Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 10, Issue, 07, pp.71149-71155, July, 2018

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

AN EXPLORATION ON CYTOTOXICITY INDUCED BY TOBACCO PRODUCTS AND CYTO-PROTECTIVE EFFECTS OF CERTAIN PLANT EXTRACTS

[1]Maji Jose, [2]Vengal Ipe Varghese, [3]Varsha Jayakar, [3]Vinayak Lokapur, [3]Srinivasa, K., [3]Pratap, G.K. and [3]*Manjula Shantaram

[1]Department of Oral Pathology and Microbiology, Yenepoya Dental College, Yenepoya University, Nityanandanagar, Deralakatta, Mangalore, Karnataka, India, 575 018
[2]Registrar and Professor of Oral Pathology, Kerala University of Health Sciences, Thrissur, Kerala, India, 680 596
[3]Department of Studies and Research in Biochemistry, PG Centre, Jnana Kaveri, ChikkaAluvara, Kodagu, Karnataka, India, 571 232

ARTICLE INFO

Article History:
Received 28th April, 2018
Received in revised form
01st May, 2018
Accepted 27th June, 2018
Published online 30th July, 2018

Key Words:

Cytotoxicity, Tobacco,
Cyto-Protection,
Mango Leaves, Coconut Husk,
Areca Husk.

ABSTRACT

Cytotoxic effect of tobacco both *in vivo* and *in vitro* is confirmed by a wealth of scientific evidences available in literature. It is established that smokeless tobacco related tissue damage is related to reactive oxygen species production in oral cells, peritoneal macrophages, hepatic mitochondria and microsomes. In the present study, it is explored to compare the cytotoxic effects of three different tobacco samples and cyto-protective effects of three different plant extracts. Cyto-toxicity induced by tobacco samples andcyto-protective effects of plant extracts, when analysed, a reduction in % cell viability of oral epithelial cells after incubating with different tobacco extract was noted in a concentration dependent manner. Viability noted with tobacco -1 exposure was 73-52 % with increasing concentration, with tobacco -2 was 75 to 32% and with tobacco-3 was 52 to 23%. Simultaneous treatment with plant extracts along with tobacco samples resulted in reduction in cytotoxic effect reflected as increase in % viability than tobacco treatment alone, indicating the cyto-protective effect of plant extracts, but not to the level of untreated controls.

***Citation: Maji Jose, Vengal Ipe Varghese, Varsha Jayakar, Vinayak Lokapur, Srinivasa, K., Pratap, G.K. and Manjula Shantaram*, 2018.** "An Exploration on Cytotoxicity Induced by Tobacco Products and Cyto-Protective Effects of Certain Plant Extracts", *International Journal of Current Research*, 10, (07), 71149-71155.

INTRODUCTION

Tobacco is a menace that has grabbed millions of people all over the world, cutting across the nation and social barriers. Tobacco was introduced into India by Portuguese traders in the late 16th or early 17th century. Since then tobacco use has spread with remarkable rapidity seeping into all sections of the society. In India, people consume tobacco in different forms. Use of smokeless tobacco in various forms particularly traditional betel quid chewing is one of the most popular tobacco habits among Indians (Yang and Wen, 1996). One of the commercial replacements for betel quid is *gutka,* where tobacco along with other ingredients is dispensed in ready to use packets.

Corresponding author: **Manjula Shantaram**
Department of Studies and Research in Biochemistry, PG Centre, Jnana Kaveri, ChikkaAluvara, Kodagu, Karnataka, India, 571 232
DOI: https://doi.org/10.24941/ijcr.31307.07.2018

The packaging revolution has made tobacco products portable, cheap and convenient, with the added advantage of a long shelf-life, making it highly popular among youngsters (Padma and Lalitha, 1989). Preparations of smokeless tobacco, 4-methyl-*N*-nitrosamino-1-(3-pyridyl)-1-butanoneand *N*-nitrosonornicotinecause oxidative lesions leading to cytotoxic damage. These lesions may be attributable to nitric oxide and peroxynitrate through nitrosative damage. Cytotoxic effect of tobacco both *in vivo* and *in vitro* is confirmed by a wealth of scientific evidences available in literature. Two oral squamous cell carcinoma cell lines and normal human gingival epithelial cells were treated with cigarette smoke total particulate matter, smokeless tobacco extracted with complete artificial saliva, or whole-smoke conditioned media. When normalized to nicotine content, cytotoxicity for whole-smoke conditioned media and total particulate matter was higher compared to that observed with smokeless tobacco extract(STE) while nicotine alone had no or only minimal cytotoxicity. This was mediated through activation of pro-apoptotic caspase-3 and thus authors

demonstrated differential responses of normal and malignant oral cells after exposure to different extracts (Hong*et al*., 2013). Electron microscopic study in liver tissue carried out by Bagchi*et al*., (1995) revealed an accumulation of indistinct filamentous material in the peri-sinusoidal spaces following 60 days of treatment with STE, occupying most of the sinusoids which was confirmed as antibody against heat shock/stress protein by Western blot analysis.By their *in vitro* experiments Bagchi*et al*., (1996) also established that oral cells, peritoneal macrophages, hepatic mitochondria and microsomes produce reactive oxygen species (ROS) following *in vitro*incubation with STE and therefore, ST related tissue damage is related to ROS production. Smokeless tobacco (ST) extracts also found to be influencing the complement system particularly activate the alternative pathway and also some measure of classical pathway suggesting this may be a mechanism for initiating inflammation of the oral mucosa (Chang *et al.,* 1998). A greater numbers of mitochondria in cells of ST-treated of the hyperplastic epithelia was noted in hamster cheek pouch (HCP) epithelium treated with ST than in the normal epithelium indicative of cytotoxic effect (Schwartz *et al*., 2010).

While analysing the possible transient disruptions in the cell membrane caused moist smokeless tobacco (MST), Joyce *et al.,* (2010) verified that MST-induced oral injury may result from a combined interaction of physical disruption of the plasma membrane by the tobacco material itself and the adverse effects of MST chemical constituents, notably high levels of calcium, that gain entry to the cell by way of MST-induced cell wounding. An *in vitro* and clinical study on harm and harm reduction in smokeless tobacco users conducted by Wallstrom(2010) showed that smokeless tobacco extract caused a significant dose dependent inhibition of proliferation of spleen cells, T cells, epithelial cells, including Langerhans' cells.A study on differential cellular/molecular responses of tobacco product preparations in short-term cell culture suggested that combustible tobacco product preparations induced higher cytotoxicity than STE, indicating that relative cytotoxic and other cell biological effects of tobacco product preparations are dose-dependent, and that ST extract is the least cytotoxic tobacco product preparations tested in this study (Arimilli*et al*., 2012). A study investigated the direct effect of reference moist STE exposure on the viability of MM6 monocyte/macrophage cell line led to a significant and dose-related decrease in cell viability which were inhibited by pre-incubation with a pan-caspase inhibitor, suggesting that the observed STE toxicity was due to the induction of apoptosis. Further evaluation confirmed that apoptosis is induced in part; by reference STE-mediated osmotic stress (Lombard *et al*., 2010). A dose-dependent induction of apoptosis that is mediated by nitric oxide by STE is reported (Mangipudyand Vishwanatha, 1999). In the present study, it is explored to compare the cytotoxic effects of three different tobacco samples and cyto-protective effects of three different plant extracts.

MATERIALS AND METHODS

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 was collected from farm where it is grown for commercial purpose while tobacco sample- 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. This selection was based on an earlier survey conducted by students of Department of Oral Pathology, Yenepoya Dental College. For the preparation of extract of fresh tobacco leaves, same procedure used for other plant materials was followed. 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. Other samples were directly subjected to extraction procedure. The crude alcoholic extracts thus prepared were used for further study.

Concentration dependent effects of different tobacco extracts on cell viability of cultured oral epithelial cells were assessed by trypan blue exclusion method (Iliya and Wallace, 2011) and MTT assay (Gerlierand Thomasset, 1986). Different concentrations of tobacco products were added to oral epithelial cells cultured in Stem line ^{TM} keratinocyte medium. Cultured oral epithelial cells were seeded at 1×10^5 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. A well with cultured cells, with no extract added was the negative control. After incubation for 24 hours, *in vitro* cytotoxicity induced by tobacco was determined by the above mentioned assays.

Trypan blue exclusion test of cell viability: For the analysis of cytotoxic effect of different tobacco samples at different concentrations, cultured oral epithelial cells were seeded at 1×10^5 cells/mL in 6-well plates for 24 hours to allow cell adherence. After incubation, cells were treated with different concentrations of various tobacco extracts ranging from 100-300µg and incubated for a period of 24 hours. After 24 hours, adherent and floating cells were collected and centrifuged for 5 minutes at 100 × g and discarded the supernatant. The cell pellet obtained was re-suspended in 1 ml PBS and 1 part of 0.4% trypan blue and 1 part dilution of cells was mixed. The mixture was incubated for 3 minutes at room temperature. A drop of the trypan blue/cell mixture was applied to a haemocytometer, and counted the unstained (viable) and stained (nonviable) cells separately in thehaemocytometer using a binocular microscope at 40X (Olympus CH 20). To obtain the total number of viable cells per ml of aliquot, the total number of viable cells was multiplied by 2, (the dilution factor for trypan blue) and to obtain the total number of cells per ml of aliquot, the total numbers of viable and nonviable cells were added up and multiplied by 2. The percentage of viable cells was calculated as follows:

$$\text{Viable cells (\%)} = \frac{\text{Total number of viable cells per ml of aliquot}}{\text{Total number of cells per ml of aliquot}} \times 100$$

MTT Assay: Cell viability was also assessed using a precise colorimetric technique i.e. MTT assay, a quantitative, more sensitive test, that measures the reduction of 3-(4,5 dimethyl thiazole – 2-yl),2,5,diphenyl tetrazolium bromide (MTT)by

mitochondrial succinate dehydrogenase. To the cell culture suspension, 30 µL MTT was added and kept for incubation at 37ºC for 4 hrs. After incubation, 200 microliter of DMSO was added to each culture plate, incubated at room temperature for 30 minutes, until homogenous colour was obtained. After scraping, the absorbance was read at 540 nm using DMSO as blank and percentage of viability was calculated.

$$\% \text{ of viability} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

To study the protective effect of plant materials, both trypan blue exclusion assay and MTT assay were repeated, after incubating the cultured oral epithelial cells simultaneously with tobacco samples and plant extracts. As tobacco sample -1 did not show significant reduction in cell viability in studied concentrations, only tobacco samples -2 and 3 were chosen for this purpose at a concentration of 300µg /ml and extracts of mango leaves, husk of coconut or areca nut in three different concentrations namely 20, 40 and 60mg /ml for 24 hours. Concentration of plant extracts were decided based on IC_{50} value obtained in previous experiments. The possibility of cytotoxic effect of selected concentrations of different plant extracts were excluded by carrying out trypan blue exclusion assay of cells treated with plant extracts alone. All the above experiments were carried out in triplicate and mean value was taken to compare the effects.

RESULTS

The % cell viability tested after incubating the cultured oral epithelial cells with different tobacco extract showed a concentration dependent reduction in cell viability as noted by trypan blue exclusion method and was confirmed by MTT assay (Table 1, Figs.1 and 2). When the results observed were statistically analysed using two way ANOVA, highly significant difference with $p < 0.001$was noted between the types of tobacco samples tested and different concentrations used as observed by both trypan blue exclusion method and MTT assay. Pair-wise comparison done using Bonferroni showed statistically significant difference in percentage viability between control and different tobacco samples at all concentrations ($p<0.05$). Similarly comparison between different tobacco samples showed significant difference in cytotoxic effect between different tobacco samples at all selected concentrations studied except tobacco 2 and 3 at a concentration of 100 as noted by MTT assay but same was not observed in trypan blue exclusion method (Table 2).

Irrespective of concentration significant difference in cytotoxic effect was noted between different tobacco samples with highest effect with tobacco sample -3 followed by tobacco sample -2 and least with tobacco sample-1. The protective effect of plant materials were tested with both tobacco sample-2 and 3 as these two samples showed significant reduction in cytotoxic effects. Both the assays performed in cell culture incubated with tobacco samples along with plant material showed significantly higher viability than incubated with tobacco samples alone, indicating the protective effects (Table 3, Figs. 3 and 4). Statistically highly significant difference was observed between activity between the plant materials and different concentrations of same plant materials while analysing using two way ANOVA. Pair-wise comparison of cell viability results obtained in both trypan blue exclusion method and MTT assay by Bonferroni also showed significant difference between activities of different plant materials except for mango leaf and coconut husk at 20mg/ml concentration (Table 3). Similarly when the cell culture were treated simultaneously with tobacco sample -3 in concentration of 300 and of different concentrations of plant extracts i.e. 20, 40 and 60 mg/mlusing MTT assay and trypan blue exclusion method, improvement in the percentage cell viability was noted when compared to cells treated with tobacco alone (Table 4, Fig.4). Statistically highly significant difference was observed between activity between the plant materials and different concentrations of same plant materials while analysing with two way ANOVA. Pair-wise comparison of cell viability results obtained in both trypan blue exclusion method and MTT assay also showed significant difference between activities of different plant materials (Table 6).

DISCUSSION

Concentration dependent effects of different tobacco extracts on cell viability of human oral keratinocytes were assessed by trypan blue exclusion method and MTT assay. The assays showed a decrease in percentage cell viability with increase in concentrations of all three types of tobacco samples studied suggesting reduction in the number of cells. The decrease in percentage cell viability caused by tobacco samples suggests the toxic effects on the cells, thus, confirming the cytotoxic effects. Statistical evaluation showed a significant difference in cytotoxic effect of three tobacco samples studied and concentration dependent reduction in cell viability ($P \leq 0.05$). Irrespective of concentration, significant difference in cytotoxic effect was noted between different tobacco samples with highest effect with tobacco sample 3 followed by sample 2. Tobacco sample 1 expressed much less cytotoxic effect than other two samples of comparable concentrations and even in the highest concentration studied, the viability had not dropped down below 50%. The difference in cytotoxicity induced by different tobacco products could be directly related to the nitrosamine content in different samples, resulted from processing or due to additives used.

Trypan blue dye exclusion assay is a rapid, simple and inexpensive, traditional viability assay which is based on cell membrane integrity of living cells, as the dyes cross the compromised cell membrane and stain cellular targets or structures in dead cells. On the other hand, MTT assay is based on a biochemical event that occurs only in living cells i.e. conversion of MTT salt into a formazan product by mitochondrial succinate dehydrogenase of active mitochondria, which can then be measured as a colorimetric readout. This assay gives an indication of intact mitochondrial and also extra mitochondrial, NADH- and NADPH-dependent redox enzyme systems. Therefore, it is a precise, quantitative, more sensitive and reliable test for cell viability. The results obtained in the present study by the two methods were almost identical with mild difference in values, to be exact, a difference of 5-6% between two assays with MTT showing less % reduction. Cytotoxic effects of STE on various cell culture systems such as hamster cheek pouch cell (HCPC-1) cultures, cultured macrophages (Mitchell *et al*., 2009) alveolar type II cell-derived cell lines and human oral keratinocyte cell lines have been reported earlier (Bagchi *et al*., 1995; Bagchi *et al.*, 2001; Coppe *et al*; 2008; Mitchell *et al.*, 2009; Mitchell *et al.,* 2010; Toklu *et al.,* 2010; Boyle *et al.*, 2010).

Table 1. Percentage cell viability after exposure to different tobacco products at selected concentrations

Dependent Variable: Cell viability

		Tobaco							
		Tobacco 1		Tobacco 2		Tobacco 3		Total	
parameter	Concentratio	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation
(MTT assay)	Control	90.33	3.215	90.33	3.215	90.33	3.215	90.33	2.784
	100	73.67	2.517	57.00	2.000	52.33	1.528	61.00	9.874
	200	63.00	2.000	50.00	2.646	34.00	3.606	49.00	12.816
	300	54.67	1.155	32.67	2.082	23.67	1.528	37.00	13.883
	Total	70.42	14.061	57.50	21.961	50.08	26.630	59.33	22.565
(Trypan blue)	Control	85.00	1.000	85.00	1.000	85.00	1.000	85.00	.866
	100	78.33	2.082	62.00	2.000	55.00	2.646	65.11	10.553
	200	67.67	2.517	55.33	2.082	39.33	1.528	54.11	12.434
	300	59.00	1.000	38.00	2.000	23.33	3.055	40.11	15.640
	Total	72.50	10.501	60.08	17.661	50.67	23.853	61.08	19.843

Table 2. Comparison of percentage cell viability between different tobacco products at selected concentrations

Parameter	Concentration (µg)	(I) Tobacco	(J) Tobacco	Mean Diff. (I-J)	S.E	p(a)
MTT assay	100	Control	Tobacco 1	16.667(*)	1.915	.000
			Tobacco 2	33.333(*)	2.068	.000
			Tobacco 3	38.000(*)	2.160	.000
	200		Tobacco 1	27.333(*)	1.915	.000
			Tobacco 2	40.333(*)	2.068	.000
			Tobacco 3	56.333(*)	2.160	.000
	300		Tobacco 1	35.667(*)	1.915	.000
			Tobacco 2	57.667(*)	2.068	.000
			Tobacco 3	66.667(*)	2.160	.000
	100	Tobacco 1	Tobacco 2	16.667(*)	1.678	.000
			Tobacco 3	21.333(*)	1.678	.000
		Tobacco 2	Tobacco 3	4.667	1.678	.096
	200	Tobacco 1	Tobacco 2	13.000(*)	2.309	.004
			Tobacco 3	29.000(*)	2.309	.000
		Tobacco 2	Tobacco 3	16.000(*)	2.309	.001
	300	Tobacco 1	Tobacco 2	22.000(*)	1.333	.000
			Tobacco 2	22.000(*)	1.333	.000
		Tobacco 2	Tobacco 3	9.000(*)	1.333	.002
	100		Tobacco 1	6.667(*)	1.453	.011
			Tobacco 2	23.000(*)	1.491	.000
			Tobacco 3	30.000(*)	1.810	.000
Trypan blue	200	Control	Tobacco 1	17.333(*)	1.453	.000
			Tobacco 2	29.667(*)	1.491	.000
			Tobacco 3	45.667(*)	1.810	.000
	300		Tobacco 1	26.000(*)	1.453	.000
			Tobacco 2	47.000(*)	1.491	.000
			Tobacco 3	61.667(*)	1.810	.000
	100	Tobacco 1	Tobacco 2	16.333(*)	1.846	.000
			Tobacco 3	23.333(*)	1.846	.000
		Tobacco 2	Tobacco 3	7.000(*)	1.846	.027
	200	Tobacco 1	Tobacco 2	12.333(*)	1.700	.001
			Tobacco 3	28.333(*)	1.700	.000
		Tobacco 2	Tobacco 3	16.000(*)	1.700	.000
	300	Tobacco 1	Tobacco 2	21.000(*)	1.785	.000
			Tobacco 2	35.667(*)	1.785	.000
		Tobacco 2	Tobacco 3	14.667(*)	1.785	.001

Pair- wise Comparisons, Dependent Variable: Cell viability, Based on estimated marginal means, * The mean difference is significant at the .05 level .a Adjustment for multiple comparisons: Bonferroni

Table 3. Percentage cell viability of tobacco sample - 2 exposed cells after treatment with different plant extract at selected concentrations

Dependent Variable: Cell viability

		Plant material							
		Mango leaf		Coconut husk		Areca husk		Total	
Parameter	Concentration	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation
(MTT assay)	Control	90.33	3.215	90.33	3.215	90.33	3.215	90.33	2.784
	Tobacco 2 300	32.67	2.082	32.67	2.082	32.67	2.082	32.67	1.803
	20	45.00	3.000	43.00	2.000	30.00	1.000	39.33	7.297
	40	61.33	1.528	49.00	2.000	40.00	2.646	50.11	9.453
	60	75.33	3.215	63.67	3.055	52.33	3.055	63.78	10.317
	Total	60.93	21.459	55.73	20.810	49.07	22.911	55.24	21.805
(Trypan blue)	Control	85.00	1.000	85.00	1.000	85.00	1.000	85.00	.866
	Tobacco 2 300	38.00	2.000	38.00	2.000	38.00	2.000	38.00	1.732
	20	41.67	1.528	39.67	1.528	36.67	1.155	39.33	2.500
	40	58.67	1.155	45.67	2.082	43.00	1.000	49.11	7.373
	60	73.33	1.528	65.00	2.000	52.67	.577	63.67	9.097
	Total	59.33	18.688	54.67	18.638	51.07	18.530	55.02	18.510

Table 4. Pair-wise comparisons of cell viability of tobacco sample - 2 exposed cells after treatment with different plant extract at selected concentrations

Parameter	Conc.(mg/ml)	(I) Plant material	(J) Plant material	Mean Diff. (I-J)	Std. Error	p(a)
	20	Mango leaf	Coconut husk	2.000	1.764	.900
			Areca husk	15.000(*)	1.764	.000
		Coconut husk	Areca husk	13.000(*)	1.764	.001
	40	Mango leaf	Coconut husk	12.333(*)	1.721	.001
			Areca husk	21.333(*)	1.721	.000
		Coconut husk	Areca husk	9.000(*)	1.721	.006
MTT assay	60	Mango leaf	Coconut husk	11.667(*)	2.539	.011
			Areca husk	23.000(*)	2.539	.000
		Coconut husk	Areca husk	11.333(*)	2.539	.013
	20	Mango leaf	Coconut husk	.000	.816	1.000
			Areca husk	1.42E-014	.816	1.000
		Coconut husk	Areca husk	3.000	1.155	.122
	40	Mango leaf	Coconut husk	13.000(*)	1.217	.000
			Areca husk	15.667(*)	1.217	.000
		Coconut husk	Areca husk	2.667	1.217	.213
Trypan blue	60	Mango leaf	Coconut husk	8.333(*)	1.217	.001
			Areca husk	20.667(*)	1.217	.000
		Coconut husk	Areca husk	12.333(*)	1.217	.000

Based on estimated marginal means, Dependent Variable: Cell viability
* The mean difference is significant at the .05 level. a Adjustment for multiple comparisons: Bonferroni

Table 5. Percentage cell viability of tobacco sample -3 exposed cells after treatment with different plant extract at selected concentrations

Dependent Variable: Cell viability

		Plant material							
		Mango leaf		Coconut husk		Areca husk		Total	
Parameter	Conc.	Mean	SD	Mean	SD	Mean	SD	Mean	SD
(MTT assay)	Control	90.33	3.211	85.00	1.000	85.00	1.000	85.00	.866
	Tobacco 3 300	23.67	1.528	23.67	1.528	23.67	1.528	23.67	1.323
	20	42.00	3.000	32.67	1.528	27.67	1.528	34.11	6.566
	40	52.67	1.528	49.00	1.000	34.33	1.528	45.33	8.485
	60	56.67	1.528	56.00	3.000	47.00	2.000	53.22	5.069
	Total	52.00	20.833	49.27	22.024	43.53	23.009	48.27	21.763
(Trypan blue)	Control	85.00	1.000	85.00	1.000	85.00	1.000	85.00	.866
	Tobacco 3 300	23.33	3.055	23.33	3.055	23.33	3.055	23.33	2.646
	20	40.00	2.000	30.00	2.000	27.67	2.517	32.56	5.981
	40	49.33	2.517	44.67	2.517	32.67	2.082	42.22	7.726
	60	53.67	1.528	53.67	2.517	42.00	3.000	49.78	6.200
	Total	50.27	21.036	47.33	22.490	42.13	23.191	46.58	22.010

Table 6. Pair-wise comparisons of cell viability of tobacco sample -3 exposed cells after treatment with different plant extract at selected concentrations

Parameter	Conc. (mg/ml)	(I) Plant material	(J) Plant material	Mean Diff. (I-J)	Std. Error	p(a)
MTT assay	20	Mango leaf	Coconut husk	2.000	1.764	.900
			Areca husk	15.000(*)	1.764	.000
		Coconut husk	Areca husk	3.94E-015	1.247	1.000
	40	Mango leaf	Coconut husk	12.333(*)	1.721	.001
			Areca husk	21.333(*)	1.721	.000
		Coconut husk	Areca husk	9.000(*)	1.721	.006
	60	Mango leaf	Coconut husk	11.667(*)	2.539	.011
			Areca husk	23.000(*)	2.539	.000
		Coconut husk	Areca husk	11.333(*)	2.539	.013
Trypan blue	20	Mango leaf	Coconut husk	2.000	1.155	.402
			Areca husk	5.000(*)	1.155	.015
		Coconut husk	Areca husk	3.000	1.155	.122
	40	Mango leaf	Coconut husk	13.000(*)	1.217	.000
			Areca husk	15.667(*)	1.217	.000
		Coconut husk	Areca husk	2.667	1.217	.213
	60	Mango leaf	Coconut husk	8.333(*)	1.217	.001
			Areca husk	20.667(*)	1.217	.000
		Coconut husk	Areca husk	12.333(*)	1.217	.000

Dependent Variable: Cell viability, Based on estimated marginal means,
* The mean difference is significant at the .05 level. a Adjustment for multiple comparisons: Bonferroni.

Hoshino *et al.,*(2001) studied the cytotoxic effects of cigarette smoke extract(CSE) on an alveolar type II cell-derived cell line and reported that CSE caused apoptosis at concentrations of 5% or less and necrosis at 10% or more and CSE and acrolein, a major volatile factors in cigarette smoke increased intracellular oxidant activity. They also have opined that the cytotoxic effect might be due to an interaction between aldehydes and oxidants present in CSE or formed in CSE-exposed cells and this cytotoxic injury of the alveolar epithelium to be an important process in the pathogenesis of smoking-related pulmonary diseases (Boyle *et al.,* 2010). Considering the previous report on striking similarities in tobacco smoke induced changes found in oral and bronchial mucosa, we can presume that the cytotoxic effect we have

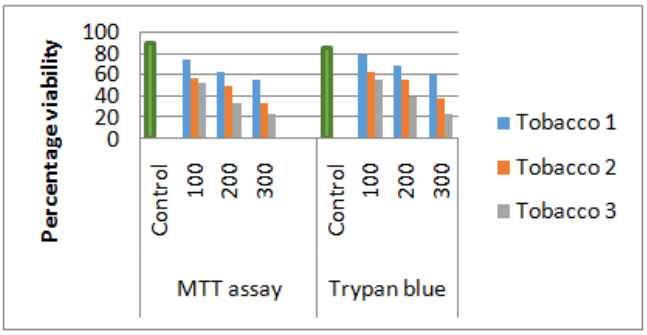

Fig. 1: Percentage cell viability of cultured oral epithelial cells after exposure to selected tobacco samples at various concentration

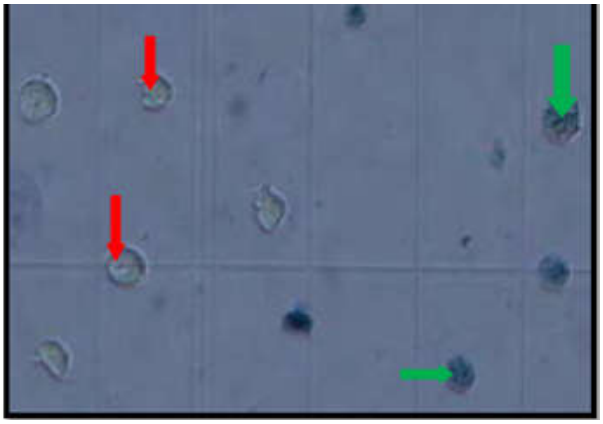

Fig. 2. Dead (green arrow) & viablecells (red arrow) observed by trypan blue exclusion method

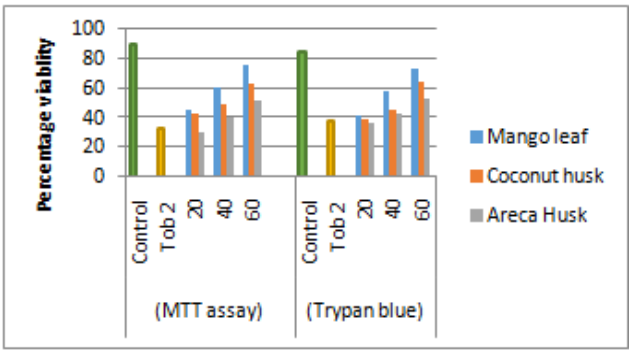

Fig. 3. Percentage cell viability of tobacco sample- 2 exposed cells after treatment with different plant extract at selected concentrations

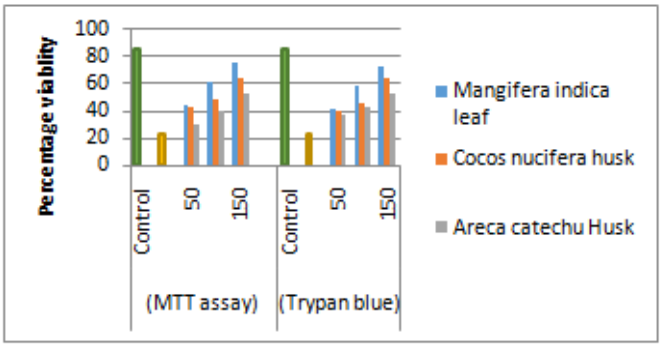

Fig. 4. Percentage cell viability of tobacco sample- 3 exposed cells after treatment with different plant extract at selected concentrations

observed in our study is the direct effect of tobacco nitrosamines through oxidative stress and this cytotoxicity is a contributing factor for tobacco related oral mucosal diseases including potentially malignant diseases and oral cancer (Agarwal *et al.,* 2012). The protective effect of plant materials were tested with both tobacco sample 2 and 3, as these two samples showed significant cytotoxic effects. Both cell viability assays performed in cell culture incubated with tobacco samples along with plant material showed significant improvement in viability than incubated with tobacco samples alone, indicating the cyto-protective effects of selected plant materials. Simultaneous treatment with plant extracts along with tobacco sample 2 resulted in reduction in cytotoxic effect caused by tobacco sample and improved the cell viability to 03, 23 and 37% with 20, 40 and 60 mg/ml of mango extract, 05,11,25% with similar concentrations of coconut extract. Areca husk extract showed relatively lesser effect. Likewise in similar experimental conditions with tobacco sample 3, mango leaf extract showed, improved cell viability to 19, 29 and 33%, and coconut husk 9, 26 and 33% and areca husk 4, 11 and 24%. None of the plant materials could enhance the cell viability to a level comparable to untreated control group. The results indicate that plant materials studied havecyto-protective effect to a certain extent against tobacco induced cytotoxicity, but not to the extent of complete protection. The difference in effect of different plant extract may be based on their chemical composition, difference between the effective components present in the extracts and their mode of action.

There are reports on reduction in tobacco induced cell death by treatment with antioxidants- Vitamin C, E and grape seed proanthocyanidine, Trolox and L-ascorbic acid etc., supporting our results (Bagchi *et al.,* 1999; Mitchell *et al.,* 2010; Toklu *et al.,* 2010). Hoshino *et al.,* (2001)proved that cytotoxic effect of tobacco is due to an interaction between aldehydes and oxidants present. They have also observed an inhibition of CSE-induced cell death by aldehyde dehydrogenase, a scavenger of aldehydes, and N-acetyl cysteine, a scavenger of oxidants and aldehydes (Boyle *et al.,* 2010). We can assume that cyto-protective effect of plant materials noted in this study is related to their radical scavenging and antioxidant property. Our observation of relatively better cyto-protective effect of mango leaf and coconut husk extract which showed more efficient radical scavenging and antioxidant activities compared to areca husk extract is another supporting evidence for this. As we have noted that the plant materials could not recover the cell viability near to that of untreated control, it can be assumed that the cytotoxic effect of tobacco is attributed to a complex interaction of oxidants and other constituents.

Conclusion

Cyto-toxicity induced by tobacco samples andcyto-protective effects of plant extracts,when analysed,a reduction in % cell viability of oral epithelial cells after incubating with different tobacco extract was noted in a concentration dependentmanner. Viability noted with tobacco -1 exposure was 73-52 % with increasing concentration, with tobacco -2 was 75 to 32% and with tobacco-3 was 52 to 23%. Irrespective of concentration, significant difference in cytotoxic effect was noted between different tobacco samples with highest cytotoxic effect with tobacco sample - 3 followed by tobacco sample - 2 and least with tobacco sample-1. Even the highest concentration of tobacco sample - 1 studied did not bring down the % viability less than 50%, while the lowest concentration of tobacco

sample - 3 caused reduced viability to nearly 50%. Simultaneous treatment with plant extracts along with tobacco samples resulted in reduction in cytotoxic effect reflected as increase in % viability than tobacco treatment alone, indicating the cyto-protective effect of plant extracts, but not to the level of untreated controls.

Conflict of Interest: The authors declare no conflicts of interest.

REFERENCES

Agarwala S, Rao BN, Mudholkar K, Bhuwania R, Rao BS. 2012. Mangiferin, a Dietary Xanthone Protects Against Mercury-Induced Toxicity in HepG2 Cells. Environ Toxicol. 27(2):117-127.

Arimilli S, Damratoski BE, Bombick B, Borgerding MF, Prasad GL. 2012. Evaluation of cytotoxicity of different tobacco product preparations. *Regulatory Toxicology and Pharmacology.* 64(3): 350-360.

Bagchi D, Hassoun EA, Bagchi M, Stohs SJ. 1995. Protective effects of free radical scavengers and antioxidants against smokeless tobacco extract (STE)-induced oxidative stress in macrophage J774A.1 cell cultures. *Arch Environ Contam Toxicol.,* 29(3):424-428.

Bagchi M, Balmoori J, Bagchi D, Ray SD, Kuszynski C, Stohs SJ. 1999. Smokeless tobacco, oxidative stress, apoptosis, and antioxidants in human oral keratinocytes. *Free RadicBiol Med.,* 26: 992-1000.

Bagchi M, Bagchi D, Adickes E, Stohs SJ. 1995. Chronic effects of smokeless tobacco extract on rat liver histopathology and production of HSP-90. *J Environ Pathol Toxicol Oncol.,* 14(2):61-68.

Bagchi M, Bagchi D, Stohs SJ. 1996. *In vitro* effects of a smokeless tobacco extract on the production of reactive oxygen species by human oral epidermal cells and rat hepatic mitochondria and microsomes, and peritoneal macrophages. *Archives of Environmental Contamination and Toxicology.,* 30(3):418-422.

Bagchi M, Kuszynski CA, Balmoori J, Joshi SS, Stohs SJ, Bagchi D. 2001. Protective effects of antioxidants against smokeless tobacco-induced oxidative stress and modulation of Bcl-2 and p53 genes in human oral keratinocytes. *Free Radic Res.,* 35(2):181-194.

Boyle JO, Gümüş ZH, Kacker A, Choksi VL, Bocker JM, Zhou XK et al. 2010. Effects of Cigarette Smoke on the Human Oral Mucosal Transcriptome. Cancer Prev Res (Phila). 3(3): 266-278.

Chang T, Chowdhry S, Budhu P, Kew RR. 1998. Smokeless Tobacco Extracts Activate Complement*in vitro*: A Potential Pathogenic Mechanism for Initiating Inflammation of the Oral Mucosa. *Clinical Immunology and Immunopathology.* 87(3):223-229.

Coppe JP, Boysen M, Sun CH, Wong BJF, Kang MK. 2008. A role for fibroblasts in mediating the effects of tobacco-induced epithelial cell growth and invasion. *Mol Cancer Res.,* 6:1085-1098.

Gerlier D, Thomasset N. 1986. Use of MTT colorimetric assay to measure cell activation. *J Immunol Methods*. 94(1-2):57-63.

Hong G, Gaddamanugu PL, Wolfgang Z. 2013. Differential cell-specific cytotoxic responses of oral cavity cells to tobacco preparations. *Toxicology in Vitro*., 27(1):282-291.

Hoshino Y, Mio T, Nagai S, Miki H, Ito I, Izumi T. 2001. Cytotoxic effects of cigarette smoke extract on an alveolar type II cell-derived cell line. *Am J Physiol Lung Cell Mol Physiol.,* 281: 509-516.

Iliya HA ,Wallace HM. 2011. Cellular growth inhibition and toxic effects of etoposide and acetyl salicylic acid. *Nigerian Journal of Pharmaceutical Sciences.,*10(1): 57-64.

Joyce AR, Hawkins W, Fariss MW, Sengupta TK. 2010. Role of plasma membrane disruption in reference moist smokeless tobacco-induced cell death. Toxicology Letters. 198(2):191-199.

Lombard C, Farthing D, Sun J, Fariss MW, McKallip RJ. 2010. Reference moist smokeless tobacco-induced apoptosis in human monocytes/macrophages cell line MM6. *Int Immunopharmacol*. 10(9): 1029-1040.

Mangipudy RS, Vishwanatha JK. 1999. Role of nitric oxide in the induction of apoptosis by smokeless tobacco extract. *Mol Cell Biochem.* 200(1-2): 51-57.

Mitchell C, Piper J, Joyce A, Fariss MW, Mc Kallip RJ. 2009. Reference moist smokeless tobacco-induced oxidative stress in human oral keratinocyte cell lines leads to cell death via JNK and p38 MAPK signaling.*FASEB J.,* 23: (Meeting Abstract Supplement) 581.10

Mitchell C, Joyce AR, Piper JT, Mc Kallip RJ. 2010. Role of oxidative stress and MAPK signaling in reference moist smokeless tobacco-induced HOK-16B cell death. Toxicology Letters. 195(1):23-30.

Padma PR, Lalitha VS. 1989. Anticarcinogenic effect of betel leaf extract against tobacco carcinogens. *Cancer Lett.,* 45(3):195-202.

Schwartz JL, Brunnemann KD, Adami AJ, Panda SP, Gordon SC, Hoffmann D, *et al*. 2010. Brand specific responses to smokeless tobacco in a rat lip canal model. *Journal of Oral Pathology and Medicine.* 39(6):453-459.

Toklu H, Sehirli O, Sahin H, SuleCetinel S, Yegen BC, Sener G. 2010. Resveratrol supplementation protects against chronic nicotine-induced oxidative damage and organ dysfunction in the rat urogenital system. *Marmara Pharmaceutical Journal.,* 14: 29-40.

Wallstrom M. Harm and harm reduction in smokeless tobacco users- An *in vitro* and clinical study. Dissertation submitted to Sahlgrenska Academy at University of Gothenburg, Sweden. Institute of Odontology, Department of Oral and Maxillofacial Surgery, ISBN 978-91-628-8202-0.

Yang MS, Wen JK. 1996. Prevalence and related risk factors of betel quid chewing by adolescent students in Southern Taiwan. *J Oral Pathol Med.* 25:69-71.

****

.