants play important role in the management of oxidative stress. An antioxidant is the molecule that inhibits oxidation of other molecules by removal of free radical intermediates and as a result formation of oxidative stress is prevented. But all the antioxidants do not work by the same mechanism. Some of them work by free radical scavenging assay (Example: Vitamin C, Vitamin E), by inhibition of free radical formation (Example: Flavonoids) and by cell damage repair.[5]

Vitamin E is a generic term for a group of tocol and tocotrienol derivatives and since the discovery that vitamin E is the major lipid soluble antioxidant in skin.[6] Due to the potent antioxidant properties of tocopherols, the impact of α-tocopherol in the prevention of chronic diseases believed to be associated with oxidative stress. Recent observation shows that the α-tocopherol transfer proteins in the liver for incorporation into plasma lipoproteins, and α-tocopherol has signaling functions in vascular smooth muscle cells. Tocopherol with similar antioxidative properties, have raised interest in the roles of vitamin E beyond its antioxidative function.[7]

Quercetin has been reported to be effective in inflammation, arteriosclerosis, bleeding, allergy and swellings. It is also known to be associated with reduced risk of certain types of cancers. However, the major problem associated with the use of Quercetin, is the very low bioavailability.[8] Quercetin (3,5,7,3′,4′-pentahydroxy

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flavone) is one of the most abundant bio-flavonoids. It is present in edible fruits and vegetables and beverages mainly as two glucosides, with the highest content in onions, apples and red wine. Epidemiological studies suggest it shows protective activity against coronary heart disease and stroke. Potential mechanisms include the inhibition of 15-lipoxygenase and LDL oxidation, chelation of metal ions and scavenging of hydroxyl and peroxy radicals.[9]

Hesperidin is a natural antioxidant flavanone having important biological features to prevent hyper glycaemia-induced teratogenic processes. Hesperidin is a bioflavonoid usually available in citrus fruits and mostly consumed in oranges.[10] Previous study suggests that plants having extracts rich in hesperidins can inhibit melanogenesis by inhibiting the transport system Rab27A melanophilin.[11-13]

Among various polyphenols, Gallic acid (3,4,5-trihydroxybenzoic acid) is a naturally occurring low molecular weight triphenolic compound, has emerged as a strong antioxidant and an efficient apoptosis inducing agent.[14] Hepato-protective as well as anti-oxidant activity of Gallic Acid has been found in paracetamol induced liver damage in mice.[15]

So our main objective was to compare the antioxidant and anti-inflammatory activities of three already established different plant derived molecules (Quercetin, Hesperidin and Gallic Acid) on human RBC by using Vitamin E as standard by using various in vitro protocols.

MATERIALS AND METHODS

Chemicals & Reagents used

Commonly used chemicals were Quercetin (Sigma Aldrich, MW = 302.24 g/mol, Purity is 95%), Hesperidin (Sigma Aldrich, MW = 610.53 g/mol, Purity is 80%), Gallic Acid (Sigma Aldrich, MW = 170.12 g/mol, Purity is 97.5%), Vitamin K. (Sigma Aldrich, MW = 450.7 g/mol, Purity is 96%). All other chemical and reagents used were high analytical grade.

Isolation of Human Red Blood Cells[16]

Blood samples were collected from the healthy human volunteer (M: F= 10:1, Age Range- 21-26; n=20) by taking consent of the patient in the vial containing anticoagulant. All the consent form had been kept in the NJIL & OMD for future references. sufficient amount of PBS were added to the blood samples & centrifuged at 2000 RPM for 5 min. Upper layer were separated out carefully by using micro-pipette & volume of the RBC were measured and solution is prepared by using RBC:PB in 1:20 ratio.

In vitro effect of H_2O_2-induced lysis of Human erythrocytes[17]

Blood-containing cells obtained from healthy human volunteer were immediately centrifuged at 2000 rpm/min for 5 min and subsequently washed with PBS (0.02 mol/L, pH 7.4) three times to remove excess plasma. RBC lysed was prepared by suspending in 20 volumes of 20 mmol/L, phosphate buffer (pH 7.4) and finally yields a hemolysate concentration of 1: 20. To 1.5 ml of freshly prepared hemolysate, 1.0 mL of different concentrations of Quercetin, Hesperidin and Gallic Acid (10–200 µg/mL) were added each time concomitantly with 0.5 ml of H_2O_2 solutions. Supernatants were collected after 50 minutes at 37°C of incubation followed by centrifugation at 2000 rpm/min for 5 minutes and extent of hemolysis were obtained spectro-photometrically at 540 nm.

In vitro effect of Hypo-saline induced lysis of Human erythrocytes[18]

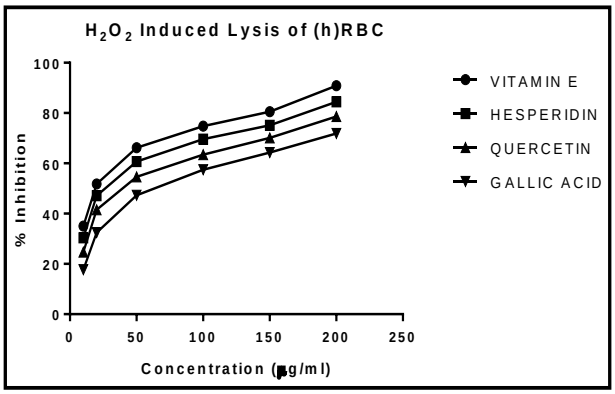
Blood-containing cells obtained from healthy human volunteer were immediately centrifuged at 2000 rpm/min for 5 min and subsequently washed with PBS (20 m.mol/L, pH 7.4) three times to remove excess plasma. RBC lysed was prepared by suspending in 20 volumes of 20 mmol/lt, phosphate buffer (pH 7.4) to yield a hemolysate concentration of 1: 20. To 1.5 ml of freshly prepared hemolysate, 1.0 ml of different concentrations of Quercetin, Hesperidin and Gallic Acid (10-200 µg/ml) were added each time concomitantly with Hypo-saline solution (0.3% NaCl solution). Supernatants were collected after 20 minutes of incubation at 37°C followed by centrifugation at 3000 rpm/min for 5 minutes and extent of protection of hRBC membrane were obtained spectro-photometrically at 560 nm.

Statistical Analysis

Results are expressed as mean ± SEM & it was calculated by using Graph Pad Prism version 4.03 software.

RESULTS

Inhibition of H_2O_2 induced lysis of human erythrocyte *(in vitro)*



(Figure 1)

[H_2O_2 produces the toxic hydroxyl radical (OH^-) via a Fe^{2+} mediated reaction leads to the decrease of the cell rigidity of the Red Blood Cells and ultimately causes cell damage. More protective activity of Hesperidin in respect to Quercetin and Gallic Acid was confirmed by IC_{50} Values of Hesperidin, Quercetin and Gallic Acid. They were 41.93 ± 0.34 µg/ml, 65.68 ± 0.72 µg/ml, 93.15 ± 0.53 µg/ml

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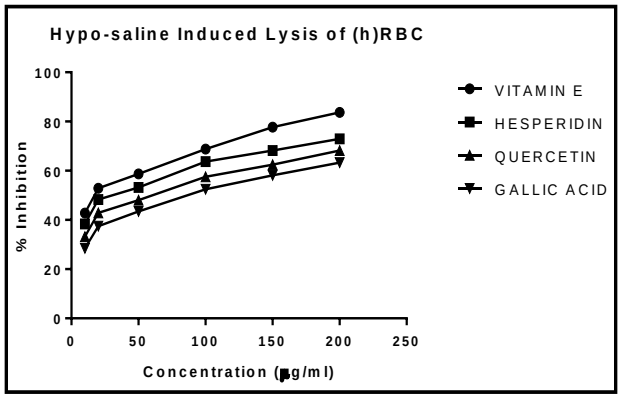
respectively whereas value of Vitamin E was 22.08 ± 0.32 µg/ml.]

The extent of hemolysis was decreased more in the erythrocytes treated with Hesperidin in comparison to the Quercetin and Gallic Acid in respect to Vitamin E (Figure 1). IC_{50} Value of Hesperidin, Quercetin and Gallic Acid in H_2O_2 models were 41.93 ± 0.34 µg/ml, 65.68 ± 0.72 µg/ml, 93.15 ± 0.53 µg/ml respectively whereas value of Vitamin E was 22.08 ± 0.32 µg/ml. So, Hesperidin can reach IC_{50} (50% inhibitory concentration) in less concentration in respect to Quercetin and Gallic Acid therefore it shows better scavenging property than Quercetin and Gallic Acid by using Vitamin E as standard.

Inhibition of Hypo-saline induced lysis of human erythrocyte *(in vitro)*

The extent of stabilization of hRBC membrane was decreased more in the erythrocytes treated with Hesperidin in comparison to the Quercetin and Gallic Acid in respect to Vitamin E (Figure 2). IC_{50} Value of Hesperidin, Quercetin, Gallic Acid in Hypo-saline model were 43.19 ± 0.34 µg/ml, 75.91 ± 0.57 µg/ml, 105 ± 0.44 µg/ml respectively whereas value of Vitamin E was 17.79 ± 0.33 µg/ml. So, Hesperidin can reach IC_{50} in less concentration in respect to Quercetin and Gallic Acid, therefore it shows better scavenging property than Quercetin and Gallic Acid by using Vitamin E as standard.



(Figure 2)

[Hypo-saline induced lysis of human erythrocyte imparts oxidative stress to the cells by the hypo-saline solution (0.3% NaCl solution) and causes damage of the cell membranes. More protective activity of Hesperidin in compare to Quercetin and Gallic Acid was confirmed by the less IC_{50} values. IC_{50} Value of Hesperidin, Quercetin, Gallic Acid in Hypo-saline model was 43.19 ± 0.34 µg/ml, 75.91 ± 0.57 µg/ml, 105 ± 0.44 µg/ml respectively whereas value of Vitamin E was 17.79 ± 0.33 µg/ml.]

DISCUSSION AND CONCLUSION

The most abundant ROS represented in living inflammatory cells, is super-oxide, as well as hydrogen peroxide and highly toxic hydroxyl radicals and all of them are highly reactive to the living cells. Natural systems, like reduced glutathione, vitamins, and free fatty acids are considered an essential pool of antioxidants.[19] Oxidative stress is the situation where the production of oxidants exceeds the capacity to neutralize them & leads to damage of cell membranes, lipids, nucleic acids, proteins, and constituents of the extra-cellular matrix, such as proteoglycans and collagens. There are different therapeutic approaches can be used to decrease the oxidative stress – which includes scavenging of free radicals, inhibition of free radical producing enzymes, enhancing the antioxidant system or targeting the signaling routes involved in the inflammatory cascade. Amongst the intracellular ROS generated, superoxide plays a pivotal role in inflammation.[20] Various plant-derived phyto-molecules are used as an important source of anti-oxidant and anti-inflammatory agents and that includes Quercetin, Hesperidin and Gallic Acid.

The interaction of the cellular immune system with endogenous and/or exogenous antigens results in increased generation of ROS and RNS, leading to the activation of signalling cascades of synthesizing pro-inflammatory cytokines and chemokines.[21,22] All ROS and RNS are constantly generated in the inflammatory cells by the initial action of NADPH oxidase enzyme. NADPH oxidation by NADPH oxidase is considered as the major source of superoxide (O_2^-) which intern again converted to less toxic hydrogen peroxide (H_2O_2) by the action of antioxidant enzyme i.e. superoxide dismutase (SOD). Subsequently H_2O_2 produces the toxic hydroxyl radical (OH^-) via a Fe^{2+} mediated reaction (known as Fenton reaction)[23] leads to the decrease of the cell rigidity of the Red Blood Cells and ultimately causes cell damage. More protective activity of Hesperidin in respect to Quercetin and Gallic Acid was confirmed by IC_{50} Values of Hesperidin, Quercetin and Gallic Acid. They were 41.93 ± 0.34 µg/ml, 65.68 ± 0.72 µg/ml, 93.15 ± 0.53 µg/ml respectively whereas value of Vitamin E was 22.08 ± 0.32 µg/ml. Therefore less concentration of Hesperidin was required to reach the 50% Inhibitory concentration than Quercetin and Gallic Acid and thus more potent antioxidant activity of Hesperidin was confirmed.

At the time of inflammation, release of lysosomal hydrolytic enzyme occurs – which causes increase of oxidative stress to the surrounding organelles, tissues along with the red blood cells.[24] It leads to the activation of proteins responsible for inflammation and ultimately triggers the membrane of the red blood cells and causes damage to the red blood cells. Various phyto-molecules have the ability to prevent the damage of the cell wall of the RBC cells. Hypo-saline induced lysis of human erythrocyte imparts oxidative stress to the cells by the hypo-saline solution (0.3% NaCl solution) and causes damage of the cell membranes. More protective activity of Hesperidin in compare to Quercetin and Gallic Acid was confirmed by the less IC_{50} values. IC_{50} Value of Hesperidin, Quercetin, Gallic Acid in Hypo-saline model were 43.19 ± 0.34 µg/ml, 75.91 ± 0.57 µg/ml, 105 ± 0.44 µg/ml respectively whereas value of Vitamin E was 17.79 ± 0.33 µg/ml. Therefore less concentration of Hesperidin was required to reach the 50% Inhibitory concentration than

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Quercetin and Gallic Acid and it confirmed the more potent anti-inflammatory activity of Hesperidin.

Taken together, the study established that Hesperidin was more effective as antioxidant and anti-inflammatory agent as compared to Quercetin and Gallic Acid on hRBC by using Vitamin E as standard. However *in vivo* experimentations are required to ultimate conclusion.

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REFERENCES

1. Betteridge DJ, "what is oxidative stress", Metabolism, 49(2), 2000, 3-8.
2. Martidale JL, "Cellular response to oxidative stress: signaling for suicide and survival" [J], cell Physiol, 192(1), 2002, 1-15.
3. Van Z, Verhoeven AJ, Roos D, "Inborn defects in the antioxidant systems of human red blood cells", Free radic Biol Med, 67, 2014, 377-86.
4. Asgary S, Naderi GH, Askari N, "Protective effect of flavonoids against red blood cell hemolysis by free radicals", Exp Clin Cardiol, 10(2), 2005, 88-90.
5. Lobo V, Patil A, Pathak A, Chandra A, "Free radical, antioxidants and functional foods: Impact on human health", Pharmacogn Rev, 4(8), 2010, 118-126.
6. Baumann LS., Spencer J., 'The effects of Topical vitamin E on the cosmetic appearance of scars', Dermatol Surg., 25(4), 1999, 311-315.
7. Regina BF., Maret GT., 'Vitamin E: Function and metabolism', The Faseb Journal, 13(10), 1999, 1145-1155.
8. Urmila JJ., Amol SG., Priscilla D., Ragini S., Sudha S., Girjesh G., 'Anti-inflammatory, Antioxidant and anticancer activity of Quercetin and its analogues', International Journal of Research in Pharmaceutical and Biomedical Sciences, ISSN: 2229-370.
9. Thomas W., Yoko O., Walle UK., Frederick AW., 'Quercetin glycosides are completely hydrolyzed in Ileostomy patients before absorption', J. Nutr., 130(11), 2000, 2658-2661.
10. Mohamed LT., Sameha M., Abdellatif B., Abdelkrim T., Kheireddine O., Mohamed AG., 'Hesperidin, a natural citrus flavanone, alleviates hyperglycaemic state and attenuates Embryopathies in pregnant diabetic mice', Journal of Medicinal Plants Research, 3(11), 2009, 862-869.
11. Heun JL., Woo JL., Sung EC., Ga YL., 'Hesperidin, A popular antioxidant inhibits Melanogenesis via Erk ½ Mediated MITF Degradation', Int J Mol Sci., 16(8), 2015, 18384-18395.
12. Kim SS., Kim MJ., Choi YH., Kim BK., Kim KS., Park SM., Lee NH., Hyun CG., 'Down regulation of tyrosinase, TRP-1, TRP-2 and MITE expression by citrus press cakes in murine B16 F10 melanoma', Asian Pac. J. Trop. Biomed., 3, 2013, 617-622.
13. Kim B., Lee JY., Lee HY., Nam KY., Park J., Lee SM., Kim JE., Lee JD., Hwang JS., 'Hesperidin suppresses melanosome transport by blocking the interaction of Rab27A-melanophilin', Biomol Ther. Seoul., 21, 2013, 343-348.
14. Bharti B., Neha S., Rita K., 'Gallic acid: A versatile antioxidant with promising therapeutic and industrial applications', RSC Adv., 5, 2015, 27540-27557.
15. Rasool MK., Sabina EP., Ramya SR., Preety P., Patel S., Mandal N., Misha PP., Samuel J., Hepato-protective and antioxidant effects of gallic acid in Paracetamol induced liver damage in mice, J Pharm Pharmacol., 62(5), 2010, 638-643.
16. Bushra HM., Munaf HZ., Ihab IA., Tavga AA., Saad AH., Free radical scavenging activity of silibinin in nitrite-induced hemoglobin oxidation and membrane fragility models, Saudi Pharm [J]., 19(3), 2011, 177–183.
17. Lalitha S, Selvum R., Prevention of H_2O_2 induced red blood cell lipid peroxidation and hemolysis by aqueous extracted turmeric, Asia Pac J Clin Nutr, 8(2), 1999, 113-114.
18. Bailly F., Zoete V., Vamecq J., Antioxidant actions of ovothiol-derived 4-mercapto-imidazoles: gluthathione pero- xidase activity and protection against peroxynitrite-induced damage, FEBS Lett, 486, 2000, 19-22.
19. Hitchon CA., El-Gabalawy HS., Oxidation in rheumatoid arthritis, *Arthritis Res Ther*, 6, 2004, 265-278.
20. Chen S., Natural products triggering biological targets-a review of the anti-inflammatory phytochemicals targeting the Arachidonic acid pathway in allergy asthma and rheumatoid arthritis, [J]. Curr Drug Targets, 12(3), 2010, 288-301.
21. Freidovich I., Fundamental aspects of reactive oxygen species, or what's the matter with oxygen, Ann N Y AcadSci, 893, 1999, 3-18.
22. Wu G., Morris SM., Arginine metabolism: nitric oxide and beyond', Biochem J, 336, 1998, 1-17.
23. Winterbourn CC., Toxicity of Iron & Hydrogen Peroxide: The Fenton Reaction', Toxicol Lett., 82(83), 1995, 969-974.
24. Sadique J., Al-Rqobahs WA., Bughaith MF, El-Gindi AR., The bioactivity of certain medicinal Plants on the stabilization of RBC membrane system, Fitoterpia, 60, 1989, 535-32.

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