



Smokeless tobacco induced biophysical and biochemical alterations in the plasma, erythrocytes, and platelets of panmasala users: Subsequent biological effects

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ABSTRACT

Aim & background: Smokeless tobacco (SLT) products are extensively consumed throughout the world including India. These products act as the primary addictive agents, due to the presence of nicotine among other tobacco products to humans and animals and its quitting is difficult. Higher the exposure of SLT products more is the toxic effects and alterations in erythrocytes and platelets.

Objectives: The products of smokeless tobacco could cause increase in the concentrations of oxidants (free radicals), decrease the activities antioxidant enzymes, activate the process of programmed cell death through enhanced expression of inducible nitric oxide synthase. Smokeless tobacco products represent a major modifiable risk factor for the development of redox imbalance through the enhanced production of reactive oxygen species and diminished activities of antioxidant enzymes in plasma, bio-membranes of erythrocytes, and platelets and induction of apoptosis in the blood.

Materials and methods: The protein expression of inducible nitric oxide synthase (iNOS) was studied by western blot and gene expression of apoptotic proteins, tumor necrosis factor-alpha (TNF- α), interleukin-6 (IL-6) was evaluated by RT-PCR technique. Membrane fluidity of erythrocytes and platelets was studied by the fluorescence method.

Results: The results of the present study revealed that significantly elevated levels of iNOS enzyme in plasma, erythrocyte, and platelet membranes of panmasala users. We found that gene expression levels of Bcl₂, Bax, IL-6, caspase proteins (Caspase 8, Caspase 10, and Caspase 12) are greater and decreased levels of TNF- α with no significant change in blood of smokeless tobacco users in comparison with normal controls. In addition, there were substantial significantly higher in concentrations of nicotine, cotinine, and epinephrine in the plasma of panmasala users than non-tobacco users. Panmasala can be caused a significant increase in nitroxidative stress marker (LPO, NO, and ONOO⁻) values and significant decrease in the levels of antioxidant enzymes in erythrocytes and platelets.

Conclusion: On the basis of the present study results, it may be concluded that the chronic use of panmasala than any smokeless tobacco products may be a contributory risk factor or may give conclusive idea and has been associated with the expansion of the development of structural and functional alterations in the erythrocyte and platelet membranes induced oxidative damage and apoptosis, possibly further enhanced by nicotine and tobacco-specific N-nitrosamines. SLT exposure had implicated a threat and enormous implications on public health and is required to prove that may not be viewed as a safe alternative to any tobacco products.

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1. Introduction

Tobacco is used mainly in the form of smoking tobacco and smokeless tobacco products [1]. These smokeless tobacco products have suggested as popular tobacco products worldwide and have attracted attention for its potent toxic effects. Nicotine is considered as a major alkaloid of all tobacco products and has greater addiction, dependency potential to users [2]. It is alarming to note that the extensive use and high persistence in the Indian tobacco market results in greater prevalence, offering multiple avenues of exposure of these SLT products to humans. The risk increases with the increase in the prevalence and duration of chewing habits. Smokeless tobacco is ingested predominantly as chewing, sucking, and inhaled through the nasal mucosa. Previous publications have shown that the common factors of sex, age, ethnic origin, and socioeconomic status are known to influence the variation in the pattern of consumption of smokeless tobacco products [3]. According to a working group of the International Agency for Research on Cancer (IARC), smokeless tobacco is regarded as a group I carcinogenic to humans. The tobacco-specific N-nitrosamines are 4-methyl nitrosamino 1, 3 pyridyl butanone (NNK), nitrososornicotine (NNN), nitrosoanatabine (NAT), and nitrosoanabasine (NAB) [4]. Hecht

and Tricker, (1999) reported that the tobacco-specific N-nitrosamines are synthesized from nicotine, nornicotine, anabasine, and anatabine respectively through the process of nitration (Fig. 1) [5]. Chronic exposure to SLT products is due to the presence of hazardous chemicals (Nicotine and TSNA) in the smokeless tobacco products is detrimental to human health [6]. Several studies have reported smokeless tobacco products contain toxicants and carcinogens, including nicotine, N-nitrosamino acids, volatile N-nitrosamines, aldehydes (formaldehyde and acetaldehyde), hydrocarbons, and polonium-210 [7].

Recently, Food and Drug Administration, (2017) have shown to reduce the concentration of nitrosamines like 4-methyl nitrosamino 1, 3 pyridyl butanone and nitrososornicotine in smokeless tobacco products than the original content [8]. Cumulative evidence was reported to provide that various brands of panmasala from nearby paan shops in Ananthapur. The Rajnigandha, Vimal, Rasikala Manikchand Dhariwal (RMD), and Hira panmasala are the most widely consumed brands markedly available in Ananthapur. Recently, People were required likely to consume this panmasala brand in combination with chewing tobacco brand (Rajnigandha with BABA 120, Vimal with V1 scented tobacco, RMD with M scented tobacco, and Hira with Royale-717 tobacco produced by the same company [9]. In addition, an increasing

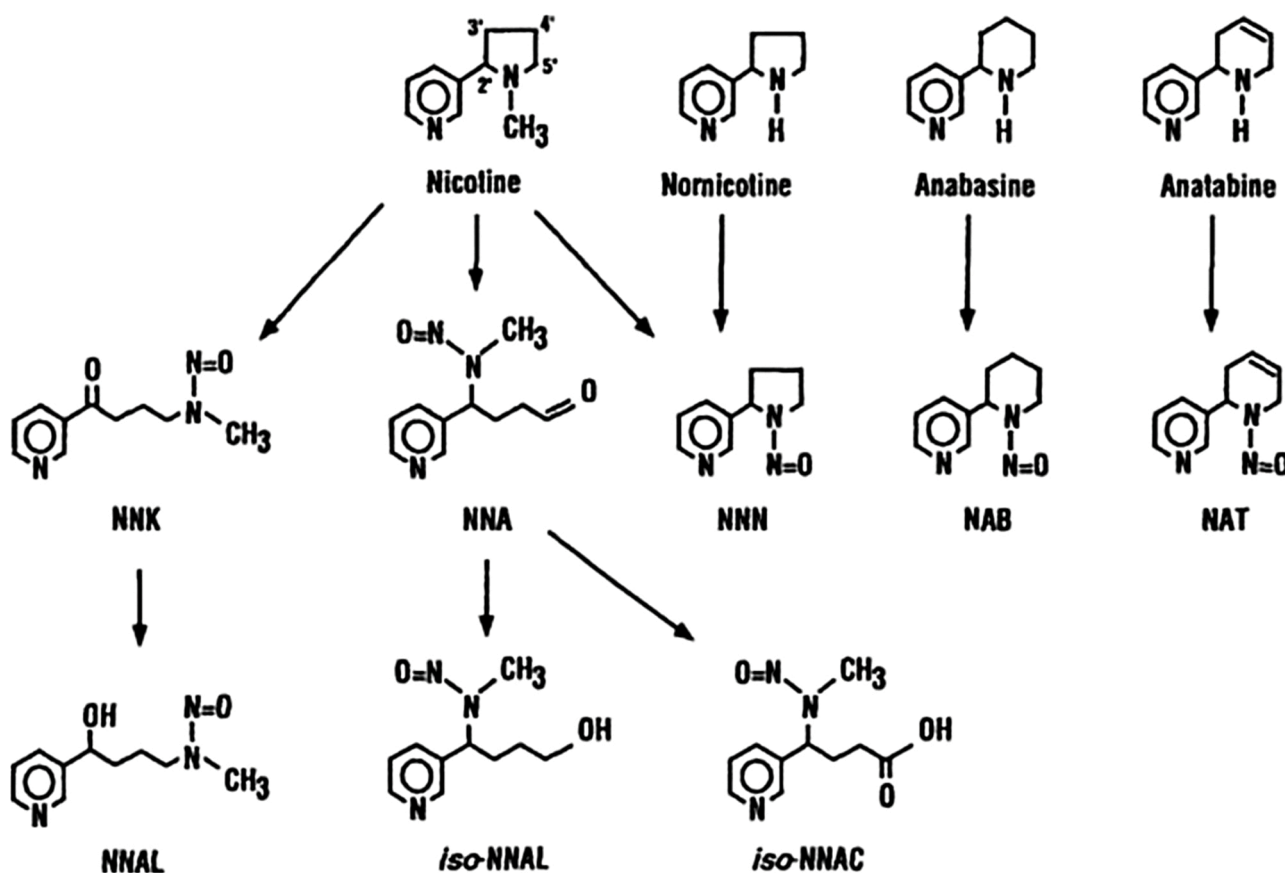


Fig. 1. Formation of tobacco-specific N-nitrosamines from nicotine and other alkaloids in smokeless tobacco products [5].

a) Status of kidney markers and blood hemoglobin.

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

b) Levels of liver marker enzymes and glycosylated hemoglobin in plasma of controls and experimental subjects.

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

Note: SGOT; Serum glutamate oxaloacetate transaminase, SGPT; Serum glutamate pyruvate transaminase, ALP; Alkaline phosphatase; HbA1c-Glycosylated hemoglobin.

c) Biochemical profile in plasma of controls and experimental subjects.

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

Note: VLDL-C; Very low-density lipoprotein cholesterol, LDL-C; Low-density lipoprotein cholesterol.

d) Biochemical profile in plasma of controls and experimental subjects.

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

Note: HDL-C; High-density lipoprotein cholesterol; CRI-Coronary risk index.

number of smokeless tobacco products are directly proportional to the consumption patterns, consumers influence with their friends, addictive behaviour and use occur highly among young adult males. There is evidence that supports that the availability of smoke-free legislation, increased taxes on smoking, high social acceptance during working periods have described directly proportional to the consumption of smokeless tobacco among adolescent males [10]. There have been many reports on chronic consumption of smokeless tobacco have suggested may be linked to the development of chronic diseases like cardiovascular disease, oral cancer [11,12]. The ingredients like lime and catechu, used in the preparation of smokeless tobacco products are indicated by involved in the production of reactive oxygen species (ROS) in the cells [13].

It has been established that the rate of smokeless tobacco consumption becomes susceptible to be a contributory factor in the elevated concentration of reactive oxygen species, the most prevalent of which are nitric oxide and peroxynitrites [14]. The reactive oxygen species of hydroperoxides and reactive nitrogen species of nitric oxide and peroxynitrites are produced during metabolic reactions in the cells. Previous publications have shown that free radicals are potent toxicants involved in the chronic damage to biomolecules like lipids, proteins, and DNA [15]. The nitric oxide was used to evaluate by measuring the levels of nitrites and nitrates, stable end products of nitric oxide (NO) metabolism. Nicotine could cause enhanced production of nitric oxide and indirectly involved in the pathogenesis of endothelium dysfunction [16]. The direct toxicity of nitric oxide is modest but is greatly enhanced by reacting with superoxide to form peroxynitrite (ONOO^-). NO have shown that acts as free radical present in greater amounts high competing with the superoxide dismutase for superoxide, precursor for the formation of peroxynitrites [17].

The majority of nicotine is metabolized into cotinine and 3-hydroxycotinine [18]. Cotinine acts a major metabolite and an important indicator of nicotine addiction and dependence [19]. However, to date, the role of smokeless tobacco in the development of -oxidative stress in membranes in humans has not been studied. Therefore, this study was to explore the underlying mechanisms of smokeless tobacco induced toxicity in erythrocyte and platelet membrane through redox imbalance, interruption of antioxidant enzymes, and induction of apoptosis.

2. Materials and methods

2.1. Chemicals

N-1-naphthylethylene diamine (NED), Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), 5, 5' -Dithio-bis (2-nitro benzoic acid) (DTNB), Antibodies iNOS, Ethidium bromide (EtBr), Enhanced chemiluminescence (ECL), Horse radish peroxidase (HRP), and all other fine chemicals were obtained from Sigma Aldrich, Bangalore.

2.2. Study area and data collection

Sixty human male volunteers were used for this purpose and each group included thirty volunteers, aged between 20–40 years residing in Ananthapuramu town, Andhra Pradesh taking local diet and using smokeless tobacco (Panmasala) were selected as experimental subjects who include mainly auto drivers, car mechanics, and house builders by using a questionnaire. Non-tobacco users, individuals who do not consume any form of tobacco products were regarded as normal healthy controls. The baseline information for the category of SLT users was that individuals used SLT products habitually, at least > 20 times per week consists of 50–60 g during the last 4 years. Socio-demographic information was collected by an interviewer with information on age, educational qualification, marital status, income, occupational status, and religion [20].

The inclusion criteria are the habitual use of only panmasala packets by the users, and choose unmarried and low economic status people. The

exclusion criteria are the consumed either alcoholic, smoking groups, and religion of the people are not preferred. All the baseline information regarding smokeless tobacco user's consumption, age, occupational status, etc are collected from human male volunteers through questionnaire sheets separately. In the present study, all volunteers were free from any chronic disease, illness, and teetotalers with no smoking habit with free from the use of any tranquilizers, drugs and anesthetics. All experiments were performed by following the approved guidelines and regulations of the Ethical Committee (No.25/1/2019-AWD) and ethical committee is Sri Krishnadevaraya University.

2.3. Blood sample collection and analysis of clinical parameters

Blood samples, drawn from human male volunteers by vein puncture between 7 and 10 AM into heparinized test tubes, were used immediately for plasma analysis. A spectrum of clinical parameters like fasting plasma glucose, urea, uric acid, creatinine, hemoglobin, glycosylated hemoglobin, total lipids and lipoprotein profile, liver marker enzymes and other plasma variables were analyzed from all studied individuals from their blood using auto analyzer kit methods. Plasma total amino acids were estimated by Moore and Stein [21], iron by the Ramsay method (1958) [22]. Nitrites and nitrates were by the method of Sastry et al. [23] and values obtained by this procedure represent the sum of nitrite and nitrate levels in the form of nitric oxide. The sample containing peroxynitrite was by using the protocol of Beckman et al. [24], lipid peroxidation was by the method of Buege and Aust [25] and glycolipids were estimated by the method by Roughan and Batt [26].

Protein oxidation of cells was measured as carbonyl group content according to the protocol of Levine et al. [27]. Erythrocytes were isolated by using the method of Beutler [28], the red blood cell membrane was prepared using the protocol of Dodge et al. [29] and protein content was determined by Lowry et al. [30]. The determination of GSH activity was by the protocol of Ellman [31]. Superoxide dismutase (SOD) activity was measured according to the protocol of Kakker et al. [32]. The catalase activity was by Aebis [33], the activity of glutathione-s-transferase was assayed by Rotruck et al. [34]. Erythrocyte membrane lipids were extracted as described previously by Folch et al. [35]. The erythrocyte/platelet membrane suspension with isopropanol and chloroform and aliquots were taken for estimation of cholesterol by Zlatkis et al. [36] and phospholipids by Connerty et al. [37]. The quantitative measurement of membrane fluidity was performed by the fluorescence polarization technique described by Choi and Yu [38].

2.4. Individual phospholipids analysis

Individual phospholipids in the erythrocyte membrane were estimated by the method of Skipski et al. [39].

2.4.1. Procedure

Individual phospholipids were separated by thin-layer chromatography technique. TLC plates were prepared using silica gel in Na_2CO_3 (1 mM) which was coated as thin layers were of 0.5 mm thickness and dried. Chromatograms were developed with chloroform: methanol: acetic acid: water (25:15:4:2 by volume respectively). The average running time was 2 h. The plates were air-dried at room temperature for 20 min. The compounds (R_f values) with different membranes phospholipids were identified and encircled with a fine dissecting needle. Most of the iodine was allowed to evaporate before the removal of spots. Spots were carefully scraped and the vapour was suspended in eluting solvent (1 N HCl in methanol) and the samples were placed in the water bath for 15 min with periodical stirring at 50–600 °C and centrifuged and supernatants were used for the estimation of phosphorus content by Fiske and Subbarow [40].

2.5. Protein determination by western blotting

Equal amounts of protein (100 µg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride filters (Protein Sequencing Membrane, BioRad). Membranes were probed with the corresponding primary antibody followed by incubation with HRP conjugated anti-rabbit antibody and blots were developed with the ECL system. Normalization of Western blot was ensured by β-tubulin and bands were quantified using a scanner and Image J software.

2.6. Total RNA isolation and cDNA preparation

Total RNA was isolated by a combination of two methods. First, total RNA was isolated from the blood samples using the Trizol method (Thermo fisher scientific). RNA was dissolved in diethylpyrocarbonate (DEPC)-treated, RNase-free, water. Purity was tested nanodrop and considered suitable for further processing at 260/280 ratios of 2. Total RNA was converted to single-stranded cDNA using a high-capacity cDNA reverse transcription kit (Sigma Aldrich) composed of reverse transcriptase (RT) buffer, RT random primers, dNTP mix, reverse transcriptase, and RNase-free H₂O.

2.7. Gene expression analysis

Target genes included Bcl₂, Bax, IL6, caspase proteins, and TNF-α. Three independent experiments with two technical replicates for each were performed using SYBR Green PCR Master Mix (Thermo Fisher scientific). All data were normalized to the housekeeping gene: glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All graphs were created using Graph Pad Prism 6.01 for Windows (Graph Pad Software Inc., San Diego, CA).

2.8. HPLC

HPLC system (Shimadzu, Japan) is equipped with a binary gradient system with a 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 a C18 reverse-phase column (5 µ) at 37 °C.

2.9. HPLC operating conditions of nicotine and cotinine

The resolution of peaks was performed with the mobile phase consisting of a mixture of 0.272 g of KH₂PO₄, 0.184 g 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 dropwise addition of orthophosphoric 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 of Massadeh et al. [41]. Nicotine and cotinine at the concentrations of 20 µM/mL were used as standards.

2.10. Sample analysis of nicotine and cotinine

Sample analysis was processed by the modified method of Massadeh et al. [41]. A 0.1 mL aliquot of plasma sample was placed into a glass test tube and addition of 20 µL of 2.5 M NaOH to the sample 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 and addition of 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 the mobile phase. A 20 µL aliquot was injected into the HPLC for analysis.

2.11. HPLC operating conditions of epinephrine

The 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 of Misra et al. [42]. Epinephrine concentrations of mg/mL were used as a stock solution.

2.12. Measurement of atherogenic index and percentage of protection

$$\text{Atherogenic index} = \frac{\text{Total cholesterol} - \text{HDL cholesterol}}{\text{HDL cholesterol}}$$

$$\text{Protection}(\%) = \frac{\text{AI of experimental control} - \text{AI of treated group}}{\text{AI of control}} \times 100$$

2.13. 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 to indicate a statistically significant. Densitometry analyses of the western blots were done using Image J software and the data are represented as mean ± SEM.

3. Results

3.1. Biochemical profile

As shown in Table 1, the panmasala users had found higher levels of total proteins, globulins and decreased levels of albumins, total amino acids, iron, and glycolipids compared to those of non-tobacco users. It was observed that mean values of amino acids and glycolipids showed a significant difference and the remaining variables did not yield a significant result. Significantly increased levels of membrane cholesterol (+38.84 %) and total phospholipids were higher among smokeless tobacco users (+23.52 %) compared to normal controls. The data revealed that experimental subjects comprised decreased levels of membrane proteins than healthy controls (-21.36 %). The values of cholesterol/phospholipids ratio were increased in panmasala users with no significant change when compared to those of normal subjects (Table 2).

3.2. Markers of diabetes, cardiovascular, liver marker enzymes, and kidney markers

Data (Fig. 1a and b) demonstrated that, smokeless tobacco users have found that mean levels of creatinine (+34.44 %) and urea (+26.91 %) were increased significantly in experimental subjects in comparison with non-tobacco users. It was observed that values of uric acid levels were higher in the non-user group, however, the differences were not statistically significant. The panmasala users showed decreased levels of hemoglobin with no significant change than non-users. Whereas, the

Table 1
Plasma biochemical profile in controls and panmasala users.

Parameter	Groups	
	Controls	Panmasala users
Total proteins (g/dl)	6.46 ± 0.17	6.53 ± 0.37 ^{NS}
Globulins (g/dl)	2.73 ± 0.17	2.86 ± 0.37 ^{NS}
Albumins (g/dl)	3.83 ± 0.15	3.70 ± 0.14 ^{NS}
Iron (mg/dl)	127.23 ± 5.52	122.98 ± 3.27 ^{NS}
Glycolipids (mg/dl)	277.26 ± 5.68	254.89 ± 5.56*
Amino acids (mg/dl)	5.12 ± 0.28	4.20 ± 0.12*

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

Note: NS-Not significant.

Table 2
Effect of smokeless tobacco on biochemical parameters of erythrocytes.

Parameter	Groups	
	Controls	Panmasala users
Proteins (mg/dl)	126.78 ± 7.04	99.69 ± 2.72*
Cholesterol (µg/mg protein)	160.35 ± 11.72	222.63 ± 18.61*
Phospholipids (µg/mg protein)	122.63 ± 6.00	151.48 ± 7.39*
C/P ratio	1.30	1.46

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

Note: C/P ratio-Cholesterol/Phospholipids ratio.

mean levels of SGOT (+28.71 %), SGPT (+33.60 %), and ALP (+45.61 %) were increased significantly in experimental subjects when compared to non-tobacco users. We have found that the panmasala user group had significantly higher levels of glycosylated hemoglobin (+36.75 %).

As we examined in Fig. 1c and d, the levels of total cholesterol, and triglycerides, (+64.27 %) low-density lipoprotein-cholesterol (LDL-C, +16.87 %), and very low-density lipoprotein-cholesterol (VLDL-C, +50.87 %), were higher in experimental subjects than normal controls. Moreover, SLT users have reported that significantly decreased levels of high-density lipoprotein cholesterol compared to the healthy controls (HDL-C, -18.81 %). The mean values of thiols, coronary risk index (CRI), and atherogenic index, were significantly higher in study subjects and CRI did not showed significant difference.

3.3. Nitroxidative stress makers in plasma

From the summary statistics in Fig. 2 have described that significantly higher levels of malondialdehyde (+20.00 %), protein carbonyls, and peroxynitrites in plasma of study subjects than non-tobacco users. The mean values of nitric oxide (+19.40 %) were higher in panmasala users and there is no significant difference observed in comparison with normal subjects.

3.4. HPLC chromatograms of nicotine, cotinine, and epinephrine

The range of retention time of standard nicotine is 5.8–6.8 min and has shown a chromatogram peak at 6.51 min. The range of retention time of standard cotinine is 3.6–4.6 min and showed that a chromatogram peak at 4.09 min. The epinephrine standard has exhibited at the range of retention time is 3.6–4.0 min and a chromatogram peak at 3.60 min (Fig. 3a). Our data demonstrated that (Fig. 3b) indicated that there are no peaks observed in chromatograms of plasma 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 the control group due to environmental tobacco exposure and some food constituents. Panmasala consumers showed that nicotine chromatogram peak at the retention time of 5.35 min and cotinine peak at 3.81 min. There is a peak observed in chromatograms of plasma in normal controls at the retention of epinephrine at 3.62 min and panmasala consumers showed that long sharp chromatogram peak observed at the retention time of 3.60 min.

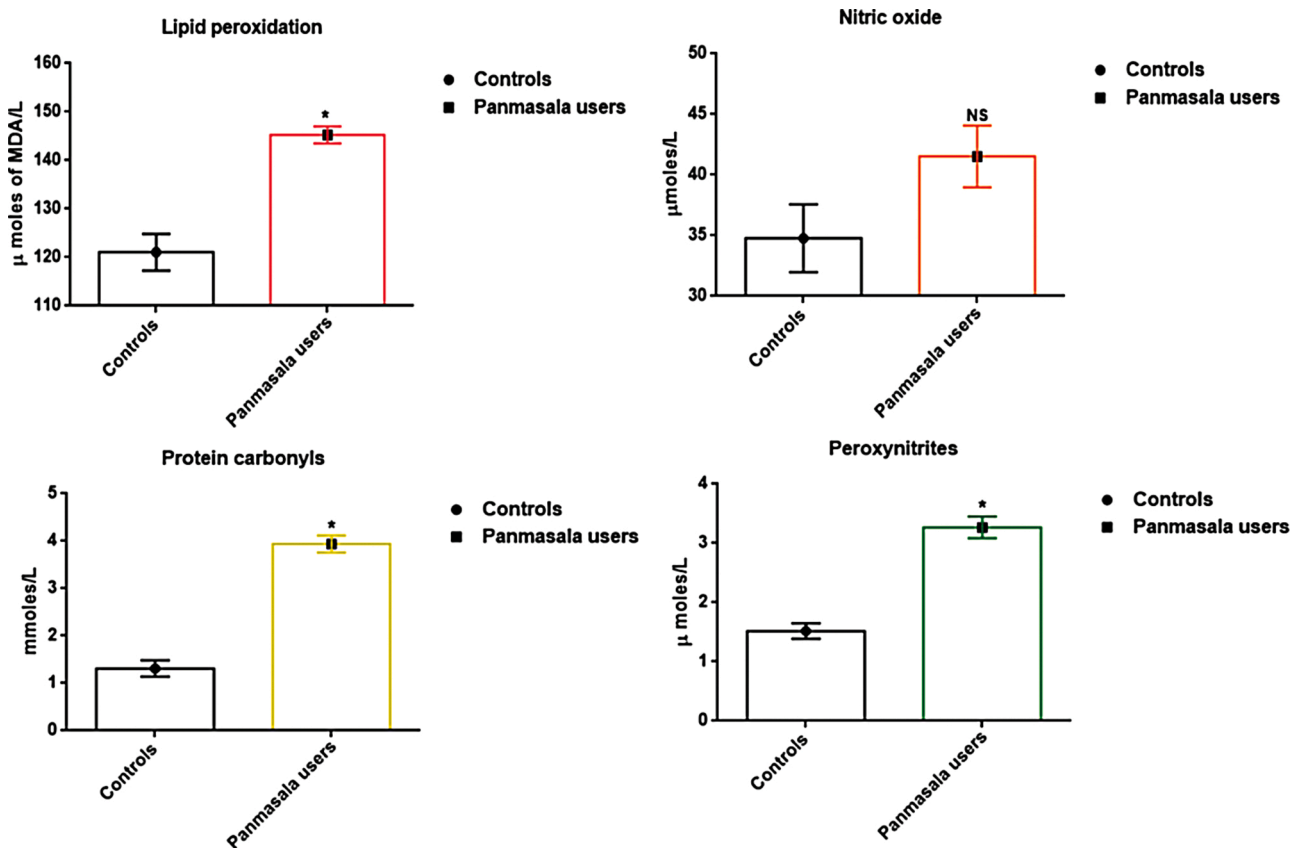
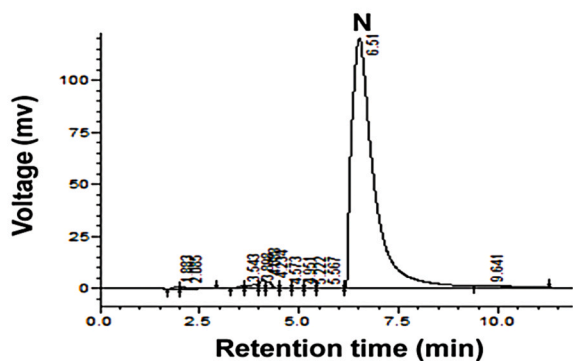
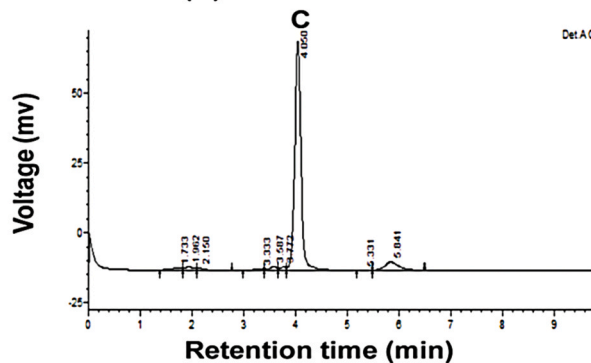


Fig. 2. Levels of nitroxidative stress markers in plasma of panmasala users. Data are represented as the mean ± SEM. * denotes that data are significantly different with the controls.

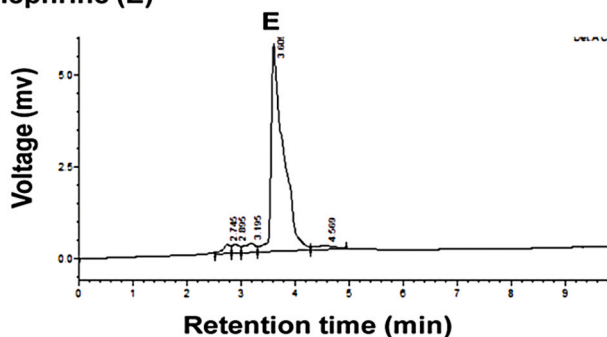
1. Nicotine (N)



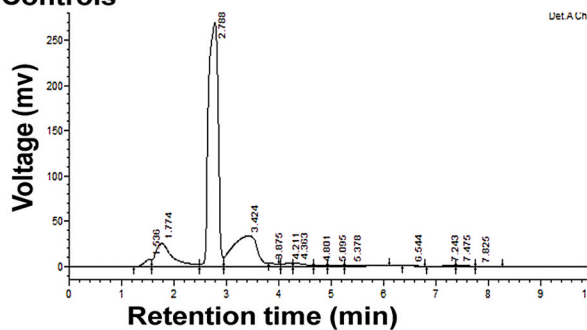
2. Cotinine (C)



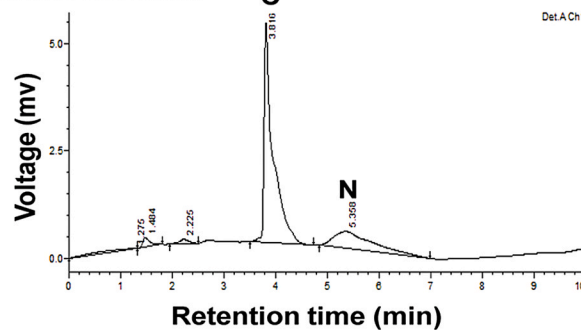
3. Epinephrine (E)



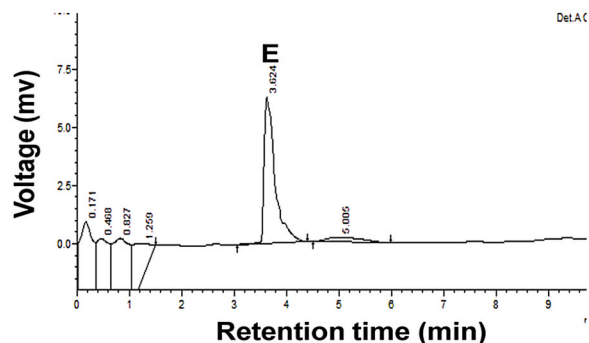
Nicotine and cotinine Controls



Panmasala users



Epinephrine Controls



Panmasala users

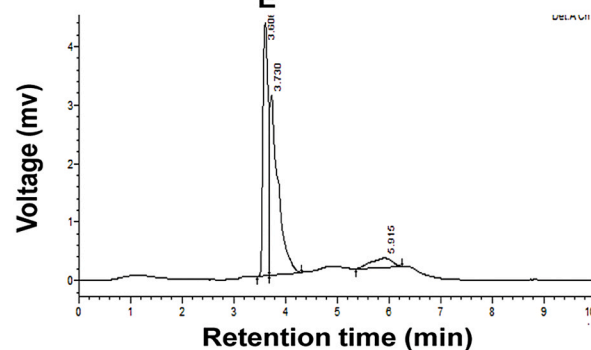


Fig. 3. a) HPLC chromatograms of nicotine, cotinine, and epinephrine standards.

b) HPLC chromatograms of nicotine, cotinine, and epinephrine in plasma.

The levels of thiols were significantly increased in panmasala users than normal controls (Fig. 1a).

c) The concentrations of nicotine, cotinine, epinephrine, and glucose in plasma.

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

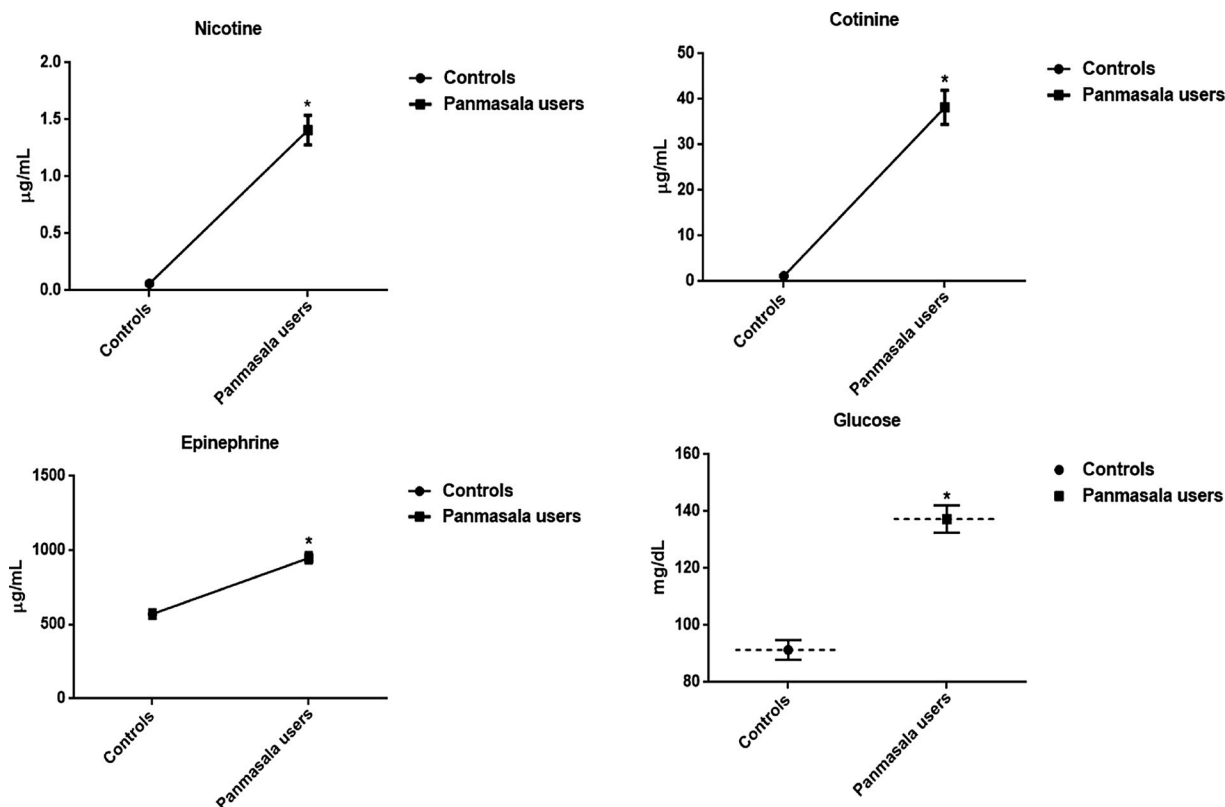


Fig. 3. (continued).

3.5. Concentrations of nicotine, cotinine, and epinephrine in panmasala users

HPLC analyses have carried out the nicotine and cotinine concentrations in smokeless tobacco users found that significantly higher levels of nicotine, cotinine, and epinephrine in plasma when compared to normal controls. We found that the panmasala user group had significantly higher levels of fasting plasma glucose (+50.27 %) (Fig. 3c).

3.6. Concentrations of reactive oxygen species and individual phospholipids in the erythrocyte membrane

The resulted data of Fig. 4a indicated that the levels of lipid peroxidation (+67.82 %), nitric oxide (+40.74 %), peroxynitrites are significantly elevated and significantly lower levels of protein carbonyls (-55.78 %) were observed in the erythrocyte membrane of experimental subjects. The mean values of phosphodityl choline (PC) and sphingomyelin (SM) were significantly increased in panmasala users and normal controls found that increased levels of phosphotidyl ethanolamine (PE), phosphotidyl serine (PS), and phosphotidyl inositol (PI) than studied groups. The mean values of PE, PS, and PI have been found that the statistically significant change with normal controls. A decreased level of membrane fluidity with a significant difference found in panmasala users (-27.73 %) than non-chewers. The mean value of osmotic fragility was increased in SLT users than non-users (Fig. 4b).

3.7. Antioxidant enzyme status in panmasala users

We have demonstrated that decreased levels of superoxide dismutase (SOD, -30.43 %), catalase (CAT, -27.35 %), glutathione peroxidase, glutathione S-transferase were observed in the erythrocyte membrane of panmasala users than normal subjects. The levels of CAT were showed a statistically significant difference with the controls. The mean values of reduced glutathione were higher in smokeless tobacco users than normal

controls (Fig. 4c).

3.8. The protein expression of iNOS in plasma, erythrocyte, and platelet membranes

It showed significantly higher levels of protein expression for iNOS in the SLT-user group of plasma, erythrocytes, and platelet membrane in comparison with normal subjects. Densitometric analysis of immune blot data of the panmasala users group and non-user group were represented in Fig. 5.

3.9. Gene expression studies in panmasala users

Smokeless tobacco users had significantly increased levels of Bcl₂, Bax, Bcl₂/Bax ratio, and IL-6 in blood compared to controls. The mean values of TNF- α were decreased in panmasala users than non-tobacco users. The mean values of caspase proteins in experimental subjects have found to be higher and comparable with normal group controls and the mean values of caspase 8 and caspase 10 exhibited a significant difference as shown in Fig. 6a and b. The primer sequence of different apoptotic genes are listed in Table 4.

3.10. Biochemical analysis in platelet membrane of experimental subjects

From the summary statistics, we observed that the significantly increased concentrations of membrane cholesterol (+41.53 %), total phospholipids (+49.24 %), and proteins (+12.96 %) with no significant change than non-tobacco users (Table 3).

3.11. The markers of oxidative stress, antioxidant enzymes, fluidity, and concentrations of individual phospholipids in platelets

Data (Fig. 7a) demonstrated that levels of nitric oxide (+43.23 %), nitrites, nitrates, malondialdehyde, protein carbonyls, and

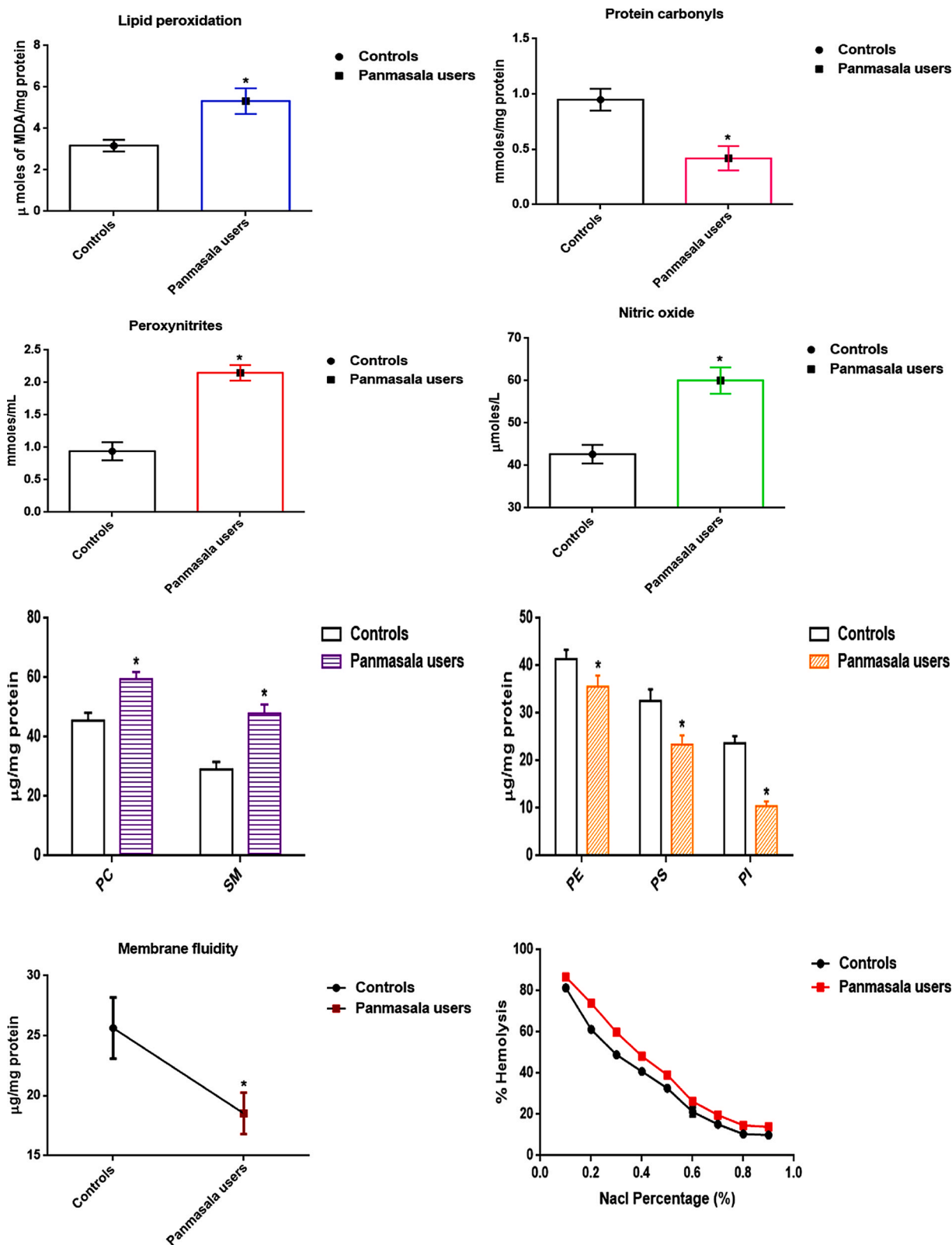


Fig. 4. a) Concentrations of reactive oxygen and nitrogen species in erythrocytes. Data are represented as the mean ± SEM. * denotes that data are significantly different with the controls.
 b) Concentrations of individual phospholipids, membrane fluidity, and osmotic fragility in erythrocytes. Data are represented as the mean ± SEM. * denotes that data are significantly different with the controls. The values of osmotic haemolysis are expressed as mean. Note: PC-Phosphotidyl choline; SM-Sphigomyelin; PE-Phosphotidyl ethanolamine; PS-Phosphotidyl serine; PI-Phosphotidyl ionisitol.
 c) The status of antioxidant enzymes in erythrocyte membrane. Data are represented as the mean ± SEM. * denotes that data are significantly different with the control. Note: GST-Glutathione S-Transferase; GPx-Glutathione peroxidase; GSH-Reduced glutathione; SOD-Superoxide dismutase; CAT-catalase.

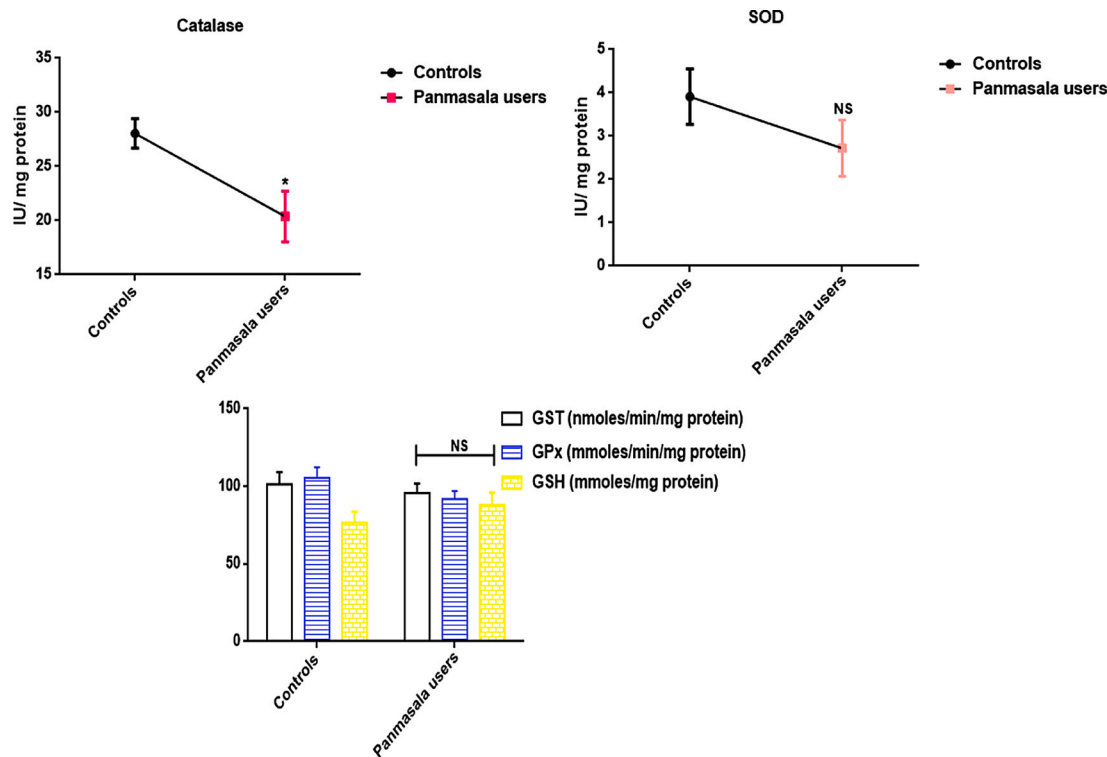


Fig. 4. (continued).

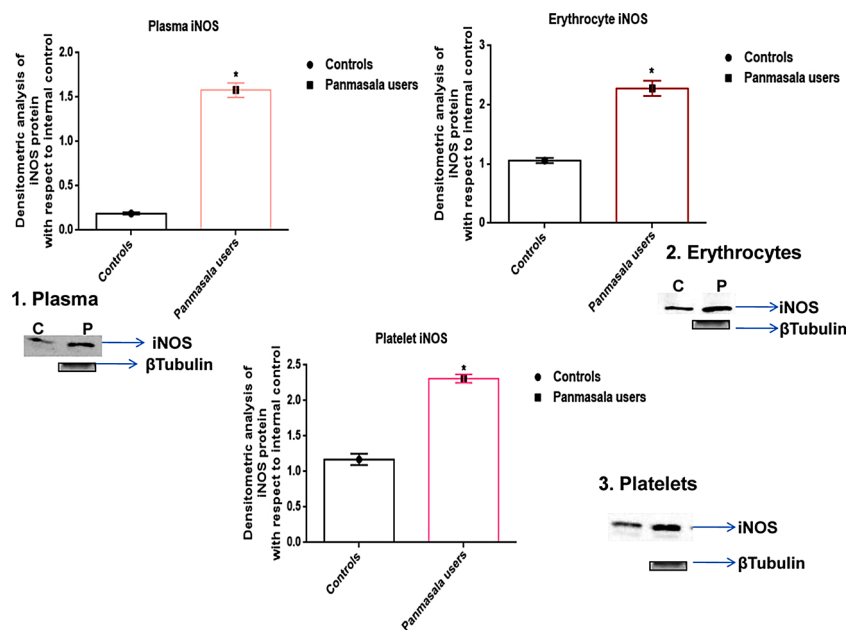


Fig. 5. The immune blots and concentrations of inducible nitric oxide synthase in plasma, erythrocytes and platelets.

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

Note: C-Controls; P-Panmasala users; iNOS-Inducible nitric oxide synthase.

peroxynitrites (+85.07 %) were significantly higher in platelet membrane of panmasala users. Our study indicated panmasala chewers were reported that the mean values of phosphodityl choline and sphingomyelin were significantly increased in panmasala users. The resulted values for phosphotidyl ethanolamine, phosphotidyl serine, and phosphotidyl ionositol were found to be significantly lower in the studied groups. Our study indicated panmasala chewers were demonstrated that significantly decreased membrane fluidity (-23.98 %) (Fig. 7b). The

mean values of glutathione (-32.74 %), and antioxidant enzymes (CAT: -31.96 % and SOD: -26.21 %) were significantly lower in platelets (Fig. 7c).

The quantile comparison plots indicated the distribution of plasma nicotine and cotinine concentrations in controls and panmasala users (Fig. 8). The different varieties of panmasala brands like rajnigandha with BABA 120, vimal with v1 scented, and RMD with M scented tobacco are the widely consumed flavours available in the Indian tobacco

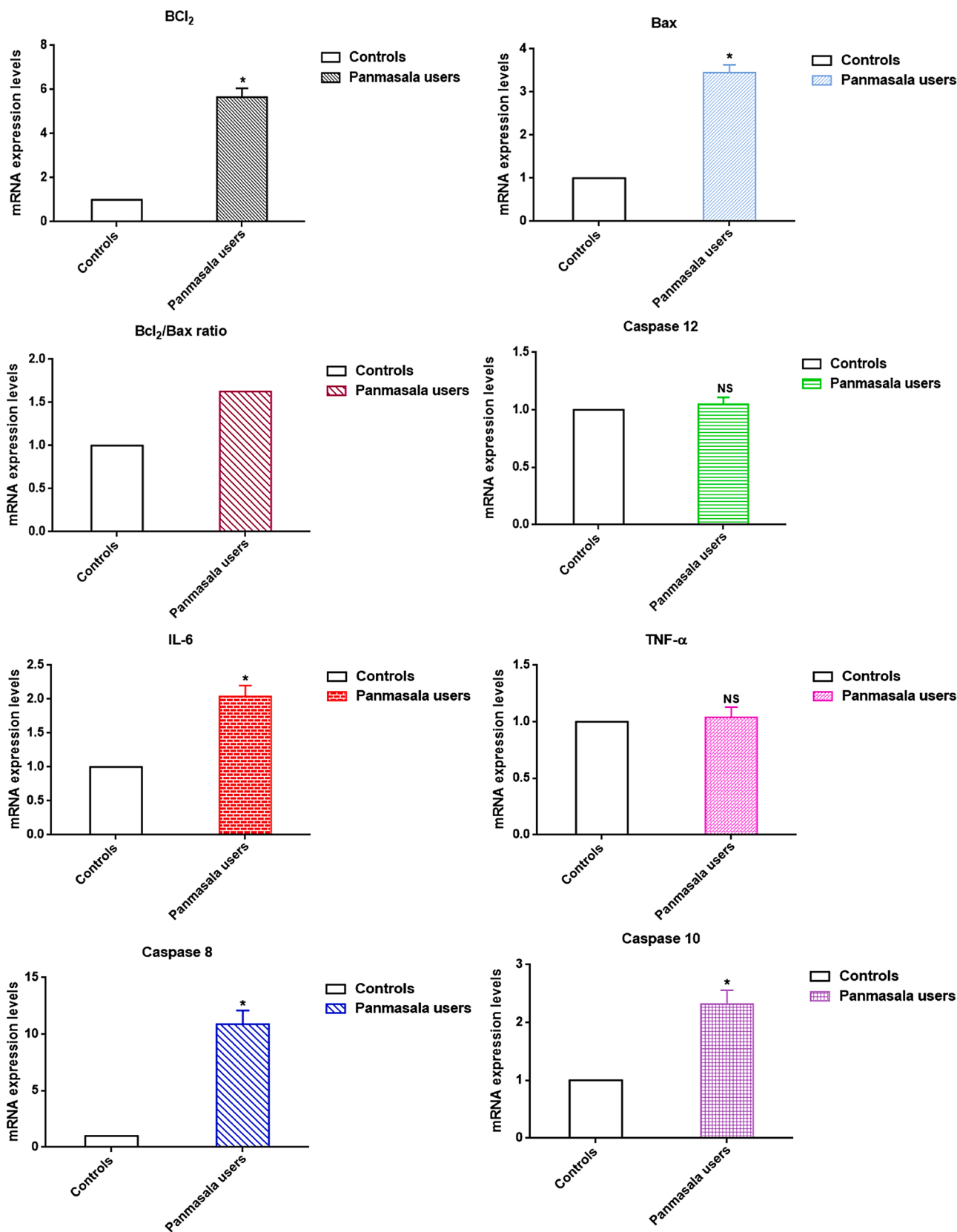


Fig. 6. a) The expression levels of apoptotic marker proteins in controls and panmasala users of plasma. Data are represented as the mean ± SEM. * denotes that data are significantly different with the controls.

b) The expression levels of IL-6, TNF-α and apoptotic marker proteins in controls and panmasala users of plasma. Data are represented as the mean ± SEM. * denotes that data are significantly different with the controls.

Note: IL-6-Interleukin; TNF-α-Tumor necrosis factor-alpha.

Table 3
Biochemical profile in platelets of panmasala users.

Parameter	Groups	
	Controls	Panmasala users
Proteins (mg/dl)	82.31 ± 3.87	92.98 ± 9.29 ^{NS}
Cholesterol (µg/mg protein)	417.94 ± 24.10	591.54 ± 25.09*
Phospholipids (µg/mg protein)	131.57 ± 4.07	196.36 ± 5.94*
C/P ratio	3.17	3.01

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

Table 4
Primers used in reverse transcription analysis.

Gene	Primer Sequence
Bcl ₂	F5'CCTGATTCATTGGGAAGTTTCAA 3' R5'AAACAAATGCATAAGGCAACGA 3'
Bax	F 5' TAATCCAGCGTTGGAA 3' R 5' TGCAGAGACCTGGATCTAGCAA 3'
TNF-α	F 5' GAAAGCATGATCCGGACGCTG 3' R 5' GATGGCAGAGAGAGGTTGAC 3'
IL-6	F 5'-CCAGCTATGAACCTCTCTC 3' R 5'-GCTTGTTCCTCACATCTCTC 3'
Caspase 8	F 5'-CTGGAAGGATCGACGATTA 3' R 5'-CATGTCCTGCATTTGATGG 3'
Caspase 10	F 5'-AATCTGACATGCCTGGAG-3' R 5'-ACTCGGCTTCTGTCTAC-3'
Caspase 12	F 5'-GCCATGGCTGATGAGAAACC-3' R 5'-CTGAGTTGCTTCTATGAG-3'
GAPDH	F 5'-GAGTCAACGATTTGGTCGT-3' R5'-GACAAGCTTCCCCTTCTCAG-3'

market. The company details and pictorial warning labels of the panmasala brands presented in the present study displayed on their packs (Fig. 9).

4. Discussion

Smokeless tobacco is a major consumption of tobacco in the human population and has become the leading source of tobacco products. Heavy metals like lead, cadmium, chromium of smokeless tobacco products could induce cancer risk and need to develop industry standards and regulation for smokeless tobacco products in the manner of reducing concentrations of heavy metals [43]. The emergence of tobacco harm reduction which results in decrease the burden of smoking and smokeless tobacco-related diseases [44]. It is of prime importance to note that, smokeless tobacco is involved in the up-regulation of iNOS enzyme and anti-apoptotic protein expression, the key player in the development of carcinogenesis. It was noticed that the reactive oxygen species are synthesized with the requirement of oxygen and exert toxic effects associated with chronic disease conditions like cardiovascular and neurodegenerative diseases [45]. People believed that individual tobacco products are less harmful and better than a mixture of all tobacco products [46].

In the current study, there were significantly increased levels of nitric oxide and peroxynitrites in plasma, erythrocyte membrane, and platelet membrane of panmasala users than non-tobacco users and nicotine was able to induce the production of NO and ONOO⁻ at the highest concentration. Our results are consistent with those from Bagchi et al. [47] reported that tobacco snuff could be capable of free radicals production, which leads to protein nitration, lipid peroxidation, the formation of DNA adducts. Overproduction of nitric oxide is correlated with the up-regulation of iNOS expression. The exposure of smokeless tobacco results in the production of reactive oxygen species and the level of iNOS/NO was significantly higher of smokeless tobacco users in plasma, erythrocytes, and platelets than that of non-tobacco users. The nitric oxide synthase is actively engaged in the formation of peroxynitrites.

The ONOO⁻ might have the functions of protein oxidation and nitrotyrosine formation in proteins, these are all specific markers for inducing cardiovascular diseases [48]. The direct or indirect effect of nicotine and TSNA might contribute to increased ROS results in the increased expression of nitric oxide synthase.

Previous studies have been shown that the smokeless tobacco extract was found to be a more toxic and potent activator of ROS production than nicotine alone [49]. Nicotine-induced the significant increase in reactive oxygen species that resulted in the activation of NF-κB by activating signal responsive kinases [50]. It is documented in the literature that tobacco-specific N-nitrosamine is known as 4-methyl nitrosamino 1, 3 pyridyl butanone proved that a strong carcinogen by an enhanced proliferation of lung cancer cells through reduced apoptosis [51]. The elevated levels of nitric oxide in chronic infections not only produce DNA alkylating agents and inhibit repair mechanisms results in chronic DNA damage [52]. More significantly, our data strongly support the hypothesis that toxic and carcinogenic activities of smokeless tobacco will be mainly due to the carcinogenic effects of tobacco-specific N-nitrosamines working together in a synergistic way. The present study illustrates that significantly increased levels of gene expression of Bcl₂, Bax, interleukin-6, caspase proteins, and decreased levels of tumor necrosis factor-alpha in panmasala users in comparison with normal controls.

Mangipudy and Vishwanatha [53] reported that smokeless tobacco extract causes a dose-dependent activation of apoptosis through the immediate actions of nitric oxide. We examined the participation of anti-apoptotic and pro-apoptotic markers, which have been described as importantly involved in the process of apoptosis. The exposure and toxicity of smokeless tobacco products play an important role in the induction of apoptosis in human macrophage cells [54]. Early studies demonstrated that the caspases have potential role in the enhancement of apoptosis and the precise elimination of excess cells [55]. Apoptosis was induced with the consequence of the generation of reactive species, induction of DNA damage, and activation of caspase-cascade [56].

Our data suggested that both panmasala users showed that significantly increased levels of nicotine and cotinine in the plasma than non-tobacco users. It was shown that previous reports revealed that blood nicotine levels were higher in cigarette smokers [57]. Cotinine acts as a biomarker of nicotine exposure and intake and both nicotine and cotinine can be responsible for the development of various pathological conditions like cardiovascular disease and metabolic alterations in the membrane. Lipids act as the structural components of membranes and peroxynitrites are the initiators of lipid peroxidation [58]. In SLT-users group, significant enhancement in the levels of malondialdehyde (MDA) of plasma, erythrocyte, and platelet membranes than non-tobacco users. The lipid peroxidation product like malondialdehyde causes alterations in the structural organization of membrane [59].

The lipid peroxidation could able to interact with oxidized LDL and indirectly induced the atherosclerosis risk [60]. The significant decrease in erythrocyte and platelet membrane fluidity was observed in panmasala chewers compared to non-chewers. The components of smokeless tobacco may able to cause alterations in the structure of the membrane by increasing the levels of PC, SM and decreasing levels of inner leaflet phospholipids (PE, PS, and PI). It is known that cholesterol is an essential component of lipoproteins and low-density lipoprotein cholesterol acts as bad cholesterol and high-density lipoprotein cholesterol act as good cholesterol. We have shown that SLT users have elevated levels of total cholesterol, triglycerides, LDL-C and decreased HDL-C are indicators for augmenting the risk of cardiovascular disease. It is widely known that nicotine has both cardioactive and cardiovascular effects have been reported and atherosclerosis risk is greatly announced in the smokeless tobacco consumers [61].

The LDL-C undergoes oxidation results in the formation of oxidized LDL and phospholipids acts as both pro-atherogenic and pro-inflammatory results in the development of atherosclerosis [62]. In response to smokeless tobacco consumption, the reactive oxygen species

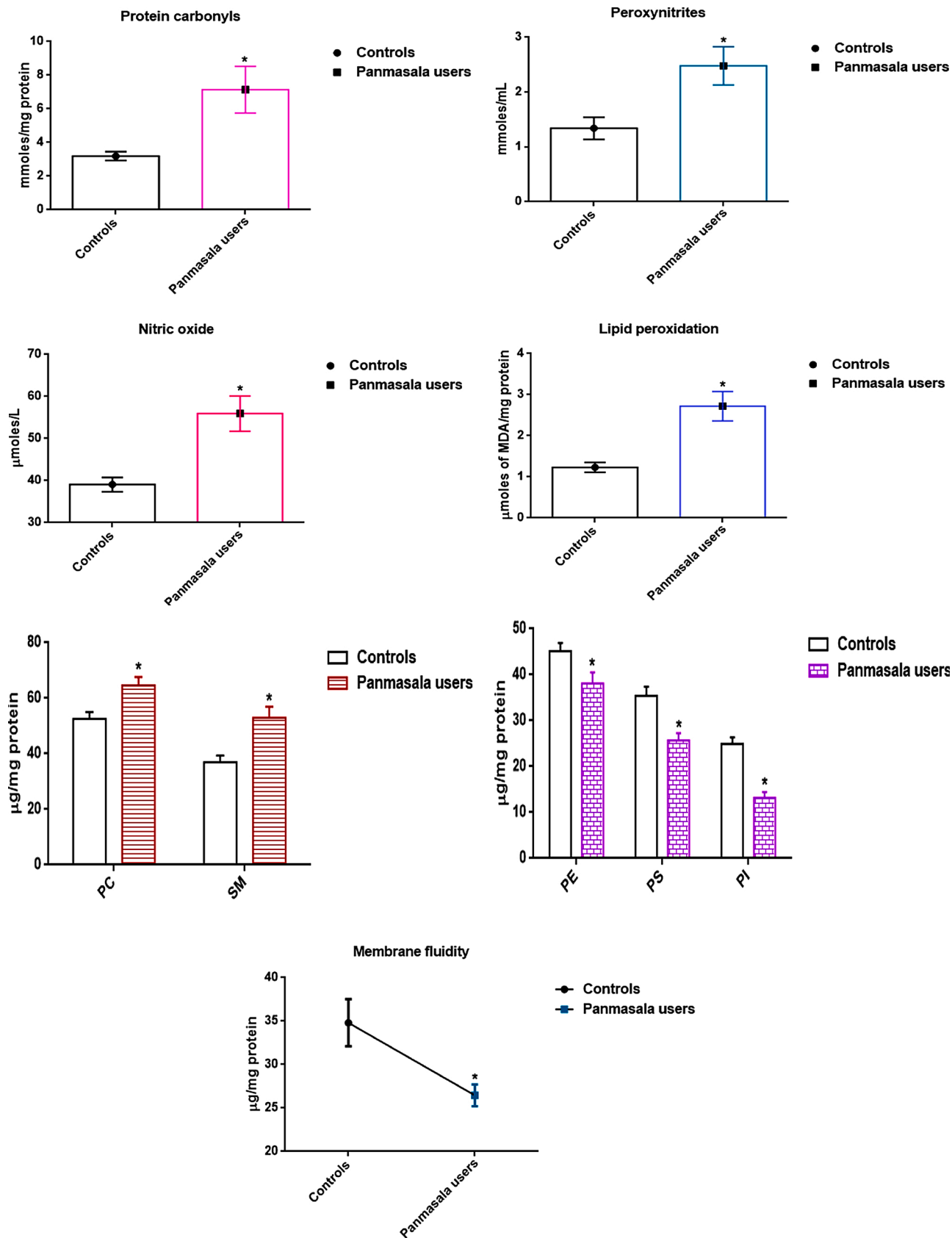


Fig. 7. a) Concentrations of protein carbonyls and peroxynitrites of platelets in panmasala users. Data are represented as the mean ± SEM. * denotes that data are significantly different with the controls. b) Concentrations of individual phospholipids and membrane fluidity of platelets. c) The status of antioxidant enzymes in platelets. Data are represented as the mean ± SEM. * denotes that data are significantly different with the controls.

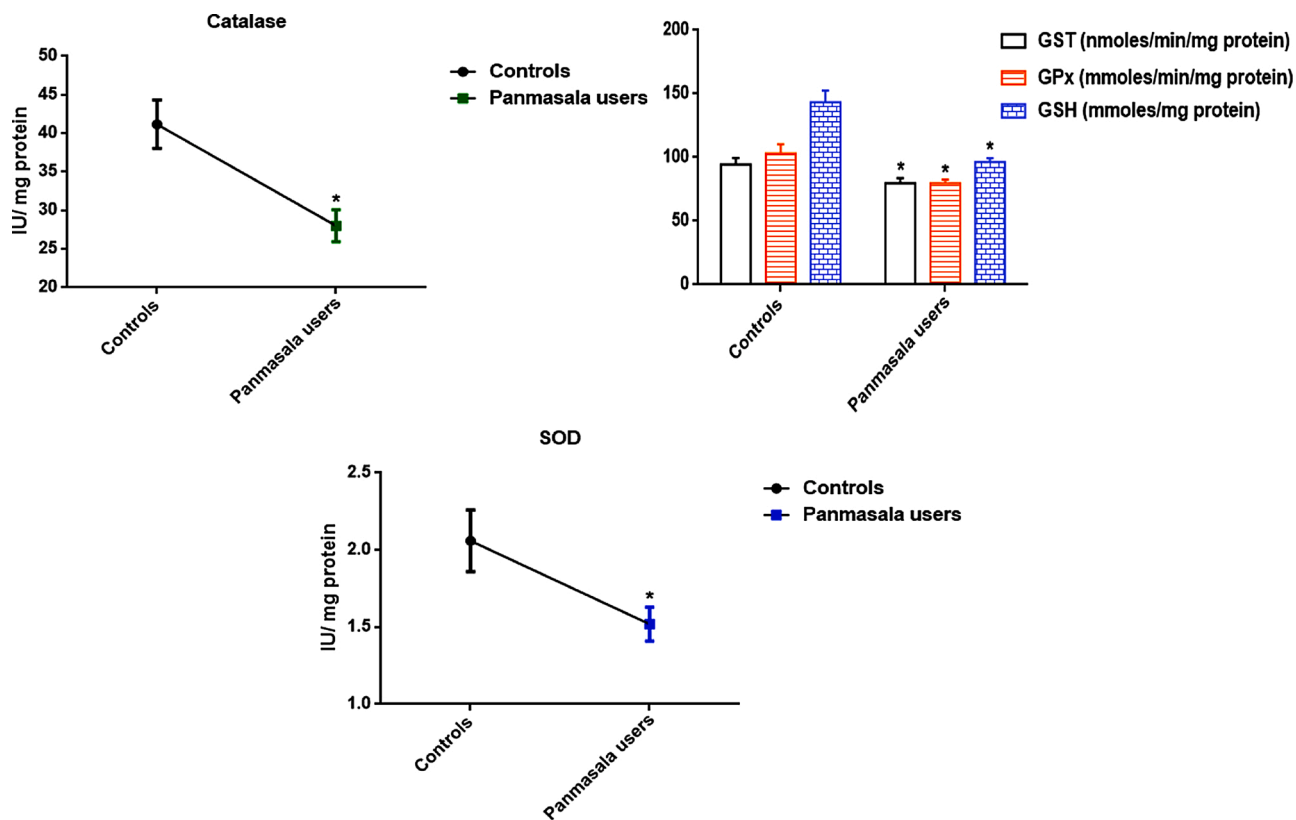


Fig. 7. (continued).

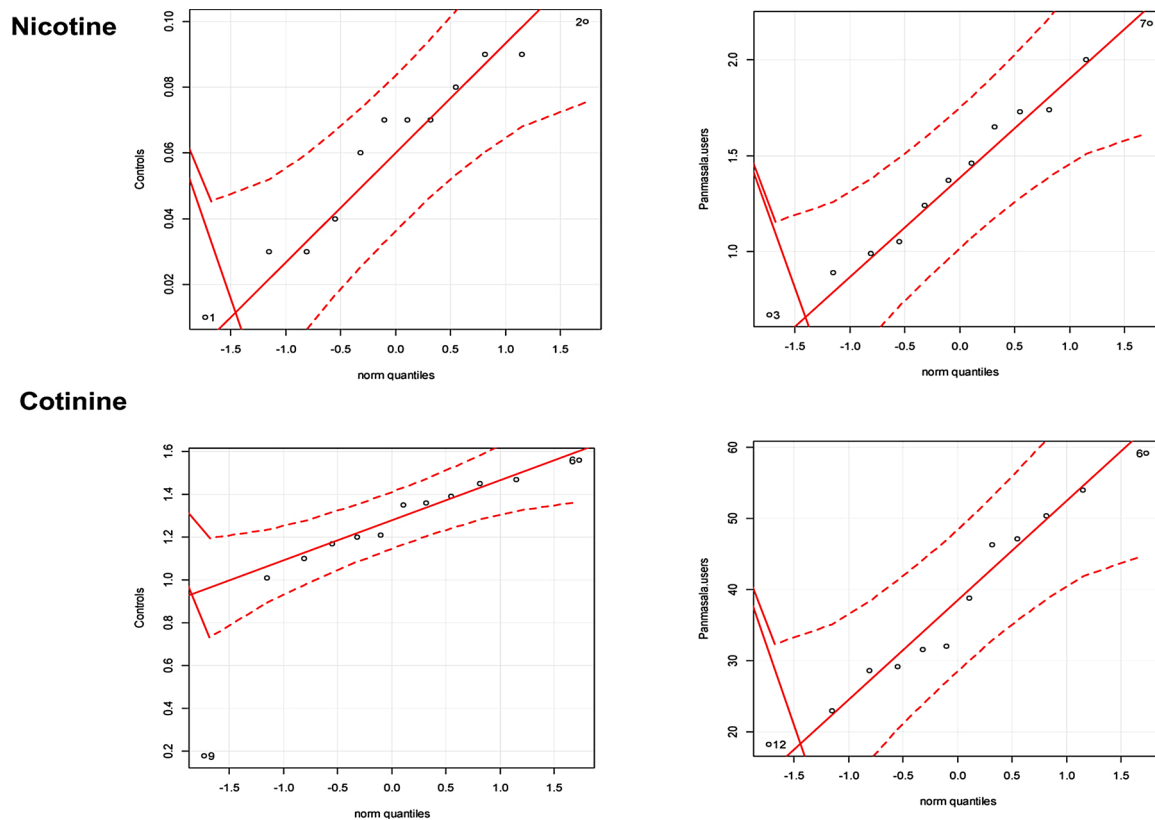


Fig. 8. Quantile comparison plots of plasma nicotine and cotinine in controls and panmasala users.

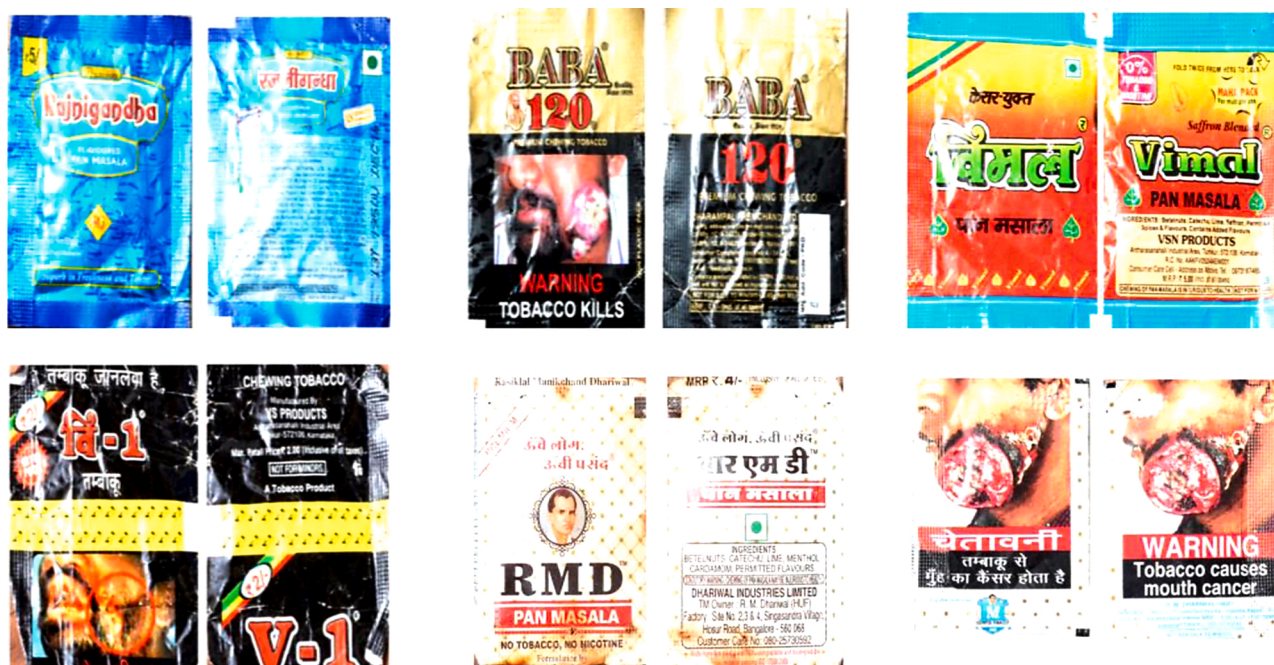


Fig. 9. The photographs of panmasala brands (Rajnigandha with BABA120, Vimal with v1 scented, and RMD with M scented tobacco).

are the predominant risk factors induce the derangements of metabolic and cellular response in the cells. In our experimental setup, we found increased levels of reduced glutathione in chewers of the RBC membrane compared to the non-chewers. Serum gamma-glutamyl transpeptidase (GGT) acts in the degradation of extracellular glutathione and making its amino acids available to the cells, results in the increased synthesis of glutathione [63]. In particular, nicotine from smokeless tobacco was able to induce gamma-glutamyl transpeptidase, resulting in increased glutathione production in erythrocytes.

We were found that decreased activities of antioxidant enzymes in erythrocytes, platelets, and decreased levels of albumin, uric acid of user group compared to the non-user group. The mean values of plasma total proteins and globulins were increased in SLT users in comparison with controls. These observations recommended insufficient antioxidant status would be more informative in plasma, erythrocytes, and platelets of panmasala chewers. In our study, we have shown that decreased levels of hemoglobin, iron and increased levels of erythrocyte glutathione than those of non-users. Earlier studies had shown that the reaction of glutathione with hemoglobin results in the formation of glutathionyl hemoglobin and it may be a marker of oxidative stress [64]. An ingredient like nicotine and tobacco-specific nitrosamines of smokeless tobacco disrupts the antioxidant system could explain conclusive association between smokeless tobacco and increased oxidative stress in cells and membranes.

The current study evidence remains sufficient to explain that smokeless tobacco which could induce significantly increased levels of protein carbonyls in plasma and platelets of panmasala users have a higher risk of protein damage related to redox imbalance. The experimental subjects had significantly increased levels of protein carbonyls in the membrane of erythrocytes. In our results, SLT users had reported that the significantly increased levels of cholesterol, total phospholipids in both erythrocyte and platelet membrane than those of non-SLT users. It is imperative that the erythrocyte membrane proteins were shown to be significantly decreased levels observed in the experimental subjects. Data have shown that smokeless tobacco use due to the presence of nicotine and tobacco-specific nitrosamines, the erythrocyte and platelet membranes undergo functional and structural alterations. Additionally, we reported that panmasala users found increased levels of platelet

membrane proteins in comparison to controls. Nicotine of tobacco products may alter the membrane composition leads to metabolic changes occur.

Interestingly, we found that smokeless tobacco users have reported that the increased levels of plasma urea, creatinine and liver marker enzymes which have been identified as risk factors in the studied groups indicated that acute toxic effects on liver and kidney functions. Furthermore, the result highlights that significantly increased levels of plasma glucose and glycosylated hemoglobin have provided from panmasala users support that nicotine could establish the risk of diabetes were reported. Accordingly, clinical studies have indicated it is important to continue regular consumption of smokeless tobacco products rich in carcinogens and toxicants have toxic effects in cells and in consequence increase the risk of atherosclerosis and apoptosis. Our results revealed that nicotine in smokeless tobacco significantly increased levels of epinephrine in plasma. These results are in agreement with that *in vitro* study intended that smoking has been shown to increase in levels of plasma catecholamines and epinephrine has been associated with the increase in blood glucose levels that result in cardiovascular diseases [65]. The duration of the smokeless tobacco use was also strongly related with risk of atherosclerosis.

Staerck et al. [66] corroborated present finding of the enzymatic antioxidants are superoxide dismutase, catalase, glutathione peroxidase, and glutathione system have been implicated in the scavenging of excess reactive oxygen and nitrogen species [66]. The present study assessed that the decreased membrane fluidity and increased osmotic fragility were revealed in SLT users. The excess malondialdehyde could lead to decrease the fluidity of the membrane lipid bilayer and increased osmotic stability [67]. The nicotine of smokeless tobacco products are further detrimental to young adult males and are responsible for an inducer of oxidative stress through the redox imbalance of free radicals and antioxidants production.

5. Conclusions

The nicotine exposure through the consumption of smokeless tobacco was confirmed by the analysis of nicotine, cotinine, oxidative stress markers, and apoptotic proteins of human male subjects.

Smokeless tobacco is a significant risk factor for reactive oxygen species production is widely proved and has been the greatest predictor of tobacco use. The overall objective of the present study was to better define the parameters associated with the chronic consumption of smokeless tobacco in inducing apoptosis by promoting ROS generation and possible mechanisms involved. With the course of biological actions, panmasala products seem to be more powerful than smokeless tobacco brands in altering the structure and functions of membranes. These data might be helpful in order to comprehensively address potential toxic and harmful health risks associated with the use of smokeless tobacco products and the present findings suggest a need for tobacco control policies and interventions. Further studies remain to verify the relationship between the mechanistic signaling pathways of specific nitrosamines of SLT products in the apoptosis. Increase awareness on harmful effects associated with the consumption of smokeless tobacco products.

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Declaration of Competing Interest

The authors report no declarations of interest.

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