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ANALYSIS OF OXIDATIVE STRESS INDUCED BY DIFFERENT TOBACCO SAMPLES AND THE PROTECTIVE EFFECT BY CERTAIN PLANT EXTRACTS

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ABSTRACT

The tobacco use has spread with significant rapidity percolating into all sections of the society. Although tobacco usage is not a new problem, it has become a source of increasing concern due to the increase in realization of its volume, awful morbidity and mortality. Numerous modes of action by which tobacco carcinogens induce cancer have been recognised, including tobacco induced oxidative stress through production of reactive oxygen species. Cellular antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase and free radical scavengers like reduced glutathione and vitamins A, C, and E protect cells and tissues against noxious radicals. To analyze the extent of oxidative stress that is induced by tobacco in different forms, selected concentrations of alcoholic extracts of various tobacco products, ranging from 100-300µgs were added to oral epithelial cell culture. Melondialdehyde and reduced glutathione were estimated as a measure of oxidative stress and lipid peroxidation. Treatment of cell culture with mango leaf extract, coconut husk extract and areca husk extract along with tobacco samples have shown a significant variation in oxidative parameters such as melondialdehyde and reduced glutathione levels and antioxidant enzymes such as superoxide dismutase, catalase and peroxidase activity indicating protective effect.

KEYWORDS: Oxidative stress, Tobacco, Mango leaves, Coconut husk, Areca nut husk.

INTRODUCTION

Tobacco is a menace that has grabbed millions of people all over the world, cutting across the nation and social barriers. The tobacco use has spread with remarkable rapidity seeping into all sections of the society. Although tobacco usage is not a new problem, it has become a source of increasing concern due to the increase in realization of its volume, awful morbidity and mortality. Several modes of action by which tobacco carcinogens induce cancer have been identified, including tobacco induced oxidative stress.[1] through production of reactive oxygen species (ROS). Oxidative damage to cellular macromolecules can arise through overproduction of ROS and faulty antioxidant and/or DNA repair mechanisms.

Cellular antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) and free radical scavengers like reduced glutathione and vitamins A, C, and E protect cells and tissues against noxious radicals. An imbalance between cellular pro-oxidant and antioxidant levels results in the oxidative stress that leads to tissue damage. The antioxidant enzymes react directly with reactive oxygen species to yield non-radical products. Superoxide dismutase, a mitochondrial as well as cytosolic enzyme, dis-mutates O_2^{--} to H_2O_2, which is decomposed by CAT to H_2O.

On the other hand, GPx removes H_2O_2 and lipid peroxides using reduced glutathione (GSH). This prevents H_2O_2-mediated damage, which is thought to be a recognized risk factor for carcinogenesis. Decreased GSH levels increase the free radical burden due to ineffective removal of ROS from the tissues, which results in increased lipid peroxidation and cell damage which has been implicated in malignant transformation and other systemic disorders.[2]

There are several experimental evidences in scientific literature, in different cell culture systems, suggesting that Smokeless tobacco induced oxidative stress and subsequent cytotoxicity. Bagchi *et al.*, has noted concentration and time-dependent release of lactate dehydrogenase (LDH) from cultured macrophages when incubated with smokeless tobacco extract and a significant reduction of the same when incubated with free radical scavengers indicating oxidative stress induced cell damage.[3] The same investigators demonstrated 1.5-7.6- fold increases in lipid peroxidation, cytochrome c reduction and DNA fragmentation

following treatment of normal human oral keratinocyte cells with 300µg smokeless tobacco extract (STE) /ml 10-54% decreases in these parameters with addition of antioxidants.[4]

Mitchell *et al.*, also used human oral keratinocyte cell lines, HOK-16B, to test the possibility of smokeless tobacco extract induced oxidative stress. Based on results of series of experiments, they conclude that Smokeless tobacco extract leads to cell death in human oral keratinocyte cell lines in part, through oxidative stress.[5]

MATERIALS AND METHODS

Selection and identification of medicinal plants

Three different plant materials namely mango leaves (*Mangifera indica)*, husk of coconut (*Cocos nucifera)* and husk of areca nut (*areca catechu),* which have been used frequently as oral hygiene measures by people of Dakshina Kannada but not investigated in detail for their relevant properties were chosen for the present study and were identified and authenticated by a botanist.

Preparation of plant extract

Fresh mango leaves, husk of ripe coconut and areca nut were collected from the native, where it is grown for non-commercial purpose. The plant materials were washed in tap water to remove the dirt, followed by distilled water, cut in to smaller pieces and dried under shade. The dried materials were powdered using household electric blender. 100 grams of the plant powder was extracted in a Soxhlet apparatus with 500 ml of ethanol as solvent and concentrated using a rotor-evaporator. The crude alcoholic extracts thus prepared were used for various analyses.

Selection of tobacco samples and preparation of extract

Three tobacco samples were chosen for the present study, namely fresh leaves of tobacco collected from farm (tobacco sample-1), tobacco processed for traditional chewing (tobacco sample -2) and tobacco dispensed in commercial sachets (tobacco sample-3). Tobacco sample-1 (Fig. 1) was collected from farm where it is grown for commercial purpose while tobacco sample- 2 and 3 (Fig. 2 and 3) were purchased from local tobacco sellers. Of different tobacco sachets sold in the local market, the brand which has been consumed maximum by local people was chosen. For the preparation of extract of fresh tobacco leaves, same procedure used for other plant materials was followed. Other samples were directly

subjected to extraction procedure. The crude alcoholic extracts thus prepared were used for further study.

Fig. 1: Fresh tobacco leaves, Sample 1.

Fig. 2: Tobacco processed for traditional chewing, Sample 2.

Fig. 3: Tobacco dispensed in commercial sachet, Sample 3.

Preparation of primary culture of human oral epithelial cells[6]

Oral mucosal tissues from retro molar area were collected from patients undergoing extraction of impacted teeth with institutional ethical committee approval, after obtaining a written consent. Subjects with history of consumption of any form of tobacco, with poor oral hygiene or with any form of and oral infection/inflammatory conditions were excluded. Once the subject was selected for the study, oral hygiene was optimized with preoperative 1% Betadine mouthwash for three days. 3 x 3 mm specimen from the redundant mucosal tissue was surgically excised from the flap raised to expose the impacted tooth during extraction procedure under local anaesthesia. The tissue was washed in Phosphate Buffered Saline to remove the blood and immediately transferred to a T-flask containing Stem line Keratinocyte media as the transport medium.

The connective tissue was excised carefully from the tissue obtained and washed thrice with PBS containing double strength antibiotics. The tissue was then cut into multiple bits and digested with collagenase and trypsin for 60 minutes at 37ºC. The cells were agitated and centrifuged at 3000 rpm for 5 minutes. The pellets were re-suspended in culture medium (10 ml of stem line keratinocyte growth supplement added to 500 ml of stem line TM keratinocyte medium II) and plated on culture flasks. The plates were incubated at 37ºC and 5% CO_2. The medium was changed once in two days until they reached confluence. Cell growth was monitored daily with an inverted phase contrast microscope. Confluent normal epithelial cells were obtained successfully after 7-10 days. Confluent cells were sub-cultured and used for further studies when they had reached 60-70% confluence. All these procedures were carried out under high aseptic conditions within the laminar air flow. Cell viability was confirmed by trypan blue exclusion assay.

Investigation of oxidative stress induced by different tobacco samples and protection from plant extracts

For the analysis of oxidative stress and antioxidant parameters, cultured oral epithelial cells were seeded at 1 × 105 cells/mL in 6-well plates for 24 hours to allow cell adherence. After incubation, cells were treated with different concentrations of various alcoholic extracts ranging from 100-300 µg and incubated for a period of 24 hours. The different concentrations of tobacco samples were selected to test the oxidative stress parameters, based on previous reports[4] after 24 hours, adherent and floating cells were collected and cell lysate were

subjected to assays for oxidative stress and antioxidant parameters. The experiments were performed in 10 groups, as follows,

Group 1: Control (untreated cells).

Group 2, 3 & 4: Cells treated with tobacco sample - 1 in concentrations 100, 200 & 300µgm/ml.

Group 5, 6 & 7: Cells treated with tobacco sample - 2 in concentrations 100, 200 & 300µgm/ml.

Group 8, 9 & 10: Cells treated with tobacco sample - 3 in concentrations 100, 200 & 300µgm/ml.

Assessments made were

a. Estimation of lipid peroxidation

Lipid peroxidation was estimated by determining melondialdehyde (MDA) levels, spectrophotometrically in cell lysate, according to the method described by Ohkawa *et al.*,[7] Briefly, the reaction mixture contained Tris-HCl buffer (50 mM, pH 7.4), ter-butyl hydroperoxide (500 l M in ethanol) and 1 mM $FeSO_4$. After incubating the samples at 37ºC for 90 minutes, the reaction was stopped by adding 0.2 ml of 8% sodium dodecyl sulphate followed by 1.5 ml of 20% acetic acid (pH 3.5). The amount of MDA formed during incubation was estimated by adding 1.5 ml of 0.8% TBA and further heating the mixture at 95ºC for 45 minutes. After cooling, samples were centrifuged, and the thiobarbituric acid reactive substances (TBARS) were measured in supernatants at 532 nm. The levels of lipid peroxidation were expressed in terms of n moles of TBARS per 90 minutes/mg protein.

b. Estimation of GSH

Reduced glutathione estimation was performed by the method of Moron *et al.*,[8] 1 ml of cell lysate was mixed with 0.5 ml of 0.2 M sodium phosphate buffer (pH 8.0). Later, 2 ml of 0.6mM, DTNB (Ellman's reagent) was added. Contents were mixed well and the optical density of the yellow-coloured complex formed by the reaction of GSH and DTNB was measured after 10 minutes at 420nm. A standard curve was obtained with standard reduced glutathione. The levels of GSH were expressed as l g of GSH/mg protein.

c. Estimation of SOD

Superoxide dismutase was estimated according to the method of Mishra and Fridovich[9] Briefly, 50mM potassium phosphate buffer (pH 7.8), 45µM methionine, 5.3mM riboflavin, and 84µM NBT were added to 500µl of 72 hours grown culture of human peripheral

lymphocytes. The tubes were incubated in 24°C for 10 minutes and read on spectrophotometer at 600 nm. The levels of SOD were expressed in terms of IU/mg protein.

d. Estimation of CAT

Catalase activity was measured by the method of Sinha[10], 0.5 ml of cell culture was taken into a test tube. To this 1.0 ml phosphate buffer (0.067M, pH 7) was added followed by 0.4 ml distilled water and 0.5 ml hydrogen peroxide. The tubes were incubated for 60 minutes and added 2.0 ml dichromate solution. The colour developed was read at 620 nm on a spectrophotometer.

e. Estimation of GPx

The GPx activity was measured by the method of Rotruk *et al*,[11] 3 ml phosphate buffer (0.1M pH 7), 0.05ml Guaicol (20mM) and 0.03ml hydrogen peroxide (12.3 mM) was added to 0.5 ml cell culture. Mixed well and absorbance at 436 nm. Values were expressed as nano moles of NADPH oxidized to NADP by using the extinction coefficient of 6.2 3 103 M_1 cm_1 at 340 nm. The levels of GPx were expressed in terms of n moles NADPH consumed/min per milligram of protein.

In order to investigate the protective effect of plant materials, the cells were simultaneously incubated with the most active concentration different tobacco samples evaluated for oxidative stress parameters i.e., 300µg /ml and extracts of mango leaves, husk of coconut or areca nut in three different concentrations namely 20, 40 & 60 mg/ml for 24 hours. Concentration of plant extracts were decided based on IC50 value obtained in previous experiments. Assays for oxidative stress and antioxidant parameters were carried out to analyse the protective effect of plant material extracts on tobacco induced intracellular oxidative stress. All the above experiments were carried out in triplicate and mean value is taken to compare the effects.

RESULTS

To analyse the extent of oxidative stress that is induced by tobacco in different forms, selected concentrations of alcoholic extracts of various tobacco products, ranging from 100-300µgms were added to oral epithelial cell culture (Fig.4 and 5) and incubated for a period of 24 hours, following which assays for oxidative stress and antioxidant parameters were carried out.

Fig. 4: Primary cells culture grown and maintained in CO_2 incubator.

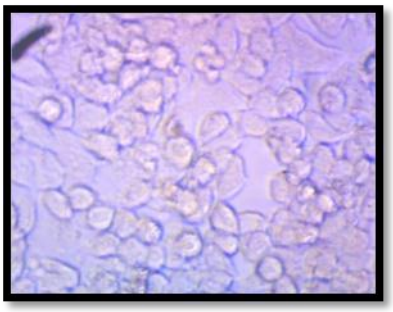
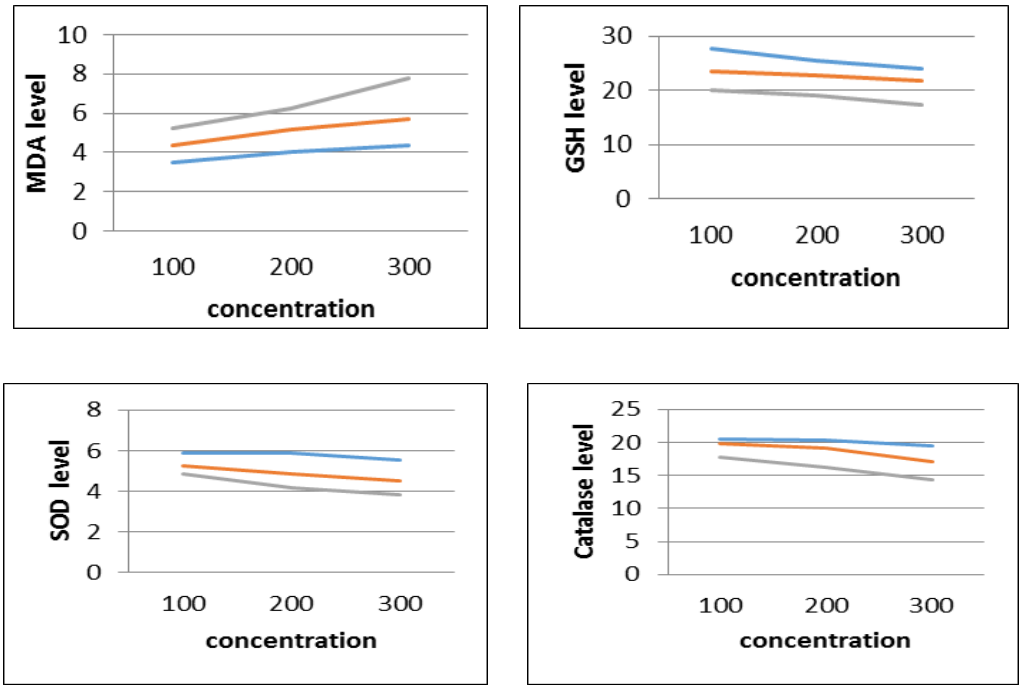


Fig. 5: Primary culture of oral epithelial cells grown in Stem line Keratinocyte growth media.

a. Lipid peroxidation

Treatment of cells with different extracts such as sample 1, 2, 3 resulted in a concentration dependent increase of lipid peroxidation expressed as increased MDA levels (Fig. 6).



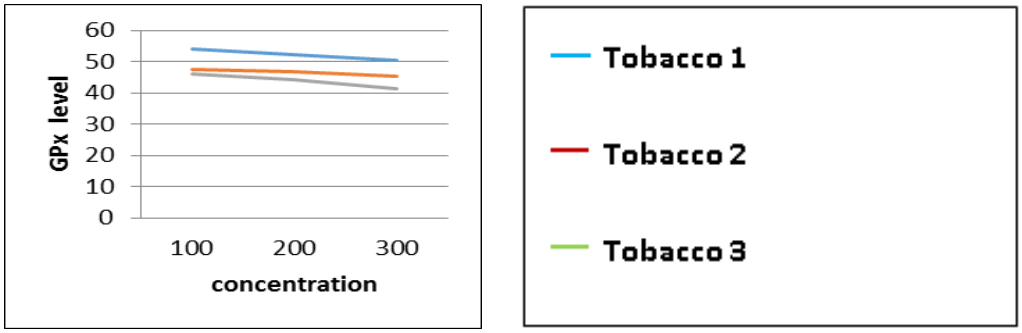


Fig. 6: Oxidative stress and antioxidant parameters after exposure of cells to different tobacco samples at various concentrations (a-MDA, b- GSH, c-SOD, d- catalase, e-GPx).

When the results obtained were statistically analysed using ANOVA a highly significant difference between the groups were noted in all parameters checked with ($p \leq 0.05$). When multiple comparison was done using Tukey HSD, The MDA levels after treatment with all different tobacco samples were significantly different from Control value($p \leq 0.001$) except for 100µg of Tobacco sample- 1(p value ≥0.060). When the MDA Values were compared between different tobacco samples and various concentrations a concentration dependent increase in MDA levels were noted. Significant difference in MDA levels was observed between different tobaccos samples with tobacco- 1 compared to other two samples with lowest values. MDA levels in cultured oral epithelial cells were significantly high after treatment with tobacco - 3 with highest MDA levels noted 300µg in comparison with all other experimental groups studied ($p \leq 0.05$, Table 1.

a. Estimation of GSH

When the GSH levels were estimated after incubating the cultured oral mucosal cell for a period of 24 hours with various alcoholic extracts of selected tobacco samples ranging from 100-300µgms and was found to be reduced with all tobacco types compared to the untreated cells details are shown in (Fig. 6).

The GSH levels were significantly different from control with p value ≤ 0.05in all experimental groups except for 100µg of tobacco sample- 1(p value ≥.409). When the GSH values were compared between different tobacco samples and various concentrations a significant lower GSH level was observed with tobacco sample-1 when compared to other two samples. All different tobacco samples exhibited concentration dependent decrease in GSH level. Higher concentration of tobacco sample-1, i.e., 300µg was able to cause a drop in

GSH comparable to that of various concentrations of tobacco-2. GSH levels in cultured oral epithelial cells were significantly low after treatment with 300µg of tobacco -3 in comparison with all other experimental groups studied ($p \leq 0.001$) except for 200µg of same sample (Table 1).

Table 1: Comparison of MDA and GSH levels caused by tobacco samples.

(I) Category	(J) Category	MDA			GSH		
		Mean Diff. (I-J)	S.E	P	Mean Diff.(I-J)	S.E	p
Tobacco 1-100	Control	.433	.1256	.060	-1.333	.5704	.409
	Tob 2 - 100	-.867(*)	.1256	.000	4.233(*)	.5704	.000
	Tob 3 - 100	-1.800(*)	.1256	.006	7.733(*)	.5704	.000
	Tob 1 - 200	-.567(*)	.1256	.000	2.167(*)	.5704	.029
	Tob 2 - 200	-1.733(*)	.1256	.000	5.000(*)	.5704	.000
	Tob 3 - 200	-2.767(*)	.1256	.000	8.700(*)	.5704	.000
	Tob 1 - 300	-.900(*)	.1256	.000	3.600(*)	.5704	.000
	Tob 2 - 300	-2.233(*)	.1256	.000	5.833(*)	.5704	.000
	Tob 3 - 300	-4.300(*)	.1256	.000	10.433(*)	.5704	.000
Tobacco 2-100	Control	.433	.1256	.060	-5.567(*)	.5704	.000
	Tob 3 - 100	-.933(*)	.1256	.000	3.500(*)	.5704	.000
	Tob 1 - 200	.300	.1256	.381	-2.067(*)	.5704	.042
	Tob 2 - 200	-.867(*)	.1256	.000	.767	.5704	.930
	Tob 3 - 200	-1.900(*)	.1256	.000	4.467(*)	.5704	.000
	Tob 1 - 300	-.033	.1256	1.000	-.633	.5704	.978
	Tob 2 - 300	-1.367(*)	.1256	.000	1.600	.5704	.200
	Tob 3 - 300	-3.433(*)	.1256	.000	6.200(*)	.5704	.000
Tobacco 3 –100	Control	2.233(*)	.1256	.000	-9.067(*)	.5704	.000
	Tob 1 - 200	1.233(*)	.1256	.000	-5.567(*)	.5704	.000
	Tob 2 - 200	.067	.1256	1.000	-2.733(*)	.5704	.003
	Tob 3 - 200	-.967(*)	.1256	.000	.967	.5704	.786
	Tob 1 - 300	.900(*)	.1256	.000	-4.133(*)	.5704	.000
	Tob 2 - 300	-.433	.1256	.060	-1.900	.5704	.076
	Tob 3 - 300	-2.500(*)	.1256	.000	2.700(*)	.5704	.004
Tobacco 1 – 00	Control	1.000(*)	.1256	.000	-3.500(*)	.5704	.000
	Tob 2 - 200	-1.167(*)	.1256	.000	2.833(*)	.5704	.002
	Tob 3 - 200	-2.200(*)	.1256	.000	6.533(*)	.5704	.000
	Tob 1 - 300	-.333	.1256	.256	1.433	.5704	.319
	Tob 2 - 300	-1.667(*)	.1256	.000	3.667(*)	.5704	.000
	Tob 3 - 300	-3.733(*)	.1256	.000	8.267(*)	.5704	.000
Tobacco 2 –200	Control	2.167(*)	.1256	.000	-6.333(*)	.5704	.000
	Tob 3 - 200	-1.033(*)	.1256	.000	3.700(*)	.5704	.000
	Tob 1 - 300	.833(*)	.1256	.000	-1.400	.5704	.347
	Tob 2 - 300	-.500(*)	.1256	.020	.833	.5704	.892
	Tob 3 - 300	-2.567(*)	.1256	.000	5.433(*)	.5704	.000
Tobacco 3 –200	Control	3.200(*)	.1256	.000	-10.033(*)	.5704	.000
	Tob 1 - 300	1.867(*)	.1256	.000	-5.100(*)	.5704	.000
	Tob 2 - 300	.533(*)	.1256	.011	-2.867(*)	.5704	.002

	Tob 3 - 300	-1.533(*)	.1256	.000	1.733	.5704	.132
Tobacco 1 –300	Control	1.333(*)	.1256	.000	-4.933(*)	.5704	.000
	Tob 2 - 300	-1.333(*)	.1256	.000	2.233(*)	.5704	.023
	Tob 3 - 300	-3.400(*)	.1256	.000	6.833(*)	.5704	.000
Tobacco 2 –300	Control	2.667(*)	.1256	.000	-7.167(*)	.5704	.000
	Tob 3 - 300	-2.067(*)	.1256	.000	4.600(*)	.5704	.000
Tobacco 3 –300	Control	4.733(*)	.1256	.000	-11.767(*)	.5704	.000

Tob– Tobacco, Dependent Variable: level, Multiple comparison -Tukey HSD Based on observed means. the mean difference is significant at the .05 level.*

c. Estimation of SOD

Exposure of cultured oral mucosal cell with various alcoholic extracts of selected tobacco samples ranging from 100-300µgms for a period of 24 hours has also altered SOD enzyme levels (Fig. 6).

Statistical comparison of the SOD enzyme levels using Tukey HSD, between control and different and various concentrations showed significant difference with all other samples studied except different concentrations of Tobacco -1 and 100µg of tobacco -2. Difference in SOD values of different concentrations of tobacco -1 and that of 100µg of tobacco sample -2 was comparable with p value $\geq .05$. Similarly SOD values with all different concentrations of tobacco -2 and 100µg of tobacco sample- 3 were statistically comparable but significant difference was noted with compared with 200 & 300µg of tobacco sample- 3. SOD levels were significantly high after treatment with 300µg of tobacco- 3 in comparison with all other experimental groups studied ($p \leq 0.001$). Details are shown in Table 2.

d. Estimation of CAT

When the effect of different tobacco samples on catalase enzyme was checked after exposing the cultured oral mucosal cell with various alcoholic extracts of selected tobacco samples ranging from 100-300µgms for a period of 24 hours, values obtained are expressed in (Fig.6).

Statistical comparison of the catalase enzyme levels using Tukey HSD, between control and different tobacco samples and various concentrations showed a highly significant difference with all other samples studied except 100 and 200µg of tobacco 1. The result of statistical comparison of catalase level between different tobacco samples and different concentrations are shown in table 2. When 100µg of tobacco 1 was compared with other samples the values obtained were comparable to that of other two concentrations of same sample studied and 100µg of tobacco sample - 2 with p value $\geq .05$, while other groups showed significance

difference. Highest alteration in catalase levels in cultured oral epithelial cells was noted after treatment with 300µg of tobacco 3 and was significantly high in comparison with all other experimental groups studied ($p \leq 0.05$, Table 2).

e. Estimation of GPx

The alterations in the levels of GPx that had occurred after exposing the cultured oral mucosal cell with various alcoholic extracts of selected tobacco samples in three different concentrations is depicted in fig. 6.

In case of cell culture exposed to all different tobacco samples, levels of GPx enzyme were significantly different from control with p value ≤ 0.05 except for 100µg of tobacco sample-1(p value ≥ 0.181). When the GPx values were compared between 100µg of tobacco-1 and other experimental groups significantly lower level was noted than all other samples studied. No significant difference was observed between GPx values obtained with different concentrations of tobacco 2 and 100µg and 200µg of tobacco 3, while all other samples showed significant difference with 100µg of tobacco 3. GPx levels in cultured oral epithelial cells were significantly high after treatment with 300µg of tobacco 3 in comparison with all other experimental groups studied ($p \leq 0.05$, Table 2).

Table 2: Comparison of antioxidant enzymes SOD, catalase and GPx levels caused by different tobacco exposure.

(I) Category	(J) Category	SOD			Catalase			GPx		
		Mean Difference(I-J)	Std. Error	P	Mean Difference(I-J)	Std. Error	p	Mean Difference (I-J)	Std. Error	p
Tobacco1-100	Control	-.200	.2906	.999	-.767	.2970	.287	-1.533	.5353	.181
	Tob 2 - 100	.667	.2906	.433	.633	.2970	.528	6.587(*)	.5353	.000
	Tob 3 - 100	1.033(*)	.2906	.049	2.633(*)	.2970	.000	8.167(*)	.5353	.000
	Tob 1 - 200	.033	.2906	1.000	.200	.2970	.999	2.033(*)	.5353	.029
	Tob 2 - 200	1.033(*)	.2906	.049	1.333(*)	.2970	.007	7.433(*)	.5353	.000
	Tob 3 - 200	1.733(*)	.2906	.000	4.300(*)	.2970	.000	9.900(*)	.5353	.000
	Tob 1 - 300	.367	.2906	.951	.933	.2970	.109	3.800(*)	.5353	.000
	Tob 2 - 300	1.367(*)	.2906	.004	3.333(*)	.2970	.000	8.700(*)	.5353	.000
	Tob 3 - 300	2.067(*)	.2906	.000	6.133(*)	.2970	.000	12.733(*)	.5353	.000
Tobacco2-100	Control	-.867	.2906	.147	-1.400(*)	.2970	.004	-8.120(*)	.5353	.000
	Tob 3 - 100	.367	.2906	.951	2.000(*)	.2970	.000	1.580	.5353	.155
	Tob 1 - 200	-.633	.2906	.499	-.433	.2970	.893	-4.553(*)	.5353	.000
	Tob 2 - 200	.367	.2906	.951	.700	.2970	.398	.847	.5353	.842
	Tob 3 - 200	1.067(*)	.2906	.038	3.667(*)	.2970	.000	3.313(*)	.5353	.000
	Tob 1 - 300	-.300	.2906	.986	.300	.2970	.988	-2.787(*)	.5353	.001
	Tob 2 - 300	.700	.2906	.370	2.700(*)	.2970	.000	2.113(*)	.5353	.021
	Tob 3 - 300	1.400(*)	.2906	.003	5.500(*)	.2970	.000	6.147(*)	.5353	.000
Tobacco3–100	Control	-1.233(*)	.2906	.011	-3.400(*)	.2970	.000	-9.700(*)	.5353	.000
	Tob 1 - 200	-1.000	.2906	.061	-2.433(*)	.2970	.000	-6.133(*)	.5353	.000
	Tob 2 - 200	.000	.2906	1.000	-1.300(*)	.2970	.009	-.733	.5353	.923
	Tob 3 - 200	.700	.2906	.370	1.667(*)	.2970	.001	1.733	.5353	.091
	Tob 1 - 300	-.667	.2906	.433	-1.700(*)	.2970	.000	-4.367(*)	.5353	.000
	Tob 2 - 300	.333	.2906	.973	.700	.2970	.398	.533	.5353	.989
	Tob 3 - 300	1.033(*)	.2906	.049	3.500(*)	.2970	.000	4.567(*)	.5353	.000
Tobacco1– 200	Control	-.233	.2906	.998	-.967	.2970	.088	-3.567(*)	.5353	.000
	Tob 2 - 200	1.000	.2906	.061	1.133(*)	.2970	.028	5.400(*)	.5353	.000
	Tob 3 - 200	1.700(*)	.2906	.000	4.100(*)	.2970	.000	7.867(*)	.5353	.000

	Tob 1 - 300	.333	.2906	.973	.733	.2970	.340	1.767	.5353	.081
	Tob 2 - 300	1.333(*)	.2906	.005	3.133(*)	.2970	.000	6.667(*)	.5353	.000
	Tob 3 - 300	2.033(*)	.2906	.000	5.933(*)	.2970	.000	10.700(*)	.5353	.000
Tobacco2– 200	Control	-1.233(*)	.2906	.011	-2.100(*)	.2970	.000	-8.967(*)	.5353	.000
	Tob 3 - 200	.700	.2906	.370	2.967(*)	.2970	.000	2.467(*)	.5353	.005
	Tob 1 - 300	-.667	.2906	.433	-.400	.2970	.930	-3.633(*)	.5353	.000
	Tob 2 - 300	.333	.2906	.973	2.000(*)	.2970	.000	1.267	.5353	.393
	Tob 3 - 300	1.033(*)	.2906	.049	4.800(*)	.2970	.000	5.300(*)	.5353	.000
Tobacco3– 200	Control	-1.933(*)	.2906	.000	-5.067(*)	.2970	.000	-11.433(*)	.5353	.000
	Tob 1 - 300	-1.367(*)	.2906	.004	-3.367(*)	.2970	.000	-6.100(*)	.5353	.000
	Tob 2 - 300	-.367	.2906	.951	-.967	.2970	.088	-1.200	.5353	.463
	Tob 3 - 300	.333	.2906	.973	1.833(*)u	.2970	.000	2.833(*)	.5353	.001
Tobacco1– 300	Control	-.567	.2906	.639	-1.700(*)	.2970	.000	-5.333(*)	.5353	.000
	Tob 2 - 300	1.000	.2906	.061	2.400(*)	.2970	.000	4.900(*)	.5353	.000
	Tob 3 - 300	1.700(*)	.2906	.000	5.200(*)	.2970	.000	8.933(*)	.5353	.000
Tobacco2– 300	Control	-1.567(*)	.2906	.001	-4.100(*)	.2970	.000	-10.233(*)	.5353	.000
	Tob 3 - 300	.700	.2906	.370	2.800(*)	.2970	.000	4.033(*)	.5353	.000
Tobacco3– 300	Control	-2.267(*)	.2906	.000	-6.900(*)	.2970	.000	-14.267(*)	.5353	.000

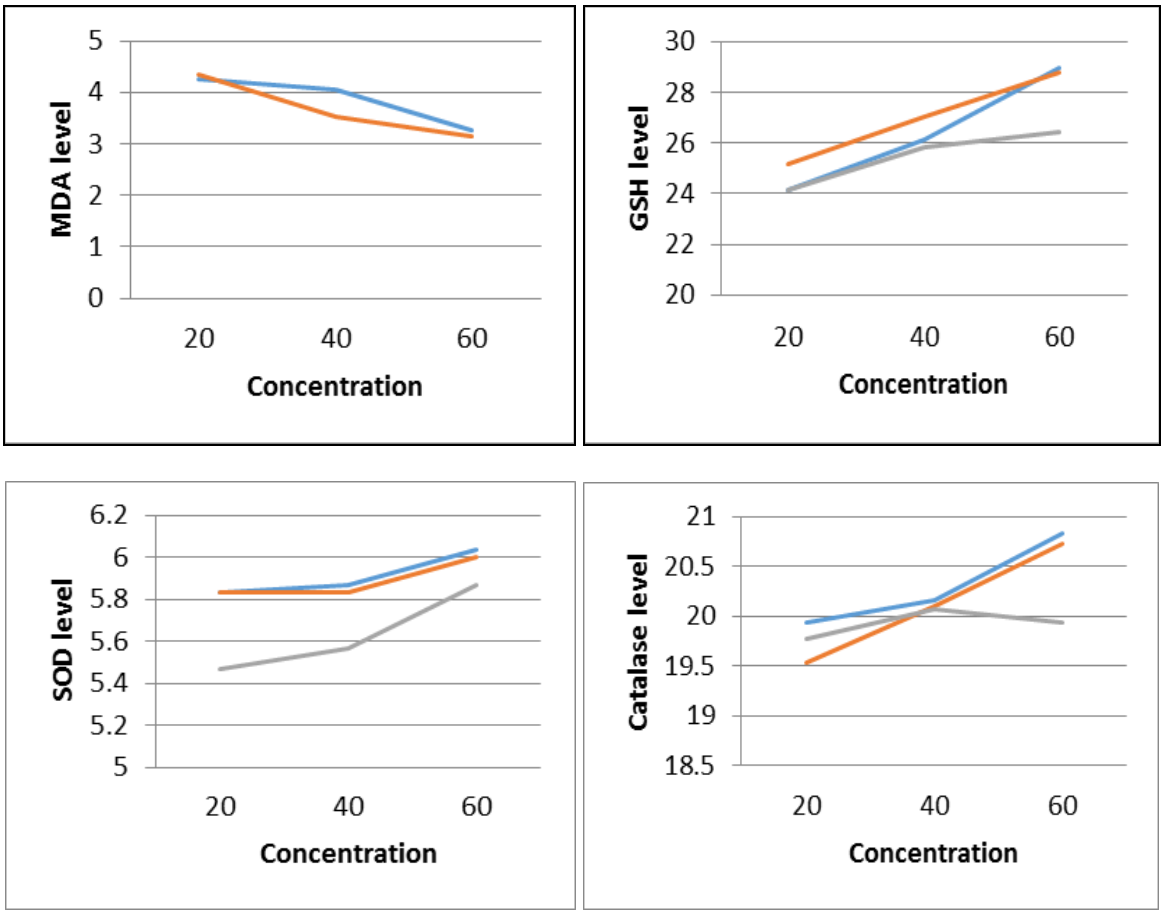
Dependent Variable: Level, Multiple comparison -Tukey HSD Based on observed means. The mean difference is significant at the .05 level. Tob - Tobacco*

Protective effect of selected plant extract on tobacco induced oxidative stress

With the purpose of analysing the protective effect of selected plant extracts such as mango leaf extract, coconut husk extract and areca nut husk extract, on tobacco induced oxidative stress; oxidative stress indicators were analysed after treating the oral mucosal cell culture simultaneously with 300µg concentrations of three tobacco samples along with various concentrations of selected plant extracts.

Study of protective effect of selected plant materials on oxidative stress induced by tobacco sample- 1

The cultured oral epithelial cells were treated with 300µg of tobacco sample-1 along with three different concentrations of mango leaf extract, coconut husk extract and areca husk extract and various parameters such as MDA, GSH, SOD, Catalase and GPx were estimated and found to be different compared to cell culture treated with tobacco alone. Details of values obtained are shown in (Fig. 7).



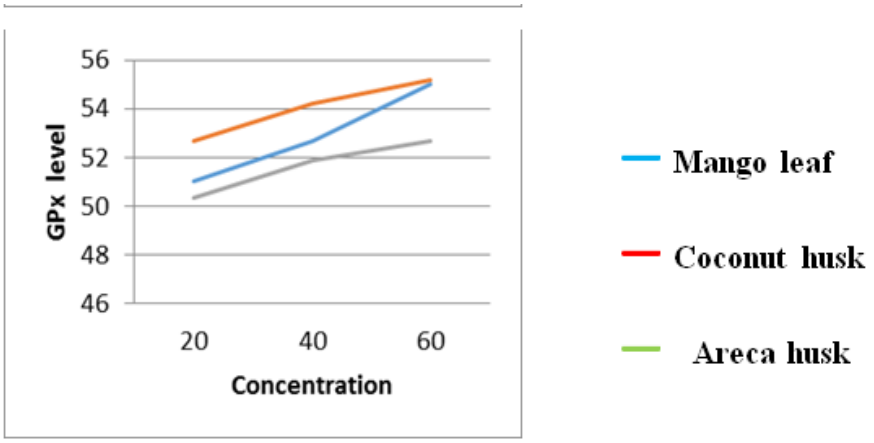


Fig. 7: Oxidative stress and antioxidant parameters in tobacco sample- 1 exposed cells after treatment with different plant materials at various concentrations (a- MDA, b- GSH, c-SOD, d- Catalase, e- GPx).

When the results of oxidative stress parameters after treating the cell culture simultaneously with tobacco 1 at concentration of 300 µg and different plant extracts of various concentrations, were statistically analysed using ANOVA a highly significant difference between the groups were noted in all parameters checked with $p \leq 0.05$. When multiple comparison was done using Tukey HSD, The MDA levels have shown a statistically significant difference compared to tobacco exposure alone, after treatment with all plant extracts in a concentration of 60mg/ml and both mango leaf and coconut husk extracts in 40mg/ml concentration. When compared to control (untreated cells) the all the values were statistically different except 60mg/ml of mango leaf and coconut husk extract. GSH levels were statistically different in all samples treated with different plant extract along with tobacco 1 of 300 µg when compared to tobacco alone except different plant extracts in 20mg/ml concentrations and also the values fail to reach a comparable level to the control even in different plant extract of highest concentration studied with a statistically significant difference except mango leaf and coconut husk extracts of 60mg/ml concentration (Table 3).

Except for the experimental samples with tobacco and mango leaf and arecanut husk extract of 60mg/ml concentration all other samples showed a statistically similar values of SOD levels as cells treated with tobacco alone and cells treated with mango leaf and coconut husk extracts of 60mg/ml concentration showed SOD levels statistically comparable to that of control. Catalase estimation showed the results similar to SOD. With respect to GPx, all plant extracts in 60mg/ml concentration, mango leaves and coconut husk in 40mg/ml concentration and coconut husk in 20mg/ml concentration could get a value statistically different from

tobacco treatment alone. Then again none of the values except mango leaves and coconut husk in 60mg/ml concentration have reached to a level which is statistically comparable to controls (Table 4).

Table 3: Comparison of MDA and GSH levels in tobacco sample- 1 exposed cells after treatment with different plant extracts.

(J) Category	(I) Category	MDA levels			GSH levels		
		Mean Diff. (I-J)	S.E.	P value	Mean Diff. (I-J)	S.E.	p value
Mango leaf - 20	Control	-1.233(*)	.0865	.000	4.900(*)	.4688	.000
	Tobacco 1	.100	.0865	.981	-.033	.4688	1.000
	Coconut husk – 20	.067	.0865	.999	1.033	.4688	.525
	Areca husk – 20	.067	.0865	.999	.000	.4688	1.000
	Mango leaf - 40	-.200	.0865	.459	2.000(*)	.4688	.011
	Coconut husk – 40	-.733(*)	.0865	.000	2.900(*)	.4688	.000
	Areca husk – 40	-.067	.0865	.999	1.667	.4688	.052
	Mango leaf - 60	-1.000(*)	.0865	.000	4.800(*)	.4688	.000
	Coconut husk –60	-1.133(*)	.0865	.000	4.667(*)	.4688	.000
	Areca husk – 60	-.633(*)	.0865	.000	2.300(*)	.4688	.003
Coconut husk – 20	Control	-1.300(*)	.0865	.000	3.867(*)	.4688	.000
	Tobacco 1	.033	.0865	1.000	-1.067	.4688	.481
	Areca husk – 20	.000	.0865	1.000	-1.033	.4688	.525
	Mango leaf - 40	-.267	.0865	.133	.967	.4688	.613
	Coconut husk – 40	-.800(*)	.0865	.000	1.867(*)	.4688	.021
	Areca husk – 40	-.133	.0865	.889	.633	.4688	.948
	Mango leaf - 60	-1.067(*)	.0865	.000	3.767(*)	.4688	.000
	Coconut husk – 60	-1.200(*)	.0865	.000	3.633(*)	.4688	.000
	Areca husk – 60	-.700(*)	.0865	.000	1.267	.4688	.260
Areca husk - 20	Control	-1.300(*)	.0865	.000	4.900(*)	.4688	.000
	Tobacco 1	.033	.0865	1.000	-.033	.4688	1.000
	Mango leaf - 40	-.267	.0865	.133	2.000(*)	.4688	.011
	Coconut husk – 40	-.800(*)	.0865	.000	2.900(*)	.4688	.000
	Areca husk – 40	-.133	.0865	.889	1.667	.4688	.052
	Mango leaf - 60	-1.067(*)	.0865	.000	4.800(*)	.4688	.000
	Coconut husk – 60	-1.200(*)	.0865	.000	4.667(*)	.4688	.000
	Areca husk – 60	-.700(*)	.0865	.000	2.300(*)	.4688	.003
Mango leaf - 40	Control	-1.033(*)	.0865	.000	2.900(*)	.4688	.000
	Tobacco 1	.300	.0865	.062	-2.033(*)	.4688	.009
	Coconut husk - 40	-.533(*)	.0865	.000	.900	.4688	.700
	Areca husk - 40	.133	.0865	.889	-.333	.4688	1.000
	Mango leaf - 60	-.800(*)	.0865	.000	2.800(*)	.4688	.000
	Coconut husk - 60	-.933(*)	.0865	.000	2.667(*)	.4688	.000
	Areca husk - 60	-.433(*)	.0865	.002	.300	.4688	1.000
Coconut husk – 40	Control	-.500(*)	.0865	.000	2.000(*)	.4688	.011
	Tobacco 1	.833(*)	.0865	.000	-2.933(*)	.4688	.000
	Areca husk - 40	.667(*)	.0865	.000	-1.233	.4688	.292

	Mango leaf- 60	-.267	.0865	.133	1.900(*)	.4688	.018
	Coconut husk - 60	-.400(*)	.0865	.005	1.767(*)	.4688	.033
	Areca husk - 60	.100	.0865	.981	-.600	.4688	.963
Areca husk - 40	Control	-1.167(*)	.0865	.000	3.233(*)	.4688	.000
	Tobacco 1	.167	.0865	.695	-1.700(*)	.4688	.045
	Mango leaf - 60	-.933(*)	.0865	.000	3.133(*)	.4688	.000
	Coconut husk - 60	-1.067(*)	.0865	.000	3.000(*)	.4688	.000
	Areca husk - 60	-.567(*)	.0865	.000	.633	.4688	.948
Mango leaf - 150	Control	-.233	.0865	.261	.100	.4688	1.000
	Tobacco 1	1.100(*)	.0865	.000	-4.833(*)	.4688	.000
	Coconut husk - 60	-.133	.0865	.889	-.133	.4688	1.000
	Areca husk - 60	.367(*)	.0865	.012	-2.500(*)	.4688	.001
Coconut husk – 60	Control	-.100	.0865	.981	.233	.4688	1.000
	Tobacco 1	1.233(*)	.0865	.000	-4.700(*)	.4688	.000
	Areca husk - 60	.500(*)	.0865	.000	-2.367(*)	.4688	.002
Areca husk - 60	Control	-.600(*)	.0865	.000	2.600(*)	.4688	.001
	Tobacco 1	.733(*)	.0865	.000	-2.333(*)	.4688	.002

Based on observed means. The mean difference is significant at the .05 level.*

Dependent Variable: Level Multiple Comparisons -Tukey HSD

Table 4: Comparison of SOD, catalase and GPx levels in tobacco sample - 1 exposed cells after treatment with different plant extracts.

		SOD levels			Catalase levels			GPx levels		
(J) Category	**(I) Category**	**Mean Diff. (I-J)**	**S.E**	**P value**	**Mean Diff. (I-J)**	**S. E**	**P value**	**Mean Diff. (I-J)**	**S.E**	**P value**
Mango leaf - 20	Control	.267	.1255	.574	1.300(*)	.2429	.001	4.667(*)	.6045	.000
	Tobacco 1	-.300	.1255	.415	-.400	.2429	.846	-.667	.6045	.987
	Coconut husk –20	.000	.1255	1.000	-.400	.2429	.846	1.633	.6045	.260
	Areca husk – 20	-.367	.1255	.179	-.167	.2429	1.000	-.667	.6045	.987
	Mango leaf - 40	.033	.1255	1.000	.233	.2429	.995	1.633	.6045	.260
	Coconut husk –40	.000	.1255	1.000	.167	.2429	1.000	3.200(*)	.6045	.001
	Areca husk – 40	-.267	.1255	.574	.133	.2429	1.000	.833	.6045	.941
	Mango leaf - 60	.200	.1255	.869	.900(*)	.2429	.038	4.000(*)	.6045	.000
	Coconut husk –60	.167	.1255	.953	.800	.2429	.089	4.167(*)	.6045	.000
	Areca husk – 60	.033	.1255	1.000	.000	.2429	1.000	1.633	.6045	.260
Coconut husk – 20	Control	.267	.1255	.574	1.700(*)	.2429	.000	3.033(*)	.6045	.002
	Tobacco 1	-.300	.1255	.415	.000	.2429	1.000	-2.300(*)	.6045	.031
	Areca husk – 20	-.367	.1255	.179	.233	.2429	.995	-2.300(*)	.6045	.031
	Mango leaf - 40	.033	.1255	1.000	.633	.2429	.302	.000	.6045	1.000
	Coconut husk –40	.000	.1255	1.000	.567	.2429	.447	1.567	.6045	.310
	Areca husk – 40	-.267	.1255	.574	.533	.2429	.530	-.800	.6045	.954
	Mango leaf - 60	.200	.1255	.869	1.300(*)	.2429	.001	2.367(*)	.6045	.024
	Coconut husk –60	.167	.1255	.953	1.200(*)	.2429	.002	2.533(*)	.6045	.013
	Areca husk – 60	.033	.1255	1.000	.400	.2429	.846	.000	.6045	1.000
Areca husk – 20	Control	.633(*)	.1255	.002	1.467(*)	.2429	.000	5.333(*)	.6045	.000
	Tobacco 1	.067	.1255	1.000	-.233	.2429	.995	.000	.6045	1.000
	Mango leaf - 40	.400	.1255	.109	.400	.2429	.846	2.300(*)	.6045	.031
	Coconut husk –40	.367	.1255	.179	.333	.2429	.943	3.867(*)	.6045	.000
	Areca husk – 40	.100	.1255	.999	.300	.2429	.971	1.500	.6045	.365
	Mango leaf - 60	.567(*)	.1255	.006	1.067(*)	.2429	.008	4.667(*)	.6045	.000
	Coconut husk –60	.533(*)	.1255	.011	.967(*)	.2429	.021	4.833(*)	.6045	.000
	Areca husk – 60	.400	.1255	.109	.167	.2429	1.000	2.300(*)	.6045	.031

Mango leaf -40	Control	.233	.1255	.735	1.067(*)	.2429	.008	3.033(*)	.6045	.002
	Tobacco 1	-.333	.1255	.280	-.633	.2429	.302	-2.300(*)	.6045	.031
	Coconut husk –40	-.033	.1255	1.000	-.067	.2429	1.000	1.567	.6045	.310
	Areca husk – 40	-.300	.1255	.415	-.100	.2429	1.000	-.800	.6045	.954
	Mango leaf - 60	.167	.1255	.953	.667	.2429	.242	2.367(*)	.6045	.024
	Coconut husk -60	.133	.1255	.990	.567	.2429	.447	2.533(*)	.6045	.013
	Areca husk – 60	.000	.1255	1.000	-.233	.2429	.995	.000	.6045	1.000
Coconut husk – 40	Control	.267	.1255	.574	1.133(*)	.2429	.004	1.467	.6045	.395
	Tobacco 1	-.300	.1255	.415	-.567	.2429	.447	-3.867(*)	.6045	.000
	Areca husk - 40	-.267	.1255	.574	-.033	.2429	1.000	-2.367(*)	.6045	.024
	Mango leaf- 60	.200	.1255	.869	.733	.2429	.150	.800	.6045	.954
	Coconut husk -60	.167	.1255	.953	.633	.2429	.302	.967	.6045	.867
	Areca husk - 60	.033	.1255	1.000	-.167	.2429	1.000	-1.567	.6045	.310
Areca husk – 40	Control	.533(*)	.1255	.011	1.167(*)	.2429	.003	3.833(*)	.6045	.000
	Tobacco 1	-.033	.1255	1.000	-.533	.2429	.530	-1.500	.6045	.365
	Mango leaf - 60	.467(*)	.1255	.037	.767	.2429	.116	3.167(*)	.6045	.001
	Coconut husk -60	.433	.1255	.064	.667	.2429	.242	3.333(*)	.6045	.001
	Areca husk – 60	.300	.1255	.415	-.133	.2429	1.000	.800	.6045	.954
Mango leaf - 150	Control	.067	.1255	1.000	.400	.2429	.846	.667	.6045	.987
	Tobacco 1	-.500(*)	.1255	.021	-1.300(*)	.2429	.001	-4.667(*)	.6045	.000
	Coconut husk -60	-.033	.1255	1.000	-.100	.2429	1.000	.167	.6045	1.000
	Areca husk - 60	-.167	.1255	.953	-.900(*)	.2429	.038	-2.367(*)	.6045	.024
Coconut husk – 60	Control	.100	.1255	.999	.500	.2429	.615	.500	.6045	.999
	Tobacco 1	-.467(*)	.1255	.037	-1.200(*)	.2429	.002	-4.833(*)	.6045	.000
	Areca husk - 60	-.133	.1255	.990	-.800	.2429	.089	-2.533(*)	.6045	.013
Areca husk – 60	Control	.233	.1255	.735	1.300(*)	.2429	.001	3.033(*)	.6045	.002
	Tobacco 1	-.333	.1255	.280	-.400	.2429	.846	-2.300(*)	.6045	.031

Based on observed means. The mean difference is significant at the .05 level. Dependent Variable: Level, Multiple Comparisons -Tukey HSD*

Study of protective effect of selected plant materials on oxidative stress induced by tobacco sample -2

Treatment of cell culture with mango leaf extract, coconut husk extract and areca nut husk extract along with 300µg of tobacco sample-2 have shown a significant variation in oxidative parameters such as MDA, GSH levels and antioxidant enzymes such as SOD, catalase and GPx compared to tobacco treatment alone. Details of observations are expressed in fig. 8.

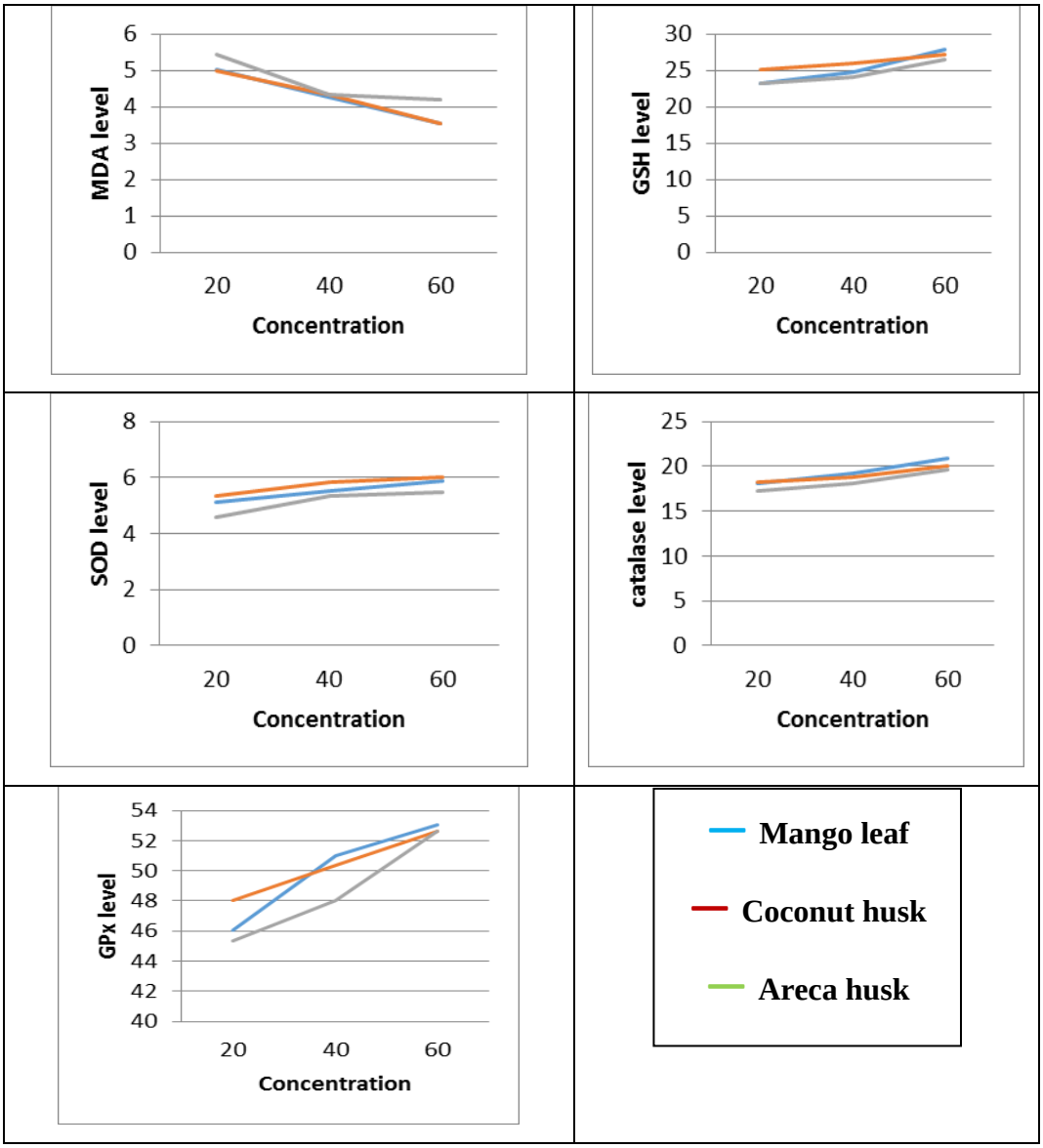


Fig. 8: Oxidative stress and antioxidant parameters in tobacco sample- 2 exposed cells after treatment with different plant materials at various concentrations (a- MDA, b- GSH, c-SOD, d- catalase, e- GPx).

When the results obtained were statistically analysed using ANOVA a highly significant difference between the groups were noted in all parameters checked with p 0.001. The MDA

values obtained after simultaneous treatment with 300 µg tobacco samples -2 and different plant extracts at concentrations 20,40 and 60mg/ml, when compared with that of 300 µg of tobacco -1 alone using Tueky HSD, all values were significantly different except that of 20mg/ml of areca nut husk extract. The MDA levels were comparable to Control value ($p \leq 0.05$) GSH levels in the same experimental conditions were statistically analysed, a significant difference was noted between the tobacco 300 µg alone and all other experimental groups except those with different plant extracts at concentration of 20mg/ml. However when the same values were compared with that of control, values were significantly different in all except those treated with mango leaf & coconut husk extract of 60mg/ml (Table 5).

Table 5: Comparison of MDA and GSH levels in tobacco sample-2 exposed cells after treatment with different plant extracts.

(J) Category	(I) Category	MDA levels			GSH levels		
		Mean Diff. (I-J)	Std. Error	P value	Mean Diff. (I-J)	Std. Error	p value
Mango leaf - 20	Control	2.000(*)	.0974	.000	-5.867(*)	.4486	.000
	Tobacco 1	-.667(*)	.0974	.000	1.300	.4486	.187
	Coconut husk – 20	.033	.0974	1.000	-2.000(*)	.4486	.007
	Areca husk – 20	-.400(*)	.0974	.016	.033	.4486	1.000
	Mango leaf - 40	.767(*)	.0974	.000	-1.633(*)	.4486	.043
	Coconut husk – 40	.700(*)	.0974	.000	-2.867(*)	.4486	.000
	Areca husk – 40	.700(*)	.0974	.000	-.967	.4486	.555
	Mango leaf - 60	1.500(*)	.0974	.000	-4.633(*)	.4486	.000
	Coconut husk –60	1.500(*)	.0974	.000	-3.967(*)	.4486	.000
	Areca husk – 60	.833(*)	.0974	.000	-3.267(*)	.4486	.000
Coconut husk - 20	Control	1.967(*)	.0974	.000	-3.867(*)	.4486	.000
	Tobacco 1	-.700(*)	.0974	.000	3.300(*)	.4486	.000
	Areca husk – 20	-.433(*)	.0974	.007	2.033(*)	.4486	.006
	Mango leaf - 40	.733(*)	.0974	.000	.367	.4486	.999
	Coconut husk – 40	.667(*)	.0974	.000	-.867	.4486	.692
	Areca husk – 40	.667(*)	.0974	.000	1.033	.4486	.465
	Mango leaf - 60	1.467(*)	.0974	.000	-2.633(*)	.4486	.000
	Coconut husk – 60	1.467(*)	.0974	.000	-1.967(*)	.4486	.008
	Areca husk – 60	.800(*)	.0974	.000	-1.267	.4486	.212
Areca husk – 20	Control	2.400(*)	.0974	.000	-5.900(*)	.4486	.000
	Tobacco 1	-.267	.0974	.246	1.267	.4486	.212
	Mango leaf - 40	1.167(*)	.0974	.000	-1.667(*)	.4486	.037
	Coconut husk – 40	1.100(*)	.0974	.000	-2.900(*)	.4486	.000
	Areca husk – 40	1.100(*)	.0974	.000	-1.000	.4486	.509
	Mango leaf - 60	1.900(*)	.0974	.000	-4.667(*)	.4486	.000
	Coconut husk – 60	1.900(*)	.0974	.000	-4.000(*)	.4486	.000
	Areca husk – 60	1.233(*)	.0974	.000	-3.300(*)	.4486	.000
Mango leaf	Control	1.233(*)	.0974	.000	-4.233(*)	.4486	.000

- 40	Tobacco 1	-1.433(*)	.0974	.000	2.933(*)	.4486	.000
	Coconut husk - 40	-.067	.0974	1.000	-1.233	.4486	.241
	Areca husk - 40	-.067	.0974	1.000	.667	.4486	.910
	Mango leaf - 60	.733(*)	.0974	.000	-3.000(*)	.4486	.000
	Coconut husk - 60	.733(*)	.0974	.000	-2.333(*)	.4486	.001
	Areca husk - 60	.067	.0974	1.000	-1.633(*)	.4486	.043
Coconut husk - 40	Control	1.300(*)	.0974	.000	-3.000(*)	.4486	.000
	Tobacco 1	-1.367(*)	.0974	.000	4.167(*)	.4486	.000
	Areca husk - 40	.000	.0974	1.000	1.900(*)	.4486	.012
	Mango leaf- 60	.800(*)	.0974	.000	-1.767(*)	.4486	.023
	Coconut husk - 60	.800(*)	.0974	.000	-1.100	.4486	.380
	Areca husk - 60	.133	.0974	.944	-.400	.4486	.997
Areca husk – 40	Control	1.300(*)	.0974	.000	-4.900(*)	.4486	.000
	Tobacco 1	-1.367(*)	.0974	.000	2.267(*)	.4486	.002
	Mango leaf - 60	.800(*)	.0974	.000	-3.667(*)	.4486	.000
	Coconut husk - 60	.800(*)	.0974	.000	-3.000(*)	.4486	.000
	Areca husk - 60	.133	.0974	.944	-2.300(*)	.4486	.002
Mango leaf - 60	Control	.500(*)	.0974	.002	-1.233	.4486	.241
	Tobacco 1	-2.167(*)	.0974	.000	5.933(*)	.4486	.000
	Coconut husk - 60	.000	.0974	1.000	.667	.4486	.910
	Areca husk - 60	-.667(*)	.0974	.000	1.367	.4486	.143
Coconut husk - 60	Control	.500(*)	.0974	.002	-1.900(*)	.4486	.012
	Tobacco 1	-2.167(*)	.0974	.000	5.267(*)	.4486	.000
	Areca husk - 60	-.667(*)	.0974	.000	.700	.4486	.882
Areca husk – 60	Control	1.167(*)	.0974	.000	-2.600(*)	.4486	.000
	Tobacco 1	-1.500(*)	.0974	.000	4.567(*)	.4486	.000

*Based on observed means.*The mean difference is significant at the .05 level. Dependent Variable: Level Multiple Comparisons -Tukey HSD*

SOD levels in plant extract treated samples showed a similar relation as other antioxidant enzymes with respect to comparison with tobacco treated samples but SOD values reached a comparable level to control in case of cell culture treated with all different plant extracts at concentrations 60mg/ml and both mango leaf and coconut husk extract at 40mg/ml. Catalase enzyme levels were significantly different compared the tobacco 300 µg alone except those treated with different plant extracts at concentration of 20mg/ml and areca nut husk extract of 40mg/ml. and when the same values were compared with that of control, values were significantly different in all except those treated with mango leaf and coconut husk extract of 60mg/ml. Like catalase, GPx levels also were significantly different compared the tobacco 300 µg alone except those treated with mango leaf and areca nut husk extracts at concentration of 20mg/ml. However the values with respect to any of the plant extracts in all different concentrations studied, could reach the level of control value, exhibiting a statistically significant difference (Table 6).

Table 6: Comparison of SOD, catalase and GPx levels in tobacco sample- 2 exposed cells after treatment with different plant extracts

(J) Category	(I) Category	SOD levels		Catalase levels			GPx levels			
		Mean Diff. (I-J)	S.E	P value	Mean Diff. (I-J)	S.E	p value	Mean Diff. (I-J)	S.E.	P value
Mango leaf - 20	Control	-.967(*)	.2035	.004	-3.133(*)	.3913	.000	-9.667(*)	.6012	.000
	Tobacco 1	.600	.2035	.171	.967	.3913	.371	.567	.6012	.996
	Coconut husk – 20	-.200	.2035	.994	-.100	.3913	1.000	-1.967	.6012	.093
	Areca husk – 20	.533	.2035	.296	.867	.3913	.518	.667	.6012	.986
	Mango leaf - 40	-.400	.2035	.672	-1.067	.3913	.250	-4.967(*)	.6012	.000
	Coconut husk – 40	-.700	.2035	.066	-.667	.3913	.819	-4.333(*)	.6012	.000
	Areca husk – 40	-.233	.2035	.982	.033	.3913	1.000	-1.967	.6012	.093
	Mango leaf - 60	-.733(*)	.2035	.047	-2.733(*)	.3913	.000	-7.000(*)	.6012	.000
	Coconut husk –60	-.867(*)	.2035	.011	-1.967(*)	.3913	.002	-6.633(*)	.6012	.000
	Areca husk – 60	-.367	.2035	.767	-1.500(*)	.3913	.029	-6.633(*)	.6012	.000
Coconut husk – 20	Control	-.767(*)	.2035	.033	-3.033(*)	.3913	.000	-7.700(*)	.6012	.000
	Tobacco 1	.800(*)	.2035	.023	1.067	.3913	.250	2.533(*)	.6012	.012
	Areca husk – 20	.733(*)	.2035	.047	.967	.3913	.371	2.633(*)	.6012	.009
	Mango leaf - 40	-.200	.2035	.994	-.967	.3913	.371	-3.000(*)	.6012	.002
	Coconut husk – 40	-.500	.2035	.378	-.567	.3913	.922	-2.367(*)	.6012	.023
	Areca husk – 40	-.033	.2035	1.000	.133	.3913	1.000	.000	.6012	1.00
	Mango leaf - 60	-.533	.2035	.296	-2.633(*)	.3913	.000	-5.033(*)	.6012	.000
	Coconut husk – 60	-.667	.2035	.092	-1.867(*)	.3913	.003	-4.667(*)	.6012	.000
	Areca husk – 60	-.167	.2035	.999	-1.400(*)	.3913	.050	-4.667(*)	.6012	.000
Areca husk – 20	Control	-1.500(*)	.2035	.000	-4.000(*)	.3913	.000	-10.333(*)	.6012	.000
	Tobacco 1	.067	.2035	1.000	.100	.3913	1.000	-.100	.6012	1.00
	Mango leaf - 40	-.933(*)	.2035	.005	-1.933(*)	.3913	.002	-5.633(*)	.6012	.000
	Coconut husk – 40	-1.233(*)	.2035	.000	-1.533(*)	.3913	.024	-5.000(*)	.6012	.000
	Areca husk – 40	-.767(*)	.2035	.033	-.833	.3913	.571	-2.633(*)	.6012	.009
	Mango leaf - 60	-1.267(*)	.2035	.000	-3.600(*)	.3913	.000	-7.667(*)	.6012	.000
	Coconut husk – 60	-1.400(*)	.2035	.000	-2.833(*)	.3913	.000	-7.300(*)	.6012	.000
	Areca husk – 60	-.900(*)	.2035	.008	-2.367(*)	.3913	.000	-7.300(*)	.6012	.000

Mango leaf - 40	Control	-.567	.2035	.227	-2.067(*)	.3913	.001	-4.700(*)	.6012	.000
	Tobacco 1	1.000(*)	.2035	.003	2.033(*)	.3913	.001	5.533(*)	.6012	.000
	Coconut husk – 40	-.300	.2035	.914	.400	.3913	.992	.633	.6012	.990
	Areca husk – 40	.167	.2035	.999	1.100	.3913	.217	3.000(*)	.6012	.002
	Mango leaf - 60	-.333	.2035	.850	-1.667(*)	.3913	.011	-2.033	.6012	.074
	Coconut husk - 60	-.467	.2035	.471	-.900	.3913	.467	-1.667	.6012	.232
	Areca husk – 60	.033	.2035	1.000	-.433	.3913	.986	-1.667	.6012	.232
Coconut husk –40	Control	-.267	.2035	.957	-2.467(*)	.3913	.000	-5.333(*)	.6012	.000
	Tobacco 1	1.300(*)	.2035	.000	1.633(*)	.3913	.014	4.900(*)	.6012	.000
	Areca husk - 40	.467	.2035	.471	.700	.3913	.774	2.367(*)	.6012	.023
	Mango leaf- 60	-.033	.2035	1.000	-2.067(*)	.3913	.001	-2.667(*)	.6012	.008
	Coconut husk - 60	-.167	.2035	.999	-1.300	.3913	.084	-2.300(*)	.6012	.029
	Areca husk - 60	.333	.2035	.850	-.833	.3913	.571	-2.300(*)	.6012	.029
Areca husk – 40	Control	-.733(*)	.2035	.047	-3.167(*)	.3913	.000	-7.700(*)	.6012	.000
	Tobacco 1	.833(*)	.2035	.016	.933	.3913	.417	2.533(*)	.6012	.012
	Mango leaf - 60	-.500	.2035	.378	-2.767(*)	.3913	.000	-5.033(*)	.6012	.000
	Coconut husk - 60	-.633	.2035	.126	-2.000(*)	.3913	.002	-4.667(*)	.6012	.000
	Areca husk - 60	-.133	.2035	1.000	-1.533(*)	.3913	.024	-4.667(*)	.6012	.000
Mango leaf - 60	Control	-.233	.2035	.982	-.400	.3913	.992	-2.667(*)	.6012	.008
	Tobacco 1	1.333(*)	.2035	.000	3.700(*)	.3913	.000	7.567(*)	.6012	.000
	Coconut husk - 60	-.133	.2035	1.000	.767	.3913	.676	.367	.6012	1.000
	Areca husk - 60	.367	.2035	.767	1.233	.3913	.117	.367	.6012	1.000
Coconut husk –60	Control	-.100	.2035	1.000	-1.167	.3913	.161	-3.033(*)	.6012	.002
	Tobacco 1	1.467(*)	.2035	.000	2.933(*)	.3913	.000	7.200(*)	.6012	.000
	Areca husk - 60	.500	.2035	.378	.467	.3913	.977	.000	.6012	1.000
Areca husk – 60	Control	-.600	.2035	.171	-1.633(*)	.3913	.014	-3.033(*)	.6012	.002
	Tobacco 1	.967(*)	.2035	.004	2.467(*)	.3913	.000	7.200(*)	.6012	.000

Based on observed means. The mean difference is significant at the .05 level. Dependent Variable: Level of activity, Multiple Comparisons - Tukey HSD*

Protective effect of selected plant materials on oxidative stress induced by tobacco sample - 3

Treatment of cell culture with mango leaf extract, coconut husk extract and areca nut husk extract along with 300µg of Tobacco sample- 3 have shown a significant variation in oxidative parameters such as MDA and GSH levels and antioxidant enzymes such as SOD, catalase and GPx compared to tobacco treatment alone. Details of observations are expressed in fig. 9.

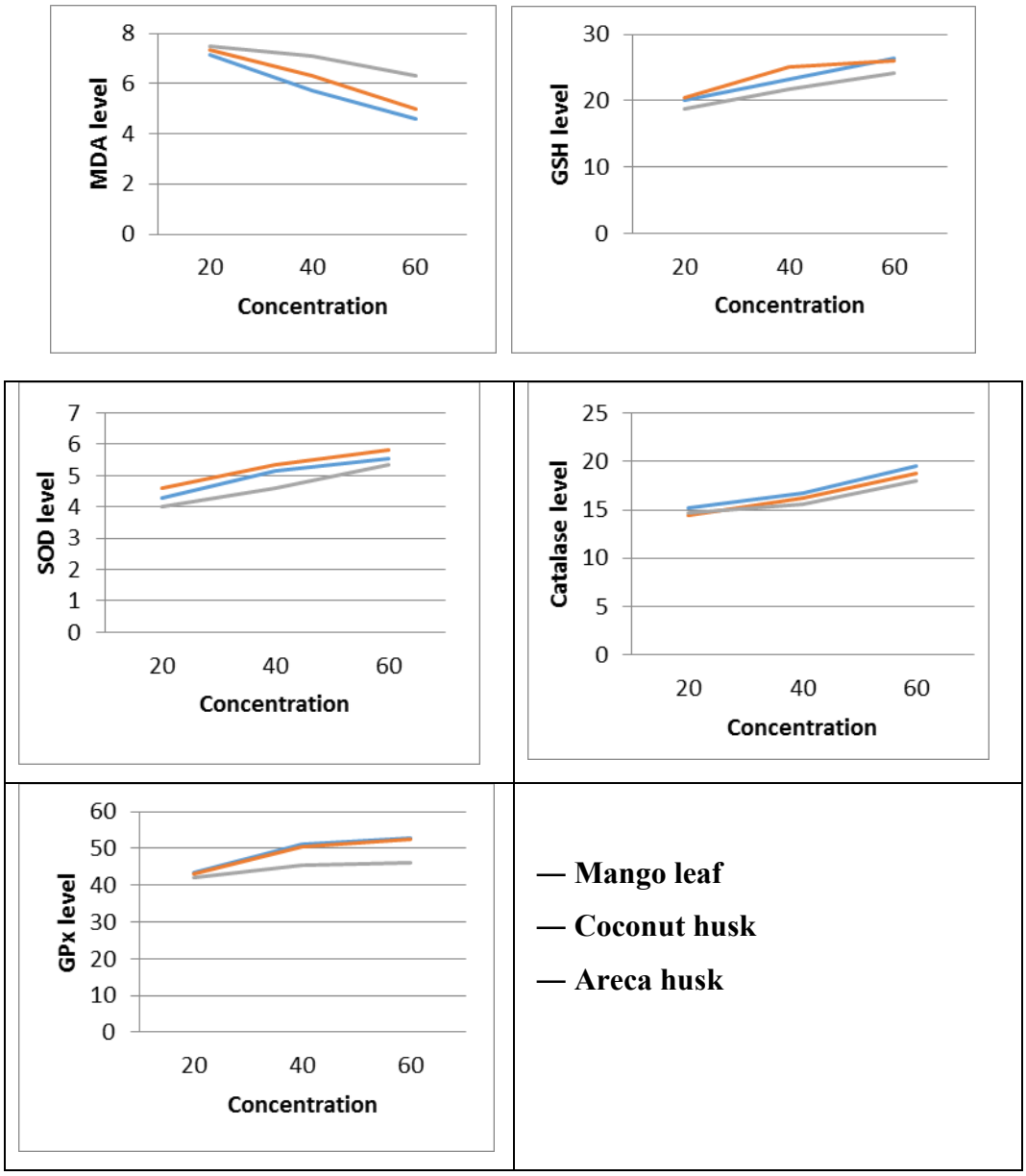


Fig. 9: Oxidative stress and antioxidant parameters in tobacco sample- 3 exposed cells after treatment with different plant materials at various concentrations (a- MDA, b- GSH, c-SOD, d- catalase, e- GPx).

When the results of oxidative stress parameters after treating the cell culture simultaneously with tobacco 3 at concentration of 300 µg and different plant extracts of various concentrations, were statistically analysed using ANOVA a highly significant difference between the groups were noted in all parameters checked with $p \leq 0.05$. When multiple comparison was done using Tukey HSD, The MDA levels have shown a statistically significant difference compared to tobacco exposure alone, after treatment with all plant extracts in a concentration of 60mg/ml and both mango leaf and coconut husk extracts in 40mg/ml concentration. When compared to control the all the values were statistically different. GSH levels were statistically different in all samples treated with different plant extract along with tobacco- 3 of 300 µg when compared to tobacco alone and also the values fail to reach a comparable level to the control even in different plant extract of highest concentration studied with a statistically significant difference (Table 7).

Except for the experimental samples with tobacco and mango leaf and areca nut husk extract of 20mg/ml concentration all other samples showed a statistically significant variation in SOD levels and those treated with mango leaf and coconut husk extracts of 60mg/ml concentration showed SOD levels statistically comparable to that of control. When the catalase levels of cell culture exposed 300 µg of tobacco sample- 3 and plant extracts were compared with that of tobacco alone, a statistically different values were noted in all samples except for plant extracts at 20mg/ml concentration. But none of the values were statistically comparable to control. With respect to GPx, all test samples showed statistically different values compared to tobacco treatment alone. Then again none of the values have reached to a level which is statistically comparable to controls (Table 8).

Table 7: Comparison of MDA and GSH levels in tobacco sample- 3 exposed cells after treatment with different plant extracts.

(J) Category	(I) Category	MDA levels			GSH levels		
		Mean Diff. (I-J)	S.E	P value	Mean Diff. (I-J)	S.E	p value
Mango leaf - 20	Control	4.133(*)	.1990	.000	-9.033(*)	.3929	.000
	Tobacco 1	-.600	.1990	.151	2.733(*)	.3929	.000
	Coconut husk – 20	-.200	.1990	.993	-.367	.3929	.996
	Areca husk – 20	-.300	.1990	.902	1.200	.3929	.141
	Mango leaf - 40	1.467(*)	.1990	.000	-3.167(*)	.3929	.000
	Coconut husk – 40	.833(*)	.1990	.013	-5.167(*)	.3929	.000
	Areca husk – 40	.067	.1990	1.000	-1.800(*)	.3929	.005
	Mango leaf - 60	2.567(*)	.1990	.000	-6.433(*)	.3929	.000
	Coconut husk –60	2.167(*)	.1990	.000	-6.033(*)	.3929	.000

	Areca husk – 60	.833(*)	.1990	.013	-4.133(*)	.3929	.000
Coconut husk – 20	Control	4.333(*)	.1990	.000	-8.667(*)	.3929	.000
	Tobacco 1	-.400	.1990	.645	3.100(*)	.3929	.000
	Areca husk – 20	-.100	.1990	1.000	1.567(*)	.3929	.021
	Mango leaf - 40	1.667(*)	.1990	.000	-2.800(*)	.3929	.000
	Coconut husk – 40	1.033(*)	.1990	.001	-4.800(*)	.3929	.000
	Areca husk – 40	.267	.1990	.951	-1.433(*)	.3929	.043
	Mango leaf - 60	2.767(*)	.1990	.000	-6.067(*)	.3929	.000
	Coconut husk – 60	2.367(*)	.1990	.000	-5.667(*)	.3929	.000
	Areca husk – 60	1.033(*)	.1990	.001	-3.767(*)	.3929	.000
Areca husk- 20	Control	4.433(*)	.1990	.000	-10.233(*)	.3929	.000
	Tobacco 1	-.300	.1990	.902	1.533(*)	.3929	.025
	Mango leaf - 40	1.767(*)	.1990	.000	-4.367(*)	.3929	.000
	Coconut husk – 40	1.133(*)	.1990	.000	-6.367(*)	.3929	.000
	Areca husk – 40	.367	.1990	.744	-3.000(*)	.3929	.000
	Mango leaf - 60	2.867(*)	.1990	.000	-7.633(*)	.3929	.000
	Coconut husk – 60	2.467(*)	.1990	.000	-7.233(*)	.3929	.000
	Areca husk – 60	1.133(*)	.1990	.000	-5.333(*)	.3929	.000
Mango leaf - 40	Control	2.667(*)	.1990	.000	-5.867(*)	.3929	.000
	Tobacco 1	-2.067(*)	.1990	.000	5.900(*)	.3929	.000
	Coconut husk – 40	-.633	.1990	.110	-2.000(*)	.3929	.002
	Areca husk – 40	-1.400(*)	.1990	.000	1.367	.3929	.061
	Mango leaf - 60	1.100(*)	.1990	.001	-3.267(*)	.3929	.000
	Coconut husk - 60	.700	.1990	.056	-2.867(*)	.3929	.000
	Areca husk – 60	-.633	.1990	.110	-.967	.3929	.376
Coconut husk – 40	Control	3.300(*)	.1990	.000	-3.867(*)	.3929	.000
	Tobacco 1	-1.433(*)	.1990	.000	7.900(*)	.3929	.000
	Areca husk - 40	-.767(*)	.1990	.028	3.367(*)	.3929	.000
	Mango leaf- 60	1.733(*)	.1990	.000	-1.267	.3929	.102
	Coconut husk - 60	1.333(*)	.1990	.000	-.867	.3929	.523
	Areca husk - 60	.000	.1990	1.000	1.033	.3929	.292
Areca husk - 40	Control	4.067(*)	.1990	.000	-7.233(*)	.3929	.000
	Tobacco 1	-.667	.1990	.079	4.533(*)	.3929	.000
	Mango leaf - 60	2.500(*)	.1990	.000	-4.633(*)	.3929	.000
	Coconut husk - 60	2.100(*)	.1990	.000	-4.233(*)	.3929	.000
	Areca husk - 60	.767(*)	.1990	.028	-2.333(*)	.3929	.000
Mango leaf - 60	Control	1.567(*)	.1990	.000	-2.600(*)	.3929	.000
	Tobacco 1	-3.167(*)	.1990	.000	9.167(*)	.3929	.000
	Coconut husk - 60	-.400	.1990	.645	.400	.3929	.993
	Areca husk - 60	-1.733(*)	.1990	.000	2.300(*)	.3929	.000
Coconut husk – 60	Control	1.967(*)	.1990	.000	-3.000(*)	.3929	.000
	Tobacco 1	-2.767(*)	.1990	.000	8.767(*)	.3929	.000
	Areca husk - 60	-1.333(*)	.1990	.000	1.900(*)	.3929	.003
Areca husk - 60	Control	3.300(*)	.1990	.000	-4.900(*)	.3929	.000
	Tobacco 1	-1.433(*)	.1990	.000	6.867(*)	.3929	.000

*Based on observed means.*The mean difference is significant at the .05 level.*

Dependent Variable: Level Multiple Comparisons -Tukey HSD.

Table 8: Comparison of SOD, catalase and GPx levels in tobacco sample -3 exposed cells after treatment with different plant extracts.

		SOD levels			Catalase levels			GPx levels		
(J) Category	**(I) Category**	**Mean Diff. (I-J)**	**S.E**	**P value**	**Mean Diff. (I-J)**	**S.E**	**P value**	**Mean Diff. (I-J)**	**S. E**	**P value**
Mango leaf - 20	Control	-1.833(*)	.1621	.000	-6.000(*)	.3101	.000	-12.367(*)	.5819	.000
	Tobacco 1	.433	.1621	.272	.900	.3101	.185	1.900	.5819	.094
	Coconut husk – 20	-.333	.1621	.616	.800	.3101	.315	.333	.5819	1.000
	Areca husk – 20	.267	.1621	.846	.600	.3101	.690	1.300	.5819	.506
	Mango leaf - 40	-.867(*)	.1621	.001	-1.467(*)	.3101	.004	-7.667(*)	.5819	.000
	Coconut husk – 40	-1.067(*)	.1621	.000	-.967	.3101	.125	-7.033(*)	.5819	.000
	Areca husk – 40	-.333	.1621	.616	-.333	.3101	.989	-2.033	.5819	.059
	Mango leaf - 60	-1.267(*)	.1621	.000	-4.367(*)	.3101	.000	-9.333(*)	.5819	.000
	Coconut husk –60	-1.567(*)	.1621	.000	-3.533(*)	.3101	.000	-9.000(*)	.5819	.000
	Areca husk – 60	-1.100(*)	.1621	.000	-2.833(*)	.3101	.000	-2.700(*)	.5819	.005
Coconut husk – 20	Control	-1.500(*)	.1621	.000	-6.800(*)	.3101	.000	-12.700(*)	.5819	.000
	Tobacco 1	.767(*)	.1621	.004	.100	.3101	1.000	1.567	.5819	.264
	Areca husk – 20	.600(*)	.1621	.038	-.200	.3101	1.000	.967	.5819	.839
	Mango leaf - 40	-.533	.1621	.089	-2.267(*)	.3101	.000	-8.000(*)	.5819	.000
	Coconut husk – 40	-.733(*)	.1621	.006	-1.767(*)	.3101	.000	-7.367(*)	.5819	.000
	Areca husk – 40	.000	.1621	1.000	-1.133(*)	.3101	.042	-2.367(*)	.5819	.017
	Mango leaf - 60	-.933(*)	.1621	.000	-5.167(*)	.3101	.000	-9.667(*)	.5819	.000
	Coconut husk – 60	-1.233(*)	.1621	.000	-4.333(*)	.3101	.000	-9.333(*)	.5819	.000
	Areca husk – 60	-.767(*)	.1621	.004	-3.633(*)	.3101	.000	-3.033(*)	.5819	.001
Areca husk – 20	Control	-2.100(*)	.1621	.000	-6.600(*)	.3101	.000	-13.667(*)	.5819	.000
	Tobacco 1	.167	.1621	.992	.300	.3101	.995	.600	.5819	.992
	Mango leaf - 40	-1.133(*)	.1621	.000	-2.067(*)	.3101	.000	-8.967(*)	.5819	.000
	Coconut husk – 40	-1.333(*)	.1621	.000	-1.567(*)	.3101	.002	-8.333(*)	.5819	.000
	Areca husk – 40	-.600(*)	.1621	.038	-.933	.3101	.153	-3.333(*)	.5819	.000
	Mango leaf - 60	-1.533(*)	.1621	.000	-4.967(*)	.3101	.000	-10.633(*)	.5819	.000
	Coconut husk – 60	-1.833(*)	.1621	.000	-4.133(*)	.3101	.000	-10.300(*)	.5819	.000
	Areca husk – 60	-1.367(*)	.1621	.000	-3.433(*)	.3101	.000	-4.000(*)	.5819	.000

Mango leaf - 40	Control	-.967(*)	.1621	.000	-4.533(*)	.3101	.000	-4.700(*)	.5819	.000
	Tobacco 1	1.300(*)	.1621	.000	2.367(*)	.3101	.000	9.567(*)	.5819	.000
	Coconut husk – 40	-.200	.1621	.971	.500	.3101	.861	.633	.5819	.988
	Areca husk - 40	.533	.1621	.089	1.133(*)	.3101	.042	5.633(*)	.5819	.000
	Mango leaf - 60	-.400	.1621	.372	-2.900(*)	.3101	.000	-1.667	.5819	.198
	Coconut husk – 60	-.700(*)	.1621	.010	-2.067(*)	.3101	.000	-1.333	.5819	.472
	Areca husk - 60	-.233	.1621	.924	-1.367(*)	.3101	.008	4.967(*)	.5819	.000
Coconut husk – 40	Control	-.767(*)	.1621	.004	-5.033(*)	.3101	.000	-5.333(*)	.5819	.000
	Tobacco 1	1.500(*)	.1621	.000	1.867(*)	.3101	.000	8.933(*)	.5819	.000
	Areca husk - 40	.733(*)	.1621	.006	.633	.3101	.625	5.000(*)	.5819	.000
	Mango leaf- 60	-.200	.1621	.971	-3.400(*)	.3101	.000	-2.300(*)	.5819	.022
	Coconut husk – 60	-.500	.1621	.133	-2.567(*)	.3101	.000	-1.967	.5819	.075
	Areca husk - 60	-.033	.1621	1.000	-1.867(*)	.3101	.000	4.333(*)	.5819	.000
Areca husk – 40	Control	-1.500(*)	.1621	.000	-5.667(*)	.3101	.000	-10.333(*)	.5819	.000
	Tobacco 1	.767(*)	.1621	.004	1.233(*)	.3101	.021	3.933(*)	.5819	.000
	Mango leaf - 60	-.933(*)	.1621	.000	-4.033(*)	.3101	.000	-7.300(*)	.5819	.000
	Coconut husk – 60	-1.233(*)	.1621	.000	-3.200(*)	.3101	.000	-6.967(*)	.5819	.000
	Areca husk - 60	-.767(*)	.1621	.004	-2.500(*)	.3101	.000	-.667	.5819	.982
Mango leaf - 60	Control	-.567	.1621	.059	-1.633(*)	.3101	.001	-3.033(*)	.5819	.001
	Tobacco 1	1.700(*)	.1621	.000	5.267(*)	.3101	.000	11.233(*)	.5819	.000
	Coconut husk – 60	-.300	.1621	.740	.833	.3101	.266	.333	.5819	1.000
	Areca husk - 60	.167	.1621	.992	1.533(*)	.3101	.002	6.633(*)	.5819	.000
Coconut husk – 60	Control	-.267	.1621	.846	-2.467(*)	.3101	.000	-3.367(*)	.5819	.000
	Tobacco 1	2.000(*)	.1621	.000	4.433(*)	.3101	.000	10.900(*)	.5819	.000
	Areca husk – 60	.467	.1621	.193	.700	.3101	.492	6.300(*)	.5819	.000
Areca husk – 60	Control	-.733(*)	.1621	.006	-3.167(*)	.3101	.000	-9.667(*)	.5819	.000
	Tobacco 1	1.533(*)	.1621	.000	3.733(*)	.3101	.000	4.600(*)	.5819	.000

*Based on observed means.*The mean difference is significant at the .05 level. Dependent Variable: Level, Multiple Comparisons -Tukey HSD*

DISCUSSION

To analyze the extent of oxidative stress that is induced by tobacco in different forms, selected concentrations of alcoholic extracts of various tobacco products, ranging from 100-300µgs were added to oral epithelial cell culture and incubated for a period of 24 hours, following which assays for oxidative stress and antioxidant parameters were carried out.

MDA and GSH were estimated as a measure of oxidative stress and lipid peroxidation. All preparations of tobacco extract exhibited a concentration dependent decrease in GSH levels and increase in MDA generation. However the levels of these parameters in tobacco sample1 (unprocessed tobacco), treated cells were statistically less compared to other samples. On the contrary, tobacco samples 2 and 3 (processed) in all concentrations caused significantly higher increase in MDA level and lower GSH than untreated control and the alteration of above parameters were maximum with tobacco sample 3. Analysis of antioxidant enzymes also showed similar effect with significant decrease in SOD, catalase and GPx after treatment with tobacco samples which was again more significant with tobacco sample 3.

A wealth of experimental evidence, both *in vivo* and *in vitro* in cell culture systems, are available in the literature indicating that tobacco in any form induces oxidative stress.[4, 12-25] Our observation of high MDA and lower GSH level and decrease in antioxidant enzymes are consistent with these earlier reports and indicate that tobacco increases lipid peroxidation and depletion of antioxidant enzymes. However considerable variation in actual values and percentage increase or decrease in the tested parameters are noted between earlier reports and with our results. This could be due to variation in the type of tobacco samples used, concentrations tested and experimental conditions and tests applied.

In the present study, the profile of oxidative/anti-oxidative status in various experimental conditions revealed marked alterations in antioxidant enzyme activities and lipid peroxidation. Low activities of antioxidant enzymes such as SOD, CAT, and GPx might be due to the overwhelming effects of free radicals, as evidenced by the elevated levels of lipid peroxidation. Cellular antioxidant enzymes such as SOD, CAT, and GPx and free radical scavengers like GSH protect cells and tissues against noxious radicals. An imbalance between cellular pro-oxidant and antioxidant levels results in the oxidative stress that leads to tissue damage. The antioxidant enzymes react directly with reactive oxygen species (ROS) to yield non-radical products. SOD, a mitochondrial as well as cytosolic enzyme, dis-mutates O_2^{--} to H_2O_2, which is decomposed by CAT to H_2O. In the present study, the activity of SOD

was decreased significantly after exposure to tobacco extracts which might have led to the inefficient removal of $O_2^{\cdot-}$ radicals from the cellular milieu, resulting in the ROS burden. Overproduction of these radicals has an inhibitory effect on other enzymes such as CAT and GPx, responsible for removal of ROS. It has been reported that superoxide radicals inhibit CAT activity and that H_2O_2 suppresses SOD activity [26], which might explain the inhibition of these enzymes after exposure to tobacco extracts. On the other hand, GPx uses GSH to remove H_2O_2 and lipid peroxides. This prevents H_2O_2-mediated damage, which is thought to be a recognized risk factor for carcinogenesis [27]. Decreased levels of antioxidant enzymes in general and of GSH in particular caused by tobacco induced radical species, results in increased lipid peroxidation due to ineffective removal of ROS and is evidenced as increased MDA. In conclusion, tobacco impairs the enzymatic antioxidant defense system and reduces glutathione levels and these alterations may be one of the responsible factors for tobacco induced cytotoxicity and genotoxic damage.

The results obtained indicate that tobacco in all forms studied could induce oxidative stress. However unprocessed tobacco is relatively safer. The variable effects of different tobacco sample could be due to the differential load of metabolites present in these samples. Tobacco sample1, i.e., fresh tobacco leaves from farm induced only minimum oxidative stress while processed tobacco and commercially dispensed tobacco products caused significant oxidative stress. This difference can be related to the difference in nitrosamine levels in different samples which in turn is related to various factors such as the post-harvest processing (e.g., curing) to which tobacco is subjected.[18,19] Although it is generally considered that green and freshly harvested tobacco leaves are virtually free of TSNA, in our sample, some amount of TSNA would have formed during the process of preparation of extract, possibly due to microbial action, in spite of all aseptic precautions taken. Tobacco used for chewing undergo curing during which the leaves are exposed to a temperature between 82-160°C for three days to ten weeks, which leads to release of TSNAs and this explains the reason for more oxidative stress caused by other two samples. While comparing the tobacco sample 2 and 3, the difference in processing techniques .i.e. additional fermentation for commercial tobacco products and also addition of flavoring agents and vegetable dyes could be the reason for difference in oxidative stress levels.

The data obtained while evaluating the protective effect of mango leaf, husk of coconut and areca demonstrated the wide range of antioxidant activity. Among these, mango leaves

showed the highest antioxidant activity followed by coconut husk and then areca husk which can be directly related to the total phenolic components. The high antioxidant activity of these extracts can be partly attributed to radical-scavenging and reducing mechanisms demonstrated in earlier section.

Thus the present study along with earlier reports confirmed that tobacco in any form induces oxidative stress in cell culture system in a dose dependent manner and antioxidants can reduce the extent of tobacco induced oxidative stress.[5,28-33] Further studies on the actual mechanism of action may elucidate how the plant materials such as mango leaf, husk of coconut and areca reduce the tobacco induced oxidative stress. This may offer therapeutic opportunities and ways to modify the adverse effects of tobacco.

CONCLUSION

When Tobacco induced oxidative stress and effect on antioxidant parameters were studied, it was noted that extracts of tobacco sample - 1, 2 and 3 caused a concentration dependent increase of lipid peroxidation in cell culture, expressed as increased MDA levels and decreased GSH and antioxidant enzyme levels. However, the effect of unprocessed tobacco sample was significantly less compared to other processed samples and lower concentration of it did not show any significant alteration in tested parameters studied compared to untreated control. Significant difference also was noted between tobacco samples - 2 & 3 with highest level of oxidative stress parameters expressed with tobacco sample – 3, i.e. commercially dispensed tobacco. Treatment of cell culture with mango leaf extract, coconut husk extract and areca husk extract along with Tobacco samples have shown a significant variation in oxidative parameters such as MDA and GSH levels and antioxidant enzymes such as SOD, catalase and GPx indicating protective effect. Although the activity levels varied between plant materials, all three showed concentration dependent increase in activity.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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