AMELIORATIVE EFFECT OF OREGANO LEAVES EXTRACT ON ASPARTAME TOXICITY BY WHOLE BLOOD CULTURE AND PBMC ASSAY, IN VITRO

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ABSTRACT

Aspartame is the most widely used artificial sweetener worldwide. Used in over 6000 products in almost 200 countries it is utilized as low calorie sweetener acting as replacement of sugar in diet supplements. Approved by FDA in 1984 with acceptable daily intake of 40mg/kg body weight, there is still concern over its toxic effects as it is known to cause several neurological and behavioral disturbances. Over the years studies have been done to study its toxic effects as lately consumption of aspartame have increased in and as dietary enhancements. In present study acute effect of aspartame on blood cells in vitro were studied at 2mM, 4mM and 10mM concentrations and oregano leaves extract were used as antidote for amelioration. Various Oxidative stress enzymatic and non enzymatic parameters were studied. Catalase, SOD, protein thiol, reduced glutathione and Protein carbonyl showed decrease in activity and GST and GPx activity increased. Oregano administration ameliorated aspartame treatment, with significant changes (p<0.01) in catalase, SOD, GPx and GST activities in WB and PBMC assays. Reduced glutathione and protein carbonyl showed non significant (p<0.05) changes with negative percentage changes.

KEYWORDS: aspartame, acute toxicity, oregano, oxidative enzymes, protein carbonyl.

INTRODUCTION

India has about 65 million diabetics and 77 million pre-diabetics and by 2030 as predicted the number will be massive. Due to change in lifestyles, even teenagers are diabetics. With growing awareness for diet, obesity and other health problems, there is concern in individuals
for attraction towards low calorie sweeteners. Hence increasing the market of artificial sweeteners and substitutes thereby by more consumption of seconds most consumed sweetener aspartame.

A dipeptide methyl ester, Aspartame L-α-aspartyl-L-phenylalanine methyl ester (C_{14}H_{18}N_{2}O_{5}) was approved by Food and Drug Administration (FDA) around 40 years ago and is consumed in more than thousand of products by hundreds of millions of people around the globe enjoying sweet sugary taste without the calories. With molecular weight of 294.3gm/mol, it is 200 times more sweet then sucrose whereas saccharin and cyclamate are 300 and 30 times sweeter, respectively.[31]

Aspartame is absorbed in the intestinal lumen and hydrolyzed by intestinal mucosal cells into its components phenylalanine, aspartic acid and methanol. These components upon absorption are are metabolized, utilized and excreted by the body in the same pathways as when they are derived from any other nutritional source.[4]

Approximately, 50% of the aspartame molecule is phenylalanine, 40% is aspartic acid, and 10% is methanol. Among the metabolites, methanol, which is released during aspartame digestion, is a toxicant that causes systemic toxicity.[2]

Large doses of aspartame and its individual metabolites have been tested in humans and other animals before its launch in the market in 1983 and even after that.[5] Methanol, after absorption converts into formate which can be excreted, or into formaldehyde, diketopiperazine (a carcinogen) and a number of other highly toxic derivatives.[11] Also, methanol intoxication is associated with mitochondrial damage and increased microsomal proliferation resulting in increased production of oxygen radicals. These factors together with the excess of formaldehyde, formed during acute methanol intoxication cause significant increase in lipid peroxidation.[12] Inspite of being accepted at Acceptable Daily Intake(ADI) of 40 mg/kg body weight aspartame is proven toxic even at 20mg/kg body weight.[33]

In this report, acute effect of aspartame was studied in blood derived from healthy individuals and then treated with Origanum Vulgare L. leaf extract. Scavenging of different types of reactive oxygen and nitrogen species, mostly free radicals, is thought to be one of the main mechanisms of the antioxidant action exhibited by phenolic phytochemicals. In the assessment of radical-scavenging properties, biologically-relevant free radicals are used.[7]
Commonly known as oregano, it is widely used in types of spices, fragrance, medicinal as well as ornamental plants in form of oil, seeds or extracts. Origanum vulgare also consists of flavonoids, galangin, vitamins, minerals, quercetin, phenolic, flavonoid and high antioxidants. And Blood because whenever substances are transported in the body, it is due to blood, lymph or urine. Every time after uptake of any substance in an organism its circulated through blood only.

MATERIAL AND METHODS

Chemicals
Aspartame was purchased from Himedia labs Pvt Ltd., Mumbai. All the other chemicals were of analytical grade obtained from Sisco research laboratory, Mumbai, and Himedia labs Pvt Ltd, Mumbai, India.

Experimental Design
The blood cultures were divided into three groups, control PBS, Aspartame Treated and Aspartame treated+oregano leaves extract. The aspartame treated cultures were incubated for 24hr with different dosages of 2mM, 4mM and 10mM and then treated with oregano extract for 24 hour.

WB and PBMC assay
Human whole blood was drawn from healthy volunteers (25±5) (n=5, in triplicates) using standard blood drawing system, collected in heparinized vials and processed immediately. Whole Blood Assay (WB) and Peripheral Blood Monocyte Cells (PBMC) were performed. For WB assay, 1:10 dilution of blood was made with sterile basal medium RPMI 1640 medium (Himedia labs Pvt Ltd) and supplemented with L-Glutamine, FBS, phytohemagglutinin (PHA-M), Penicillin, Streptomycin and Sodium bicarbonate. PBMC were purified using density gradient separation (Ficoll-Hypaque, Himedia lab’s Pvt Ltd, Mumbai, India). They were both cultured for 24 hours with same complete cell culture medium with fetal bovine serum at 2 × 10^5 cells/ml. An equal volume of ice cold 10% metaphosphoric acid was added to the samples after 24 hours. Supernatants were collected after centrifugation and analyzed further for different parameters. Protein estimation was carried out using method of Lowry et al., 1951. Fresh Oregano leaf aqueous extract (2000 μg/ml) was prepared.
Catalase (E.C. 1.11.1.6)(CAT)
Catalase assay was performed by method of Sinha\textsuperscript{30} using Phosphate buffer(pH:7.0; 0.06M), hydrogen peroxide and potassium dichromate acetic acid. Change in the reaction was noted at various intervals (in minutes) at 570 nm. The activity was expressed as amount of H$_2$O$_2$ utilized/min/mg protein.

Superoxide Dismutase (E.C 1.15.1.1) (SOD)
Superoxide dismutase assay was performed by method of Marklund and Marklund\textsuperscript{17} with ethanol- chloroform mixture and Tris HCl buffer(pH: 8.2). The change in activity was noted at 450 nm and expressed as units/min/mg protein.

Glutathione Peroxidase (E.C. 1.11.1.9) (GPx)
The activity of Glutathione peroxidase was measured by method of Rotruck\textsuperscript{25} using Tris HCL buffer (pH:7.0), sodium azide and reaction was stopped using 10% TCA. Change in absorbance was noted at 430 nm and activity was expressed as μmol/ mg protein.

Glutathione S Transferase (E.C. 2.5.1.18) (GST)
The activity of this enzyme was estimated by method of Mannervik\textsuperscript{16} and Boyland\textsuperscript{3} using 1-chloro, 2,4-dinitrobenzene (CDNB), Phosphate buffer saline(pH:6.5) and Glutathione. The activity was measured at 340 nm and expressed by following formula GST activity = [((absorbance 340/min)/ molar extinction coeffiecient μM$^{-1}$/cm) x (1.0 ml /0.1 ml) x sample dilution= U/ml Results.

Protein Carbonyl and Protein Thiol
Protein carbonyl was analyzed by 2,4-dinitrophenylhydrazine (DNPH) method as described by Levine\textsuperscript{14} at 385 nm and expressed in μg/ mg protein. Protein Thiol assay was done by modified method of Sedlack and Lindsay\textsuperscript{28} with Tris HCl(pH:8.2), DTNB(5,5’-dithiobis-(2-nitrobenzoic acid) or Ellman’s reagent and methanol. The absorbance was noted at 412 nm and level of thiol was expressed as μg/ mg protein.

Reduced Glutathione
Reduced Glutathione assay was performed by method of Moron\textsuperscript{20} using Ellman’s reagent and Phosphate buffer(pH:8.0) and absorbance of the mixture was read at 412 nm. The concentration of Glutathione reduced was expressed as μg of GSH/ mg protein.
Statistical analysis

The results were documented after repeating the experiments in triplicates for five times (n=5). The experimental results were expressed as mean ± Standard error (SE). The statistical analysis of the data were carried out by one way ANOVA and the results were considered significant when p<0.05 and highly significant when p<0.01.

RESULTS

Total protein estimation was performed by method of Lowry.[15] Linear graph was obtained with y=0.015x and $R^2=0.990$, using these values of protein the further calculation of estimation of activity of the given enzymes were done. The data represents control, control and oregano, aspartame treated at 2mM, 4mM and 10mM concentrations for 24 hours and subsequent treatment with oregano (2000µg/ml) in WB and PBMC assays. The graphs presented here are 2D graphs with primary and secondary Y axis to inculcate values of WB and PBMC assay of same parameter in single chart. Following is the table for percent different changes in aspartame treated and oregano treated cells as compared to control.

In catalase activity assay, there was significant decrease in activity after aspartame treatment and administration of oregano increased the activity (Fig:1). The increase in activity after oregano treatment in WB assay at 2 and 4mM concentrations was non significant while in PBMC assay it was non significant at 2mM but significant at 4 and 10mM concentrations (Table:1).

Table: 1 Percentage difference in aspartame treated and oregano treated oxidative stress and non oxidative stress cell parameters with respect to control

<table>
<thead>
<tr>
<th>Oregano Treated</th>
<th>2mM</th>
<th>WB assay 4mM</th>
<th>10mM</th>
<th>2mM</th>
<th>PBMC assay 4mM</th>
<th>10mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase*</td>
<td>0.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.43&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.21&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-0.98&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.47&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SOD*</td>
<td>1.94&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.68&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gpx*</td>
<td>3.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-2.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.04&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>GST*</td>
<td>2.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.98&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.28&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Protein Thiol*</td>
<td>0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.76&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-0.29&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.65&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.39&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Reduced Glutathione*</td>
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<td>-3.18&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-2.92&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.28&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-0.55&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Protein Carbonyl*</td>
<td>0.68&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.29&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>-0.31&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-0.56&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*All the data presented here are in percentage (n=5, in triplicates).

<sup>a</sup>represents that the values are highly significant; p<0.01,  
<sup>b</sup>represents values are significant; between p<0.01 to p<0.05,  
<sup>c</sup>represents values are non significant; p>0.05.
Fig: 1 Changes in catalase activity (H$_2$O$_2$ utilized/min/mg protein) after administration of aspartame and oregano treatments in WB and PBMC cultures (n=5, in triplicates).

Superoximde Dismutase (SOD) activity of blood cells showed decrease in their activity after aspartame dosing and no subsequent increase was observed after oregano treatment (Fig:2). Although, the increase was non significant at 2mM concentration in WB assay and significant at 4 and 10mM concentrations, in PBMC assay it was highly significant at 2 and 4mM concentrations and significant at 10mM concentrations (Table:1).

Fig: 2 Changes in Superoxide Dismutase (SOD) activity (units/min/mg protein) after administration of aspartame and oregano treatments in WB and PBMC cultures (n=5, in triplicates).
Glutathione Peroxidase (GPx) and Glutathione S Transferase (GST) are major oxidative stress parameters of cells. Aspartame administration showed increase in their activity and oregano treatment helped them to retain back their activity close to normal cells (Fig:3 and Fig:4). In WB and PBMC assay, the changes were significant only in PBMC assay at 10mM concentration GPx and 4mM concentration of GST the values were non significant (Table:1).

Fig:3 Changes in Glutathione(GPx) activity(μmol/mg protein) after administration of aspartame and oregano treatments in WB and PBMC cultures(n=5, in triplicates).

Fig: 4 Changes in Glutathione S Transferase(GST) activity after administration of aspartame and oregano treatments in WB and PBMC cultures(n=5, in triplicates).

Protein thiol, Reduced Glutathione (GSH) and protein Carbonyl are non enzymatic oxidative stress parameters. Aspartame treatment in cells showed decrease in their concentration which increased gradually after oregano administration on comparison to control (Fig 5, 6,7). In
protein thiol, Increase in PBMC assays were significant and in WB assay at 4 and 10mM concentrations values were nonsignificant with negative percentage change at 10mM concentration with respect to control values (Table:1). It indicates the increase was far more as compared to control. In reduced Glutathione assay, in PBMC assay at 2mM concentration the change was significant while at all concentrations in WB assay and at 4mM and 10mM concentration the changes were non significant and percentage change was negative (Table:1). Protein carbonyl concentrations were significant in WB assay and in PBMC assay the changes were non significant and percentage difference was negative concerning to Control (Table:1).

![Protein Thiol](image1)

**Fig: 5** Changes in protein thiol concentration (µg/mg protein) after administration of aspartame and oregano treatments in WB and PBMC cultures (n=5, in triplicates).

![Reduced Glutathione](image2)

**Fig: 6** Changes in Reduced Glutathione concentration (µg of GSH/mg protein) after administration of aspartame and oregano treatments in WB and PBMC cultures (n=5, in triplicates).
Fig: 7 Changes in Protein Carbonyl concentration (μg/mg protein) after administration of aspartame and oregano treatments in WB and PBMC cultures (n=5, in triplicates).

DISCUSSION
Aspartame has been approved as a sweetener for liquid carbonated beverages, food applications. In a review by Butchko[4] aspartic acid, phenylalanine and methanol have not been proven toxic. But, Phenylalanine plays an important role in neurotransmitter regulation, whereas aspartic acid is also thought to play a role as an excitatory neurotransmitter in the central nervous system so change in their levels might affect the transport of large neutral amino acids across blood brain barrier.

Also, methanol if excreted won’t cause any harm but if any of its by products like accumulates it results in methanol toxicity and absorption metabolism mechanism of methanol formaldehyde might result in synergistic damage.[11] Aspartame also triggered the generation of ROS (reactive oxygen species) and loss of cell proliferation in Human endothelial cells even at low concentrations of 20μM.[11]

In present study, effect of acute toxicity of aspartame was studied on blood cells. The extent of interaction of aspartame metabolites influences the way it gets distributes to tissues in body and eventually affecting the cells. Here in present research we checked the effect oxidative enzymatic and non enzymatic parameters and its amelioration with oregano fresh leaf aqueous extract. Oregano leaves consists of Polyphenols and polyphenols have many favourable effects on human health such as inhibiting the oxidation of low-density lipoproteins, thereby decreasing the risk of heart disease and having highest antioxidant
Carvacrol, present in oregano is a bioactive compound with antioxidant properties which interfere with propagation reactions and inhibit the enzymatic systems involved in initiation reactions. Also, due to the presence of phenolic compounds, rich in terpenoids, such as carvacrol, thymol and rosmarinic acid, in the oregano plant increase in SOD and GPx activity was noted providing efficient scavenging of free reactive radicals in ducks. Aquoeus extracts have phenolic compounds which can act as therapeutic agents against diseases involving radical damage.

Superoxide Dismutase is important in the biological defence mechanism as it breaks endogenous cytotoxic superoxide radicals to H$_2$O$_2$ and O$_2$ these are further degraded by catalese enzyme and Glutathione peroxidase. If catalese amount is reduced it won’t be able to degrade H$_2$O$_2$ and this accumulation of H$_2$O$_2$ would cause oxidative stress, and removal of polyunsaturated fatty acids and proteins due to presence of methanol which are deleterious to polyunsaturated fatty acids and proteins. The decline in the activities of these enzymes might be due to their inactivation caused by excess reactive oxygen species production. Thus, SOD, CAT and GPx act mutually and constitute the enzymatic anti oxidative defense mechanism against reactive oxygen species. After treatment with oregano there was increase in SOD and catalyse enzyme activity as oregano is rich in phenolics. The effect of oregano have also been studied in ameliorating the adverse effects of oxidative stress caused by H$_2$O$_2$ by mediating an antioxidant enzyme response in porcine muscle tissue.

Glutathione S transferase involves in the detoxification of xenobiotics and signaling cascades. Glutathione is an endogenous substance that protect cell suffering from oxidative stress, GSH can function as antioxidant by catalyzing the reduction of H$_2$O$_2$ to water through GPx. The antioxidant enzyme GPx catalyzes the reduction of hydrogen peroxide and hydroperoxides formed from fatty acids, thereby effectively removing toxic peroxides from living cells. It plays the important role of protecting cells from potential damage by free radicals, formed by peroxide decomposition and thereby oxidation of Glutathione. It has a high potency in scavenging reactive free radicals in response to oxidative stress. So the decrease in cellular glutathione content increases cell vulnerability to oxidative stress. Glutathione also helps in binding with –SH groups (Thiol). Oxidation of thiol groups is major manifestation of protein oxidation might hinder with amino acid synthesis.

The decrease in Glutathione and protein thiol and increase in glutathione peroxidase in the present study seems to have been caused by methanol, because methanol metabolism depends
upon glutathione.[12] Also, protein carbonyl has been proven as effective oxidative stress marker,[6] so decrease may affect the stress levels of the cells. In addition, a decrease in glutathione content would also be caused by its rapid reaction with the highly reactive compound, formaldehyde, which is generated during methanol. Antioxidant capacity of water extracts of medicinal plants, also proved a very high antioxidant capacity and simultaneously, a very high content of polyphenol compounds in oregano.[26] this might have helped in maintaining the antioxidant capacity of cells.

CONCLUSION
The observed results support the toxic nature of aspartame even at shorter period of time in in vitro. The phenolic antioxidants, flavanoids and antioxidants present in oregano have reversed the toxic effect of aspartame in blood cells by maintaining the levels of oxidative stress parameters.

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REFERENCES


WEBSITES
