Global Journal of Biotechnology & Biochemistry 7 (1): 19-25, 2012
ISSN 2078-466X

DOI: 10.5829/idosi.gjbb.2012.7.1.1101

Effect of Quercetin and Epicatechin on the Transcript Expression and Activity of Antioxidant Enzymes in Tobacco Seedlings

Monika Mahajan and Sudesh Kumar Yadav

Biotechnology Division, CSIR-Institute of Himalayan Bioresource Technology,
CSIR, Palampur-176061 (HP), India

Abstract: The aim of this study was to evaluate the effect of flavonoids quercetin and epicatechin on the transcript expression and activity of antioxidant enzymes. Tobacco seedlings were exposed to 50 and 100 µM quercetin and epicatechin. The transcript expression level and the activity analysis of various antioxidant enzymes were monitored in their root and shoot. Interestingly, 50 µM epicatechin and 100 µM quercetin exposures were found to increase the expression of genes encoding antioxidant enzymes in shoot. In tobacco root, only GST and GPx expression were increased with 50 µM epicatechin and 100 µM quercetin exposures. Activity assay of all the enzymes showed similar trend to that of the transcript expression in shoot tissue. While in root, except CAT and SOD other enzymes activity also showed similar trend to that of expression pattern. Results have suggested the possible regulation of antioxidant enzymes by these two flavonoids at transcriptional and post-transcriptional level. Additionally, appropriate levels of such flavonoids seem to be essential for such regulations.

Key words: Antioxidant enzymes · Epicatechin · Quercetin · Tobacco · Transcript expression

INTRODUCTION

Flavonoids represent a large family of low molecular weight polyphenolic secondary metabolites. They are widespread throughout the plant kingdom [1]. In nature, they are involved in wide range of functions. These polyphenolic compounds are well documented for their antioxidant properties. The term antioxidant refers to free radical scavengers, inhibitors of lipid peroxidation and chelating agents [2]. These antioxidant properties of flavonoids have been suggested to provide the protection against the oxidative damage, coronary heart diseases, certain cancers and other age related diseases [3-5]. Their chemical structures appear to be ideal for free radical scavenging. The *in vitro* studies have shown that majority of flavonoids like quercetin and epicatechin gallate have five fold higher total antioxidant activities than vitamins E and C [6, 7]. For this reason, there is currently a growing interest in the development of agronomical important food crops with the optimized levels and composition of such flavonoids.

The free radical scavenging property of flavonoids also provides protection to plants against the different stresses. Stresses on the plants lead to the generation of reactive oxygen species (ROS) such as superoxides, H_2O_2 and hydroxyl molecules. These ROS cause rapid cell damage by triggering off a chain reaction [8, 9]. Under stress, plants produce some defense mechanisms to protect themselves from harmful effects of oxidative stress. ROS are detoxified either directly by non-enzymatic antioxidants reduced glutathione (GSH), ascorbate (ASH), tocopherols and carotenoids etc. or by antioxidant enzymes like ascorbate peroxidase (APx), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST), Catalase (CAT), Superoxide dismutase (SOD) etc [10-12].

There are many reports relating to the reactivity of flavonoids with active oxygen species, thus emphasizing their antioxidant potential via a direct radical scavenging mechanism in animals [13, 14]. Flavonoids have been well characterized for both *in vitro* and *in vivo* antioxidant activity [15-17]. However, the relevance of this property for flavonoid function in planta is still a topic of debate [18]. The vascular localization of many flavonoids allows for their light-screening, photoprotective and pigmentation functions but probably not their antioxidative functions [19]. Therefore, we studied the effect of flavonoids on the expression of genes encoding

Corresponding Author: Sudesh Kumar Yadav, Biotechnology Division, CSIR-Institute of Himalayan Bioresource Technology, CSIR, Palampur-176061 (HP), India.

antioxidant enzymes in plants to see relation of these two pathways. For this, tobacco seedlings were exposed with 50 and 100 µM quercetin and epicatechin and thus the transcript expression level and the activity analysis of various antioxidant enzymes was monitored in their root and shoot.

MATERIALS AND METHODS

Plant Material: For germination, tobacco seeds (*Nicotiana tabacum* var. Xanthi) were treated with 10% Tween-20 for 5 min and then with 70% ethanol for 30 sec. Thereafter, seeds were surface sterilized with 0.001% mercuric chloride for 3 min and washed thrice with sterile distilled water. Seeds were germinated on Murashige and Skoog (MS) medium (Sigma) in petri dishes at 25±2°C for 7 days, until cotyledons had emerged and roots reached the length of 1-1.5 cm. Seedlings at this stage were transferred to the plates containing either 50µM or 100µM quercetin and epicatechin (Sigma). The stock solution of 10 mM quercetin and epicatechin were prepared by dissolving in 50% dimethyl sulfoxide (DMSO). Finally, MS medium contained 50µM or 100µM quercetin and epicatechin with 1% DMSO. For controls, seedlings were transferred to MS medium containing 1% DMSO alone. The pH of the medium was adjusted to 5.8 with NaOH/HCl. Seedlings were allowed to grow for the next 21 days. Thereafter, seedlings were carefully removed from the plates and root and shoot were separated and frozen in liquid nitrogen for further use.

Transcript Expression Analysis: Total RNA was isolated from 100mg of treated and untreated tissues by using RNAeasy mini kit (Qiagen). cDNA was synthesized using 1µg of RNA in the presence of 200 U reverse transcriptase SuperscriptTM III (Invitrogen, USA), 1 µl of 10 mM dNTPs and 250 ng oligo (dT)12–18. Resulting cDNA was used to carry out the PCR reactions with gene specific primers encoding for GR (Forward 5'-CATTGCCAATAAAAATGCCGAGT-3' and Reverse 5'-ATGATATGAGAGAAACCTTCAAC-3'), GST (Forward 5'-GTTTGTCCCTGTTGATATGGCCT-3' and Reverse 5'-CACAGCAGCATCATCTGTGGTC-3'), APx (Forward 5'GAAGCTTAAGATTTGAAGTTGAA-3' and Reverse 5'CTTAAAGTAGGAATTGTCAAAC-3'), GPx (Forward 5'-GAAATTTTAGCATTTCCTTGT-3' and Reverse 5'- ACGTGGTGAAATGTTCAAX(GA)AAX(CT)-3'), CAT (Forward 5' CTGGCCTGAGGATATCTTGCC-3' and Reverse 5' GACGACAAGGATCAAACCTTGA-3'), SOD (Forward 5'- GTCACGGGACCACATTACAAT-3' and Reverse 5'- CCACAAGCAACCCTTCCACC-3'). These genes encoded enzymes as APx, GR and Cu/Zn SOD are present in chloroplast, GST is in mesophyll protoplast, while GPx and catalase are in peroxisomes. The various gene specific primers used for gene expression were analyzed for linearity between the amount of input RNA and the final RT-PCR products was verified and confirmed. After standardizing the optimal amplification at exponential phase, PCR was carried out under the conditions of 94°C-4min, 94°C-30s, 50 to 58°C-40s, 72°C-1min for 25 cycles and fractionated on agarose gel electrophoresis and visualized with ethidium bromide staining. The 26S rRNA-based gene primers were used as internal control for expression studies [20]. The intensity of bands was analyzed densitometrically and presented in the form of bar diagram.

Antioxidant Activity Assay: Activity analysis of different antioxidant enzymes was also conducted in tobacco shoot and root. APx activity was assayed following the oxidation of ascorbate to dehydroascorbate at 265 nm ($e = 13.7\ mM^{-1}\ cm^{-1}$) by the modified method of Nakano and Asada [21]. GST and GR activities were determined by the standard methods described earlier [22, 23]. GPx activity was also followed by decrease in A_{340}, resulting from NADPH oxidation [24]. CAT activity was measured following the standard method [25]. SOD activity was determined by nitro blue tetrazolium (NBT) photochemical assay method [26].

Statistical Analysis: All the measurements were made in triplicate and all values are represented as means±standard deviation (SD). The $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

The influence of exogenous application of quercetin and epicatechin was studied on the transcript level and enzymatic activities of six antioxidant enzymes i.e. GST, GPx, APx, GR, CAT and SOD in tobacco shoot and root. In tobacco shoot, 50 µM epicatechin exposures enhanced the expression of all the six enzymes GST, GPx, APx, GR, CAT and SOD. In contrast to other enzymes, GR expression was still increased with 100 µM epicatechin. The 50 µM quercetin either decreased or has no effect on the expression of genes encoding antioxidant enzymes in tobacco shoot. However, application of 100 µM quercetin increased the expression of all six genes encoding antioxidant enzymes (Fig. 1). Results suggest that these

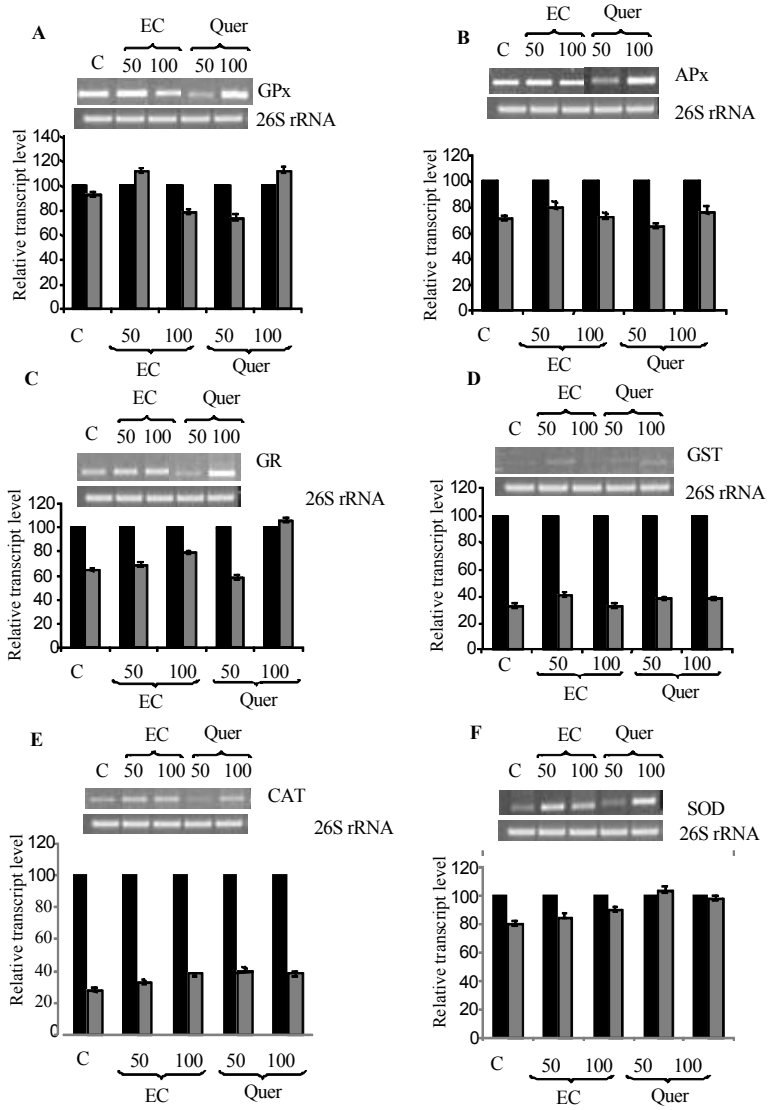

Fig. 1: Changes in the transcript level of A) Glutathione peroxidase (GPx), B) Ascorbate peroxidase (APx), C) Glutathione reductase (GR) and D) Glutathione S- transferase, E) Catalase (CAT) and F) Superoxide dismutase (SOD) in tobacco shoots in response to epicatechin (EC; 50 and 100 µM) and quercetin (Quer; 50 and 100 µM) different treatments. Below gel picture, bar diagram shows relative transcript levels of the respective amplified bands. Expression analysis was repeated at least three times and representative one time gel pictures are presented. Data are means of three measurements±SD. Black and grey bars showed 26S rRNA and antioxidant enzyme transcript levels, respectively. C, control; EC, epicatechin; Quer, quercetin.

two flavonoids have effected transcript expression of genes encoding antioxidant enzymes. However, they were effective at different concentrations. Epicatechin was effective at lower concentration while quercetin was at higher concentration.

In tobacco root, GR expression was not affected upon application of either of flavonoids. The APx expression was not affected by epicatechin. But the expression of APx was decreased upon quercetin application. While GST and GPx expression was increased in tobacco root with 50 µM epicatechin and 100 µM quercetin exposures, whereas CAT and SOD expression showed reverse behaviour (Fig. 2). Earlier literature has suggested differential level of expression of genes encoding antioxidant enzymes in various parts of the plant [27, 28]. Our results support this and further documented that this differential expression seem to be under the regulation of flavonoids. Results also documented that expression is dependent on the concentration and nature of flavonoids.

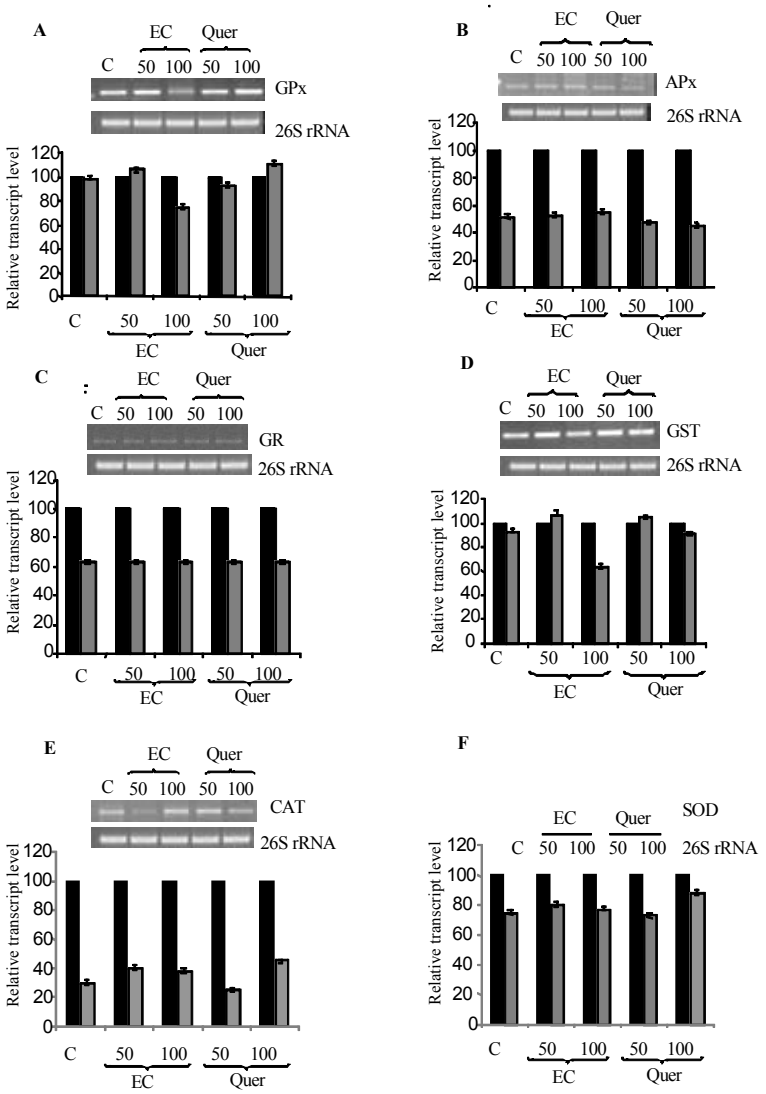

Fig. 2: Changes in the transcript level of A) Glutathione peroxidase (GPx), B) Ascorbate peroxidase (APx), C) Glutathione reductase (GR) and D) Glutathione S- transferase E) Catalase (CAT) and F) Superoxide dismutase (SOD) in tobacco roots in response to epicatechin (EC; 50 and 100 μM) and quercetin (Quer; 50 and 100 μM) different treatments. Below gel picture, bar diagram shows relative transcript levels of the respective amplified bands. Expression analysis was repeated at least three times and representative one time gel pictures are presented. Data are means of three measurements±SD. Black and grey bars showed 26S rRNA and antioxidant enzyme transcript levels, respectively. C, control; EC, epicatechin; Quer, quercetin.

To check whether transcriptional or post-transcriptional regulation, activity analysis of all these enzymes was conducted in tobacco shoot and root tissues exposed to epicatechin and quercetin. In tobacco shoot, activity of all the enzymes showed similar trend to that of the transcript expression (Table 1). This has suggested the transcriptional regulation of all the antioxidant enzymes in tobacco shoot. In root, APx, GR, GST and GPx enzyme activity pattern was found to be similar to that of respective transcript expression data with both the treatments. However, CAT and SOD activity showed the reverse trend to that of expression pattern in tobacco root (Table 1). This has suggested the transcriptional regulation of APx, GR, GST and GPx and post transcriptional regulation of CAT and SOD enzymes in tobacco roots. Further, results have suggested that higher mRNA production does not always correlate with higher activity. There could be two reasons for this, either the rate of such mRNA degradation is higher or inactive protein enzyme is produced.

Table 1: Antioxidant enzyme activity in tobacco shoots and roots in response to epicatechin and quercetin treatments

		Epicatechin		Quercetin	
Samples	Control	50 µM	100 µM	50 µM	100 µM
Shoot					
GPx	[a]967.0± 10.8	[b]1680± 50.24	[a]940±58.2	[a]884±34.4	[b]1760± 62.8
APx	[a]54.2±1.2	[b]88.6± 1.4	[c]62.9±1.8	[b]89.0±1.2	[d]58.0±1.3
GR	[a]10.3±0.4	[b]12.9±0.7	[a]10.96±0.6	[a]11.0±0.7	[c]25.4±0.9
GST	[a]31.7±0.9	[b]50.2±1.0	[a]34.9±0.8	[c]64.8±1.3	[c]60.0±1.2
CAT	[a]17.0±0.8	[b]22±1.2	[c]8.8±0.2	[a]15.2±0.5	[d]25±1.0
SOD	[a]5.8±0.2	[b]13.5±0.9	[c]8.5±0.1	[b]15.8±0.6	[d]21.7±0.9
Root					
GPx	[a]325±21.2	[a]373±22.5	[b]273±14.8	[a]305±20.7	[a]334±25.8
APx	[a]137±2.5	[b]152.1±4.8	[c]162.8±6.3	[a]125.1± 5.9	[d]110.4±5.2
GR	[a]10.96±0.6	[b]9.16±0.4	[a]11.6±0.6	[c]15.0±0.8	[c]13.8±0.9
GST	[a]14.58±0.9	[b]24.2±1.0	[c]9.99±0.4	[d]30.4±0.7	[a]12.8±0.8
CAT	[a]9.2±0.2	[b]11.3±0.4	[c]13.4±0.7	[d]20.5±1.2	[c]15.3±0.8
SOD	[a]10.2±0.7	[a]11±0.5	[b]15.16±0.8	[c]20.8±1.2	[b]15.6±0.9

*Enzyme activity of APx, GST and GR is expressed in µmoles $min^{-1}g^{-1}FW$, CAT and GPx is expressed in nmoles $min^{-1}g^{-1}FW$ and SOD is expressed in U $min^{-1}g^{-1}FW$. All results are presented as mean±SD (n=3). Different alphabet superscript to numeric in the same row represents signi?cant difference in the mean values of estimates at 5% level.

Though several independent studies have been conducted documenting the functions of flavonoid and antioxidant systems in plants, studies pertaining to the effect of flavonoids on antioxidant systems are lacking. Perhaps one of the greatest challenges is to prove the molecular mechanism(s) through which these flavonoid compounds exert beneficial activity in plants itself. Flavonoids are synthesized mainly in the cytosol by multi-enzymatic complexes that are linked to the membrane of endoplasmic reticulum [29]. From the site of synthesis, flavonoids are transported to their subcellular destinations. Owing to their potent redox activities, this subcellular trafficking is tightly regulated to avoid undesired chemical or enzymatic reactions. It is presumed that inside the plants either these flavonoids undergo modification by glycosylation and prenylation, or undergo conjugation with glutathione. Interestingly, specific flavonoid-conjugate transporters move them across the membranes and through intracellular transport reaches to the vacuoles. From vacuole, these flavonoids get remobilised and bind to the targets or receptors directly or indirectly to activate ROS-scavenging genes [19]. Results of this study show that flavonoids have affect on antioxidant enzymes at transcription levels.

Studies have documented the role of flavonoids in redox maintenance. In chloroplast, flavonol glycosides particularly kaempferol glycoside is oxidized to semiquinones by ROS and recycled back to the reduced form by ascorbate. In nuclei, flavonoid glycosides chelate transition metal such as iron, screen UV radiation and avoid photooxidative reactions. Flavonoids are also known to prevent oxidative damage to nucleic acid [18, 30]. Furthermore, few biochemical studies have been conducted that showed the interaction of flavonoid and antioxidant pathways. The GPx enzyme activity has been reported to be activated by the action of flavonoids, quercetin and catechin [31]. The quercetin and its derivatives have been found to prevent oxidative cell damage by either increasing glutathione or reducing the activity of glutathione peroxidase [16]. Flavonoids have also been reported to protect cells from glutathione depletion with the cooperation of ascorbic acids [32]. However, the results of this study documented that flavonoids might be regulating antioxidant system by acting at transcriptional as well as post-transcriptional levels.

In this study, exogenous application of quercetin and epicatechin flavonoids has resulted in the influence on expression of antioxidant enzymes. Effect of these flavonoids was concentration dependent and tissue specific. Results have first time documented the effect of flavonoids on the antioxidant system of plants. This study would help in further understanding the cross talk between flavonoid and antioxidant pathways.

ACKNOWLEDGEMENTS

Authors thank the Director, IHBT for providing the necessary facility to conduct the research and suggestions throughout this work. We would like to acknowledge the financial support from Council of Scientific and Industrial Research (CSIR), Govt of India under NMITLI programme. The IHBT communication number for this study is 2240.

REFERENCES

1. Koes, R.E., F. Quattrocchio and J.N.M. Mol, 1994. The flavonoid biosynthetic pathway in plants: function and evolution. Bioassays, 16: 123-132.
2. Lee, S.R., K.J. Im, S.I. Suh and J.G. Jung, 2003. Protective effect of green tea polyphenol (-) epigallocatechin gallate and other antioxidants on lipid peroxidation in gerbil brain homogenates. Phytother. Res., 17: 206-209.
3. Kuo, S.M., 1997. Dietary flavonoids and cancer prevention: evidence and potential mechanism. Crit. Rev. Oncogen., 8: 47-69.
4. Yang, C.S., J.M. Landau, M.T. Huang and H.L. Newmark, 2001. Inhibition of carcinogenesis by dietary polyphenolic compounds. Annu. Rev. Nutr., 21: 381-406.
5. Ross, J.A. and C.M. Kasum, 2002. Dietary flavonoids: bioavailability, metabolic effects and safety. Annu. Rev. Nutr., 22: 19-34.
6. Rice-Evans, C.A., N.J. Millier and G. Paganga, 1995. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biol. Med., 20: 933-956.
7. Rice-Evans, C.A., N.J. Miller and G. Paganga, 1997. Antioxidant properties of phenolic compounds. Trends Plant Sci., 2: 2152-2159.
8. Rao, M.V., G. Paliyath and D.P. Ormrod, 1996. Ultraviolet-B and ozone induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. Plant Physiol., 110: 125-136.
9. Jayakumar, K., C.A. Jaleel and P. Vijayarengan, 2007. Changes in growth, biochemical constituents and antioxidant potentials in radish (*Raphanus sativus* L.) under cobalt stress. Turkish J. Biol., 31: 127-136.
10. Xiong, L. and J.K. Zhu, 2002. Molecular and genetic aspects of plant responses to osmotic stress. Plant Cell Environ., 25: 131-139.
11. Kumar, S., S.L. Singla-Pareek, M.K. Reddy and S.K. Sopory, 2003. Glutathione: biosynthesis, homeostasis and its role in abiotic stresses. J. Plant Biol., 30: 179-187.
12. Wang, W., B. Vinocur and A. Altman, 2003. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta, 218: 1-14.
13. Sichel, G., C. Corsaro, M. Scalia, A.J. Di Bilio and R.P. Bonomo, 1991. *In vitro* scavenger activity of some flavonoids and melanins against OH2. Free Radical Biol. Med., 11: 1-8.
14. Hanasaki, Y., S. Ogawa and S. Fukui, 1994. The correlation between active oxygens scavenging and antioxidative effects of flavonoids. Free Radical Biol. Med., 16: 845-850.
15. Hertog, M.G.L., D. Kromhout, C. Aravanis, H. Blackburn, R. Buzina, F. Fidanza, S. Giampaoli, A. Jansen, A. Menotti, S. Nedeljkovic, M. Pekkarinen, B.S. Simic, H. Toshima, E.J.M. Feskens, P.C.H. Hollman and M.B. Katan, 1995. Flavonoid intake and long term risk of coronary heart disease and cancer in the seven countries study. Arch. Intern. Med., 155: 381-386.
16. Cruz, T., J. Gálvez, M.A. Ocete, F. Crespo, S. Medina and A. Zarzuelo, 1998. Oral administration of rutoside can ameliorate inflammatory bowel disease in rats. Life Sci., 62: 687-695.
17. Schroeter, H., C. Heiss, J. Balzer, P. Kleinbongard, C.L. Keen, N.K. Hollenberg, H. Sies, C. Kwik-Uribe, H.H. Schmitz and M. Kelm, 2006. (-)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. Proc. Natl. Acad. Sci., 103: 1024 -1029.
18. Hernandez, I., L. Alegre, F. Van Breusegem and S. Munne-Bosch, 2009. How relevant are flavonoids as antioxidants in plants? Trends Plant Sci., 14: 125-132.
19. Dixon, R.A. and G.M. Pasinetti, 2010. Flavonoids and Isoflavonoids: from plant biology to agriculture and neuroscience. Plant Physiol., 154: 453-457.
20. Singh, K., J. Raizada, P. Bhardwaj, S. Ghawana, A. Rani, H. Singh, K. Kaul and S. Kumar, 2004. 26S rRNA-based internal control gene primer pair for reverse transcription-polymerase chain reaction based quantitative expression studies in diverse plant species. Anal. Biochem., 335: 330-333.
21. Nakano, Y. and K. Asada, 1981. Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplast. Plant Cell Physiol., 22: 867-880.
22. Habig, W.H., M.J. Pabst and W.B. Jakoby, 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem., 246: 7130-7139.
23. Carlberg, I. and B. Mannervik, 1985. Glutathione reductase. Method Enzymol., 113: 484-490.
24. Griger, P.G., F. Lin and A.W. Girotti, 1993. Selenoperoxidase-mediated cytoprotection against the damaging effects of tert-butyl hydroperoxide on leukemia cells. Free Radical Biol. Med., 14: 251-266.

25. Dhindsa, R.S., P. Plumb-Dhindsa and T.A. Thorpe, 1981. Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation and decreased levels of superoxide dismutase and catalase. J. Exp. Bot., 32: 93-101.
26. Beyer, W.F. and I. Fridovich, 1987. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Anal. Biochem., 161: 559-566.
27. Mohanpuria, P., N.K. Rana and S.K. Yadav, 2007. Cadmium induced oxidative stress influence on glutathione metabolic genes of *Camellia sinensis* (L.) O. Kuntze. Environ. Toxicol., 22: 368-374.
28. Gill, S.S. and N. Tuteja, 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem., 48: 909-930.
29. Hrazdina, G. and G.J. Wagner, 1985. Metabolic pathways as enzyme complexes: evidence for the synthesis of phenylpropanoids and flavonoids on membrane associated enzyme complexes. Arch. Biochem. Biophys., 237: 88-100.
30. Van Acker, S.A., G.P. van Balen, D.J. van den Berg, A. Bast and W.J. van der Vijgh, 1998. Influence of iron chelation on the antioxidant activity of flavonoids. Biochem. Pharmacol., 56: 935-943.
31. Nagata, H., S. Takekoshi, T. Takagi, T. Honma and K. Watanabe, 1999. Antioxidative action of flavonoids, quercetin and catechin, mediated by the activation of glutathione peroxidase. Tokai J. Exp. Clin. Med., 24: 1-11.
32. Skaper, S.D., M. Fabris, V. Ferrari, M.D. Carbonare and A. Leon, 1997. Quercetin protects cutaneous tissue-associated cell types including sensory neurons from oxidative stress induced by glutathione depletion: cooperative effects of ascorbic acid. Free Radical Biol. Med., 22: 669-678.