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Protection of Precision Cut - Goat Liver Slices By *Zea Mays* Leaf Extracts From Hydrogen Peroxide Induced Oxidative Stress *In Vitro*

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ABSTRACT

Plants play an indispensable part in the environment, especially as a medication to protect man from intrinsic and extrinsic ill effects imparted by stress agents. In tune with the search of antioxidant-rich plants, the leaves of *Zea mays* were analyzed for its antioxidant potential at three different stages of growth. The effect of the leaf extract on the oxidant stress induced in *in vitro* systems, which simulated *in vivo* conditions, were studied. The *in vitro* model used as a means to replace or minimize the use of live animals in experiments was goat liver slices. The aim of the present study was to assess the extent of oxidative stress, the activities and levels of antioxidants by the extracts of *Zea mays* in the presence and absence of oxidative stress. Our findings suggest that the hepatoprotection by *Zea mays* leaves against hydrogen peroxide induced oxidative stress could be due to its antioxidant property.

Keywords: liver slices, oxidative stress, antioxidants, *Zea mays* leaf extracts.

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INTRODUCTION

A majority of the present day diseases are reported to be due to the shift in the balance of the pro-oxidant and the antioxidant homeostatic phenomenon in the body. Pro-oxidant conditions dominate either due to the increased generation of the free radicals caused by excessive oxidative stress of the present day life, or due to the poor scavenging/quenching in the body caused by depletion of the dietary antioxidants (1). The harmful effects of free radicals are counterbalanced by the antioxidant action of both enzymic and non-enzymic antioxidants. (2).

Consumption of dietary antioxidants from plant materials has been associated with a lowered incidence of diseases due to the oxidative stress from free radicals (3). Plants are widely acclaimed as rich sources of antioxidants. Several studies have analyzed the antioxidant potential of a variety of herbs (4).

With this backdrop, the present study was formulated to analyze the antioxidant potential of *Zea mays* leaves. *Zea mays* is commonly known as makkacholam or maize. It belongs to the family Gramineae. Although it has a history of usage in treating various disorders, no systematic study has been undertaken on analyzing the extracts of *Zea mays* leaves on oxidant challenged events *in vitro*.

Traditionally, testing of drug candidates are being performed using animal models – an expensive and slow process. Nowadays the use of *in vitro* hepatocyte models for drug metabolism and hepatotoxicity studies are rapidly increasing (5). These *in vitro* models play an important role, especially in the earlier part of the pre-clinical studies.

Precision-cut liver slices have proved useful for several pharmacological and toxicological investigations. Liver was the organ of choice because it is the metabolic organ and is responsible for the metabolic clearance of many xenobiotics. Liver slice is a microcosm of the intact liver and therefore it is an *in vitro* technique that offers the advantages of *in vivo* situation and hence is a more suitable model for the experimental analysis of antioxidant protection studies (6).

However, the applicability of these slices to study the H_2O_2 induced oxidative stress and to test the antioxidant effect of *Zea mays* leaves has not previously been evaluated. The aim of this study was therefore, to evaluate precision-cut liver slices generated from goat liver as a tool to test the protective effects of the *Zea mays* leaves *in vitro* against H_2O_2 induced toxicity. The presence and functionality of the liver cell types in the liver slice provides a multicellular milieu *in vitro* that resembles the *in vivo* situation more closely than current *in vitro* models.

MATERIALS AND METHODS

In vitro model system used

The *in vitro* model system used in the study was precision-cut goat liver slice. Fresh goat liver was obtained from a local slaughterhouse and transported to the laboratory on ice. The liver was washed with isotonic KCl and processed for the assays. Very thin slices (1mm thick) were cut from the liver using a sterile scalpel. The slices were taken in sterile Hanks Balanced Salt Solution (HBSS) at a proportion of 0.25g in 1ml. HBSS simulated the peritoneal fluid in live animals.

Hydrogen peroxide was used as the oxidant to induce oxidative stress in the liver slices. H_2O_2 was used at a final concentration of 500µM, which is the dose used in *in vivo* studies for intraperitoneal administration. 20µl of the plant extract (juice) corresponding to 20mg of plant extract was used to study the antioxidant effect on the cells.

Plant material

The plants of *Zea mays* were grown within the University campus. The leaves of the plantlets

were collected fresh for every parameter to be analyzed. They were cleaned of surface contaminants by washing thoroughly in running tap water and gently blotted dry between folds of tissue paper**.** The antioxidant content was assessed in the leaves of *Zea mays* at six different time periods of growth namely 5, 10, 15, 20, 25 and 30 days after sowing, in order to determine whether any change in the antioxidant status was observed as the age of the plant increased. The results revealed that the leaves on the 10^{th} day of growth was found to have maximum content of all the enzymic and non-enzymic antioxidants, followed closely by the leaves on their 5^{th} day and 15^{th} day of growth, compared to other time points tested. Therefore, the 10^{th} day leaves were selected for further studies.

In order to throw light on the nature of the bioactive component responsible for the antioxidant potential of the leaves of 10^{th} day plant, extracts were prepared in solvents of differing polarity namely water, methanol and chloroform and all the three extracts were taken for further analyses of the study.

Preparation of Aqueous extract

The leaves were homogenized in water (1g/ml) using a micropestle in a microfuge tube. It was centrifuged at low speed to clarify the extract. The supernatant corresponding to the concentration of 20mg/20µl was used for the assay.

Preparation of methanolic /chloroform extract

Zea mays leaves (1.0g) were homogenized in approximately 1ml of the solvents (methanol/chloroform). The supernatant was collected and dried at 60°C well protected from light. The residue obtained after drying the chloroform and methanol extracts were weighed and dissolved in a known amount of DMSO to yield a concentration of 20mg/5µl, DMSO was maintained at a minimum level to avoid DMSO-induced events, if any.

Experimental Design

The experiment was planned to further probe the antioxidant property of *Zea mays* leaves in goat liver slices. The extracts of the leaves were administered to the oxidatively stressed goat liver slices and the antioxidant status was analyzed in them.

After addition of the respective agents, the tissue slices were incubated at 37°C for one hour with mild shaking. After the incubation period, the tissue was homogenized in a teflon homogenizer in the HBSS used for the incubation of the slices. This was done to take into consideration any leaching during the incubation period. The estimations of various parameters indicative of antioxidant potential were carried out in the homogenate.

Enzymic antioxidants

SOD uses the photochemical reduction of riboflavin as oxygen-generating system and catalyses the inhibition of NBT reduction. The SOD activity was analyzed based on the measurement of increase in absorbance at 560 nm due to the reduction of NBT to $NBTH_2$ (7). Catalase activity was estimated by measuring the rate of decomposition of H_2O_2 at 280nm (8). The peroxidase activity was assayed by the method of Reddy *et al*. (1985)(9).GST activity can be determined spectrophotometrically by monitoring the thioester formation at 340nm using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate (10).Glutathione reductase activity was followed as per the method of David and Richard (1983)(11) where the conversion of oxidized glutathione to reduced glutathione employing NADPH as a substrate by GR.

Non-enzymic antioxidants

The tocopherol content in the liver slices were estimated using Emmerie-Engel reaction and the absorbance can be measured spectrophotometrically at 540nm (12). Vitamin A content was measured by the method of Bayfield and Cole (1980) method (13). The amount of reduced glutathione is measured by

its reaction with DTNB (5, 5'-dithiobis-2-nitro benzoic acid) (Ellmans reaction) to give a yellow colored product that absorbs at 412 nm (14). The ascorbic acid levels were estimated by the method of Roe and Keuther (1943) (15).

Statistical analysis

The biochemical parameters studied were subjected to statistical analysis using Agress (Version 3.01) statistical software. Statistical significance was determined by two-way analysis of variance with $P<0.01$ considered significant was adapted to all the parameters under study to test the level of statistical significance.

RESULTS AND DISCUSSION

Precision-cut liver slices are described as a valuable tool for *in vitro* metabolism studies of potential drug candidates. Some papers have reported successful cryopreservation conditions for liver slices, facilitating a broader and more efficient use of the tissue (particularly of human origin) (16).

In the current study, by employing the precision-cut liver slices generated from goat liver as a tool, the protective effects of the *Zea mays* leaves *in vitro* against hydrogen peroxide-induced oxidative stress was evaluated. Enzymic and non-enzymic antioxidants were assessed in the liver slices subjected to oxidative stress in the presence and the absence of the leaf extracts.

ENZYMIC ANTIOXIDANTS

The activities of SOD, CAT and POD in the slices exposed to H_2O_2 and/or *Zea mays* leaf extracts are represented in Table 1. The goat liver slices treated with the different extracts of *Zea mays* leaves showed a significant increase in SOD activity, compared to untreated control. Hydrogen peroxide caused a considerable decrease in the SOD activity. The co-treatment with the methanolic and aqueous extracts resulted in a significant increase compared to untreated control. Similar trend was observed in catalase.

H_2O_2 is considered as a reactive oxygen metabolite as it can cause damage to cells at a relatively low concentration. Direct activities of H_2O_2 include degradation of haem proteins, release of iron, inactivation of enzymes and oxidation of lipids and DNA (17). The decreased activities of SOD and CAT in H_2O_2 treated group may be reflective of the oxidative stress, wherein the balance between reactive oxygen species production and the antioxidant defenses may be lost.

The peroxidase activity in the H_2O_2 treated liver slices decreased compared to the untreated control. The toxic effect of H_2O_2 was negated by the concordant treatment with *Zea mays* leaf extracts. *Zea mays* leaf extracts, by themselves, significantly ($P<0.01$) increased the peroxidase activity compared to control, except chloroform extract.

The exposure of hepatocytes to *S. cumini* peel extract rich in anthocyanins after CCl_4 treatment was found to elevate GSH and GPx activities by 2-folds, whereas the activities of catalase and superoxide dismutase were not significantly affected (18).

CAT or GPx catalyze the transformation of H_2O_2 to harmless byproducts, thereby curtailing the quantity of cellular destruction (19). During the reaction of H_2O_2 scavenging, GSH is oxidized to GSSG by the enzyme GPx (20). The reduction of GSSG to GSH is catalyzed by GR using NADPH as reducing potential. The reduced activities of these enzymes points out the damage associated with the administration of H_2O_2

The effect of *Zea mays* leaf extracts on the GR activities of H_2O_2 exposed liver slice homogenates was analysed and the results are presented in Table 2. The GR activity was decreased slightly by H_2O_2, but this decrease was not statistically significant. When the liver slices were exposed to the different extracts of *Zea mays* leaves, an increase in GR activity was observed, with methanolic extract eliciting the maximum increase.

TABLE -1

Effect of Zea mays leaf extracts on the SOD, CAT and POD activities in goat liver slices exposed in vitro to hydrogen peroxide

Sample	Superoxide oxidase (Units$/g tissue)		Catalase (Units#/g tissue)		Peroxidase (Units@/g tissue)	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
No extract	13.2 ± 0.10	11.0 ± 0.50 [a]	212.0 ± 1.0	121.4 ± 1.2 [a]	23.3 ± 1.02	21.4 ± 1.0
Aqueous extract	17.0 ± 0.10 [a]	6.0 ± 0.40 [a,b,c]	377.0 ± 1.0 [a]	200.0 ± 1.0 [a,b,c]	29.2 ± 1.06 [a]	26.0 ± 2.0 [b]
Methanol extract	18.0 ± 0.05 [a]	16.9 ± 0.05 [a,b,c]	425.0 ± 1.0 [a]	308.0 ± 1.0 [a,b,c]	30.8 ± 1.40 [a]	24.8 ± 1.0 [c]
Chloroform extract	15.4 ± 0.05 [a]	13.6 ± 0.50 [b,c]	242.0 ± 2.0 [a]	147.8 ± 1.4 [a,b,c]	25.8 ± 1.20	23.2 ± 1.0

The values are Mean ± S.D. of triplicates
CD value = 2.84

- ***Statistically significant (P<0.01) compared to untreated control***
- ***b - Statistically significant (P<0.01) compared to H_2O_2 alone treated group***
- ***c - Statistically significant (P<0.01) compared to the respective plant extract treated group***

The GSTs are active in the detoxification of numerous products, including reactive oxidant damage to DNA and lipids, such as organic epoxides, lipid hydroperoxides and unsaturated aldehydes (21). The activities of GST decreased upon exposure to H_2O_2. Treatment with aqueous or methanolic extracts of *Zea mays* leaves caused an increase in GST activity over the control. They also caused the reversal of the decrease caused by H_2O_2 in GST activity, to a considerable extent. The chloroform extract of the leaves did not increase GST activity by itself. However, it effectively counteracted the effect of H_2O_2, raising the activity to near control values.

TABLE 2

Effect of Zea mays leaf extracts on the GST and GR activities in goat liver slices exposed in vitro to hydrogen peroxide

SAMPLE	Glutathione S-transferase (Units@/g tissue)		Glutathione reductase (mmoles NADPH oxidized/g tissue)	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
No extract	0.025 ± 0.003	0.020 ± 0.001	2.00 ± 0.2	1.60 ± 0.3
Aqueous extract	0.028 ± 0.004	0.024 ± 0.002	3.38 ± 1.3	2.86 ± 1.4
Methanol extract	0.030 ± 0.002	0.024 ± 0.003 [c]	4.18 ± 2.1	3.12 ± 1.1
Chloroform extract	0.024 ± 0.001	0.023 ± 0.002	3.08 ± 1.0	2.24 ± 1.0

The values are Mean ± S.D. of triplicates
CD value = 2.84

- ***a - Statistically significant (P<0.01) compared to untreated control***
- ***b - Statistically significant (P<0.01) compared to H_2O_2 alone treated group***
- ***c - Statistically significant (P<0.01) compared to the respective plant extract treated group***

Yousef *et al*. (2009) (22) have reported that grape seed proanthocyanidin extract significantly elevated the hepatic GST activity during cisplatin intoxication. Kundu *et al*. (2008) (23) demonstrated that the methanol-aqueous fraction of *Cajanus cajan* leaf extract could prevent the chronically treated alcohol-induced rat liver damage by augmenting the antioxidant enzyme activities.

The administration of *Zea mays* leaf extracts in the present study, improved the GR activities from the effect of the oxidant assault. This observation shows that the leaf extracts are effective in ensuring GSH homeostasis in the cell, as GR replenishes GSH (reduced) from GSSG (oxidized). GSH, apart from being a strong antioxidant by itself also acts as a substrate for antioxidant enzymes like GPx and GST (24).

Non-enzymic antioxidants

The defense of cells against oxidative stress also involves non-enzymatic antioxidants such as ascorbic acid, tocopherol, vitamin A and GSH. In our present study, the levels of these antioxidants were decreased in the liver slices by H_2O_2 and *Zea mays* prevented this decrease (Table 3 and 4).

The goat liver homogenate when treated with hydrogen peroxide showed a slight decrease in the levels of vitamin E, which, however, escaped statistical significance. This depleting effect was counteracted by the co-treatment with the leaf extracts. The vitamin E levels showed a significant ($p<0.01$) increase in the methanolic and aqueous extract treated groups (Table 3). The levels were found to be higher in the methanolic extract treated group. The chloroform extract reverted the depleted vitamin E levels to control level in the oxidant treated group. It is, thus, conceivable from the results, that the treatment with the leaf extracts of *Zea mays* can improve the vitamin E status in the liver, thereby preventing H_2O_2-induced damages.

Bansal *et al*. (2005) (25) reported that pretreatment with vitamin E to NDEA induced rats provide protection against oxidative stress in liver caused by the carcinogen. El-Shenawy *et al*. (2010) (26) demonstrated that the co-treatment of vitamin E with diazinon prevents the oxidative stress-induced liver tissue injury in mice.In line with these reports; it is conceivable that the *Zea mays* leaf extracts can render protection to the membranes by increasing the levels of vitamin E, the major antioxidant present in the membrane.

The levels of vitamin A observed in the different treatment groups are tabulated in Table 3. H_2O_2 treatment caused a significant depletion in the levels of vitamin A compared to untreated controls. This depleting effect was reversed by the co-treatment with the leaf extracts. The extent of increase was maximum with the methanolic extract followed by the aqueous and chloroform extracts. The liver slice homogenates prepared after exposure to the leaf extracts alone also showed a marked increase in vitamin A levels with all the three extracts. Similar trend was observed in vitamin C levels.

TABLE 3

Effect of Zea mays leaf extracts on the vitamin E and vitamin A levels in the goat liver slices exposed in vitro to hydrogen peroxide

SAMPLE	Vitamin E (□g/g tissue)		Vitamin A (□g/g tissue)	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
No extract	18.37 ± 1.8	16.58 ± 1.2	121.0 ± 2.0	96.70 ± 2.2 [a]
Aqueous extract	35.73 ± 2.0 [a]	30.15 ± 1.0 [a,b,c]	166.5 ± 2.2 [a]	130.0 ± 1.0 [a,b,c]
Methanol extract	43.15 ± 3.4 [a]	34.45 ± 2.4 [a,b,c]	195.2 ± 2.1 [a]	159.3 ± 2.1 [a,b,c]
Chloroform extract	28.09 ± 2.1 [a]	20.59 ± 1.0 [c]	137.4 ± 2.1 [a]	118.8 ± 1.4 [b,c]

The values are Mean ± S.D. of triplicates
CD value = 0.00572
a - Statistically significant (P<0.01) compared to untreated control
b - Statistically significant (P<0.01) compared to H_2O_2 alone treated group
c - Statistically significant (P<0.01) compared to the respective plant extract treated group

The *Casearia esculenta* root extract improved the levels of vitamin C, vitamin E and GSH of the liver and kidney of streptozotocin diabetic rats (27). *Ganoderma lucidum* polysaccharides treatment significantly and dose-dependently increased non-enzymic and enzymic antioxidants in STZ-induced diabetic rats (28).

Table 4 presents the levels of GSH in the oxidant treated goat liver slices in the presence and the absence of *Zea mays* leaf extracts. The oxidant (H_2O_2) treated liver slices showed a significant (P<0.01) decrease in the levels of reduced glutathione, compared to the untreated control. The methanolic and aqueous extracts of *Zea mays* leaves were effective in mitigating the toxicity caused by H_2O_2. The chloroform extract did not exert any protective effect, albeit increasing the GSH values in the absence of H_2O_2. The GSH levels in the liver slice homogenates prepared after exposure to the leaf extracts.

TABLE 4

EFFECT OF Zea mays LEAF EXTRACTS ON THE ASCORBIC ACID LEVELS IN THE GOAT LIVER SLICES EXPOSED in vitro TO HYDROGEN PEROXIDE

SAMPLE	Glutathione (nmoles/g tissue)		Ascorbic acid (mg/g tissue)	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
No extract	116.8 ± 2.6	88.0 ± 2.0 [a]	2.08 ± 0.002	0.196 ± 0.002 [a]
Aqueous extract	159.2 ± 3.0 [a]	144.0 ± 2.0 [a,b,c]	0.233 ± 0.002 [a]	0.304 ± 0.002 [a,b,c]
Methanol extract	200.0 ± 2.0 [a]	173.6 ± 1.8 [a,b,c]	0.233 ± 0.003 [a]	0.249 ± 0.004 [a,b,c]
Chloroform extract	131.2 ± 1.4 [a]	88.0 ± 3.0 [a,c]	0.250 ± 0.002 [a]	0.303 ± 0.001 [a,b,c]

The values are Mean ± S.D. of triplicates
CD value = 0.00572
a - Statistically significant (P<0.01) compared to untreated control
b - Statistically significant (P<0.01) compared to H_2O_2 alone treated group
c - Statistically significant (P<0.01) compared to the respective plant extract treated group

Wills and Asha (2006) (29) observed that the treatment with *n*-hexane extract of *Lygodium flexuosum* (L.) significantly improved the levels of GSH in liver in CCl_4 intoxicated rats, indicating the hepatoprotection. Tirkey *et al*. (2005) (30) have reported that hesperidin, a potential citrus bioflavonoid improved the levels of GSH in the liver and kidney homogenates of the rats administered with CCl_4. The ethanolic extract of root and root derived callus extracts of *Premna serratifolia L* prevents the damaging effects of paracetamol induced oxidative stress and hepatotoxicity in blood and liver of male albino rats (31).

CONCLUSION

Our results demonstrate that the leaf extracts of *Zea mays* can improve the antioxidant status in the goat liver slices exposed *in vitro* to oxidative stress. Since the model was carefully planned to simulate *in vivo* conditions, it is perceivable that the effects also occur in the intact system. Increased activity of antioxidant enzymes observed in this study may be due to the increased availability of antioxidants from *Zea mays*.

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