ORIGINAL ARTICLE

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Rec. Nat. Prod. 3:1 (2009) 68-75

records of natural products

Antigenotoxic Activity of Polyphenolic Rich Extracts from *Aegle marmelos* (L.) Correa in Human Blood Lymphocytes and E.coli PQ 37

Prabhjit Kaur[1], Amandeep Walia[1], Subodh Kumar[2] and Satwinderjeet Kaur[1*]

[1]*Department of Botanical & Environmental Sciences, Guru Nanak Dev University, Amritsar-143005 (Punjab) India*

[2]*Department of Chemistry Guru Nanak Dev University, Amritsar-143005 (Punjab) India*

(Received July 1, 2008; Revised December 6, 2008; Accepted December 12, 2008)

Abstract: The present paper deals with the antigenotoxic activity of *Aegle marmelos* fruit extracts employing short term assays i.e. the SOS chromotest using *Escherichia coli* PQ37 and the Comet assay in peripheral human blood lymphocytes. Methanol extract and Acetone extract were quite effective in decreasing the SOS response induced by hydrogen peroxide and aflatoxin B1 in the SOS chromotest. Methanol extract inhibited the genotoxicity of H_2O_2 by 70.48% and that of AFB1 by 84.65%. The extracts showed significant decrease in the tail moment induced by hydrogen peroxide (9µM) in the Single Cell Gel Electrophoresis (SCGE) assay. The antigenotoxic activity exhibited by the extracts may be attributed the various polyphenolic constituents present in these extracts.

Keywords: *Aegle marmelos;* SOS chromotest; antigenotoxic activity, Comet assay

1. Introduction

The dietary constituents have received much attention among researchers as chemopreventive agents. One of the ways by which dietary ingredients provide protection is by destroying certain reactive oxidative species (ROS) that damage DNA and initiate diseases as cancer. Natural antioxidants such as polyphenolic compounds are found to play protective roles against many human chronic diseases which are associated with oxidative stress. Epidemiological studies showed that a high intake of antioxidants rich foods is inversely related to cancer risk [1]. Among the possible causes of cancer, damage to DNA and other cellular molecules by reactive oxygen species ranks high as a major culprit in the onset and development of diseases. These by-products of normal metabolism; which increase in inflammation and in exposure to exogenous sources including nitrogen oxide pollutants, smoking, certain drugs and radiations, can induce cancer causing mutations oxidize lipids,

[*] Corresponding author: E-Mail: sjkaur@rediffmail.com ; sjkaur2001@yahoo.co.in ; Phone: 91-183-2259732, 2451048. Fax: 91-183-2258819, 20

The article was published by Academy of Chemistry of Globe Publications
www.acgpubs.org/RNP © Published 01/26/2009 EISSN:1307-6167

proteins and alter signal transduction pathways that enhance cancer risk [2,3]. The implication of oxidative stress in the etiology and progression of several acute and chronic clinical disorders has led to the suggestion that the antioxidants can have health benefits as prophylactic agents.

Since ancient times several diseases have been treated by administration of plant extracts based on traditional medicine [4]. The tree *Aegle marmelos* (Rutaceae) commonly known as Bael is indigenous to India and found wild all over the Sub-Himalayan forests, in Central, and South India. It is a rich source of coumarins, vitamin C, and riboflavin. The bark as well as fruit is reputed to be a valuable Ayurvedic medicine for dysentery and various intestinal complaints [5,6]. It possesses potent microfilarial [7], radioprotective [8], analgesic [9], antihyperglycemic, antidyslipidemic [10], anticancer, [11, 12] and antidiabetic activity [13,14]. As *in vitro* antigenotoxicity/antimutagenicity studies may be useful for discovering chemopreventive phytochemicals, the present study was planned to investigate antigenotoxic activity of *A. marmelos* fruits extracted with different organic solvents against oxidative stress induced DNA damage using *E.coli* PQ37 in the SOS chromotest and by using human blood lymphocytes in the Comet assay. The polyphenolic content of each extract was also measured to estimate the relationship between antigenotoxic activity and polyphenolic content.

2. Materials and Methods

2.1. Chemicals and Plant material

Normal melting point agarose (NMPA), low melting point agarose (LMPA), ethidium bromide, ortho-nitrophenyl β-D-galactopyranoside (ONPG), Nicotinamide adenine dinucleotide phosphate, (NADP) and Glucose-6-phosphate (G6P) were purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India. Histopaque 1077, Aflatoxin B1 from Sigma Chemicals (St Louis, MO, USA). Para-nitrophenylphosphate (PNPP) were procured from Sisco Research Laboratories Pvt. Ltd., Mumbai, India; polyethyleneglycol-4-tetraoctylphenolether (Triton X-100), hydrogen peroxide, dimethyl sulphoxide from Qualigens Fine Chemicals, Mumbai, India. All the chemicals used were of analytical grade. *Escherichia coli* PQ37 strain was purchased from Institut Pasteur, France. The fruits of *Aegle marmelos* were purchased from local market at Amritsar, India. A voucher specimen has been kept in the herbarium of Departmental of Botanical and Environmental Sciences for future reference.

2.2. Preparation of Plant Extracts

The dried fruits were crushed to coarse powder. The powder was macerated with acetone, methanol, and distilled water separately for 48 h with continuous stirring. They were then filtered and dried using a rotary evaporator to obtain acetone extract, methanol extract, and water extract respectively.

2.3. Total Phenolic Content (TPC)

TPC of all the extracts of *Aegle marmelos* (fruits) were analysed by Folin-Denis colorimetric method [15] using Tannic acid as a standard. The total phenolic content was expressed as tannic acid equivalents (mg/100 g of fruits).

2.4. SOS Chromotest

Inhibition of bacterial genotoxicity was tested in *Escherichia coli* PQ37 by the SOS chromotest [16]. The hydrogen peroxide (without exogenous metabolic activation) and Aflatoxin B1 (with exogenous metabolic activation) were used to induce SOSIP. The extracts were also tested alone (without mutagens) in order to check the genotoxicity. The Induction factor (IF) was calculated as the

ratio of Rc/Ro where Rc is equal to β-galactosidase activity/alkaline phosphatase activity determined for the test compound at concentration c, and Ro is equal to β-galactosidase activity / alkaline phosphatase activity in the absence of test compound. Anti-genotoxicity was expressed as percentage inhibition of genotoxicity according to the formula:

$$\text{Inhibition (\%)} = 100 - (IF_1 - IF_0/IF_2 - IF_0) \times 100$$

where
IF_1 is the induction factor in the presence of the test compound and mutagen,
IF_2 the induction factor in the absence of the test compound (only mutagen) and
IF_0 is the induction factor of the blank.
Data was collected (Mean ± Standard Error) of triplicate experiments.

2.5. Single cell gel electrophoresis assay (Comet assay)

The alkaline comet assay was performed by using human blood lymphocytes [17]. Blood samples were obtained by healthy male donor aged 25-40 years. Lymphocytes were isolated [18] and their viability was determined [19]. Lymphocytes (2×10^6 cells/ml) suspended in 1 ml phosphate buffer saline (PBS), were incubated for 30 min at 37 ^{0}C with 20 µl of mutagen, hydrogen peroxide (9 µM) in the presence 20 µl of different concentrations of extracts. Each test compound/mutagen combination was tested thrice in each experiment along with positive controls.

To evaluate the extent of DNA damage, images of 100 randomly selected cells were analysed from each sample using an Epifluorescent Nikon microscope connected with a digital camera. Imaging was performed by using a computerized image analysis system (Lucia Comet Assay Software 4.8 of Laboratory Imaging Ltd.) which acquires images, computes the integrated intensity profile for each cell, estimates the comet cell components (head and tail) and evaluates a range of derived parameters. These include tail length, % DNA in migrated in comet tail and tail moment (an index of DNA damage which considers both tail length & the fraction of DNA in comet tail). To check for toxicity or an effect on DNA, all the extracts were tested alone (without mutagen).

The percentage inhibition was calculated by the formula:

$$\text{Inhibition (\%)} = (T_1 - T_C) / (T_1 - T_0) \times 100$$

Where
T_1 = Tail moment indued by H_2O_2
T_C = Tail moment of extracts in presence H_2O_2
T_0 = Tail moment of negative control

2.6. Statistical Analysis

The results are presented as the average and standard error of triplicate experiments. The data in all the experiments were analyzed for statistical significance using analysis of variance (one-way ANOVA). The difference among average values was compared by high-range statistical domain (HSD) using Tukey's Test [20]. The statistical significant was checked at $p<0.05$.

3. Results and discussion

The hallmark of oxidative stress is increased production of reactive oxygen species (ROS) in amounts that exceed cellular antioxidant defenses. The consequence of oxidative stress may result in

the oxidative damage to lipids, proteins, and DNA with subsequent disease development. DNA damage plays a very important role in carcinogenesis and any agent which is capable of chemically modifying DNA could be carcinogenic. Hydroxyl radicals attack upon DNA generating a whole series of modified purine and pyrimidine bases, many of which are known to be mutagenic. Attack of ˙OH upon deoxyribose also yields a multiplicity of products [21, 22]. A possible mechanism for the protective effects of fruits and vegetables with respect to disease is that bioactive compounds in these reduce oxidative stress by scavenging the ROS. In the SOS chromotest (Table 1,2) the extracts from *Aegle marmelos* fruits were effective in reducing the IF induced by H_2O_2, a direct acting mutagen as well as that of Aflatoxin B1, a metabolically activated mutagen. At the highest concentration of 200 µg/assay, methanol extract showed significant effect in modulating the DNA damaging effect of both H_2O_2 and Aflatoxin B1. The extract decreased the SOSIP (SOS inducing potency) of H_2O_2 by 70.48% and that of Aflatoxin B1 by 84.65%. Acetone extract also showed inhibition of IF induced by H_2O_2 by 58.78%. The genotoxicity induced by S9 dependent AFB1 was reduced by 71.46 %. Water extract showed moderate antigenotoxic activity. The SOSIP induced by H_2O_2 and AFB1 were inhibited by 50.89% and 58.94% respectively. All the concentrations of methanol, acetone and water extracts were not toxic, as the IF ≤ 1.5. The compounds are classified as non-genotoxic if the IF remains < 1.5 and genotoxic if IF exceeds 2.0 [23].

Table 1. Effect of extracts of *Aegle marmelos* on the genotoxicity induced by Hydrogen peroxide (without metabolic activation) in SOS chromotest using *E.coli* PQ37 as a tester stain.

Dose		β-gal units	AP units	IF	Inhibition of genotoxicity (%)
Positive control	H_2O_2 (1.0 mM)	4.183 ± 0.08	10.86 ± 0.08	8.953	-
	0 [a]	0.471 ± 0.01	10.94 ± 0.05	1	-
Acetone extract (µg/assay)	50	4.069 ± 0.02	11.44 ± 0.03	8.255	8.78
	100	3.705 ± 0.03	11.44 ± 0.06	7.511*	18.14
	150	2.440 ± 0.01	11.47 ± 0.06	4.930*	50.59
	200	2.107 ± 0.02	11.45 ± 0.05	4.279*	58.78
Methanol extract (µg/assay)	50	3.819 ± 0.05	11.69 ± 0.01	7.581*	17.26
	100	2.670 ± 0.07	11.67 ± 0.09	5.302*	45.91
	150	2.129 ± 0.05	11.62 ± 0.05	4.255*	59.08
	200	1.675 ± 0.03	11.60 ± 0.07	3.348*	70.48
Water extract (µg/assay)	50	3.175 ± 0.03	9.58 ± 0.05	7.697*	15.80
	100	2.671 ± 0.02	9.60± 0.07	6.465*	28.96
	150	2.507 ± 0.02	9.65 ± 0.05	6.023*	36.85
	200	2.042 ± 0.04	9.66 ± 0.03	4.906*	50.89

[a] Negative Control (without any test sample).

Data represents Mean ± S.E. of β-galactosidase (β-gal) units and alkaline phoshatase (AP) units of triplicate experiments.

Induction Factor ($IF=R_C/R_0$).

* $p < 0.05$ by comparison with hydrogen peroxide (H_2O_2).

Table 2. Effect of extracts of *Aegle marmelos* on the genotoxicity induced by Aflatoxin B1 (with metabolic activation) in SOS chromotest using *E.coli* PQ37 as a tester stain.

Dose		β-gal units	AP units	IF	Inhibition of genotoxicity (%)
Positive control	AFB1 (10µg/assay)	6.110 ± 0.06	11.10 ± 0.02	10.18	-
	0 [a]	0.720 ± 0.03	13.13 ± 0.01	1.00	-
Acetone extract (µg/assay)	50	5.662 ± 0.01	13.09 ± 0.04	8.00*	23.75
	100	4.694 ± 0.02	12.09 ± 0.05	7.18*	32.68
	150	3.110 ± 0.02	13.00 ± 0.03	4.43*	63.73
	200	2.359 ± 0.01	12.15± 0.03	3.62*	71.46
Methanol extract (µg/assay)	50	4.665 ± 0.03	11.90± 0.02	7.25*	31.92
	100	3.190 ± 0.02	12.76 ± 0.01	4.62*	60.57
	150	2.000 ± 0.03	12.10 ± 0.01	3.06*	77.56
	200	1.621 ± 0.01	12.44 ± 0.02	2.41*	84.65
Water extract (µg/assay)	50	6.109 ± 0.04	13.00 ± 0.04	8.70*	16.13
	100	5.420 ± 0.01	12.96 ± 0.07	7.74*	26.58
	150	4.109 ± 0.03	13.00 ± 0.06	5.85*	47.17
	200	3.226 ± 0.02	12.50 ± 0.02	4.70*	58.94

[a] Blank (without any test sample).
Data represents Mean ± S.E of β-galactosidase (β-gal) units and alkaline phoshatase (AP) units of triplicate experiments.
Induction Factor ($IF=R_C/R_0$).
* $p < 0.05$ by comparison with Aflatoxin B1 (AFB1).

In the comet assay as well, the extracts alone did not cause any genotoxicity in the tested dose range (5-100µg/ml). The DNA damage induced by H_2O_2 was reduced by methanol extract by 75.34% as measured by the decrease in tail moment. Acetone extract and water extract decreased the tail moment induced by H_2O_2 moderately. The antigenotoxic activity was observed to be 58.72% and 51.80% respectively (Fig. 3).

Table 3. Percentage yield and total phenolics present in various extracts of *Aegle marmelos*.

Extract	Yield (g/100g of fruits)	Phenolics as tannic acid equivalents (mg/100g of fruits)
Acetone extract	2.5	1841.20
Methanol extract	4	3288.56
Water extract	14	1508.92

The antigenotoxic activity of the extracts of *A.marmelos* may be attributed to the presence of polyphenolic constituents in these extracts. As measured by Folin-Denis method, methanol extract showed total phenolic content 3288.56 mg as tannic acid equivalents/100g of fruits, followed by Acetone extract 1841.20 mg and water extract 1508.92 mg (Table 3). The high activity of methanol extract in comparison to acetone extract and water extract may be associated to the concentration of polyphenols in these extracts. Various studies have established a positive correlation between the

antioxidant activity and total phenolic content [24-30]. The bark of *Aegle marmelos* has been reported to possess nitric oxide (NO) quenching capacity due to the high phenolic content [31]. The extracts of *A. marmelos* have been reported to inhibit the interactions between nuclear factor and specific target DNA sequences [32]. The fruit extracts of *A. marmelos* have also been shown to possess the antioxidant and hepatoprotective activity against CCl_4-induced hepatic oxidative stress, toxicity, tumor promotion in Wistar rats [33]. Pretreatment with hydroalcoholic fruit extract of *A.marmelos* protected mice against the gastrointestinal as well as bone marrow deaths induced by γ radiations [34].

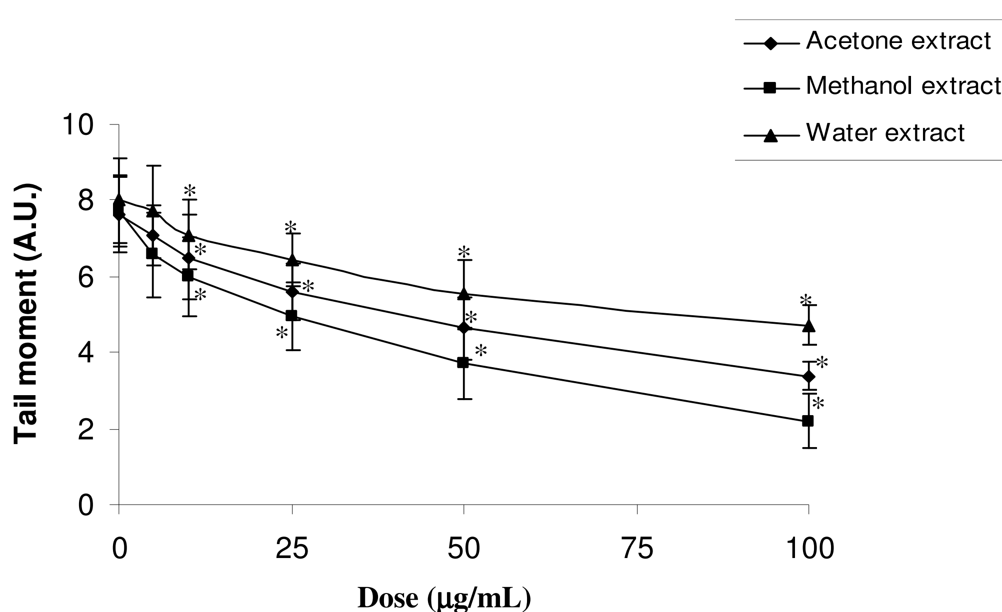

Figure 1. Decrease in tail moment by acetone extract, methanol extract and water extract of *Aegle marmelos* against the DNA damage induced by H_2O_2 (9 µM) in comet assay. Data showing the Mean ± SE of triplicate experiments.*$p < 0.05$ by comparison with hydrogen peroxide (H_2O_2).

Phenolics such as quercetin and myricetin have been reported to inhibit H_2O_2-induced DNA damage in human peripheral blood lymphocytes as evaluated by comet assay [35]. Black tea polyphenols possessed marked antigenotoxic potential against hetrocyclic amine-induced DNA damage in human lymphocytes. The antioxidant activity of the fraction was attributed to the hydrogen donating ability of phenolics present in extracts [36]. Methanol and Acetone extract also effectively suppressed the genotoxicity of AFB1 an S9 dependent mutagen. The marked inhibitory effect against AFB1 may be due to inhibition of activity of cytochrome P450 dependent enzymes involved in the activation of AFB1. Various reports showed that polyphenolic rich extracts can reduce the activity of enzymes involved in AFB1 metabolism. Extracts from *Rhamnus alaternus* are rich in polyphenolic compounds, which have been shown to inhibit the Induction factor induced by AFB1 in SOS chromotest [37]. Myricetin-3-o-galactoside and myricetin-3-o-rhamnoside isolated from the leaves of *Myrtus communis* have been reported to reduce the IF induced by the AFB1 [38].

The present results eventually lead us to conclude that antigenotoxic activity exhibited by the extracts of *A.marmelos* may be attributed in part to the polyphenolic constituents, which possess the potential to protect DNA from reactive oxygen species and S9 dependent mutagens. Further studies

are required to isolate these constituents and decipher their mode of action for their eventual application in cancer chemoprevention.

Acknowledgements

The financial assistance from the Department of Science and Technology, New Delhi is thankfully acknowledged.

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