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ANTIOXIDANT POTENTIAL OF PUNICALAGIN IN AN *IN-VITRO* SYSTEM OF PRECISION CUT GOAT LIVER SLICES SUBJECTED TO OXIDATIVE STRESS

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ABSTRACT: Oxidative stress has been implicated in various pathological diseases like neurodegenerative disorders, stroke, cancer, inflammation, atherosclerosis, *etc*., which upshots when the production of reactive oxygen species overwhelms the antioxidant defense mechanism. Nowadays, development of drugs with antioxidant potential gained utmost importance therapeutically to treat many pathological diseases. Plant-derived anti-oxidants were proven to be more effective than synthetic antioxidants. With this as focus, the present study investigated the antioxidant potential of punicalagin (PG) in an *in-vitro* system of goat liver slices imperiled to oxidative stress. Precision cut goat liver slices are used as an *in-vitro* model for the evaluation of anti-oxidant activity because it crafts *in-vivo* tissue environment and helps in curtailing the use of intact animals. In our study, hydrogen peroxide is used as an oxidant to create oxidative stress to the liver slices. Hydrogen peroxide treated liver slices showed a significant decrease in both enzymatic and non-enzymatic antioxidant levels as compared with normal liver slices. PG treated liver slices showed significant improvement in both enzymatic and non-enzymatic antioxidant levels as compared with untreated control group. From our results, it was observed that PG showed significant anti-oxidant activity in an *in-vitro* system of goat liver slices subjected to oxidative stress which confirms the antioxidant potential of punicalagin.

INTRODUCTION: Reactive oxygen species (ROS) or free radicals are generated during a typical metabolic process in our body. Our body nurses defensive mechanism in the form of antioxidants against generated ROS and eliminates them from our body, thereby upholding the homeostasis. If any over production of ROS than defensive mechanism upshots in oxidative stress.

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The generated ROS may cause lipids, proteins and DNA damage. Such damage may lead to the development of many pathological diseases such as cancer, inflammation, atherosclerosis, stroke and neurodegenerative disorders [1, 2].

Nowadays, research has been focused on the development of drugs with antioxidant potential from herbs rather than synthetically for the therapy of many pathological diseases. With this as focus, the present study investigates the antioxidant potential of punicalagin (PG) in an *in-vitro* system of precision cut goat liver slices (PCGLS) subjected to oxidative stress. In our study hydrogen peroxide (H_2O_2) is used as an oxidant to create oxidative stress to the liver slices.

Punicalagin (PG) is a hydrolyzable tannin found in *Punica granatum* as a major component and is accountable for pomegranate health benefits. PCGLS are an apt model for evaluating the antioxidant potential of drugs *in-vitro* because of its simplicity, ease of preparation and parodists the *in-vivo* tissue environment both structurally and functionally as well [3]. PCGLS is used as an alternative model against an intact animal model which would aid in curtailing the use of intact animals [4].

MATERIALS AND METHODS:

Preparation of PG Extract: Punicalagin was purchased from Natural Remedies, Bangalore, Karnataka, India. 20 mg of PG dissolved in 50 µl of dimethyl sulfoxide (DMSO) and was used for the study. The concentration of Punicalagin used for the antioxidant assay was 100 µg.

***In-vitro* Model:** Fresh goat liver was obtained from the local slaughterhouse in cold phosphate buffer saline (PBS) and maintained at 4 °C till use. Thin slices (1 mm thickness) of the liver were cut using a sterile scalpel, and the slices were placed in PBS at a proportion of 0.25 g in 1 ml, in broad, flat bottomed flasks. H_2O_2 was used as the oxidizing agent to induce oxidative stress at a final concentration of 200 mM (4 ml). The treatment groups are:

Group 1: Untreated control containing the liver slices alone.

Group 2: Positive control in which the liver slices were treated with H_2O_2.

Group 3: Treatment control in which the liver slices were treated with PG 100 µg in the presence of oxidant H_2O_2.

Group 4: Negative control in which the liver slices were treated with PG 100 µg in the absence of oxidant H_2O_2.

The liver slices were treated with H_2O_2 both in the presence and the absence of the PG and incubated at room temperature for 1 h with mild shaking. After incubation, the mixture was homogenized followed by centrifugation and the supernatant was used for the analysis.

Analysis of Enzymatic Antioxidant Activity: The superoxide dismutase (SOD) activity estimated by the method of Misra and Fridovich [5]. Catalase activity (CAT) was estimated by the method of Aebi [6]. The glutathione peroxidase (GPx) activity was assayed using the method proposed by Jagetia *et al*. [7] Glutathione reductase (GR) activity was assayed as per the method of Carlberg and Mannervick [8].

Analysis of the Levels of Non-Enzymatic Anti-Oxidants: Ascorbic acid (Vitamin C) levels were estimated based on the method of Roe and Keuther [9]. The reduced glutathione (GSH) level was estimated by the method of Ellman [10].

Statistical Analysis: Data were expressed as the Mean ± standard error of the mean (SEM; n=3). Statistical significance was determined by one-way analysis of variance (ANOVA) with $P<0.05$ considered to be significant using GraphPad Prism (Version 5.0).

RESULTS AND DISCUSSION:

Effect of AA on Enzymatic Antioxidants: In our study, the liver slices are treated with H_2O_2, an oxidant which induces oxidative damage to the liver slices. H_2O_2 is a stable ROS. It undergoes reaction with iron and readily converts to highly reactive free radicals like hydroxyl radical (OH^*) and superoxide radical, which can pledge degradation of heme proteins, inactivation of enzymes, oxidation of lipids and DNA damage [11]. There is an overproduction of these free radicals which overwhelms the antioxidant defense mechanism and cause cellular, DNA damage [12]. The oxidative damage can be assessed by computing the antioxidant enzyme Levels, *viz.*, SOD, CAT, GPx, GR, GSH in the liver slices.

SOD, CAT, GPx are three primary enzymes which are involved in direct elimination of reactive oxygen species (hydroxyl radical, superoxide radical, hydrogen peroxide). SOD is a metalloprotein and is deliberated as a first-line defensive enzyme. It acts against ROS by lowering the steady state level of superoxide radical [13]. It converts superoxide radicals to H_2O_2 and molecular oxygen. This H_2O_2 can be thwarted by catalase or GPx reactions, thereby tumbling the level of cellular damage [14]. CAT undergoes catalytic reaction and decomposes H_2O_2 to water and molecular oxygen, and further oxidative damage

can be reduced. Thus, it acts as oxidative stress regulator [15]. Glutathione peroxidase reduces hydrogen peroxide to water, along with that the oxidation of GSH [16]. Later, the oxidized glutathione (GSSG) is converted to reduced glutathione (GSH) by glutathione reductase (GR) in the presence of NADPH [17].

In our study, enzymatic antioxidant levels were assessed in liver slices subjected to oxidative stress in the presence and the absence of PG. We observed that the positive control group (liver slices treated with H_2O_2) showed decreased levels of SOD, CAT, GPx, GR significantly (P<0.001) as compared with untreated control group **Table 1** indicating the development of oxidative stress in the positive control group.

No significant change in these enzymatic antioxidant levels was observed in the negative control group (liver slices treated with PG 100 µg in the absence of H_2O_2) as compared with untreated control group. The treatment control group showed significant (P<0.001) improvement in these enzymatic antioxidant levels as compared with positive control group

TABLE 1: EFFECT OF PG ON ENZYMATIC ANTIOXIDANTS IN GOAT LIVER SLICES EXPOSED *IN-VITRO* TO H_2O_2

Groups	SOD (U/g tissue)	CAT (U/g tissue)	GPx (U/g tissue)	GR (U/g tissue)
Liver slices + vehicle	15.44±1.22	76.06±0.88	30.12±1.45	2.53±1.55
Liver slices + H_2O_2	10.42±0.98***	31.09±1.02***	18.88±0.66***	1.19±1.31***
Liver slices + H_2O_2 + PG	14.54±1.22^{*+}	71.17±1.67^{+++}	27.49±1.59^{+++}	2.39±2.01^{+++}
Liver slices + PG	15.12±1.17^{+++}	77.33±1.41^{+++}	31.82±2.11^{+++}	2.55±1.76^{+++}

Values are expressed as mean ± SEM (n=3); enzyme activity was expressed as units/g liver tissue. Analysed by one way ANOVA followed by Dunnett 't' test. *(P < 0.05), *** (P < 0.001) *vs.* untreated control group; +++ (P < 0.001) *vs.* H_2O_2 control group;

Effect of PG on Non-Enzymatic Anti-Oxidants: Apart from enzymatic antioxidants, non-enzymatic antioxidants are also found to play a major role in rendering the oxidative stress to maintain homeostasis. Vitamin C, also known as ascorbic acid, is a water-soluble antioxidant, which prevents oxidative damage to the cell membrane induced by aqueous radicals [18]. It is a powerful antioxidant, acts as a scavenger of ROS and alleviates the deleterious effects caused by ROS [19]. Glutathione (GSH), a tripeptide, free thiol and most abundant, non-protein antioxidant in the cells, plays a pivotal role in the defense mechanism against oxidative stress-induced cell injury and mitochondrial damage [20].

It is an important defense mechanism against potentially toxic hydrogen peroxide by glutathione peroxidase, which reduces hydrogen peroxide to water, and along with that, the oxidation of GSH [16]. It maintains the intracellular thiol redox status and detoxifies exogenous and endogenous reactive molecules [21]. The protective effect of PG against oxidative damage may be due to improving the antioxidant levels by eliminating reactive free radicals and thereby maintaining homeostasis between the antioxidant defense system and reactive oxygen species. Vitamin C and GSH were assessed in goat liver slices subjected to oxidative stress. We observed that the positive control group (liver slices treated with H_2O_2) showed significantly (P<0.001) decreased levels of vitamin C and GSH as compared with the untreated control group **Table 2** indicating the development of oxidative stress in the positive control group. No significant change in these non-enzymatic antioxidant levels was observed in the negative control group (liver slices treated with PG 100 µg in the absence of H_2O_2) as compared with untreated control group.

TABLE 2: EFFECT OF PG ON NON-ENZYMATIC ANTIOXIDANTS IN GOAT LIVER SLICES EXPOSED *IN-VITRO* TO H_2O_2

Groups	Vitamin C (mg/g tissue)	GSH (mg/g tissue)
Liver slices + vehicle	0.53±1.09	2.93±0.25
Liver slices + H_2O_2	0.17±1.54***	1.27±1.07***
Liver slices + H_2O_2 + PG	0.48±0.89^{+++}	2.81±1.06^{+++}
Liver slices + PG	0.52±1.09^{+++}	2.91±1.11^{+++}

Values are expressed as mean ± SEM (n=3); enzyme activity was expressed as units/g liver tissue. Analysed by one way ANOVA followed by Dunnett 't' test. *(P < 0.05), *** (P <0.001) *vs.* untreated control group; +++ (P < 0.001) *vs.* H_2O_2 control group

In our study, we observed that the treatment control group showed significant (P<0.001) improvement in vitamin C and GSH levels as compared with positive control group, which combats the

oxidative stress by scavenging the reactive species, keeping the cellular redox state in balance.

CONCLUSION: In our present study, precision cut goat liver slices are used as an alternative *in-vitro* model for evaluating the antioxidant potential of punicalagin against hydrogen peroxide-induced stress *in-vitro*. This *in-vitro* model parodist the *in-vivo* system and aids in minimizing the use of live animals. Enzymatic and non-enzymatic antioxidant levels were analyzed in the goat liver slices subjected to oxidative stress in the presence and absence of punicalagin.

From our results, it was observed that H_2O_2 exposed liver slices showed a significant decrease in antioxidant levels which was reverted significantly with the administration of punicalagin. Punicalagin improve the antioxidant status in an oxidatively stressed tissue, observations from our present study confirm the antioxidant potential of punicalagin.

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CONFLICT OF INTEREST: Nil

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