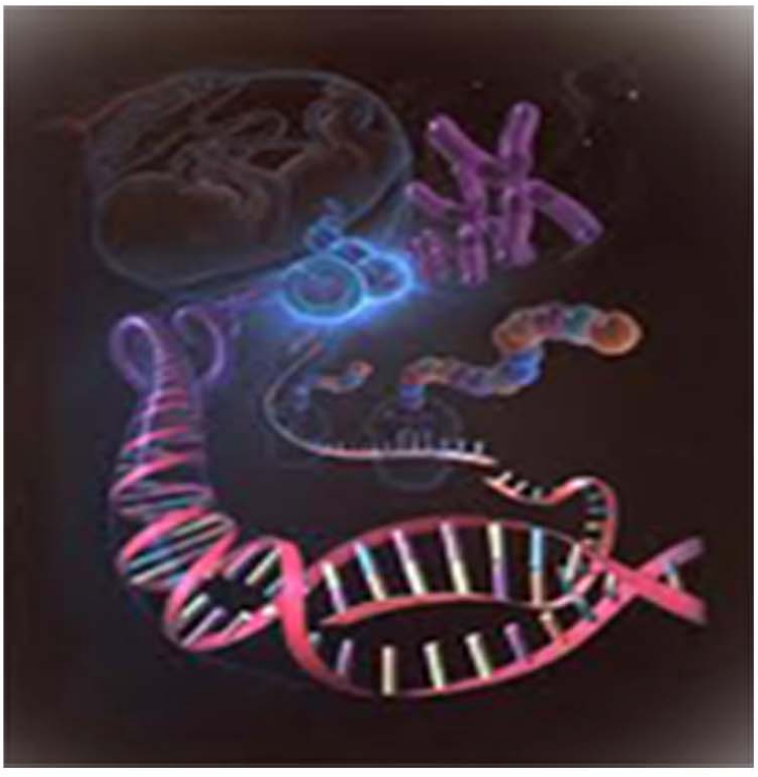
IJLBPR

ISSN 2250 - 3137
Vo1. 2, No. 3, July 2013

International Journal of

Life Sciences Biotechnology and Pharma Research



International Journal of Life Sciences Biotechnology and Pharma Research
Hyderabad, INDIA

www.ijlbpr.com

Email : editorijlbpr@gmail.com or editor@ijlbpr.com

International Journal of Life Sciences Biotechnology and Pharma Research

ISSN 2250-3137 www.ijlbpr.com
Vol. 2, No. 3, July 2013

Research Paper

EVALUATION OF ANTIMUTAGENIC ACTIVITY OF ORANGE PEEL EXTRACT USING AMES SALMONELLA MICROSOME ASSAY

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In the present investigation, antimutagenic effect of petroleum ether, chloroform, methanol and water extracts of orange peel 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 10 µl and 6 µl 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 orange peel to 10 µl of sodium azide and 6 µl of daunomycin treated plates resulted in the inhibition of number of histidine revertant colonies. Furthermore, supplementation with all the four extracts of orange peel 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 orange peel was found to be higher than that of the other extracts. Hence the study revealed that orange peel has protective efficacy in sodium azide and daunomycin induced mutagenicity in the test microbial system.

Keywords: Orange peel, Antimutagenic activity, Ames assay, Salmonella typhimurium

INTRODUCTION

Diet rich in fruits and vegetables are associated with a decreased risk of cancer and many chronic diseases. Citrus fruits are have long been valued as part of a nutritious and tasty diet. The flavors provided by citrus are among the most preferred in the world, and it is increasingly evident that citrus not only tastes good, but it is also good for people.

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, (McCord, 1994; and Adegoke, 1998) which are involved in the onset of much disease (Ames *et al.*, 1993). These free radicals attack the unsaturated fatty acids of bio membranes which results in lipid peroxidation and the destruction of proteins and DNA, which causes

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a series of deteriorative changes in the biological system leading to cell inactivation. Thus the identification of anti oxidants, which can retard the process of lipid peroxidation by blocking the generation of free radical chain reaction, has importance in recent years.

Analysis of fruits demonstrated that citrus fruits contained very high concentrations of antioxidants. Antioxidants in dietary plants may explain all of the protective effects against oxidative stress related chronic diseases. Antioxidants protect the body against damaging effects of free radicals generated in the body during conversion of glucose and fat to energy, exercise and by the action of sunlight on the skin. It protects cell membranes and DNA from oxidative damage.

Flavanoids, a broad class of polyphenolic compounds present in citrus fruits are strong antioxidants with significant anticancer and anti cardiovascular disease activity. They scavenge free radicals by forming a stable radical that can react with another flavonoid radical to produce two non radicals.

During fruit maturation, flavonoid levels have been reported to increase, decrease or fluctuate. Peel flavonoids form a part of the resistance mechanisms in citrus, possessing antimicrobial and antiviral activity and protecting peel by absorbing UV radiation.

There are four classes of flavonoids in citrus fruits. Flavonones, flavones, flavols and anthocyanins. The predominance of flavones and flavanones is a distinctive characteristic of citrus fruits. More than 60 different flavonoids have been identified in citrus species.

Oxidative damage to DNA promotes mutations and thus enhancing the risk of carcinogenesis. Damage to DNA is likely to be a major cause of cancer and other diseases (Ames, 1971). The salmonella microsome assay (Ames *et al.*, 1975) along with other short term assays always being extensively used to survey a variety of substances in our environment for antimutagenic effects. Homogenates of rat liver (or other mammalian tissue) is added to the bacterial suspension as an approximation of mammalian metabolism (Ames *et al.*, 1975). Using this system several antimutagens were detected. Hence the present investigation has been conducted to evaluate the antimutagenic effect of orange peel extracted using four different solvents viz., petroleum ether, chloroform, ethanol and water in Salmonella microsome assay.

MATERIALS AND METHODS

Soxhlet Extraction

10 g of orange peel powder was weighed using an electrical balance and made into 8 packets using xerohaze filter paper. Soxhlet extraction of powdered orange peel 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 °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 1 mg/kg 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 °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 homogenate was centrifuged for 10 minutes 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, 10 ml/plate of petroleum ether, chloroform, ethanol and water extracts of orange peel plus the mutagen (daunomycin at the concentration of 6 ml/plate and sodium azide at the concentration of 10 ml/plate) and 10 ml of metabolically activated S9 mix plus 10 ml 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 hours 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

The present investigation depicts the antimutagenic potential of orange peel extracts in Salmonella typhimurium reverse mutation assay (Tables 1 and 2). The number of spontaneous revertants was found to be 39 and 145 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 62 and 185 of TA 98 and TA 100 strains respectively. However supplementation with different extracts of orange peel resulted in the inhibition of induction of histidine revertant colonies either by daunomycin or sodium azide.

The percentage inhibition of daunomycin was 23%, 18%, 27.9% and 6.6% for orange peel in petroleum ether, chloroform, ethanol and water extracts respectively in TA 98 strains. The percentage inhibition of different extracts of orange peel towards sodium azide induced histidine reversion was found to be 13%, 14.7%, 14.2% and 3.3% respectively for petroleum ether, chloroform, ethanol and water extracts in TA 100 tester strains. The number of histidine revertants

Table 1: Antimutagenic Effect of Petroleum Ether, Chloroform, Ethanol and Water Extract of Orange Peel in Salmonella Typhimurium TA 98 and TA 100 Strains

Strains	Treatment	Petroleum Ether	Chloroform	Ethanol	Water
TA 98	SR + OP^{NS}	40 ± 3.8 ^{NS}	40 ± 4.5^{NS}	36 ± 4.6^{NS}	37 ± 3.5^{NS}
	SM + OP	50 ± 2.3** (23%)	44 ± 4.1** (18%)	57 ± 4.8** (27.9%)	47 ± 6.5^{NS} (6.6%)
TA 100	SR + OP^{NS}	146 ± 7.2^{NS}	148 ± 6.1^{NS}	143 ± 3.7^{NS}	146 ± 4.1^{NS}
	SM + OP**	160 ± 4.7** (13%)	157 ± 9.6** (14.7%)	158 ± 7.8** (14.2%)	178 ± 4.5** (3.3%)

Note: Results are the average of two independent experiments: spontaneous revertant rate of TA 98 was 39 ± 4.1 and TA 100 was 145 ± 3.7; standard mutation rate for TA 98 was 62 ± 2.1 and TA 100 was 185.0 ± 3.2; NS – Not Significant; **: 1% significance: per cent inhibition of revertant frequency with the addition of different extracts of orange peel to standard mutagen induced plates is given in parenthesis.

Table 2: Antimutagenic Effect of Petroleum Ether, Chloroform, Ethanol and Water Extract of Orange Peel 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 + OP	105 ± 8.7^{NS}	105 ± 8.5^{NS}	107 ± 6.2^{NS}	107 ± 11.3^{NS}
	SM + OP**	130 ± 2.9** (16.2%)	135 ± 5.2** (13%)	112 ± 5.9** (27.8%)	144 ± 4.6** (7.2%)
TA 100	SR + OP	207 ± 6.3^{NS}	208 ± 8.1^{NS}	210 ± 3.7**	205 ± 8.0^{NS}
	SM + OP	320 ± 8.8** (10.9%)	322 ± 10.8** (10.4%)	309 ± 7.09** (14%)	337 ± 7.6** (6.2%)

Note: Results are the average of two independent experiments: spontaneous revertant rate of TA 98 was 107 ± 8.6 and TA 100 was 204 ± 5.8; standard mutation rate for TA 98 was 156 ± 4.9 and TA 100 was 360.0 ± 5.5; NS – Not Significant; **: 1% significance: per cent inhibition of revertant frequency with the addition of different extracts of orange peel to standard mutagen induced plates is given in parenthesis.

per plates for TA 98 and TA 100 tester strains in orange peel was found to reduce 50, 44, 57 and 47 and 160, 157, 158 and 178 respectively. In the presence of S9 fraction the number of spontaneous revertants for petroleum ether, chloroform, ethanol and water extracts of orange peel were found to be 107 and 204 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 156 and 360 respectively. However in plates supplemented with petroleum ether, chloroform, ethanol and water extracts of orange peel 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 orange peel were found to be reduced to 130, 135, 112 and 144 and 320, 322, 309 and 337 respectively.

DISCUSSION

Recent studies shows that antioxidant fractions of Citrus sinensis (Blood orange) shows variable radical scavenging activity and establish the antioxidant potency of citrus fruit extracts. In the present study addition of orange peel extracts to the Sodium azide and Daunomycin treated plates resulted in the significant inhibition of number of colonies formed in TA 100 and TA 98 respectively. The present study falls in line with the studies conducted by Bala and Grover (1989). They studied the antimutagenicity of some citrus fruits in Salmonella typhimurium. They evaluated the

antimutagenic effect of 10 citrus fruit juices against the mutagenicity of N-Nitro-o-phenylenediamine (NPD) in TA 97 and sodium azide in TA 100 tester strains of salmonella using Ames test. It was noticed that the juices of all these fruits reduced significantly the NPD and sodium azide induced revertant colonies.

Similar studies were carried out by Jayaprakasha *et al.* (2006). They studied the antimutagenicity of hexane and chloroform extracts from the fruit rinds of Garcinia pedunculata against the mutagenicity of direct acting mutagen sodium azide by the Ames test. Both the extracts showed strong antimutagenicity at or above 1250 µg/plate in the tester strains of Salmonella typhimurium (TA 100 and TA 1535). However the hexane extract showed higher antimutagenic potential than the chloroform extract. The present study is also supported by the observation of Kazimierz *et al.* (1997) who isolated anthocyanins from fruits of Aronia melanocarpa which markedly inhibited the mutagenic activity of benzo-a pyrene and 2-amino fluorine in the Ames test. This investigation corroborates with the study of Calle and Sullivan (1982) who screened antioxidants and other compounds for antimutagenic properties towards benzo-a-pyrene induced mutagenicity in strain TA 98 of Salmonella typhimurium and reported strong antimutagenic activity of Vitamin A towards Benzo-a-pyrene.

Similar studies were conducted by Raphael *et al.* (2002) who observed the antimutagenic activity of methanolic extract of Phylanthus amarus in Salmonella typhimurium strains TA 1535, TA 100 and TA 102 (Ames test) and reported the inhibitory effect of methanolic extract towards 2-acetamino fluorine and aflatoxin B1 at concentrations of 0.25-2 mg/plate.

Most carcinogens are inactive when present in the environment, upon entering the system, they are converted into active metabolites by the carcinogen metabolizing enzymes, Ames *et al.* (1975) have improvised a method for detecting chemicals which are potential human carcinogens or mutagens by adding homogenates of rat liver directly to the Petri plates thus incorporating an important aspect of mammalian metabolism into the in vitro test. In the present study antimutagenicity of orange peel were studied by adding homogenates of rat liver to the Petri plates along with the extracts to understand the important aspects of mammalian metabolism in the in vitro testing. Orange peel extracts when subjected to antimutagenicity test in the presence of S9 fraction showed inhibition of mutagenicity.Similar studies were carried out by Pina *et al.* (1998) who used the Salmonella micro suspension assay to examine the antimutagenicity of ellagic acid (major constituent in Pomegranate) against the potent mutagen aflatoxin B1 using tester strains TA 98 and TA 100. They reported that ellagic acid as significantly inhibited the mutagenicity of all AFB1 doses in both tester strains with the addition of S9 fraction.

According to Silalahi (2000) a high intake of citrus fruits may reduce the risk of degenerative diseases. This protective effect is assumed to be associated mainly with the antioxidant activities of bio active components present in citrus fruits such as Vitamin C, beta carotene, flavonoids and limnoids.

CONCLUSION

Our findings point to higher antimutagenic activity of ethanolic extracts of orange peel 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 consuming orange regularly.

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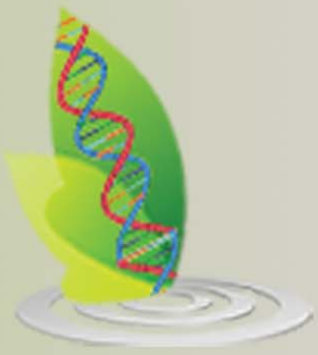

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ISSN 2250-3137