ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 17, Issue 12, 2024

Online - 2455-3891
Print - 0974-2441

Research Article

FROM PLEASURE TO PERIL: EXAMINING THE TOXIC AND POTENTIAL CARCINOGENIC IMPLICATIONS OF PREMIUM PAN MASALA CONSUMPTION

PRERONA BORUAH*, ADITYA B YADAV, MALA PARAB, PRAMODKUMAR P GUPTA

School of Biotechnology and Bioinformatics, DY Patil Deemed to be University, Navi Mumbai, Maharashtra, India.
***Corresponding author: Prerona Boruah; Email: boruahprerona@yahoo.com**

Received: 04 September 2024, Revised and Accepted: 27 October 2024

ABSTRACT

Objective: This study aims to assess the phytochemical composition and toxicity profile of pan masala, with and without tobacco, through cellular toxicity analysis. The specific objectives are to analyze the cytotoxicity of pan masala extracts on fibroblast and keratinocyte cells and to investigate DNA fragmentation and apoptosis induced by these products.

Methods: Two popular brands of pan masala, one with and one without tobacco, were selected for phytochemical and toxicity evaluation. Aqueous extracts of both products were tested on mice fibroblast cells for cytotoxicity analysis. In addition, keratinocyte cell lines were employed to assess DNA fragmentation and apoptosis through standard molecular assays.

Results: The study revealed a significant increase in toxicity in both types of pan masala. Pan masala extracts caused marked cytotoxic effects on fibroblast cells and induced DNA fragmentation and apoptosis in keratinocytes. These toxicological impacts were observed in both tobacco-containing and tobacco-free samples, with heightened damage noted in the presence of tobacco.

Conclusion: The findings confirm that pan masala, with or without tobacco, has substantial cytotoxic effects, posing serious risks to normal cellular functions. Both varieties are implicated in DNA damage and apoptosis, underscoring the urgent need for public health interventions to reduce the consumption of these products due to their potential to cause severe oral and systemic health issues.

Keywords: Pan masala, Cytotoxic, Keratinocytes, Apoptosis.

© 2024 The Authors. Published by Innovare Academic Sciences Pvt Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/) DOI: http://dx.doi.org/10.22159/ajpcr.2024v17i12.52580. Journal homepage: https://innovareacademics.in/journals/index.php/ajpcr

INTRODUCTION

The pan, a traditional chewing mixture, is widely consumed in Southeast Asia and India as part of cultural practices and social rituals [1]. This preparation, as a rule, includes a mixture of stored lime, bétel, katha (Acacia catechu), and an arecanut, and some users prefer to add tobacco for its psychoactive effects. Despite the fact that the traditional use of betel remains common, there is a major change in the consumption of pan masala, a product that provides convenience and certain quality. Pan masala often contains similar components to betel but is sold in a more sophisticated preparation.

Arecanut, a major ingredient in both betel and pan masala, has long been used for its medicinal properties such as aiding digestion and antibacterial effects [2]. However, growing evidence from *in vitro* and *in vivo* studies has highlighted the possible carcinogenicity of arecanut [3]. Compounds such as arecoline, an alkaloid found in arecanut, have been shown to induce genotoxicity and cytotoxicity, potentially leading to malignant transformations in cells [4]. In addition, lime and catechu, common components of both traditional betel quid and modern pan masala, have been found to generate reactive oxygen species (ROS), which can cause oxidative DNA damage, leading to mutations and cellular dysfunction [4].

The health risks are further compounded by the presence of tobacco in some variations of pan masala. Tobacco contains nicotine, an addictive alkaloid that makes pan masala more addictive [5]. Besides addiction, tobacco has been shown to significantly increase oxidative stress in the body, promoting the formation of ROS and exacerbating DNA damage [5]. The resultant oxidative stress can lead to various pathological conditions, including cancer and cardiovascular diseases.

Although some ingredients, such as cardamom, are added for their beneficial properties, such as balancing pH and supporting kidney function, these beneficial effects may be overshadowed by the toxicological risks associated with other ingredients [6]. The complex blend of ingredients in pan masala requires a comprehensive assessment of its overall safety, particularly with respect to its impact on cellular health and genetic stability [7].

The aim of this study is to evaluate the potential toxicity of aqueous extracts of pan masala from two high-quality Indian brands currently available on the market. By investigating the effects on normal fibroblast cells and assessing possible DNA damage using an apoptosis assay, this study aims to gain a better understanding of the health risks associated with the consumption of pan masala, especially in the context of the growing popularity of pan masala as an alternative to traditional betel liquid.

METHODS

Sample material

The two of the most premium brands of pan masala for the study were obtained from the local pan shop of Sector 15, CBD Belapur, Navi Mumbai, Maharashtra.

Extract preparation

One gram of the sample was ground to a fine powder using liquid nitrogen. The ground powder was stirred in the distilled water for 24 h, centrifuged at 10,000 revolutions per minute, and the supernatant was filtered using the 0.22-micron filter to prepare an aqueous extract of stock concentration of 100 mg/mL. A 1:1 ratio was used for the preparation of the extract of pan masala with the tobacco. A total of four extracts (with and without tobacco) were prepared (Extract 1:

Brand 1, Extract 2: Brand 1+tobacco, Extract 3: Brand 2, Extract 4; Brand 2+tobacco) [8]. The pan masala brand used in this study has been provided in a supplementary document.

Cell lines

Mouse embryonic fibroblast (MEF) and human immortalized keratinocytes (HaCaT) were obtained from the National Centre for Cell Science, Pune. They were maintained in Dulbecco's Modified Eagle's Medium (MP Biomedicals) with 10% fetal bovine serum (Gibco) at 37°C at 5% CO_2.

Phytochemical screening of bioactive compounds present in pan masala

Phytochemical screening was done to screen the extracts for the presence of bioactive compounds: Alkaloids, flavonoids, tannins, phenols, saponins, and glycosides as per the process described by Rajkumar *et al.* [9].

Quantitative analysis of bioactive compound

Phytochemicals found in the qualitative analysis were further quantitatively analyzed as per the method described in the previous study [10].

Antioxidant activity (1,1-diphenyl-2-picrylhydrazyl [DPPH] scavenging assay)

The DPPH free radical scavenging method was conducted to evaluate the antioxidant properties of the pan masala extracts. Different concentrations of gallic acid (standard) and extract (0.1 mg/ml, 0.5 mg/mL, 1 mg/mL, and 5 mg/mL) were mixed with 1 mL of methanolic DPPH (SRL Pvt) and incubated in the dark for 30 min at room temperature (RT) and absorbance was measured at 517 nm [11].

***In vitro* cytotoxicity assay using mouse embryo fibroblast**

It is a spectrophotometric assay that involves the quantification of reduced 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by living cell mitochondrial dehydrogenase enzyme to purple-blue colored formazan crystals within the cells. MEF cells were cultured and used, to measure the cell cytotoxicity, A 96-well plate was seeded with 5000 cells/well with the complete media with 10% FBS (Gibco) were treated with doses (0.5, 1, 5, 10 mg/mL) of pan masala extracts and incubated further for 24 h with 5% CO_2. Upon incubation, MTT reagent was added and incubated for 4 h, and then 30% dimethyl sulfoxide was added. Ciprofloxacin served as a positive control. The absorbance was measured at 570 nm using a microplate reader [12].

Apoptosis analysis on Hacat (keratinocyte cell) cell lines

DNA damage by pan masala extracts in HaCaT cell lines was determined using the Hoechst staining method described by [13]. A 96-well plate was seeded with the 2000 cells/well and treated with the pan masala extracts of concentration 5 mg/mL. The cells were fixed with 4% paraformaldehyde for 20 min. At RT, washed the cells with phosphate-buffered saline (PBS) and incubated the cells with Hoechst 33342 (100 ng/mL in PBS) (SRL, Pvt) in the dark for 15 min. Upon incubation wash the cells with PBS and mount the cells on a coverslip using dibutyl phthalate polystyrene xylene and dry the coverslip and the image was analyzed using a fluorescence microscope with emission 350 nm.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8. The data were analyzed by Student's t-test and one-way analysis of variance all the results were expressed as mean±standard error of the mean for triplicate determinations. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$ were considered statistically significant.

RESULTS

Phytochemical screening of bioactive compounds present in Indian pan masala

The present study was carried out to screen the extracts of pan masala for the presence of phytochemicals such as alkaloids, flavonoids, saponins, glycosides, phenol, and tannins. The results of the qualitative analysis are represented in Table 1. Where (+) indicates presence and (-) indicates absence.

Quantitative analysis of bioactive compounds

Total alkaloid content

The dry weight of the alkaloids was noted after the extraction and purification. The average alkaloid content for extract 1, extract 2, extract 3, and extract 4 was found to be 59 mg/g, 79 mg/g, 76 mg/g, and 79 mg/g, respectively. The alkaloid content was found to be comparable in the pan masala extract with tobacco and pan masala without tobacco. The total alkaloid content is represented in Fig. 1.

Total flavonoid content

The total flavonoid content of aqueous extract of pan masala was estimated using rutin as standard. The average value of total flavonoid concentration of aqueous extracts for extract 1, extract 2, extract 3, and extract 4 was found to be 18.872 μg/mg, 7.372 μg/mg, 17.5 μg/mg, and 14.8 μg/mg, respectively. The highest flavonoid content was found in extract 1 followed by extract 3 and the least amount of flavonoid was found in extract 2 pan masala with tobacco.

Total phenolic content

The total phenolic content of the aqueous extract of pan masala was estimated using the Follin–Ciocalteau method. The figure results clearly indicated the highest amount of average phenolic content in extract 3 (58.52 μg/mg) and the least amount of phenolic content reported in extract 2 (4.98 μg/mg). A decrease in phenolic content was observed when the pan masala was mixed with tobacco (extract 2, extract 4).

Total tannin content

The total tannin content was estimated using the Follin–Ciocalteau method using Tannic acid as a standard. Fig. 1 clearly indicates that the highest amount of tannin is present in extract 3 extract (19.901 μg/mg) and the least amount was found in extract 2 (0.87 μg/mg).

Radical scavenging activity (RSA)

The premium Indian pan masala extracts were evaluated for antioxidant activity using a DPPH radical scavenging assay. Gallic acid was used as a standard. Results revealed that all the pan masala extract (extract 1, extract 2, extract 3, and extract 4) exhibited RSA with IC_{50} values of 3.59 mg/mL, 5.07 mg/mL, 2.98 mg/mL, and 2.983 mg/mL. It is evident from the graph that extract 3 was found to have high antioxidant activity with increasing dose. Both the pan masala extract with tobacco (extract 2 and extract 4) have lower antioxidant properties at higher doses. The high antioxidant activity of extract 3 extract is due to the high phenolic, tannin, and flavonoid content, which are associated with the antioxidant capacity. The result is represented in Fig. 2.

Cytotoxicity assay

Cytotoxicity of all pan masala extract on chick embryo fibroblast with doses (1 mg/mL, 5 mg/mL, and 10 mg/mL), respectively, was determined using MTT assay. Since antioxidant activity was very low in a lower dose of 0.1 mg/mL, it was not considered for further analysis. Ciprofloxacin which is a potent toxic agent against fibroblast cells served as a positive control. Results were displayed as % cell toxicity.

Table 1: Phytochemical screening of bioactive present in Indian pan masala

Compounds	Extract 1	Extract 2	Extract 3	Extract 4
Alkaloids	+	+	+	+
Glycosides	-	-	-	-
Flavonoids	+	+	+	+
Saponins	-	-	-	-
Phenols	+	+	+	+
Tannins	+	+	+	+

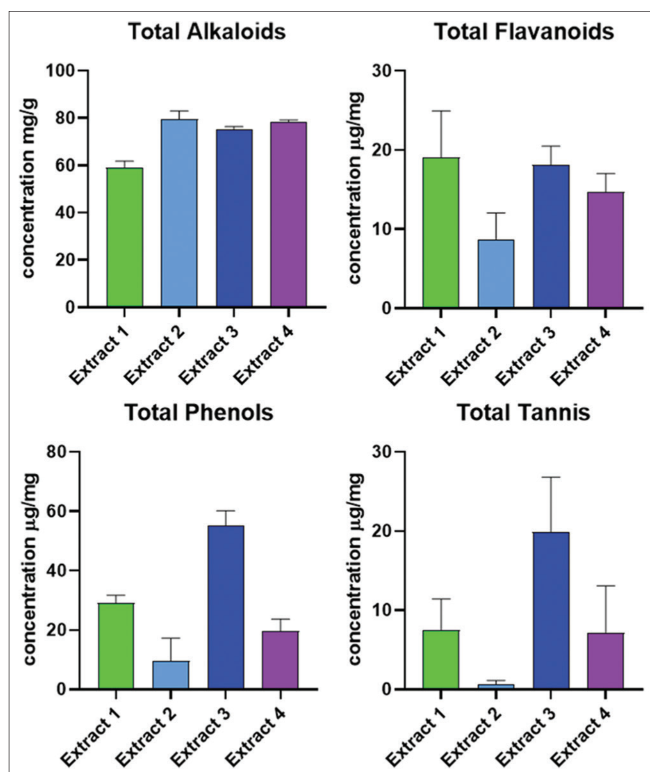

Fig. 1: Quantitative analysis of phytochemicals present in the extracts. Values represented are mean±standard deviation of the phytochemicals present per gram/milligram of sample used

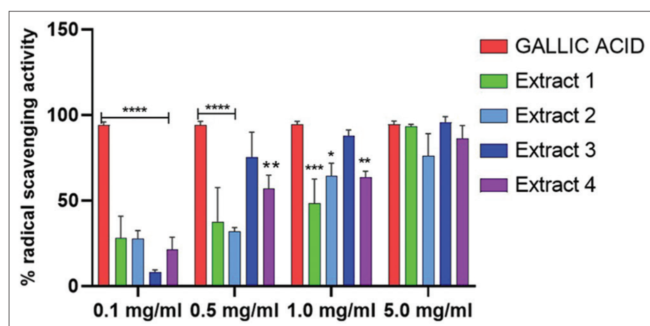

Fig. 2: Radical scavenging activity of the pan masala extracts. n=2, values represented as mean±standard deviation, *denotes significance compared to control

With higher concentrations of extracts, the cell toxicity was found to be significantly higher in all pan masala extracts. It was also found that the pan masala extract with tobacco was toxic even at lower concentrations. The IC_{50} values of extract 1, extract 2, extract 3, and extract 4 were found to be 7.72 mg/mL, 5.23 mg/mL, 4.76 mg/mL, and 10.2 mg/mL, respectively. The results are represented in Fig. 3.

Apoptosis analysis on HaCaT cell lines

Since the pan masala extracts were found to be toxic to normal cells, we further evaluated if the extract could be involved in DNA fragmentation and apoptosis in keratinocytes as the oral mucosa also consists of keratinocytes [14].

As 10 mg/mL was highly toxic to fibroblast cells therefore a moderate dose of 5 mg/mL was used. Hoechst staining of pan masala extract at a concentration of 5 mg/mL was performed on HaCaT cells (Fig. 4a). Indicating that as compared to the control, significant apoptosis was seen in the cells treated with extract 3 and extract 4. Fig. 4b indicates the

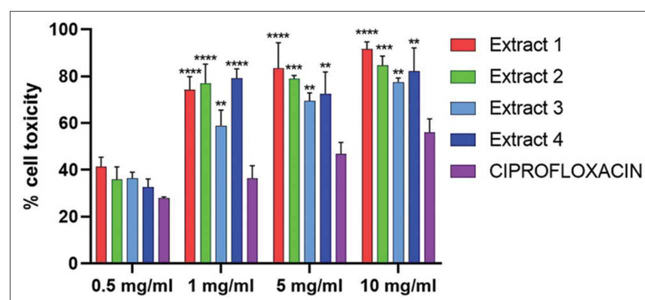

Fig. 3: Cytotoxic activity of the pan masala extracts in mouse fibroblast cells. n=2, values represented as mean±standard deviation, *denotes significance compared to control

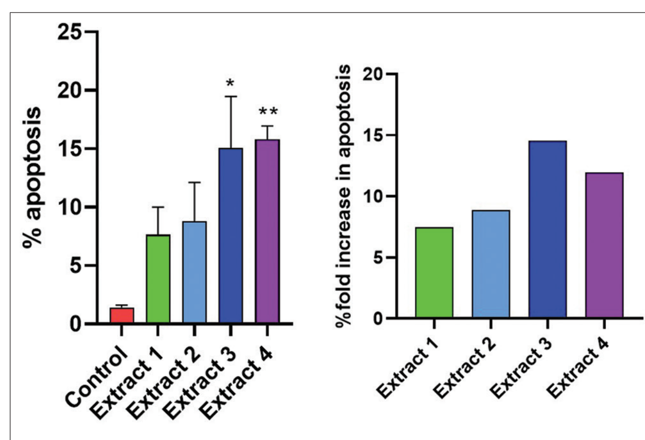

Fig. 4: Apoptosis observed in human immortalized keratinocyte cells (left), fold increase in apoptosis after pan masala treatment (right). n=2, values represented as mean±standard deviation, *denotes significance compared to control

% fold increase in apoptosis is higher in the cells treated with extract 3 (14.12 times) followed by extract 3 (13.7 times). The representative images of apoptosis by pan masala extract are given in Fig. 5.

DISCUSSION

The present study investigated the phytochemical profiles of two popular pan masala brands using aqueous extracts. Both samples contained alkaloids, flavonoids, phenols, and tannins. Previous studies have similarly identified polyphenols, such as catechin and tannins such as gallic acid and D-catechol, as major phenolic compounds in pan masala [2], supporting our findings that these specific classes are likely to present in the analyzed samples. Among the alkaloids, arecoline, and nicotine were notable for their psychoactive and addictive properties, which contribute to the chronic consumption of pan masala [5].

Our analysis of the antioxidant potential revealed that the pan masala variant without tobacco exhibited increased RSA in a dose-dependent manner. In contrast, the tobacco-containing variant showed decreased RSA, likely due to oxidative stress induced by tobacco, which enhances the formation of ROS and reduces antioxidant activity [15]. Despite the significant antioxidant properties, pan masala consumption has been linked to cytotoxic effects.

To further understand the potential health risks, we conducted cytotoxicity assays on mouse fibroblast cells. Both tobacco-containing and tobacco-free pan masala extracts demonstrated dose-dependent toxicity. This observation aligns with prior research that attributes pan masala's cytotoxicity primarily to arecanut and tobacco constituents [16]. Arecoline and arecaidine, compounds in arecanut, inhibit protein synthesis, deplete intracellular thiols, and possess

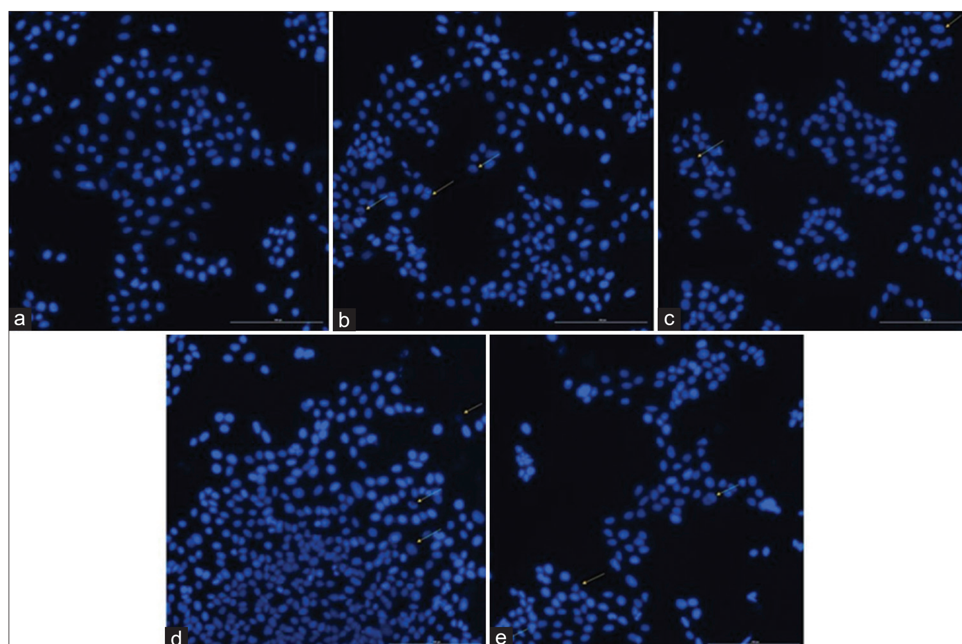

Fig. 5: Representative images of apoptosis induction in human immortalized keratinocyte cells post-treatment with pan masala extracts; (a) control, (b) extract 1, (c) extract 2, (d) extract 3, (e) extract 4

cytotoxic properties. Moreover, the nitrosamine derivatives of arecoline (arecoline-specific nitrosamines) and tobacco (tobacco-specific nitrosamines) are potent carcinogens, capable of causing DNA adducts and strand breakage [17]. Previous studies have reported cytotoxicity in L929 fibroblast cells following exposure to arecanut extracts and oxidative damage to HaCaT cells caused by nicotine [18]. Both arecoline and nicotine appear to act synergistically, exacerbating cytotoxic effects.

In our study, DNA damage was further evaluated using Hoechst staining on HaCaT cells treated with pan masala at a concentration of 5 mg/mL, a dosage based on the IC_{50} values. Significant apoptosis was observed in keratinocytes post-treatment, evidenced by chromatin condensation, apoptotic bodies, and nuclear fragmentation. These results are consistent with previous findings that highlight the dose- and time-dependent increases in free radicals, DNA damage, lipid peroxidation, and apoptotic cell death in human oral keratinocytes upon short-term exposure [19,20].

The detection of phytochemicals such as alkaloids, flavonoids, phenols, and tannins indicates that pan masala may possess bioactive compounds with both beneficial (antioxidant) and harmful (cytotoxic) effects. The presence of arecoline and nicotine, along with their nitrosamine derivatives, underscores the carcinogenic potential of these products, especially with chronic use. Notably, the increase in ROS and subsequent decrease in RSA in tobacco-containing variants of pan masala suggest that these products could exacerbate oxidative stress, leading to DNA damage and potential progression to malignancies. Chronic ingestion of these substances is also associated with systemic toxicity, including liver fibrosis and damage to other internal organs.

CONCLUSION

Since eons, people have been consuming varieties of pan masala as a nerve stimulant. Despite statutory warnings on the pouches, its demands are flourishing. We conducted a noteworthy study on the toxic potential of pan masala brands. Our findings suggested that pan masala constitutes various phytochemicals such as polyphenols, tannins, and flavonoids. These compounds were attributed to the antioxidant potential of the pan masala. Interestingly, despite significant RSA pan masala extracts exhibited significant cytotoxic effects on the fibroblast and HaCaT cells even at the lower dose of 5 mg/mL more so in the extract containing tobacco. In light of the above data, it can be concluded that irrespective of phytochemicals, some of the components of pan masala such as catechin, lime, arecoline, and nicotine potentially generate ROS that damages DNA and alters the DNA repair system. Hence, pan masala with or without tobacco is toxic to cells and can lead to apoptosis and chronic consumption may lead to oral malignancies.

ACKNOWLEDGMENT

We take this opportunity to thank other members of our team Miss Sejal R Yadav and Miss Aishwarya R Rathod for their help and support in completing the work.

AUTHOR'S CONTRIBUTION

PB: Conceptualization and wrote the first draft, AY: Carried out the experiments, MP: Reviewed, PPG: Reviewed.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR FUNDING

No authors received funding for this work.

REFERENCES

1. Mehrotra D, Kumar S, Mishra S, Kumar S, Mathur P, Pandey CM, *et al*. Pan masala habits and risk of oral precancer: A cross-sectional survey in 0.45 million people of North India. J Oral Biol Craniofac Res. 2017;7(1):13-8. doi: 10.1016/j.jobcr.2016.12.003, PMID 28316915
2. Sari EF, Prayogo GP, Loo YT, Zhang P, McCullough MJ, Cirillo NJ Sr. Distinct phenolic, alkaloid and antioxidant profile in betel quids from four regions of Indonesia. Sci Rep. 2020;10(1):16254.
3. Mojidra BN, Archana K, Gautam AK, Verma Y, Lakkad BC, Kumar S. Evaluation of genotoxicity of pan masala employing chromosomal aberration and micronucleus assay in bone marrow cells of the mice. Toxicol Ind Health. 2009;25(7):467-71. doi: 10.1177/0748233709345939, PMID 19783571
4. Garg A, Chaturvedi P, Mishra A, Datta S. A review on harmful effects of pan masala. Indian J Cancer. 2015;52(4):663-6. doi: 10.4103/0019-509x.178449, PMID 26960511
5. Nair J, Ohshima H, Friesen M, Croisy A, Bhide SV, Bartsch HJC. Tobacco-specific and betel nut-specific N-nitroso compounds: Occurrence in saliva and urine of betel quid chewers and formation

in vitro by nitrosation of betel quid. *Carcinogenesis*. 1985;6(2):295-303.

6. Sharifi-Rad J, Dey A, Koirala N, Shaheen S, El Omari N, Salehi B, *et al*. *Cinnamomum* species: Bridging phytochemistry knowledge, pharmacological properties and toxicological safety for health benefits. Front Pharmacol. 2021;12:600139. doi: 10.3389/fphar.2021.600139, PMID: 34045956
7. Jaju RJ, Patel RK, Bakshi SR, Trivedi AH, Dave BJ, Adhvaryu SG. Chromosome damaging effects of pan masala. Cancer Lett. 1992;65(3):221-6. doi: 10.1016/0304-3835(92)90235-n, PMID 1516036
8. Pradheeba M, Pugalenthi M, Deepa MA, Kumar SV, Vasukipridharshini G. Evaluation of phytochemical profile and *in vitro* antioxidant, anti-bacterial and anti-inflammatory activity of piper Schmidtii hook. fil. A wild edible fruit. Asian Pac J Health Sci. 2022;9(3):191-7. doi: 10.21276/apjhs.2022.9.3.39
9. Rajkumar G, Panambara PA, Sanmugarajah V. Comparative analysis of qualitative and quantitative phytochemical evaluation of selected leaves of medicinal plants in Jaffna, Sri Lanka. Borneo J Pharm. 2022;5(2):93-103. doi: 10.33084/bjop.v5i2.3091
10. Mathew S, Raju R, Zhou X, Bodkin F, Govindaraghavan S, Münch G. A method and formula for the quantitative analysis of the total bioactivity of natural products. Int J Mol Sci. 2023;24(7):6850. doi: 10.3390/ijms24076850, PMID 37047821
11. Zakaria Z, Aziz R, Lachimanan YL, Sreenivasan S, Rathinam X. Antioxidant activity of *Coleus blumei*, *Orthosiphon stamineus*, *Ocimum basilicum* and *Mentha arvensis* from *Lamiaceae* family. Int J Nat Eng Sci. 2019;2(1):93-5.
12. Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods. 1986;89(2):271-7. doi: 10.1016/0022-1759(86)90368-6. PMID: 3486233
13. Crowley LC, Marfell BJ, Waterhouse NJ. Analyzing cell death by nuclear staining with hoechst 33342. Cold Spring Harb Protoc. 2016;2016(9):779-80.
14. Turabelidze A, Guo S, Chung AY, Chen L, Dai Y, Marucha PT, *et al*. Intrinsic differences between oral and skin keratinocytes. PLoS One. 2014;9(9):e101480. doi: 10.1371/journal.pone.0101480, PMID 25198578
15. Bagchi M, Balmoori J, Bagchi D, Stohs SJ, Chakrabarti J, Das DK. Role of reactive oxygen species in the development of cytotoxicity with various forms of chewing tobacco and pan masala. Toxicology. 2002;179(3):247-55. doi: 10.1016/s0300-483x(02)00357-8, PMID 12270596
16. Chioran D, Sitaru A, Macasoi I, Pinzaru I, Sarau CA, Dehelean C, *et al*. Nicotine exerts cytotoxic effects in a panel of healthy cell lines and strong irritating potential on blood vessels. Int J Environ Res Public Health. 2022;19(14):8881. doi: 10.3390/ijerph19148881, PMID 5886732
17. Nair U, Bartsch H, Nair J. Alert for an epidemic of oral cancer due to use of the betel quid substitutes gutkha and pan masala: A review of agents and causative mechanisms. Mutagenesis. 2004;19(4):251-62. doi: 10.1093/mutage/geh036
18. Sari LM, Subita GP, Auerkari EI. Potential antioxidant and cytotoxic activities of areca nut (*Areca catechu* Linn.) extract in human oral squamous cell carcinoma and keratinocyte cells. Asian J Pharm Clin Res. 2017;10(10):286-91.
19. Theilig C, Bernd A, Ramirez-Bosca A, Görmar FF, Bereiter-Hahn J, Keller-Stanislawski B, *et al*. Reactions of human keratinocytes *in vitro* after application of nicotine. Skin Pharmacol. 2009;7(6):307-15. doi: 10.1159/000211311
20. Joshi MS, Verma Y, Gautam AK, Parmar G, Lakkad BC, Kumar S. Cytogenetic alterations in buccal mucosa cells of chewers of areca nut and tobacco. Arch Oral Biol. 2011;56(1):63-7. doi: 10.1016/j.archoralbio.2010.08.012