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Research Article

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Novel Scanometric Assay for Charantin in Bitter Melon (*Momordica charantia*) Extract Based on Immobilized Silver Nitrate and Methylene Blue as Colorimetric Paper

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Abstract

Bitter melon (*Momordica charantia*) contains charantin that responsible for the antidiabetic activity. Therefore, it is important to determine charantin in the extracts for the purposed of its formulation or quality control. Herein, we proposed a novel scanometric assay for charantin determination in the bitter melon extracts based on immobilized silver nitrate and methylene blue as a colorimetric paper. The presence of charantin can be detected simply via the color change from blue to green and can be quantified using a scanner via the ImageJ software. The proposed charantin sensor has a response time of 8 mins with the linear range of 300 to 1000 µg/mL and the detection limit was 216.25 µg/mL. The sensor has good reproducibility (RSD = 2.86%) with the stability up-to 5 months. The sensor was applied to determine charantin in the sample extracts, and the result shown in a good agreement with TLC method.

Keywords

Colorimetric sensor, Charantin, Silver nitrate, Methylene blue, Bitter melon

Introduction

Momordica charantia or bitter melon commonly known as a fruit that is widely reported to have anticancer, antihyperlipidemic, analgesic, anti-inflammatory, antitumor, antiulcer, hepatoprotective, antioxidant activities [1], antidiabetic [2-8]. Charantin is a mixture of steroidal glycoside (1:1), and as an active compound or chemical marker contained in bitter melon that responsible for antidiabetic activity [4, 9]. Since charantin has the capability of lowered blood sugar equivalent to insulin [10]. Therefore, charantin has been used as an active compound or chemical marker for antidiabetic in the herbal medicine that has been formulated from the bitter melon extract [11]. Therefore, it is important to determine the charantin concentration in bitter melon extracts for the purposed of the antidiabetic formulation or quality control of charantin concentration as active compounds in the bitter melon extract used either as pharmaceutical or nutraceutical products purposes.

The conventional methods for determination of charantin are thin layer chromatography (TLC) [12], high performance thin layer chromatography (HPTLC) [13], high performance liquid chromatography (HPLC) [14], gases chromatography tandem mass spectroscopy (GC-MS) [15]. However, these methods are needed sample preparation, long procedure, and skilled personnel, as well as relatively expensive [16]. Certainly, for the big company or industry using conventional standard methods would not be an issue to comply with all

requirements needed, since they have enough resources, setting, and budget. However, for small and medium enterprises (SME), particularly home industry, this issue becomes a big problem for them, as they have a low resource, setting, and budget. Therefore, the development of the low-cost, portable and disposable method is needed for charantin detection in the bitter melon extracts particularly that can be used for SME including home industries. The aim of this study is to develop a simple method and low-cost for determination charantin as alternative compare to conventional standard methods. One alternative to the simple methods and low-cost is a chemical sensor. This is due to the fact that it can be developed as a colorimetric paper for simple strip or spot test by immobilization of suitable reagent solution for charantin onto a filter paper.

The reagents that can be used as an alternative that suitable for charantin detection is silver nitrate ($AgNO_3$) coupled with methylene blue (MB). Since charantin could reduce Ag^+ from $AgNO_3$ into Ag^0 as a silver nanoparticle (AgNPs), where AgNPs act as an electron transfer mediator and donates to MB as a redox catalyst termed as electron relay effect [17] and then reduce MB into leucomethylene blue (LMB) [18]. Based on this reaction, herein for the first time, we developed a novel scanometric assay for charantin in the bitter melon extract based on immobilized $AgNO_3$ coupled with MB as colorimetric paper for simple detection of charantin in the bitter melon extracts. The colorimetric paper was fabricated simply by immobilizing the reagents mixture of $AgNO_3$ and MB on the filter paper via adsorption method. The colorimetric paper results in the determination of charantin in the real sample of bitter melon extracts show a good agreement with the standard method using TLC. Thus, the results show that the proposed sensor can be used as a simple and rapid method for the determination of charantin in the bitter melon extracts.

Materials and Methods

Material

All reagents were used as purchased without further purification. Charantin was obtained from Xi'an Le Sen Biotechnology Co., Ltd (Shaanxi, China), methanol, ethanol chloroform, and hexane were purchased from Merck (Germany), silver nitrate ($AgNO_3$), methylene blue (MB) and filter paper (Whatman cat No 1001 150) were purchased from Sigma Aldrich. Polyacrylic, nitrocellulose, aluminum foil obtained from Indomaret (Indonesia). Fresh *M. charantia* was purchased from Materia Medica (Malang, Indonesia). All chemicals used were of analytical reagent grade without further purification.

Preparation of colorimetric paper

A colorimetric paper as charantin sensor was developed by immobilizing reagent solutions mixture of $AgNO_3$ and MB onto a filter paper (⌀ 0,8 cm). The reagent immobilization was simply by using absorption method. This procedure was carried out by dipping the paper, that already cut into a circular shape (i.d. 5 mm), into 10 ml of the solution mixture overnight (~12 hrs) at ambient temperature with a slow stirrer. Then, the immobilized reagent on the filter paper was dried at room temperature. Afterward, the sensor membrane was attached to white polyacrylic using double transparent tape (3M), to form a strip test for charantin. Then, the colorimetric paper was ready to be used as strip test for charantin detection as presented in figure 1A.

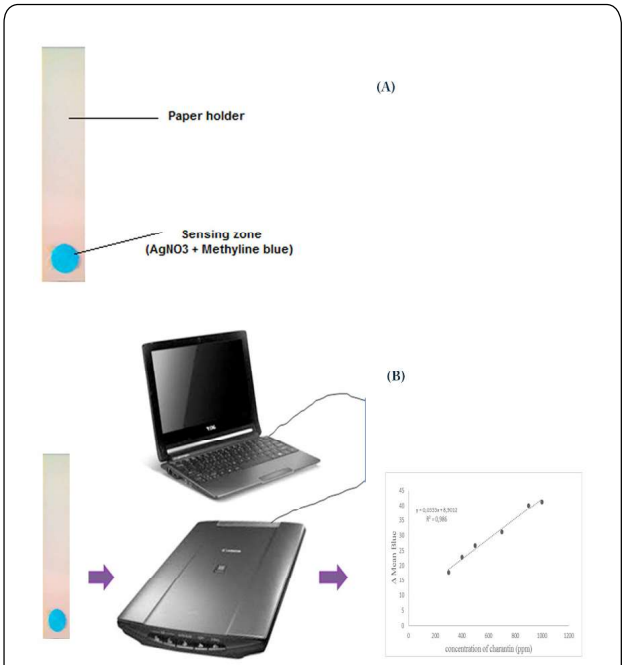

Figure 1: The colorimetric paper based on immobilized $AgNO_3$ and methylene blue as a strip test is ready for use **(A)**, and scanometric assay for charantin determination using the colorimetric paper as strip test **(B)**.

Sample preparation

The sample preparation was performed using previous method [19]. About 2 g sample was placed in conical flask extracted with 10 ml solvent consisting of methanol-water (80:20; %v/v). Then the sample was extracted using ultrasonic (Elmasonic, Germany) at 45 ^{0}C with ultrasonic power at 240 W for 2 hr. Afterword, the extract was filtered, then added with the methanol-water mixture until 10 ml. Then, the sample was ready to be used using the colorimetric paper.

Optimization of colorimetric paper

The reagents concentration of $AgNO_3$ and MB used in the colorimetric paper was according to previous method [18], from which the reagent concentrations were optimized. Here, the $AgNO_3$ solution at concentration of 169 and 1690 µg/mL were used, while MB at concentration of 32 and 1000 µg/mL were used to make four combinations (1) 169:32; (2) 1690:32, (3) 169:1000, and (4) 1690:1000. The volume ratio (v/v) of selected reagents combination then optimized at the different volume ratio i.e. 8:2, 9:1 and 9.8:0.2 (v/v). Then, the sensor response time was also studied at the period from 0 to 20 min. For this study, 1000 µg/mL of charantin was introduced into the sensor membrane. Lastly, the effect of the pH on the sensor response was also optimized between pH 5 to 8.

Scanometric assay

For determination of charantin concentration, the scanometric assay was employed. Firstly, charantin solution was introduced into the sensor membrane, afterword waits at the desired time for the color change of the sensor to occur. Secondly, the color change of the sensors was captured by the flatbed scanner (CanoScan LIDE 110, Japan) at 300 dpi resolution. Finally, the scanned image was analyzed with ImageJ program (https://imagej.nih.gov/ij/). The intensity of the sensor color change is expressed as mean red, green and blue as well as RGB and presented as the intensity value of Δ color unit of the sample subtracted with the blank in order to determine charantin concentration as given in figure 1B. All of the measurements were carried out in triplicate.

Result and Discussion

Sensing mechanism

The charantin detection mechanism is based on the reaction between charantin with $AgNO_3$, which in turn reduced MB. Herein, the reduction of MB is mediated by AgNPs formed as results of $AgNO_3$ reduction by charantin in the bitter melon extract. This is according to Ajitha et al. [18], wherein this scheme, as a result of the decrease in MB absorbance, the increase of SPR peak of AgNPs was observed. In the electron transfer process, when there is a large redox potential difference between acceptor and donor, then there might be the restriction of electrons transfer. However, the electron transfer becomes easy, if an effective catalyst has intermediate redox potential value between acceptor and donor [20, 21]. Therefore, in this case, it reveals that AgNPs act as an electron transfer mediator since it accepts the electrons from extract and donates to MB by acting as a redox catalyst, which in turn termed as electron relay effect [17].

Similarly, in this sensing scheme, the charantin reduces Ag^+ from $AgNO_3$ into Ag^0 as AgNPs, where AgNPs act as an electron transfer mediator and donates to MB as a redox catalyst termed as electron relay effect [17] and reduced MB into leucomethylene blue (LMB) [18] as presented in figure 2A. Based on this reaction scheme due to reduced MB into LMB, hence the sensor color change was from blue to green (Figure 2B). The color change was then analyzed using the ImageJ program for quantitative measurements, and the results show that the color value change or Δ color unit in term of blue gave the best correlation coefficient (r) compared to another color, i.e. red, green and RGB value (Figure 2C). Where Δ color unit presents color of the sensor before and after reaction with charantin at various concentrations. In addition, the color change of the sensor in the Δ blue was found to be the most proportional to the increasing concentration of charantin, followed by ΔRGB. Therefore, the Δ blue value was used for further measurements.

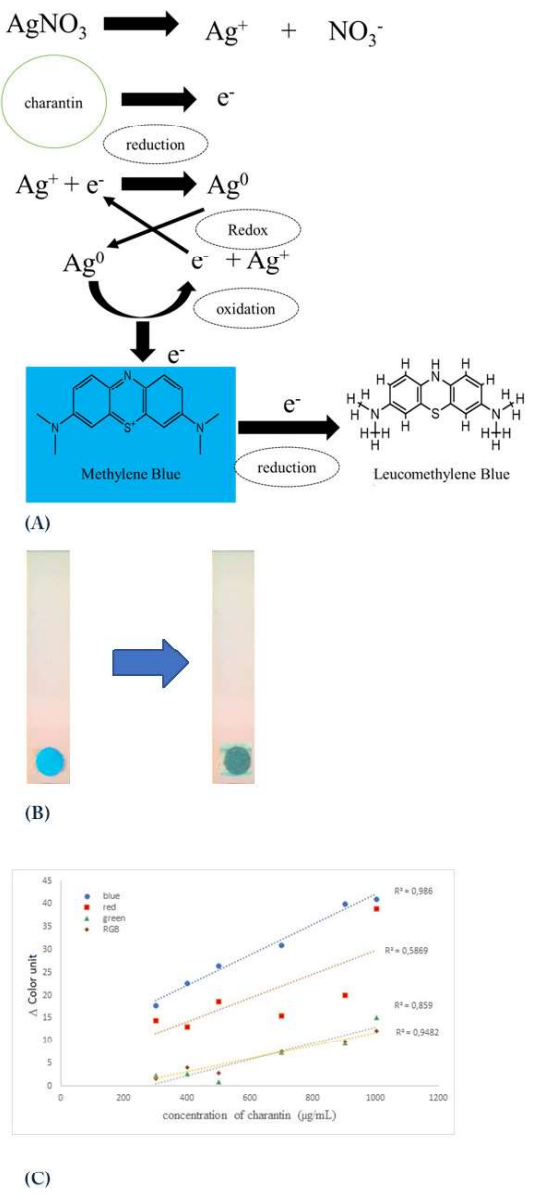

Figure 2: The sensing mechanism of the charantin sensor based on $AgNO_3$ and methylene blue, where Ag^0 (AgNPs) act as an electron transfer mediator, since it accepts the electrons from extract and donates to methylene blue by acting as a redox catalyst **(A)**; the color change of the colorimetric sensor, the sensor before reaction; and after interaction with charantin **(B)**; and the sensor response in the range of 300–1000 µg/mL charantin, where the Δmean blue show the best correlation coefficient (r = 0.993) **(C)**. Here, the Δ colour unit present color of the sensor before and after reaction with charantin at various concentrations. The experiments were carried out in triplicate.

Optimization of experimental parameters

Since the color change of the sensor response was from blue to green, when it tested with charantin (1000 µg/mL). In addition, the adsorption method of immobilization was employed in this case. Then, the optimizations of $AgNO_3$ and MB concentrations for each of them in the mixture were needed to be performed as well as their volume ratio, as these value could be affecting the sensor response. In the case of concentration, the optimum concentration was found at 1690 µg/mL and 1000 µg/mL for $AgNO_3$ and MB respectively as it gave optimum of the sensor response as given in figure 3A.

Furthermore, the optimization of volume ratio for $AgNO_3$ and MB in the mixture was found at the volume ratio of 9.8:0.2 (v/v) for $AgNO_3$ and MB respectively as this volume ratio gave optimum of the sensor response as shown in figure 3B.

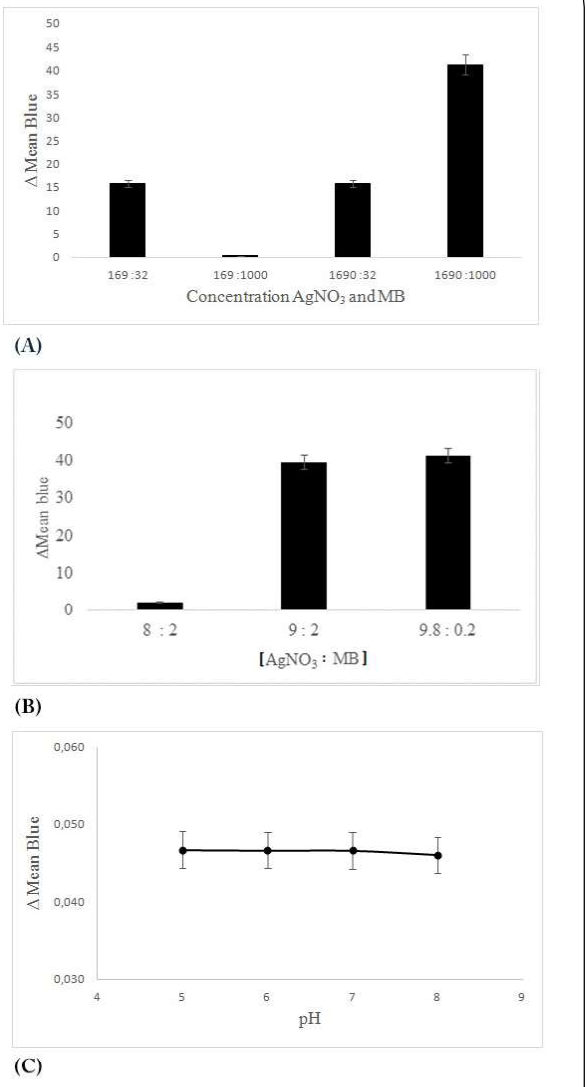

Figure 3: The colour intensity change with the combination of concentration between $AgNO_3$ and MB on the colorimetric paper **(A)**; the colour intensity change with the different volume ratio (v/v) of $AgNO_3$ and MB on the colorimetric paper **(B)**; and the effect of pH buffer on the color change of the charantin sensor between pH 5 to 8 **(C)**. All of the experiments were carried out in triplicate.

Beside the above parameters that would affect the charantin sensor prepared, another parameter that might affect the sensor response is pH. Here, the pH 5-7 was tested using pH buffer solution. In the case of pH, the charantin sensor response was not affected by the pH (5-7) as shown in figure 3C. Here, the pH 5 is similar to the pH of the charantin extract. Therefore, the sample tested no need to be conditioned with the pH buffer is usually used in many assays and simply can be used to determine the charantin concentration directly from the extract. This due to the fact that in the green synthesis of AgNPs, the pH buffer is not required [17, 18]. Thus, for further experiment, the sample extract was used directly without any addition of buffer or other reagent and solution. In addition, this feature would add to the advantages of the proposed sensor, since make the sensor applications simpler and more practical particularly when it applied in the field or for small and home industries.

Analytical characteristics

The response time of the sensor was observed within 20 minutes, where it gave a stable response toward charantin (Figure 4A). Here, all the time response selected (3, 5, 8 and 10 min) were give a good response as it gives correlation coefficient (R) close to 1. However, the response time of the sensor at 8 min was found to be optimum, where it gives the best linear sensor response towards charantin concentration as given in figure 4B. Based on this response time, the calibration curve was constructed and the sensor was found to have a linear concentration range in 300 to 1000 µg/mL of charantin (Figure 4C), with coefficient correlation (r) was 0.993. The detection limit of the sensor response was

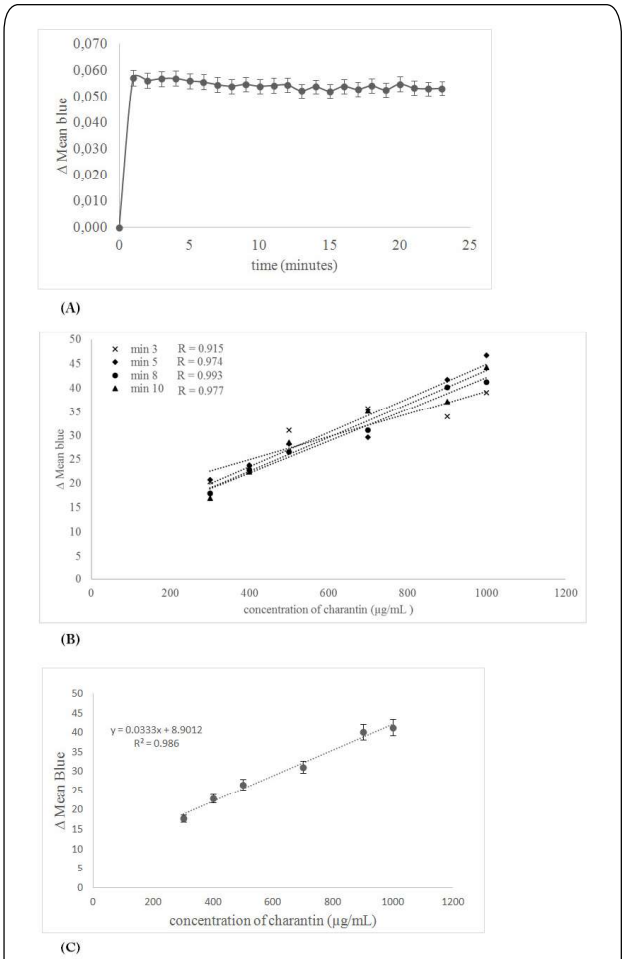

Figure 4: The time response of the charantin sensor within 20 min **(A)**; the response time of the sensor at various time at 3, 5, 8 and 10 min, where 8 min was give the best linear correlation for the charantin sensor **(B)**; and the calibration plot of the sensors response vs. charantin concentration **(C)**.

216.25 µg/mL of charantin, and the quantification limit of the sensor was 648.76 µg/mL of charantin. This limit of detection and the linear range are suitable to be used for the quality control of charantin in the bitter extracts, since the in-house herbal formulation was found to contain 0.361 ± 0.014 of charantin (% w/w dry weight) or equal to 361 µg/mL, while in the charantin content in *M. charantia* fruit extract was 1.053 ± 0.032 (% w/w dry weight) or equal to 1053 µg/mL [22] In addition, the maximum yield of charantin on the dry weight basis of bitter melon was 3.18 mg/g or equal to 3180 mg/L. [1]. Thus, the proposed charantin sensor has a low limit of detection for quality control of charantin either in the herbal formulation or the dry bitter melon extracts.

In order to demonstrate the selectivity of the sensor, a selectivity study on the potential effect of other active compounds in the bitter melon extracts that may interfere the sensor response was studied, such as quercetin. The concentration ratio of 1:1 between charantin and quercetin were used (500 µg/mL). The interfering signal, calculated by subtracting the color intensity change of interfering substances added-sample with that of the original sample, and divided by color intensity change of original sample. The result shows that quercetin interferes charantin with the interference value of 43.769%. This is due to the fact that quercetin also reduces $AgNO_3$ to become AgNPs [23, 24] Therefore, the sample preparation has to be separate quercetin from the extract. Here, the quercetin could be separate from the extract by degradation of quercetin via ultrasonication of the extract, so that its reduction activity will be reduced [25]. Thus, the sample could be prepared by ultrasonication during 2 hrs at 45 ^{0}C, and then extracted with the methanol-water mixture (80:20% v/v). After this degradation step, the quercetin interference was less than 5%, and the other hand interfering signal from pharmaceutical excipient used sucrose, nipagin, starch respectively as show in figure 5. The interference of pharmaceutical excipient less than 5% and this value was acceptable for this type of assay [26].

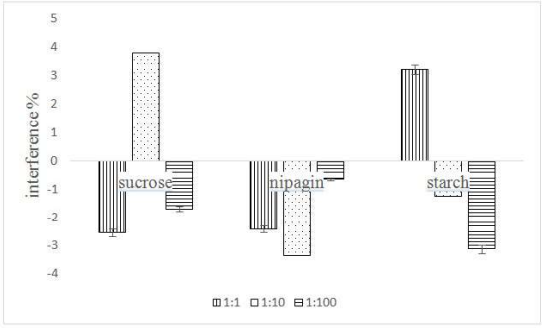

Figure 5: The possible interferences arised from pharmaceutical excipients.

In term of the reproducibility using six sample at the concentration 700 µg/mL, the sensor shows good reproducibility with the RSD value was calculated to be 2.868%. This reproducibility value was indicated that the sensor has the good precision for the charantin determination [26]. The accuracy of the sensor was performed using standard addition method, where the recovery values (%) for the accuracy of the charantin sensor was calculated to be 100.605%. Thus, this value complies with the recovery values within 0.1% of sample concentration tested (95-105%) [26]. Therefore, this value indicated that this method has a good accuracy in determination of charantin concentration.

Stability

The stability study of the sensor response was carried out at the room temperature (25 ^{0}C). The sensor response was evaluated every day until 10% of the sensor response decreased from the initial response value. The result shows that the sensor was very stable at room temperature (25 ^{0}C) up to 5 months, since afterword, the sensor response was observed to be decreased more than 10% at room temperature.

Application of the real samples

In order to demonstrate the applicability of the proposed sensor in the determination of charantin in the real bitter melon extracts. The sensor was applied for the detection of charantin in the real sample extracts. The content of charantin in the bitter melon extracts was calculated based on the calibration curve constructed above. The results of the proposed charantin sensor were also compared with the result determined using the standard method, i.e. TLC – densitometry method [27] and the results are summarized in table 1. Based on these two results are found to be no difference between the two methods, since the calculated p-value were more than $\alpha = 0.05$. Thus, it can be stated that the proposed charantin sensor is in good agreement with the standard method (TLC), and can be suggested that the proposed charantin sensor can be used as an alternative method for the charantin determination in the bitter melon extract samples.

Table 1: The results of charantin determination in the bitter melon extract samples using the proposed charantin sensor and TLC-densitometric method ($n = 3$, $\alpha = 0.05$).

Sample	Charantin Sensor	TLC - Densitometer	P(T ≤ t) two-tail
A	0.286 ± 0.010	0.288 ± 0.0035	0.723
B	0.241 ± 0.006	0.232 ± 0.0035	0.135
C	0.313 ± 0.010	0.314 ± 0.0007	0.869

Conclusion

The novel scanometric assay based on a colorimetric paper of immobilized reagent mixture of $AgNO_3$ and MB has been developed for measuring charantin in the bitter melon extract. Here, the charantin sensor has a linear response at 300-700 µg/mL with the limit of detection at 216.25 µg/mL and the results were found to be reproducible. The proposed method is simple, easy to operate, and low-cost, since it is fabricated as a colorimetric paper in the form of the strip test, making it as a simple and rapid method for determination charantin in the bitter melon extracts.

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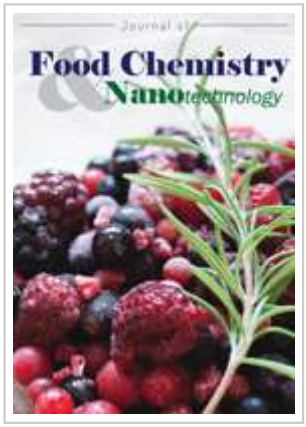

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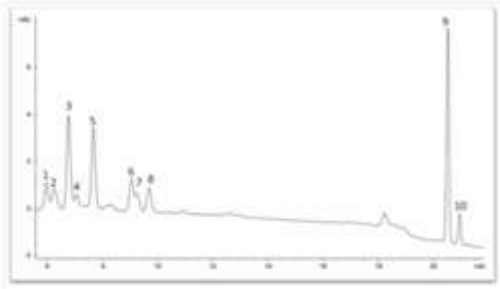

JANUARY 3, 2020

Foam Mat Drying of Tommy Atkins Mango: Effects of Air Temperature and Concentrations of Soy Lecithin and Carboxymethylcellulose on Carotenoid Compounds and Colorimetric Parameters

Francine Albernaz Teixeira Fonseca Lobo, Josiane Domingues, Deborah Falcão, Carla Stinco, Francisco Rodríguez-Pulido, Carlos Eduardo Faria, Francisco Heredia, Katia Gomes de Lima Araujo and Dolores Vila

Mango is an important tropical fruit and a source of bioactive compounds. In this work foam mat drying was used for Tommy Atkins mango pulp.

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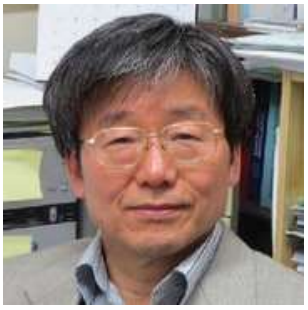

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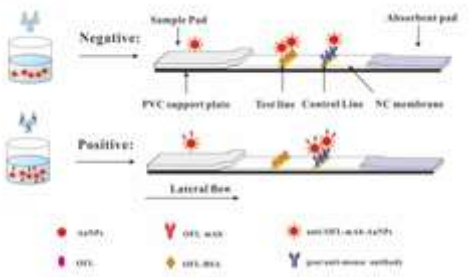

JANUARY 3, 2020

Gold Nanoparticle-Based Paper Sensor for Highly Specific Detection of Ofloxacin in Beef

Cristiane Patrícia de Oliveira, Nilda de Fátima Ferreira Soares, Alvaro Vianna Novaes de Carvalho Teixeira, Taíla Veloso de Oliveira and Antônio Manoel Maradini Filho

Ofloxacin (OFL), a third-generation fluoroquinolone, is widely used as an antibacterial drug to prevent diseases in livestock and poultry. In this study, a paper sensor based on gold nanoparticle and a highly specific monoclonal antibody (mAb) against OFL was prepared to detect OFL residues in beef.

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Biomimetic System Characterization: pH-Induced Chromatic Transition and Nanostructural Transformation of Polydiacetyle and Dimyristoylphosphatidylcholine Vesicles Under pH Variation Using Dynamic Light Scattering (DLS) Technique

Cristiane Patrícia de Oliveira, Nilda de Fátima Ferreira Soares, Alvaro Vianna Novaes de Carvalho Teixeira, Taíla Veloso de Oliveira and Antônio Manoel Maradini Filho

Blue polydiacetylene (PDA) vesicles were prepared and their response to pH

changes was investigated using UV-Vis absorption. The effect of the H+ and OH- presence on the size of vesicles was also studied using the dynamic light scattering (DLS) technique

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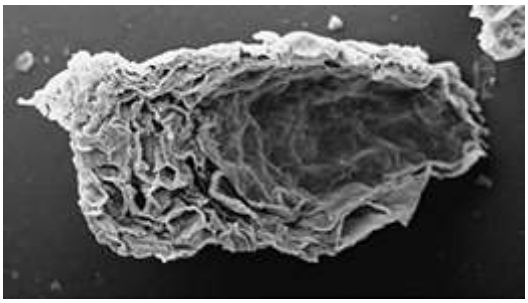

SEPTEMBER 12, 2019

Comparative Evaluation of Soxhlet and Ultrasonics on the Structural Morphology and Extraction of Bioactive Compounds of Lemon (*Citrus limon* L.) Peel

A. Jagannath and Ravikumar Biradar

The relative recoveries of bioactive compounds and nutritional ingredients from lemon peel by ultrasound-assisted extraction (USAE/UAE) and traditional soxhlet extraction methods under different conditions of extraction were evaluated.

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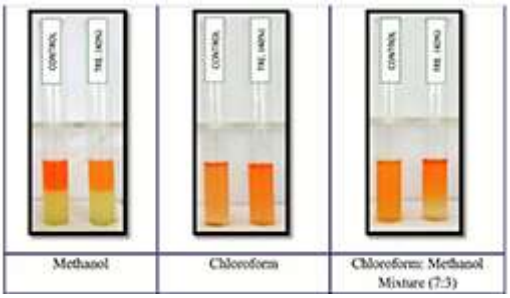

AUGUST 23, 2019

Development of a Rapid Qualitative Test Using Ferroin Dye for Detection of Rancid Ghee Mixed with Fresh Ghee

Bhavesh N. Chaudhary, Keyursinh D. Vaghela, Bhavbhuti M. Mehta, Amit Kumar Jain, Vijaykumar B. Darji and Kishorkumar D. Aparnathi

In the present study, a rapid ferroin dye-based test was developed for detection of rancid ghee mixed with

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Muhammad Hilmi Afthoni, Mariana Wahjudi and Bambang Kuswandi

Bitter melon (*Momordica charantia*) contains charantin that responsible for the antidiabetic activity. Therefore, it is important to determine charantin in the extracts for the purposed of its formulation or quality control.

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Novel Scanometric Assay for Charantin in Bitter Melon (*Momordica charantia*) Extract Based on Immobilized Silver Nitrate and Methylene Blue as Colorimetric Paper

Muhammad Hilmi Afthoni[1,2], Mariana Wahjudi[3] and Bambang Kuswandi[2*]

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Abstract

Bitter melon (*Momordica charantia*) contains charantin that responsible for the antidiabetic activity. Therefore, it is important to determine charantin in the extracts for the purposed of its formulation or quality control. Herein, we proposed a novel scanometric assay for charantin determination in the bitter melon extracts based on immobilized silver nitrate and methylene blue as a colorimetric paper. The presence of charantin can be detected simply via the color change from blue to green and can be quantified using a scanner via the ImageJ software. The proposed charantin sensor has a response time of 8 mins with the linear range of 300 to 1000 µg/mL and the detection limit was 216.25 µg/mL. The sensor has good reproducibility (RSD = 2.86%) with the stability up-to 5 months. The sensor was applied to determine charantin in the sample extracts, and the result shown in a good agreement with TLC method.

Keywords

Colorimetric sensor, Charantin, Silver nitrate, Methylene blue, Bitter melon

Introduction

Momordica charantia or bitter melon commonly known as a fruit that is widely reported to have anticancer, antihyperlipidemic, analgesic, anti-inflammatory, antitumor, antiulcer, hepatoprotective, antioxidant activities [1], antidiabetic [2-8]. Charantin is a mixture of steroidal glycoside (1:1), and as an active compound or chemical marker contained in bitter melon that responsible for antidiabetic activity [4,9]. Since charantin has the capability of lowered blood sugar equivalent to insulin [10]. Therefore, charantin has been used as an active compound or chemical marker for antidiabetic in the herbal medicine that has been formulated from the bitter melon extract [11]. Therefore, it is important to determine the charantin concentration in bitter melon extracts for the purposed of the antidiabetic formulation or quality control of charantin concentration as active compounds in the bitter melon extract used either as pharmaceutical or nutraceutical products purposes.

The conventional methods for determination of charantin are thin layer chromatography (TLC) [12], high performance thin layer chromatography (HPTLC) [13], high performance liquid chromatography (HPLC) [14], gases chromatography tandem mass spectroscopy (GC-MS) [15]. However, these methods are needed sample preparation, long procedure, and skilled personnel, as well as relatively expensive [16]. Certainly, for the big company or industry using conventional standard methods would not be an issue to comply with all

requirements needed, since they have enough resources, setting, and budget. However, for small and medium enterprises (SME), particularly home industry, this issue becomes a big problem for them, as they have a low resource, setting, and budget. Therefore, the development of the low-cost, portable and disposable method is needed for charantin detection in the bitter melon extracts particularly that can be used for SME including home industries. The aim of this study is to develop a simple method and low-cost for determination charantin as alternative compare to conventional standard methods. One alternative to the simple methods and low-cost is a chemical sensor. This is due to the fact that it can be developed as a colorimetric paper for simple strip or spot test by immobilization of suitable reagent solution for charantin onto a filter paper.

The reagents that can be used as an alternative that suitable for charantin detection is silver nitrate ($AgNO_3$) coupled with methylene blue (MB). Since charantin could reduce Ag^+ from $AgNO_3$ into Ag^0 as a silver nanoparticle (AgNPs), where AgNPs act as an electron transfer mediator and donates to MB as a redox catalyst termed as electron relay effect [17] and then reduce MB into leucomethylene blue (LMB) [18]. Based on this reaction, herein for the first time, we developed a novel scanometric assay for charantin in the bitter melon extract based on immobilized $AgNO_3$ coupled with MB as colorimetric paper for simple detection of charantin in the bitter melon extracts. The colorimetric paper was fabricated simply by immobilizing the reagents mixture of $AgNO_3$ and MB on the filter paper via adsorption method. The colorimetric paper results in the determination of charantin in the real sample of bitter melon extracts show a good agreement with the standard method using TLC. Thus, the results show that the proposed sensor can be used as a simple and rapid method for the determination of charantin in the bitter melon extracts.

Materials and Methods

Material

All reagents were used as purchased without further purification. Charantin was obtained from Xi'an Le Sen Biotechnology Co., Ltd (Shaanxi, China), methanol, ethanol chloroform, and hexane were purchased from Merck (Germany), silver nitrate ($AgNO_3$), methylene blue (MB) and filter paper (Whatman cat No 1001 150) were purchased from Sigma Aldrich. Polyacrylic, nitrocellulose, aluminum foil obtained from Indomaret (Indonesia). Fresh *M. charantia* was purchased from Materia Medica (Malang, Indonesia). All chemicals used were of analytical reagent grade without further purification.

Preparation of colorimetric paper

A colorimetric paper as charantin sensor was developed by immobilizing reagent solutions mixture of $AgNO_3$ and MB onto a filter paper (⌀ 0,8 cm). The reagent immobilization was simply by using absorption method. This procedure was carried out by dipping the paper, that already cut into a circular shape (i.d. 5 mm), into 10 ml of the solution mixture overnight (~12 hrs) at ambient temperature with a slow stirrer. Then, the immobilized reagent on the filter paper was dried at room temperature. Afterward, the sensor membrane was attached to white polyacrylic using double transparent tape (3M), to form a strip test for charantin. Then, the colorimetric paper was ready to be used as strip test for charantin detection as presented in figure 1A.

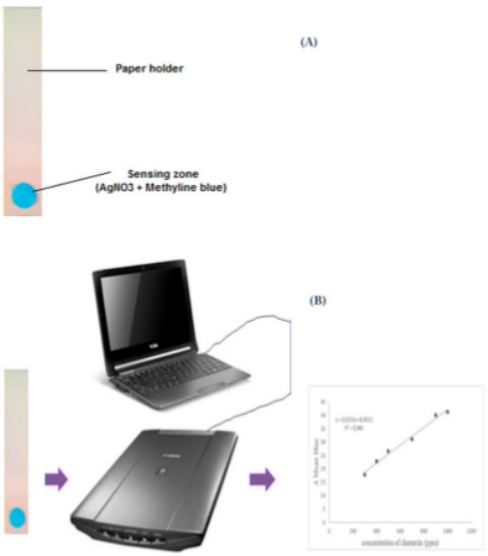

Figure 1: The colorimetric paper based on immobilized $AgNO_3$ and methylene blue as a strip test is ready for use **(A)**, and scanometric assay for charantin determination using the colorimetric paper as strip test **(B)**.

Sample preparation

The sample preparation was performed using previous method [19]. About 2 g sample was placed in conical flask extracted with 10 ml solvent consisting of methanol-water (80:20; %v/v). Then the sample was extracted using ultrasonic (Elmasonic, Germany) at 45 ^{0}C with ultrasonic power at 240 W for 2 hr. Afterword, the extract was filtered, then added with the methanol-water mixture until 10 ml. Then, the sample was ready to be used using the colorimetric paper.

Optimization of colorimetric paper

The reagents concentration of $AgNO_3$ and MB used in the colorimetric paper was according to previous method [18], from which the reagent concentrations were optimized. Here, the $AgNO_3$ solution at concentration of 169 and 1690 µg/mL were used, while MB at concentration of 32 and 1000 µg/mL were used to make four combinations (1) 169:32; (2) 1690:32, (3) 169:1000, and (4) 1690:1000. The volume ratio (v/v) of selected reagents combination then optimized at the different volume ratio i.e. 8:2, 9:1 and 9.8:0.2 (v/v). Then, the sensor response time was also studied at the period from 0 to 20 min. For this study, 1000 µg/mL of charantin was introduced into the sensor membrane. Lastly, the effect of the pH on the sensor response was also optimized between pH 5 to 8.

Scanometric assay

For determination of charantin concentration, the scanometric assay was employed. Firstly, charantin solution was introduced into the sensor membrane, afterword waits at the desired time for the color change of the sensor to occur. Secondly, the color change of the sensors was captured by the flatbed scanner (CanoScan LIDE 110, Japan) at 300 dpi resolution. Finally, the scanned image was analyzed with ImageJ program (https://imagej.nih.gov/ij/). The intensity of the sensor color change is expressed as mean red, green and blue as well as RGB and presented as the intensity value of Δ color unit of the sample subtracted with the blank in order to determine charantin concentration as given in figure 1B. All of the measurements were carried out in triplicate.

Result and Discussion

Sensing mechanism

The charantin detection mechanism is based on the reaction between charantin with $AgNO_3$, which in turn reduced MB. Herein, the reduction of MB is mediated by AgNPs formed as results of $AgNO_3$ reduction by charantin in the bitter melon extract. This is according to Ajitha et al. [18], wherein this scheme, as a result of the decrease in MB absorbance, the increase of SPR peak of AgNPs was observed. In the electron transfer process, when there is a large redox potential difference between acceptor and donor, then there might be the restriction of electrons transfer. However, the electron transfer becomes easy, if an effective catalyst has intermediate redox potential value between acceptor and donor [20, 21]. Therefore, in this case, it reveals that AgNPs act as an electron transfer mediator since it accepts the electrons from extract and donates to MB by acting as a redox catalyst, which in turn termed as electron relay effect [17].

Similarly, in this sensing scheme, the charantin reduces Ag^+ from $AgNO_3$ into Ag^0 as AgNPs, where AgNPs act as an electron transfer mediator and donates to MB as a redox catalyst termed as electron relay effect [17] and reduced MB into leucomethylene blue (LMB) [18] as presented in figure 2A. Based on this reaction scheme due to reduced MB into LMB, hence the sensor color change was from blue to green (Figure 2B). The color change was then analyzed using the ImageJ program for quantitative measurements, and the results show that the color value change or Δ color unit in term of blue gave the best correlation coefficient (r) compared to another color, i.e. red, green and RGB value (Figure 2C). Where Δ color unit presents color of the sensor before and after reaction with charantin at various concentrations. In addition, the color change of the sensor in the Δ blue was found to be the most proportional to the increasing concentration of charantin, followed by ΔRGB. Therefore, the Δ blue value was used for further measurements.

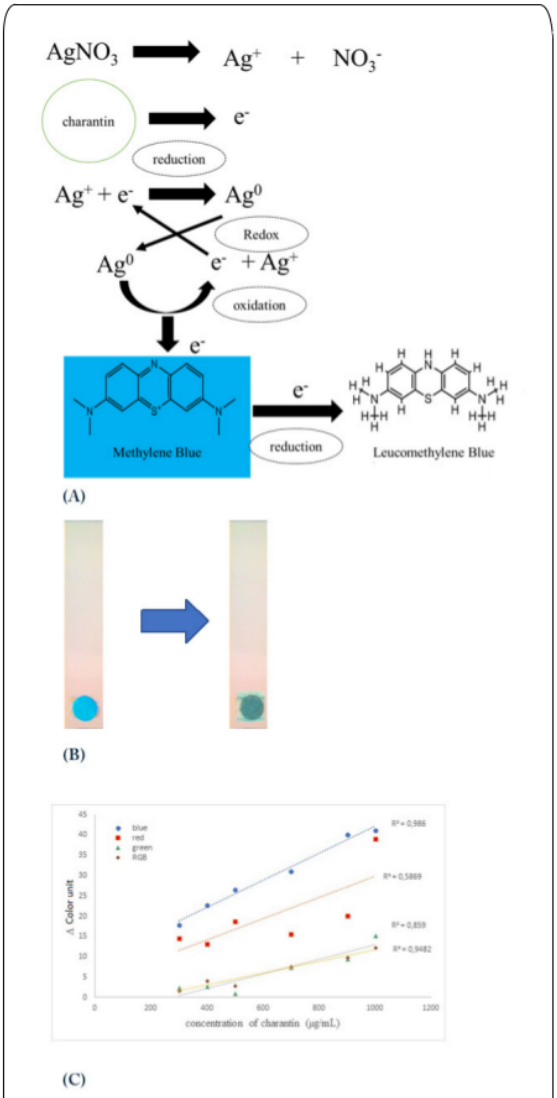

Figure 2: The sensing mechanism of the charantin sensor based on $AgNO_3$ and methylene blue, where Ag^0 (AgNPs) act as an electron transfer mediator, since it accepts the electrons from extract and donates to methylene blue by acting as a redox catalyst **(A)**; the color change of the colorimetric sensor, the sensor before reaction; and after interaction with charantin **(B)**; and the sensor response in the range of 300–1000 µg/mL charantin, where the Δmean blue show the best correlation coefficient (r = 0.993) **(C)**. Here, the Δ colour unit present color of the sensor before and after reaction with charantin at various concentrations. The experiments were carried out in triplicate.

Optimization of experimental parameters

Since the color change of the sensor response was from blue to green, when it tested with charantin (1000 µg/mL). In addition, the adsorption method of immobilization was employed in this case. Then, the optimizations of $AgNO_3$ and MB concentrations for each of them in the mixture were needed to be performed as well as their volume ratio, as these value could be affecting the sensor response. In the case of concentration, the optimum concentration was found at 1690 µg/mL and 1000 µg/mL for $AgNO_3$ and MB respectively as it gave optimum of the sensor response as given in figure 3A.

Furthermore, the optimization of volume ratio for $AgNO_3$ and MB in the mixture was found at the volume ratio of 9.8:0.2 (v/v) for $AgNO_3$ and MB respectively as this volume ratio gave optimum of the sensor response as shown in figure 3B.

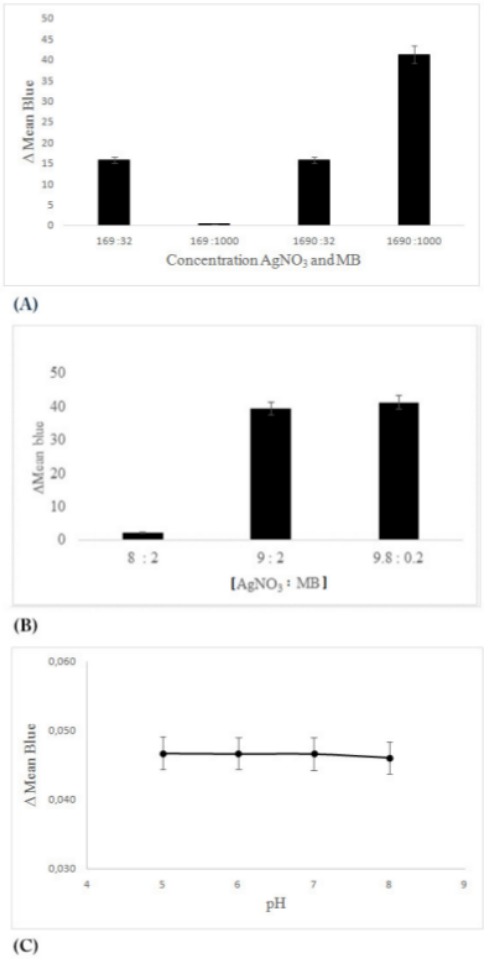

Figure 3: The colour intensity change with the combination of concentration between $AgNO_3$ and MB on the colorimetric paper **(A)**; the colour intensity change with the different volume ratio (v/v) of $AgNO_3$ and MB on the colorimetric paper **(B)**; and the effect of pH buffer on the color change of the charantin sensor between pH 5 to 8 **(C)**. All of the experiments were carried out in triplicate.

Beside the above parameters that would affect the charantin sensor prepared, another parameter that might affect the sensor response is pH. Here, the pH 5-7 was tested using pH buffer solution. In the case of pH, the charantin sensor response was not affected by the pH (5-7) as shown in figure 3C. Here, the pH 5 is similar to the pH of the charantin extract. Therefore, the sample tested no need to be conditioned with the pH buffer is usually used in many assays and simply can be used to determine the charantin concentration directly from the extract. This due to the fact that in the green synthesis of AgNPs, the pH buffer is not required [17, 18]. Thus, for further experiment, the sample extract was used directly without any addition of buffer or other reagent and solution. In addition, this feature would add to the advantages of the proposed sensor, since make the sensor applications simpler and more practical particularly when it applied in the field or for small and home industries.

Analytical characteristics

The response time of the sensor was observed within 20 minutes, where it gave a stable response toward charantin (Figure 4A). Here, all the time response selected (3, 5, 8 and 10 min) were give a good respons as it gives correlation coefficient (R) close to 1. However, the response time of the sensor at 8 min was found to be optimum, where it gives the best linear sensor response towards charantin concentration as given in figure 4B. Based on this response time, the calibration curve was constructed and the sensor was found to have a linear concentration ran in 300 to 1000 µg/mL of charantin (Figure 4C), with coefficient correlation (r) was 0.993. The detection limit of the sensor response was

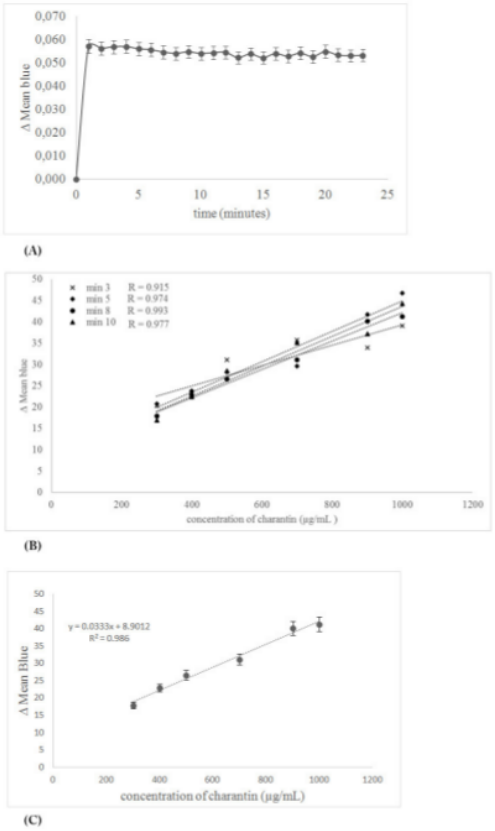

Figure 4: The time response of the charantin sensor within 20 min **(A)**; the response time of the sensor at various time at 3, 5, 8 and 10 min, where 8 min was give the best linear correlation for the charantin sensor **(B)**; and the calibration plot of the sensors response vs. charantin concentration **(C)**.

216.25 µg/mL of charantin, and the quantification limit of the sensor was 648.76 µg/mL of charantin. This limit of detection and the linear range are suitable to be used for the quality control of charantin in the bitter extracts, since the in-house herbal formulation was found to contain 0.361 ± 0.014 of charantin (% w/w dry weight) or equal to 361 µg/mL, while in the charantin content in *M. charantia* fruit extract was 1.053 ± 0.032 (% w/w dry weight) or equal to 1053 µg/mL [22] In addition, the maximum yield of charantin on the dry weight basis of bitter melon was 3.18 mg/g or equal to 3180 mg/L. [1]. Thus, the proposed charantin sensor has a low limit of detection for quality control of charantin either in the herbal formulation or the dry bitter melon extracts.

In order to demonstrate the selectivity of the sensor, a selectivity study on the potential effect of other active compounds in the bitter melon extracts that may interfere the sensor response was studied, such as quercetin. The concentration ratio of 1:1 between charantin and quercetin were used (500 µg/mL). The interfering signal, calculated by subtracting the color intensity change of interfering substances added-sample with that of the original sample, and divided by color intensity change of original sample. The result shows that quercetin interferes charantin with the interference value of 43.769%. This is due to the fact that quercetin also reduces $AgNO_3$ to become AgNPs [23, 24] Therefore, the sample preparation has to be separate quercetin from the extract. Here, the quercetin could be separate from the extract by degradation of quercetin via ultrasonication of the extract, so that its reduction activity will be reduced [25]. Thus, the sample could be prepared by ultrasonication during 2 hrs at 45 °C, and then extracted with the methanol-water mixture (80:20% v/v). After this degradation step, the quercetin interference was less than 5%, and the other hand interfering signal from pharmaceutical excipient used sucrose, nipagin, starch respectively as show in figure 5. The interference of pharmaceutical excipient less than 5% and this value was acceptable for this type of assay [26].

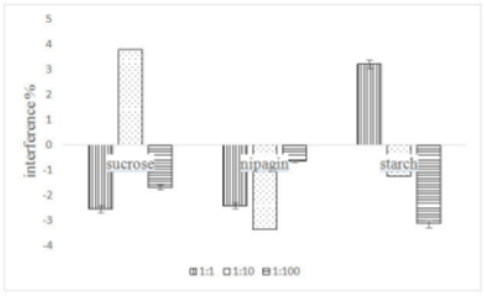

Figure 5: The possible interferences arised from pharmaceutical excipients.

In term of the reproducibility using six sample at the concentration 700 µg/mL, the sensor shows good reproducibility with the RSD value was calculated to be 2.868%. This reproducibility value was indicated that the sensor has the good precision for the charantin determination [26]. The accuracy of the sensor was performed using standard addition method, where the recovery values (%) for the accuracy of the charantin sensor was calculated to be 100.605%. Thus, this value complies with the recovery values within 0.1% of sample concentration tested (95-105%) [26]. Therefore, this value indicated that this method has a good accuracy in determination of charantin concentration.

Stability

The stability study of the sensor response was carried out at the room temperature (25 °C). The sensor response was evaluated every day until 10% of the sensor response decreased from the initial response value. The result shows that the sensor was very stable at room temperature (25 °C) up to 5 months, since afterword, the sensor response was observed to be decreased more than 10% at room temperature.

Application of the real samples

In order to demonstrate the applicability of the proposed sensor in the determination of charantin in the real bitter melon extracts. The sensor was applied for the detection of charantin in the real sample extracts. The content of charantin in the bitter melon extracts was calculated based on the calibration curve constructed above. The results of the proposed charantin sensor were also compared with the result determined using the standard method, i.e. TLC – densitometry method [27] and the results are summarized in table 1. Based on these two results are found to be no difference between the two methods, since the calculated p-value were more than $\alpha = 0.05$. Thus, it can be stated that the proposed charantin sensor is in good agreement with the standard method (TLC), and can be suggested that the proposed charantin sensor can be used as an alternative method for the charantin determination in the bitter melon extract samples.

Table 1: The results of charantin determination in the bitter melon extract samples using the proposed charantin sensor and TLC-densitometric method ($n = 3$, $\alpha = 0.05$).

Sample	Charantin Sensor	TLC - Densitometer	P(T ≤ t) two-tail
A	0.286 ± 0.010	0.288 ± 0.0035	0.723
B	0.241 ± 0.006	0.232 ± 0.0035	0.135
C	0.313 ± 0.010	0.314 ± 0.0007	0.869

Conclusion

The novel scanometric assay based on a colorimetric paper of immobilized reagent mixture of $AgNO_3$ and MB has been developed for measuring charantin in the bitter melon extract. Here, the charantin sensor has a linear response at 300-700 µg/mL with the limit of detection at 216.25 µg/mL and the results were found to be reproducible. The proposed method is simple, easy to operate and low-cost, since it is fabricated as a colorimetric paper in the form of the strip test, making it as a simple and rapid method for determination charantin in the bitter melon extracts.

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