

Research Article

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The Critical Functions of Nicotine and Cotinine in Panmasala Consumers Contribute to the Oxidative Stress in Saliva and Urine

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Abstract

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Panmasala is one of the form of Smokeless Tobacco product (SLT) and is available in different brands like Rajnignadha, Vimal, Hira, and RMD (Rasikala Manikchand Dhariwal) manufactured by various smokeless tobacco industries.

Aim: Smokeless is a type of tobacco and acts as inducer for the formation of oxidative stress in saliva and urine.

Materials and methods: People consumed specifically rajnigandha panmasala with BABA 120 chewing tobacco for the period of 4 years. The concentrations of nicotine and cotinine are evaluated by HPLC technique, levels of nitric oxide, lipid peroxidation, creatinine, and status of antioxidant enzymes were measured by spectrophotometric method. The urea, uric acid, albumins, and total proteins were determined by kit methods and remaining variables were calculated by respective formula.

Results: Our results reported that significantly increased concentrations of nicotine and cotinine in saliva and urine of smokeless tobacco users. The nitroxidative stress includes higher levels of nitric oxide (Nitrites and nitrates) and lipid peroxidation was exhibited in the saliva and urine of panmasala users. The significant decrease in concentrations of glutathione, uric acid, and antioxidant enzymes was observed in the saliva of experimental subjects. The smokeless tobacco users have shown significantly decreased levels of uric acid and creatinine in the urine.

Conclusion: Panmasala is more toxic and harmful than any smokeless tobacco product and acts as greater risk of inducer of oxidative stress in saliva and urine of SLT users for prolonged consumption.

Keywords: Panmasala, Nicotine, Cotinine, Saliva, Urine, Oxidative stress

Introduction

Nicotine is considered as a major source of all tobacco products and has the greater addictive potential to users [1]. Smokeless tobacco products consist of toxicants and carcinogens, including nicotine, Tobacco-Specific N-nitrosamines (TSNA), N-nitrosamino acids, volatile N-nitrosamines, aldehydes like formaldehyde and acetaldehyde, hydrocarbons, and polonium-210 [2]. Nearly 91% of a total 356 million adult SLT users are present in low and low-middle income countries, with

81.6% in countries of South-East Asia region [3]. There is evidence which supports that the enactment of smoke-free legislation has been directly proportional to the consumption of smokeless tobacco among adolescent males [4]. The Rajnigandha, Vimal, RMD-Rasikala Manikchand Dhariwal, and Hira panmasala are the most widely brands available in Andhra Pradesh. People more likely to consume this in combination with a chewing tobacco brand like BABA120, V1 scented, M-scented, and Royale 717 tobacco respectively are produced by the

same company of the panmasala brand. The contents of panmasala are betel nuts, catechu, lime, saffron and added flavors. The prevalence of smokeless tobacco has been increasing steadily and there is a need of placing restrictions on smokeless tobacco consumption is drive forward the gains of the anti-tobacco movement [5].

There is a strong policy for monitoring and reducing the amount of tobacco specific N-nitrosamines in SLT products [6]. Different people convert different percentages of nicotine to cotinine and metabolize cotinine differently at different rates [7]. Data (Table 1) reported that the half-life nicotine is 2 h which is less than cotinine half-life of 18 h. The total clearance includes renal and non-renal clearance is higher of nicotine compared to cotinine clearance [8,9]. Cotinine is the active metabolite of nicotine metabolism and its levels in plasma, saliva, and urine acts a marker of nicotine exposure indicates nicotine dependence [10]. The cotinine levels of saliva and urine were strong positively correlated with blood cotinine [11]. The state level monitoring and socio-economic inequalities, taxation of SLT consumption were reported in India [12]. Therefore, the purpose of this study was to explore the biochemical alterations in biological fluids of saliva and urine due to chronic consumption of smokeless tobacco products for prolonged duration in human male volunteers.

Materials and Methods

Study subjects and data collection

Sixty human male volunteers were selected and each group consisting of thirty volunteers, aged between 20-40 years residing in Ananthapuramu town, Andhra Pradesh is taking local diet. The entire studied population was subdivided into two groups. Panmasala users- Individuals consume only panmasala brand (Rajnigandha with BABA 120 chewing tobacco) consist of 5-6 packets per week for last 4 years. Non-tobacco users or healthy controls individuals do not consume any form of tobacco products.

Saliva and urine collection

Five milliliter's of un-stimulated whole salivary samples were obtained by expectoration, in the absence of chewing movements, in dry plastic vials with the test subject sitting in a relaxed position. The collected saliva samples were centrifuged at 3000 rpm for 10 min. The supernatants were stored at -70°C until further analysis. Two milliliter's of urine samples in morning were collected in a sterile flask covered with aluminium foil to keep out stray light and processed within 2 h of the collection. The collected samples were centrifuged at 3,000 rpm for 10 min for further analysis.

Biochemical analysis

A spectrum of clinical parameters like albumins, total proteins, urea, uric acid, creatinine, and other variables were analyzed from all studied individuals from their saliva and urine using auto analyzer kit methods. Nitrites and nitrates were estimated by the method [13] and values obtained by this procedure represent the sum of nitrite and nitrate levels in the form of nitric oxide. The levels of malondialdehyde were determined by the method [14] and determination of GSH activity was measured by the protocol [15]. Superoxide Dismutase (SOD) activity was measured according to the protocol [16]. The catalase activity was determined by method [17], the activity of glutathione-s-transferase and glutathione peroxidase was measured by method [18].

HPLC

HPLC system (Shimadzu, Japan) is equipped with a binary gradient system with variable UV/VIS detector (SPD-20A) and Rheodyne injector with a 20 μL loop and LC-20AD pumps and Integrator. Reversed phase chromatographic analysis was performed in isocratic condition using C18 reverse phase column (5 μ) at 37°C .

HPLC operating conditions of nicotine and cotinine

Resolution of peaks was performed with the mobile phase consisting a mixture of 0.272 g of KH_2PO_4 , 0.184 g

Table 1: Pharmacokinetic effect of nicotine and cotinine in humans [8,9].

	Nicotine	Cotinine
Half-life	120 min	18 hr
Volume of distribution	180 L	88 L
Total clearance	1300 mL/min	72 mL/min
Renal clearance	200 mL/min	12 mL/min
Non renal clearance	1100 mL/min	60 mL/min

of sodium n-heptane sulfonate, 820 mL of water (HPLC-grade), and 180 ml of methanol (HPLC grade). The pH of the mobile phase was adjusted by drop wise addition of ortho phosphoric acid (pH=3.2). The flow rate used was 1.0 mL/min, and the wavelength was fixed at 256 nm for nicotine and 262 nm for cotinine as per the modified method [19]. Nicotine and cotinine at the concentrations of 20 µM/mL were used as standards.

Sample analysis of nicotine and cotinine

Sample analysis was processed by the modified method [19]. A 0.1 mL aliquot of plasma was placed into a glass test tube was alkalinized with 20 µL of 2.5 M NaOH for plasma and then vortex mixed at 2800 rpm for 1 min. Equal amounts of dichloromethane–diethyl ether (1:1 v/v) was used for one-step single extraction, then vortex mixed at 2800 rpm for 2 min. The organic layer, after being centrifuged at 3500 rpm for 3 min, was transferred to a new glass tube containing 4 µL of 0.25 M HCl. The organic layer, after being centrifuged at 3500 rpm for 3 min, was transferred to a new glass tube containing 4 µL of 0.25 M HCl. The organic phase was then evaporated under a stream of nitrogen at 35°C until dryness and reconstituted in 50 µL of mobile phase. A 20 µL aliquot was injected into the HPLC for analysis.

HPLC operating conditions of epinephrine

Resolution of peaks was performed with the mobile phase composed of a mixture of acetic acid and 50 mm ammonium acetate buffer pH at 3.1 (1:99 v/v). The flow rate used was 1.0 mL/min, and the wavelength was fixed at 285 nm for epinephrine as per the modified method [20]. Epinephrine concentrations of mg/ml were used as a stock solution.

Statistical analysis

All the quantitative data were expressed as mean ± SEM and Students t-test was used to determine the significance of the parameters between the groups. *P* < 0.05 was considered statistically significant.

Results

Biochemical profile in saliva

The results of the present study were indicated that the smokeless tobacco users had significantly higher levels of nicotine and cotinine in saliva and urine compared to normal controls (Figure 1). It was observed that the mean values of glutathione and uric acid shown to be significantly decreased in panmasala users of saliva with normal subjects. The smokeless tobacco users demonstrated that significantly increased levels of salivary

malondialdehyde and nitric oxide in saliva compared to non-SLT users (Figure 2). Data (Figure 3) reported that the experimental subjects have been found that significantly decreased levels of antioxidant enzymes like superoxide dismutase, catalase, glutathione peroxidase, and glutathione S-transferase in saliva in comparison with normal controls. Data demonstrated (Table 2) that the total proteins, globulins, and albumins were significantly decreased in the saliva of the panmasala group and mean values of globulins showed no significant change. From the summary statistics that salivary nitrites and nitrates were significantly increased in panmasala groups than the control group.

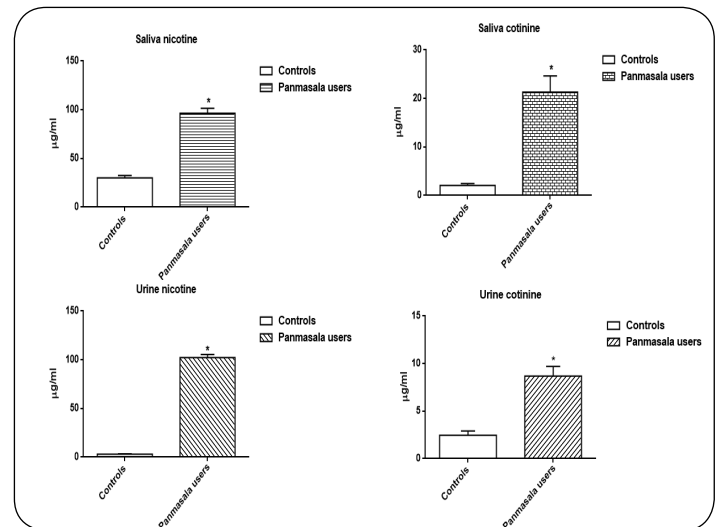


Figure 1: Concentrations of nicotine and cotinine in saliva and urine of panmasala consumers.

Data are represented as the mean ± SEM. * denotes that data are significantly different with the controls.

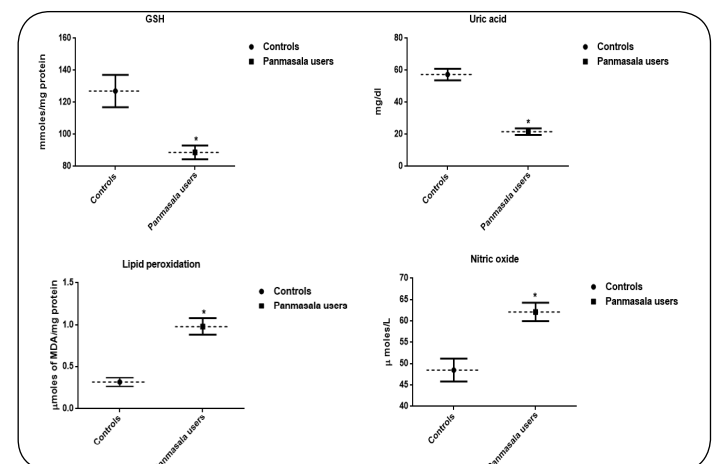


Figure 2: Levels of glutathione, uric acid, lipid peroxidation and nitric oxide in saliva.

Data are represented as the mean ± SEM. *denotes that data are significantly different with the controls.

Note: GSH-Glutathione.

Urine biochemical profile

The Albumin-creatinine ratio, creatinine clearance, urea clearance, and glomerular filtration rate found that there was a decreased level in panmasala group of smokeless tobacco consumers in comparison with non-consumers and showed no statistical difference. The mean values of urea clearance in experimental users showed a statistically significant difference (Figure 4). Our data presented that significantly decreased levels of protein creatinine ratio in urine of panmasala group consumers with non-tobacco users. The SLT consumers found that significantly increased levels of lipid peroxidation and nitric oxide in the urine (Figure 5).

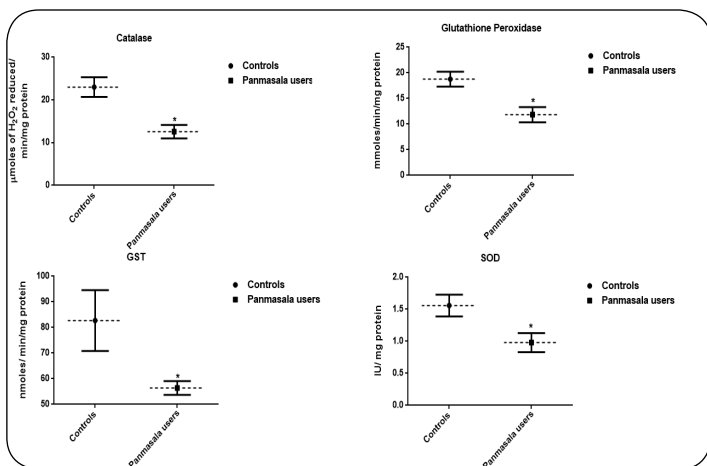


Figure 3: Activities of antioxidant enzymes in saliva of experimental subjects.

Data are represented as the mean ± SEM. *denotes that data are significantly different with the controls.

Note: SOD-Super oxide dismutase; GST-Glutathione S-Transferase.

We found that, the panmasala users group had significantly higher levels of uric acid, creatinine, and epinephrine than normal controls. The experimental user groups showed diminished levels of urine urea in comparison with non-users (Figure 6). Our results concluded (Table 3) that the levels of albumins, nitrates, and nitrites were increased significantly in smokeless tobacco users of urine in comparison with non-tobacco users and mean values of albumins showed no statistically significant difference. The experimental users found that the significantly decreased levels of total proteins and globulins compared to the normal subjects.

HPLC chromatograms of nicotine and cotinine

Our data demonstrated that the range of retention time of standard nicotine is 5.2-6.0 min and shown a chromatogram peak at 5.25 min. The range of retention

time of standard cotinine is 3.6-4.8 min and showed that a chromatogram peak at 4.01 min (Figure 7a). There are no peaks observed in chromatograms of saliva and urine in normal controls at the retention of 4.01 and 6.00 min of nicotine and cotinine. The normal control group had no nicotine intake and tobacco exposure. Small concentrations of nicotine and cotinine levels are observed in control group due to environmental tobacco exposure and some food constituents. Panmasala consumers showed that nicotine chromatogram peak at retention time of 5.93 min and cotinine peak at 3.67 min in samples of saliva. There is a peak observed in chromatograms of nicotine in urine at the retention of 5.39 min and cotinine showed chromatogram peak observed at retention time of 4.79 min of smokeless tobacco users (Figure 7b). Data (Figure 8a and 8b) depicted that the distribution of nicotine and cotinine levels in the saliva and urine of controls and panmasala users.

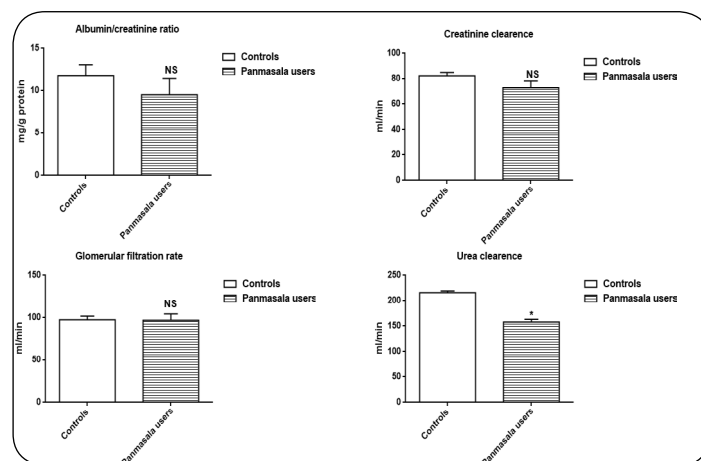


Figure 4: Biochemical profile in urine of SLT users.

Data are represented as the mean ± SEM. *denotes that data are significantly different with the controls.

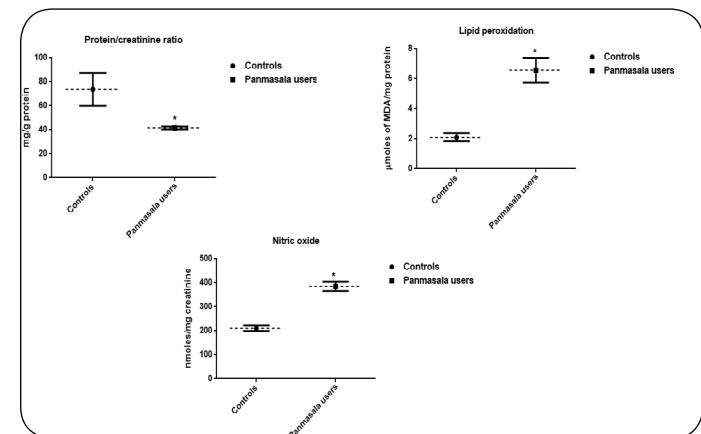


Figure 5: Levels of malondialdehyde and nitric oxide in urine.

Data are represented as the mean ± SEM. *denotes that data are significantly different with the controls.

Note: MDA-Malondialdehyde.

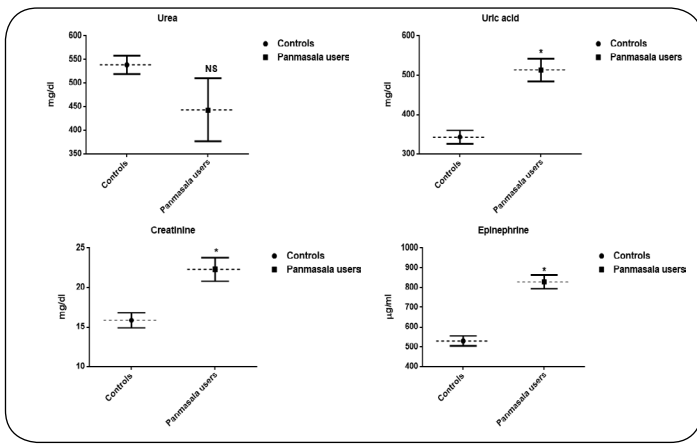


Figure 6: Levels of urea, uric acid, creatinine, and epinephrine in urine of panmasala users. Data are represented as the mean \pm SEM. *denotes that data are significantly different with the controls.

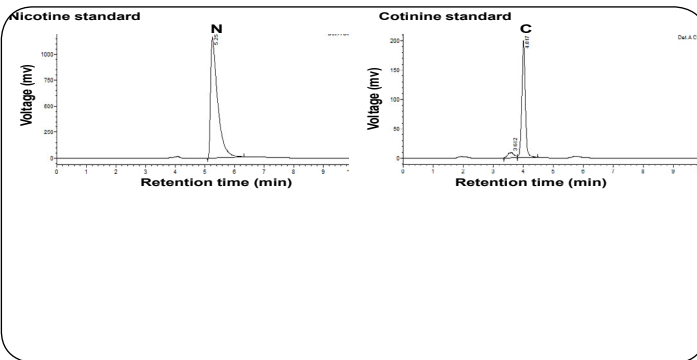


Figure 7a: HPLC chromatograms of nicotine and cotinine standards.

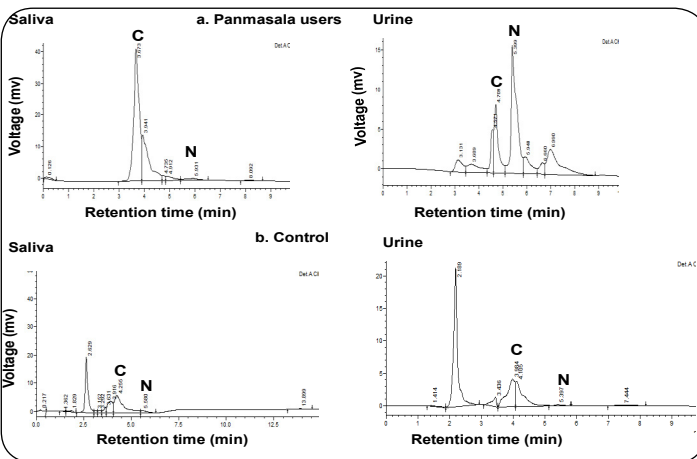


Figure 7b: HPLC chromatograms of nicotine, cotinine in saliva and urine of panmasala users and controls.

Correlation analysis

The nicotine levels of saliva have been shown that there is a positive correlation with lipid peroxidation and negative with superoxide dismutase and catalase. There Adv Clin Endo Met, 2(1): 66-74 (2019)

is a negative correlation of cotinine with nitric oxide, SOD, CAT and positive with lipid peroxidation. Urinary concentrations of nicotine exhibited that the negative with lipid peroxidation and positive with nitric oxide. Cotinine in the urine showed that there is a negative correlation with nitric oxide in panmasala users. All the variables do not show any significant difference with the nicotine and cotinine of saliva and urine (Table 4a and 4b).

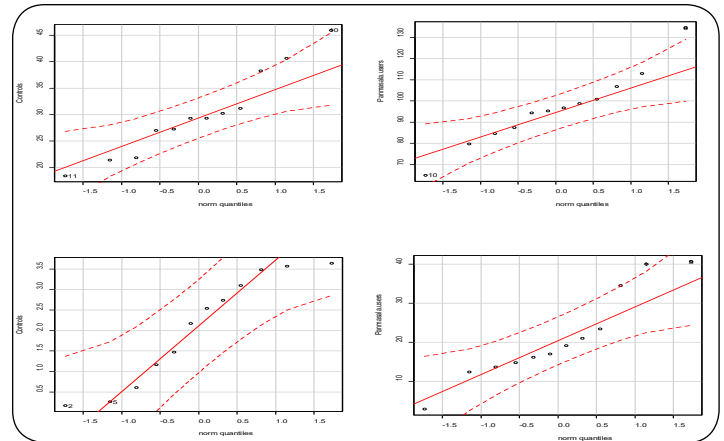


Figure 8a: Quantile comparison plots indicate distribution of nicotine and cotinine in saliva.

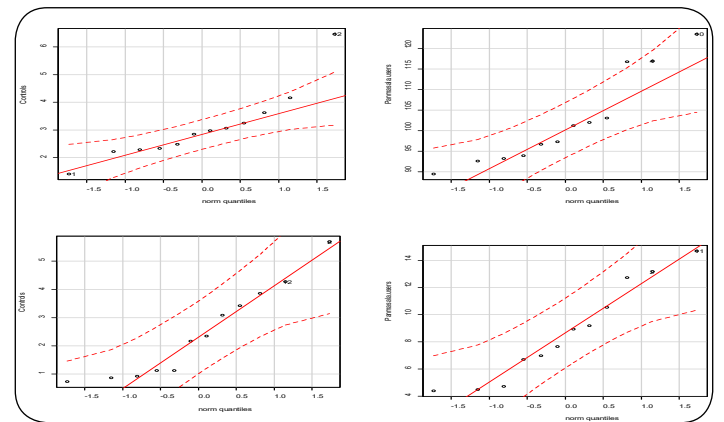


Figure 8b: Quantile comparison plots indicate distribution of nicotine and cotinine in urine.

Discussion

The consumption of tobacco is the specific marker of nicotine addiction and its exposure [21]. The chewing habits of smokeless tobacco are the major contributor of detectable concentrations of nicotine and its metabolite in saliva and urine. Our results reported that the levels of nicotine and cotinine in samples of saliva and urine were increased in panmasala users. Begum et al. [22] reported that the smokeless tobacco users have shown to be significantly increased levels of salivary nicotine

Table 2: Saliva biochemical profile in controls and panmasala users.

Parameter (g/dl)	Controls	Panmasala users
Albumins	0.29±0.04	0.16±0.008*
Total proteins	1.14±0.04	0.90±0.05*
Globulins	0.88±0.08	0.73±0.04NS
NO ₂ (µmoles/L)	3.78±0.27	6.24±0.57*
NO ₃ (µmoles/L)	44.63±1.91	55.87±1.98*

Data are represented as the mean ± SEM. * denotes that data are significantly different with the controls.

Note: NS-Not significant; NO₂-Nitrites; NO₃-Nitrates.

Table 3: Effect of smokeless tobacco on urine biochemical parameters.

Parameter	Controls	Panmasala users
Albumins (g/dl)	0.16±0.008	0.19±0.02NS
Total proteins (g/dl)	1.35±0.13	0.91±0.04*
Globulins (g/dl)	1.20±0.12	0.72±0.05*
NO ₂ (nmoles/mg creatinine)	21.87±2.10	53.02±4.00*
NO ₃ (nmoles/mg creatinine)	180.08±18.03	332.36±19.60*

Data are represented as the mean ± SEM. * denotes that data are significantly different with the controls.

Table 4a: Correlation of nicotine and cotinine with LPO, NO, and antioxidant enzymes in saliva of panmasala users.

Parameter	Nicotine		Cotinine	
	r	P	r	P
Lipid peroxidation	0.16	0.61	0.18	0.58
Nitric oxide	-0.063	0.84	-0.12	0.7
SOD	-0.46	0.13	-0.23	0.46
CAT	-0.48	0.11	-0.31	0.32

Table 4b: Correlation of nicotine and cotinine with LPO and NO in urine of SLT users.

Parameter	Nicotine		Cotinine	
	r	P	r	P
Lipid peroxidation	-0.31	0.31	-0.081	0.8
Nitric oxide	0.49	0.1	-0.24	0.45

r= Correlation coefficient; P < 0.05 was considered statistically significant.

and cotinine compared to normal healthy controls. The significant increase in the concentrations of nicotine and cotinine in urine samples of SLT consumers [23]. The major portion of nicotine is metabolized into cotinine and remaining amounts of nicotine is metabolized into trans3'-hydroxy cotinine, nicotine glucuronide, and others etc [24]. The global consumption of smokeless tobacco products is prevalent in India and amounts of tobacco-specific nitrosamines in SLT products were greater compared to nitrosamine levels present in food [25].

in the levels of nitric oxide (Nitrites and nitrates), and malondialdehyde in saliva and urine of experimental subjects. The enhanced production of superoxide anion, lipid peroxidation, DNA fragmentation occur significantly in primary cultures of human oral keratinocytes by the administration of smokeless tobacco extracts [26]. Previous reports revealed that the habitual users with lower antioxidant enzymes and higher oxidative stress markers are associated with higher lifetime tobacco exposure [27]. The earlier reports stated the major aqueous antioxidant component of whole saliva is uric acid, with lesser contributions from ascorbic acid and

albumin [28]. Nitric oxide is considered to be detectable concentrations in certain pathologic conditions and serves as a biomarker of health and disease [29] (Figure 9).

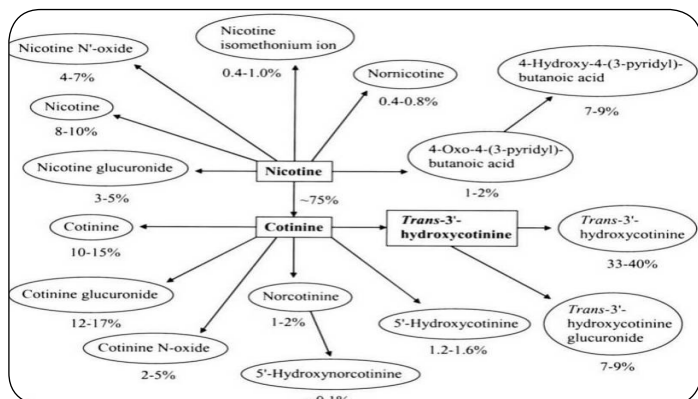


Figure 9: The major metabolic pathway of nicotine alkaloid in smokeless tobacco consumers [24].

The antioxidant status was decreased in saliva of experimental subjects was observed. The cytotoxic effects of the pan masala are mediated through the production of the reactive oxygen species in the cells [30]. The various types of oxidative stress like nitroxidative and glycol oxidative stress which may be associated with the modification of redox regulation and cellular function [31]. The glutathione and glutathione-dependent enzymes play a central role in cellular defense against toxicants and carcinogens of the environment [32]. Oral smokeless tobacco causes a duration-dependent increase in oxidative stress through significant decrease in erythrocyte malondialdehyde with significant decreased in the enzyme activities of antioxidant enzymes [33]. Panmasala users have shown that the significantly decreased activities of glutathione and antioxidant enzymes in the saliva are evaluated. The habitual users with lower antioxidant enzymes and higher oxidative stress markers are associated with higher lifetime tobacco exposure [27]. The antioxidant enzyme of superoxide dismutase is involved in the conversion of super oxide anion into hydrogen peroxide and oxygen [34].

Our results reported that salivary levels and urinary excretion of albumin are increased in panmasala users. The albumin constitutes extracellular antioxidant defenses in blood plasma and during many pathological conditions, biomarkers of oxidative protein damage increase with considerable oxidation of human serum albumin [35]. The concentrations of nicotine in the body results from smokeless tobacco can lead to nicotine addiction and dependence [36]. Increases in the number

of pack of smokeless tobacco used, were associated with increased salivary levels of cotinine and associated with higher in smokeless tobacco consumers [37]. The mean values of salivary uric acid are significantly decreased/diminished in smokeless tobacco users and earlier reports have stated the major aqueous antioxidant component of whole saliva is uric acid, with lesser contributions from ascorbic acid and albumin [28].

Our study demonstrated that elevated levels of urine albumins in panmasala users than controls. Urinary albumin excretion is measured as albumin creatinine ratio and it reflects the vascular process which affects the glomeruli and albumin acts as an index of renal damage [38]. The mean values of urea were decreased in the urine of smokeless tobacco users and are main metabolite of nitrogen excretion. The urea was inversely related to blood pressure and hypertension [39]. The determination of creatinine has been reported to be useful in evaluating the renal handling of uric acid and as concentrations of this parameter is highly dependent on endogenous production as well as on renal excretion [40].

Conclusion

The chronic consumption of panmasala is actively engaged in the development of oxidative stress in saliva and urine through enhanced formation of free radicals like nitric oxide, products of lipid peroxidation and decreased activities of glutathione, uric acid, albumin, and antioxidant enzymes. The nicotine is the main leading role and active component of ROS formation in saliva and urine of panmasala users. This work provides as an understanding of the physiological impact of panmasala products on induction of oxidative stress through the imbalance of antioxidant enzymes in biological fluids of saliva and urine leads to all disease chronic complications in chronic users of smokeless tobacco.

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