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CLINICAL MICROBIOLOGY - RESEARCH PAPER

Anthocyanin-rich black carrot (*Daucus carota* ssp. sativus var. atrorubens Alef.) and red cabbage (*Brassica oleracea*) extracts incorporated biosensor for colorimetric detection of *Helicobacter pylori* with color image processing

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Abstract
In this work, we developed novel colorimetric biosensors consisting of anthocyanin-rich either black carrot (*Daucus carota* ssp. sativus var. atrorubens Alef.) or red cabbage (*Brassica oleracea*) extracts for rapid, sensitive, and economic detection of *Helicobacter pylori* (*H. pylori*). We comparatively prepared two test solutions as biosensors including anthocyanin-rich black carrot extract (Anth@BCE) and red cabbage extract (Anth@RCE), both of which fixed to pH 2.5 and investigated their colorimetric responses based on electronic structure and electron density of anthocyanins. We successfully used anthocyanin-rich BCE and RCE as natural pH indicators in detection of *H. pylori* and introduced their advantages like non-toxicity, easy accessibility, and high stability compared to synthetic indicators. The BCE and RCE tests gave the best color change in the presence of 10^3 CFU/mL (at 60 min) and 10^4 CFU/mL (at 75 min) *H. pylori* suspensions prepared in an artificial gastric fluid. The limit of detection was down to 10 CFU/mL for RCE and BCE tests by increasing incubation time (≥ 5 h). We further made an additional study that color differences in the colorimetric responses observed by naked eyes were supported by digital image processing with RGB (Red Green Blue) and Delta-E (ΔE) analysis. It is confirmed that results evaluated by naked eyes and digital image processing are well consistent with each other. These findings proposed that these colorimetric tests can be implemented to pH dependent detection of various microorganisms and can be effectively transferred from laboratory work to clinics in the near future.

Keywords Anthocyanin · Biosensor · Colorimetric detection · *Helicobacter pylori* · Color image processing

Introduction

The millions of people regardless of gender and age have been affected by *Helicobacter pylori* (*H. pylori*) each year in the world. Prevalence of *H. pylori* infection in populations can be varied based upon societies due to their living regions and/or socioeconomic status [1, 2]. It is worthy to mention that *H. Pylori* has shown quite a high prevalence rate in almost every country and it has caused serious life-threatening diseases [3, 4]. The first action of *H. pylori is* usually to colonize in human stomach, and then it can cause ulcers or fatal diseases as like "cancer" in where it colonized. Especially, stomach-related cancers have caused hundreds of thousands of deaths every year based upon their degree or severity [5–8].

For these reasons, researchers have developed several invasive and/or noninvasive methods in order to detect *H. pylori* in clinics. In terms of invasive methods, for instance, polymerase chain reaction (PCR) and culture and histology provide accurate detection of *H. pylori*, but need of expensive instruments and expert users are major drawbacks [9–11]. The urea breath test (UBT), the stool antigen test (SAT), and the *H. pylori* saliva antigen test (HPS) have been known as well documented and easy-to-use noninvasive methods. However, these noninvasive methods also hold

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some serious limitations in aspect of specificity, reproducibility, sensitivity, availability, and even cost-effectiveness [12, 13].

The anthocyanins termed "cyanidin-based pigments" were identified as major compounds in BCE and RCE [14–16]. While the core of cyanidin-based anthocyanin compounds are cyanidin-3-xylosyl-glucosyl glucoside and cyanidin 3-O-diglucoside-5-O-glucoside in BCE and RCE, respectively, the slight structural differences in anthocyanins can be considered as key point on color profiling at different pH values (pHs) [17–19]. Benefiting from biocompatibilities, stabilities, and pH-dependent color properties, anthocyanins have been devised as a new colorimetric biosensor system for sensing pH value of reaction environment [20, 21]. In our previous work, the *H. pylori* was detected by preparing RCE incorporated colorimetric urease assay at pH 8 (blue color-adjusted urease test) [22]. Various biosensors including the RCE incorporated-tests have been also used to detect the presence of antibiotics [23–25] and the growth of antibiotic-resistant bacteria producing acidic organic volatile compounds, which make culture media acidic [26, 27].

Herein, we introduced rational and novel phenotypic colorimetric urease tests containing anthocyanin-rich black carrot extract (Anth@BCE) and red cabbage extract (Anth@RCE). The anthocyanins as agricultural by-products are key components in Anth@BCE and Anth@RCE tests for detection of *H. pylori*. The working principle of these tests relied on pH-dependent colorimetric response of anthocyanins in the presence of *H.pylori*. The reason is that *H.pylori* releases urease enzymes to hydrolyze urea existing in the test environment to generate ammonium molecule (NH_3). The NH_3 generation leading to the growth of *H.pylori* in the stomach makes microenvironment of test solutions alkaline, and then, this situation respectively triggers deprotonation of anthocyanins and results in color change of the test solutions. We utilized color image processing system by calculating Delta-E and G/B values of the test solutions to both quantitatively support visual color change and to effectively detect color change which cannot distinguished by human eye. This image processing software can be easily integrated on a smartphone for developing point-of-care (POC) testing.

Experimental section

Materials Phosphate-buffered saline (PBS) tablet (1 tablet dissolved in 200 mL water to prepare a 137 mM NaCl, 2.7 mM KCl and pH 7,4; 10 mM PBS at 25 °C), sodium azide(NaN_3), urea, and hydrochloric acid %37 (HCl) were purchased from Sigma-Aldrich (Germany). We purchased tryptic soy agar (Merck, Germany), Columbia agar (CampyGen, Oxoid, UK), and skimmed milk medium (Difco, USA). The Pharmaceutical Microbiology Research Laboratory ATCC culture collection in the Faculty of Pharmacy at Erciyes University, Turkey, was a source for *H. pylori* ATCC 43,504.

Preparation of black carrot and red cabbage extracts Both black carrot and red cabbage extracts were prepared by following a modified method [28–32]. For RCE extract preparation, red cabbage leaves are cut into small pieces. One hundred grams of material is weighed and taken into a beaker containing 100 mL of distilled water. The mixture is boiled for 30 min for extraction; the obtained anthocyanin extract is filtered and kept at − 20 °C for further use. For BCE extract preparation, black carrots are sliced and frozen at − 20 °C. The 150 g of frozen carrot slices was mixed with 150 mL solvent (acidified ethanol) without thawing. Ethanol acidified with citric acid is used as the extraction solvent. The extraction is achieved by grinding and mixing frozen carrot slices in a blender for 20 min. After extraction process, the solvent is removed by using the lyophilizer through filtration. The obtained powder extract is stored at 4 °C until used [33]. The initial pH of both BCE and RCE extract solutions were around 7.4 and 1 M hydrochloric acid (37% HCl) was added in each extract solution drop by drop under stirring till to adjust to pH of 2.5.

H. pylori culture The jar with a gas-generating kit for microaerophilic conditions was used to culter the *H. pylori* in 3 days. The culture media contains tryptic soy agar and Columbia agar base with 5% sterile sheep blood [22].

Preparation of black carrot and red cabbage extracts-based colorimetric tests Anth@BCE and Anth@RCE tests were prepared in 10 mM PBS at pH 2.5. The color of each Anth@BCE and Anth@RCE test was adjusted to red or pink. Both tests containing 5% w/w BCE and RCE, 2 g of urea, and 20 mg of NaN_3in 100 mL of 10 mM PBS were placed in an autoclave at 121 °C for 15 min sterilization prior to use. After autoclave, Anth@BCE test and Anth@RCE test solutions were filtered through sterile membrane filters with a pore size of 0.45 µm. The best distinctive color change was seen at the 5% BCE and RCE concentration.

Digital image processing To support colorimetric response visualized by naked eyes, we used ImageJ software (National Institutes of Health) to analyze the color changes in images of Anth@BCE and Anth@RCE test solutions during *H. pylori* detection. In digital image processing, RGB (Red Green Blue) and Delta-E (ΔE) as two analytical tools were utilized to calculate value of red, green, and blue components and color differences via Euclidean distance from the CIE 1976 Lab color difference formula, respectively [26, 27, 34–36].

Results and discussion

In this current work, we rationally used anthocyanin molecules predominantly found in BCE and RCE extracts as water-soluble pigment molecules owing their natural indicators property in preparation of colorimetric diagnostic tests. The Food and Drug Administration (FDA) approved anthocyanin pigments as color additives used in foods, drugs, cosmetics, and medical devices the USA [37]. The main advantages of anthocyanins compared to synthetic organic indicators are low cost, biocompatibility, easy availability, high stability, and showing pH-dependent color changes [38].

The pH-dependent color changes of anthocyanins in BCE and RCE were demonstrated at pH scale in Fig. 1. The BCE solution gives red colors with slightly different tones below pH 7, the purple-like colors are seen in between pH from 7 to 11, and finally dark and light yellow colors are obtained in between pH 11 and 13. The RCE solution has both some similarities and differences in terms of color compared to BCE. For instance, while RCE solution gives pinkish and reddish colors at acidic environment below pH 2.5 as like BCE, the original purple colors (in between pH 4 and pH 7), blue color (in between pH 7 and pH 10), green color (at pH 11), and yellow colors (above pH 12) are witnessed.

Potential equations of anthocyanins of BCE (left) and RCE (right) were given in Fig. 2. As seen that structural differences in anthocyanins based upon protonation and/or deprotonation and electron density can be considered as a main driving forces on color changes. We demonstrated how pHs of reaction solutions (from pH 2.5 to above pH 12) predominantly affect deprotonation of anthocyanins. Although deprotonation of anthocyanins in BCE and RCE occurred on the same aromatic group or on the same carbon atom, the differences in structure and electron density of anthocyanins result in different colors at the same pHs.

The BCE-based colorimetric tests including 0.01 M PBS, urea, NaN_3, and 5% w/w BCE were prepared at pH 2.5 for detection of *H. pylori* (2×10^5 CFU/mL) in the different periods of time. We worked on single concentration of *H. pylori* to be 2×10^5 CFU/mL, which is meaningful for clinic. Simply, the 50 µL of *H. pylori* (2×10^5 CFU/mL) suspension was added into 1 mL of BCE test, and then it was incubated to observe the color change. During the incubation, *H. pylori* strain continuously releases urease enzymes to hydrolyze urea for producing NH_3 molecules, which made test environment alkaline. As we mentioned above, the principle of color change relies on the number of the deprotonated anthocyanin molecules in an alkaline environment. For instance, Fig. 3 shows that the initial red color of BCE test solution turned to reddish-purplish color (pH 7–8) in

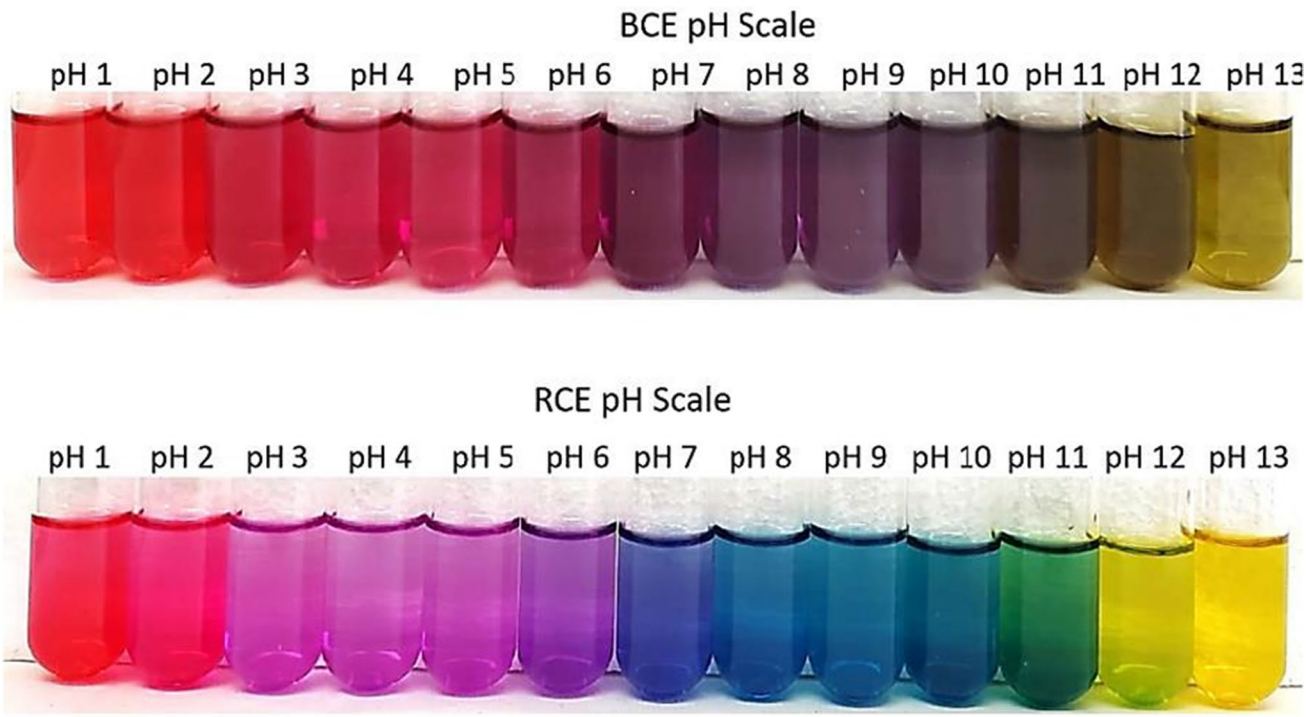

Fig. 1 pH-dependent color changes of BCE solution (above) and RCE solution (above) in between pH 1 and pH 13

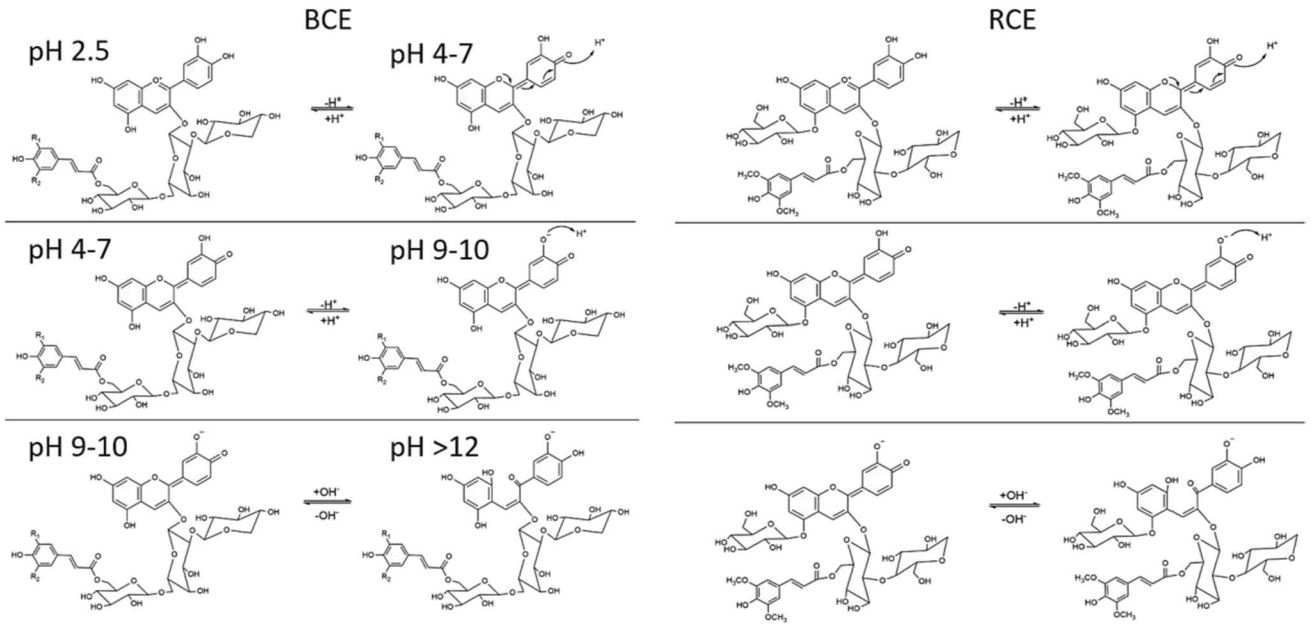

Fig. 2 The deprotonation of anthocyanins in BCE and RCE at different pH values

30 min, which can be an indication of anthocyanin deprotonation (presented in the right column in Fig. 3). The number of produced NH_3 molecules is increased with proportion to the incubation time, and then, the environment becomes more alkaline in which the number of deprotonated anthocyanin molecules increases, and then color change activity is rapidly continued. For instance, after 60-min incubation, the BCE test solution became more alkaline (pH 8–10), its color turned to purple. It is worthy to mention that the BCE solution is quite stable at room temperature (RT:25 °C). We incubated the BCE test solution without *H. pylori* as a control test; no change in initial red color of the BCE solution was observed in any incubation time as presented in the left column in Fig. 3. In addition to that, the BCE test solution was left incubated for several weeks, but its initial color was not changed (data not shown here) which can represent the stability of BCE.

We also run both tests for detection of *Escherichia coli* (*E. coli*) as a urease-negative control bacterium; no color change was observed (data not shown here). We confirmed that the both BCE and RCE test acted as rapid urease tests working on urease activity of urease-positive bacteria. We added model antibiotic (colistin sulfate) into BCE and RCE tests for only detection of *H. pylori* because *E. coli* was not grown owing to antibiotic in all tests, and then no color change was observed (data not shown here).

As a further study, the colorimetric results of the BCE test solutions in Fig. 3 were quantitatively supported by RGB and ΔE analysis. The initial red color of the BCE test solution was used as a reference, and then RGB analysis was performed. A distinct increase in B/R values of the BCE test solution was observed with proportional to incubation time as shown in Fig. 4A. As a complementary study, ΔE values were calculated to differentiate the cutoff point of two colors. As it is well known that the degree of accurate detection of the target can be increased proportional to the large gap in ΔE values. Figure 4B exhibits that the highest ΔE value of the BCE test solution was reached based upon increase in incubation time.

As a comparison study, the RCE-based colorimetric test (including 0.01 M PBS, urea, NaN_3, and 5% w/w RCE prepared at pH 2.5) was used for detection of *H. pylori* (presented in right column in Fig. 5) by following the same protocol used for the BCE colorimetric test. The initial pink color (pH 2.5) of the 1 mL RCE test solution turned to purple (pH 6) in the first 30 min of incubation with the 50 µL of *H. pylori* (2×10^5 CFU/mL) suspension. As it is seen that the RCE test solution became more alkaline based upon incubation time and its color turned to green (pH 11). In terms of the stability of the RCE based colorimetric test solution, it was left for incubation for 105 min as a control experiment without the addition of *H.pylori* suspension, but the initial pink color of the RCE solution did not turn to any color as presented in the left column in Fig. 5. We also witnessed that no color change was seen on RCE test solution even incubated for several months (data not shown here).

As an additional study, color image processing system including RGB and ΔE analysis was also utilized to quantitatively support colorimetric data of the RCE test solutions. Both RGB and ΔE analysis clearly demonstrated distinct

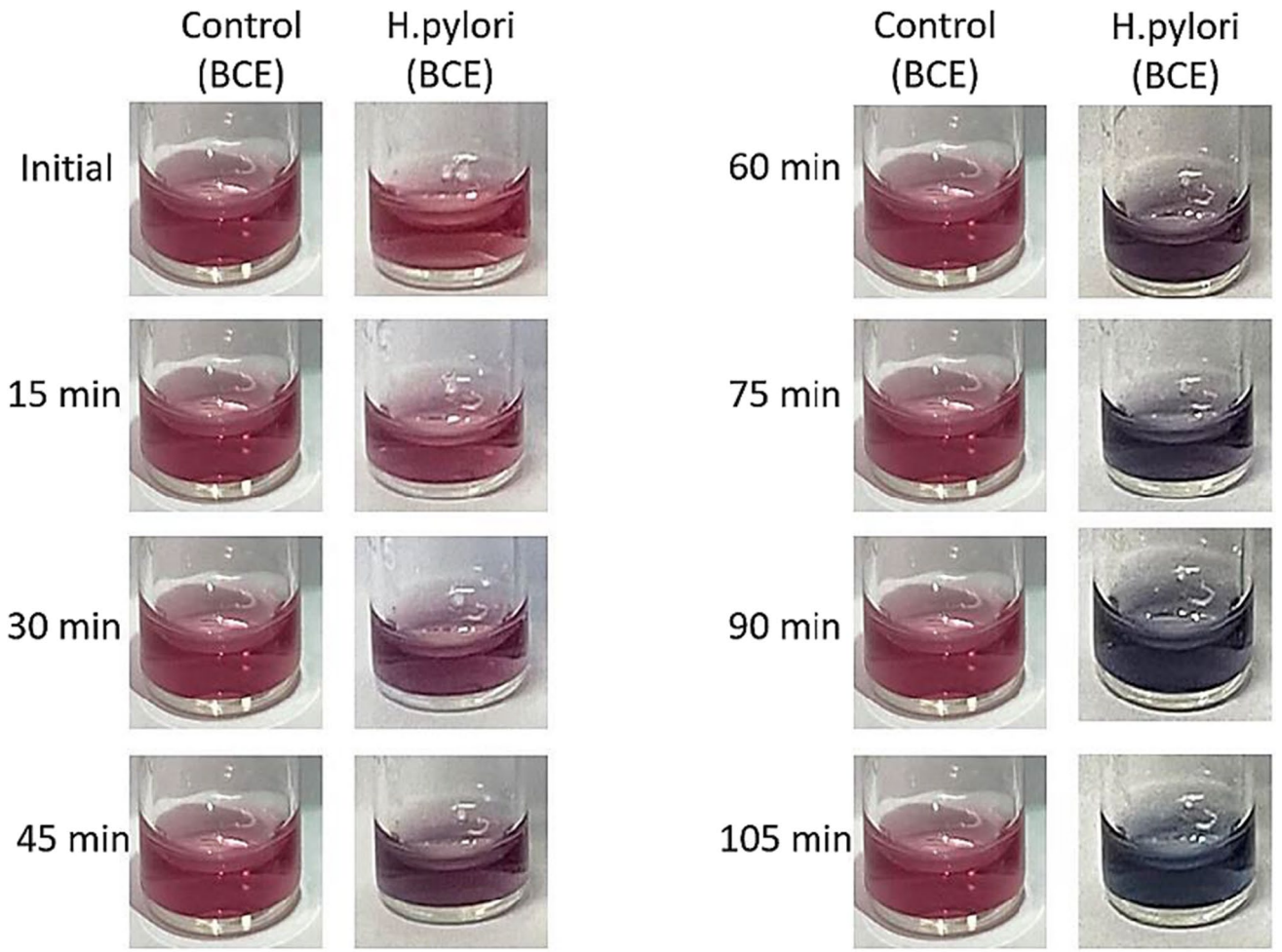

Fig. 3 BCE test solution prepared at pH for time-dependent detection of *H. pylori*. The left and right columns represent the test solutions without and with the addition of *H. pylori*, respectively

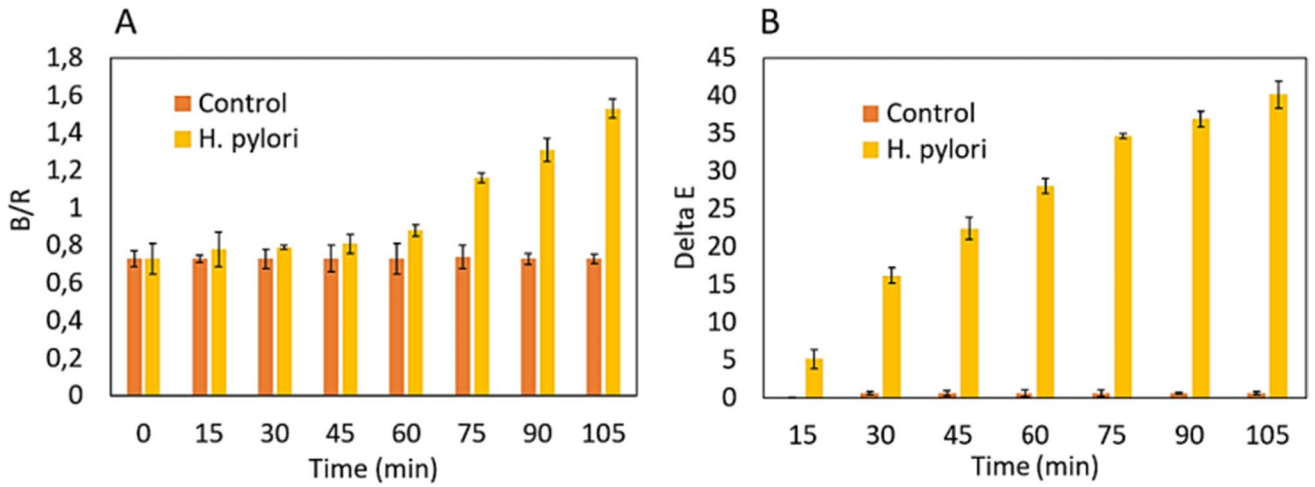

Fig. 4 The digital image processing of the BCE test solution. **A** RGB analysis and **B** ΔE analysis

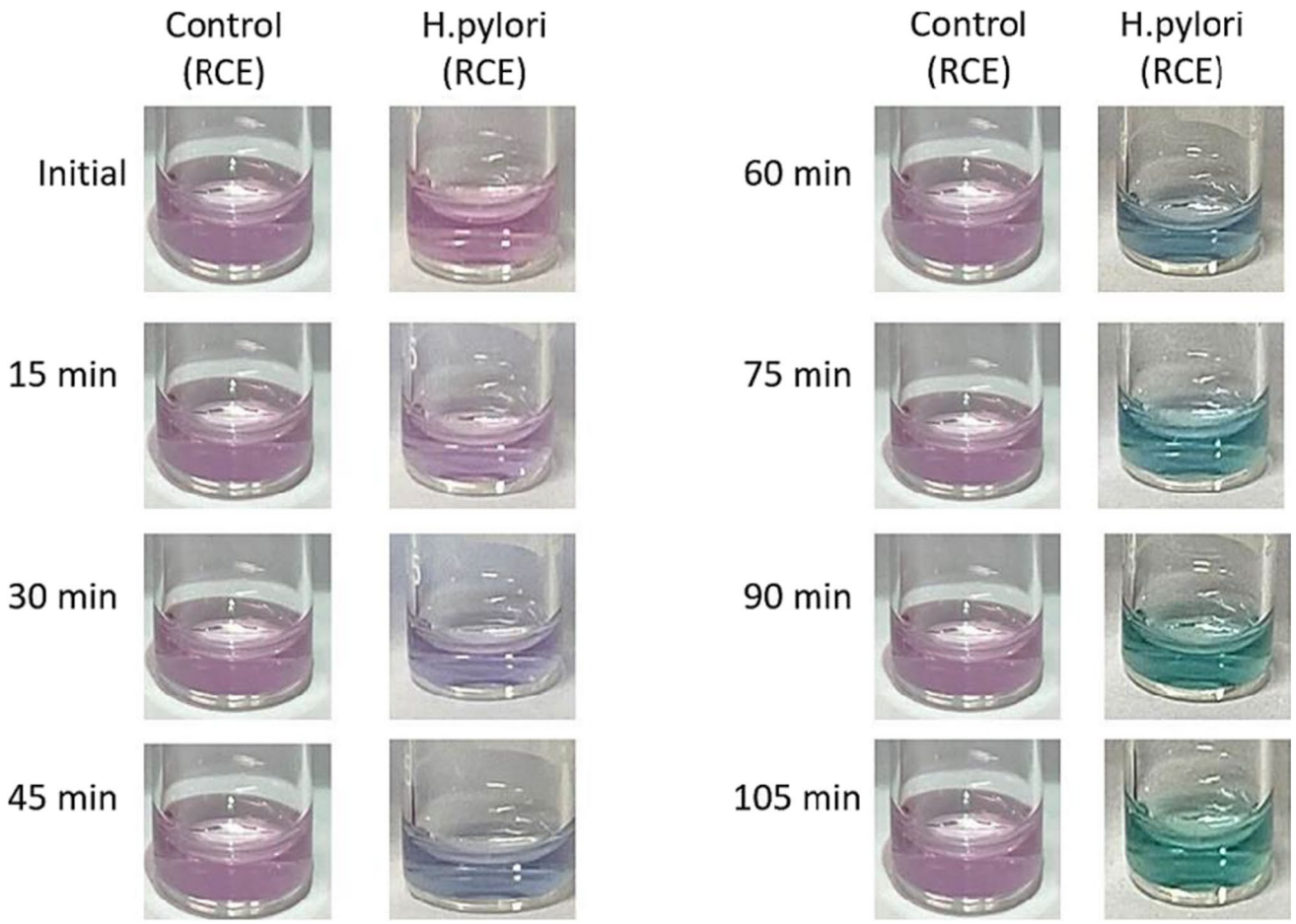

Fig. 5 RCE test solution prepared at pH for time-dependent detection of *H. pylori*. The left and right columns represent the test solutions without and with the addition of *H. pylori*, respectively

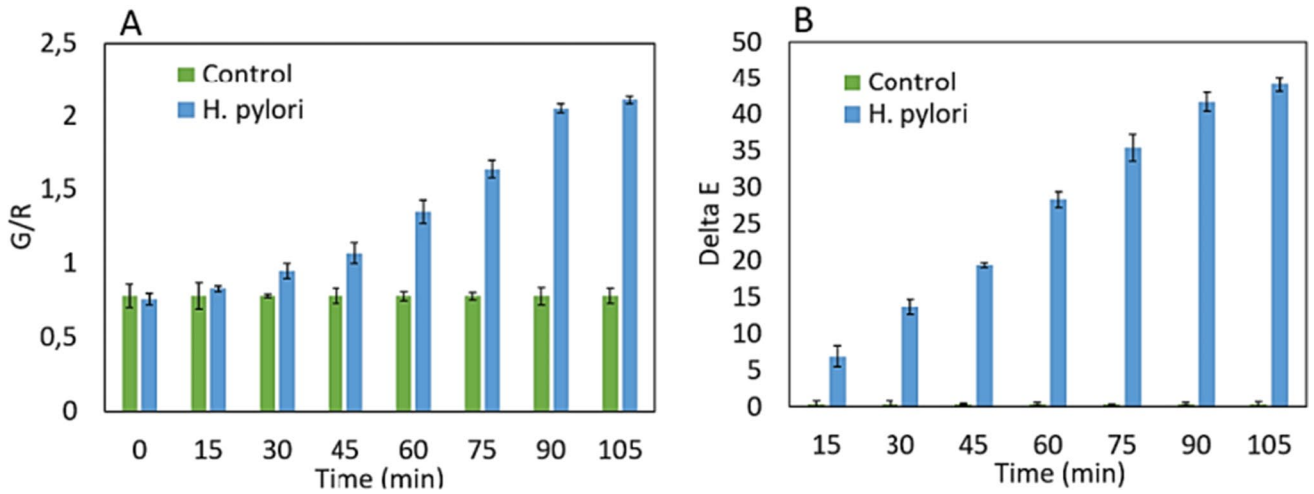

Fig. 6 The digital image processing of the RCE test solution. **A** RGB analysis and **B** ΔE analysis

Commercial Test	Indicator	Detection Time	Reference
CLO test	Phenol red	≥4-24 hour	30, 31, 32
Ploritek	Phenol red	1 hour	32
Hp One	Bromothymol blue	1 hour	30
Hp Fast	Bromothymol blue	≥4-24 hour	31
Pronto Dry	Phenol red	1 hour	30, 31
Anthocyanin test	Anthocyanin	< 1 hour	Current study

Fig. 7 Comparison of anthocyanin test with commercial urease tests

color changes on the RCE test solutions (Fig. 6). The G/R values of the RCE test substantially increased with elongation of incubation time (Fig. 6A). Increase in the G/R values of the RCE test is well consistent with increase in ΔE values (Fig. 6B). We state that large differences in ΔE values may provide accurate detection.

We provided a comparison table with other commercial urease tests in Fig. 7. It is demonstrated that anthocyanin-based tests are quite rapid compared to all other commercial urease tests [38–40]. In addition to that, anthocyanin molecules acting as pH indicators are biocompatible compared to bromothymol blue and phenol red indicators used in commercial urease tests [41]. While no *H.pylori* concentration was reported in Ploritek, Hp one, Hp fast, and Pronto Dry used as commercial urease tests, but CLO test works with 10^4 CLF/mL within ≥ 4–24 h. Initial concentration of *H. pylori* was used as 2×10^5 CFU/mL in this current study and in previous work as well. Although the BCE and RCE tests showed great performance (data not shown here) in the presence of 10^4 CLF/mL as used in CLO test, 2×10^5 CFU/mL concentration of *H. pylori* was selected as an initial concentration because this concentration is meaningful and acceptable in clinics and gave a clear color change in the tests.

We also demonstrated how pathophysiological environment influences the detection performance of BCE and RCE tests by preparing 30 negative (without *H. pylori*) and 30 positive (containing *H. pylori*) samples in an artificial gastric fluid. Firstly, the bacterial suspensions were prepared with a dilution series from 10^{-1} to 10^{-8} CFU/mL. While the distinct color change for RCE test was obtained in the presence of 10^3 CFU/mL *H. pylori* suspension in 60 min, 10^4 CFU/mL *H. pylori* suspensions induced clear color change in BCE test in 75 min. After 5-h incubation and more, the limit of detection was reached to 10 CFU/mL for both RCE- and BCE-based tests. We drew the fitted curve for sensitivity and specificity, the regression equations were $Y=-0.489X+0.514$ ($R^2=0.987$) and $Y=-0.845X+0.816$ ($R^2=0.918$) for RCE (Fig. 8A) and BCE (Fig. 8A), respectively. Each study was performed in 3 repetitions.

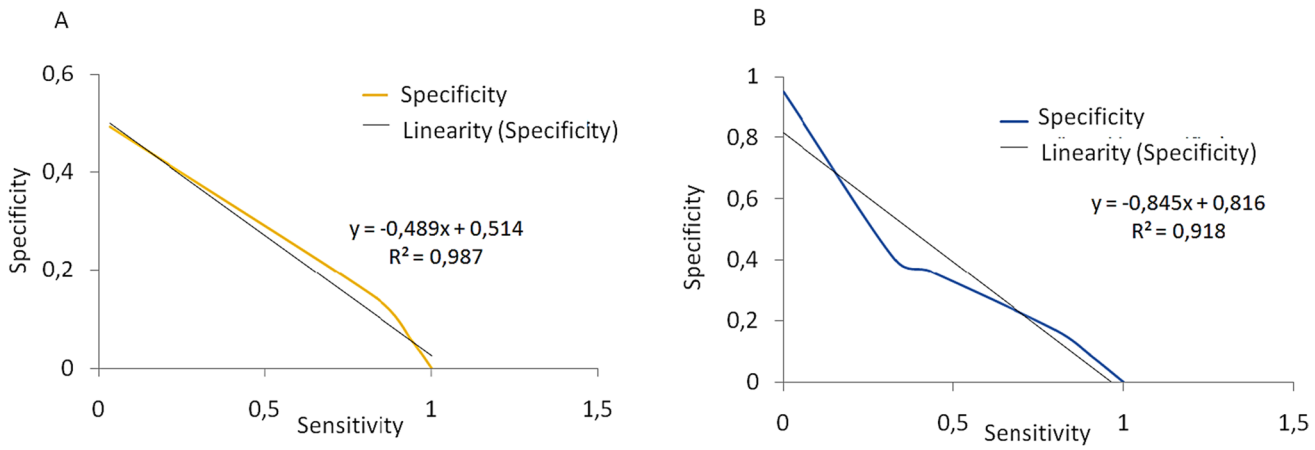

Fig. 8 Fitted curve for sensitivity and specificity for **A** RCE test and **B** BCE test

We claim that pH-dependent color activities of the BCE test solution and RCE test solution are quite similar to each other. We also demonstrated that the BCE can be prepared as a colorimetric biosensor for detection of model pathogens, an alternative to the RCE owing to its proper anthocyanin source. However, slight differences in structure of anthocyanin in RCE enable it to show more variety of color based upon pHs of reaction environments. Interestingly, although pomegranate extracts (*Punica granatum*) contain anthocyanin as major compounds, no color change on pomegranate extract solution was observed at any pHs including strong acidic and basic conditions. It can be attributed to structural properties of anthocyanin molecules and differences in their protonation and deprotonation.

Conclusion

We conclude that both the BCE test and RCE test solutions were successfully used for colorimetric detection of *H. pylori*. We prepared the BCE and CE test solutions at pH 2.5 as an acidic condition to start initial color of the test red or pink and to provide distinct and rapid color change. We hypothesize that the anthocyanins in the BCE and RCE test solutions were positively charged at pH 2.5, then they were rapidly deprotonated when reaction media becomes alkaline. Our hypothesis was supported by colorimetric response and color image processing software. For instance, the red color of the BCE test and pink color of the RCE test turned to blue-purple and green, respectively, when incubated with *H. pylori*. In addition to that, quite large differences in B/R, G/R, and ΔE values were observed in this current tests compared to our previous reports in which the colorimetric test solutions were prepared at pH 7 and above. We propose that this BCE-incorporated colorimetric test can be devised and accordingly implemented into clinic for the detection of various pathogens.

Author contribution The project was conveyed and designed by G.C.S. and I.O. G.C.S. run all the experiments as a first author. I.O managed the project as a corresponding author. All authors wrote the manuscript.

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Declarations

Competing interest The authors declare no competing interests.

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