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RESEARCH ARTICLE

Paper-based biosensor for early detection of oral cancer

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The significance of paper-based biosensors for the early stage detection of oral cancer and their compatibility with affordable and low-cost diagnostics have been discussed in this article. Oral cancer is a major public health concern in India, and early-stage detection can be a great boon to the public. Among all the available methods for diagnosis, paper-based biosensors have proven to be very efficient in all aspects. It requires a very small volume of sample and very little time, and the process of detection is very simple. The diagnostic platform described here employs a hydrophobic material (wax) to pattern microfluidic channels on a hydrophilic substrate (paper) to detect the desired biomarker in saliva as biological fluid. Biomarker detection in saliva is advantageous because it is a noninvasive and potentially diagnostic sample. One of the primary benefits of using saliva in diagnosis is that sample collection is simple and noninvasive, significantly reducing the discomfort associated with blood collection and the privacy concerns associated with urine collection. The presence of biomarkers such as salivary cotinine and nitrite can be detected on a single paper-based analytical device, allowing for parallel quantification of the biomarkers. Calorimetric assays are used to analyze the biomarkers qualitatively, and ImageJ software is used to analyze the biomarkers quantitatively.

Keywords: salivary nitrite, salivary cotinine, paper based analytical device, calorimetric assay, ImageJ software.

Introduction

Biomarkers are the principle target analytes in clinical diagnostics because they can be detected quickly, easily, accurately, and inexpensively. Recent immunoassay methods, such as enzyme-linked immunosorbent assay (ELISA) for biomarker detection in body fluids like urine, blood, and serum, produce reliable and sensitive results. However, the lengthy procedures that require large amounts of reagents and samples, as well as skilled personnel to perform the work and analyze the results, preclude the use of these devices in resource-limited settings (1). As a result, there is a pressing need for simple, low-cost detection systems that are user-friendly and capable of detecting and analyzing biomarkers in resource-constrained environments. One alternative approach is to use paper-based biosensor applications for disease diagnosis, health monitoring, pathogen detection, and other purposes.

Paper-based biosensor methods enable the development of low-cost, flexible, simple, and portable diagnostic devices (2). This inexpensive technology employs patterning to create hydrophobic microfluidic channels from hydrophilic paper (3). A clinical sample containing a biomarker, such as urine, blood, sweat, tears, or saliva, is introduced to the diagnostic tool and flowed to a sensing zone via capillary force with no external power (4, 5). The electrochemical and optical properties of the sample solution are altered by a biochemical reaction, allowing biomarker detection. Calorimetric detection, which is based on optical detection, is the most commonly used detection technique among these.

The results can be easily analyzed by a color change caused by ligand-analyte interaction, such as antibody antigen binding, which can be measured using commercial scanners, cameras, or smartphones (6, 7). As a result, paper-based analytical devices are the most user-friendly and portable diagnostic tools for the rapid and on-site detection of a variety of diseases (8).

Oral cancer starts with a small, unusual, unexplained growth or sore in the mouthparts, which include the lips, cheeks, sinuses, tongue, hard and soft palate, base of the mouth, and oropharynx (9). Because there is evidence that stage is an important prognostic factor in cancer, interventions are being developed.

Cancer treatments aimed at "down-staging" are part of a comprehensive cancer control strategy. Many cancer patients could be saved from death and suffering if they had access to effective detection programs and appropriate treatment in a timely manner.

Another site that responds well to early detection is oral cancer. Treatment and tumor stage at the time of diagnosis, as well as the detection of small, early-stage malignant tumors, have been linked to overall survival (9). The ability of medical practitioners, nurses, and other health care providers to detect oral cancer early in primary care has significantly reduced mortality and morbidity from this disease. In countries with good access to primary care clinics, health care workers emphasize the general need for and feasibility of early cancer detection (10). Oral cancer is the world's sixth most common type of cancer. India has the most cases of oral cancer, accounting for one-third of the global burden. The low-income population is the most vulnerable due to extensive exposure to various risk factors (10). Despite recent advances in diagnostics and therapeutics, patients with oral carcinoma have a 50% chance of survival. Tobacco and alcohol use account for more than 90% of oral cancer cases, with other dental, genetic, and environmental factors accounting for 10% (11). Tobacco, in its various forms, contains multiple carcinogens, such as alkaloids, as well as N-nitrosonornicotine, a nitrate-based compound (NNN). In the oral cavity, salivary nitrates are converted to nitrites (NO_2). Nitrosamines, which are carcinogenic, are formed when nitrite reacts with amines and amides. As a result, salivary nitrite can be used as a biomarker to detect oral carcinoma (12).

Nicotine is one of the chemicals found in almost all tobacco products. Cotinine is its primary proximate metabolite. As a result, the presence of cotinine in the oral cavity can be used as a biomarker. This is useful for distinguishing tobacco users from non-users and also reflects a person's level of tobacco exposure (13).

Nitrite concentrations in saliva range from 0 to 210 μg/ml in an individual (0 to 100 μg/ml in healthy individuals and 100 to 210 μg/ml in cancer patients), and salivary cotinine concentrations range from 0 to 100 ng/ml (0 to 10 ng/ml in healthy individuals and 10 to 100 ng/ml in smoking individuals). Individuals with salivary cotinine levels less than 10 ng/ml are also considered healthy (14). This can be estimated qualitatively using a calorimetric method in a paper-based analytical device (15) and quantitatively using ImageJ software (16). For quantification, a gray scale analysis is performed.

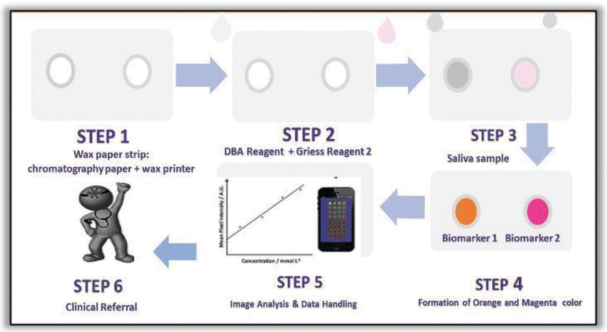

FIGURE 1 | The overall methodology for the working of the paper-based sensor.

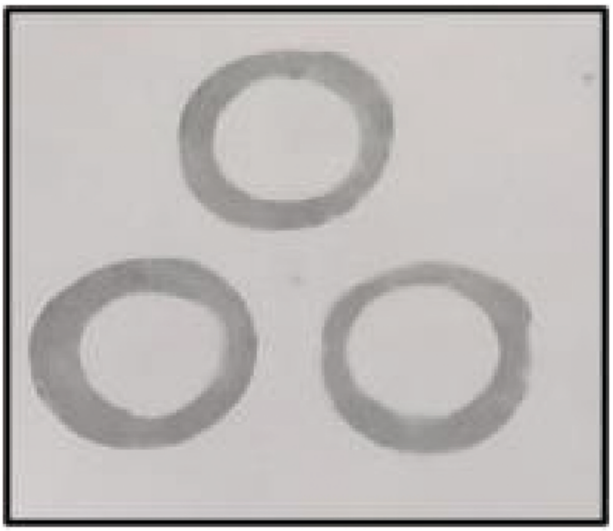

FIGURE 2 | Fabricated paper-based sensor.

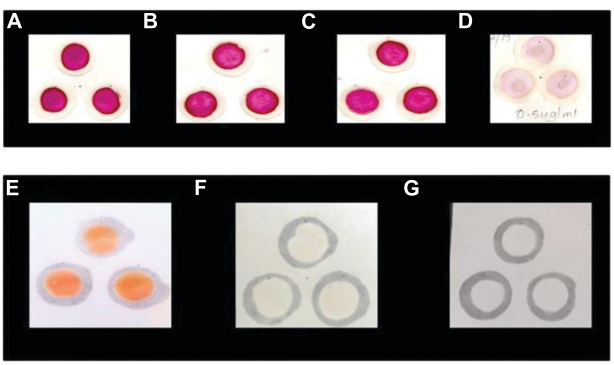

FIGURE 3 | The standard concentration of salivary nitrite: **(A)** 250 g/ml, **(B)** 210 g/ml, **(C)** 100 g/ml, and **(D)** 0.5 g/ml. The standard concentration of salivary cotinine: **(E)** 100 ng/ml, **(F)** 10 ng/ml, and **(G)** 0 ng/ml.

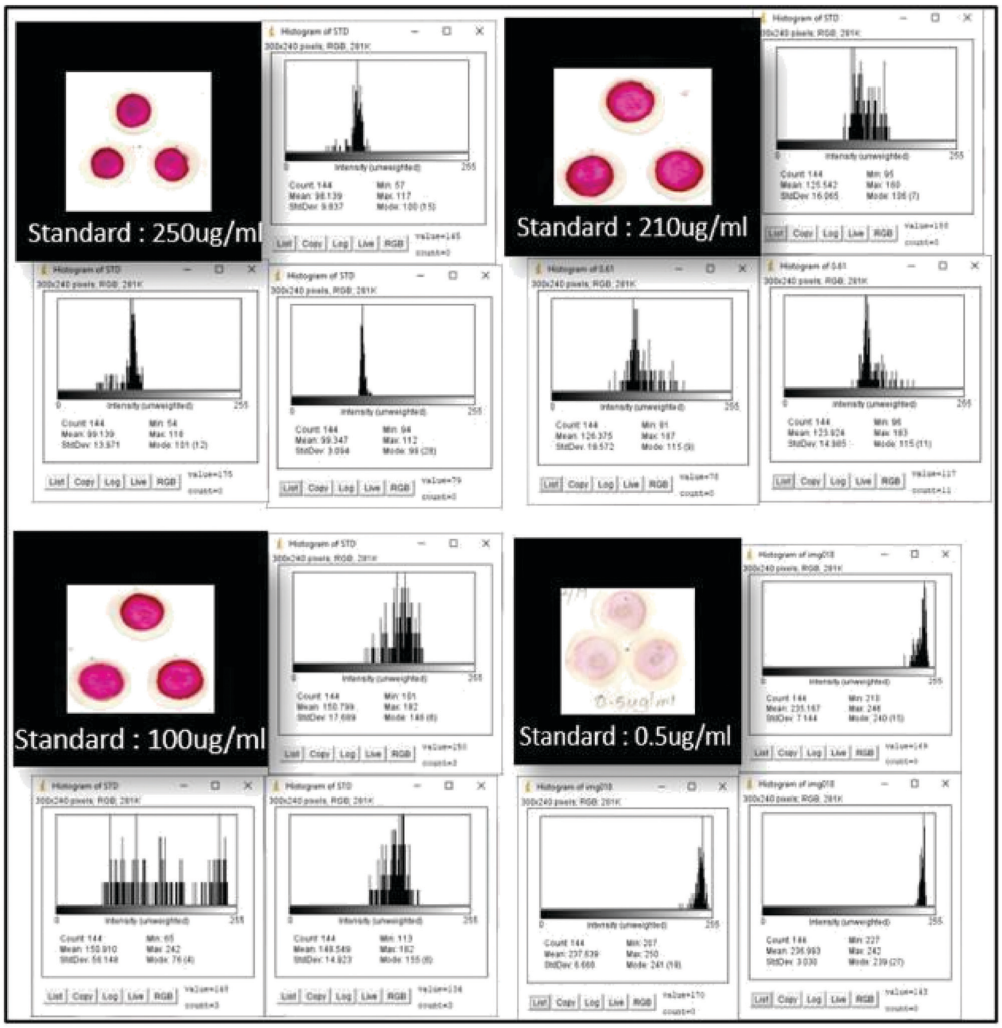

FIGURE 4 | Images of the histograms obtained in the ImageJ software for the quantitative analysis of standard concentration nitrite for 250, 210, 100, and 0.5 g/ml.

As a result, we created a single paper-based sensor that can detect and quantify the presence of two salivary biomarkers, salivary nitrite and salivary cotinine, for the rapid and early detection of oral carcinoma and tobacco exposure.

Materials and methodology

Reagent and standard preparations for salivary nitrite

The Griess reagent was made by combining distilled water with a 1:1 ratio of 1% (w/v) sulfanilamide (Research Lab Fine Chem Industries) in a 5% (v/v) phosphoric acid solution (Karnataka Fine Chemicals Ltd.) and 0.1% (w/v) N-naphthyl ethylenediamine dihydrochloride (Oxford Lab Fine Chem LLP). 1.232 g in 1000 ml of distilled water containing 250 g/ml, diluted to standard concentrations of 0.5, 100, and 210 g/ml.

Reagent and standard preparation for salivary cotinine

A total of 4 mol/L sodium citrate buffer, pH 4.1, potassium thiocyanate (Karnataka Fine Chemicals Ltd.) in distilled water, 0.4 mol/L chloramine-T (Oxford Lab Fine Chem LLP) in distilled water, and 78 mmol/L barbituric acid (Oxford Lab Fine Chem LLP) in acetone: H_2O (50% v/v) The standard concentrations of (-)-cotinine (Sigma, 74003) were prepared by diluting with distilled water to 10 and 100 ng/ml, respectively.

Detection of biomarkers on the fabricated paper-based sensor

A 1 L droplet of individual samples (non-smoker and smoker samples) or standard solution is dispensed onto the testing zone. After collecting the sample, the paper is allowed to dry for 15 min. The presence of the Griess reagent causes the

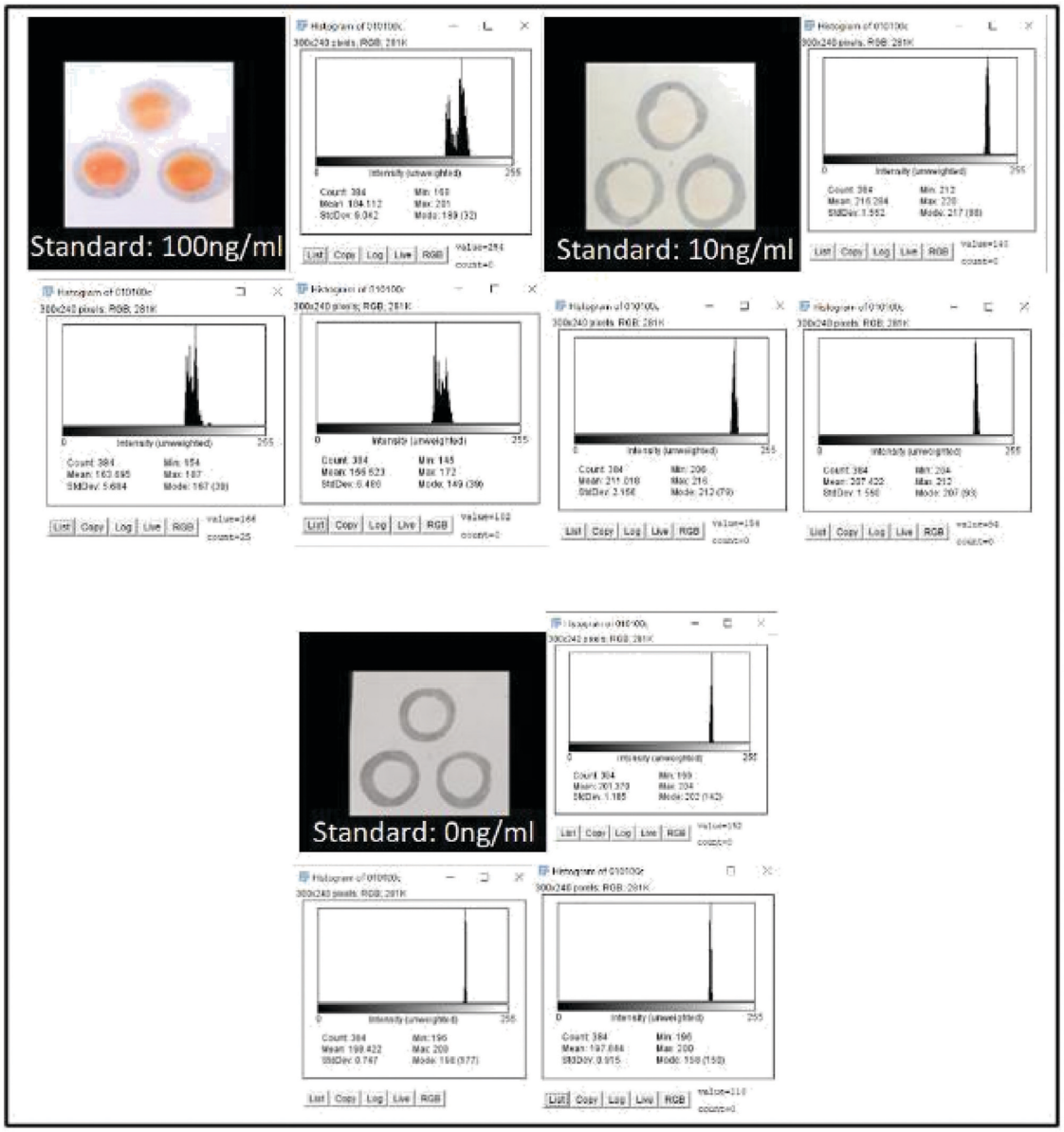

FIGURE 5 | Images of the histograms obtained in the ImageJ software for the quantitative analysis of standard concentration cotinine for 100, 10, and 0 ng/ml.

formation of a magenta azo compound for salivary nitrite, and the presence or absence of cotinine causes the formation of an orange-reddish pink color spectrum. The intensity of these color spectra (magenta and orange-reddish pink) varies with the concentration of cotinine in the sample.

Quantitative analysis—Gray scale analysis

The color image captured by the paper-based analytical device is converted to gray scale as a blend of black and white shades. In gray scale, each pixel's value only contains intensity information, with black representing the weakest gray minimum intensity at 0 and white representing the strongest gray maximum at 255. The gray intensity of a gray image rises and falls in proportion to its brightness and darkness. In image processing, these data correspond to the number of pixels in terms of the various intensities found in an image and are plotted with respect to their intensity, resulting in a gray image histogram that is used to depict the distribution of numerical data. Because a histogram's gray intensity varies with the concentration of nitrite and cotinine in saliva, the gray intensity can be used to analyze salivary nitrite and cotinine. The darker the image, the higher the concentration of salivary nitrite and cotinine, resulting in a lower gray intensity. As a result, the concentration of the salivary biomarker can be effectively quantified using a histogram and ImageJ software by the gray intensity value of an image.

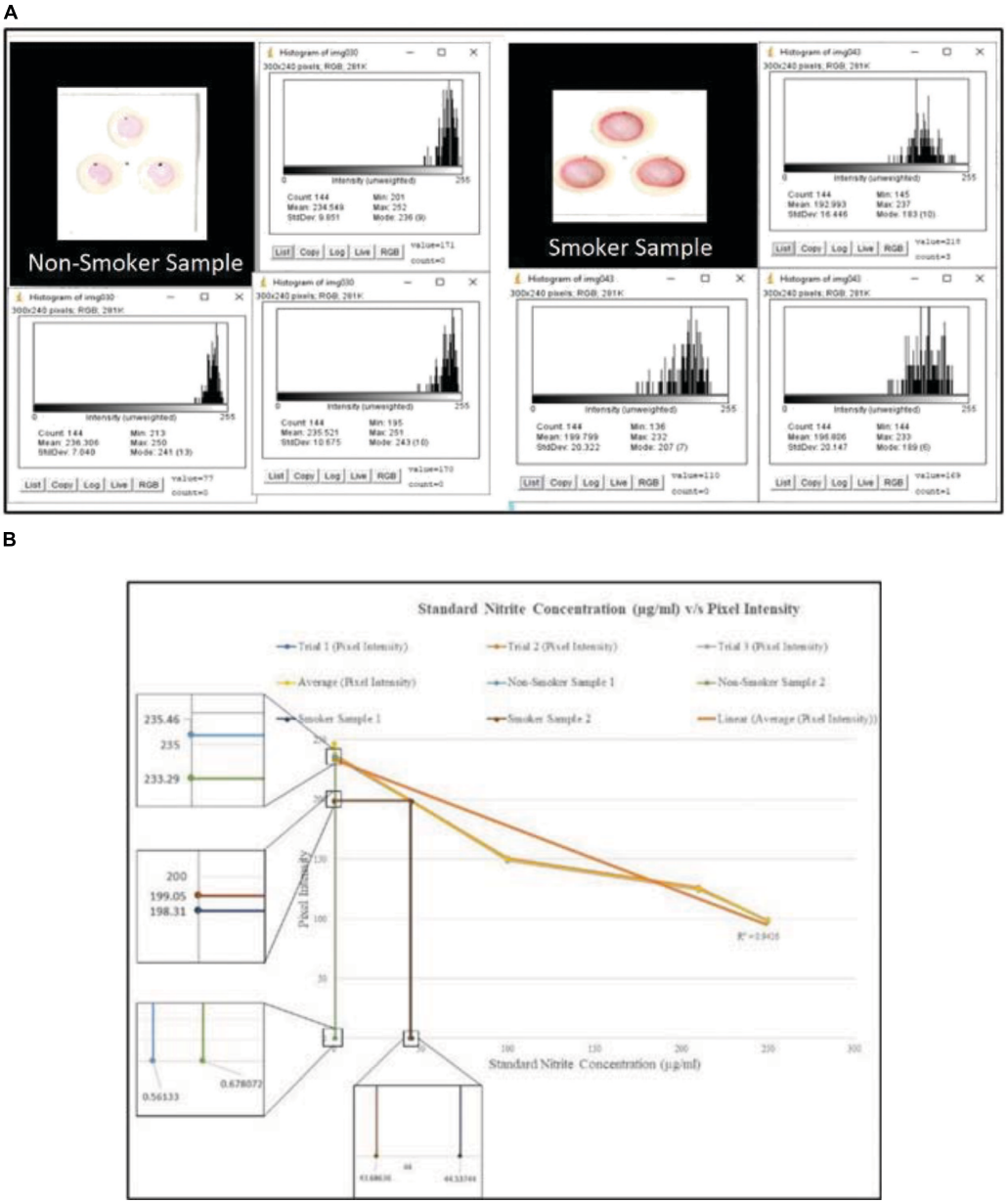

FIGURE 6 | (A) Images of the histograms obtained from ImageJ software for quantitative analysis of non-smoker sample and smoker sample for nitrite estimation. **(B)** Graph illustrating the standard nitrite concentration versus pixel intensity curve along with the interpolated values of non-smoker and smoker samples.

The overall methodology for analyzing and quantifying the biomarker is shown in **Figure 1**.

Results and discussion

The "ease-of-use" and "portability" of the paper-based analytical device were demonstrated

Whatman filter paper was used for the fabrication of the device and hence less expensive. The fabrication of the device requires no sophisticated equipment, and its size (7 cm × 4 cm) allows the device to be cost-effective and portable, as shown in **Figure 2**.

Analyzed the presence of biomarkers using the calorimetric method

The intensity of the color on the strip (magenta and orange-reddish pink) varies with the concentration of nitrite and cotinine in the sample. The presence of the color, in this case magenta for nitrite and orange-reddish pink for cotinine, indicates a positive result. The absence of the color (magenta for nitrite and orange-reddish pink for cotinine) indicates

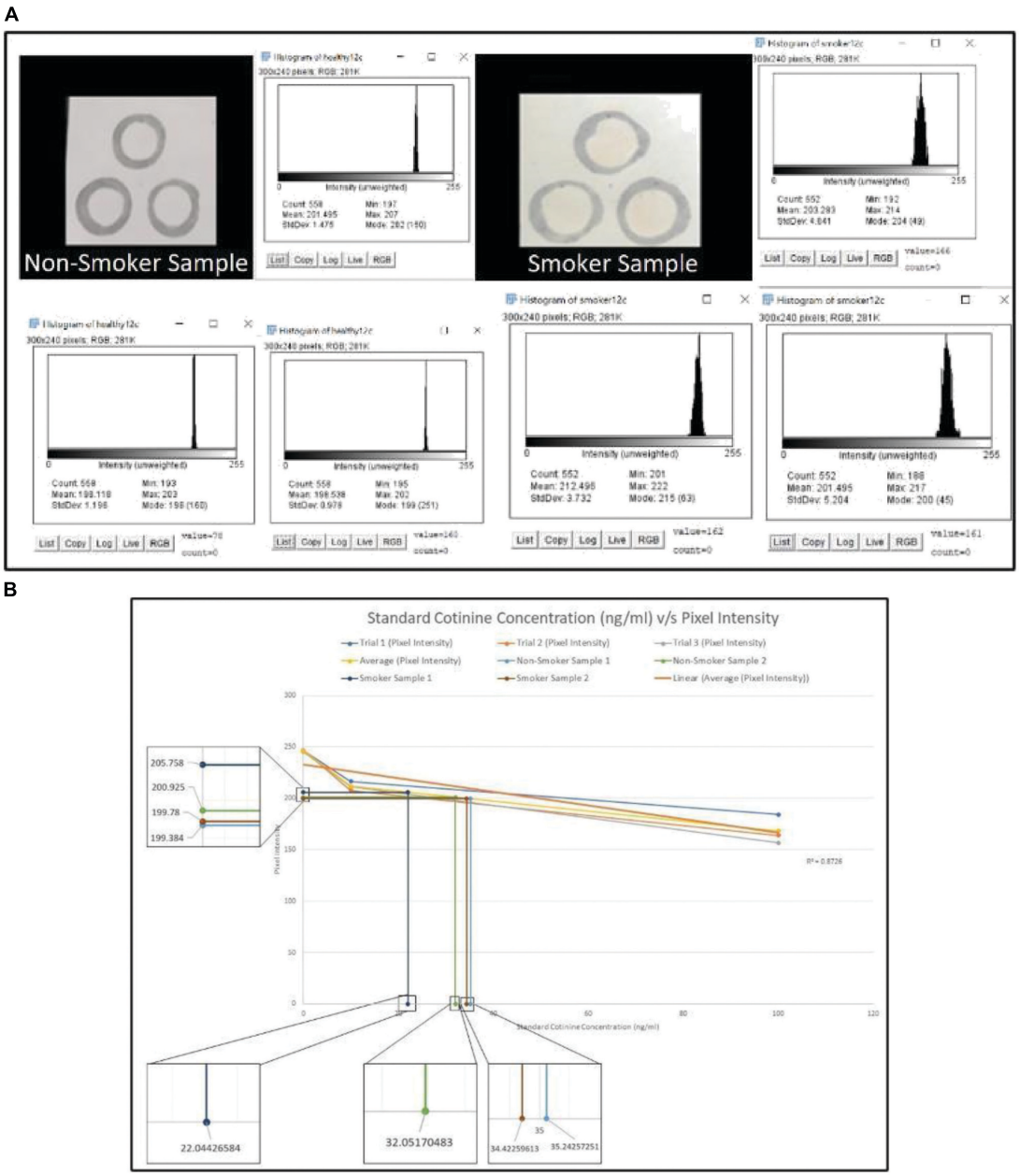

FIGURE 7 | (A) Images of the histogram obtained from ImageJ software for quantitative analysis of non-smoker sample and smoker sample for cotinine estimation. **(B)** Graph illustrating the standard cotinine concentration versus pixel intensity curve along with the interpolated values of non-smoker and smoker samples.

a negative result. As shown, the standard concentrations of salivary nitrite and cotinine are qualitatively analyzed (**Figures 3A, B**).

Image processing of captured images of the paper-based sensor using ImageJ software

The color image captured for the standard concentration of salivary nitrite is converted to gray scale in the quantitative analysis. The maximum number of pixels corresponded to the same gray intensity of 99 for a nitrite concentration of 250 g/ml, implying that the maximum number of pixels corresponded to the same gray intensity of 99 for a nitrite concentration of 250 g/ml. Similarly, the highest number of pixels was obtained for 210, 100, and 0 g/ml of nitrite levels at the 125, 150, and 245-pixel intensities, which correspond to the respective gray intensities of the 210, 100, and 0 g/ml of nitrite concentration in the form of a histogram as shown in **Figure 4**. As a result, the higher the gray intensity of pixels, the lower will be the level of salivary nitrite (**Figure 4**). From a color image, the maximum number of pixels obtained for the standard concentration of salivary cotinine is converted

to gray scale. The maximum number of pixels corresponding to 100 ng/ml of cotinine concentration was discovered at a pixel intensity of 168. Similarly, at pixel intensities of 211.5 and 246, cotinine levels of 10 and 0 ng/ml were obtained, as shown in the form of a histogram (**Figure 5**).

Parallel detection and estimation of biomarkers using the calorimetric method

Nitrite estimation

The salivary concentration of the tested RNS: NO_2 was measured in healthy controls using a calorimetric assay method. The quantitative analysis of smoker and nonsmoker samples for salivary nitrate estimation, as well as the graph of the standard nitrite concentration vs. pixel intensity curve, have been illustrated, along with the interpolated values of non-smoker and smoker samples (**Figures 6A, B**).The non-smoker samples had pixel intensities of 235.46 and 233.29, while the smoker samples had pixel intensities of 199.05 and 198.31. As expected, the pixel intensities obtained in smoker samples are lower than those obtained in non-smoker samples. These pixel intensities correspond to salivary nitrite concentrations of 0.56, 0.67l, 43.68, and 44.53 g/ml, respectively, which are less than the threshold of 100 g/ml of salivary nitrate, indicating that none of the samples collected are from a diseased individual. Furthermore, because the salivary nitrate concentrations are significantly lower than the threshold, none of the individuals are at immediate risk of developing oral carcinoma.

Cotinine estimation

The salivary concentration of cotinine in the sample was compared to a known concentration standard using a calorimetric assay method. The quantitative analysis of smoker and non-smoker samples for salivary cotinine estimation, as well as the graph of the standard cotinine concentration versus pixel intensity curve and interpolated values of non-smoker and smoker samples, are shown in **Figures 7A, B**. The non-smoker samples had pixel intensities of 200.925 and 199.384, while the smoker samples had pixel intensities of 205.758 and 199.78. These pixel intensities correspond to 32.05, 35.24, 22.04, and 34.42 ng/ml of salivary cotinine, which is between 10 and 44 ng/ml of salivary cotinine, indicating that the samples collected could be from individuals at a lower risk of developing oral carcinoma.

Conclusion

The proposed paper-based sensor was "user-friendly" and "portable" for detecting salivary nitrite and cotinine biomarkers for early-stage oral cancer diagnosis. Due to its lower fabrication cost, the fabricated paper-based sensor provided a cost-effective diagnostic tool, and the proposed device employs the calorimetric assay as the analytical detection technique, which evaluates whether the color formation or color change is qualitatively and quantitatively detected, providing accurate results at a lower cost.

The results indicated that smokers (ranging from 100 to 230 μg/ml) had a higher concentration of salivary nitrite than healthy controls (from 0 to 100 μg/ml). Similarly, smokers' salivary cotinine levels (ranging from 10 to 100 ng/ml) were higher than those of healthy controls (ranging from 0 to 10 ng/ml). The salivary concentrations of nitrite in oral squamous cell carcinoma (OSCC) samples will be analyzed further; these values are expected to be higher than in healthy controls.

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