RESEARCH PAPER

Synergistic Amelioration of Smoking-Induced metabolic disregulations by Amla and Ginger: An NMR based metabolomics study by Amla and Ginger: Insights from ¹H NMR Profiling

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ABSTRACT
Cigarette smoking exposure is known to disrupt metabolic homeostasis and induce oxidative stress, leading to organ dysfunction. The current study uses ¹H NMR spectroscopy to examine the protective effects of *Zingiber officinale* (ginger) and *Phyllanthus emblica* (Amla) against Cigarette smoking-induced metabonomic changes in male albino rats. Rats were divided into control, Cigarette smoking (6mg/kg/bw), Cigarette smoking + Amla (200mg/kg/bw), Cigarette smoking + Ginger (200mg/kg/bw), and combined supplementation groups (Cigarette smoking + Amla + ginger 200mg/kg/bw) for a time period of 90 days. When comparing experimental rats urine profiles to those of control rats, the study finds that the concentrations of citrate, creatinine, allantoin, trans-aconitate succinate, and acetate are significantly lower in the former group. These changes reflect renal dysfunction, oxidative stress, and antioxidative enzymes. Supplementation with Amla and Ginger partially restored the metabolic profile, while their combined administration exhibited a more pronounced normalization of perturbed metabolites. The protective effects attributed to their antioxidant, anti-inflammatory, and detoxifying properties, which counteract Cigarette smoking-induced oxidative damage and maintain metabolic integrity. This study demonstrates the potential of Amla and Ginger as natural interventions to prevent or attenuate Cigarette smoking-mediated metabolic disturbances.

Key words: *Phyllanthus emblica* (Amla), *Zingiber officinale* (Ginger), Cigarette smoking and NMR spectroscopy.
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.Introduction:

The main addictive agent in tobacco smoke is Cigarette smoking [1,2]. Worldwide, a third of the population is Cigarette smoking-dependent, according to the WHO [3]. While a minority of heavily-addicted smokers attempt to quit, fewer than 3–5% are able to do so without aid of Cigarette smoking replacement therapy products (NRTs), and only a third of those who do use NRTs will succeed in quitting for good.

So far metabonomics has been applied to various neuropsychiatric studies including drug addiction, motor neuron disease, schizophrenia, and Parkinson's disease etc. [5,6]. It is also known that smoking is associated with so called plasmalogen-deficiency disorders as supported by multiple-variate analysis of metabolic information. Analyzing all metabolic products in a sample at the same time enables to understand the pathophysiology of the sample and to identify biomarkers that relate to its pathology. In contrast, genomics, transcriptomics, and proteomics are analytical methods that can reveal potential. Among various analytical techniques that have been successfully applied to metabolomics studies of brain tissues using NMR spectroscopy [8,9], this report employs the approach to search for biomarkers associated with the pathophysiology of smoking-induced damage in brain.

About one in four people with chronic obstructive pulmonary disease (COPD), a condition brought on by exposure to tobacco smoke, have muscle atrophy and weakness. People who smoke but do not have other chronic illnesses brought on by exposure to tobacco smoke also exhibit muscle atrophy and a change from slow-twitch to fast-twitch muscle [10-12].Similarly, muscle from subjects with other chronic diseases caused by tobacco smoke exposure (chronic bronitis, emphysema and asthma) has a long history of severe mitochondrial dysfunction characterized by increased mitochondria (ROS) and decreased oxidative capacity [13-15]

This study aimed to examine changes in metabolic profile of rat urine and kidney by 1H NMR-based metabonomics as well as principal components of

urine metabolites. Cigarette smoking-induced changes in rat urine and kidney metabolites were also investigated [16,17]. Biochemical changes were observed in oxidative stress, mitochondrial dysfunction, membrane damage, disturbance of energy metabolism and amino acid disorders, which can provide new insights into biochemical and neurobiological changes associated with Cigarette smoking rewarding effects [16-19].

Methodology

Experimental Animal: From the animal facility at NIMS University, thirty Wistar strain of rats weighing 150 ±3.6 g and 24 meters in length were removed. The creatures were housed in individual polypropylene cages in an atmosphere with a cycle of 12-hours of light and darkness, 22±2°C, and 50±10% humidity. They were provided with a limitless supply of water and commercial pellet feed. The institution's Animal Ethics Committee approved the study and all the experimental processes prior to the experiment's commencement.

Study period – This study was performed in March 2025 for 90 days.

Experimental Groups:

All of the test animals were split up into five groups, with 6 rats in each group.

1. **Group I (control):** For ninety days, six members of this group were fed a regular meal and given tap water.
2. **Group II (Cigarette smoking):** Rats were given cigarettes by inhalation every day with 0.6 mg of Cigarette smoking per kilogram of body weight.
3. **Group III (amla + Cigarette smoking):** The animals were given smoking by inhalation with 6 mg/kg body weight concurrently with oral dose of amla (200mg/kg/bw) daily for 90 days.
4. **Group IV (ginger + Cigarette smoking):** The animals received an oral dose of 200 mg/kg/bw of ginger and 6 mg/kg of Cigarette smoking by inhalation.
5. **Group V (Cigarette smoking + amla + ginger):** The animals were given smoking by inhalation with 6 mg, Cigarette smoking/kg body weight concurrently with an oral dose of amla (200mg/kg/bw) and o ginger (200mg/kg/bw) daily for 90 days.

FTIR analysis - The Raman, emission, and absorption infrared spectra of any solid, liquid, or gas can be obtained using Fourier Transform Infrared spectroscopy. Additionally, it is used to simultaneously record spectra across a very wide spectral range. Fourier Transform Infrared spectroscopy was applied to dried extracts of the aonla varieties. KBr pellets were used to conduct IR-spectral analyses using a Shimadzu IR affinity-I 8000 FT-IR spectrometer in dry air at room temperature. Approximately 300 mg of KBr and one mg of the sample were combined to create tablets. After that, the samples were placed straight into the sampling unit on top of the attenuated reflectance KBr crystal. The samples' spectra were captured at a resolution of 4 cm−1 in the 4000–400 cm−1 range, with the signal averaged over 32 scans. [20].

Urine collection - Using sterile pipettes, spot urine samples from rats kept in a metabolic cage were collected into Eppendorf tubes, which were then kept at -40 °C until analysis [21].

Nuclear Magnetic Resonance Experiments:

A 5-mm broad band inverse probehead was used to obtain 1H NMR spectra of all urine samples at 300 K using a Bruker Biospin Avance 400 MHz spectrometer. Five-mm NMR tubes were used to collect 500 µL urine samples (each). Before the NMR spectra were obtained, the NMR tube was filled with a sealed coaxial capillary tube that contained 0.375% TSP D4 in 35 µL D2O. For "field frequency-locking," deuterium oxide served as the solvent, and TSP served as the standard signal and chemical shift reference for the metabolites' absolute quantitative determination.

Using a one-pulse sequence and water resonance suppression through presaturation to eliminate the broad resonances originating from macromolecules, one-dimensional 1H NMR spectra were acquired for the samples. Relaxation latency (5 s), spectral width (8000 Hz), number of scans (128), spectrum size (32 K), time domain points (32 K), pulse angle (90◦), and line broadening (0.3 Hz) were among the standard parameters. 420 echoes with a total echo time of 0.64 ms were used in the CPMG experiment. The integral area of TSP in CPMG spectra was compared with the integral area of the corresponding metabolite marker signal to determine the metabolite concentrations [22].

Histopathology:

The experimental and control rats were killed by cervical dislocation following a 90-day period. After being surgically removed, the kidneys were washed with physiological saline. A section of the renal cortex was embedded in paraffin, dried using varying ethyl alcohol concentrations, and fixed with neutral formalin. To histologically grade renal and hepatic damage, tissue slices of 4 µm were cut and stained with hematoxylin and eosin.

Results

1.1. **FTIR Analysis** - In Fig. 1 a broad peak at 3367 cm^{-1} due to O–H stretching hydroxyl groups is observed. Assignments for a peak at 2918 cm^{-1} and a strong absorption at 1736 cm^{-1} are due to aliphatic C–H stretching and C=O stretching of carbonyl groups, respectively. Absorptions at 1606, 1366, 1215 and 1022 cm^{-1} are assigned to aromatic C=C bonds and combinations/ring vibrations, as well as C–H bending and C–O stretching.

In Fig. 2 the O–H bond stretches at 3286 cm^{-1}. The broad C–H stretch appears at 2934 cm^{-1}. The aromatic C=C bonds stretches at 1639 cm^{-1}.

C–H bending, as well as several C–O stretches, are observed at 1387, 1231, and 1052 cm^{-1}. These can be indicative of alcohols, ethers, or esters.

In Fig. 3 FT-IR spectrum of combination shows all characteristic bands of both samples. Broad O–H stretching band of water appeared at ~3300–3400 cm^{-1}, while narrow bands of C–H stretch appeared at ~2920 cm^{-1}. C=O and C=C double bond overlapping absorptions are observed in 1700–1600 cm^{-1} region. C–O bands are visible in 1200–1000 cm^{-1} range. There are slight shift in some peaks and broadening of some bands, which might be due to some intermolecular interaction including hydrogen bonding.

All spectra obtained show signals due to O–H, C=O and aromatic functionalities. The combined spectrum confirms that the spectra of P1 and P2 have maintained major functionalities with only slight changes in the intensity of peaks.

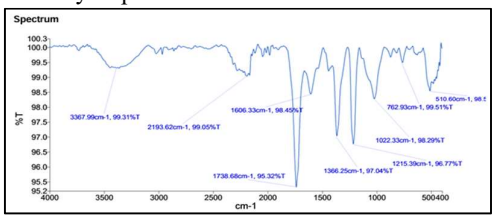

Fig. 1 – FTIR spectrum of *Phyllanthus emblica* illustrating characteristic absorption peaks, corresponding functional groups and intensity variations across analysed wavenumber regions.

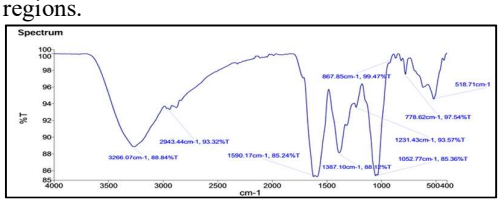

Fig. 2 – FTIR spectrum of *Zinziber officinale* exhibiting broad O-H stretching, prominent carbonyl peaks, and pronounced variations in functional groups.

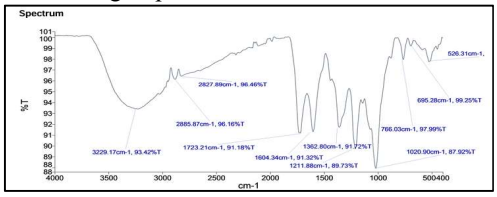

Fig. 3 – Combined FTIR spectrum of *Phyllanthus emblica* & *Zinziber officinale* showing merged functional groups, peak shifts, and interactions between bioactive phytochemical constituents.

1.2. Urine samples from experimental and control animals were subjected to proton NMR spectrum analysis: Figure 4 displays the typical 1H NMR spectra of the urine of control and experimental animals after experimental period, together with the signal labeling. Citrate, Acetate, allantoin, succinate, creatinine, and trans-aconitate are the six metabolites that were identified and measured (table 1). Table 1 displayed the levels of these urine metabolites in the experimental rats and control rats of the same age group after experimental period, along with a statistical analysis.

When compared to the controls, the Cigarette smoking-treated rats' citrate concentrations were found to be considerably ($p<0.001$) lower by 60%. In contrast, rats treated with Cigarette smoking + amla + ginger showed significant ($p<0.001$) recovery by 84%, after treatment. After experimental period, it was discovered that the creatinin levels in Cigarette smoking-treated rats were significantly lower by 41% than in the control group. On the other hand, after experimental period, the groups that received Cigarette smoking + amla + ginger showed a substantial increase of 70%, in comparison to the rats that received Cigarette smoking.

After experimental period, it was shown that rats given Cigarette smoking had much lower amounts of allantoin—by 66%—than the controls. After ninety days of treatment, rats treated with Cigarette smoking with amla and ginger had a 120% higher concentration of allantoin than rats treated with Cigarette smoking alone. After ninety days of treatment, rats given Cigarette smoking had significantly higher levels of acetate than the controls. However, after experimental period, the concentration was 80% higher in rats given with Cigarette smoking plus amla and ginger than in rats treated with Cigarette smoking alone.

After experimental period, trans-aconitate levels were greatly decreased by 70% in Cigarette smoking-treated rats compared to the controls. When compared to rats treated with Cigarette smoking, it rose in rats treated with Cigarette smoking plus amla and ginger during a experimental period. After experimental period of therapy, it was discovered that Cigarette smoking-treated rats had 85% lower urine succinate concentrations than the controls. On the other hand, after experimental period of therapy, it was much higher in rats treated with Cigarette smoking plus amla and ginger (678%) than in rats treated with Cigarette smoking alone as shown in Fig 4.

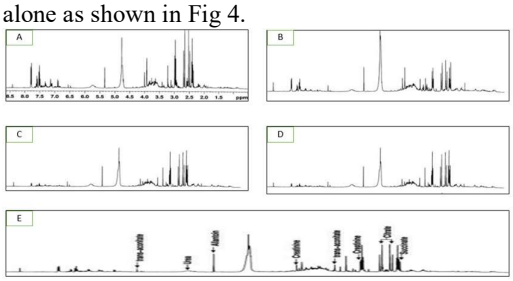

Fig-4: After experimental period of treatment, ¹H NMR spectroscopy was used to analyze the urine

metabolic profiles of rats from the following experimental groups: (a) control, (b) Cigarette smoking exposed, (c) Cigarette smoking with *Phyllanthus emblica* supplementation, (d) Cigarette smoking with *Zingiber officinale* supplementation, and (e) Cigarette smoking with combination of *Phyllanthus emblica* and *Zingiber officinale* supplementation.

Table-1. Rats used in the experiment and control groups had semi-quantitative urine metabolite concentrations in mg/dL.

	90 Days				
	Control	**Cigarette smoking**	**Cigarette smoking+ Amla**	**Cigarette smoking + Ginger**	**Cigarette smoking + Amla + Ginger**
Citrate	394.1 □109.3	156.4 □ 80.7[a*]	408.3 □106.3	288.2 □ 102.3[c]	302.6 □ 41.4[c]
Creatinine	96.9 □ 33.7	37.7 □ 12.5[a]	101.6 □ 3.8a	63.9 □12.1[c]	54.1 □ 10.3[ns]
Allantoin	419.4 □115.5	149.19 □45.7[a]	405.14 □ 135.1	328.2 □89.7[c]	242.6 □74.2[c]
Acetate	ND	5.0 □3.5[a*]	ND	1.01 □0.5[c]	2.99 □ 0.49[ns]
trans-Aconitate	123.00 □73.8	43.78 □ 10.4[a]	116.8 □ 50.2	74.6 □ 16.3[c]	35.6 □ 3.5[ns]
Succinate	18.97 □ 10.5	2.75 □ 1.7[a*]	19.0 □ 5.2	21.4 □8.7[c]	33.8 □ 14.1[c]

1.3. Histopathology:

Rats administered Cigarette smoking exhibited characteristic changes, such as vacuolar (hydropic) degradation of tubular cells, mild glomerular congestion, and localized congestion, when compared to the control group. The control group showed no changes in histopathology. When compared to rats treated with Cigarette smoking, the kidneys of the co-administered Cigarette smoking+amla+ginger did not exhibit any aberrant cellular alterations as shown in Fig.5.

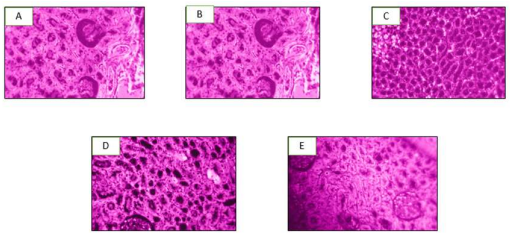

Fig 5 - The cortical kidney (H&E staining) of the control group (A), Cigarette smoking induced rats (B), Cigarette smoking+amla-treated rats (C), Cigarette smoking+ginger-treated rats (D), and the combination of amla and ginger-treated (E) rats after experimental period (40x). Rat renal glomeruli treated with amla and ginger exhibited normal lobular organization, flat cellular lining of the glomerular capsule, and normal structure. However, vascular glomeruli enlargement, vaculate, and degenerated glomerular epithelia were observed in rats treated with Cigarette smoking, whereas rats treated with a combination

Discussion

The primary goal of the current study was to examine how ginger and amla together might protect against Cigarette smoking-induced endogenous metabolic alterations in the urine. Previous research has demonstrated that a variety of physiological changes occur, which are mirrored in the animal's physical and biochemical disturbances. These changes lead to a variation in the percentage of endogenous intermediate metabolites expelled in the urine [22]

In the current investigation, after treatment, the concentrations of Succinate, citrate, creatinine, trans-aconitate and allantoin were found to be gradually reduced in rats treated with Cigarette smoking in comparison to the controls (table 1). Conversely, after treatment, these metabolites were recovered in close proximity to controls when amla and ginger were combined (figure 1).

The pH of the urine is reflected in the decreased citrate concentration Additionally, pH inside the cell and bicarbonate concentration control the amount of citrate that renal tubular cells use and the speed at which citrate is transferred across the mitochondria's inner membrane using the TCA cycle's aconitase enzyme [23,24]. Furthermore, during renal tubular acidosis, a lower citrate level was observed [25]. This could be because the TCA cycle converts less oxaloacetate to citrate. Additionally, kidney stones of oxalate are indicated by oxaloacetate. Rats supplemented with amla and ginger showed reversal of citrate changes in rats treated with Cigarette smoking. These findings are consistent with those of Siener et al. [26], who suggested that supplementing with omega fatty acids raises the amount of citrate in urine.

Due to its high blood concentration and easy measurement as a by-product of muscle metabolism that the kidneys eliminate, the increased

concentration of creatinine found in urine is a significant indicator of renal performance [27,28]. Furthermore, in both diabetes and cardiovascular disease, the level of creatinine has also been linked to reduced renal blood flow [29, 30]. The present study found that supplementing with a combination of amla and ginger increased the concentration of creatinine, indicating that amla and ginger have ameliorative properties. Consistent with our findings, the studies by Rizwan et al. [31] and Palaniswamy et al. [32] discovered that omega-3-fatty acid reduces the risk of nephrotoxicity.

Supplementing with amla and ginger was found to significantly increase the level of allantoin in the current study. The indicator of oxidative stress is allantoin. Human purine metabolism ends with uric acid, which can be converted to allantoin by a variety of ROS, which are the characteristic of oxidative damage and can harm DNA, lipids, and proteins [33]. Our results align with the findings published by Fukuhara and colleagues [34] who found that exposure to oxidative conditions in a neurodegenerative animal model resulted in a high level of allantoin. However, there has been conflicting research regarding the overall effects of amla and ginger on lipid peroxidation. The LPO products cause ATP depletion and DNA damage, which ultimately results in cell death [35].

It's possible that mitochondrial dysfunction or Cigarette smoking-induced oxidative stress of the mitochondria in the kidney is the cause of the notable drop in trans-aconitate in Cigarette smoking-treated rat urine in this study [36,37]. Rats treated with a combination of ginger and amla showed notable improvement when compared to control rats. The results demonstrated that the combination of both amla and ginger is the most efficient n-3 fatty acid for lowering Cigarette smoking-induced oxidative damage at the mitochondrial level [38]. The current study showed that Cigarette smoking-treated rat urine had significantly lower levels of succinate, a crucial elimination end product of the Krebs cycle, and that there were also pathophysiological alterations in the kidney and liver [39].

A thorough recovery from the metabolic disruption caused by Cigarette smoking in the rat renal system, an animal model of kidney disease, was demonstrated by the current metabolic profiles study using 1H NMR. Additionally, this study shows that metabolism profiling is a valuable technique for researching the medicinal benefits of ginger and amla. It's interesting to note that a combination of ginger and amla was found to protect the kidneys. In fact, the amla and ginger combination was successful in reversing the cellular alterations in the Cigarette smoking-treated group (figure 2). Additionally, it has greatly enhanced the histological parameters and metabonomic changes.

In addition to a notable change in serum metabolites, this study demonstrated Cigarette smoking-induced kidney damage by altering kidney tissue histological features that were reminiscent of certain known diseases. In this rat model, the Cigarette smoking supplement was also able to enhance histological changes and induce neurotoxicity by combining amla and ginger. In summary, the overall findings have unmistakably demonstrated that ginger and amla can provide protection against certain aspects of Cigarette smoking consumption in the kidney and serum, most likely as a result of a synergistic effect of numerous compounds. As a result, amla and ginger together can be taken regularly to help reduce the negative effects of ingesting Cigarette smoking while exposed to it, particularly for patients on dialysis who are more vulnerable to dialysis dementia and Cigarette smoking-induced nephrotoxicity.

Conclusion

This study revealed the changes in metabolism, oxidative stress, and kidney injury in rats exposed to Cigarette smoking through alterations in urinary metabolites and FTIR spectral components, as well as histological changes. ^{1}H NMR analyses of urine from Cigarette smoking-treated rats revealed significantly decreased levels of a number of metabolites, including citrate, creatinine, succinate, trans-aconitate and allantoin, that are indicators of energy metabolism, mitochondrial function and oxidative stress. Supplementation of *Phyllanthus emblica* (Amla) and *Zingiber officinale* (Ginger) reversed partially the impaired metabolic fingerprint of STZ-induced diabetic rats. Their combination was found to have synergistic effect on reestablishment of the metabolite profile. Remarkable improvement was observed in the histomorphology of diabetic kidney following combined supplementation. FTIR study supported the presence of compatible bioactive functional groups and suggestive of improved intermolecular interactions in the combined sample. Present findings indicate a synergistic effect of Amla and Ginger, possibly through their antioxidant, anti-inflammatory, and detoxifier actions. Such synergism enhances their protective effects against toxicity induced by Cigarette smoking. Hence, supplementation of Amla and Ginger can serve as a natural approach to prevent metabolic and renal dysfunction caused by Cigarette smoking.

Disclosure of Interest – The Authors report there are no competing interest to declare.

Author's contribution – **Divya Darak** carried out the experiment and wrote manuscript**. Devesh Kumar Joshi** designed the model and the computational framework and analysed the data. **Sandeep Tripathi** contributed to the interpretation of results.

Availability of data and materials – Upon reasonable request, the corresponding author will provide the data supporting the study's conclusions.

Ethical Consideration- All experimental procedures involving animals were conducted in strict accordance with the internationally accepted guidelines for the care and use of laboratory animals, as outlined in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH, 2011). Prior to the initiation of the experimental study, the experimental protocol was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of NIMS University Rajasthan, Jaipur, under approval number NIMSUR/IAEC-01/2024/07. All efforts were made to minimize animal suffering and to reduce the number of animals used throughout the experimental procedures.

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