International Journal of Pharma Medicine and Biological Sciences

ISSN 2278 – 5221 www.ijpmbs.com
Vol. 2, No. 1, January 2013

Research Paper

ANTIMUTAGENIC EFFECT OF GREEN TEA EXTRACTS IN REVERSE MUTATION ASSAY

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In the present investigation, antimutagenic effect of petroleum ether, chloroform, methanol and water extracts of green tea was evaluated in *Salmonella typhimurium* TA-98 and TA-100 strains. Well known mutagens like sodium azide and daunomycin were added at a concentration of 10µl and 6µl per plate respectively resulted in the induction of histidine revertant colonies. However addition of 10 µl of petroleum ether, chloroform, ethanol and water extracts of green tea to 10 µl of sodium azide and 6 µl of daunomycin treated plates resulted in the inhibition in the number of histidine revertant colonies. Furthermore, supplementation with all the four extracts of green tea at a concentration of 10 µl per plate respectively in the presence of S9 fraction also led to significant inhibition in sodium azide and daunomycin induced colony formation. The antimutagenic activity of ethanolic extract of green tea was found to be higher than that of the other extracts. Hence the study revealed that green tea has protective efficacy in sodium azide and daunomycin induced mutagenicity in the test microbial system.

Keywords: Green tea, Antimutagenic activity, Ames assay, *Salmonella typhimurium*

INTRODUCTION

Damage to DNA is likely to be a major cause of cancer and other diseases (Ames, 1975). In modern times man is exposed to a multitude of environmental poisons (Renner, 1990). Repeated exposures to small doses of natural toxicants or carcinogens may lead to most hazardous situations (Iverson, 1991). It is difficult to eliminate mutagenic or carcinogenic factors present in our environment, but it is possible to diminish the risk of cancer through simple dietary and herbal recommendations (Block *et al.*, 1992).

Plant contains many natural substances that can promote health and alleviate illness. Flavonoids, a broad class of polyphenolic compounds, widely distributed among photosynthesizing cells, possess an impressive array of pharmacological activity (Hasten, 1983).Oxidation is a metabolic process that leads to energy production necessary for essential cell activities. However, metabolism of oxygen in living cells also leads to the unavoidable production of oxygen derived free radicals, commonly known as Reactive Oxygen Species (ROS) (McCord,

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1994 and Adegoke, 1998) which are involved in the onset of much disease (Ames *et al.*, 1993).

Flavonoids found in tea show 20 times more powerful antioxidant activity. Antioxidants protect the body against the damaging effects of free radicals generated in the body during the conversion of glucose and fat to energy. Oxidative stress induced by the over production of reactive oxygen species like hydroxyl radical disrupt the cellular function. Over production of free radical occurs with smoking, environmental pollution, chronic inflammation, external action of toxic substances, microbial attacks and ozone (Diplock *et al.*, 1998 and Jackson *et al.*, 1998).

The tea leaves contain four major catechins as colourless water soluble compounds namely Epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG).Green tea has received much attention as chemo preventer for promotion and progression of various forms of tumour and EGCG with five phenolic hydroxyl groups is claimed to be the most potent tea antioxidant (Ho *et al.*, 1996). EGCG has been shown to act as inhibitor of urokinase, the enzyme crucial for tumour growth (Lin *et al.*, 1996).

In the present study an attempt was made to evaluate the antimutagenic effect of green tea extracted using four different solvents viz., petroleum ether, chloroform, ethanol and water in *Salmonella* microsome assay.

MATERIALS AND METHODS

Soxhlet Extraction

10 g of green tea powder was weighed using an electrical balance and made into 8 packets using xerohaze filter paper. Soxhlet extraction of powdered green tea was carried out to obtain its extract. Petroleum ether, chloroform, ethanol and water were used as solvents for soxhlet extraction in the increasing order of polarity. The distillation process was carried out at a low temperature of 40 ^{0}C. After evaporation of solvents; corresponding residues obtained were stored in the refrigerator for further use. 100 mg of soxhlet extract was dissolved in 2 ml of DMSO and then mixed with 100 ml distilled water and this formed 1000 ppm solution. From this stock solution, solutions of required concentrations were prepared and used in this study.

Bacterial Tester Strains

Salmonella typhimurium TA 98 and TA 100 were kindly provided by Professor Bruce N. Ames, Berkley, USA. The strains were checked routinely for amphicillin resistance, ultra violet sensitivity and spontaneous revertants.

Preparation of S9 Fraction

Swiss albino mice weighing about 25 g were obtained from animal laboratory, Food Science and Nutrition Department, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore and kept in plastic cages with husk bedding and a stainless steel lid suitable for feeding and watering. The mice were fed on by standard rodent diet pellet. Mice were injected with phenobarbitone at a dose of 1mg/g body weight intraperitoneally for 3 consecutive days. On day six, no food was provided to the mice for fasting. The mice were sacrificed on the seventh day for preparation of liver S9 fraction. All the steps were performed at 0-4 ^{0}C with cold and sterile solutions and glass wares. The liver was excised out after dissecting the animal. The excised liver was then washed in an equal volume of 0.15 M KCl. Then it was mixed in 0.14 M KCl and homogenized with a homogenizer. The homo-

genate was centrifuged for 10 min at 9000 g and the supernatant so collected was the S9 mix fraction. The freshly prepared S9 fraction was quickly frozen in dry ice and stored at -20°C.

For plate incorporation assay, top agar (2 ml) was distributed into each small test tubes held in a water bath. In different set groups of experiments in above tubes, 10ml/plate of petroleum ether, chloroform, ethanol and water extracts of green tea plus the mutagen (daunomycin at the concentration of 6ml/plate and sodium azide at the concentration of 10ml/plate) and 10ml of metabolically activated S9 mix plus 10ml of standardized bacterial cultures of TA 98 and TA 100 strains were added to the top agar and then poured into minimal glucose agar plates. The plates were then inverted and placed in an incubator at 37°C for 48 h and counted for the number of histidine revertant colonies.

Similar experiments were carried out for positive controls (taking daunomycin and sodium azide) and negative controls (untreated groups) for identifying spontaneous culture for both the strains concurrently.

Statistical analysis

The mean values of number of histidine revertants/ plate for different groups were subjected to statistical analysis using student 't' test.

RESULTS AND DISCUSSION

The present investigation depicts the antimutagenic potential of green tea extracts in *Salmonella typhimurium* reverse mutation assay (Tables 1 and 2). The number of spontaneous revertants was found to be 38 and 144 in TA 98 and TA 100 strains respectively. Addition of daunomycin and sodium azide to the minimal glucose plates resulted in significant induction in the number of histidine revertants and was found to be 61 and 184 of TA 98 and TA 100 strains respectively. However supplementation with different extracts of green tea resulted in the inhibition of induction of histidine revertant colonies either by daunomycin or sodium azide.

The percentage inhibition of daunomycin was 26%, 28%, 33% and 19% for green tea in petroleum ether, chloroform, ethanol and water extracts respectively in TA 98 strains. The percentage inhibition of different extracts of green tea towards sodium azide induced histidine reversion was found to be 8% , 9% , 10% and 5% respectively for petroleum ether, chloroform, ethanol and water extracts in TA 100 tester strains. The number of histidine revertants per plates for TA 98 and TA 100 tester strains in green tea was found to reduce 45, 44, 41 and 50 and 171, 169, 167 and 175 respectively. In the presence of S9 fraction the number of spontaneous revertants for petroleum ether, chloroform, ethanol and water extracts of green tea were found to be 106 and 203 in TA 98 and TA 100 strains respectively. In addition of daunomycin and sodium azide to the above extracts in TA 98 and TA 100 strains the number of histidine revertant were found to be increased to 155 and 359 respectively. However in plates supplemented with petroleum ether, chloroform, ehanol and water extracts of green tea resulted in inhibition of induction of histidine revertant colonies either by daunomycin or by sodium azide. The number of histidine revertants/plate of TA 98 and TA 100 tester strains of green tea were found to be reduced to 141, 134, 127 and 136 and316, 315, 311 and 338 respectively.

Table 1: Antimutagenic Effect of Petroleum Ether, Chloroform, Ethanol and Water Extract of Green tea in Salmonella Typhimurium TA 98 and TA 100 strains

Strains	Treatment	Petroleum ether	Chloroform	Ethanol	Water
TA 98	SR + GT	39.0 ±3.29^{NS}	41.0± 3.23^{NS}	40.0 ±2.79^{NS}	37.0± 5.19^{NS}
	SM + GT	45.0±2.61**(26%)	44.0±3.60**(28%)	41.0±3.88**(33%)	50.0±2.66**(19%)
TA 100	SR + GT	146.0±5.65^{NS}	143.0±9.08^{NS}	141.0±10.53^{NS}	145.0±8.18^{NS}
	SM + GT	171.0 ±4.99**(8%)	169.0±3.87**(9%)	167.0±3.37**(10%)	

Note: Results are the average of two independent experiments: Spontaneous Revertant rate of TA 98 was 38 ±4.1 and TA 100 was 144±3.7; Standard Mutation rate for TA 98 was 61±2.1 and TA 100 was 84.0 ±3.2; NS – Not Significant;**:1% Significance: per cent inhibition of revertant frequency with the addition of different extracts of green tea to standard mutagen induced plates is given in parenthesis.

Table 2: Antimutagenic Effect of Petroleum Ether, Chloroform, Ethanol And Water Extract of Green Tea in Salmonella Typhimurium TA 98 and TA 100s Strains in the Presence of S9 Fraction

Strains	Treatment	Petroleum ether	Chloroform	Ethanol	Water
TA 98	SR + GT	108.0±8.08NS	105.0±6.48 NS	104.0 ±6.55 NS	112.0± 8.60NS
	SM + GT	141.0±2.61**(9%)	134.0±6.11**(14%)	127.0±3.15**(18%)	136.0±4.05**(13%)
TA 100	SR + GT	204.0±8.12 NS	210.0±6.84 NS	208.0±5.86 NS	202.0±3.15 NS
	SM + GT	316.0 ±7.06**(12%)	315.0±5.74**(13%)	311.0±6.45**(14%)	338.0±7.25**(6%)

Note: Results are the average of two independent experiments: Spontaneous Revertant rate of TA 98 was 106 ±8.6 and TA 100 was 203±5.8; Standard Mutation rate for TA 98 was 155±4.9 and TA 100 was 359.0±5.5; NS – Not Significant; **: 1% Significance: per cent inhibition of revertant frequency with the addition of different extracts of green tea to standard mutagen induced plates is given in parenthesis.

The *Salmonella typhimurium* reverse mutation assay is most commonly used method to assess mutagenic potential of test chemicals, which may cause base pair and frame shift mutations in the genome of this organism (Maron and Ames, 1983). Its applicability in screening the antimutagenic potential of green tea has been performed in the present study. Daunomycin and sodium azide are well known genotoxicants in mammalian and microbial test systems. Addition of daunomycin and sodium azide to the minimal glucose plates resulted in the significant induction of histidine revertant colonies. In the present study addition of green tea extracts to sodium azide and daunomycin treated plates resulted in the significant inhibition of number of colonies formed in TA 98 and TA 100 strains respectively.

Earlier reports states that Catechin component including epicatechin gallate (ECG) and epigallo catechin gallete (EGCG) provide a significant protection against mutagenicity of Trp – P – 2 and the N-OH-Trp – P – 2 using *Salmonella typhimurium* TA 98 and TA 100 (Hayatsu *et al.*, 1992 and Kuroda and Hara, 1999). EGCG also have been reported to provide strong inhibitory effect against mutagenicity of Ba P diol epoxide in TA 100 strain (Hour *et al.*, 1999).Using *Salmonella typhimurium* TA 98 and TA 100 strains, the tea catechins ECG and EGCG have been shown to inhibit the mutagenic activity of direct acting mutagens (Okuda *et al.*, 1984). In another study the extracts of green tea decreased the mutagenic activity on N-methyl – N – Nitro – N – Nitroso guanidine (MNNG) in *Escherichia coli*

WP2 in a dismutagenic manner (Kuroda and Hara, 1999).

Green tea extracts were also effective against the mutagenicity of PAH Benzo (a) pyrene, DMBA with S9 activation (Kuroda and Hara, 1999). The antimutagenic effect of tea involves interaction between the reactive genotoxic species of various promutagens and polyphenolic tea component present in the tea (Kuroda and Hara, 1999). The anti genotoxic properties of tea include induction of DNA repair and binding of activated carcinogens (Yang *et al.*, 2002).

CONCLUSION

Our findings point to higher antimutagenic activity of ethanolic extracts of green tea when compared to other three extracts using petroleum ether, chloroform and water. This study throws possibility of reduction of mutagenicity and there by carcinogenicity in people drinking green tea regularly.

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Red yeast rice stimulates osteoblast proliferation and increases alkaline phosphatase activity in MC3T3-E1 cells

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Received 1 April 2010; revised 5 June 2010; accepted 17 June 2010

Abstract

Red yeast (*Monascus purpureus*) is used as a traditional hypocholesterolemic dietary food component in Asia due to its bioactive component, lovastatin. Recently, new evidence suggesting that the statins in red yeast enhance bone formation has been reported, but more research is still needed in order to support these claims of osteogenic effects. Therefore, in this study, we hypothesized that red yeast rice (in which red yeast is fermented) can improve osteogenic function through osteoblast cell proliferation and differentiation. We studied the effect of methanol extract of red yeast rice powder (RYRP) on osteoblast proliferation and differentiation by measuring mitochondrial enzyme activity and bone marker alkaline phosphatase (ALP) activity, respectively. Osteoblast-like MC3T3-E1 cells were cultured in various concentrations of RYRP methanol extract (0.001-1 mg/mL) during the osteoblast differentiation period (1, 5, 10, and 15 days). As measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay, RYRP extracts stimulated cell proliferation during a 24-hour period, compared to cooked white rice powder extract. The most pronounced effect was observed at the concentration range between 0.075 and 0.1 mg/mL. This RYRP stimulatory effect for cell proliferation was observed during the whole osteogenic period. Cellular (synthesized) ALP activity was increased at a RYRP extract concentration of 0.075 mg/mL during 15 days of culture, but the medium (secreted) ALP activity did not show any significant change. This cellular ALP activity stimulation by RYRP extract was confirmed by the staining of ALP activity on cell matrix layers for matrix calcification. The results imply that RYRP extract may increase osteogenic effect by stimulating cell proliferation and ALP activity in osteoblastic cells.

Keywords: Red yeast rice powder; *Monascus purpureus*; Osteoblast-like MC3T3-E1 cells; Proliferation; ALP activity

Abbreviations: ALP, Alkaline phosphatase; CWRP, Cooked white rice powder; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; OSM, osteogenic differentiation media; PBS, phosphate-buffered saline; Pi, inorganic phosphate; RWRP, Raw white rice powder; RYRP, Red yeast rice powder.

This work was supported by the Regional Innovation Center, the Ministry for Industry and Economics and, in part, by the Technology Development Program for Agriculture and Forestry, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.
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0271-5317/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.nutres.2010.06.011

1. Introduction

Red yeast rice (red koji), on which red yeast (*Monascus purpureus*) is fermented, is a dietary staple in Asian countries and has been used as a food preservative, food colorant and flavoring agent for many centuries [1,2]. Red yeast rice powder (RYRP) is considered a potential health and medicinal food with neutraceutical activity due to its anti-atherogenic [3,4], anti-lipogenic [5-7], and antioxidant effects [8]. These antiatherogenic and hypolipidemic effects of red yeast rice are feasible since monacolins (which are naturally occurring in red yeast rice) are hydroxymethylglutaryl-CoA reductase inhibitors [3,6] that block the activity of hydroxymethylglutaryl-CoA reductase (a key enzyme for cholesterol synthesis). Therefore, one can conclude that red yeast rice favorably affects lipid profiles of hypercholesterolemia [9,10].

Monacolin K, which is naturally present in red yeast rice and produced by cultivating the yeast strain, *Monascus purpureus*, is reported to be identical in chemical structure to Lovastatin, a drug widely used for lowering blood cholesterol [11]. The types of antihyperlipidemic agents that are present in Lovastatin were also isolated from monacolins that are strains of *Monascus*, which is the same yeast used to make red yeast rice [10]. Interestingly, several recent studies reported that statins, as a pure biocompound, enhanced bone formation in both in vitro and in vivo models. Lovastatin produced strong bone anabolic effects in rat models [12], in rats with bone repair from fractures [13], and in mice [11]. This anabolic effect of statins may be mediated through stimulating bone morphogenic protein 2 expression and synthesis in bone cells [14]. Simvastatin also showed this stimulatory effect on bone formation in rats [11]. The stimulatory effect of simvastatin on bone formation was also shown in vitro using osteoblastic MC3T3-E1 cells [15], human bone marrow stromal cells [16], HOS-NY cells, and human osteosarcoma cell lines [14]. Ohnaka et al [17] reported the stimulatory effect of statin on osteocalcin mRNA levels in a primary culture of human osteoblasts prepared from femur bone fragments. All of these studies suggest that statin, as a pure biocompound, can be an active agent for promoting bone formation.

Despite the accumulating evidence of statin's effect on bone formation as single biocompound, only a few reports concerning the effect of red yeast rice, in which statins are present by Monacolin K fermentation, on bone formation have been reported so far. Gutierrez et al [18] confirmed that a few strains of red yeast rice methanolic extract included statins as the active components, and they showed increased bone mineral density in rat femur after consuming red yeast rice methanolic extract. Red yeast rice methanolic extract also increased new bone formation in the cranial defects in the parietal bones in rabbits [19].

Some evidence for statins in red yeast to enhance bone formation is reported, but the stimulatory osteogenic effect of red yeast rice as a food component is very limited, especially in osteoblastic cell models. More confirmation is required for clarifying the osteogenic effect induced by red yeast rice. Bone is continuously remodeled through a repeated cycle of resorption and formation controlled by osteoclasts and osteoblasts, respectively. Therefore, osteoblast proliferation and differentiation which mature the extracellular matrix and, eventually, matrix calcification are critical factors for osteogenesis [20].

In this study, we hypothesized that red yeast rice in which red yeast is fermented can improve osteogenic function through osteoblast proliferation and differentiation. To test this hypothesis, we determined whether the extract of RYRP increased cell proliferation and osteoblast differentiation by using mouse calvaria origin osteoblastic MC3T3-E1 cells and measuring mitochondrial enzyme activity and alkaline phosphatase (ALP). Alkaline phosphatase is one of the most representative osteoblast differentiation marker proteins and has a stimulatory effect for matrix calcification in bone tissue [21,22]. We believe that these study results can be used to support the idea that red yeast rice effects bone formation.

2. Methods and materials

2.1. Preparation of red yeast rice

Red yeast rice was prepared by culturing the fungus, *Monascus purpureus* (which is red in color), on white rice. The red yeast rice was then dried and crushed in a

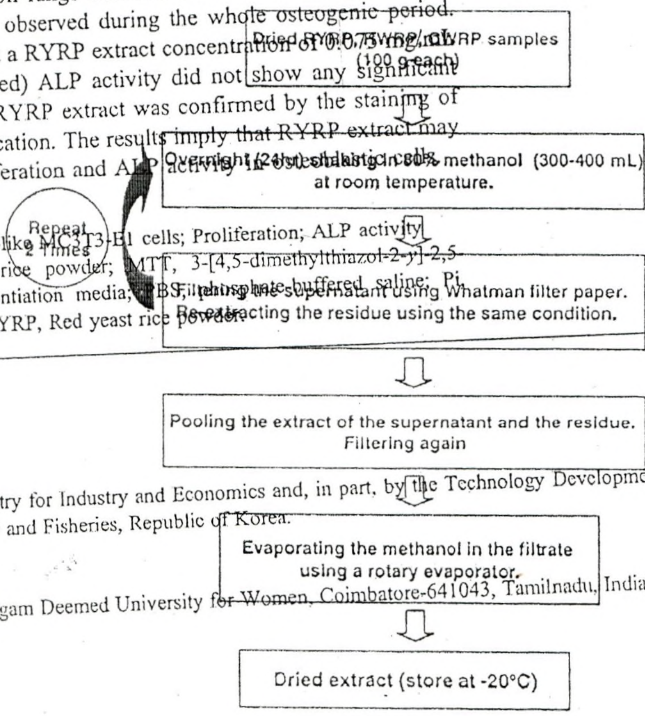

Fig. 1. The method of extraction of RYRP, RWRP and CWRP samples.