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TOBACCO INDUCED APOPTOSIS IN CULTURED ORAL EPITHELIAL CELLS AND PROTECTIVE EFFECT OF SELECTED PLANT EXTRACTS

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ABSTRACT

Tobacco is a menace that has grabbed millions of people all over the world, cutting across the nation and social barriers. Apoptosis is a form of programmed cell death that occurs in multicellular organisms. Biochemical events lead to characteristic cell changes and death. Enhanced cell death can be caused by either necrosis or apoptosis or a combination of these mechanisms. To determine whether the tobacco induced cell death observed by cell viability assays was due to the induction of apoptosis, double staining technique with ethidium bromide and acridine orange was performed which is highly specific, simple, time efficient that can quantify live, apoptotic and necrotic cells simultaneously. All the three tobacco samples caused reduction in number of viable cells in varying degrees compared to untreated control with concomitant increase in number of apoptotic and necrotic cells in cultured oral epithelial cells. Relative effect of alcoholic extract of both mango leaf and coconut husk was comparable and areca husk was found to be least effective among all plant materials tested. This indicates that tobacco induces cell death through apoptosis and plant materials protect the cells from this effect.

Keywords: .Tobacco, Apoptosis, Mango Leaves, Coconut Husk, Areca Nut Husk

Introduction

Natural products from plants have provided immense application in biological and pharmacological activity. Over the past two decades researchers have turned into many of the traditional folk medicines to uncover the scientific basis of their medicinal effects. Phenols and polyphenols, the flavonoids and their derivatives, are ubiquitous in plants and more than 8,000 different compounds are included in this group.[1] The action of phytochemicals isolated from folk medicines include, regulation of growth factor-receptor interactions and cell signalling cascades, including kinases and transcription factors, that determine the expression of genes involved in cell cycle arrest, cell survival and apoptosis.

Apoptosis is a form of programmed cell death that occurs in multicellular organisms. [2] Biochemical events lead to characteristic cell changes and death. These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation. The average adult human loses between 50 and 70 billion cells each day due to apoptosis.[3] For an average human child between the ages of 8 and 14, approximately 20 to 30 billion cells die a day.[4] The mechanisms of apoptosis are highly complex and sophisticated, involving an energy-dependent cascade of molecular events. Apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue and are quickly phagocytosed by macrophages or adjacent normal cells and so there is essentially no inflammatory reaction.[5,6] Apoptotic cells exhibit several biochemical modifications such as protein cleavage, protein cross-linking, DNA breakdown, and phagocytic recognition that together result in the distinctive structural pathology.[7]

The alternative to apoptotic cell death is necrosis, which is a form of traumatic cell death that results from acute cellular injury. However, apoptosis is a highly regulated and controlled process that confers advantages during an organism's lifecycle. Apoptosis produces cell fragments called apoptotic bodies that phagocytic cells are able to engulf and remove before the contents of the cell can spill out onto surrounding cells and cause damage to them.[8]

Although the mechanisms and morphologies of apoptosis and necrosis differ, there is overlap between these two processes. Evidence indicates that necrosis and apoptosis represent morphologic expressions of a shared biochemical network described as the “apoptosis-necrosis

continuum".[9] Enhanced cell death can be caused either by necrosis or apoptosis or a combination of these mechanisms. Failure to induce apoptosis is the important reason leading to cancers.

The present comparative study is aimed at observing whether different tobacco samples induce cell death through apoptosis and investigating the protective effect of the cells by various plant extracts.

Materials and Methods

Preparation of plant extract

Fresh mango leaves, husk of ripe coconut and areca nut (Fig. 1- 3) 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 into 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.

Fig. 1 Mango leaf (*Mangifera indica*)

Fig. 2 Coconut (*Cocos nucifera*)

Fig. 3 Areca (*Areca catechu)*

Fig. 4 Fresh tobacco leaves (sample-1)

Fig. 5 Tobacco processed for traditional chewing (sample-2)

Fig. 6 Tobacco dispensed in commercial sachet (sample-3)

To ascertain whether the tobacco induced cell death noted by cell viability assays was due to the induction of apoptosis, ethidium bromide (EB) and acridine orange (AO) double staining was carried out.[10] For the AO-EB staining, 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 the most active concentration of tobacco extracts i.e. 300µg of tobacco sample -2 and 3 for 24 hours. No extract was added to the well with negative control. At the end of the treatment, cells were trypsinized with 0.025% (w/v) trypsin solution and rinsed with PBS once. 25µL of the cell suspension was then mixed together with 1µL of AO-EB dye cocktail, containing 100 mg/mL of each dye. Cells were immediately visualized under a fluorescence microscope at 400X magnifications. The cells were divided into four categories as follows: living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation) and necrotic cells (uniformly orange-stained cell nuclei). In each experiment, 100 cells /sample were counted.

To study the protective effect of plant materials, EB and AO double staining was repeated, after incubating the cultured oral epithelial cells simultaneously with tobacco samples and plant extracts. Only tobacco samples -2 and 3 were chosen for this purpose as these had shown significant effect on cultured cells, 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. All the above experiments were carried out in triplicate and mean value was taken to compare the effects**.**

Results & Discussion

Results

Oral epithelial cells treated with tobacco extracts exhibited apoptotic changes as detected by AO/EB double staining. The number of viable cells and apoptotic cells including early apoptotic (AO +ve, EB –ve) and late apoptotic cells (AO +ve, EB +ve) and necrotic cells after incubating the cultured oral epithelial cells with different types of tobacco samples in various concentrations were recorded (Fig. 7 and 8). Between different concentrations of tobacco 1 samples studied, 7-

10% reduction in number of viable cells was noted with 7-10% increase in apoptotic cells, where most of apoptotic cells were in early apoptotic stage. No difference in number of necrotic cells was observed with increasing concentrations. With respect to tobacco sample-2, 16-21% reduction in viable cell number was noted which is accounted by 5-8% increase in apoptotic cells and 7-11% increase in necrotic cells. Among the apoptotic cells late apoptotic cells were predominant. Although tobacco-3 showed significant reduction in viable cells and increase in apoptotic cells compared to control and other tobacco samples, variation with increasing concentration was only 5-10%. Late apoptotic cells and necrotic cells were predominant.

Fig. 7: Percentage of viable, early apoptotic, late apoptotic and necrotic cells in tobacco exposed cultured cells at various concentrations

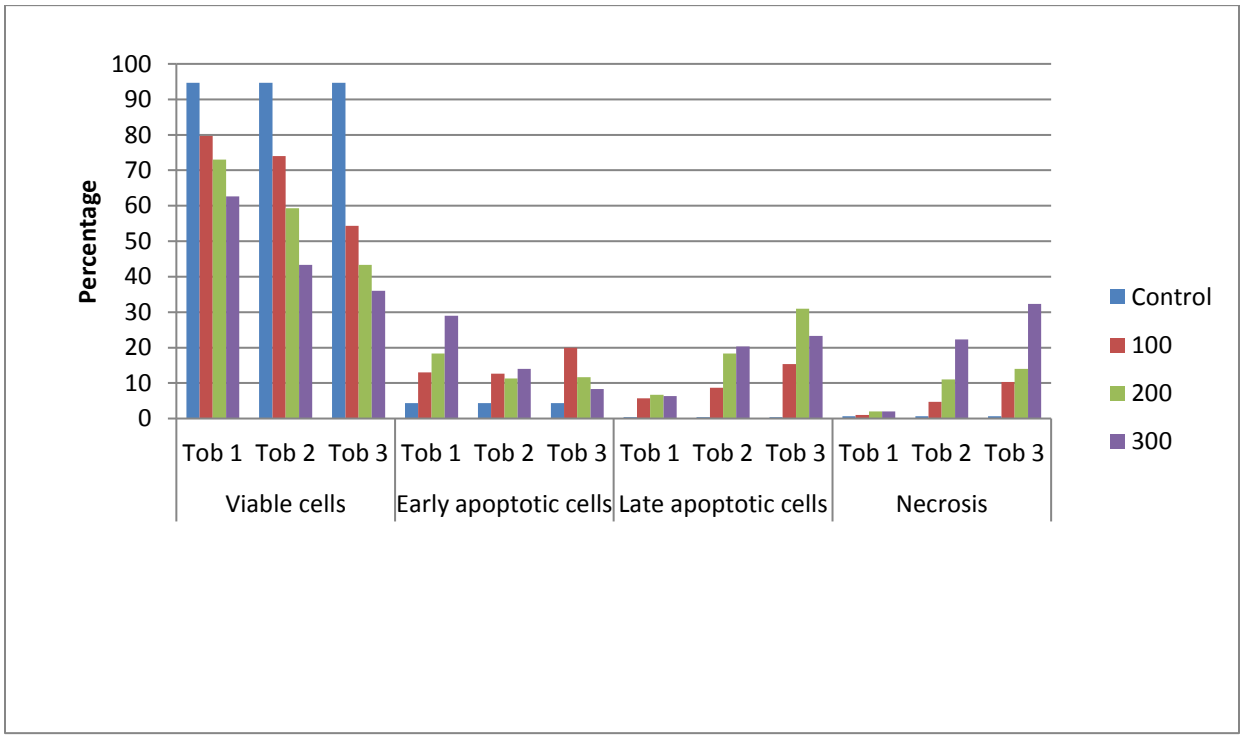

Fig. 8: Oral epithelial cells in different stages of apoptosis/necrosis following tobacco exposure (AO-EB stain, Short yellow arrow-viable cells, long yellow arrows-early apoptotic cells, Short red arrows-late apoptotic cells and long red arrows-necrotic cells)

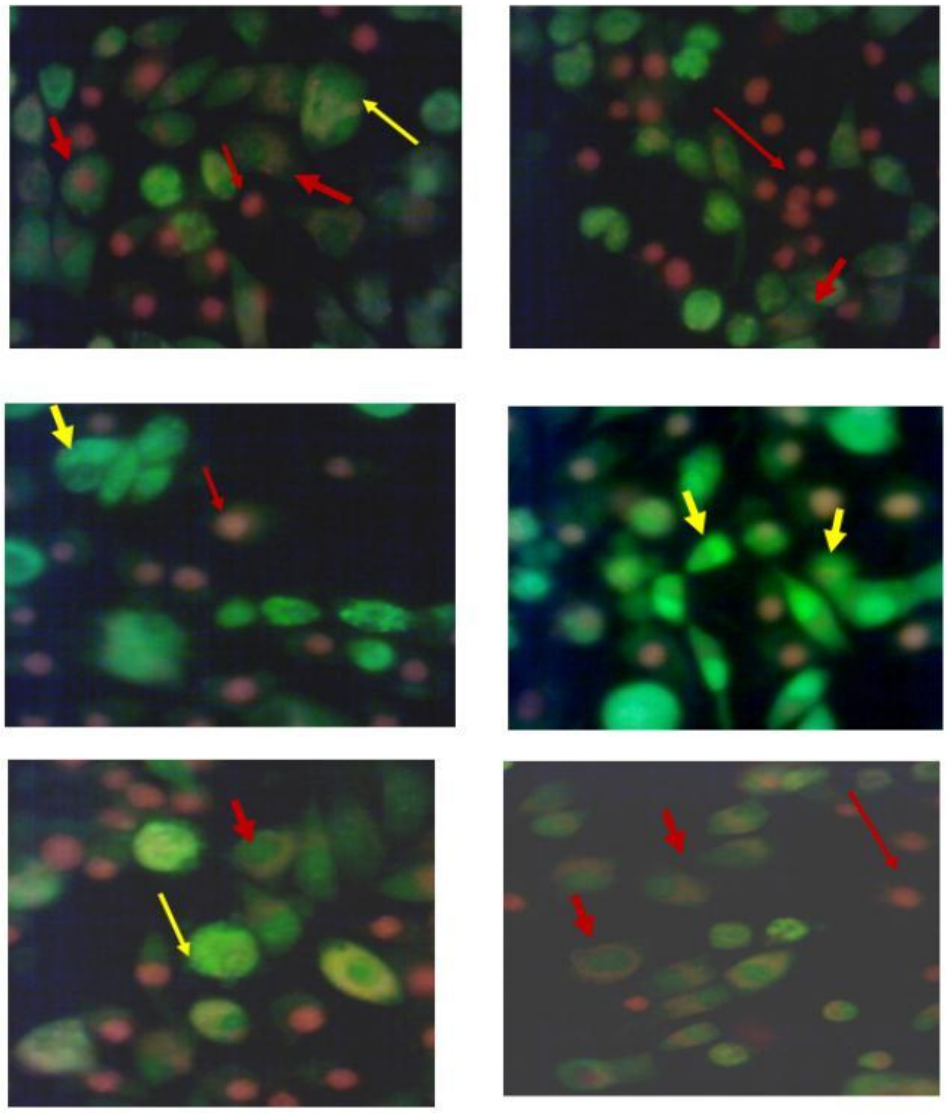

Fig. 9: Percentage of viable, early apoptotic, late apoptotic and necrotic cells in tobacco- 2 exposed cultured cells after treatment with plant extract at various concentrations

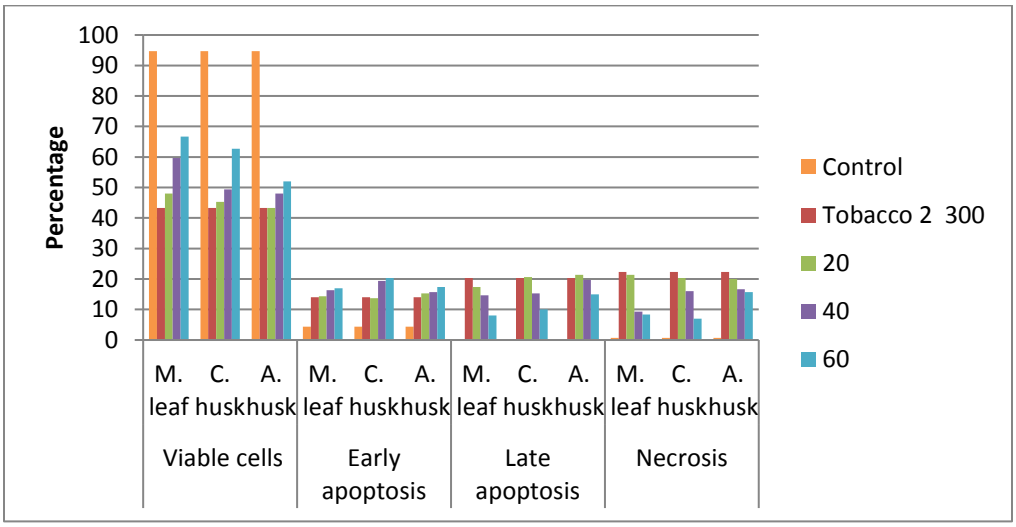

Table 2: Pair-wise comparison of percentage of viable, early apoptotic, late apoptotic and necrotic cells in tobacco- 2 exposed cultured cells after treatment with different plant extracts

Conc. mg/ml	(I) Plants	(J) Plants	Parameter											
			Viable cells			Early apoptotic cells			Late apoptotic cells			Necrotic cells		
			Mean Diff. (I-J)	SE	p(a)	Mean Diff. (I-J)	SE	p(a)	Mean Diff. (I-J)	SE	p(a)	Mean Diff. (I-J)	S.E	p(a)
20	Mango leaf	Coconut husk	2.667	.903	.076	.667	.667	1.000	-3.333(*)	.667	.007	1.000	.385	.122
		Areca husk	4.667(*)	.903	.006	-1.000	.667	.553	-4.000(*)	.667	.003	1.333(*)	.385	.040
	Coconut husk	Areca husk	2.000	.903	.206	-1.667	.667	.140	-.667	.667	1.000	.333	.385	1.000
40	Mango leaf	Coconut husk	10.333(*)	1.018	.000	-3.000(*)	.471	.002	-.667	.471	.621	-6.667(*)	.770	.000
		Areca husk	11.667(*)	1.018	.000	.667	.471	.621	-5.000(*)	.471	.000	-7.333(*)	.770	.000
	Coconut husk	Areca husk	1.333	1.018	.715	3.667(*)	.471	.001	-4.333(*)	.471	.000	-.667	.770	1.000
60	Mango leaf	Coconut husk	4.000(*)	.903	.013	-3.333(*)	.609	.005	-2.000	.000	.	1.333(*)	.385	.040
		Areca husk	14.667(*)	.903	.000	-.333	.609	1.000	-7.000	.000	.	-7.333(*)	.385	.000
	Coconut husk	Areca husk	10.667(*)	.903	.000	3.000(*)	.609	.008	-5.000	.000	.	-8.667(*)	.385	.000

*Dependent Variable: Effect, Based on estimated marginal means, * the mean difference is significant at the .05 level.*

a Adjustment for multiple comparisons: Bonferroni

When the protective effect of mango leaf extract, coconut husk extract and areca husk extract on oral epithelial cells treated with tobacco 3 of 300 µg/ml and was analysed, percentage of the viable cells, early apoptotic cells, late apoptotic cells and necrotic cells were noted. When the results obtained were analysed using two way ANOVA, highly significant difference in viable cells, early apoptotic cells, late apoptotic cells and necrotic cells was noted between different types of plant extracts used and different concentrations studied($p<0.01$). When statistical comparison was made keeping viable cells as dependent variable, highly significant difference ($p<0.001$) was noted between cell culture treated with tobacco sample- 3 alone and simultaneous treatment of tobacco 3 and all different concentrations of mango leaf extract, coconut husk and areca husk extract with $p<0.05$ except for 20mg/ml concentration of areca husk extract. However, the effect was statistically different from control. Observation was same with respect to early apoptotic cells except 20mg/ml concentration of mango leaf extract. No significant alteration in late apoptosis was noted in samples treated with areca husk extract in all concentrations studied and 20mg/ml concentrations of other two extracts. But all the concentrations of all plant extracts significantly reduced the number of necrotic cells compared to sample treated with tobacco 3 alone ($p<0.001$). However even in this effect was not comparable to untreated cells (Table 3 & Fig. 10).

Fig. 10: Percentage of viable, early apoptotic, late apoptotic and necrotic cells in tobacco- 3 exposed cultured cells after treatment with plant extract at various concentrations

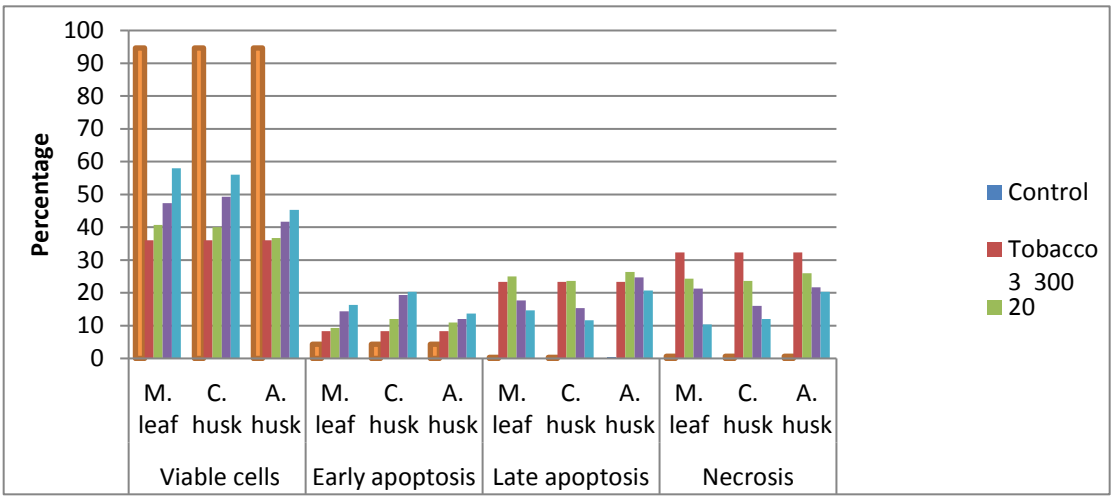

Table 3: Pair-wise comparison of percentage of viable, early apoptotic, late apoptotic and necrotic cells in tobacco- 3 exposed cultured cells after treatment with different plant extracts

Conc. Mg/ml	(I) Plants	(J) Plants	Parameter											
			Viable cells			Early apoptotic cells			Late apoptotic cells			Necrotic cells		
			Mean Diff.(I-J)	S.E	p(a)	Mean Diff. (I-J)	S.E	p(a)	Mean Diff. (I-J)	S.E	p(a)	Mean Diff.(I-J)	S.E	p(a)
50	Mango leaf	Coconut husk	.667	1.217	1.000	-2.667	.861	.063	1.333	.770	.402	.667	.770	1.000
		Areca husk	4.000	1.217	.050	-1.667	.861	.303	-1.333	.770	.402	-1.667	.770	.221
	Coconut husk	Areca husk	3.333	1.217	.101	1.000	.861	.868	-2.667(*)	.770	.040	-2.333	.770	.069
100	Mango leaf	Coconut husk	-2.000	1.247	.480	-5.000(*)	.385	.000	2.333	.816	.087	5.333(*)	.770	.001
		Areca husk	5.667(*)	1.247	.012	2.333(*)	.385	.003	-7.000(*)	.816	.000	-.333	.770	1.000
	Coconut husk	Areca husk	7.667(*)	1.247	.003	7.333(*)	.385	.000	-9.333(*)	.816	.000	-5.667(*)	.770	.001
150	Mango leaf	Coconut husk	2.000	.720	.096	-4.000(*)	.471	.000	3.000(*)	.471	.002	-1.667	.770	.221
		Areca husk	12.667(*)	.720	.000	2.667(*)	.471	.004	-6.000(*)	.471	.000	-10.000(*)	.770	.000
	Coconut husk	Areca husk	10.667(*)	.720	.000	6.667(*)	.471	.000	-9.000(*)	.471	.000	-8.333(*)	.770	.000

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

Discussion

To establish whether the tobacco induced cell death noted by cell viability assays was due to the induction of apoptosis, EB and AO double staining was carried out. EB-AO double staining is highly specific, simple, time efficient technique that can quantify live, apoptotic and necrotic cells at the same time. Multiple endpoints of cell death and apoptosis can be measured in a single experiment with very small amounts of cells and therefore is valuable for the cells, difficult to grow in large amounts i.e., the short term cultures as our oral epithelial cell culture.

In this part of the study, it is shown that all tobacco samples studied, caused reduction in number of viable cells in varying degrees compared to untreated control with concomitant increase in number of apoptotic and necrotic cells in cultured oral epithelial cells. But effect was significantly high with respect to tobacco sample 3, followed by tobacco sample 2 than tobacco sample 1. However, all the three samples showed a dose dependent decrease in viability and increase in apoptosis. Between different concentrations of tobacco 1 samples studied, 7-10% reduction in number of viable cells was noted with 7-10% increase in apoptotic cells, where most of the apoptotic cells were in early apoptotic stage. No difference in the number of necrotic cells was observed with increasing concentrations. With respect to tobacco sample 2, 16-21 % reduction in viable cell number was noted which is accounted by 5-8% increase in apoptotic cells and 7-11% increase in necrotic cells. Among the apoptotic cells late apoptotic cells were predominant. Although tobacco 3 showed significant reduction in viable cells and increase in apoptotic cells compared to control and other tobacco samples, variation with increasing concentration was only 5-10%. As in case of tobacco sample 2, late apoptotic cells and necrotic cells were found to be increasing with increasing concentrations. This observation is consistent with the report of Hashino *et al., and* Boyle *et al.,* and we confirm that tobacco, particularly processed types at higher concentration is cytotoxic and cell death is mediated through apoptosis. [11,12] The difference in findings between different tobacco samples and different concentrations could be due to the variation in TSNA (tobacco specific nitrosamines) and other chemical

constituents which in turn is related to different processing, the selected tobacco materials were subjected to and presence of additives such as in tobacco sample 3.

Although the ability of tobacco to induce apoptotic cell death is well established, understanding of the molecular mechanisms involved is still very fragmentary. The observations in different cell systems indicate the existence of several alternative cell death pathways. Earlier reports indicate that exposure to smokeless tobacco causes release of cytochrome c from the mitochondria leading to apoptotic cell death probably due to activation of the effector caspases. [13] Alternate mechanism suggested is oxidative stress via activation of ASK1 and the JNK1/2 and p38 MAPK pathways. [14,15] In brief, intracellular oxidation/oxidative state may play a central role in tobacco induced apoptotic cell death. Oxidative stress-induced apoptosis seems to be the product of multiple pathways resulting in the oxidation of membranes, loss of Ca^{2+} homeostasis, diffused activation of enzyme systems including activation of endonucleases, disruption of metabolic processes including the changes in the mitochondrial transmembrane potential, ATP loss, and, alterations in certain proteins and transcription factors. A growing body of work has also suggested that oxidative stress-induced apoptosis involves alterations in the expression of the tumour suppressor (*p53*) and the promoter genes (*Bcl-2* and *c-myc*). Thus, apoptosis induced by oxidative stress is also subjected to many checks and balances between different genes. [16] Nevertheless, much of the details of tobacco induced cell death pathways are still to be resolved.

When the percentage of viable cells, early apoptotic cells, late apoptotic cells and necrotic cells were counted after incubating the cultured oral epithelial cells with selected concentrations of tobacco samples along with plant extracts in different concentrations, protective effect of plant extracts was noted. When pairwise comparison was made using Tukey HSD, significant difference was noted between cell culture treated with tobacco sample 2 alone and simultaneous treatment of tobacco 2 and all different concentrations of mango leaf extract, coconut husk and areca husk extract with $p<0.05$. However, none of the samples got values statistically similar to control. When the effect of different plant extracts is compared, significant difference in effect was noted between them in all parameters such as viable cells, early and late apoptosis and necrotic cells, with mango leaf exhibiting highest effect followed by coconut husk and areca husk.

Our observation can be compared to the earlier report of Bagchi *et al.,* who studied the effect of oxidative stress and apoptosis caused by smokeless tobacco in human oral keratinocytes. [13] As in the present study, these investigators isolated and cultured oral epithelial cells and treated with smokeless tobacco extracts of different concentrations. They have observed 9-35% increase in apoptotic cell death following treatment with increasing concentrations of extract and 51-85% of reduction in apoptotic cell death with antioxidants. In the present study, we have observed an increase of apoptosis by 27-31% after treatment with different tobacco extracts and is consistent with observations of Bagchi *et al*., However, the reduction in apoptosis noted in our study after treatment with plant extracts was much less. The difference in value obtained could be due to the reason that Bagchi *et al.,* used pure antioxidants such as vitamin C, E and grape seed proanthocyanidine, while we have used crude plant extracts. [13] Similarly, Mitchell et *al* demonstrated a reduced apoptotic cell death in a human oral keratinocyte cell line, HOK-16B after treatment with the antioxidants, Trolox and L-ascorbic acid and an iron chelator, deferoxamine. [14,15].

Though the number of viable, early apoptotic, late apoptotic and necrotic cells were significantly different after treatment with plant extracts from cells treated with tobacco alone, we could not demonstrate values comparable to untreated controls. This suggests that the plant materials studied are not efficient to completely revert the adverse effects of tobacco. On the other hand, because the tobacco effect was only partly attenuated by plant extracts, other mechanisms of tobacco toxicity in addition to oxidative stress also must be considered and we presume that different mechanisms may act synergistically to induce apoptosis of oral epithelial cells.

The exact mechanism of action of the plant materials used was not analyzed as it was beyond the scope of this study. However, from the experimental evidences from the literature we can presume that antioxidant property is a key factor. There are sufficient evidences that mangiferin is an effective antioxidant and an efficient anti-apoptotic agent. Cyto-protective potential of mangiferin is reported by many investigators, which may be attributed to quenching of the ROS (reactive oxygen species) generated in the cells due to oxidative stress, restoration of mitochondrial membrane potential and normalization of cellular antioxidant levels as well as mutual regulation of Bcl-2/Bax. Furthermore, mangiferin is shown to inhibit activation of

mitogen-activated protein kinases (MAPKs-phospho-ERK 1/2, phospho-JNK phospho- p38), nuclear translocation of NF-κB and apoptotic cell death. [17-21] Since mangiferin is the major component present in mango leaf, we would like to suggest that our observation of cyto-protective and anti-apoptotic effect of this plant material can be attributed to mangiferin.

Likewise, there are reports in literature on properties of catechin and epicatechin as protective for oxidative stress and mitochondria-induced apoptosis. Yu *et al*., has reported that the molecular mechanisms of anti-apoptotic effects of epicatechin is associated with inhibition of p38, [21] MAPK phosphorylation and Bax expression, and reduction of ROS production. [22] Similar mechanism of action of catechin is also reported earlier with more efficient activity than epicatechin. [22-25] As per earlier reports, the major polyphenol in the coconut and areca husk extract is catechin and epicatechin, [21] we would like to relate our observation to catechin and epicatechin.

Further analysis of cell cycle events following tobacco exposure and plant extract treatments may give insight into the molecular mechanisms involved so that possibility of utilizing the beneficial effects of these plant materials in fighting against tobacco induced cell damage can be established.

Conclusion

AO-EB double staining exhibited presence of cells in different stages of apoptosis along with viable and necrotic cells in tobacco exposed oral epithelial cells, with significant variation in the count between different tobacco samples studied. 7-10% increase in apoptotic cells with no alteration in necrotic cells was noted with different concentrations of tobacco sample - 1, while tobacco sample - 2 and 3 showed increase in apoptotic cells as well as necrotic cells. After incubating the oral epithelial cells with tobacco samples 2 and 3 along with plant extracts, a highly significant increase in cell viability with reduction in apoptotic cells and necrotic cells was noted. Relative effect of both mango leaf and coconut husk was comparable and areca husk was found to be least effective among all plant material tested. This indicates that tobacco induces cell death through apoptosis and plant materials protect the cells from this effect.

Conflict of Interest

The authors declare no conflicts of interest.

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