

Communication

An Improved Purification Method for Removing Colour Interference from 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide (MTT) Antibacterial Assays

Danxia Shi ^{1,2}, Wenliang Xu ¹, Marie Wong ³ and David G. Popovich ^{1,*} 

¹ School of Food and Advanced Technology, College of Sciences, Massey University, Palmerston North 4442, New Zealand

² Alpha-Massey Natural Nutraceutical Research Centre, Massey University, Palmerston North 4442, New Zealand

³ School of Food and Advanced Technology, Massey University, Auckland 0745, New Zealand

* Correspondence: d.g.popovich@massey.ac.nz; Tel.: +64-6356-9099

Abstract: The MTT antibacterial assay is an important method in natural antibacterial component discovery. Researchers can use the MTT antibacterial assay to quickly and efficiently evaluate the antibacterial activity of natural compounds. The aim of this research was to find a method to reduce background colour and bacterial cell interference when using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay to study the antibacterial effect of phytochemicals. This study used NZ ‘Hass’ ripe avocado seed extract as an example to develop a silica gel column chromatography method that could combine with the MTT assay to remove the background colour and bacterial cells before the formazan measuring stage. This method is particularly suitable for MTT-based antibacterial inhibition studies when the tested phytochemical extracts have an interfering colour.

Keywords: background colour; bacterial cell; MTT; silica gel column chromatography method; formazan



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1. Introduction

Tetrazolium salts are used to evaluate the antibacterial effect of phytochemical extracts [1]. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is the most common tetrazolium salt used in bioassays [2]. It can be used in colorimetric assays to assess the viability and proliferation of cells [3,4] or microorganisms [5,6]. MTT is converted into a purple water-insoluble formazan crystal (Figure 1) by the mitochondrial dehydrogenases of living bacterial cells. Therefore, the in vitro cell viability can be tested using the MTT colorimetric assay [7]. The MTT colorimetric assay has been used to show the antibacterial properties of several phytochemicals [8,9].

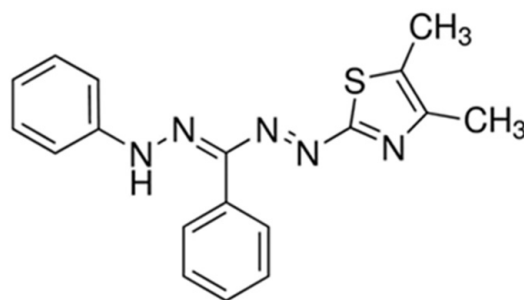


Figure 1. The chemical structure of formazan.

Phenolic compounds (anthocyanins, phenolic acids, stilbenes, flavanols, and tannins) make up the majority of phytochemicals in plant crude extracts [10,11]. Phenolic compounds have shown strong UV light absorption due to the π -conjugated systems with hydroxyl-phenolic groups [12]. Most phenolic compounds have intense absorption bands in the 280 nm and 320 nm regions. Some phenolic compounds have shown a wide-UV-light absorption range from 250 to 600 nm [13,14]. Flavonoids and anthocyanins are two phenolic compounds that have a wider range of UV absorption (250–600 nm) due to the complex molecular structures and the multiple conjugated double bonds [15–17]. Crude plant extracts not only contain phenolic compounds, but also have several other chemical components (multi-vitamins, proteins, alkaloids, and others) [18,19]. These components can potentially interfere with the absorbance measurement. For example, proteins can absorb light at various wavelengths due to the amino acid residues with aromatic rings [20]. In addition, the colour intensity, tonality, and molecular structures of compounds can all influence the UV light absorption ranges of crude extracts [13]. Crude extracts with darker colours or intense tonality may absorb more strongly at specific wavelengths, making it more challenging to accurately measure absorption. Therefore, in MTT colorimetric assays, the tested crude extracts may have overlapping UV light absorption ranges with formazan. Furthermore, floating bacterial cells also have significant absorption in the wavelength range used to assess formazan concentrations after treating with MTT (usually 30 min) [21]. The cells can scatter or absorb light, leading to inaccurate readings [22]. These interferences can reduce the accuracy of the final colorimetric assessment used to determine the phytochemical antibacterial effect. To the best of our knowledge, few reports have tried to remove the interference of plant crude extracts and bacterial suspensions within MTT colorimetric assays.

Avocado seed extract expresses good antibacterial activity against many species of bacteria (*E. coli*, *P. aeruginosa*, *E. faecalis*, *P. vulgaris*, and *S. aureus*) [23,24]. The current study used New Zealand (NZ) ‘Hass’ avocado crude ripe seed extracts (RSE) as a crude plant extract example to explore a developed MTT colorimetric assay. The aim of this study was to find a method to remove the interference of background colours (broth and plant extracts), and bacterial cells before formazan microplate testing while ensuring accurate and reproducible results. With this modified method, the aim was then to increase the accuracy of formazan measurement in phytochemical antibacterial results.

2. Materials and Methods

2.1. Chemicals and Reagents

Methanol, isopropanol (IPA), n-hexane, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), formazan, and hydrochloric acid (HCl) were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). Brain heart infusion broth (BHI) was purchased from Remel™ (Lenexa, KS, USA). All chemical reagents were of analytical grade or higher. Normal phase silica gel plates (silica gel F254, 0.2 mm in thickness, aluminium sheets) were purchased from Merck (Darmstadt, Germany).

2.2. Materials and Extraction Process

The extraction method was the same as reported previously [25]. ‘Hass’ avocados were purchased from a local supermarket (Palmerston North, NZ) during the period November 2018–February 2019. The seeds (pits) were immediately removed. The seeds were ground with a high-power grind mill (Flour Milling, Wuyi Hainan Electric Appliance Company Ltd., Jinhua, China) and then freeze-dried at -80 °C. Powdered seed (80 g) was extracted in a Soxhlet extractor (Buchi, Flawil, Switzerland) with 1500 mL of methanol. Then, n-hexane was used in liquid–liquid extraction to separate the lipid-soluble compounds. Both n-hexane and methanol layers were further concentrated and collected through a rotary evaporator under 50 °C (Buchi, Flawil, Switzerland). The phytochemicals obtained from the methanol layer were used as the final extracts for this study.

2.3. Microorganism and Growth Conditions

Staphylococcus aureus ATCC 25923 (*S. aureus*) strains were obtained from the School of Food & Advanced Technology microbiology laboratory of Massey University, cultured in BHI broth (Hi-media, M002) at 37 °C and stored in BHI agar slants at 4 °C.

2.4. Preparation of Reagents

For stock reagents, MTT (AK Scientific, Union City, CA, USA) was dissolved in distilled water to a concentration of 5 mg/mL and stored in a 4 °C cold room in darkness. The formazan standard was dissolved in the hexane to a concentration of 1 mg/mL and stored in a 4 °C cold room. Acidic isopropanol was prepared as a 1.5% (*v/v*) solution of hydrochloric acid (37%) in isopropanol and stored in a 4 °C cold room in the dark. NZ 'Hass' ripe avocado seed extract (RSE) (Figure S1A) was dissolved in distilled water to a concentration of 5 mg/mL and stored at −20 °C in the dark.

2.5. MTT Assay Conditions

Staphylococcus aureus ATCC 25923 was incubated with 500 mL of BHI broth overnight at 37 °C in an incubator. The final concentration of the prepared *S. aureus* BHI broth was at OD₆₀₀ = 1.0 by microplate reader reading. One millilitre of RSE (5 mg/mL) was added into the prepared *S. aureus* BHI broth (1 mL) to perform the RSE treatment incubation (37 °C, 24 h). Then, 200 µL of MTT water solution (5 mg/mL) was immediately added into the RSE-treated *S. aureus* suspension (Figure S1B) and incubated at 25 °C for 30 min for the MTT to completely convert to formazan. The formazan solution from the RSE-treated *S. aureus* suspension was named 'RSE-formazan' in this study. Working formazan content standards from 7.8125 to 125 µg/mL were prepared for RSE-formazan content analysis.

2.6. Before and after Column Chromatography Filtering Comparison Study

Whether silica gel could remove the possible background interferences (broth, bacteria, and plant extracts) was studied in this comparison study. The tested background interferences were: (1) pure BHI broth; (2) *S. aureus* suspension (OD₆₀₀ = 1.0), and (3) 5 mg/mL of RSE solution. The absorbance values were measured using a microplate reader (Varioskan™ LUX, Thermo Scientific™, Waltham, MA, USA) at an absorbance of 570 nm. Absorbance was measured at 570 nm using distilled water as the blank. The absorbance values of before and after column chromatography filtering interferences were compared with the absorbance value of pure IPA.

The comparison solution preparation:

1. Before the filtering comparison (isopropanol dissolving): two hundred microliters of each interference solution: (1) pure BHI broth; (2) *S. aureus*; and (3) RSE were added directly and separately into 2000 µL of IPA. Then, these reagents were vortexed for 1 min to mix completely. The final mixture reagents were diluted with IPA to a volume of 2500 µL.
2. After a filtering comparison (column chromatography): two hundred microliters of each interference solution: (1) pure BHI broth; (2) *S. aureus*; (3) RSE were loaded separately onto a normal phase silica gel column (200–300 mesh, 0.3 g, pre-packed in a 3 mL glass dropper) (Merck, Darmstadt, Germany). Then, 2000 µL of IPA was used as the eluant for each interference solution. The eluted isopropanol solutions were diluted with IPA to a volume of 2500 µL.

The dissolving method of each sample was performed in independent triplicate to validate the results.

2.7. Formazan Dissolving Comparison Study

To identify the final formazan content, RSE-formazan was prepared using three different dissolving methods. The absorbance values of all samples were measured using a microplate reader (Varioskan™ LUX, Thermo Scientific™, Waltham, MA, USA) at an

absorbance of 570 nm. The absorbance of distilled water was measured at 570 nm as the blank.

Preparing the Formazan-Dissolving Solutions

1. Common method (acidic isopropanol dissolving): acidic isopropanol has been reported to dissolve formazan crystals in an MTT-antibacterial study (*v/v*, 1:1) [14]. Two hundred microliters of acidic isopropanol was directly added into 200 μL of test solution (RSE-formazan), and further incubated for one hour at 25 °C to completely dissolve the formazan. The final mixture of acidic isopropanol and formazan reagent was diluted with IPA to a volume of 2500 μL .
2. Isopropanol-dissolving method (before silica gel filtering): Two hundred microliters of RSE-formazan was added directly into 2000 μL of IPA. The mixture reagent was further incubated for one hour at 25 °C to dissolve the formazan. The final formazan reagent mixture was diluted with IPA to a volume of 2500 μL .
3. Column chromatography method (after silica gel filtering): Two hundred microliters of RSE-formazan was loaded onto a normal phase silica gel column (200–300 mesh, 0.3 g and pre-packed in a 3 mL glass dropper) (Merck, Darmstadt, Germany). A 3 mL glass dropper was used with the glass column. Then, 2000 μL of IPA was used as the eluant. The eluted isopropanol formazan solution was diluted with IPA to a volume of 2500 μL .

Each formazan dissolving procedure was performed in independent triplicate to validate the results.

2.8. Thin-Layer Chromatography (TLC)

Aluminium-backed silica gel 60 F254 plates (Merck, Darmstadt, Germany) were used to examine the final RSE-formazan solution components from the common method and the column chromatography method following thin-layer chromatography (TLC) analysis with a spotting volume of 4.8 μL . Pure n-hexane was used as the developing agent. Normal phase silica gel plates were developed in a rectangular TLC developing chamber. The chamber was shaken with the developing agent and sealed with parafilm before each experiment. The plates were removed when the solvent front had moved along 90% of the total plate length (10 cm) and subsequently allowed to dry.

2.9. Statistical Analysis

Means and standard deviations were calculated. Statistical analysis was performed using SPSS version 23 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (one-way ANOVA) was performed to address the differences. The correlation coefficients between the variables were determined using Pearson's analysis at two significance levels ($p < 0.05$ and $p < 0.001$).

3. Results and Discussion

Silica gel is a widespread material used for column chromatography, which is used for phytochemical purification [26]. The column chromatography method separates compounds based on the different polarities of each compound. Therefore, in the current study, we tried to use column chromatography to separate the formazan, bacterial broth, and the red RSE. The expected result was that, after the column chromatography, the bacterial cells, redundant MTT, and plant extracts would firmly adsorb to the silica gel, while the formazan would be completely eluted by isopropanol.

In the before-and-after column chromatography filtering comparison study, we explored whether silica gel chromatography could remove the colour interference (RSE and BHI broth) (Figure 1). The microplate reader results show that column chromatography-filtered BHI broth solutions (Figure S2B) had lower OD₅₇₀ values than isopropanol dissolving solutions (before column chromatography filtering) ($p < 0.05$). We considered that most of the protein, amino acids, and coloured components in the broth were left in the silica

gel column after column chromatography filtration. Notably, the column chromatography-filtered RSE showed a significantly lower OD₅₇₀ value than the isopropanol dissolving (before column chromatography filtering) RSE mixture solution. The column chromatography-filtered RSE showed nearly the same OD₅₇₀ value as the pure isopropanol (0.347) ($p > 0.05$). Thus, the current study demonstrates that the seed extracts from NZ ‘Hass’ ripe avocado are left in the silica gel column (Figure 2).

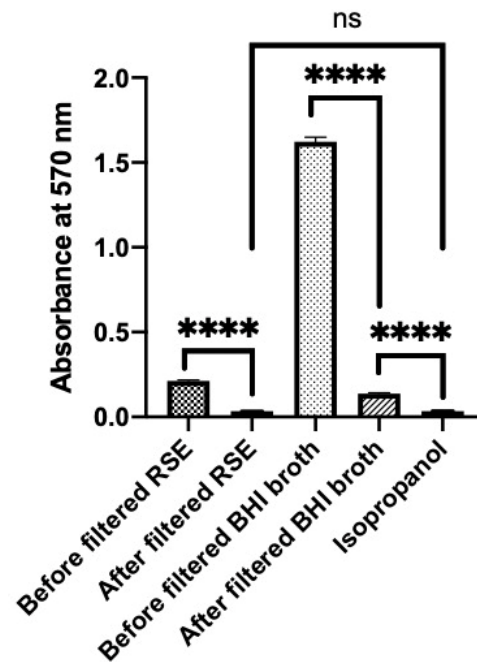


Figure 2. The optical density results of the NZ ‘Hass’ avocado ripe seed extracts (RSE), and the BHI broth before (isopropanol dissolving) and after (column chromatography) filtering. RSE before filtering: avocado ripe seed extract dissolved in isopropanol; BHI broth before filtering: pure BHI broth dissolved in isopropanol; RSE after filtering: avocado ripe seed extract solution obtained after column chromatography filtration; BHI broth after filtering: pure BHI broth obtained after column chromatography filtration. Results are expressed as the mean \pm SD. Quadruple asterisks (****) indicate $p < 0.0001$. ns indicates $p > 0.05$.

Additionally, this comparison study evaluated whether silica gel chromatography could remove the interference of bacterial cells (Figure 3). The microplate reader results showed that isopropanol-dissolving (before column chromatography filtering) BHI broth solutions had lower OD₅₇₀ values than the isopropanol-dissolving (before column chromatography filtering) *S. aureus* suspension (Figure S3A). The results supported the hypothesis that bacterial cells can block the light signals produced by microplate reader. The results demonstrate that the cells of *S. aureus* cannot be dissolved by isopropanol. After column chromatography filtering, both the BHI broth and *S. aureus* suspension showed significantly lower OD₅₇₀ values than the isopropanol-dissolving (before column chromatography filtering) BHI broth and *S. aureus* suspension ($p < 0.0001$). After column chromatography filtering, the OD₅₇₀ value of the BHI broth solution showed a non-significant difference with the *S. aureus* suspension (Figure S3B). Therefore, most of the bacterial cells were left in the silica gel column after column chromatography filtration (Figure 3).

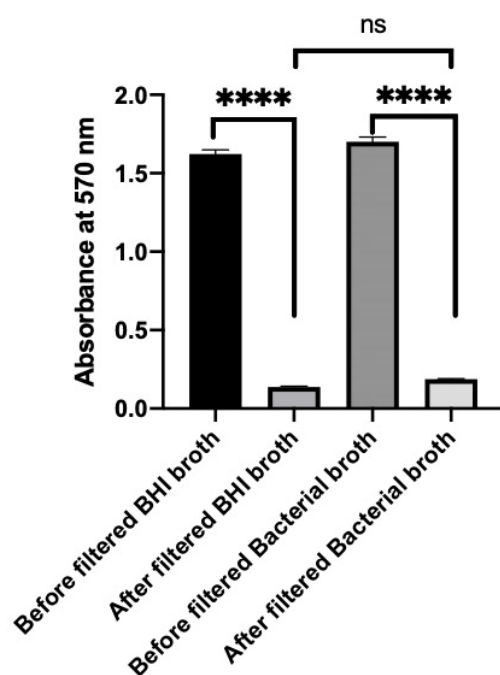


Figure 3. The optical density results of the BHI broth before (isopropanol dissolving) and after (column chromatography) filtering, and *S. aureus* suspension. Bacterial broth before filtering: *S. aureus* suspension dissolved in isopropanol; BHI broth before filtering: pure BHI broth dissolved in isopropanol; bacterial broth after filtering: *S. aureus* suspension obtained after column chromatography filtration; BHI broth after filtering: pure BHI broth obtained after column chromatography filtration. Results are expressed as the mean \pm SD. Quadruple asterisks (****) indicate $p < 0.0001$. ns indicates $p > 0.05$.

The formazan that was produced by NZ ‘Hass’ ripe avocado seed extract treated in the *S. aureus* suspension (RSE-formazan) was dissolved by three different methods (Figure S5). The final RSE-formazan concentration was evaluated by microplate reader at 570 nm through the formazan standard curve. The final RSE-formazan content from 1 common method (acidic isopropanol dissolving); 2 before column chromatography filtering (isopropanol dissolving); 3 after column chromatography filtering was compared (Figure 4). The results show that there is a significant difference among the final RSE-formazan content of these three methods ($p < 0.0001$). The isopropanol-dissolving RSE-formazan showed the highest OD₅₇₀ values over acidic isopropanol dissolving and after column chromatography-filtered RSE-formazan solutions. The final RSE-formazan content from the column chromatography-filtered method is significantly lower than the final RSE-formazan content from the common method. Then, we used TLC plates to verify the components of the final RSE-formazan solutions from the common method and the column chromatography method. In Figure 5, the TLC plate shows that there is a yellow dot at the bottom of A (the acidic isopropanol RSE-formazan) ($R_f = 0.00$). However, in the column chromatography-purified RSE-formazan (B), there are no visible component residues in the bottom. Therefore, the TLC plate results support the hypothesis that coloured components of BHI broth, RSE, and bacterial cells were adsorbed by the silica gel during the eluent procedure (Figure 5).

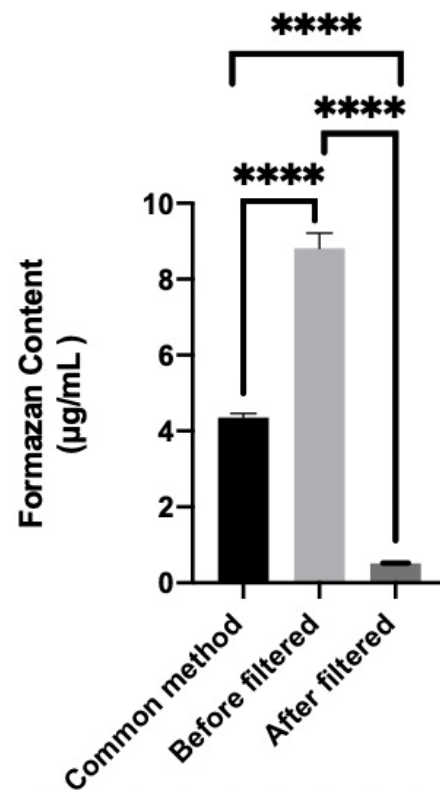


Figure 4. The final formazan content of NZ ‘Hass’ ripe avocado seed extract treated in the *S. aureus* suspension (RSE-formazan) using three dissolving methods. Common method: acidic isopropanol dissolving method; before filtering: isopropanol dissolving method; after filtering: after column chromatography filtering method. Results are expressed as the mean \pm SD. Quadruple asterisks (****) indicate $p < 0.0001$. ns indicates $p > 0.05$.

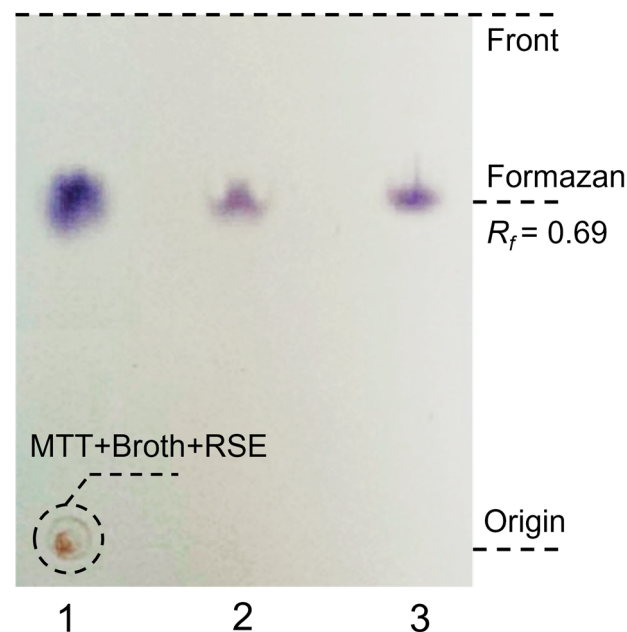


Figure 5. The TLC plate of formazan that was produced from NZ ‘Hass’ ripe avocado seed extract treated in the *S. aureus* suspension. 1 indicates common method: The formazan dissolved using the acidic isopropanol dissolving method; 2 indicates after filtering: The formazan dissolved using the column chromatography filtering method; 3 indicates standard: The formazan standard solution.

NZ ‘Hass’ avocado ripe seed extract has been reported to contain a high phenolic content [25]. Additionally, several phytochemical studies showed that phenolic extracts contain antibacterial activities [27–29]. TLC is a well-established analytical technique to be used to separate and identify the components in complex plant extracts [30]. It is a valuable method to guide the column chromatography by providing mobility, purity, and polarity information on the components in plant extracts [31]. We considered the polarity character of the ripe avocado seed crude extracts, IPA, and formazan. The polarity of these three materials is: formazan < IPA < avocado ripe seed crude extracts (phenolic compounds). Current TLC results (Figure 5) show that IPA can selectively partition different compounds with different polarities, allowing them to be separated on the TLC plate. The results from the TLC plate suggest that IPA is a suitable solvent for separating the formazan and RSE (phenolic compounds) in column chromatography. The RSE (phenolic compounds) was strongly adsorbed to the stationary phase (silica gel) in the column.

As the first column chromatography and MTT colorimetric combination method, it removed the background colour (broth and plant extracts), and bacterial cell interferences before formazan microplate testing. These interferences could affect the formazan measurement and lead to false-positive or false-negative results. This column chromatography removal interference step improved the accuracy of the formazan measurement in the MTT antibacterial studies. Therefore, this developed column chromatography and MTT colorimetric combination method could be used in the antibacterial activity studies of other plant crude extracts. Column chromatography combined with the MTT colorimetric method was a valuable tool for the accurate and reliable measurement of the antibacterial activity of plant extracts. This could provide a new prospect of MTT antibacterial phytochemical studies.

4. Conclusions

In conclusion, in the current study, we prepared formazan crystals using *S. aureus* cells and conducted a systematic investigation of the effects of the column chromatography method combined with the MTT method in an antibacterial study. The results show that avocado ripe seed extract, BHI broth, and bacterial cells can be adsorbed by silica gel when using isopropanol as an eluent, while the formazan can be separated through this method. The column chromatography method could exclude major absorbance interference from plant crude extracts, broth, and bacterial cells in MTT bacterial studies. Therefore, the combination of column chromatography and the MTT colorimetric method can greatly improve the accuracy of formazan measurement.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app13085067/s1>, Figure S1: The NZ Hass avocado ripe seed extract in water solution (A) and *S. aureus* suspension (B); Figure S2: The final tested reagents from before (A) and after (B) column chromatography-filtering the BHI broth; Figure S3: The final tested reagents from before (A) and after (B) the column chromatography filtering *S. aureus* suspension; Figure S4: The isopropanol eluted silica gel column of MTT water solution, BHI broth, *S. aureus* suspension, and NZ Hass avocado ripe seed extract (RSE); Figure S5: The final dissolving formazan reagent from three different formazan dissolving methods. (A) isopropanol dissolving method (before silica gel filtering); (B) column chromatography method (after silica gel filtering); and (C) common method (acidic isopropanol dissolving). Figure S6: Spectral scan of before and after column chromatography filtered bacterial suspension, NZ Hass avocado ripe seed extract, and BHI broth from 500 to 600 nm.

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