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Effects of various plant polyphenols on bladder carcinogen benzidine-induced mutagenicity

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Abstract

Benzidine (Bz), a human bladder carcinogen, was strongly mutagenic to *Salmonella* TA102 tester strain in the Ames *Salmonella* microsome/mutagenicity assay in the presence of rat liver S9 mix. Various non-mutagenic plant polyphenols were included in the assay to test their inhibitory effects on the Bz-induced mutations. Coumestrol, ellagic acid (EA), (−)-epicatechin (EC), (−)-epichatechingallate (ECG), gallic acid (GA), (−)-gallocatechin (GC), plumbagin, propyl gallate (PG), taxifolin, and 2,2′,4′-trihydroxychalcone were found to have a strong inhibitory effect on Bz-induced mutations. (−)-Epigallo-catechingallate (EGCG), fisetin, (−)-gallocatechingallate (GCG), and piceatannol were moderately inhibitory to the mutations; whereas, (−)-catechin, (−)-catechingallate (CG), and reseveratrol were weakly inhibitory to the mutations. (−)-Epigallocatechin (EGC) and 7,3′,4′-trihydroxy isoflavon were not inhibitory to the Bz-induced mutations. Isoliquirtigenin, quercetin dihydrate, and rhein were found to be mutagenic in tester strain TA102. Benzidine mediated lipid peroxidation was conducted employing the thiobarbituric acid reactive substances (TBARS) assay using linoleic acid as a substrate. In the presence of rat liver S9 mix, Bz could cause lipid peroxidation as an outcome of production of oxygen free radicals. Incorporation of the above mentioned non-mutagenic plant polyphenols significantly inhibited benzidine mediated lipid peroxidation in a time dependent manner. These polyphenols also effectively reduced the iron mediated lipid peroxidation. Thus, it is concluded that the inhibition of oxidative mutagenicity of Bz by plant polyphenols could be due to an inhibitory effect of plant polyphenols on the bioactivating enzymes such as cytochrome P-450 and peroxidase and the chelation of iron present in the cytochrome P-450 in the S9 mix. Thus, these plant polyphenols play a significant inhibitory role on Bz-induced mutagenicity.

Keywords: Benzidine; Polyphenols; Genotoxicity; Lipid peroxidation

1. Introduction

Free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) which are constantly generated *in vivo* to cause cellular damage and hence disease. Oxidative stress resulting from ROS or RNS is associated with an imbalance of oxidant/antioxidant status of the cell, which leads to a cascade of reactions in the cell and affects the structural and functional integrity at the cell membrane level (Bast et al., 1991; Gey, 1994). The human body has primary and secondary defenses against free radicals. The primary defense consists of enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. The secondary defense system consists of vitamins A, C, E, β-carotenes, urates, bilirubin, and many others, which can act as antioxidants (Muggli, 1993).

Over the past two decades, plant polyphenols increasingly attracted researchers, food manufacturers, as well as consumers due to their antioxidant properties. The abundance of polyphenols in our diet such as vegetables, fruits, green tea, black tea, red wine, and medicinal plants

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(Giovanna et al., 2000) and their role in the prevention of various diseases such as cancer, cardiovascular, and neurodegenerative diseases has increased their prominence (Claudine et al., 2004). Polyphenols and other dietary reducing agents, such as vitamin C, vitamin E, and carotenoids guard the body's tissues against oxidative stress (Augustin and Gary, 2000). Polyphenols can be classified according to their chemical structures into phenolic acids, flavonoids, stilbenes, and lignans (Lynnette and Ferguson, 2001). According to Augustin and Gary (2000), phenolic acids can be subdivided into derivatives of benzoic acid and derivatives of cinnamic acid. Flavonoids can be divided into six subclasses: flavonols, flavones, isoflavones, flavanones, anthocyanidins, and chalcones (Adele et al., 2004). Stilbenes are organic compounds that contain 1,2-diphenylethylene as a functional group. Lignans are made up of two phenylpropane units. Much of the early research on polyphenolic compounds concerned toxic effects associated with the ability of certain of these to bind and precipitate macromolecules including protein and carbohydrates, thereby reducing the digestibility of foods (Singleton, 1981). Recently, attention has been focused on the identification of antioxidative, anti-inflammatory, antiestrogenic, antiviral, antibacterial, antimutagenic, and/or anticarcinogenic effects of these polyphenols in animal systems (Bravo, 1998; Zhang and Rock, 2004). They were reported as anticancer agents that could reduce carcinogenesis in various experimental models (Yang and Wang, 1993; Wang et al., 1992). Furthermore, polyphenols controls the activity of a wide range of enzymes in cells (Middleton et al., 2000).

Benzidine (CAS No.: 92-87-5) is also called (1,1′-biphenyl)-4,4′-diamine, or 4,4′-diaminobiphenyl or *p*-diaminodiphenyl. Numerous epidemiologic studies have shown that occupational exposure to benzidine resulted in an increased risk of bladder cancer. Oral exposure to Bz in animal studies revealed that it can affect blood, liver, kidney, and the central nervous system (Agency for Toxic Substances and Disease Registry (ATSDR), 1995).

Benzidine was reported as a mutagenic moiety of many azo dyes (Chung and Cerniglia, 1992). The mutagenicity of Bz and its analogues was identified using Ames *Salmonella*/microsomal mutagenicity assay (Bos et al., 1982; Prival et al., 1984; Savard and Josephy, 1986; Chung et al., 2000, 2006). Benzidine has been used for the production of azo dyes, which are extensively used in the textile, paper, and leather industries (Chung et al., 1998a). Although Bz was banned in the USA, benzidine-based dyes and other mutagenic dyes are still synthesized and used in many parts of the world (Schneider et al., 2004). The contamination of water sources by Bz occurs from the release of waste water discharged by the dye industries (Choudhary, 1996). Benzidine may gain entry into the human body by ingestion of contaminant food or water.

There is increasing evidence that Bz causes mutations in the genome with the bioactivation of cytochrome P-450 or peroxidases such as myeloperoxidase and prostaglandin H synthase (PHS) (Geng and Strobel, 1995; Josephy, 1986). Benzidine can be oxidized to its N-hydroxy derivative in the liver by CYP1A2 isozyme of P-450 (Murata et al., 2001). Benzidine and/or its *N*-hydroxy derivatives will be esterified by phases I and II enzymes such as *N*-acetyltransferases (NAT-I, NAT-II) or uridine diphosphate glucuronidase (UDPGA) to form *N*-acetylbenzidine, *N*-acetoxybenzidine, and *N*-glucuronides. These intermediates can be converted to DNA reactive arylnitrenium ions, which can covalently bind to DNA to form DNA adducts (Kadlubar et al., 1990). It was reported that Bz can be oxidized to its *N*-cation by PHS. The free radical cation of Bz may be the electrophilic intermediate responsible for PHS-catalyzed binding of Bz to proteins and nucleic acids (Wise et al., 1983). Josephy (1986) reported that the oxidative activation of Bz may proceed by peroxidase catalyzed one electron oxidation via free radical intermediates or by *N*-acetylation followed by monooxygenase such as horseradish peroxidase or prostaglandin synthase dependent *N*-hydroxylation. It is evident that Bz could cause DNA damage either by covalent binding of electrophilic forms of Bz to DNA or by free radical mediated oxidative DNA damage. In the presence of S9, Bz was found to induce lipid peroxidation (Makena and Chung, 2007).

In this paper, effects of various plant polyphenols on benzidine-induced mutagenicity was analyzed using *Salmonella* tester strain TA102. The effects of these antimutagenic polyphenols were also tested to see whether they can inhibit the lipid peroxidation.

2. Materials and methods

2.1. Chemicals

Benzidine, dimethyl sulphoxide (DMSO), nicotinamide adenine dinucleotide phosphate monosodium salt (NADP), D-glucose phosphate, butylated hydroxytolune, H_2O_2, trichloroactic acid, thiobarbituric acid, and *n*-butonol were obtained from Sigma Chemical Company (St.Louis, MO). All Bz concentrations were freshly prepared by dissolving in DMSO just before the assay. All the plant polyphenols used in this work were a kind gift from Dr. Charles Rock, St. Jude Children's Research Hospital; these were originally obtained from Sigma Chemical Company (St.Louis, MO). The names, hydroxyl substitutions, chemical formulas and molecular weights of polyphenols were shown in Table 1 and the chemical structures were represented in Fig. 1. The rat liver enzyme S9 (1254 Aroclor induced in 0.154 M KCl) was purchased from Molecular Toxicology Inc. (Boone, NC).

2.2. Overnight culture of Salmonella TA102

Salmonella typhimurium TA102 was provided by Dr. B.N. Ames of the Department of Biochemistry, University of California, Berkeley. DMSO was added to an overnight culture as a cryoprotective agent; aliquots of the culture were frozen and stored at −80 °C as stocks. The phenotypic features of the tester strain were regularly monitored using the procedures outlined by Maron and Ames (1983). The TA102 tester strain was cultured overnight in oxoid nutrient broth [25 g in 1000 ml dH_2O] in a shaker at 125 RPM for 10–12 h at 37 °C.

Table 1
Names, OH substitution patterns, chemical formula and molecular weights of various plant polyphenols used in our study

Polyphenol	Chemical name	Formula	MW
(−)-Catechin	(2*S*,3*R*)-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-1(2*H*)-benzopyran-3,5,7-triol	$C_{15}H_{14}O_6$	290.27
(−)-Catechin gallate	(2*S*,3*R*)-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-1(2*H*)-benzopyran-3,5,7-triol 3-(3,4,5-trihydroxybenzoate)	$C_{22}H_{18}O_{10}$	442.37
Coumestrol	7,12-Dihydroxycoumestan	$C_{15}H_8O_5$	268.22
Ellagic acid	4,4′,5,5′,6,6′-Hexahydroxydiphenic acid	$C_{14}H_6O_8$	302.19
(−)-Epicatechin	(−)-*cis*-3,3′,4′,5,7-Pentahydroxyflavane	$C_{15}H_{14}O_6$	290.27
(−)-Epicatechingallate	(−)-*cis*-3,3′,4′,5,7-Pentahydroxyflavane 3-gallate	$C_{22}H_{18}O_{10}$	442.37
(−)-Epigallocatechin	(−)-*cis*-3,3′,4′,5,5′,7-Hexahydroxyflavane	$C_{15}H_{14}O_7$	306.27
(−)-Epigallocatechingallate	(−)-*cis*-3,3′,4′,5,5′,7-Hexahydroxy-flavane-3-gallate	$C_{22}H_{18}O_{11}$	458.37
Fisetin	3,3′,4′,7-Tetrahydroxyflavone	$C_{15}H_{10}O_6$	286.24
Gallic acid	3,4,5-Trihydroxybenzoic acid	$(HO)_3C_6H_2CO_2H$	170.12
(−)-Gallocatechin	(2*S*,3*R*)-2-(3,4,5-Trihydroxyphenyl)-3,4-dihydro-1	$C_{15}H_{14}O_7$	306.27
(−)-Gallocatechin gallate	(2*S*,3*R*)-2-(3,4,5-Tri-hydroxyphenyl)-3,4-dihydro-1(2*H*)-benzopyran-3,5,7-triol 3-(3,4,5-trihydroxybenzoate)	$C_{22}H_{18}O_{11}$	458.37
Isoliquirtigenin	4,2′,4′-Trihydroxychalcone	$C_{15}H_{12}O_4$	256.25
Piceatannol	3,3′,4,5′-Tetrahydroxy-*trans*-stilbene	$C_{14}H_{12}O_4$	244.24
Plumbagin	5-Hydroxy-2-methyl-1,4-naphthoquinone	$C_{11}H_8O_3$	188.18
Propyl gallate	3,4,5-Trihydroxybenzoic acid propyl ester	$3,4,5\text{-}(HO)_3C_6H_2CO_2CH_2CH_2CH_3$	212.20
Quercetin dihydrate	3,3′,4′,5,7-Pentahydroxyflavone dihydrate	$C_{15}H_{10}O_7 \cdot 2H_2O$	338.27
Resveratrol	3,4′,5-Trihydroxy-*trans*-stilbene	$C_{14}H_{12}O_3$	228.24
Rhein	4,5-Dihydroxyanthraquinone-2-carboxylic acid	$C_{15}H_8O_6$	284.22
Taxifolin	(2*R*,3*R*)-3,3′,4′,5,7-Pentahydroxyflavanone	$C_{15}H_{12}O_7$	304.25
2,2′,4′-Trihydroxychalcone	2,2′,4′-Trihydroxychalcone	$C_{15}H_{12}O_4$	256.25
7,3′,4′-Trihydroxy isoflavon	7,3′,4′-Trihydroxy isoflavon	$C_{15}H_{10}O_5$	270.24

2.3. Cytotoxicity assay-trypan blue dye exclusion

The experimental procedure follows standard trypan blue exclusion using hemocytometer. One hundred μl of overnight culture (10–12 h) of *Salmonella* TA102 was incubated at 37 °C with 100 μl of each test chemical in the presence and absence of 500 μl of 4% rat liver S9 mix. After 4 h of incubation, 50 μl of the mixture was mixed with 50 μl of 0.04% trypan blue dye and 20 μl of this mixture was loaded on to the hemocytometer and counted the viable cells (unstained) and dead cells (stained) under microscope (40× magnification) to estimate cytotoxicity caused by the test chemicals. One hundred μl of overnight culture of *Salmonella* TA102 (without tested chemical) was stained for the viable cell count as a control and the value was taken as 100%. The percent viability was calculated using the formula given below:

$$\%\ \text{Cell viability} = \frac{\text{Total viable cells (unstained)}}{\text{Total cells (stained plus unstained)}} \times 100.$$

2.4. Ames Salmonella/microsomal mutagenicity inhibition test

The assay was performed using the preincubation method in the absence and presence of rat liver S9 mix described by Maron and Ames (1983), with minor modifications. The assay procedure involved adding 500 μl of rat liver S9 mix, 100 μl of the test chemical in DMSO, and 100 μl of an overnight culture of TA102. One hundred μl of Bz was added to 100 μl of *Salmonella* TA102 tester strain in the presence or absence of 500 μl of S9, then incubated with 100 μl of plant polyphenols such as (−)-catechin, (−)-catechingallate (CG), coumestrol, ellagic acid (EA), (−)-epicatechin (EC), (−)-epicatechingallate (ECG), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG), fisetine, gallic acid (GA), (−)-gallochatechin (GC), (−)-gallocatechin gallate (GCG), isoliquirtigenin, piceatannol, plumbagin, propyl gallate (PG), quercetin dehydrate, (−)-rhein, resevetrol, taxifolin, 2,2′,4′-trihydroxy chalcone, and 7,3′,4′-trihydroxy isoflavon. The final concentration of each polyphenols was 50 μg/plate. The reactions were then incubated in a shaker at 250 RPM, maintained the temperature at 37 °C. After 20 min, the reactions were mixed with 2 ml of molten (48 °C) top agar containing biotin and a trace amount of histidine plated on minimal glucose plates, and incubated at 37 °C for 48 h. The revertant colony numbers were counted. All the above polyphenols were tested without Bz (polyphenols alone) on TA102 to study whether any of the above polyphenols could cause significant mutations in TA102 tester strain. Benzidine was used as a positive control and DMSO was used as a negative control.

2.5. Measurement of inhibition of Bz-mediated lipid peroxidation by polyphenols

The assay protocol was obtained from Jeongmin et al. (2005). In brief, 100 μl of a 66% solution of linoleic acid were added to 200 μl of TWEEN 20 and 19.7 ml of distilled water. This emulsion was prepared just before the assay and stored in the dark in order to avoid the autoxidation of linoleic acid. To 500 μl of 0.02 M phosphate-buffered saline (PBS), 200 μl of the linoleic acid emulsion and 400 μl (50 μg/ml) of Bz were added, in the presence and absence of S9 mix. This mixture was used as a positive control. Four hundred μl of 0.01% of plant polyphenols were added to 400 μl of Bz with S9 mix and this mixture added to 500 μl of 0.02 M PBS and 200 μl of the linoleic acid emulsion. To check the effect of S9 on linoleic acid peroxidation, 400 μl of S9 mix was mixed with 500 μl of 0.02 M PBS and 200 μl of the linoleic acid emulsion as a control. The reaction mixtures were incubated at 37 °C for 3 and 6 h. The reactions were analyzed for lipid peroxidation by adding 200 μl of 4% trichloroacetic acid, 2 ml of 0.8% thiobarbituric acid, and 200 μl of 0.4% BHT, and incubating the mixture at 100 °C for 30 min, followed by cooling on ice. Two ml of butanol were added, and the absorbance of thiobarbituric acid reactive substances in the butanol extract was measured at 532 nm. The percent inhibition of lipid peroxidation was calculated by using the following formula:

$$\frac{A_{532}(\text{blank}) - A_{532}(\text{sample})}{A_{532}(\text{blank})} \times 100$$

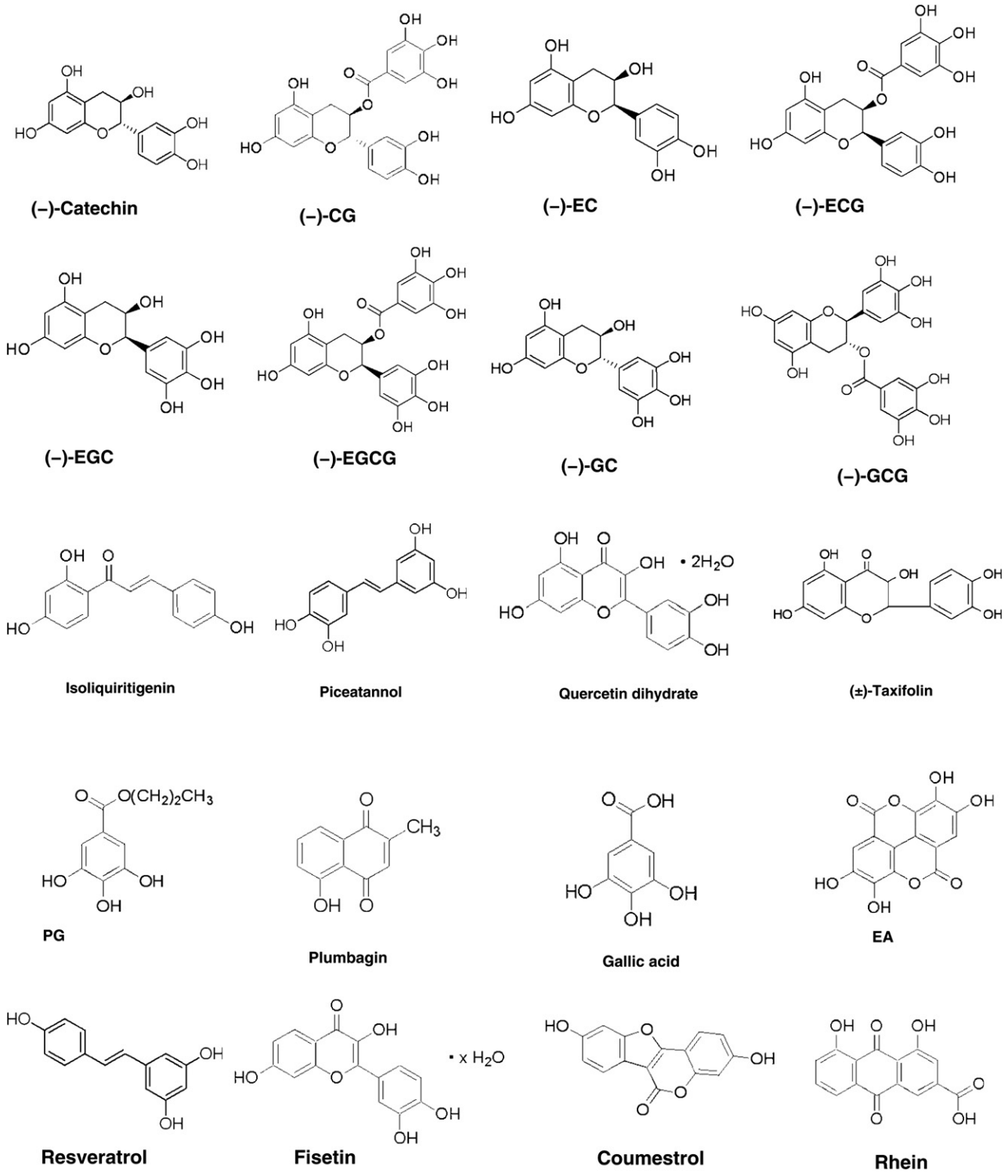

Fig. 1. Chemical structures of various plant polyphenols. CG = catechingallate; EC = epicatechin; ECG = epicatechingallate; EGC = epigallocatechin; EGCG = epigallocatechingallate; GC = gallocatechin; GCG = gallocatechingallate; PG = propyl gallate; EA = ellagic acid.

2.6. Measurement of inhibition of iron + ascorbate-mediated lipid peroxidation by polyphenols

The experimental procedure is same as described above. However, the positive control in this experiment was iron in combination with ascorbic acid. Various plant polyphenols were incorporated in the test system to measure inhibition of lipid peroxidation via metal ion chelation.

2.7. Statistical analysis

All experiments were repeated for the reproducibility. The values are means of revertants/plate ± standard deviations (Using Microsoft Excel) of one or two experiments, each with three plates/dose. The statistical significance was calculated using student *T*-test (using the TTEST program in Microsoft Excel software: Microsoft, Redmond, WA), and all p values were less than 0.05.

3. Results

3.1. Cytotoxicity of test chemicals on Salmonella TA102 tester strain

Benzidine, DMSO, H_2O_2 and all tested plant polyphenols were tested for cytotoxicity on *Salmonella* TA102 tester strain. The tested concentrations of Bz and plant polyphenols were the same in the cytotoxicity test. No test chemicals were found to be cytotoxic to *Salmonella* TA102. After 4 h incubation, all tested chemicals have shown more than 90% cell viability in trypan blue dye exclusion assay (data were not shown).

3.2. Mutagenicity of plant polyphenols

Mutagenicity of various plant polyphenols at the 50 μg/plate was tested employing the *Salmonella*/microsomal mutagenicity assay using tester strain TA102. Table 2 shows that all these tested polyphenols were not mutagenic except isoliquirtigenin, quercetin dihydrate, and (−)-rhein, which had a significantly higher number of revertants than the control. Isoliquirtigenin and quercetin dihydrate were mutagenic only in the presence of S9 mix; whereas, (−)-rhein was mutagenic both in the presence and absence of S9 mix. (−)-Epigallocatechin showed a slightly higher number of revertants than the control; thus could be very weakly mutagenic on TA102 (Table 2).

Table 2
Mutagenicity of plant polyphenols on *Salmonella* TA102 tester strain

Test chemicals (50 μg/plate)	Number of revertants	
	With S9	Without S9
Benzidine (positive control)	1022 ± 24	383 ± 55
DMSO (negative control) (100 μl)	311 ± 11	293 ± 9
Hydrogen peroxide		781 ± 20
Non-mutagenic		
(−)-Catechin	420 ± 22	425 ± 16
(−)-Catechin gallate	419 ± 8	404 ± 6
Coumestrol	428 ± 21	366 ± 21
Ellagic acid	393 ± 25	355 ± 36
(−)-Epicatechin	329 ± 51	308 ± 17
(−)-Epicatechin gallate	372 ± 43	330 ± 10
(−)-Epigallocatechin gallate	420 ± 26	285 ± 8
Fisetine	421 ± 16	322 ± 20
Gallic acid	378 ± 17	395 ± 7
(−)-Gallocatechin	400 ± 32	317 ± 20
(−)-Gallocatechin gallate	334 ± 11	295 ± 15
Piceatannol	441 ± 8	314 ± 13
Plumbagin	382 ± 12	335 ± 18
Propyl Gallate	393 ± 10	310 ± 11
Reseveratrol	440 ± 12	331 ± 14
Taxifolin	373 ± 43	325 ± 8
2, 2′,4′-Trihydroxychalcone	182 ± 53	226 ± 27
7,3′,4′-Trihydroxy isoflavon	396 ± 36	305 ± 19
(−)-Epigallocatechin	669 ± 37	608 ± 9
Mutagenic		
Isoliquirtigenin	808 ± 31	524 ± 35
Quercetin dihydrate	1060 ± 24	826 ± 32
Rhein	1010 ± 20	2568 ± 11

Effect of various polyphenols such as flavonoids, stilbenes, and phenolic acids on TA102 with and without S9 mix. The values are means of revertants/plate ± standard deviations of one experiment, each with three plates/dose.

3.3. Effect of plant polyphenols on benzidine-induced mutations

The effect of the non-mutagenic polyphenols on the oxidative genotoxicity mediated by Bz was examined using tester strain TA102 and results were shown in Figs. 5–8. In the presence of S9 mix, coumestrol, ellagic acid (EA), (−)-epicatechin (EC), (−)-epichatechingallate (ECG), gallic acid (GA), gallocatechin (GC), (−)-plumbagin, propyl gallate (PG), taxifolin, and 2,2′,4′-trihydroxychalcone significantly decreased the Bz-induced revertant numbers (Fig. 5). (−)-Epigallocatechingallate (EGCG), fisetin, (−)-gallocatechingallate (GCG), and piceatannol were moderately inhibitory to the Bz-induced mutagenicity (Fig. 6). (−)-Catechin, (−)-catechingallate (CG), and reseveratrol were weakly inhibitory to the Bz-induced mutations (Fig. 7). 7,3′,4′-Trihydroxyisoflavon and (−)-epigallocatechin (EGC) were not inhibitory to the Bz-induced mutations (Fig. 8). In the presence of S9, coumestrol, ellagic acid (EA), (−)-epicatechin (EA), (−)-epicatechingallate (ECG), gallic acid (GA), gallocatechin (GC), (−)-gallocatechin gallate (GCG), piceatannol, plumbagin, propyl gallate (PG), taxifolin, and 2,2′,4′-trihydroxychalcone were further tested on Bz for their dose–response using different concentrations as 50, 100 and 200 μg/plate, and results were shown in Table 3. 2,2′,4′-Trihydroxychalcone was found to have the strongest inhibitory effect among the polyphenols tested (Table 3).

3.4. Inhibitory effect of polyphenols on benzidine-induced lipid peroxidation

The polyphenols that exerted a strong inhibition on Bz-induced mutations were selected for the inhibition of lipid peroxidation assay. Table 4 shows that (−)-catechingallate (CG), coumestrol, ellagic acid (EA), (−)-epicatechin (EC), (−)-epichatechingallate (ECG), (−)-epigallocatechingallate (EGCG), gallic acid (GA), gallocatechin (GC), (−)-gallocatechingallate (GCG), piceatannol, plumbagin, propyl gallate (PG), taxifolin, and 2,2′,4-trihydroxychalcone had shown significant inhibitory effects on Bz-mediated lipid peroxidation in a time dependent manner.

3.5. Inhibitory effect of polyphenols on Fe/ascorbate mediated lipid peroxidation

The Fe/ascorbate system was used for the induction of free radical formation by causing lipid peroxidation (Decker and Faraju, 1990). The effect of polyphenols on the Fe/ascorbate mediated lipid peroxidation was tested and results are in Table 5. All the selected polyphenols had been shown to have a significant inhibition on iron-mediated lipid peroxidation.

Table 3
Polyphenol dose–response on benzidine-induced mutations on *Salmonella* TA102 tester strain

Test chemicals	No. of revertants		
	50 μg/plate	100 μg/plate	200 μg/plate
Benzidine	1049 ± 12[a]	–	–
DMSO	325 ± 21[b]	–	–
Coumestrol	567 ± 35	501 ± 12	457 ± 12
Ellagic acid	498 ± 6	391 ± 7	260 ± 18
(−)-Epicatechin	483 ± 19	393 ± 28	217 ± 13
(−)-Epigallocatechingallate	620 ± 8	444 ± 33	296 ± 44
Gallic acid	483 ± 17	393 ± 21	287 ± 13
(−)-Gallocatechin	526 ± 13	482 ± 57	338 ± 25
(−)-Gallocatechingallate	706 ± 10	612 ± 38	352 ± 10
Piceatannol	605 ± 16	474 ± 33	301 ± 20
Plumbagin	536 ± 34	338 ± 54	218 ± 12
Propyl gallate	420 ± 8	361 ± 18	278 ± 7
Rhein	520 ± 8	368 ± 18	178 ± 7
Taxifolin	596 ± 91	451 ± 39	288 ± 48
2,2′,4′-Trihydroxychalcone	92 ± 5	91 ± 6	160 ± 18

The values are means of revertant/plate ± standard deviations of one experiment, each with three plates/dose.
[a] Fifty micrograms/plate concentration of benzidine was used as a positive control.
[b] Hundred microliters of DMSO was used as a negative control.

Table 4
The percent inhibition of benzidine mediated lipid peroxidation by plant polyphenols

Polyphenols	% Inhibition of benzidine mediated lipid peroxidation[a]	
	3 h	6 h
(−)-Catechin Gallate	55.9	76.5
Coumestrol	42.1	71.1
Ellagic acid	64.1	81.4
(−)-Epigallocatechin gallate	56.4	76.8
(−)-Epicatechin	64.5	81.7
(−)-Epicatechin gallate	53.1	77.7
Gallic acid	67.4	83.4
Gallocatechin	61.7	82.0
(−)-Gallocatechin gallate	59.3	78.5
Piceatannol	64.5	82.8
Propyl gallate	60.7	79.4
Plumbagin	70.3	85.4
Taxifolin	55.9	76.5
2,2,4′-Trihydroxychalcone	75.5	90.8

[a] The percent inhibition of benzidine mediated lipid peroxidation by plant polyphenols. The percent inhibition was calculated by

$$\frac{A_{532}(\text{blank}) - A_{532}(\text{sample})}{A_{532}(\text{blank})} \times 100.$$

Table 5
The percent inhibition of Fe/ascorbate mediated lipid peroxidation by plant polyphenols

Polyphenols	% Inhibition of iron/ascorbate mediated lipid peroxidation[a]	
	3 h	6 h
(−)-Catechin gallate	71.8	83.1
Coumestrol	69.9	83.1
Ellagic acid	69.7	82.8
(−)-Epicatechin	69.7	82.8
(−)-Epigallocatechin gallate	70.2	83.4
(−)-Epicatechin gallate	66.2	82.1
Gallic acid	68.1	82.0
Gallo catechin	71.8	83.1
(−)-Gallocatechin gallate	70.2	83.4
Piceatannol	70.0	83.4
Plumbagin	61.7	78.5
Propyl gallate	66.2	82.2
Taxifolin	70.7	83.2
2,2,4′-Trihydroxychalcone	68.1	82.0

[a] The percent inhibition of Fe/ascorbate mediated lipid peroxidation by plant polyphenols. The percent inhibition was calculated by

$$\frac{A_{532}(\text{blank}) - A_{532}(\text{sample})}{A_{532}(\text{blank})} \times 100.$$

4. Discussion

Under normal physiological conditions, a variety of ROS e.g. superoxide anion, hydroxyl free radical, hydrogen peroxide, and singlet oxygen are formed and decomposed by enzymatic and non-enzymatic reactions. Increased levels of ROS can initiate lipid peroxidation, and damage proteins and DNA. ROS and RNS may also be a causative factor for chemically induced cancers (James et al., 1998).

Polyphenols have been reported to have antioxidant property (Hertog et al., 1993; Zhang and Rock, 2004). Our results indicate that some of the flavonoids such as coumestrol, ellagic acid (EA), (−)-epicatechin (EC), (−)-epichatechingallate (ECG), gallic acid (GA), gallocatechin (GC), (−)-plumbagin, propyl gallate (PG), taxifolin, and 2,2′,4′-trihydroxychalcone were shown to have a strong inhibitory effect on Bz-induced mutations in TA102 tester strain at the tested concentration, which was non-cytotoxic to the *Salmonella* TA102 tester strain. There are a considerable number of reports of antimutagenic effects of polyphenols (Heo et al., 1992). There are various mechanisms to be implicated in antimutagenic effects of polyphenols. Some of these are non-specific; for example, polyphenols inhibited the uptake of several mutagens (Hatch et al., 2000). Polyphenols could diminish the production of the active metabolites of the xenobiotic through downregulation of the relevant enzymes, and/or directly interfere with DNA adduct formation (Hatch et al., 2000).

Smith et al. (1988) employed an *in vitro* system to show that DNA adduction by dibenzo[*a*,*l*]pyrene (DBP) could be strongly inhibited by ellagic acid. Moreover, studies with the active metabolites suggested that ellagic acid acted independently of enzymatic activation processes. Dixit and Gold (1986) demonstrated that ellagic acid inhibition of *N*-nitroso methylurea (NMU)-induced mutagenicity was due to inhibition of methylation at the O_6 position of guanine, through an ellagic acid-duplex DNA affinity-binding mechanism. Polyphenols may also inhibit cytochrome P-450s or peroxidase enzymes such as cyclooxygenase or lipoxygenase that can produce free radical stress.

Yang and Strickhart (1974) reported that propyl gallate did not inhibit the NADPH-dependent reduction of cytochrome P-450; therefore, the site of inhibition is not on NADPH-cytochrome c reductase. Baer-Dubowska et al. (1998) studied the inhibitory effect of several phenolic acids on cytochrome P-450 (CYP1A1, 1A2, and 2B) and found that dodecyl gallate and propyl gallate were inhibitory to CYP1A2 and 2B. The authors found that the inhibition in most cases was non-competitive in nature from the analysis of the kinetics of CYP inhibition. Our Ames *Salmonella*/microsomal mutagenicity assay results were in correlation with the Baer-Dubowska et al. (1998). The phenolic acids, gallic acid, ellagic acid, and propyl gallate have shown a very strong inhibition to the mutagenicity of Bz in the presence of S9 mix. The order of the relative inhibitory effect on Bz-induced mutations was as follows: gallic acid > ellagic acid > propyl gallate. Thus, the inhibition of mutations could be either due to inhibition of the P-450 isozymes or direct inhibition of the formation of DNA adducts, interference with the active metabolite of Bz, by chelation of the iron, and quenching the ROS generated by the Bz N-cation formation or cooxidation with NADPH. Therefore, mechanisms involved in the phenolic acid mediated inhibition of Bz-induced mutations might follow multiple pathways and hence it requires further investigation.

Siess et al. (1989) demonstrated that flavonoids inhibited CYP1A-mediated 7-ethoxyresorufin *o*-deethylase activity in rat and human liver microsomes. Breinhold et al. (1999) administered a variety of flavonoids including tangeretin, chrysin, apigenin, naringenin, genistein, and quercetin over two consecutive weeks to female rats, which were then injected with 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) and found that hepatic PhIP-DNA adduct formation was not affected by any of the administered flavonoids.

Zhai et al. (1998) studied flavonoids and several derivatives that differ only in the number and position of hydroxyl groups on the flavone nucleus and found that the inhibition of CYP1A2 activity was significantly increased by polyphenols with 3- and 5-hydroxylation; whereas, a remarkable decrease was observed by polyphenols with 7-hydroxylation. Furthermore, Zhai et al. (1998) also found that polyphenols with 3,5,7-trihydroxylation inhibited CYP1A2 activity to a greater extent than did hydroxylation at position 3 or 5 alone. In contrast, 7-hydroxyflavone was a potent inhibitor of CYP1A1 and exhibited sixfold higher selectivity for CYP1A1 over CYP1A2. They suggested that the binding environment of the CYP1A1 active site has a selection for the polyphenols with 7-hydroxyl substituent.

We have also observed the similar structure–activity relationships among these polyphenols. Our *Salmonella*/microsomal mutagenicity assay results of flavonoids (Figs. 5–8) were in correlation with Zhai et al. (1998). The position and number of hydroxyl groups and presence of methyl and acid groups on these polyphenols are crucial in their inhibitory effect. Polyphenols with polyhydroxyl groups have been shown to have a significant inhibitory effect. In addition, polyphenols with hydroxyl groups present at second, third, fourth, fifth, and seventh positions have exerted greater inhibitory effect; especially, the combination of 2–4, 3–4–5 and 3–4–5–7 position of hydroxyl groups were highly inhibitory. The position and number of hydroxyl group for these were presented in Table 1 and Fig. 1. Coumestrol and rhein are dihydroxy polyphenols; 2,2′,4′-trihydroxychalcone and (−)-gallocatechin (GC) are trihydroxy polyphenols; ellagic acid (EA) and piceatannol are tetra hydroxy polyphenols, (−)-epicatechin (EC), (−)-epicatechingallate (ECG), and taxifolin are pentahydroxy polyphenols; whereas plumbagin is monohydroxy polyphenol but it contains a methyl group in the second position. According to our data, the relative flavonoid-mediated inhibitory effect on Bz-induced mutations as follows: 2,2′,4′-trihydroxy-chalcone > (−)-epicatechin (EC) > coumestrol > plumbagin > (−)-gallocatechin (GC) > (−)-epicatechingallate (ECG) > taxifolin (Fig. 5).

The spatial arrangement of substituents on polyphenols could be more important than the backbone alone in antioxidant activity. It is consistent with most polyphenolic antioxidants; both the configuration and total number of the hydroxyl group significantly influence several mechanisms of antioxidant activity. Thus, free radical scavenging capacity is primarily attributed to the high reactivities of hydroxyl substituents (Cao et al., 1997; Sekher et al., 2001). Our results indicate that piceatannol, fisetin, (−)-gallocatechingallate (GCG), and (−)-epigallocatechingallate (EGCG) were moderately inhibited Bz-induced mutations in the presence of S9 mix. Sengupta et al. (2004) reported that the C and B rings of fisetin appear to play a more important role than the A-ring in scavenging free radicals. It is found that the 3-OH fisetin radical has the lowest calculated Bond Dissociation Enthalpies (BDE) value followed by the 3′-OH and 4′-OH fisetin radicals while the corresponding value of the 7-OH fisetin radical is predicted to be the highest; therefore, a lower BDE value is usually attributed to a higher ability to donate a hydrogen atom from the hydroxyl group and thereby scavenge free radicals. (−)-Epigallocatechin gallate (EGCG) and (−)-gallocatechin gallate (GCG) were implicated in the inhibition of the monooxygenase activity associated with cytochrome P-450 activation of carcinogen precursors (Wang et al., 1988; Robert and Huong, 1998). Chen and Ho (1994) reported that the scavenging potency of free radicals of various green tea polyphenols; their radical scavenging effect follow the order: (−)-epigallocatechin gallate (EGCG) > (−)-epicatechin gallate (ECG) > (−)-epigallocatechin (EGC) > (−)-epicatechin (EC). Our results indicated that (−)-epigallocatechin gallate (EGCG) inhibited 37%, and (−)-gallocatechingallate (GCG) inhibited 35% of Bz-induced mutation; whereas, catechingallate (CG) and catechin exerted 27% and 28% inhibition, respectively (Figs. 6 and 7). On the other hand, epigallocatechin (EGC) and 7,3′,4′-trihydroxy isoflavon did not show any

inhibitory effect on Bz-induced mutations on TA102 tester strain (Fig. 8). Careful observation of structure–activity relationships of our results indicates that the presence of galloyl and gallate groups to catechins enhanced their inhibitory effects. Thus, our findings suggest that the addition the galloyl and gallate groups could have a greater inhibitory effect on CYP1A2 and also free radical quenching ability.

Coumestrol, ellagic acid (EA), (−)-epicatechin (EC), (−)-epichatechingallate (ECG), gallic acid (GA), gallocatechin (GC), (−)-gallocatechingallate (GCG), piceatannol, plumbagin, propyl gallate (PG), taxifolin, and 2,2′,4-trihydroxychalcone were selected to estimate the inhibitory effect of Bz-induced and Fe/ascorbate-induced lipid peroxidation (Tables 4 and 5). All the above plant polyphenols have significantly reduced the degree of lipid peroxidation caused by Bz in a time dependent manner. Although there was no significant variation in the degree of inhibition of Bz-mediated and Fe/ascorbate-mediated lipid peroxidation, the iron chelation capabilities of polyphenols appeared to be slightly greater than the Bz-mediated lipid peroxidation. 2,2,4′-Trihydroxychalcone was shown to have the strongest inhibition of lipid peroxidation (90%) after 6 h and also inhibited Bz-induced mutations to 95% in the *Salmonella* test (Table 4 and Fig. 5). Hence, we suggest that the inhibition of lipid peroxidation could be due to inactivation of P-450 by chelation of the iron from the heme in the cytochrome by these plant polyphenols.

In contrast to the above polyphenols, isoliquirtigenin quercetin, and rhein were found to be mutagenic to TA102 (Table 2). Rhein was found to be a direct mutagen on TA102. The structure of rhein consists of three rings with two quinone groups and could be a DNA cross-linking agent. Quercetin was reported as mutagen in several studies (Pamukcu et al., 1980; Chung et al., 1998b). Quercetin and taxifolin have similar chemical structures except a double bond at 2, 3 position (Fig. 2). Lee et al., 1998 reported that quercetin was a strong inhibitor of CYP1A2. We found that quercetin was mutagenic in TA102 (Table 2); whereas, taxifolin exerted a great inhibitory effect on Bz-induced mutations. This indicates that only an extra double bond in the chemical structure would significantly affect the biological activity such as antimutagenicity. Isoliquirtigenin and piceatannol show structural similar (Fig. 3). Piceatannol was found to have a significant inhibitory effect on the Bz-induced mutations; whereas, isoliquirtigenin has no effect on Bz-induced mutations;

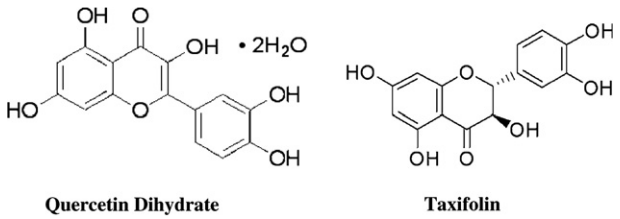

Fig. 2. Chemical structures of quercetin and taxifolin.

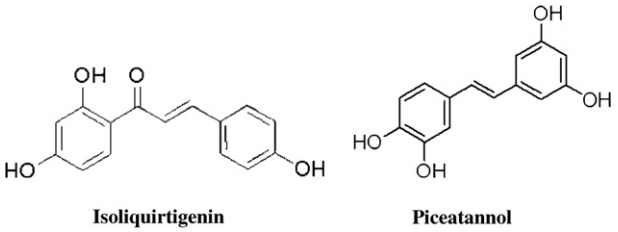

Fig. 3. Chemical structures of isoliquirtigenin and piceatannol.

whereas, (−)-epigallocatechin (EGC) was not. Isoliquirtigenin has an extra keto group in the structure compared to piceatannol. Likewise, epigallocatechin and gallocatechin show a high structural similarity (Fig. 4); however, gallocatechin has shown significant inhibition on Bz-induced mutations. Among six gallocatechins, only (−)-epigallocatechin did not have any inhibitory effect on Bz-induced mutations. From our observations, a minor structural variation in the chemical structure could cause a significant difference in the antimutagenic properties. Each functional group in these chemical structures certainly plays a significant function in their biological activity. Further studied on the structure–activity relationships are highly necessary.

In conclusion, the antimutagenic effect of polyphenols could follow several pathways such as direct antioxidant or prooxidant effect, inactivation of carcinogen activation

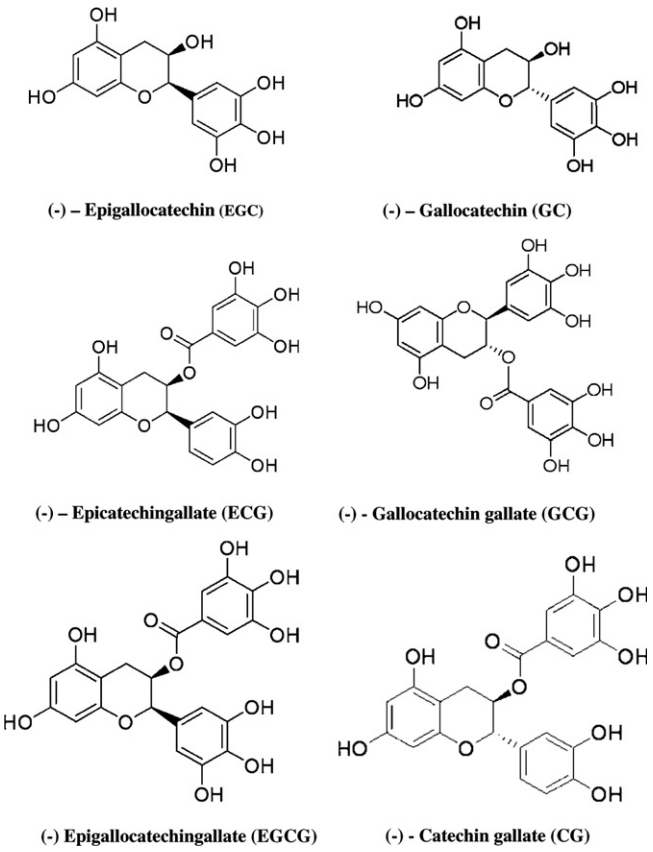

Fig. 4. Chemical structures of various catechins with galloyl and gallate groups.

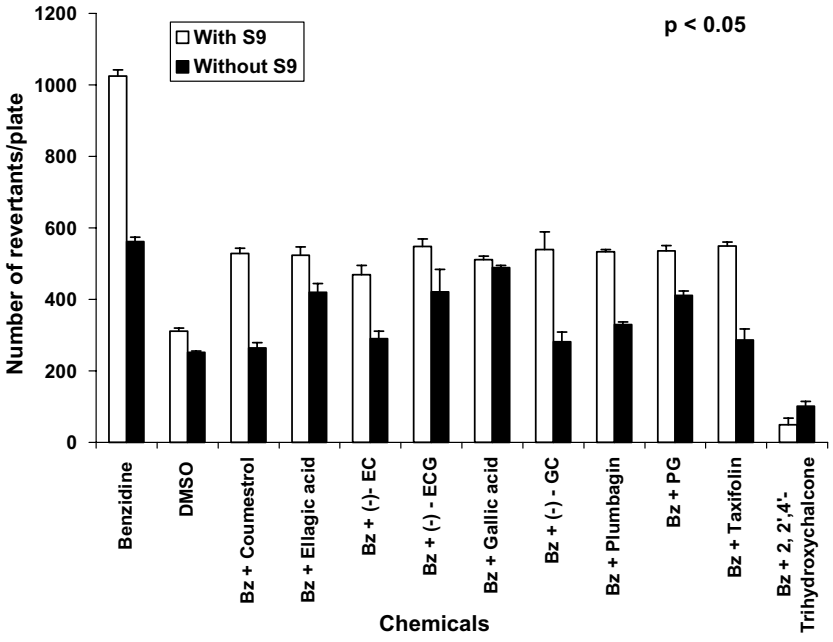

Fig. 5. Polyphenols showing strong inhibitory effect on Bz-induced mutations. Effect of polyphenols on the mutagenicity of benzidine in the presence and absence of S9 mix. The concentration of all the tested chemicals was 50 μg/plate. All the above polyphenols has shown significantly inhibited the revertant numbers (≈50%). Benzidine = positive control; DMSO = negative control. (□) With S9; (■) Without S9. EC = epicatechin; ECG = epicatechingallate; GC = gallocatechin; PG = propyl gallate. $p < 0.05$.

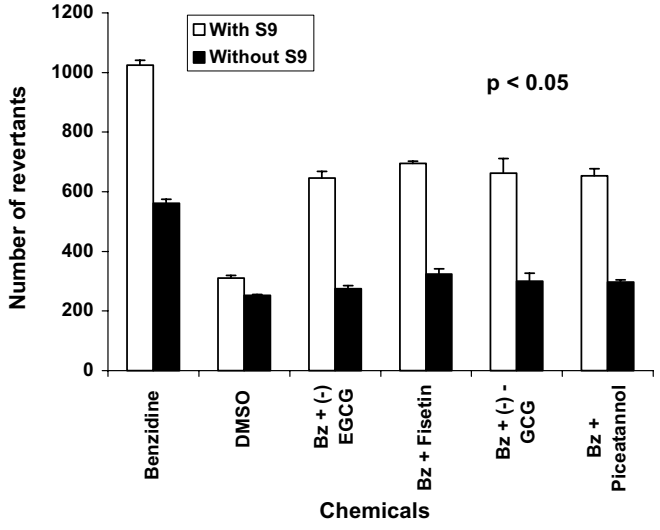

Fig. 6. Polyphenols showing moderate inhibitory effect on Bz-induced mutations. Effect EGC, fisetine, GCG and piceatannol on the mutagenicity of benzidine in the presence and absence of S9 mix. The above polyphenols have shown moderate effect on the mutagenicity of benzidine (≈35%). Benzidine is a positive control; DMSO is a negative control. (□) With S9; (■) Without S9. EGCG = epigallocatechingallate; GCG = gallocatechingallate. $p < 0.05$.

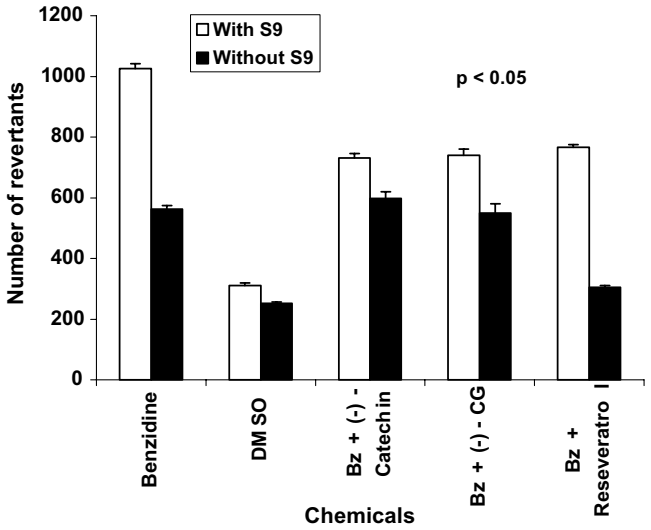

Fig. 7. Polyphenols showing weak inhibitory effect on Bz-induced mutations. Effect of catechin, catechingallate and reseveratrol on the mutagenicity of benzidine in the presence and absence of S9 mix. The above polyphenols were weakly inhibitory (≈25%) on the mutagenicity of benzidine. Benzidine is a positive control; DMSO is a negative control. (□) With S9; (■) Without S9. CG = catechingallate. $p < 0.05$.

enzymes like CYP and peroxidases, inactivating the mutagen or inhibiting the formation of DNA adducts. The degree of hydroxylation of polyphenols could contribute to their antimutagenic effects and plays a significant role in determining the antimutagenic efficiency. However, the special arrangements of different chemical groups, degree of unsaturation also account for the inhibitory effect of polyphenols. We have observed an increased inhibitory effect with compounds having methyl and acid (gallate) groups. All the selected polyphenols in our investigation have shown strong inhibition on lipid peroxidation caused by Bz could also suppress the lipid peroxidation caused by Fe/ascorbate system. Therefore, we speculated that the

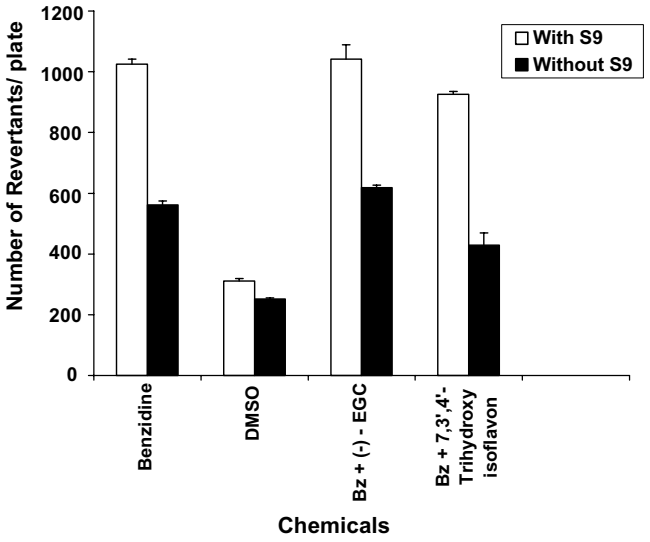

Fig. 8. Polyphenols with no inhibitory effect on Bz-induced mutations. Epigallocatechin (EGC) and 7,3′,4′-trihydroxyisoflavone on the mutagenicity of benzidine in the presence and absence of S9 mix. Benzidine is a positive control; DMSO is a negative control. (□) With S9; (■) Without S9. EGC = epigallocatechin.

inhibition of Bz-induced lipid peroxidation could be due to inactivation of P-450 and thereby inhibiting the ROS production. We also suggest that the inactivation of P-450 could be due to chelating iron from heme complex of cytochrome. This investigation would give basic understanding of the roles of various plant polyphenols on Bz-mediated genotoxicity. Our future perspective is to examine extensively the structure–activity relationships of polyphenols such as quercetin, taxifolin, isoliquirtigenin, piceatannol, (−)-epigallo-catechin (EGC) and (−)-gallocatechin (GC). Such a study will be instrumental to further understand their modes of actions, which may have value in the use of these polyphenols in preventing the genotoxic damage of carcinogenic compounds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fct.2007.04.007.

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