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RATIONAL PREPARATION OF POWDER ANTHOCYANIN INCORPORATED UREASE TEST FOR DETECTION OF *K. PNEUMONIAE* AND *P. MIRABILIS*

K. PNEUMONIAE VE *P. MIRABILIS* TESPİTİ İÇİN TOZ ANTOSİYANİN İÇERİKLİ-ÜREAZ TESTİNİN RASYONEL HAZIRLANMASI

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ABSTRACT

In this present study, we developed *Brassica oleracea* var. Capitata (red cabbage) extract (RCE) powder-incorporated colorimetric media for rapid, selective, sensitive detection of urease-postive bacteria, *Klebsiella pneumoniae* ATCC 10031 and *Proteus mirabilis* ATCC 25933. The major component of RCE is anthocyanin groups which are in charge of pH dependent color change. The ureas test contains certain concetration of anthocyanin, urea prepated in 0.01 M sodium phosphate buffer and sodium azide. The urea in the test was hydrolyzed by urease enzyme released from *K. pneumoniae* and *P.mirabilis* and produce ammonium (NH_3) molecules which sequentially made the test media alkaline, caused deprotonation of anthocyanin and induced the color change from pink to green. Also, *Escherichia coli* ATCC 25922 was used a urease-negative control bacteria. The detection of *K. pneumoniae* and *P. mirabilis* was examined as a function of anthocyanin concentration and incubation time. The anthocyanin was used lyophilized powder form in the urease test to discard the in interference of free proton in liquid form owing to addition of hydrochloric acid (HCl). We also performed the digital image analysis, Delta E (ΔE) for quantitative confirmation of the visual results. It was observed that the results showed that the color change from pink to green in 150 and 210 minutes of incubaiton for *K. pneumoniae* and *P. mirabilis*, respectively. Here in, we successfully prepared anthocyanin-incorporated urease test and perform colorimetric detection of *K. pneumoniae* and *P. mirabilis* with naked eyes and digital image analysis.

Keywords: Anthocyanin, digital image analysis, rapid urease test, urease-postive bacteria

ÖZ

Bu çalışmada, üreaz-pozitif bakteriler olan *Klebsiella pneumoniae* ATCC 10031 ve *Proteus mirabilis* ATCC 25933'ün hızlı, seçici, hassas tespiti için *Brassica oleracea* var. Capitata (kırmızılahana) lliyofilize ekstresi ilize ekstresi (RCE) içeren kolorimetrik ortam geliştirilmiştir. Ana bileşen kırmızı lahana, pH'a bağlı renk değişiminden sorumlu antosiyanin gruplarıdır. Üre testi, 0.01 M sodium fosfat tampon ve sodium azit içinde hazırlanmış belirli antosiyanin, üre konsantrasyonu içerir. Testteki üre, *K. pneumoniae* ve *P. mirabilis*'ten salınan üreaz enzimi ile hidrolize edilir ve sırasıyla test ortamını alkalin yapan, antosiyaninin proton kaybetmesine neden olan ve pembeden yeşile renk değişimini indükleyen amonyum (NH_3) molekülleri üretir. Ayrıca *Escherichia coli* ATCC 25922, üreaz negative control bakterisi olarak kullanıldı. K. *pneumoniae* ve *P. mirabilis*'in tespiti, antosiyanin konsantrasyonu ve inkübasyon süresinin bir fonksiyonu olarak incelenmiştir. Antosiyanin, hidroklorikasit (HCl) ilavesi nedeniyle sıvı formdaki serbest protonun girişimini atmak için üreaz testinde liyofilizetoz formunda kullanıldı. Görsel sonuçların kantitatif teyiti için dijital görüntü analizi olan Delta E (ΔE) ölçümleri ile sağlanmıştır. Sonuçlar, *K. pneumoniae* ve *P. mirabilis* için sırasıyla 150 ve 210 dakika inkübasyonda pembeden yeşile renk değişiminin gözlendiğini göstermektedir. Burada, antosiyanin içeren üreaz testini başarıyla hazırlayarak sonuçların çıplak göz ve dijital görüntü analizi ile *K. pneumoniae* ve *P. mirabilis*'in kolorimetrik tespitini gerçekleştirdik.

Anahtar kelimeler: Antosiyanin, dijital görüntü analizi, hızlı üreaz testi, üreaz pozitif bakteri

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INTRODUCTION

Urinary tract infections (UTIs), as frequently encountered in daily practice in the clinic, are the most common infection in the world. Although this infection is seen at a rate of 40-60% in women, it is 30 times more common in men. Normal urine pH is 5.8, and there is no erythrocyte, leukocyte and bilirubin in biochemical evaluation (1). Increased urination frequency and biochemical changes indicate problems with the kidney, ureter, urethra, bladder and genital organs. The most likely cause of symptomatic conditions is *Escherichia coli, Klebsiella spp., Proteus spp., Pseudomonas spp., Enterococcus faecalis, Staphylococcus aureus, Citrobacter species, Morganella spp., Providencia spp., Serratia spp., Mycoplasma spp*., etc. Bacteria such as *Escherichia coli, Klebsiella spp., Proteus spp.* can settle in the urinary tract (2).

Diagnosis must not only be accurate but also fast, and early diagnosis is an important step in the control of UTIs caused by urogenital pathogens. In a typical diagnostic method, middle urine is collected from the first administration in the morning in a sterilized container, and then biochemical and microbiological examinations are performed (3). Urine culture is performed using selective media such as Mac Conkey Agar and Cysteine Lactose Electrolyte Deficiency (CLED) agar. After seeding on these media, the inoculated plates are kept at 37^0C for 18–24 hours (hrs) and the number and characteristics of the breeding colonies are evaluated. The Dip Slide Method is a modified or rapid version of the culture method. There is no need to use the Mac Conkey or CLED medium as Hi Media, Oxoid etc. provided by different manufacturers (1,4). The Nitrite Test can be also used to detect bacteriuria and uropathogens (5).

K. pneumoniae and *P. mirabilis* are urease positive microorganisms. If ure as enzyme secreted by the microorganism is present in the environment, ammonia is released as a result of hydrolysis of urea in the environment, and pH changes occur. Here in, we developed powder anthocyanin-based urease test for colorimetric detection of *K. pneumoniae* and *P. mirabilis*. Anthocyanins obtained from red cabbage (*Brassica oleracea* var. Capitata) arethe main components of this plant and can act as pH sensitive indicators in the extract medium (6). The pH-dependent color changing function of anthocyanins is utilized in different areas (7,8).

The color image processing has been employed for providing quantitative study. The test media color has been captured, then the differences in Delta-E (ΔE) values were presented.

MATERIALS and METHODS

Materials and Instrumentations

Tryptic soy agar (Merck, Germany), skimmed milk medium (Difco, USA), agar (Merck, Germany), Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$, Merck, Germany), Anhydrous sodium phosphate dibasic (Na_2HPO_4, Merck, Germany), Sodium azide (NaN_3, Merck, Germany), and urea (Merck, Germany) were all purchased from the companies indicated. Microorganisms: *E. coli* ATCC 25922, *K. pneumoniae* ATCC 10031, *P. mirabilis* ATCC 25933 were obtained from Erciyes University, Faculty of Pharmacy, and the Pharmaceutical Microbiology Research Laboratory ATCC culture collection. Bacterial strains were stored in skim milk medium at -20°C and regenerated prior to the experiments. The optical density of all bacterial suspensions was determined by spectrophotometer (AzureAo, Azure Biosystems, Inc.).

Brassica oleracea var. Capitata (Red Cabbage) *Extract Preparation*

Red cabbage is purchased from local markets for extraction. The leaves are sliced and added in distilled water 3:2 by weight/volume. The mixture is boiled in the hot plate for 10 minutes. The slices are removed and the aqueous solution filtered through a funnel and filter paper and stored at -20 ° C until use. It is taken from refrigerator 24 hours before use and stored at 4 ° C in portions.

Bacterial Strains and Culture Conditions

Bacterial strains were grown at 37°C in tryptic soy agar for about 18-24 hours incubation. All strains were suspended in 3 mL saline at different turbidities.

Preparation of Anthocyanins-Based Colorimetric Test

The test solution is prepared as a buffer medium and 0.01 M sodium phosphate buffer is sterilized in autoclave at 121 ° C for 15 minutes. Urea and sodium azide (NaN_3) filtered through a membrane filter with 0.45 µm pore diameter are added to both buffers at 2 % and 1 mM, respectively. Anthocyanin powder extract is added as 5% -25% -50% in the test solution as a pH indicator. The prepared test solution was dark pink in color.

Digital Image Processing

For digital image processing, samples which contain anthocyanin-based colorimetric test were placed ona piece of white paper. The colorimetric test photos were taken using a mobile phone camera. All the photos of the sample were analyzed by Image J software to obtain quantitative result. Red Green Blue mean values of indicated areas were calculated in Image J. Delta-E (DE) value was calculated according to CIE Lab Formula based on the difference between the colors red, green, and blue (9).

RESULTS

Herein, we utilized red cabbage extract (RCE) as indicator for colorimetric detection of model pathogens *K. pneumoniae* and *P. mirabilis*. Basically, the major components or pigments in RCE are anthocyanins groups, which is in charge of color change based upon the pH of the reaction environment. The anthocyanins have great advantages over synthetic indicators. For instance 1) they can easily be preprared without complex experimetal procedures, 2) they are biocompatible, 3) they are quite senstive for pH-dependent color change and 4) they give a variety of colors (pink, purple, blue, green, andyellow) based upon pH value of reaction media. Figure I shows that anthocyanins give pink color at pH 2 owing to their protonation. However, when they are deprotonated, the color is shifted to purple between at pH 4-7, to blue at pH 8 (Figure I). The further deprotonation of anthocyanins increases their negative charges, the color of reaction media becomes green at pH 9-10 and yellow over pH 12, respectively.

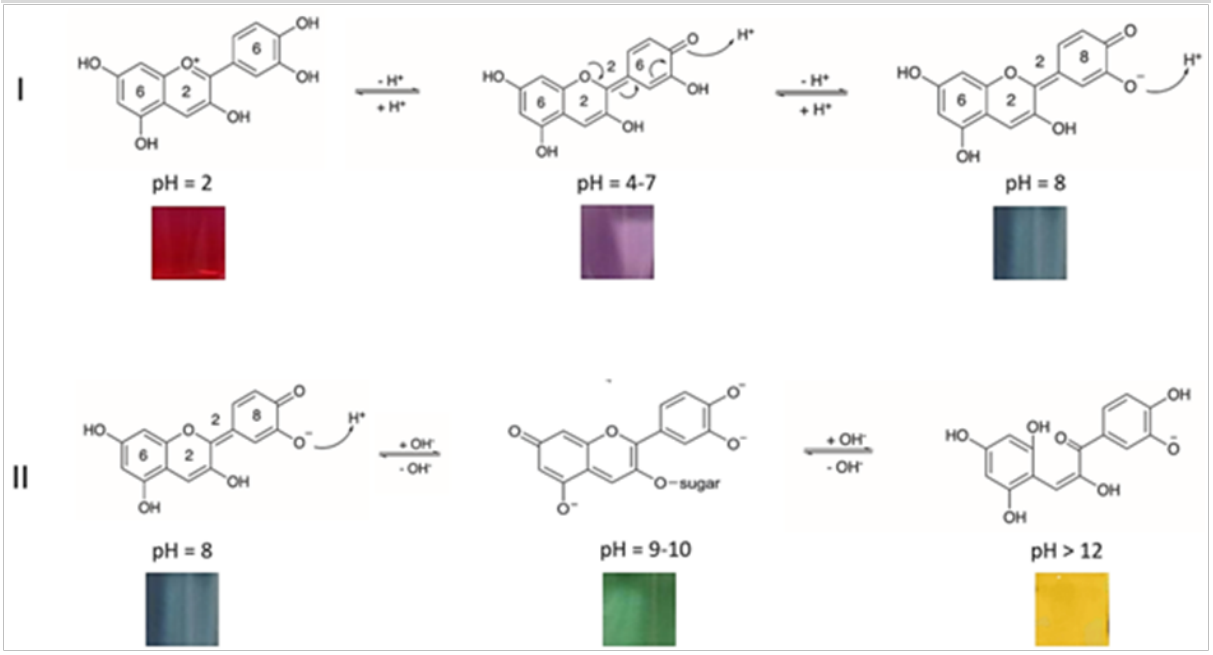

Figure I. Proposed mechanism to show pH dependent electronic structure changes and changes on color of anthocyanin solution

The working principle of the anthocyanin incorporated-urease test relies on secretion of urease enzyme by *K. pneumoniae* and *P.mirabilis* and on hydrolysis of urea, which releases ammoniac (NH_3). The NH_3 playeda major role to make the reaction media alkaline by increasing pH values, and then color change is observed accordingly. In is worth mentioning that pH value and color of RCE is 7 and purple, respectively. However, in order to chage the color of RCE solution to pink the RCE is acidified bythe addition of hydrochloricacid (HCl). This acidified RCE solution at pH2 including urea used as a test media reacted with *K. pneumoniae* and *P. mirabilis,* as ureasepostive bacteria, in the action media. Although urea in reaction media was hydrolzed by urease enzyme secreted by these bacteria, and NH_3 molecules were produced, interestingly no color change was observed on test media. We rationally hypothesize that addition of HCl not only protonated anthocyanin molecules in RCE but also caused excess amount of free H^+ ions in RCE media. And then, produced NH_3 molecules in the test media in the presence of *K. pneumoniae* and *P. mirabilis* reacted with H^+ ions instead of anthocyanin molecules for their deprotonation and color change.

To address this issue, acidified pink color RCE solution at pH 2 was lyophilized and pink color RCE powder was obtained. In the lyophilization process, free H^+ ions in RCE solution was successfully removed with water. Additionally, lyophilization known as freeze drying relies on low temperature dehydration process, which preserved anthocyanins from any damage or perish. To prove that, the lyophilized pink color RCE powder was dispersed in water and ideal pick color solution was seen.

We systematically prepared pink color RCE powder–incorporated urease tests and used them as a function of RCE concentration and reaction time for colorimetric detection of *K. pneumoniae* and *P. mirabilis*. In Figure II, *K. pneumoniae* and *P. mirabilis* were colorimerrically detected with naked eyes (Figure IIA) and quantitatively detected with Delta-E (ΔE) analysis (Figure IIB) by using RCE powder–based urease containing 5% RCE powder. Essentially, 100 µL (0.5 McFarland) of each bacterial suspension (*E.coli, K. pneumoniae* and *P. mirabilis*) was separately added into RCE-urease test, andthen each reaction mixture was left for incubation. The results show that the color change from pink to green was observed at 150 and 210 minutes (min) of incubaiton for *K. pneumoniae* and *P. mirabilis*, respectively (Figure IIA). By way of contrast, no color change occurred in the presence and growth of *E. coli*. We interpreted that although all the bacterial strains were grown in reaction to test media, the gowth of *K. pneumoniae* and *P. mirabilis* resulted in color change due to their urease enzyme secretion properties. In order to provide quantitative results, the color change was analyzed with process imaging system. Figure IIB presents that the remarkable differences in ΔE analysis can bethe indication of precise *K. pneumonia* and *P. mirabilis* detection compared to ΔE analysis of *E.coli* used as a control one.

In order to demonstrate the effect of anthocyanin concentration in this urease test, 25% anthocyanin powder was used. This experimental protocol of Figure IIA was applied in Figure III. A certain amount of *E. coli, K. pneumoniae* and *P. mirabilis* bacterial suspensions were separately added into this urease test and incubated till 240 min. However, no color change for each bacterial strain was witnessed. Although *K. pneumoniae* and *P. mirabilis* bacteria released urease enzyme and hydrolysis of urea, and production of NH_3 molecules occurred, the number of deprotonated anthocyanin molecules was not enough for color change of reaction media during the incubation (Figure IIIA). The *E. coli* was again used as urease-negative bacteria for control experiment and no color change was seen as expected (Figure IIIA). In addition, the visual results of Figure IIIA were supported with ΔE analysis as presented in Figure IIIB. No

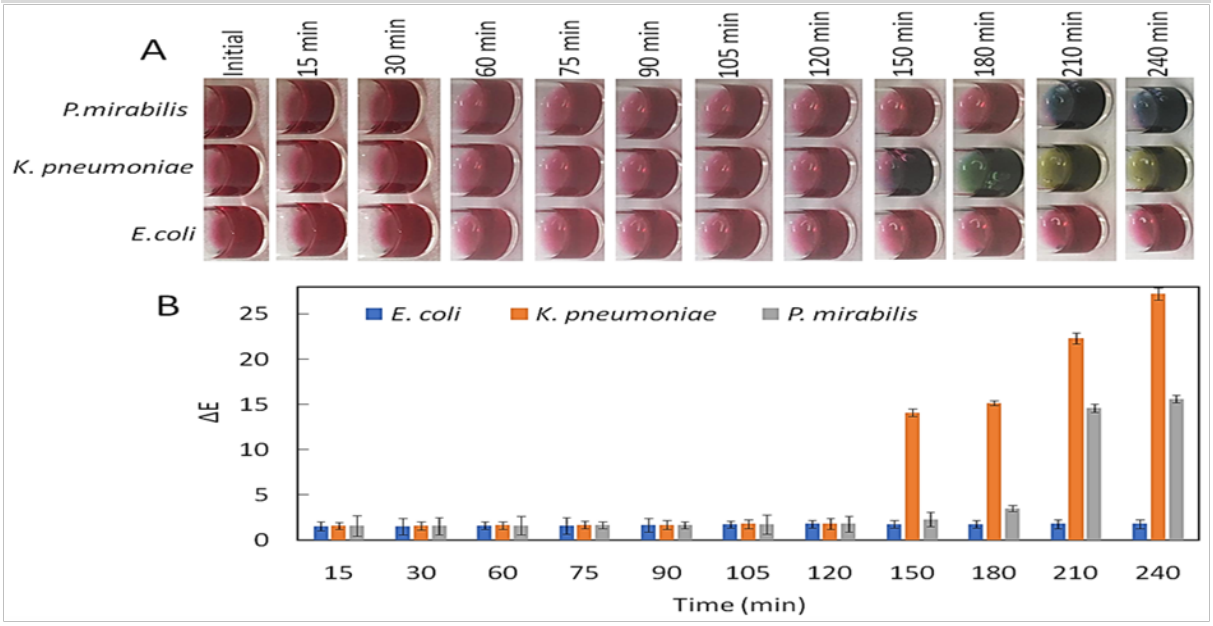

Figure II. Preperation of 5% anthocyaninin corporated-urease test at pH 2 for colorimetric detection of *K. pneumonia* and *P. mirabilis*

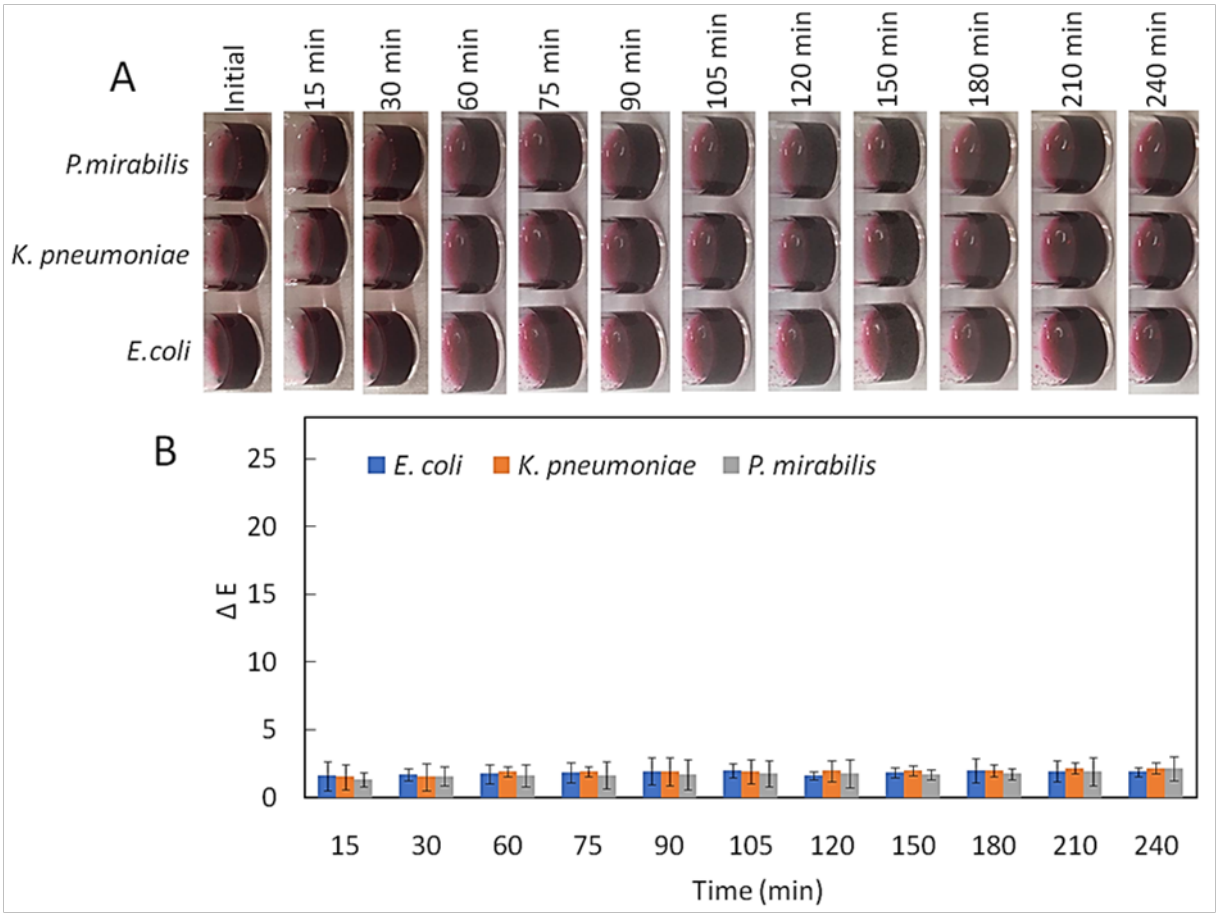

Figure III. Preperation of 25% anthocyanin incorporated-urease test at pH 2 for colorimetric detection of *K. pneumonia* and *P. mirabilis*

difference in ΔE analysis for each test was obtained, which was perfectly consistent with Figure IIIA.

For further study on anthocyanin concentration, the urease test containing 50% anthocyanin powder was used to detect *K. pneumoniae* and *P. mirabilis.* We followed the protocol used in Figure III. The model pathogens were incubated in the urease test but neither *K. pneumoniae* nor *P. mirabilis* resulted in any color change

in test media at any incubation time (Figure IVA). As we hypothesized in Figure III the color change in the test media is dependent upon the number of deprotonated anthocyanin molecules. Accordingly, no color change in the test media was detected with naked eyes and digital image analysis as presented in Figure IVA and IVB, respectively. We claim that higher concentration of *K. pneumoniae* and *P. mirabilis* and/or much higher incubation time may be required to deprotonate more anthocyanin molecules and to see color change of the urease test media contaning 25% and 25% anthocyanin molecules for colorimetric detection of *K. pneumoniae* and *P. mirabilis* with naked eyes and digital image analysis as well.

In terms of digital image analysis,we created an android -based mobile application to show differences in color of the test media (Figure V). We believe that a smart

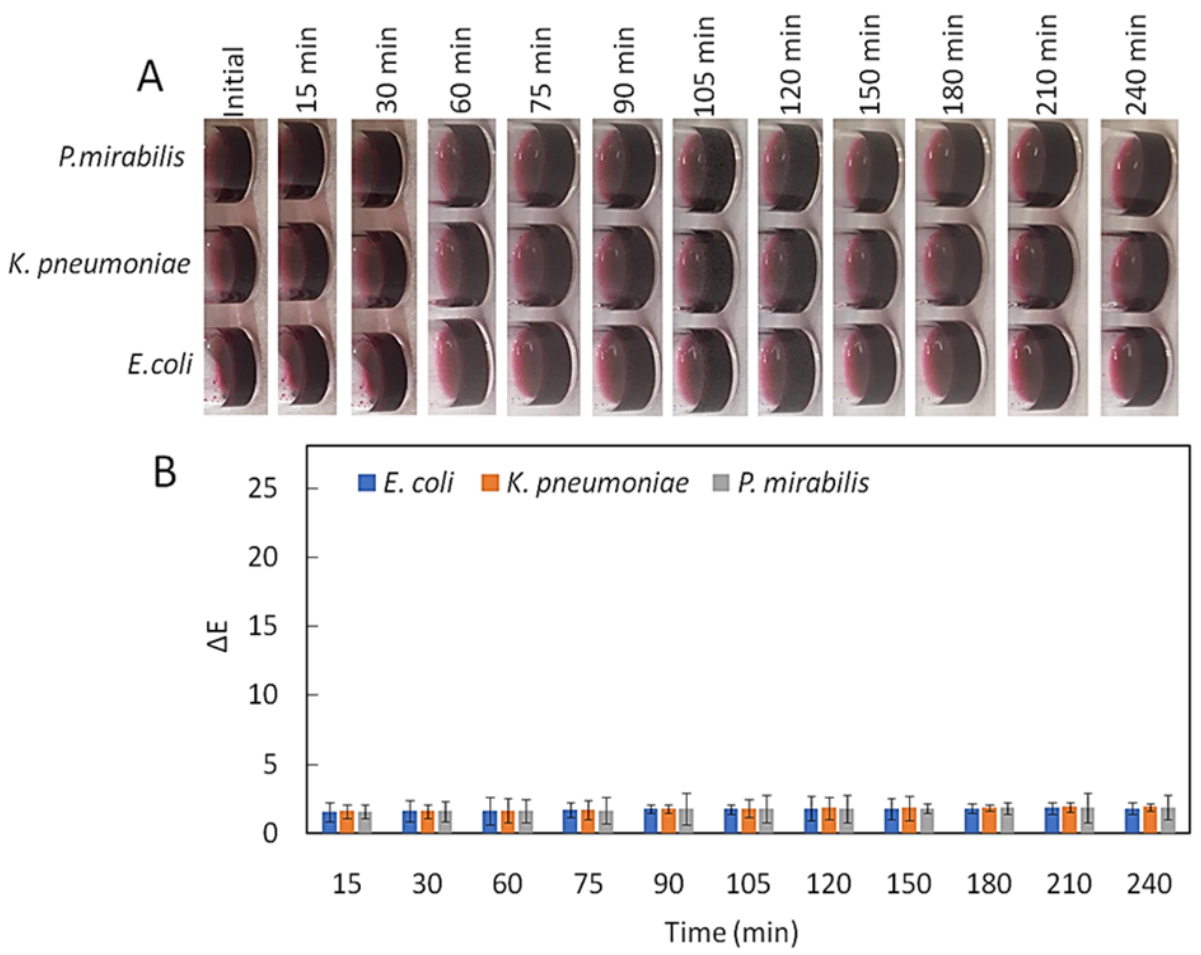

Figure IV. Preperation of 50% anthocyanin incorporated-urease test at pH 2 for colorimetric detection of *K. pneumonia* and *P. mirabilis*

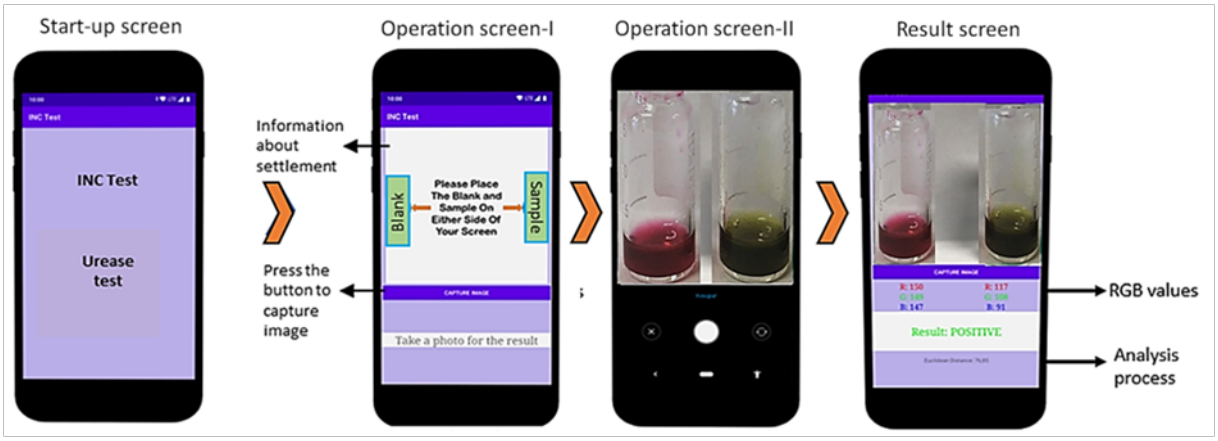

Figure V. Illustration of smart phone-based *K. pneumonie* and *P. mirabilis* detection with main screen, test process, image capture, and test result screen panels.

phone based application may facilitate detection of *K. pneumoniae* and *P. mirabilis* in clinics.

DISCUSSION

UTIs are a serious public health problem, often associated with complications, sequelae, relapse, and poor quality of life. It is known that only the treatment and care of these patients is an economic burden (10-12). It has been shown that the theoretical formation of pathogens causing UTIs is facilitated by prolonged catheterization and immune suppression in nosocomial environments (13, 14). Although the most common causative agents of UTIs are the Enterobacteriaceae family (15), some Gram-positive bacteria such as *Staphylococcus* spp., atypical microorganisms, nonfermentative Gram negative bacteria and *Candida* spp. follow them (10-12,16,17). The presence of urease enzyme, which catalyzes urea hydrolysis to form ammonium ions and carbonic acid, is known as a key virulence factor for many urinary tract pathogens such as *Proteus mirabilis, Staphylococcussaprophyticus*, some plasmid-containing strains of *Escherichia coli* and most commonly *Klebsiella* species (18-21). Biochemical tests and various strip tests are used in routine laboratories to identify these microorganisms. Biotype-100 strip tests result in 2-4 days, and API 20E strip tests in 20–24 hours (22,23). The method described in this study is much faster than other conventional methods. The present study revealed that the anthocyanin-based method could detect *Klebsiella* spp. and *Proteus* spp. from bacteria culture.

Here in, we successfully prepared anthocyanin-incorporated urease test and perform colorimetric detection of *K. pneumoniae* and *P. mirabilis* with naked eyes and digital image analysis. We demonstrated that the urease test containing 5% anthocyanin powder have color change from pink to green at 150 and 240min of incubation for in the presence of *K. pneumoniae* and *P. mirabilis*, respectively. We have demonstrated that color change of the urease test is directly attributed to the number of deprotonated anthocyanin molecules. For instance, although *K. pneumoniae* and *P. mirabilis* bacteria were in the test media, no color change was observed with the tests contaning 25% and 50% anthocyanin powder due to the absence of enough deprotonated anthocyanin molecules.

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