JOURNAL OF
EMERGING INVESTIGATORS

Article

QuitPuff: A Simple Method using Lipid Peroxidative Changes in Saliva to Assess the Risk of Oral Pre-cancerous Lesions and Oral Squamous Cell Carcinoma in Chronic Smokers

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SUMMARY

Smoking carries a risk of oral cancer. Smoking generates free radicals, which are responsible for the high levels of cellular lipid peroxidation along the oral mucosa. Free-radical-induced lipid peroxidation has been implicated in the pathogenesis of oral cancer. Malondialdehyde (MDA) is the end-product of lipid peroxidation and can serve as a marker of the degree of lipid peroxidation. This study aimed to determine the salivary MDA levels in smokers and to use salivary MDA levels to assess the risk of developing oral pre-cancer and cancer. We hypothesized that heavier smokers would exhibit greater levels of salivary lipid peroxidation which in turn would correlate with a greater risk of oral pre-cancer and cancer. To test our hypothesis, we devised QuitPuff, a simple, home-based test consisting of a diagnostic reagent that reacts with MDA and produces a color change in a sample of saliva. We measured the MDA level in each sample by matching the color change with a colorimetric Lipid Peroxidation Index (LPI) chart. This method was tested on 125 people and the results were validated by UV Spectroscopy. The test detected the degree of salivary lipid peroxidation with 96% accuracy. We found that heavier smokers exhibited greater levels of salivary MDA. QuitPuff, a simple and inexpensive home-based test, can serve as an early, safe, non-invasive test for smokers to measure their degree of salivary lipid peroxidation and thereby assess their risk of developing oral pre-cancer and cancer.

INTRODUCTION

The high mortality rate associated with oral cancer is attributed mainly to late diagnosis

Oral cancer, also known as mouth cancer, is any cancerous tissue growth located in the oral cavity (1). It most commonly involves the tongue, floor of the mouth, cheek lining, gums, lips, or roof of the mouth. More than 90% of all oral cancers are squamous cell carcinoma (2).

By country, the incidence of oral cancer is the highest in India, which accounts for almost one-third of cases found in the world (1). Over five people in India die every hour because of oral cancer (3). Public health centers and private hospitals have recognized oral cancer as a grave problem and efforts towards early detection and prevention can help reduce this burden.

The high prevalence of oral cancer in India is mainly due to the influence of tobacco and betel quid chewing (4). Greater than 90% of patients with oral cancer report using tobacco products (5). The incidence of oral cancer in patients who smoke and chew tobacco is 8.4 times greater than that of patients who do not (6).

Globally, the 5-year mortality rate of oral cancer is approximately 50% and has not improved despite advances in diagnostic techniques and improvements in treatment modalities (4). The high mortality rate in oral cancer is attributed to late diagnosis, which is either due to lack of knowledge or access to medical care (1,2,7). Most patients seek help only in later stages when symptoms like pain, ulceration, or a neck mass appear (1).

Detection of an oral cancer at stage I carries a prognosis of 80% survival, while the same lesion at stage III carries a prognosis of 20% survival (8). This difference could affect not only the quality of life for the patients, but also the cost of the medical treatment. Thus, there is a need for improvement in early risk detection of oral carcinomas, because in the initial and pre-cancerous stages, treatment is more effective and morbidity is minimal.

Link between salivary malondialdehyde and oral pre-cancer and cancer

Free-radical-induced lipid peroxidation has been implicated in the pathogenesis of oral cancer (9-11, 14, 15). Smoking generates free radicals and reactive oxygen species (ROS). ROS-induced cell damage causes lipid peroxidation. It most commonly affects polyunsaturated fatty acids, which causes alteration in the structure and function of cell membranes and also initiates and promotes the multistep process of carcinogenesis (10).

Malondialdehyde (MDA) is the end-product of lipid peroxidation and can be used as a marker for assessing the extent of lipid peroxidation (9-11, 14, 15). MDA is mutagenic and genotoxic, as it readily reacts with deoxynucleosides to produce adducts that cause DNA damage (11). An increase in MDA concentrations in saliva have been widely reported in various oral pre-cancers and cancers in the early stages (9-11, 14, 15). Additionally, previous studies have shown that salivary MDA could serve as a potential diagnostic marker

Group Name	N	Mean LPI by Colorimetric method	Mean LPI by UV Spectroscopy method
Non-smokers	25	0.24	0.2
Smokers (<10 cig/day)	25	3.56	3.68
Smokers (10-20 cig/day)	25	3.64	3.72
Smokers (>20 cig/day)	25	4.44	4.48
Smokers with oral pre-cancer & cancer	25	4.48	4.52

Table 1. Comparison of mean LPIs (Lipid Peroxidation Index) between the Colorimetric and UV Spectroscopy methods with standard deviation.

and that by measuring the salivary MDA level we can measure the extent of lipid peroxidation and thereby assess the risk of a smoker towards developing oral pre-cancer and cancer (9, 10, 12-15).

With this background, we were motivated to investigate the relationship between smoking, lipid peroxidation and oral cancer and hence we carried out our study. We hypothesized that the heavier the smoker, the greater the level of salivary lipid peroxidation and the higher the risk of developing oral pre-cancer and cancer. To test our hypothesis we devised a simple, quick, colorimetric, home-based test named QuitPuff to determine the salivary malondialdehyde levels in smokers.

RESULTS

The mean lipid peroxidation index (LPI) of non-smokers was 0.24. In the smokers group, the mean LPI of smokers who smoked less than 10 cigarettes a day was 3.56, that of smokers who smoked 10 to 20 cigarettes a day was 3.64 and that of smokers who smoked more than 20 cigarettes a day was 4.44. The mean LPI of smokers with oral pre-cancer and oral squamous cell carcinoma was 4.48 (**Table 1**).

The mean LPI was consistently and significantly elevated (p<0.001) in smokers with oral pre-cancer and cancer and smokers who smoked more than 20 cigarettes a day, 10-20 cigarettes a day and less than 10 cigarettes a day as compared to non-smokers.

The mean lipid peroxidation index (LPI) obtained by QuitPuff colorimetric method was compared with the mean LPI obtained by the validation method of UV Spectroscopy. The mean LPIs from both methods were in agreement (**Table 1**). Variations in LPI were observed in 20 of 125 samples, but only 4 out of 125 (3.2%) resulted in misclassification error when compared to the UV Spectroscopy results. Thus, the diagnostic test was able to detect the degree of lipid peroxidation in the saliva of smokers, patients with pre-cancerous mouth lesions, and Oral Squamous Cell Carcinoma (OSCC) patients with 97% accuracy.

Among smokers, 24 of 25 smokers with oral pre-cancer and cancer were found to have high degree of lipid peroxidation, an increase compared to that of otherwise healthy smokers (22/25 subjects who smoked more than 20 cigarettes a day, 18/25 who smoked 10-20 cigarettes a day, and 16/25 who smoked less than 10 cigarettes a day). In contrast in the non-smokers group, 20 of 25 subjects (80%) had no detectable lipid peroxidation, and none showed a high degree of lipid peroxidation (**Table 2**).

The color change in each sample was matched with the colorimetric lipid peroxidation index (LPI) chart (**Figure 1**). It was found, the higher the number of cigarettes smoked per day, greater the levels of salivary MDA and deeper the color produced. In the absence of salivary MDA, there was no color change found. The color change results correlated with the readings performed by UV Visible Spectroscopy, performed to validate the results. Results of the spearman correlation indicated there was a significant positive association between the color changes and the readings of the UV Spectroscopy (r=0.86, p<0.001)

DISCUSSION

Cancer is caused by the accumulation of multiple lesions occurring in a single cell. It can be described by three stages: initiation, promotion, and progression. Previous reports indicate that ROS not only initiate, but also promote multistep carcinogenesis (10). ROS-induced lipid peroxidation is implicated in the pathogenesis of oral cancer (9-11, 14, 15). Malondialdehyde (MDA) is the end-product and the most widely studied product of lipid peroxidation (9-11, 14, 15). By measuring the level of salivary MDA we can determine the degree of salivary lipid peroxidation and thereby assess the risk of a smoker developing oral pre-cancer and oral cancer.

In our study, smokers with oral pre-cancer and oral squamous cell carcinoma were found to have a higher degree of salivary lipid peroxidation as compared to non-smokers. Within the smoker's category, the heavier the smoker, the greater the degree of salivary lipid peroxidation. This is in line with other studies (9-11, 14, 15) on oral pre-cancer and cancer, which have reported similar findings, validating the relationship between free radical activity, lipid peroxidation, and cancer.

We have also observed in our study that saliva can be used as a suitable diagnostic medium, as its collection is

Group Name	N	Degree of Lipid Peroxidation			
		Zero	Low	Moderate	High
Non-smokers	25	20	4	1	0
Smokers (<10 cigarettes/day)	25	0	0	9	16
Smokers (10-20 cigarettes/day)	25	1	0	6	18
Smokers (>20 cigarettes/day)	25	0	0	3	22
Smokers with oral pre-cancer & cancer	25	0	0	1	24

Table 2. Degree of Lipid Peroxidation in Study Groups

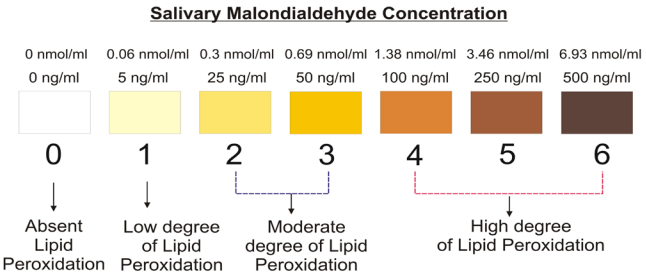


Figure 1. The Colorimetric Lipid Peroxidation Index Chart: The numbers from 0 to 6 denote the Lipid Peroxidation Index (LPI). These were interpreted as LPI 0 representing no risk, LPI 1 denoting low risk, an LPI between 2 and 3 corresponding with moderate risk, while an LPI of 4-6 respresenting high risk.

easy, non-invasive, not time-consuming, and inexpensive. Our findings are thus in accordance with studies that have shown that salivary MDA could serve as a potential diagnostic marker in potentially malignant disease and OSCC (9, 10, 12-15). Our diagnostic test was able to detect the salivary MDA level as an indicator of the degree of lipid peroxidation in the saliva of smokers, pre-cancerous mouth lesions, and OSCC patients with 97% accuracy, and therefore could serve as an early, safe, non-invasive test for smokers to assess their risk of developing oral pre-cancer and cancer.

The simplicity of the procedure does not rule out scope for systematic errors. Inappropriate saliva collection, contamination, or possible degradation of saliva after collection, as well as errors in measurement of saliva or reagent and inconsistent temperatures of the water bath, could lead to false positive results. One way to overcome these errors would be to repeat the test on different occasions. Furthermore, as the test is colorimetric, interpretation of results is largely subjective. In application, QuitPuff salivary diagnostic test does not aim to substitute conventional diagnostic tests, but is meant to act as a qualitative, self-diagnostic test for flagging early risk, and to be followed by laboratory tests for confirmation of diagnosis.

There is ample awareness that cigarette smoking can lead to cancer. For years, cigarette packets have been portraying images of cancer lesions, but this has not discouraged people from smoking. The number of smokers in India has risen from 79 million in 1998 to 108 million in 2015 (16). As the oral cavity is more accessible to complete examination, it could be used in early detection of oral precancerous and cancerous lesions. The disease however gets detected in the later stages, due to reasons such as lack of knowledge and inaccessibility of medical care (7). Careful annual examination of the oral cavity in persons above the age of 40 years can result in significant improvement in the rate of early detection of oral cancer with all the therapeutic advantages. However, a great proportion of those at risk of oral cancer do not attend annual healthcare check-ups (4).

Our aim was to devise a test that could detect the early risk of oral pre-cancer and cancer in smokers. We propose that if such a simple, do-it-yourself, home-based test could be provided with every cigarette pack, then more people might be willing to check their risk and make behavioral changes prior to the development of oral cancer. More people taking these tests would mean more people finding out their risks in the early stages. Early detection could enable better treatment outcome and improvement in the quality of life. It could reduce healthcare costs and the economic burden of treating oral cancer. The test kit requires no elaborate storage conditions and could be easily transported to remote locations and stored in small pan-beedi shops (tobacco selling shops & kiosks) in rural areas. At per sample cost of Rs. 38.15 (approximately 50 cents), it could offer an inexpensive and affordable option especially to the lower income populations, where such a test is often most needed. On account of its simplicity, non-invasive nature, low-cost and easy accessibility, QuitPuff may have great potential as a point-of-care test for oral pre-cancer and oral cancer. The test could be useful as a mass screening tool not only for routine clinics, but also for rural areas and remote locations with limited laboratory facilities or minimally trained health workers.

METHODS

In this study, a simple home-based diagnostic method named QuitPuff was developed for the determination of lipid peroxidation in saliva by simple means i.e. through a visible color change which can be observed by naked eye.

2-Thiobarbituric Acid (TBA) 98% was purchased from Sigma Aldrich; Malondialdehyde Tetrabutylammonium salt (MDA) 96% pure was purchased from Sigma Aldrich. Extra pure distilled water was used. Trichloro Acetic Acid 1% and Ortho Phosphoric Acid 85% pure were purchased from Emplura.

The study was conducted on patients from the Bangalore Medical College and Research Institute, Victoria Hospital and Dr. Health Clinic. Informed consent was obtained from all groups. The Ethics Approval was granted by the Institutional Ethics Committee, Gurushree Hi-Tech Multi-Speciality Hospital. The sample analysis and experiments were conducted at Indian Institute of Science, Bengaluru.

Preparation of MDA standards in saliva of healthy non-smokers

As our diagnostic medium was saliva, the MDA standards were also prepared in saliva. 10 healthy subjects were selected in the age group of 30 to 45 years, with no prior history of smoking or tobacco chewing. To rule out any pre-existing MDA, their saliva samples were sent to Indian Institute of Science, Bengaluru for MDA detection through Liquid Chromatography Mass Spectroscopy (LCMS). The LCMS report confirmed the absence of MDA in all 10 samples collected from the healthy people.

10 mL of unstimulated saliva was collected from each of the ten healthy subjects. MDA standards using saliva of each of the 10 healthy subjects were prepared in the concentrations of 500 ng/mL, 250 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL

and 5 ng/mL.

Preparation of the diagnostic QuitPuff reagent

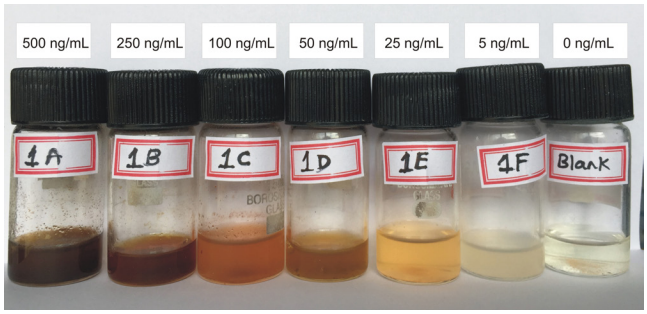
One molecule of MDA reacts with two molecules of TBA under high temperature and acidic conditions to produce a colored MDA-TBA adduct. This is the basic principle of TBARS assay which is the most widely employed assay used to determine lipid peroxidation. Although being accurate, these tests are not routinely done in laboratories. They are expensive, require technical skills, laboratories and instrumentation and sometimes need complex methods like High Performance Liquid Chromatography (HPLC) or Liquid Chromatography Mass Spectrometry (LCMS). Thus, they are fairly out of reach of ordinary people.

As the aim of our study was to develop a simple, home-based test, we explored the possibility of converting a complex laboratory based TBARS assay into a simple colorimetric test that could be self-conducted by the user and the results could be interpreted by simply matching the color change in a sample of saliva to a color chart.

To formulate the most sensitive TBA reagent that would detect MDA concentrations as low as 5 ng/mL, by producing a visible color change, MDA standards using saliva of 10 healthy subjects were prepared in the concentrations of 500 ng/mL, 250 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL and 5 ng/mL and TBA reaction was performed using various TBA formulations (**Table 3**).

As seen in **Table 3**, the formulation used in method 8 proved to be most sensitive, detecting MDA levels as low as 5 ng/mL by producing a visible color change. Thus, the QuitPuff TBA Reagent was prepared by dissolving 0.375 g of TBA in 85% Ortho-Phosphoric acid (1 ml) and 1% Trichloro Acetic Acid (1 ml).

A



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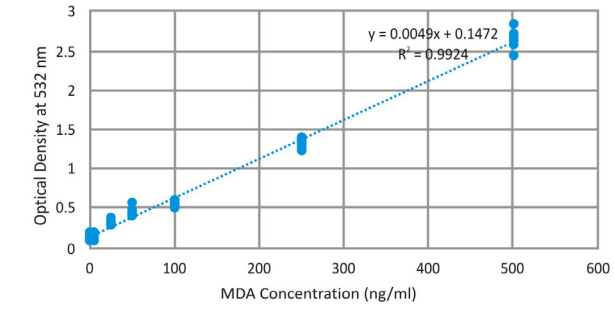

Figure 2. TBA reaction on MDA saliva standards of Healthy Non-Smokers: (**A**) The color change is observed in a gradient with A being the darkest and F being the lightest. No color change is seen in the control. (**B**) A standard curve was plotted based on optical density and MDA concentration.

TBA-Reaction on known standards using QuitPuff

Using the QuitPuff diagnostic TBA reagent, TBA reaction was carried out on the known standards of MDA to generate a visibly colored MDA-TBA adduct. 2 mL of TBA reagent was added to 1 mL of each of the six MDA standards as well as a saliva control. The mixtures were then heated in a boiling water bath for 15 minutes and the color change was observed. The color change was observed in a gradient, with A being the darkest and F being the lightest. The color of control in the saliva control remained unchanged (**Figure 2A**).

The colored samples were analyzed through UV Visible Spectroscopy at Indian Institute of Science (IISc), Bengaluru and the absorbances were measured at 532 nm. Based on the readings a standard curve was prepared (**Figure 2B**). The optical density was plotted along the Y-axis and the MDA concentration in ng/mL was plotted along the X-axis. The following linear equation was derived: [y = 0.0049x + 0.1472]

Preparation of colorimetric chart or the Lipid Peroxidation Index

Based on the color change and UV spectroscopy readings, a colorimetric chart was prepared (**Figure 1**). The colors are numbered from zero to six and denote the Lipid Peroxidation Index (LPI). The results can be interpreted by noting the LPI, which indicates the degree of lipid peroxidation, and thereby the risk status.

Testing the method on 125 subjects

The QuitPuff saliva kit was tested on 125 subjects (aged 30-60 years) from Bangalore Medical College and Research Institute, Victoria Hospital and Dr Health clinic. These subjects were divided into 5 groups. Each group consisted of 25 subjects. Informed consent was obtained from all groups.

Inclusion Criteria: The etiology of oral cancer in India, is dominated mainly by tobacco use, alcohol consumption and smoking (3-7). As the aim of our study was to investigate the relationship between smoking and oral cancer, subjects with significant alcohol consumption and tobacco chewing habits were excluded.

Non-smokers (n=25): No history of alcohol consumption or tobacco chewing habit, absence of long-term systemic diseases, and no oral lesions.

Smokers who smoked less than 10 cigarettes per day for a minimum of 5 years (n=25): no significant alcohol consumption or tobacco chewing habit, absence of long-term systemic diseases, and no oral lesions.

Smokers who smoked 10-20 cigarettes per day since for a minimum of 5 years (n=25): no significant alcohol consumption or tobacco chewing habit, absence of long-term systemic diseases, and no oral lesions.

Method No	TBA Formulation	TBA Reaction	Detection limit of color change
Method 1	Formulation adapted from Ohkawa et al. (17), 0.2mL of 8.1% sodium dodecyl sulphate (SDS), 1.5mL 20% acetic acid at pH3.5, 1.5mL of 0.8% of Thiobarbituric Acid (TBA).	0.2 mL TBA reagent was added to 0.2 ml of each of the MDA standards as well as a saliva control and boiled in water bath for 1 hour at 95 degree Celsius.	100 ng/mL
Method 2	Formulation adapted from Buege J.A and S.D. Aust et al. (18), 0.5% Thiobarbituric Acid, 20% Trichloro Acetic acid, 2.5N Hydrochloric acid.	0.5 mL TBA reagent was added to 0.5 mL of each of the MDA standards as well as a saliva control and heated in a boiling water bath for 20 minutes.	100 ng/mL
Method 3	Formulation adapted from Esterbauer H. et al. (19),1 mL 0.37% Ethylenediaminetetraacetic-acid (EDTA), 1 mL 2% Butylated hydroxytoluene (BHT), 2 mL 10% Trichloro Acetic Acid, 1 ml 0.67% Thiobarbituric Acid.	5 ml TBA reagent was added to 1ml of each of the MDA standards as well as a saliva control and heated in a boiling water bath for 10 minutes.	100 ng/mL
Method 4	Formulation adapted from Uchiyama M and Midori Mihara M. (21), 3 mL 1% Phosohoric acid, 1 mL 0.6% Thiobarbituric acid aqueous solution.	TBA reagent was added to 0.5 mL of each of the MDA standards as well as a saliva control and heated in a boiling water bath for 45 minutes.	25 ng/mL
Method 5	Formulation adapted from Asakawa T. and Matsushita S. (20), 2 mL of 20% TCA containing 20 umol Ferrous Sulphate, 1 mL 0.67% TBA.	TBA reagent was added to 1 mL of each of the MDA standards as well as a saliva control and heated in a boiling water bath for 30 minutes.	25 ng/mL
Method 6	Formulation adapted from experimentation, 0.1875 g of TBA in 0.4562 mL of Acetic Acid, added distilled water to bring the volume to 50 mL.	2 mL TBA reagent was added to 1 mL of each of the MDA standards as well as a saliva control and heated in a boiling water bath for 15 minutes.	5 ng/mL, color change observed in control.
Method 7	Formulation adapted from experimentation,1 mL 0.375 g of TBA in 1 mL 85% Ortho-Phosphoric Acid.	2 mL TBA reagent was added to 1 mL of each of the MDA standards as well as a saliva control and heated in a boiling water bath for 15 minutes.	25 ng/mL
Method 8	Formulation adapted from experimentation, 0.375 g of TBA in 1 mL 85% Ortho-Phosphoric Acid, 1 mL 1% Trichloro Acetic Acid.	2 mL TBA reagent was added to 1 mL of each of the MDA standards as well as a saliva control and heated in a boiling water bath for 15 minutes.	5 ng/mL
Method 9	Formulation adapted from experimentation, 0.375 g of TBA in 1 mL 1% Ascorbic Acid, 1 mL 1% Trichloro Acetic Acid.	2 mL TBA reagent was added to 1 mL of each of the MDA standards as well as a saliva control and heated in a boiling water bath for 15 minutes.	5 ng/mL, Color change observed in control.
Method 10	Formulation adapted from experimentation, 0.3 g of TBA dissolved in 3 mL of Dimethyl Sulfoxide (DMSO).	2 mL TBA reagent was added to 1 mL of each of the MDA standards as well as a saliva control and heated in a boiling water bath for 15 minutes.	25 ng/mL

Table 3: Detection limit of color change seen in MDA standards with various TBA formulations

Smokers who smoked above 20 cigarettes per day since for a minimum of 5 years (n=25): no significant alcohol consumption or tobacco chewing habit, absence of any long-term systemic diseases and having no oral lesions.

Smokers who smoked 10-20 cigarettes per day since for a minimum of 5 years with recently diagnosed pre-cancerous mouth lesions and oral cancer stage 1-2 (n=25): yet to start on treatment (oral erosive lichen planus, n=12; oral leukoplakia [raised, indurated, white lesion in the oral mucosa more than 5mm in diameter with dysplastic changes in the epithelium], n=6; sub-mucous fibrosis, n=2; and oral squamous cell carcinoma [OSCC], n=5). Of the five subjects with OSCC, four were diagnosed with Stage 1 OSCC and one was diagnosed with Stage 2 OSCC.

Patients with chronic alcohol addiction, tobacco chewing habits and patients with advanced stages of oral squamous cell carcinoma and/or already on treatment or operated upon were excluded from the study.

The tests were performed on fresh samples of saliva. Subjects were asked not to eat, drink, smoke or chew tobacco an hour before the collection. Subjects rinsed their mouths with 10 mL of water and thereafter saliva was collected in sterile bottles. The QuitPuff TBA reaction was performed as previously described. The colour change was matched with the colorimetric chart (**Figure 2**) and the Lipid Peroxidation Index (LPI) was noted.

Validation by UV Visible Spectroscopy

For further validation, all 125 samples were sent to Indian Institute of Science for MDA determination through UV Spectroscopy (Supporting Information). The readings of UV Visible Spectroscopy were then plotted on the standard curve and the linear equation [y = 0.0049x + 0.1472] was used to determine the MDA concentration values. A spearman correlation analysis was performed to understand the correlation between the color change in the samples and the readings of of UV Visible Spectroscopy. On the basis of the MDA concentration, the LPI was again derived and noted. A detailed report of these 125 samples, including photos, UV Spectroscopy results, MDA concentrations, and Lipid Peroxidation Index classification is available in this article's Supporting Information.

ACKNOWLEDGEMENTS

We wish to thank the following people without whom this study would not have been completed: Mr. Aloysius D'Mello, Principal, Greenwood High School for writing recommendation letters to hospital authorities and lab facilities, Dr. H.S Satish, Director & Dean, Bangalore Medical College and Research Institute, Victoria Hospital, Dr. Tania Das, Research fellow at ICAR-National Bureau of Agricultural Insect Resources for sharing experiences from her work on the effects of lipid peroxidation on plants, Ms. Madhavi Londhe, Research Fellow, BARC for her overall guidance, Mr. Muralidhar from IISc for helping us with the UV Spectroscopic analysis of the saliva samples, Dr. Arnab Roy for making the LCMS procedure easier to understand.

Received: October 18, 2018
Accepted: February 7, 2019
Published: March 27, 2019

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