Journal of
Food and Pharmaceutical Sciences

Original Article

SARS-CoV-2 Oral Vaccine Design Based on Nanoparticle Encapsulation with a Combination of Chitosan and Alginate

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Received: 31 July 2024; Revised: 6 September 2024; Accepted: 25 September 2024; Published: 31 December 2024

Abstract: Vaccines are the most effective intervention in reducing COVID-19 mortality rates. Compared to parenteral vaccines, oral vaccines offer a more convenient process with dual immune responses (systemic and mucosal). Nanoparticle encapsulation is a strategic method used to enhance the efficiency of oral vaccines, antigen stability, and the effectiveness of immune induction. Combining chitosan and alginate as encapsulating polymers interacts through an ionic gelation process, which protects the vaccine from gastrointestinal disturbances. Our research aims to perform nanoencapsulation of the intravenous "Inavac" vaccine using chitosan and alginate polymers to create an oral vaccine. The optimal formulation was obtained using Design Expert 13, determined by the parameters of % transmittance and % encapsulation entrapment. The optimal formula consists of 1.75% chitosan, 0.1% NaTPP, and 0.05% alginate, with a stirring speed of 1150 rpm and a duration of 60 minutes. The PSA characterization results show that 94.45% of particles are sized at 83.81 nm with a zeta potential of (+32.86), indicating that the COVID-19 vaccine nanoencapsulation formula has a nanometer size with homogeneous distribution and system stability, correlates with good mucoadhesive strength. The good stability of the design is also indicated by the absence of significant changes in formula concentration (p-value = 0.69), the presence of appropriate functional groups as observed through FTIR, spherical surface morphology as seen through SEM, and the highest vaccine release in the intestines (Simulated Intestine Fluid medium). The oral SARS-CoV-2 vaccine using chitosan and alginate polymers with the optimal formula shows great potential for development as an alternative option for the public.

Keywords: Alginate, Chitosan, COVID-19, Nanoencapsulation, Oral Vaccine

1. INTRODUCTION

As of January 2024, the pandemic is far from over, with a cumulative total of 774,469,939 global COVID-19 cases. The WHO emphasized the pandemic has entered an endemic phase, meaning the virus continues to circulate indefinitely and may still pose a significant public health threat. Vaccines remain one of the most effective public health interventions in significantly reducing new cases and COVID-19-related mortality [1]. Indonesia's COVID-19 vaccine, such as Inavac, is administered through the parenteral route (needle-based), which has drawbacks in management, including pain/swelling, risk of infection, and discomfort. Unlike parenteral administration, oral immunization can stimulate both cellular and humoral immune responses at systemic and mucosal levels to induce broad and long-lasting immunity, especially since mucosal surfaces are the primary site of SARS-CoV-2 infection [2]. Moreover, the Omicron variant of COVID-19 has shown gastrointestinal manifestations [3]. Therefore, mucosal immunity is crucial for long-term protection against the virus. Efforts are underway to develop an oral COVID-19 vaccine, with probiotic-based oral vaccines already designed [1], [4], [5].

The current goal of oral vaccine development is to enhance antigen delivery to Gut Associated Lymphoid Tissue (GALT), triggering a strong immune response [1]. However, oral vaccines face significant challenges, such as acidic pH (especially in the stomach), poor epithelial cell absorption in the digestive tract, and low immunogenicity. Oral vaccination efficiency can be improved through nanoencapsulation [6]. Nanoencapsulation is a technique used to package active substances, like drugs or vaccines, into a nanoscale carrier (10-6 cm). Nanoencapsulation protects its payload from early degradation in biological environments, increases bioavailability, and prolongs its presence in the bloodstream and cell absorption [7],[8]. Biodegradable and biocompatible polymer particle systems can address oral vaccine challenges. The natural polymer chitosan has been used as an alternative material for oral vaccine delivery compared to synthetic polymers. Chitosan has been extensively studied for the delivery of therapeutic proteins and antigens, especially through the mucosal route due to its excellent mucoadhesive properties and enhanced absorption into M cells from the Follicle-associated epithelium (FAE). Research has shown dendritic cell, macrophage, and lymphocyte activation by chitosan-mediated oral vaccine delivery systems [2] . According to studies, immunizing mice with chitosan NPs alone provided 47% protection against parasitic infection, demonstrating chitosan's key role in inducing a protective immune response [9].

Chitosan particles coated with alginate can effectively protect acid-sensitive drugs from degradation at acidic pH compared to chitosan particles alone and enhance antigen absorption by mucosal lymphoid tissue [10]. Research has shown that oral administration of poultry typhoid vaccine in chitosan-alginate-coated microparticles can induce innate and adaptive immune responses comparable to the subcutaneous route and provide protection against virulent strains of *S. gallinarum* [11]. Additionally, the administration of alginate-chitosan-coated NPs significantly increased mucosal IgA responses and serum IgG antibodies compared to naked OVA [7]. Therefore, the development of an oral COVID-19 vaccine involves the direct nanoencapsulation of COVID-19 vaccine (Inavac) suspensions, which already contain adjuvants, using polysaccharides like chitosan and alginate to enhance mucosal penetration and oral antigen delivery. Our research aims to formulate the optimal nanoencapsulation of an oral SARS-CoV-2 vaccine using chitosan and alginate polymers and to evaluate the effectiveness and efficiency of the oral vaccine in the digestive tract.

2. MATERIALS AND METHODS

2.1. Materials

The materials used in the research were COVID-19 Vaccine (Inavac) obtained from the Badung Regency Health Office, Chitosan (Shrimp SHELL extract), Sodium alginate (Sigma aldrich), Glacial acetic acid, Sodium tripolyphosphate (Xilong scientific), Bovine Serum Albumin (BSA) (Himedia MB083-25G), Sodium chloride, Potassium chloride, Sodium phosphate dibasic, Potassium phosphate monobasic, NaOH, DI Water (Cleo). Tools used include glassware, syringes, analytical balance (Radwag), magnetic stirrer (Thermo Scientific), pH meter (Mettler Toledo), sonicator (Branson), centrifugator (Eppendorf 5702), and freeze dryer (Lab Freeze). The instruments used were UV-Vis spectrophotometer (Shimadzu UV Mini-1240), Particle Size Analyzer/PSA, FTIR spectrophotometer (Shimadzu; IRPrestige-21), Scanning Electron Microscopy/SEM (JSM-6510LA), Design Expert software.

2.2. Preparation of Nano Encapsulation Process of Chi-Alg@Vaccine

2.2.1. Formula Development with Design Expert

Design Expert was used to determine the design and interpretation of multifactor experiments of COVID-19 vaccine nanoencapsulation. The multifactors used were stirring duration (minutes), stirring speed (rpm) and concentration (%) of chitosan, NaTPP and alginate.

2.2.2. Preparation of Chitosan, Na TPP and Alginate Solution

The formula for chitosan solution was made at a concentration of 1.75% and 0.2% dissolved in 1% glacial acetic acid solvent, with a volume of 10 mL. NaTPP was made at concentrations of 1%

and 0.1% in demineralized water solvent, with a volume of 5 mL and sodium alginate was made at concentrations of 0.6% and 0.05% in demineralized water solvent with a volume of 5 mL.

2.3. Nano-encapsulated Chi-Alg@Vaccine Formulation

The COVID-19 vaccine nanoencapsulation formulation design using chitosan and alginate has been made in 16 different formulas based on Design Expert analysis. Each formula has varying concentrations of chitosan, alginate and NaTPP. The Design Expert also determined different stirring speed and duration for each formula. Each formula contains 1.5 mL of vaccine. The stages of formulation work are as follows: (a) The vaccine was mixed with 10 mL of chitosan with stirring for 2 minutes; (b) The mixture was added with 5 mL of NaTPP and 5 mL of sodium alginate, stirred for 60 minutes or 15 minutes, at a speed of 600 rpm or 1150 rpm, according to the variation of formula from Design Expert.

2.4 % Transmittance and % Efficiency Encapsulation Capacity

2.4.1. Maximum Wavelength Determination

Standard series were made using Bovin Serum Albumin (BSA) in phosphate buffered saline solvent pH 7.4 with series concentrations of 50 ppm, 200 ppm, 400 ppm, 600 ppm, 800 ppm, and 1000 ppm. The maximum wavelength used was 277 nm. A standard curve of nanoencapsulated vaccine was made to obtain a linear equation.

2.4.2. % Transmittance Evaluation

Transmittance test was conducted to see the clarity of the preparation using UV-Vis spectrophotometry with distilled water as a blank. A total of 16 nanoencapsulated vaccine preparation formulas were homogenized and then put ± 3 mL into a cuvette and measured the percent transmittance at a wavelength of 650 nm.

2.4.3. Vaccine Encapsulation Entrapment Evaluation

A total of 16 nano encapsulated formulations of COVID-19 Chi-Alg vaccine were centrifuged for 30 min at 4000 rpm to separate the nanoparticles from the supernatant solution. The supernatant was analyzed by UV-Vis spectrophotometry at a wavelength of 277 nm to measure the level of unencapsulated vaccine in the supernatant with phosphate saline buffer as a blank. The total amount of vaccine added in the initial formulation was recorded. Encapsulation entrapment (%EE) was calculated using the following formula [12]:

$$\%EE = \frac{Total\ Drug\ Concentration - Supernatant\ Drug\ Concentration}{Total\ Drug\ Concentration} \times 100\%$$

2.5. Optimum Formula Determination

Determination of the optimum formula was carried out using Design Expert. The % transmittance and %EE values of 16 formulas were entered, then the best formula was determined based on the evaluation results.

2.6. Characterization of Nano Encapsulated Vaccines

2.6.1. Particle Size Analyzer

The samples were first homogenized and tested for particle size, poidispersity index, and zeta potential measured using a Malvern Instrument Zetasizer Advance conditioned at 25^{o}C.

2.6.2. Chemical and Physical Stability

Physical and chemical stability tests of the nanoencapsulated vaccine formula were carried out by organoleptic analysis and measuring the levels of the liquid form on days 0, 3, 5, 7. The samples were stored in a refrigerator at 40C. Measurement of encapsulation levels was carried out by UV-Vis spectrophotometry at a wavelength of 277 nm using ethanol as a blank. Then, the absorbance value until day 7 will be compared to determine the stability level of the formula.

2.6.3. Scanning Electron Microscopy (SEM)

The morphology of the encapsulated structure was characterized using Scanning Electron Microscopy (SEM) with magnifications of 35, 500, 3000, 5000 to 10,000 times after being coated with platinum under vacuum conditions, taken with an acceleration voltage of 20 kV. The samples were freeze dried for 24 hours before analysis.

2.6.4. Fourier transform Infrared Spectroscopy (FTIR)

The microscopic characterization performed was qualitative analysis with FTIR to characterize the functional groups of nanoencapsulated samples based on specific wave numbers. The sample ratio with KBr is 1:4.

2.6.5. In Vitro Release

Nanoencapsulation was weighed 10 mg each and then dissolved in SGF (Simulated Gastric Fluid) media pH 1.2 in 20 mL volume; SIF (Simulated Intestinal Fluid) media pH 6.8 in 20 mL volume; SCF (Simulated Colonic Fluid) media pH 7.4 in 20 mL volume. In SGF media, the nanoencapsulated vaccine was stirred at 75 rpm for 2 hours and at a temperature of 37±0.5°CAfter 2 hours, the sample was centrifuged at 3000 rpm for 15 minutes and the supernatant was measured for absorbance. Next, the SIF media was stirred at 50 rpm for 2 hours and 37±0.5°CIn SCF media, stirring was carried out at 50 rpm for 4 hours, temperature 37 ± 0.5 °CSamples from SIF and SCF media were centrifuged for 15 minutes at 3000 rpm and the absorbance was measured.

3. RESULTS AND DISCUSSION

Chitosan is a cationic polysaccharide derived from the N-deacetylation of chitin, which is a linear copolymer composed of 2-amino-2-deoxy-β-d-glucan repeating units with glycosidic bonds, where the amine group gives chitosan special properties, such as high charge density, readiness for chemical reactions, and salt formation. The solubility of chitosan depends on the position of the amino and N-acetyl groups and can be enhanced by aqueous acids such as formic acid and acetic acid. Chitosan has significant adsorption and mucoadhesive properties, as well as antifungal activity [13], [14]. These properties make chitosan very promising for applications in the food, environmental, and pharmaceutical industries [15].

Alginate is a linear biopolymer consisting of two uronic acids, namely 1,4-linked-β-d-mannuronic acid (M) and α-l-guluronic acid (G). The carboxylic group of the uronic acid is responsible for the negative charge of alginate. In drug carrier systems, polymers such as chitosan and alginate are often used because they are non-toxic, biocompatible, and biodegradable. Chitosan and alginate can react together because they have opposite charges, the ease of solubility of chitosan at low pH can be prevented by the alginate network because alginate is insoluble at low pH conditions. The possible disintegration of alginate at higher pH is prevented by chitosan, which is stable at higher pH ranges [15]. The structure of chitosan and alginate interacts ionically between the carboxyl residues of alginate and the amino terminals of chitosan. This complexation reduces the porosity of alginate and decreases the leakage of encapsulated substances [16]

3.1. Optimum Formulation Determination

In determining the optimum formulation, this research used the software, Design Expert with a factorial design which is the application of regression equations to model the relationship between the response variable and one or more independent variables. The independent numerical factors that affect the output are chitosan concentration (%), NaTPP (%), alginate (%), stirring speed (rpm) and stirring duration (minutes). The effects or response changes associated with these factors are transmittance (%) and EE (Encapsulation Entrapment) (%) which can be quantified. Based on this process, a total of 16 experiments were found to be conducted to obtain the response factors which can be seen in table 1. Based on the Design Expert analysis, there are 3 factors that affect transmittance, namely chitosan concentration, NaTPP/stirring speed, and NaTPP concentration. Figure 1 shows that chitosan affects transmittance positively so that an increase in chitosan concentration will be followed by a higher transmittance value. Meanwhile, the second and third

factors affect negatively so that the greater the NaTPP concentration and stirring speed will make the transmittance value smaller. For the second response, %EE, was not influenced by any factor because of the sixteen formulas used, all formulas succeeded in producing a large %EE (more than 85%). Through this data, the optimum formulation obtained was chitosan 1.75% volume 10 mL, NaTPP (crosslinked agent) 0.1% volume 5 mL and alginate 0.05% volume 5 mL. The Inavac vaccine used as much as 1.5 mL has a desirable value of 0.918 which can be interpreted as the high ability of the formulation to meet the optimal %transmittance and %EE criteria.

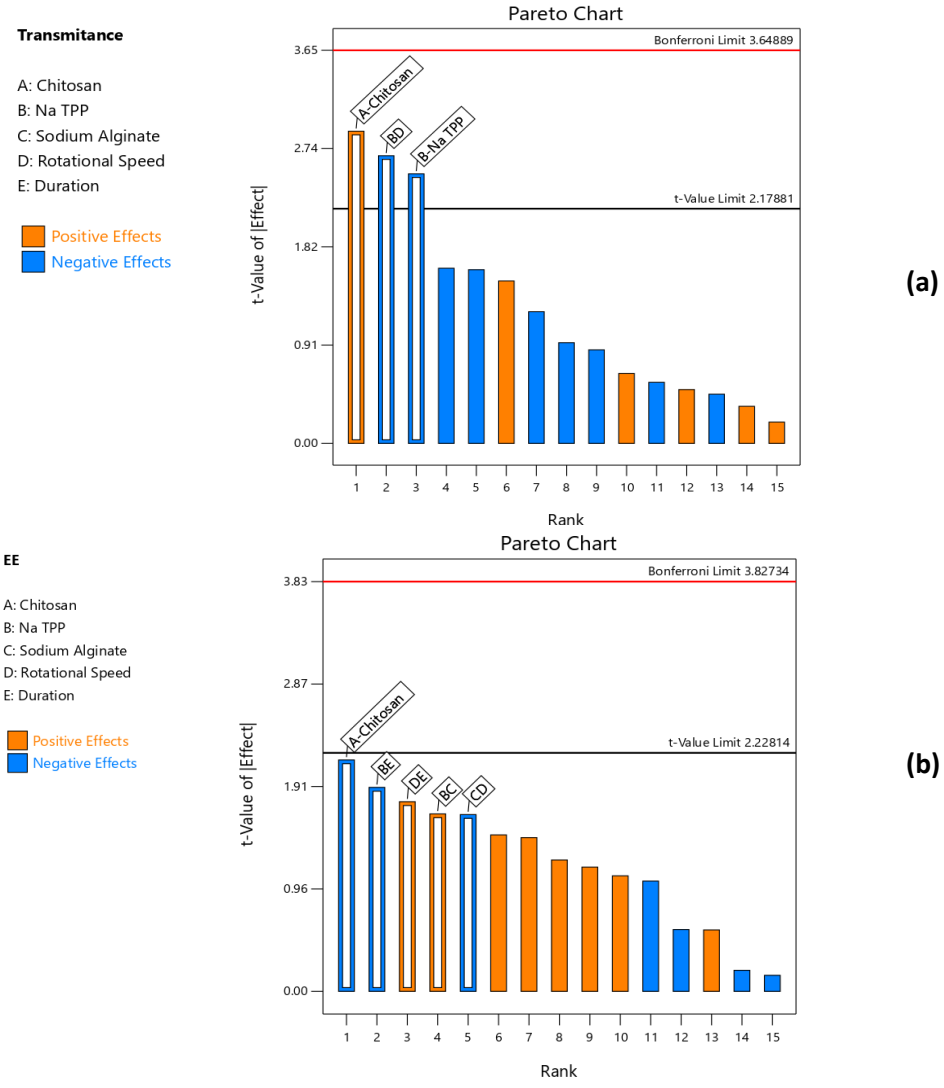


Figure 1. (a) Relationship of % Transmittance to Independent Factors; (b) Relationship of % EE to Independent Factors. Independent factors: Chitosan Concentration, NaTPP Concentration, Alginate Concentration, Stirring Speed, and Stirring Duration

Table 1. Vaccine Nanoencapsulation Formulation Design from Design Expert

Formula	Chitosan (%v/v)	NaTPP (%b/v)	Sodium alginate (%b/v)	Stirring Speed (rpm)	Stirring Duration (menit)
1	0.2	1	0.05	600	15
2	1.75	0.1	0.6	600	60
3	0.2	0.1	0.05	1150	15
4	1.75	0.1	0.6	1150	15
5	0.2	1	0.6	600	60

continued Table 1…

6	1.75	0.1	0.05	600	15
7	0.2	0.1	0.05	600	60
8	0.2	1	0.05	1150	60
9	0.2	0.1	0.6	1150	60
10	1.75	1	0.6	600	15
11	1.75	1	0.6	1150	60
12	1.75	1	0.05	600	60
13	0.2	0.1	0.6	600	15
14	1.75	1	0.05	1150	15
15	1.75	0.1	0.05	1150	60
16	0.2	1	0.6	1150	15

3.2. Particle Size Analyzer (PSA)

Particle size analyzer is a tool used to determine the size distribution of nanometer-sized particles with a measurement principle based on the scattering of laser light by particles in the sample. Light is emitted through a small needle that is sent towards the sample particles and scattered back by the particles towards the detector to be converted into a digital signal [17]. The resulting measurement results showed a particle size of 83.81 nm for 94.4%. The size and shape of nanoparticles can significantly influence their cellular uptake. Spherical nanoparticles with diameters between 50 and 100 nm are particularly effective in cellular internalization, with 50 nm being the most efficient size within this range [18] .The measured Polydispersity (PDI) value is 0.43 so that the particles can be said to be homogeneously dispersed and is an appropriate number for biological polymers. The zeta potential value obtained is +32.86 with a conductivity value of 2.79 with one peak, where the phase graph shows the electrophoresis process and the electro-osmosis process. The resulting zeta potential value illustrates the stability of the nanoparticle system and has a mucoadhesive effect on absorption [19]. The charge on the particle surface generates electrostatic forces that are positively correlated to the mucoadhesive strength. Although there is a lack of research on the correction of zeta potential with mucoadhesion so as to support the enhancement of oral absorption. In addition, a positive zeta potential may favor nanoparticle and mucosal attachment compared to a negative zeta potential [20] [21].

Table 2. Particle Size Distribution Statistics

No.	Name	Mean
1	Z-average (nm)	415.6
2	Polydispersity index (PI)	0.43
3	Intercept	0.97
4	Mean Count Rate (kcps)	229.5
5	Peak 1 Mean by Number orders by size (nm)	83.81
6	Peak 1 Area by Number ordered by size (%)	94.4
7	Peak 2 Mean by Number ordered by size (nm)	359.8
8	Peak 2 Area by Number ordered by size (%)	5.60

Table 3. Zeta Potential Statistical Data.

No.	Name	Mean
1	Zeta Potential (mV)	32.86
2	Zeta Deviation (mV)	4.55
3	Conductivity (mS/cm)	2.79
4	Quality Factor	6.38
5	Mean Count Rate (kcps)	309.3

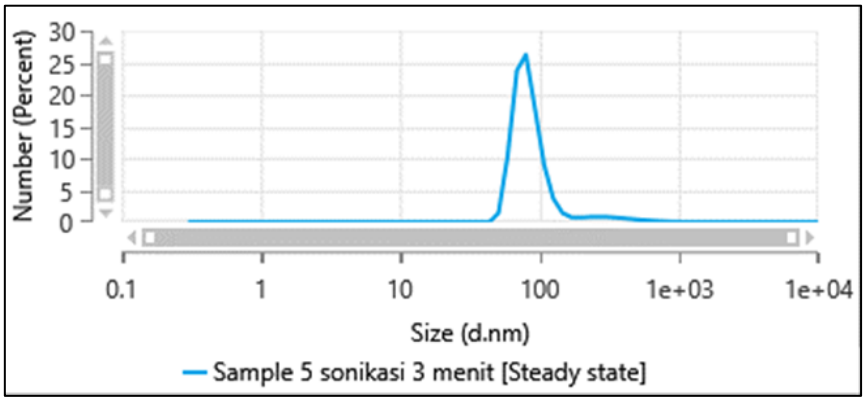


Figure 2. Distribution of Nanoencapsulation Vaccine Particle Chart

3.3. Physical and Chemical Stability

The nanoencapsulated preparation of the COVID-19 vaccine in liquid form with storage at 4°C for 7 days was physically evaluated based on its organoleptic state. Meanwhile, chemical stability was measured through vaccine nanoencapsulation levels. Organoleptically, the samples from day 0 to 7 were liquid, cloudy and contained fine particles as shown in table 4, so they had good stability in terms of organoleptics because they did not show changes during the test time. Figure 3 shows that the absorbance of the sample changed, although not significantly, which could be due to the sample being less homogenized and light fluctuations resulting in variations in the intensity of light reaching the instrument detector. The difference in levels obtained during the test showed a very low F-value (0.213) and a very high p-value (0.69) by one-way ANOVA test. This illustrates that the nanoencapsulated levels obtained were not significantly different from day 0 to 7. The figure 3 also shows an R square value of 0.0962, meaning that storage duration has very little effect on the nanoencapsulation content.

Table 4. Vaccine Nanoencapsulation Stability

Hari ke-	Amount of Vaccine	Organoleptics
0	223.8	Transparent cloudy, fine particles present
3	246.2	Transparent cloudy, fine particles present
5	181.0	Transparent cloudy, fine particles present
7	222.2	Transparent cloudy, fine particles present

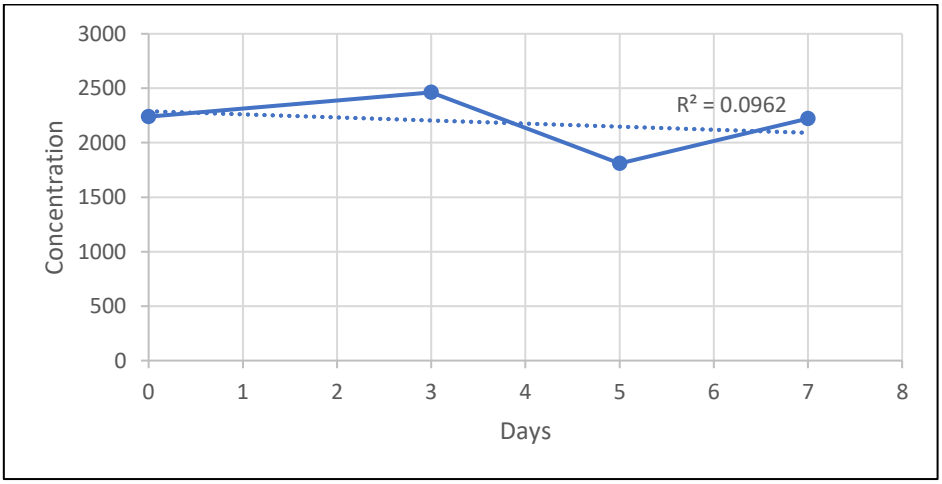


Figure 3. Vaccine Nanoencapsulation Level Chart for 7 Days

3.4. Morphological Characterization of Nanoencapsulation with SEM

The surface morphology of vaccine nanoencapsulated particles was evaluated using Scanning Electron Microscopy (SEM). SEM is capable of providing high resolution images of the surface of a sample. SEM works by utilizing the backscattering of electrons that appear on the surface of the object and taking pictures by detecting electrons that appear on the surface of the object [22]. Evaluation carried out at 5000x magnification shows a tendency to be spherical, less solid, and less uniform. SEM results are less uniform and less solid can be caused because the sample is less homogenized before drying and the sample drying time is less long. Spherical nanoparticles are often found to have higher cellular uptake rates compared to non-spherical shapes like rods or discs [23]. For example, in studies using Caco-2 cells, the uptake of rods and discs was significantly higher than spheres, but there was no difference between the uptake of rods and discs [24].

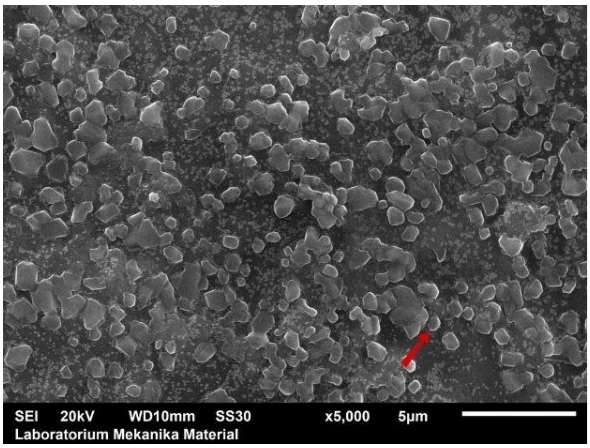
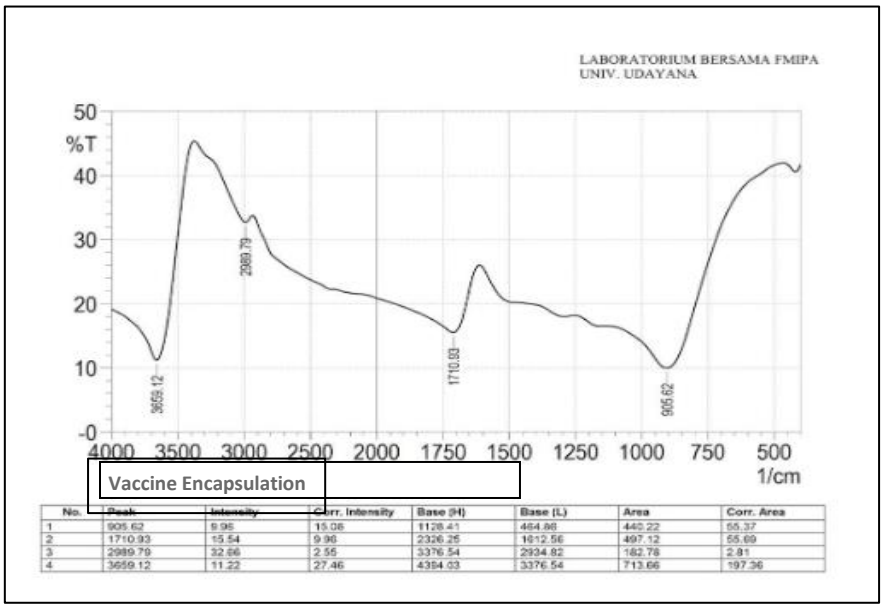


Figure 4. Scanning electron microscopy 5000x SEM results nanoencapsulation

3.5. Characterization of Nanoencapsulated Functional Groups with FTIR

Fourier Transform Infrared (FTIR) is one of the tools to represent molecular structures based on atomic vibrations. FTIR shows the specific nature of chemical bonds and molecular structures in a material, especially when analyzing specimens related to polymers and organic components [25]. FTIR peaks and spectra act as fingerprints of specific molecular structures and chemical bonds. Qualitatively, FTIR is used to identify chemical functional groups in vaccine nanoencapsulation, including components such as chitosan, and alginate.



No.	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	905.62	9.96	15.08	1128.41	464.86	445.22	55.37
2	1710.93	15.54	9.96	2326.26	1612.56	497.12	55.69
3	2989.79	32.66	2.55	3376.54	2934.82	182.78	2.81
4	3659.12	11.22	27.46	4384.03	3376.54	713.66	197.36

Figure 5. FTIR Spectra of Nanoencapsulated Vaccine

Table 5. Infrared Data Interpretation

No.	Functional Group	Wave Number (cm^{-1})	Reference Wave Number (cm^{-1})	Interpretation
1	O-H *stretching*	3659.12	3700-3584	chitosan; alginate; *spike* protein vaccine
2	C-H *stretching*	2989.79	3000-2840	chitosan
3	C=O *stretching*	1710.93	1725-1705	chitosan; *spike* protein vaccine;
4	N-H *bending*	905.62	910-665	interaction of chi-NaTPP-alg

Based on Figure 5 and Table 5, the FTIR spectra results show the following: (a) functional groups of chitosan, alginate, namely O-H stretch; (b) functional groups of chitosan, namely aliphatic C-H stretch; (c) C=O stretch bond of acetamide group which is also present in the main chain of chitosan; (d) vaccine protein spike functional groups (amino acids) namely COOH (C=O and O-H); (e) secondary N-H groups with sharp absorption bands, where there is interaction between chitosan with alginate and NaTPP through N atoms in NH and O groups in OH and C=O groups [26].

3.6. In Vitro Release

In vitro release studies are an important evaluation to assess the safety, efficacy, and quality of nanoparticle-based drug delivery systems [27]. In this evaluation, release simulations were performed in three types of fluids that simulate the stomach, intestine, and colon. The release of COVID 19 vaccine nanoencapsulation is expected to be well absorbed in GALT (Associated Lymphoid Tissue) in the intestine. Simulation of the release of samples containing antigens through in vitro release tests in the digestive tract using SGF solution pH 1.2. The absorbance measured was 0.0639, indicating that the vaccine was able to pass through the extreme pH in the stomach because the low absorbance is proportional to the low level of vaccine released. Furthermore, the highest absorbance measured at SIF liquid pH 6.8 was 0.1266 and at SCF liquid pH 7.4 was 0.104. This shows that the release of nanoencapsulated vaccine occurs most in the intestine and decreases in the colon. Based on the graph in appendix 7, the release of nanoencapsulated vaccine is in the right location, namely in the intestine targeting GALT (Gut-Associated Lymphoid Tissues) which is sensitive and an important area for immune response.

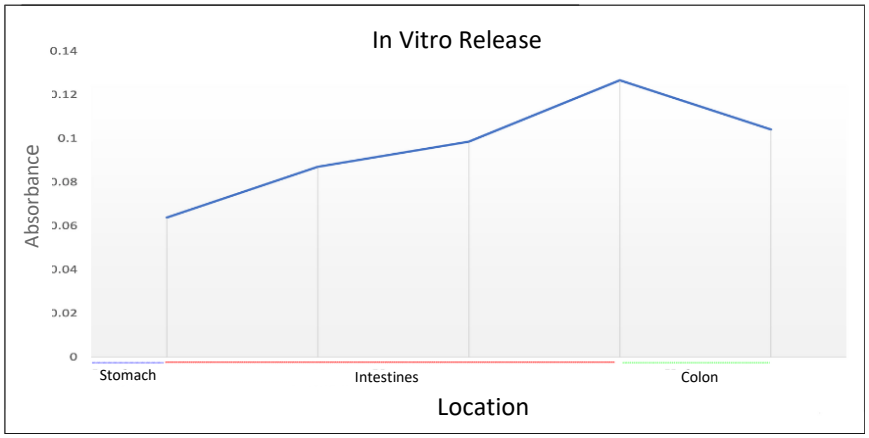


Figure 6. In Vitro Release Chart

4. CONCLUSION

Nanoencapsulation of SARS-CoV-2 oral vaccine with a combination of chitosan and alginate polymers has an optimal formula at a concentration of 1.75% chitosan; 0.1% NaTPP, and 0.05% alginate with a stirring speed of 1150 rpm and a duration of 60 minutes as measured by

%transmittance and %EE. PSA evaluation results showed 94.4% had a size of 83.81 nm with homogeneous particle distribution and zeta potential indicating a stable system that supports mucoadhesive power. Physicochemical stability evaluation from day 0 to 7 showed no significant changes in organoleptics and formula content. SEM showed spherical particle morphology but less uniform. FTIR showed the presence of chitosan, alginate, and vaccine protein functional groups. The release profile of the formula was in accordance with the target achievement, namely having the highest release in the intestine and being able to pass through acidic pH conditions well. The SARS-CoV-2 oral vaccine with chitosan and alginate polymers with the optimum formula has great potential to be developed so that it can be an alternative choice for the community.

Funding: We would like to thank the Ministry of Education, Culture, Research and Technology; the Directorate of Learning and Student Affairs; and Udayana University for funding our research.

Acknowledgments: We are also grateful to all those who have helped in the collection of data and information needed in writing this journal. Finally, we hope that this article can provide benefits and inspiration for readers who are interested in the topic of nanoencapsulation, vaccines, and pharmaceutical innovation.

Conflicts of interest: The authors declare no conflict of interest

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Journal of
Food and Pharmaceutical Sciences

Original Article

Analysis of Rhodamine B in Lip Creams from Marketplace

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Received: 15 June 2024; Revised: 21 September 2024; Accepted: 25 September 2024; Published: 31 December 2024

Abstract: Lip cream is a cosmetic product with a creamy texture that provides long-lasting color for the lips. One of the cosmetic colorants often misused as a lip colorant is Rhodamine B, which is supposed to be used for textile coloring. Rhodamine B may cause irritation, is carcinogenic, and can even cause liver damage in high concentrations. This study aims to determine whether lip creams sold in marketplaces contain Rhodamine B and measure the concentration of Rhodamine B in these products. Qualitative analysis of the Rhodamine B content in lip cream was carried out using the thin layer chromatography (TLC) method with the stationary phase silica gel GF254 and the mobile phase n-butanol: ethyl acetate: 25% ammonia with a ratio of 10:4:5. Of the 5 samples that were qualitatively analyzed using TLC, 3 samples were positive for containing rhodamine B, samples number 1, 2, and 3. Quantitative analysis was conducted to determine Rhodamine B concentrate using the spectrophotometry UV-Vis method with a maximum wavelength of 546 nm. The results of assay determination showed that the average concentration containing rhodamine B was 0.044% in sample number 1, 0.060% in sample number 2, and 0.052% in sample number 3.

Keywords: Lip cream; Rhodamine B; Spectrophotometry UV-Vis; Marketplace

1. INTRODUCTION

The development of cosmetics has undergone significant transformation over time, reflecting changes in trends, technology, and consumer needs [1]. The majority of cosmetic purchases through online stores are because the prices are cheaper. There are many cosmetic products circulating on the market and online, one of which is lip cream. Along with the times and increasing lifestyles, the use of cosmetics has increased nowadays, especially for lip coloring. Cosmetic products such as lip cream have become very popular because they can beautify the appearance of the lips with various colors and textures [2]. Lip cream is a cosmetic product for the lips that has a creamy texture and usually provides a matte or satin finish. Lip cream is known for its high pigmentation, so it can provide intense color [3]. However, along with increasing demand, there is also the risk of dangerous ingredients in cosmetic products, especially through online platforms [4].

Currently, there are many misuses of synthetic colors, especially for cosmetics. Synthetic colors are usually used by cosmetic manufacturers because they are stronger, more affordable, and more stable, but they can have bad effects on health [5]. Rhodamine B is a synthetic dye whose use in cosmetics is prohibited and is declared a dangerous substance according to the Regulation of Indonesian Food and Drug Authority Number HK.03.1.23.08.11.07517 of 2011 concerning Technical Requirements for Cosmetic Ingredients because it can cause liver, kidney and lymph damage followed by anatomical changes in the form of organ enlargement [6]. Rhodamine B in high

concentrations can cause liver damage. Even though it has been banned by the government, the use of dangerous synthetic dyes is still not widely used, especially for cosmetic dyes. The distribution of lip cream through marketplaces adds to the challenges in monitoring the quality of cosmetic products. Cosmetic products often do not go through adequate due diligence and product safety. This raises concerns about the existence of lip cream products containing Rhodamine B circulating in marketplaces

Based on this description, researchers are interested in conducting research with the title analysis of rhodamine B in lip cream circulating via marketplaces using spectrophotometry UV-Vis. This research is important to ensure the safety of cosmetic products on the market, protect consumers from health risks posed by dangerous substances, and provide information that can be used by authorities to improve regulation and supervision of cosmetic products. Samples obtained from marketplaces are selected with the cheapest and best-selling prices in the shop and do not have well-known brands or registration numbers.

2. MATERIALS AND METHOD

2.1. Materials

Tools used in this research include analytical scales (Ohaus), volume pipettes (Pyrex Iwaki), tweezers, water baths, metal spatula, and spectrophotometry UV-Vis (Shimadzu). The materials used in this study were lip cream samples sold in marketplaces with criteria no brand and have no BPOM license., lipstick samples, 2% ammonia, 10% ammonia, 25% ammonia pro analysis, silica gel GF254, ethanol 70%, ethanol 96% (pro analysis), Rhodamine B, ethyl acetate, n -butanol, acetic acid 10%.

2.2. Quantitative Analysis of Rhodamine B

Qualitative analysis was carried out using the thin-layer chromatography (TLC) method. The stationary phase of silica gel GF 254 is activated by heating at a temperature of 105^0C for 5 minutes. The mobile phase in the identification of n-butanol-ethyl acetate - ammonia 25%) with a ratio of 10:4:5. The results were visually observed, the stain appeared pink, and under UV light 254 nm and 366 nm, it fluoresced or orange. Then the Rf value is calculated, and the result is declared positive if the spots between the sample and the standard are the same or close to each other with a difference of ≤0.2 [7].

2.3. Qualitative Analysis of Rhodamine B

The first stage carried out in determining the maximum wavelength. Pipet 3 ml of 100 ppm rhodamine B solution (parts per million) using a volume pipette and put it into a 100 ml volumetric flask (concentration 3 ppm), then add 96% ethanol to the mark and homogenize. The maximum absorption was measured at a wavelength of 400-800 nm using a blank. The blank used is 96% ethanol [8]. Next, determine the calibration curve by making a standard solution of rhodamine B with a concentration of 7,9,11,13,15 ppm for each solution. Then the absorbance is measured at the maximum wavelength obtained and 96% ethanol is used as a blank to obtain a calibration vs absorbance curve.

Sample preparation with 1 gram of sample put into an Erlenmeyer and then soaked in 10 ml of 2% ammonia solution dissolved in 70% ethanol with a ratio of 1:2. The solution is filtered and then heated over a water bath. The residue from evaporation was dissolved in 10 ml of 10% acetic acid solution. Next, the wool thread is put into the solution and heated for 10 minutes. Then the wool yarn is washed with distilled water and placed in a 10% ammonia base solution. The solution is heated until the color of the wool thread dissolves, then the alkaline solution obtained is used as a sample in

the spectrophotometric UV-Vis reading. The test solution was extracted, put into a cuvette then put into a spectrophotometer and measured at the maximum wavelength obtained, and as a blank 96% ethanol was used [9].

2.4. Data Analysis

Quantitative analysis was used to calculate rhodamine B levels using UV-Vis spectrophotometry, then statistical analysis was carried out by determining linear regression. From the UV-Vis spectrophotometry method data in the form of absorbance data which is calculated using the equation y = bx + a. Calculation of Rhodamine B levels in the preparation sample using the formula below:

$$C = \frac{X \, x \, V \, x \, F}{w}$$

Information

C = Rhodamine B content in the sample (mg/L)

X = Rhodamine content after dilution (mg/L)

V = Sample volume (ml)

F = Dilution Factor

W = Sample weight (mg)

3. RESULTS AND DISCUSSION

Sampling was carried out on lip cream samples that were red in color, cheap in price, and did not have number registration with the Indonesian Food and Drug Authority (BPOM), totaling 5 samples with various brands and shops sold through the marketplace. Organoleptic tests use the five senses based on shape, color, smell, and texture. The organoleptic test results can be seen in Table 1.

Table 1. Organoleptic test results

Parameter test	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Shape	Cream	Thick liquid	Cream	Cream	Thick liquid
Smell	fragrant	Fragrant	Fragrant	Not smell	fragrant
Texture	Smooth and slightly oily	Smooth and lumpy	Smooth, color does not fade easily	Smooth and slightly oily	a little sticky

The characteristics of lip dyes that contain rhodamine B are that the color is bright and more striking, sometimes the color does not look homogeneous, there is a strong odor, there are lumps of color on the product, it does not include a code, label, brand, content information or other complementary identity [5]. Rhodamine B can be detected by a color change that is easily observed visually. A pink color change occurs when Rhodamine B is dissolved in an aqueous or ethanol solution, and the more concentrated the color, the higher the concentration of Rhodamine B [10].

3.1. Qualitative Analysis of Rhodamine B

The method used is the thin layer chromatography method which is a technique for separating compounds using the principles of adsorption and partition coefficient [11]. The TLC method was chosen because it is affordable, specific in separating one component from other components, and

simple to work with [12]. The principle of TLC is to separate samples based on differences in polarity known as the "like dissolve like" principle, meaning that polar likes polar and non-polar likes non-polar. The results of the TLC analysis can be seen in Figure 1 and Table 2.

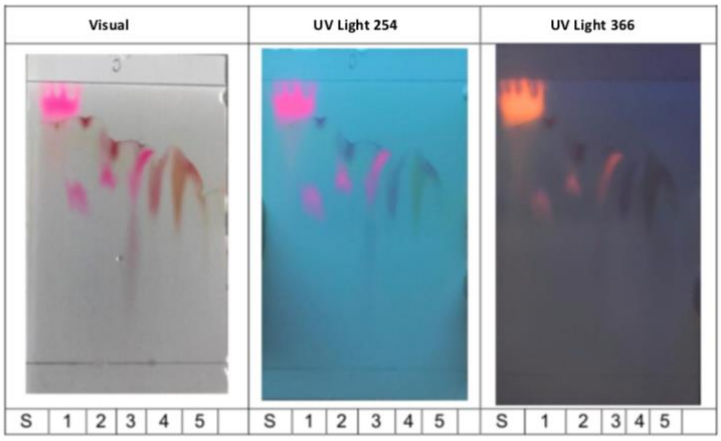

Figure 1. TLC Profile. (S) Standart of Rodhamin B (1) sample 1 (2) Sample 2 (3) Sample 3 (4) Sample 4 (5) Sample 5

Table 2. The results of the TLC analysis

Sample	Spotting			RF	Result
	Visual	**UV 254**	**UV 366**		
Rhodamine B Standard	Pink	Fluorescent	orange	0.93	Positive
Sample 1	Pink	Fluorescent	Orange	0.73	Positive
Sample 2	Pink	Fluorescent	Orange	0.75	Positive
Sample 3	Pink	Fluorescent	Orange	0.87	Positive
Sample 4	Purple	Does not fluoresce	Purple	0.74	Negative
Sample 5	Purple	Does not fluoresce	Purple	0.87	Negative

Rhodamine B exhibits a bright red color, which intensifies under UV light. Based on research if the stain containing the rhodamine B compound is visually seen it is pink, when viewed under UV light with a wavelength of 366 nm it will fluoresce yellow or orange [13].

3.2. Quantitative Analysis

The quantitative test using UV-Vis spectrophotometry aims to determine the levels of Rhodamine B contained in lip cream circulating via marketplaces. This spectrophotometric method has a principle, namely the Lambert-Beer law, where Lambert-Beer law states that the concentration of a substance is directly proportional to the amount of light absorbed, or inversely proportional to the logarithm of the light transmitted [11].

Substances that can be analyzed using spectrophotometry UV-Vis are substances in solution form and substances that appear colored because Rhodamine B is a compound or molecule that gives color due to the presence of a chromophore group, where the chromophore group is quinine. The quantity of color produced by Rhodamine B is very sharp. This is caused by the presence of an autochrome group, where the autochrome group is dimethyl amine. So a quantitative examination of the sample was carried out using UV-Vis spectrophotometry [14]. In quantitative analysis, the maximum wavelength in the rhodamine B solution is determined first. The results of determining

the maximum wavelength with a wave range of 400-800 nm obtained a wavelength of 546 nm. The results of this research are almost similar to previous research, namely that a maximum wavelength of 545 nm was obtained [8]. The purpose of making a standard curve is to determine the linearity of the concentration of the rhodamine B standard solution with the absorbance value [15]. From the absorbance results of the standard solution of rhodamine B, a curve was then created which is the relationship between absorbance (y) and concentration (x) to determine the linear equation and relationship coefficient values. The linearity of the rhodamine B solution calibration curve can be seen in Figure 2.

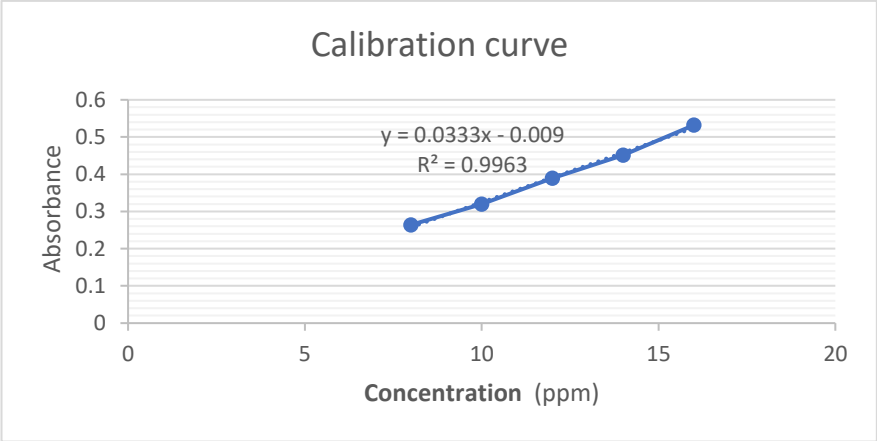

Figure 2. Linearity of the rhodamine B solution calibration curve

The calibration curve shown shows the relationship between environmental concentration (ppm) and absorption. Since the R^2 value is close to 1, it can be concluded that the data has very good linearity; that is, the relationship between concentration and absorption is almost completely linear.

Sample preparation was carried out using the wool yarn absorption method. The principle of the wool yarn absorption method is to draw the dye from the sample into the wool yarn in an acidic atmosphere with heating followed by dissolving it with a base.[14]. The binding mechanism for Rhodamine B in the wool yarn is because the wool yarn is composed of peptide bonds which contain cystine, glutaric acid, aspartic acid lysine, and arginine bonds. Rhodamine B can pass through the cuticle layer by converting the sestine into cysteine by an acid. Cysteine is formed by breaking down the SS bonds of cystine in an acidic environment. The opening of these bonds causes the entry of Rhodhamin B into the wool thread. Rhodamine B enters the wool yarn and binds to COO- from aspartic acid and also binds to +NH3 from Arginine [11]. The binding mechanism of rhodamine B in wool yarn can be seen in Figure 3.

The wool thread will release the dye, and the dye will enter the alkaline solution. This solution will be a sample sample for quantitative analysis using spectrophotometry UV- Vis [16]. In the TLC results, 3 of the 5 lip cream samples contained rhodamine B, so the samples containing rhodamine B were continued with quantitative analysis using the Uv-Vis spectrophotometric method. From the results, it is known the percentage of rhodamine B found in lip cream-type cosmetics, where 3 out of 5 lipstick samples do not meet cosmetic requirements because there is the synthetic dye Rhodamine B. The concentration of rhodamine B levels can be seen in Table 3.

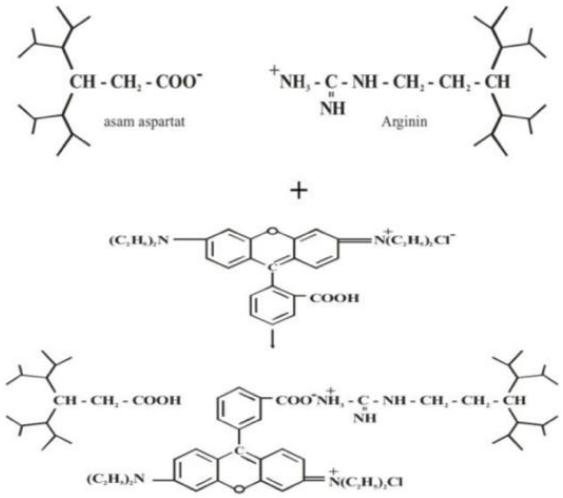

Figure 3. Mechanism of rhodamine B in wool

Table 3. Results of Rodhamine B concentration in lip cream samples

Samples	Replication	Concentration (%)	Average Concentration	SD
1	1	0.044	0.044%	0.00047
	2	0.045		
	3	0.044		
2	1	0.057	0.060%	0.00245
	2	0.063		
	3	0.060		
3	1	0.052	0.052%	0.00082
	2	0.054		
	3	0.053		

Based on Indonesian Food and Drug Authority regulation No. 23 of 2019 concerning technical cosmetic ingredients, cosmetics distributed must meet the requirements, namely, cosmetics must use ingredients that meet standards, and be registered and obtain a distribution permit from Indonesian Food and Drug Authority. Rhodamine B dye is prohibited from being added to the manufacture of cosmetics according to the Regulation of the Head of the Indonesian Food and Drug Authority regulation No. 18 of 2015 concerning Technical Requirements for Cosmetic Ingredients because it can cause negative effects on health. Rhodamine should not be used in cosmetics, especially as a dye. Rhodamine B can cause acute effects if ingested at 500mg/kg BW, which is a toxic dose. The possible toxic effect is gastrointestinal irritation [6].

Many studies and investigations have found that lip cream or lipstick products sold online, especially inexpensive ones that are not registered with Indonesian Food and Drug Authority, carry a higher risk of containing Rhodamine B. Additionally, many cosmetics such as face creams sold in marketplaces contain hazardous ingredients like mercury and hydroquinone [12]. Therefore, Indonesian Food and Drug Authority plays a crucial role in supervising the safety of cosmetics, especially those sold online.

4. CONCLUSION

Based on the research results of Rhodamine B analysis on lip cream samples sold online, it was found that 3 out of 5 samples were identified as positive for containing rhodamine B, in samples number 1, 2, and 3. The results of quantitative analysis, using UV-Vis spectrophotometry, obtained

average levels. The average content containing rhodamine B was 0.044% in sample number 1, 0.060% in sample number 2, and 0.052% in sample number 3.

Acknowledgments: This research was supported by the Institute for Research and Community Service (LPPM), Nusaputera College of Pharmaceutical Sciences

Conflicts of interest: The authors reported no potential conflicts of interest.

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Journal of
Food and Pharmaceutical Sciences

Research Article

Irritation Assessment of Herbal Deodorant from Essential Oil of Pomelo (*Citrus maxima*) Peel on Rabbit Skin

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Received: 9 February 2024; Revised: 12 September 2024; Accepted: 30 September 2024; Published: 31 December 2024

Abstract: A deodorant manages an unpleasant body odor through both reducing and suppressing antibacterial activity and it is formulated generally using antibacterial agents and fragrances. Nowadays, quaternary ammonium compounds like triclosan, aluminum salts, and odor eliminators are used as antibacterial agents and fragrance in deodorant products. A nature-based deodorant potentially replace the synthetic deodorant with less irritating effect. Pomelo (*Citrus maxima*) is one of the Indonesian plants which has some bioactive compounds, including limonene. Essential oil of Pomelo peel (EOPP) exhibits an antibacterial activity as well as fragrance. The aim of this research is to observe the irritation effect of EOPP deodorant on rabbit skin. The irritation assay of herbal deodorant of EOPP with various concentrations (F1: 1.25%; F2: 2.5% and F3: 5%) was carried out based on protocol for dermal acute irritation test on rabbit skin. All the formulations performed the same grades (negligible criteria) with the irritation index of F1, F2 and F3: 0; 0.037 and 0.148, respectively. The herbal deodorants with various concentrations of EOPP did not show an irritating effect on rabbit skin.

Keywords: herbal deodorant; essential oil; skin irritation; Pomelo peel; Citrus maxima

1. INTRODUCTION

Perspiration induces the interaction between bacteria, (e.g. *Staphylococcus epidermidis*) and the dead cells in the skin and lead to bromhidrosis axilla (BA) [1], [2], [3], [4]. Additionally, BA is possibly a result of the activity of microbial enzymes [5], [6]. This body odor may cause discomfort [1], [7]. A deodorant manages an unpleasant body odor through both reducing and suppressing antibacterial activity [8]. Deodorant is formulated generally using antibacterial agents and fragrances [9], [10]. Quaternary ammonium compounds like triclosan (TCS), aluminium salts, and aromatic odor-masking agents are used as antibacterial agents in deodorant products [11]. Although some of these agents have bad effects, not only irritating or sensitizing the skin, but also may improve Alzheimer's disease condition, breast and prostate cancers. Also, the risk of antibiotic resistance may occur. Thus, a herbal deodorant is an alternative for treatment of BA due to its potency against *Staphylococcus sp*. [12], [13].

Pomelo or *Citrus maxima* is one of the Indonesian plants which has some bioactive compounds. For instance, lycopene; pectin; flavonoids; vitamin C; limonene; 9,10-anthracenedione;

and 1,8-diethoxy-antraquinon [14]. Furthermore, the essential oil of Pomelo peel (EOPP) exhibits the MIC and MBC against *Staphylococcus epidermidis* were 0.3125 % v/v and 1.25 % v/v, respectively. In addition, EOPP may also act as a fragrance and thus EOPP has functions both as an antibacterial agent and fragrance in deodorant formulations [15]. Therefore, EOPP was formulated as a deodorant product in stick form whereas till date, there is no deodorant product developed using EOPP.

Toxicity study needs to be conducted in order to observe the safety of new products, including cosmetics before used. In this study, we performed a nonclinical toxicity assay, particularly an acute irritation test on rabbit skin [16].

2. MATERIALS AND METHODS

2.1. Materials

Pomelo fruit (*Citrus maxima*) obtained from local market and determination number conducted in Pharmacognosy and Phytochemistry Laboratory with voucher number 0126/C/UD-FF/UMI/VII/2024, sodium sulfate anhydrous (PA, CP), distilled water, sodium hydroxide (PA, Merck), propylene glycol (Technical), stearic acid (cosmetic grade), cetyl alcohol (cosmetic grade), and litmus paper (NESCO®).

2.2. Methods

2.2.1. Sample Preparation

The essential oil of Pomelo peel was extracted by hydro-distillation. Three hundreds mililitres (300 mL) of distilled water was added to 200 g of fresh pomelo peel. The extraction process was done for ± 3 hours and it was repeated until 500 g of pomelo peel was extracted. The pure essential oils were collected by separating the water using sodium sulfate anhydrous.

2.2.2. Stick Deodorant Formulation

The essential oils were formulated to make herbal deodorants using various concentrations with propylene glycol as a solvent (F1 = 1.25%, F2 = 2.5 %, and F3 = 5 %). The basis formulations (stearic acid 25%; sodium hydroxide 2.5%; cetyl alcohol 19%, and Propylene glycol 50%) were then added with the essential oils (F1, F2 and F3).

2.2.3. The Characteristic Assay of Stick Deodorant

a. Organoleptic Test
   The color, shape and smell changes of stick deodorants were observed on day 0 and 14.
b. pH Test
   The pH test was carried out once all the materials of stick deodorant were put in a roll up case (before hardening).
c. Homogeneity test
   The stick deodorants were spread onto a transparent glass to see whether all the ingredients were homogenous once the glass looked clear without coarse particles.

2.2.4. Animal Preparation

We used 3 male albino rabbits in this study. The rabbit hair was cut in the back area about 10 x 15 cm for the testing area, including the control. Before using the animal for this research, it was

approved by the Ethical Committee of Universitas Muslim Indonesia, Makassar (No. 105A.1/KEPK-UMI/IV/2023).

2.2.5. Application and Observation of the Herbal Deodorant

Each of the deodorant formulas (F1, F2, F3) was applied in the testing area (on skin surface at the back area) of rabbits at about ± 6 (2 x 3) cm^2 and a control area, which was an empty area (without applying the deodorant formula). After spreading the formula, all the area, including the controls, were covered with non-irritant gauze and bandage for 4 hours. In the next 4 hours, the gauze and bandage were removed, all the testing area, including the control area, was removed with water. The skin reaction (erythema and edema) was done in 1 hour after the application, and continued in hour 24, 48 and 72 (Table 1).

Table 1. Skin reaction scoring [16]

Erythema reaction	**Score**
None erythema	0
Very little erythema	1
Clear erythema	2
Moderate erythema	3
Heavy erythema (Flesh red)	4
Edema reaction	**Score**
Non edema	0
Very little edema	1
Mild edema (border area looks clear)	2
Moderate edema (the edema area become wider about 1 mm)	3
Heavy edema (the edema area become wider more than 1 mm until out of the application area)	4

2.3. Data Analysis

The Index of Primary Irritation was calculated based on the skin reaction score using the following formula:

$$\text{Index of Primary Irritation} = \frac{A - B}{\text{C}}$$

A: the average score of erythema and edema in all observation time (sample)
B: the average score of erythema and edema in all observation time (control)
C: the total number of tested animal

3. RESULTS AND DISCUSSION

Pomelo (*Citrus maxima*) is an Indonesian plant with numerous bioactive components, including essential oil [14]. EOPP has an excellent antibacterial effect to inhibit the bacteria causing body odor [15]. Furthermore, it also has a pleasant aroma and therefore is suitable to be developed as an herbal deodorant.

EOPP was extracted from Pomelo peel by hydro-distillation and formulated to stick deodorants in different concentrations, F1, F2, and F3 and made triplicate. All herbal formulations with various concentrations (F1, F2, and F3) exhibit good stability both before and after storage until day 14 based on the organoleptic result (Table 2). Organoleptic test was aimed to identify the

stability of the formulations, whether alterations occur, like the physical appearance (solid to liquid), color, and the smell after being stored[17], [18].

Table 2. Results of physical examination of EOPP deodorant

Parameters	**F1**			**F2**			**F3**		
pH	7	7	7	7	7	7	7	7	7
Homogeneity	H	H	H	H	H	H	H	H	H
Organoleptic result									
• **Physical appearance**									
Day 0	Solid	Solid	Solid	Solid	Solid	Solid	Solid	Solid	Solid
Day 14	Solid	Solid	Solid	Solid	Solid	Solid	Solid	Solid	Solid
• **Color**									
Day 0	White	White	White	White	White	White	White	White	White
Day 14	White	White	White	White	White	White	White	White	White
• **Smell**									
Day 0	Weak	Weak	Weak	Moderate	Moderate	Moderate	Strong	Strong	Strong
Day 14	Weak	Weak	Weak	Moderate	Moderate	Moderate	Strong	Strong	Strong

Note: H: Homogeneous

Table 3. Irritation response category of herbal deodorant formula

Formulation	**Index of Primary Irritation**	**Response criteria**
F1	0	Negligible
F2	0.037	Negligible
F3	0.148	Negligible

Aligned with the homogeneous test, all the deodorant formulations perform a satisfactory result (Table 2). Homogeneity test was also done to observe the stability of deodorants [13], [17], [18], [19]. Moreover, pH results showed a non-irritant result and thus a further irritation assay can be conducted using rabbit skin (Table 2), not extreme (pH or pH $\geqslant$ 11.5) and also aligned with the criteria for armpit skin (pH 4-8) [13], [20].

control
erythema score: 0
edema score: 0

F3 (h-24)
erythema score: 0
edema score: 0

F3 (h-48)
erythema score: 0
edema score: 0

F3 (h-72)
erythema score: 0
edema score: 0

Figure 1. Skin reaction score of herbal deodorant on rabbit skin. Notes: h-24: observation in 24 hour after deodorant application; h-48: observation in 24 hour after deodorant application; h-72: observation in 24 hour after deodorant application

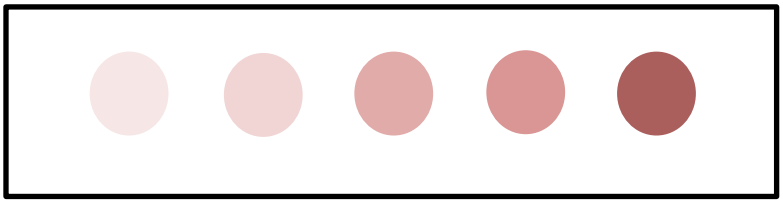

Figure 2. Kit Scoring for erythema in irritation assay on rabbit skin (EC00202305017, 2023; BPOM, 2014)

Since the pH result for all the formulations is 7, it meets the protocol standard for further irritation assay using rabbit skin. This assay was carried out based on an in vivo toxicity guide for acute dermal irritation [16]. The irritation index was determined by scoring the erythema and edema reaction (0-4) of each formulation on rabbit skin (Figure 1). The erythema score was decided using kit scoring due to the possibly subjective decision. This kit scoring (Figure 2) was made from an interpretation of the guide description from an in vivo toxicity guide for acute dermal irritation (Table 2). All the formulations performed the same grades (negligible criteria) with the irritation index of F1, F2 and F3: 0; 0.037 and 0.148, respectively (Table 3). Hence, all deodorant formulations did not show irritating effect on rabbit skin.

4. CONCLUSION

F1 (EOPP 5%), F2 (EOPP 10%), and F3 (EOPP 15%) have the same results for acute dermal irritation test on rabbit skin (negligible). Therefore, the herbal deodorant with various concentrations of essential oils from pomelo peel did not exhibit an irritation effect. Based on organoleptic result, F3 gave the best result due to the good smell. Thus, F3 (EOPP 15%) is the best formulation to be developed as a herbal deodorant.

Acknowledgments: This research was funded by LP2S (Lembaga Penelitian dan Pengembangan Sumberdaya), Universitas Muslim Indonesia (UMI), Makassar.

Conflicts of interest: The authors declared no potential conflict of interest.

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Journal of
Food and Pharmaceutical Sciences

Original Article

Effectiveness of Apple Cider Vinegar Immersion with and Without Mother as a Decrease in Lead (Pb) Levels in Green Mussels (*Perna viridis*)

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Received: 18 August 2024; Revised: 9 October 2024; Accepted: 11 October 2024; Published: 31 December 2024

Abstract: Lead is a heavy metal that often pollutes aquatic biota such as green mussels. Green mussels that contain lead if consumed in humans can pose health risks such as long-term assumptions or in high levels can cause poisoning. To reduce the lead level, we can use apple cider vinegar with and without mother which contains acetic acid and citric acid. The purpose of this study is to observe lead levels of green mussels after soaking with apple cider vinegar with and without mother, percentage decrease in green mussels lead content and the effectiveness of apple cider vinegar with and without mother in reducing lead levels. This study was a quasi-experiment by soaking green mussels using apple cider vinegar with and without mother for 5, 10, 15 minutes. The sample is prepared by drying the sample in the oven and extracting the sample using wet destruction so that a clear liquid is obtained that can be measured using an atomic absorption spectrophotometer. The results showed that the average lead levels sample soaked by apple cider vinegar with mothers were 0.219 mg/L, 0.103 mg/L, 0.088 mg/L, without mothers were 0.205 mg/L, 0.173 mg/L, 0.125 mg/L. The percentage of lead decrease was 2.6%, 49%, 51% used apple cider vinegar with mother while without mother is 8%, 15%, 30%. After the one-way ANOVA test, a p-value of > 0.05 was obtained with no effect of lead reduction. Based on research apple cider vinegar with and without mother can reduce lead levels, but statistically, the decrease is not significant.

Keywords: Lead, Green Mussels, Apple Cider Vinegar

1. INTRODUCTION

Lead is a toxic metal whose use is widespread in the industrial world such as the battery, oil, and paint industries [3]. The use of lead in the industrial field causes the spread to the human environment and poses health risks. Lead exposure in children will decrease intelligence (IQ) while in adults it will cause hypertension and immunotoxicity [10]. Lead can not only cause disturbances in humans but can cause disturbances in waters, especially the sea. The sea is a route of maritime industry activity and the final destination of waters. The activity generates residues, including ship oil spills, paint disposal, and uncontrolled disposal of lead waste [5]. The way to detect pollution can be using marine animals such as green clams.

Green mussels are biota that are susceptible to heavy metal pollution including lead because green mussels have a very high ability to accumulate lead where, in addition to filtering microscopic

particles such as plankton, green mussels absorb lead for consumption [20].In the study [12], green mussels could absorb 10 times more lead, ranging from <5 to 59-64 μg, compared to blood mussels (Anadara antiquata) and simping mussels (Amusium pleuronectes). On the other hand, green mussels are used for consumption by the public because of their delicious taste and economical price [2]. In addition, green mussels have a high nutritional content such as protein 21.9%, fat 14.5%, carbohydrates 18.5%, ash 4.3%, and water 40.8%. The high nutritional content is proportional to the high level of lead [16].

To reduce lead levels in green clams, metal-binding agents such as acetic acid and citric acid compounds are needed. The acid content is found in one of the food additives, namely apple cider vinegar. Apple Vinegar is a fermented fruit vinegar that converts sugar into alcohol and is processed into acetic acid [19]. There are 2 types of apple cider vinegar circulating on the market, namely apple cider vinegar with and without mother. Apple cider vinegar with mother does not go through the pasteurization process so it produces apple cider vinegar that is rich in probiotics and high in acid, while apple cider vinegar without mother has gone through a pasteurization process that has the potential to reduce probiotics and acid content in it [15]. The mother vinegar in apple cider vinegar is the substance resulting from the process of making apple cider vinegar itself. This substrate forms like jelly or a thin layer consisting of a group of enzymes, acetic acid bacteria and cellulose. This vinegar is without a filtering process so it is still present in it and is often considered more useful [4].

According the research [1], acetic acid and citric acid can reduce lead levels whereas blood mussels soaked with acetic acid for 1 and 3 hours can reduce lead levels by 4% and 23%. Meanwhile, soaking with citric acid for 1 and 3 hours can reduce lead by 2% and 20%. The purpose of this study is to see a decrease in lead levels using apple cider vinegar with and without mother that contains these acid compounds. This research was carried out with a variation of 5, 10, 15 minutes because in the community soaking green mussels before cooking requires a minimum of time so that the cooking process becomes efficient and does not change the taste or texture of the green clams. In previous studies, there was an efficient time of 15 and 30 minutes. So, researchers want to see a decrease in lead levels with minimal time.

2. MATERIALS AND METHODS

This study is a quasi-experimental study that analyzes the decrease in lead levels using apple cider vinegar with and without mother. This research was conducted at the Samarinda Industrial Research and Standardization Center (Baristand) from December 2023 - May 2024. The sample used was in the form of 150 grams of green clam meat where each examination was carried out in a duplo with 3 variations (5, 10, and 15 minutes) and 2 treatments (apple cider vinegar with mother and apple cider vinegar without mother). Each examination uses 5 grams of samples and then a duplicate is carried out so that 15 grams of samples are needed. The sampling technique used is purposive sampling with the criteria of green mussel samples measuring 4-6 cm in a fresh state.

2.1. Materials

The tools used in this study were the flame Atomic Absorption Spectrophotometer (SSA), measuring flask, erlenmeyer, glass funnel, porcelain cup, desiccator, oven, volume pipette, analytical scale, and stopwatch. The materials used in this study were green clam meat, apple cider vinegar with and without mother, HNO_3 65%, H_2O_2, $NH_4H_2PO_4$, and lead standard solution.

2.2. Methods

This research includes three stages, namely pre-analytical, analytical, and post-analytical. The first stage is the pre-analytical stage which includes preparation of tools and materials. Next is the analytical stage. The main standard solution has a concentration of 1000 mg/L which is diluted to 100 mg/L using aqua dest. Then a standard series solution was made with concentrations of 0, 0.25, 0.5, 0.75, and 1 mg/L from the raw solution of 100 mg/L. Then the lead level was tested using an atomic absorption spectrophotometer and the absorbance of each solution was recorded and a curve was made to determine the straightline equation. If the linear regression correlation coefficient r > 0.995 is obtained, the AAS tool is ready to use.

Sample preparation is carried out by separating the green scallop meat to be used then the sample grinding process is carried out using a blender. Then, a fine sample was followed by 3 duplications weighing 15 grams with a classification of three parts without treatment and six parts of apple cider vinegar treatment soaked with and without a parent for 5, 10, and 15 minutes. After that, the drying process is carried out in an oven at a temperature of 70-80^{o}C for 6 hours and put on a desiccant. The dried samples were subjected to a wet destruction process where 5-10 ml of HNO_3 65% and 2 ml of H_2O_2 were added and reheated using an oven at a temperature of 180^{o}C for 30 minutes until the color of the solution became clear. The solution is transferred to a 50 ml measuring flask, then a modifier matrix and aquades are added to the border.

Lead testing begins with a standard reading to see the calibration curve that aims to obtain a regression line equation of the lead standard curve. After obtaining the standard curve, the sample was tested with SSA with a wavelength of 283.3 nm so that the lead content was obtained. After the analytical stage, it is followed by the post-analytical stage where the calculation of the lead level obtained is carried out.

The calculation of lead levels is:

$$\text{Pb concentration mg/kg} = \frac{(D-E)xFpxV}{W}$$

Information:

D : Concentration of microgram samples per l from SSA

E : Concentration of microgram blanks per l of SSA

Fp : Dilution factor

V : The final volume of the sample solution (ml), must be changed to liter units

2.3. *Data Analysis:*

The results of the data obtained were analyzed descriptively and statistically. Descriptively, lead levels are presented as percentages and result in a decrease in lead levels. Furthermore, a statistical test was carried out which began with a normality test with a significance of 0.070 > 0.05 and a homogeneity test of 0.100 > 0.05 which showed normal and homogeneous data. After the data is normal and homogeneous, it is continued with the one-way ANOVA test. One-way ANOVA test and obtained a p value of 0.388 > 0.05 which means that there is no effect of reducing lead levels after soaking apple cider vinegar with and without mother.

3. RESULTS AND DISCUSSION

3.1. Average Lead (Pb) Levels in Green Mussels (Perna viridis)

Green mussels are soaked and after soaking apple cider vinegar with and without mother for 5, 10, and 15 minutes to see the lead level. The results of the lead level check can be seen in Table 1 as follows:

Table 1. Average Lead Levels in Green Mussels

		Lead Levels After Soaking	
	Initial Lead Levels (mg/L)	**Apple Vinegar with *Mother* (mg/L)**	**Apple Vinegar Without *Mother* (mg/L)**
W1 (5 minutes)	0.225	0.219	0.205
W2 (10 minutes)	0.205	0.103	0.173
W3 (15 minutes)	0.180	0.088	0.125

Based on the results of the study in Table 1 and Figure 1, the average results of green clam lead content with apple cider vinegar soaking with and without mother were obtained. Soaking apple cider vinegar with mother with the highest average lead content for 5 minutes is 0.219 mg/L and the lowest for 15 minutes is 0.088 mg/L. Soaking apple cider vinegar without mother with the highest average lead content for 5 minutes is 0.205 and the lowest for 15 minutes is 0.125 mg/L. Where the longer the soaking time, the lower the lead level, this result is in line with the study [11] that soaked green clams using tomato filtrate for 30 minutes, the highest lead content was 0.65 mg/L and the lowest for 90 minutes was 0.39 mg/L. This is because the long soaking time can make citric acid and acetic acid compounds in apple cider vinegar and citric acid in tomatoes interact with the samples. This is supported by the theory [6] that acetic acid can bind lead at a certain time, the longer the soaking time affects the lead level. This shows a reduction in lead levels either using apple cider vinegar with or without mother, according to the General Standard for Contaminants and Toxins in Food and Feed Codex Stan 193-1995 amended in 2019 the limit of lead metal contamination in fish commodities that can be consumed is 0.3 mg/L which results in lead levels after immersion below the set limit so that it is safe to consume.

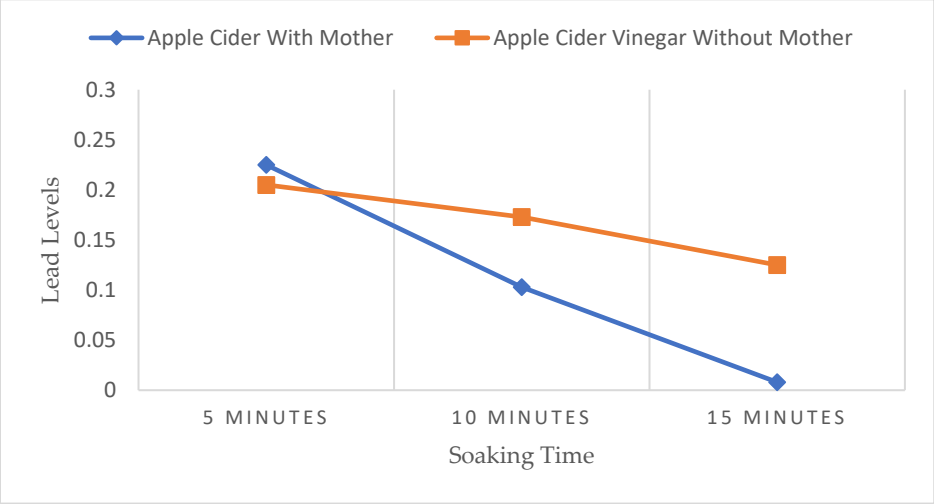

Figure 1. The Decrease in Lead (Pb) Levels

3.2. Percentage Decrease in Lead (Pb) Levels in Green Mussels (Perna viridis)

The percentage of lead reduction was obtained by reducing the lead level before treatment by the level after treatment divided by the level before treatment and multiplied by 100%. The results of the calculation of lead reduction can be seen in Table 2 as follows:

Table 2. Percentage Decrease in Lead Levels in Green Mussels

Time	Drop-down margin			
	Until		Percentage	
	With *Mother* (mg/L)	Without *Mother* (mg/L)	With *Mother* (%)	Without *Mother* (%)
5 Minutes	0.006	0.020	2.6	8
10 Minutes	0.102	0.032	49	15
15 Minutes	0.092	0.055	51	30

According to Table 2, the highest percentage of lead content reduction was soaking apple cider vinegar with the mother for 15 minutes by 51% and the lowest percentage was soaking apple cider vinegar with the mother for 5 minutes by 2.6%. The decrease in lead levels was relatively large from 2.6% to 51%, this can occur due to the use of apple cider vinegar with mother, which in addition to containing organic acids also contains more probiotics, enzymes, and organic acids than apple cider vinegar without mother. Enzymes and bacteria increase the biological and chemical activity of apple cider vinegar so that it can lower lead even more [15]. According to the time interval where from 5 minutes to 15 minutes can make a longer interaction of the content of the organizer lower the lead. According to the theory [7] where acetic acid can bind lead at a given time, the longer the immersion time affects the lead content.

These results are not in line with a previous [1] that soaking blood clam meat using acetic acid and 25% citric acid separately for 1 and 3 hours. The decrease in using acetic acid for 1 hour was 4% and for 3 hours was 23%. Meanwhile, the decrease using citric acid for 1 is 2% and for 3 hours is 2% and 20%. This result is different from the results obtained by the researcher due to the difference in the solution used as a sample soak. The research [1] uses acetic acid and citric acid separately while the researcher uses apple cider vinegar which contains 2 compounds, namely acetic acid and citric acid. According to theory [17] the decrease in metal content can be caused by the ability of chelating agents or substances that bind metals to the sample soaking solution. When the chelating agents, namely acetic acid and citric acid in apple cider vinegar, are used to lower lead, the lead level will decrease to the maximum.

Based on the theory of the content of citric acid and acetate, it can bind lead (chelator agent) so that the lead level is lower. The binding mechanism of citric acid to lead is due to the ability of carboxylate groups to bind lead. According to the theory [7] citric acid, it has a structure of 3 carboxylic acids that can form complexes with metals such as lead. The carboxyl group will release protons (H^+) and remain citrate ions (-COO-). The theory [13] states that when the proton acid is released, then the lead ion (Pb^{2+}) will be released from its complex and bind to citric ions to form citric

salts and dissolve in the citric acid solution so that the lead level decreases. In addition to seeing a decrease in lead levels, a test of the effectiveness of soaking apple cider vinegar was also carried out.

As for acetic acid (CH_3COOH) is the main constituent in apple cider vinegar which functions as an effective settling agent. Acetic acid can interact and bind lead through a chemical process, resulting in a complex that is insoluble in water. Research explains that citric acid compounds can act as heavy metal carriers [14]. When citric acid interacts with heavy metals, they form a complex bond. In this bond, atoms that have free ions will bond with heavy metals [18].

According to the theory [8], acetic acid dissolved in water will give hydrogen ions (H^+) and enlarge the hydrogen ions in the solution. This reaction will form a lead acetate compound that breaks down into separate ions. Since acetic acid is a weak acid, it will release a small amount of hydrogen ions (H^+) in the solution and make lead acetate tend to be intact without fully decomposing into separate ions. In addition to seeing a decrease in lead levels, an effectiveness test of apple cider vinegar soaking was also carried out.

3.3. Effectiveness of Apple Vinegar with and Without Mother in Reducing Lead (Pb) Levels in Green Mussels (Perna viridis)

Based on the results of the study, each shellfish that was soaked experienced a decrease. To determine the effectiveness of apple cider vinegar with and without mother in lowering lead, statistical tests were carried out. Statistical tests were carried out using the one-way ANOVA test which began with testing normality and homogeneity tests. The normality test was carried out with the Shapiro-Wilk test to see the normality of the distribution of research data. A homogeneity test was also performed, which used the Levene test to see if the data were homogeneous. The results of the normality test with a significance of 0.070 > 0.05 and the homogeneity test were met with a significance of 0.100 > 0.05 which showed normal and homogeneous data. After the data is normal and homogeneous, it is continued with the one-way ANOVA test.

The results of the one-way ANOVA test obtained a significance value of 0.388 > 0.05 which showed that Ho was accepted, namely there was no decrease in lead content by soaking apple cider vinegar with and without mother within 5, 10, and 15 minutes. The results showed that apple cider vinegar with and without mother was not significant in lowering lead levels. This is due to the limited variability of the data obtained where this study requires a larger number of samples to obtain precision so that it can detect differences in each treatment. This result is not in line with research [9] where there is a significant influence between soaking green mussles and the length of soaking using lime in lowering lead. Lime contains citric acid, which can dissolve green clam lead, as well as apple cider vinegar with and without mother. Apple cider vinegar with and without mother contains citric acid and acetic acid which can lower lead.

4. CONCLUSION

Based on the research that has been carried out, the average results of lead content before 5, 10, and 15 minutes of immersion were 0.225 mg/L, 0.205 mg/L and 0.180 mg/L respectively; apple cider vinegar treatment with mother 5, 10, 15 minutes sequentially 0.219 mg/L, 0.103 mg/L and 0.088 mg/L. The treatment of apple cider vinegar without mother for 5, 10, 15 minutes was 0.205 mg/L, 0.173 mg/L and 0.125 mg/L. Percentage decrease in lead content after apple cider vinegar treatment with mother

5, 10, 15 minutes respectively 2.6%, 49%, 51%; Motherless apple cider vinegar treatment 5, 10, 15 minutes sequentially 8%, 15%, 30%. Soaking apple cider vinegar with and without mother for 5, 10, 15 minutes can reduce lead levels but is not statistically effective in reducing green mussels lead levels.

Funding: This research received no external funding

Acknowledgments: -

Conflicts of interest: The authors declare no conflict of interest.

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Journal of
Food and Pharmaceutical Sciences

Original Article

Formulation and Effectiveness of Herbal Hair Dye from Ethanol Extract of Banyan Bark (*Ficus benjamina* L)

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Received: 14 November 2024; Revised: 1 December 2024; Accepted: 4 December 2024; Published: 31 December 2024

Abstract: The banyan plant (*Ficus benjamina* Linn.) is one of the plants that has benefit as a traditional medicine. Almost all parts of the plant have efficacy as a source of medicine and furniture or decoration. However, banyan bark is often considered waste. Based on the results of phytochemical screening that has been carried out, banyan bark contains chemical compounds tannins, saponins, flavonoids, alkaloids and glycosides. Flavonoids are a group of flavanols derived from benzene compounds that can be used as basic compounds of natural dyes. Therefore, the aim of this research is to try to make herbal hair dye using banyan bark (*Ficus benjamina* Linn.) as a natural dyeing ingredient. The research method used is experimental with 80% ethanol solvent. The formulation of herbal hair dye consists of extract of banyan bark (*Ficus benjamina* Linn), Piragalol, Xanthan gum, Methyl parabens, and BHT with concentration of 4%, 6% and 8%, respectively. Tests on the preparations made include organoleptic tests, homogeneity tests, viscosity tests, pH tests, stability tests, irritation tests, color stability tests produced, color stability tests against washing, color stability tests against sunlight and preference tests. The formulation of herbal hair dyes uses ethanol extract of banyan bark which can give color to gray hair with concentrations of ethanol extract of banyan bark 4%, 6% and 8%, Pyroganol 1%, Xanthan gum 1%, Methyl paraben and BHT. Banyan bark ethanol extract (*Ficus benjamina* Linn.) can be used in herbal hair dye by producing a blonde to dark brown color in gray hair. The best dye is obtained from the formula with concentration of 8% which consists of ethanol extract of banyan bark (EKBB) which produces a dark brown color, the pH of the preparation in the range of 5.8 – 6.1, the viscosity in the range of 2800 – 4800 cps, and the preparation is stable in several washes and exposure to direct sunlight and does not cause irritation reactions to the skin.

Keywords: Ethanol 80%, *Ficus benjamina* L, flavonoid, hair dye, phenolic.

1. INTRODUCTION

Hair has been known since ancient times with the nickname "crown" for women [1]. Hair is the part that grows on the dermis layer and follicle ducts of the skin. Hair color will change in old age which is referred to as gray hair whose existence is less favorable. Gray hair occurs because the hair does not receive oxygen and nutrients from the ends of the blood vessels, resulting in poor melanin formation, which can lead to the formation of gray hair in today's modern era, coloring is a hair trend that is popular with both men and women regardless of age. Herbal hair dyes are cosmetics used in hair makeup to dye hair, either used to restore the original hair color or other colors [2]. Dyeing hair not only changes the color of the hair from white (grey) to black or other

colors but can also nourish the hair better. Many hair dyes in circulation contain synthetic ingredients that can cause reactions and allergies. A good hair dye is a hair dye whose function is not only to dye hair, but can also nourish hair so that hair is well maintained and does not become damaged [3]. Therefore, researchers want to make herbal hair dyes using natural ingredients. One of the supplements that can be used is the banyan stem [4]. The selection of the right natural ingredients in the manufacture of cosmetics can reduce the negative effects on cosmetics and minimize the occurrence of allergic reactions on the skin. The type of plant that has never been formulated as a natural hair dye is the bark of the banyan stem. Content of flavonoid and tannin in the bark of banyan stems is a natural dye compound that is effective in giving brown color to hair because flavonoid and tannin have a conjugation system of the khalkon group when reacting with acids, so it usually produces a brick-red color to a blackish brown, in addition to that flavonoid can be used as antioxidants [5]. In addition, it is hoped that banyan bark extract (EKBB) can provide benefits as a substitute for synthetic dyes in cosmetics [6]. Therefore, the use of synthetic dyes can be replaced by natural dyes.

2. MATERIALS AND METHODS

2.1. Materials

The tools used in this study are laboratory glass tools (Pyrex), aluminum foil, blender (cosmos FP-32), desicator (Pyrex), hot plate (joan lab), lumpang, refrigerator (sharp), dryer cabinet, microscope, filter, knife, pH meter (MeterLab®), rotary evaporator (Eyela®), tamper, analytical scale (BB Adam®), viscometer (Brookfield RV).

The ingredients used are banyan bark ethanol extract (*Ficus benjamina* L.), aquaades, 80% ethanol, pyrogalenol, methyl paraben, xanthan gum, BHT, potassium iodide, iodine, mercury(II) chloride, concentrated sulfuric acid, nitric acid, anhydrous acetic acid, hydrochloric acid, iron(III) chloride, chloralhydrate reagent, iron(lll) chloride 1%, 2N hydrochloric acid, magnesium, amyl alcohol, Mayer reagent, Bouchardat reagent, Dragendroff reagent, ethanol 96%, lead(ll) acetate 0.4 M, chlorophorm, isopropanol, Molis reagent, Fehling A, Fehling B, methanol, Libermen-Bouchard reagent and n-hexane.

2.2. Methods

2.2.1. Determination

The bark of the banyan stem (*Ficus benjamina* L) was obtained in Riau Province, Kampar Regency, precisely in Plora Village. The determination was made at the Medanense Herbamium Plant Systematics Laboratory (MEDA) of the University of North Sumatra, Medan.

2.2.2. Extract manufacturing

The extraction is made by percolation using 80% ethanol solvent. Maximization of 1000 gr of simplisia powder for ± 3 hours. In a glass jar as much as 2000 ml of 80% ethanol. After 3 hours, 1000 gr of simplisia powder is put into the percolator. An 80% ethanol solution is added little by little. Close the percolator faucet and insert a new solvent. The percolator faucet is opened for extraction, the faucet is set so that the dripping speed is 1 ml per minute. To determine the end of percolate, a qualitative examination of the active substance can be carried out at the final percolat or it can be seen when the juice is already clear. When the percolation has ended, the percolat is evaporated

with the help of a rotary evaporator at a temperature of 40°C at a speed of 100 rpm, until a viscous extract is obtained. And the viscous extract is stored inside the desiccator.

2.2.3. Phytochemical screening

a. Alkaloid identification

A total of 0.5 g of fresh banyan bark sample, simplisia and ethanol extract were put into 3 test tubes added 1 ml of 2N hydrochloric acid and 9 ml of distilled water, heated for 2 minutes, cooled and filtered. Taken 1 mL plus 2 drops of Mayer's reagent, a white or yellow + alkaloid precipitate was formed. Taken 1 mL plus 2 drops of Bouchardat reagent, a blackish-brown + alkaloid precipitate was formed. Taken 1 mL plus 2 drops of Dragendrof reagent, a red to brown precipitate was formed + alkaloids. If there is a precipitate or turbidity, at least two of the three experiments indicate the presence of alkaloids [7].

b. Flavonoid identification

A total of 1 gr sample of fresh banyan bark, simplisia and its ethanol extract are put into 3 test tubes, 10 ml of hot water is added, boiled for 5 minutes and filtered, into 5 ml of filtrate added 0.1 g of magnesium powder and 1 ml of concentrated hydrochloric acid and 2 ml of amyl alcohol. The formation of orange to red colors with flavonoid [8].

c. Saponin identification

A total of 0.5 grams of fresh banyan bark sample, simplisia and ethanol extract were put into a test tube, then 10 ml of hot water was added, cooled, and beaten vigorously for 10 seconds. If a stable 1-10 cm high foam is formed and not less than 10 minutes and the foam does not disappear with the addition of 1 drop of HCl 2 N [9].

d. Tannin identification

A total of 1 gr of fresh banyan bark sample, simplisia and its ethanol extract are boiled for 3 minutes in 100 mL of distilled water, cooled and filtered, 2 ml is taken added 1-2 drops of 1% iron(III) chloride reagent. If a blackish-blue or blackish-green color occurs, it indicates the presence of tannins [10].

e. Glycoside identification

A total of 4 grams of fresh banyan bark sample, simplisia and ethanol extract were put into the Erlenmeyer added 96% ethanol 84 mL and 36 mL of aqueous acid and 4 drops of concentrated sulfuric acid were diffused for 10 minutes, cooled and filtered. Take 20 mL of filtrate add 10 mL of 0.4 M lead(II) acetate, and 10 mL of aquades are shaken, let stand for 5 minutes filtered. Filtrate is extracted with 20 mL of a mixture of chloroform and isopropanol (3:2), and is further tested as follows:

i. Assay for sugar compounds

- As much as 1 mL of the top layer is taken and evaporated on top of a water bath. The remaining evaporation is added 2 mL of water and 5 drops of Molish reagent solution, and carefully added concentrated sulfuric acid, forming a purple ring at the liquid border, this reaction indicates the presence of sugar bonds.
- A total of 1 mL of the top layer is taken and evaporated on a water bath. The rest of the evaporation is added Fehling A and Fehling B (1:1), then heated. The formation of brick-red

deposits indicates the presence of reducing sugar.

ii. Assay for non-sugar compounds

As much as 1 mL of the bottom layer is taken, evaporated on a water bath with a temperature of no more than 60oC, the remaining evaporation is dissolved in 2 mL of methanol. Furthermore, 20 drops of glacial acetic acid and 1 drop of concentrated sulfuric acid (Liberman-Bouchard reagent) are added, in case of blue, green, purplish-red or purple color, positive for non-sugar. The formation of brick red deposits indicates the presence of glycosides [7].

f. Identification steroids/triterpenoids

As much as 1 g of fresh banyan bark sample, simplisia and ethanol extract were macerated with 20 ml of n-hexane for 2 hours then filtered and 5 ml of filtrate was vaporized in a vaporizer dish until dry. To the residue was added the Liebermann-Burchard reagent. If a purple or reddish-purple color is formed, it indicates the presence of triterpenoids, while steroids are marked as blue or turquoise [11].

2.2.4. Formula preparation

The formula is selected based on the standard formula in the Indonesian Cosmetic Formulary (1985) as shown in Table 1. Based on this study, the preparation to be made is a herbal hair dye using a formula like Table 2 as follows:

Table 1. Standard formula

Composition	Light brown	Dark brown	Black
Henna powder	30	83	73
Pyroganol	5	10	15
Copper (II) sulphate	5	7	12

Table 2. Hair dye formula

Composition	Formulation (%)				Function
	F0	F1	F2	F3	
Banyan stem bark extract	0	4	6	8	Dye
Pyroganol	1	1	1	1	Color Generator
Xanthan gum	1	1	1	1	Emulsifier
Methyl paraben	0.2	0.2	0.2	0.2	Preservatives
BHT	0.05	0.05	0.05	0.05	Antioxidant
Aquadest Ad	100	100	100	100	Solvent

Information: F0 = Blank; F1= EKBB Concentration 4%; F2 = EKBB Concentration 6%; F3 = EKBB Concentration 8%

Working procedure: The active ingredient of ethanol extract of banyan bark was weighed with a concentration variation of 4%, 6%, 8%. Dissolve the xanthan gum in a hot pot until it forms transparent (M1). Eroded with pyroganol, ethanol extract of banyan stem bark into mortar is homogeneously grinded (M2). M1 and M2 are mixed into the homogeneous grind. The mass is transferred into the glass beaker, then BHT and methyl parabens are added that have been dissolved. Then add little by little the aquadest up to 100 ml of Ad, stir homogeneously, then put into a container and evaluate. Wash your hair with shampoo and dry, after which the hair is put

into a mixture of hair dye, soaked for 1 to 4 hours. After being lifted, the hair is washed and then dried and observe the color that forms according to the soaking time.

2.2.5. Evaluation of the quality of herbal hair dyes

a. Organoleptic test

The organoleptic test is intended to see the physical appearance of the preparation which includes shape, color and aroma [12].

b. Homogeneity test

The homogeneity test is carried out by visual observation. Good homogeneity is characterized by a uniform color in the herbal hair dye, in addition to Homogeneity testing ensures the absence of hard or clumpy particles [13].

c. pH test

pH measurement was carried out for 4 weeks to determine the acidity level of hair dye from banyan bark extract, measured using a pH meter in the following way: Calibrate the device first using a solution of pH 7 and pH 4. Then the herbal hair dye is prepared and the pH value obtained is recorded [14].

d. Viscosity test

Viscosity measurement is carried out using a Brookfield viscometer, namely by attaching a spindle No.4 to the tool then dipping it into the preparation to a certain extent and setting the speed to 60 rpm. Each measurement is read on the scale when the red needle has stabilized, the viscosity value is obtained from the multiplication of the dial reading with a special correction factor at each spindle speed [12].

e. Stability test of the preparation

The stability test of the preparation is carried out by paying attention to the shape, color and smell during storage. The process of storing the herbal hair dye is put into a small ointment Pot. Observed changes every 2 days for 1 month [12].

f. Color stability test produced

Gray hair is washed with shampoo and then put into the hair dye formula, soaked. Each preparation is observed the color formed [12].

g. Color stability test against washing

Gray hair is given a pre-made herbal hair dye, washed with 1 drop of shampoo and dried. Washing is done once every 2 days for 1 month [12].

h. Sunlight color stability test

The sunlight stability test is carried out to see the durability of the hair dye made, gray hair that has been colored is dried in the sun for 5 hours in the sun after which the color change is observed, carried out every 2 days for 1 month [12].

h. Irritation test

Irritation test is carried out to determine whether in the formulation of herbal hair dyes there is a reaction between components so that irritant or toxic substances are formed [15].

i. Preference test

The preference test is carried out to find out the level of consumer preference for the dye preparations made [1].

2.3. Data Analysis

The analysis method used in this study is experimental, with a quantitative approach. This experiment was to see the results of the comparison of hair dyes from 3 concentrations, namely: 4%, 6% and 8%.

3. RESULTS AND DISCUSSION

3.1. Determination

The results of plant identification or determination carried out at the Medanense Herbarium Laboratory (MEDA) of the University of North Sumatra stated that the plants used in this study were banyan bark plants (Ficus benjamina Linn.) with the Moraceae family.

3.2. Formulation extract

From 1000 gr of banyan bark simplisia powder, extracted by percolation method using 80% ethanol solvent. Then the percolat is evaporated in a rotary evaporator with a temperature of 40°C at a speed of 100 rpm and concentrated so that a thick extract of 128 grams is obtained.

3.3. Phytochemical screening

Table 3. Results of phytochemical screening of fresh banyan bark, simplisia and ethanol extract

No	Examination	SE	SI	E
1	Alkaloid	+	+	+
2	Flavonoid	+	+	+
3	Saponin	+	+	+
4	Tanin	+	+	+
5	Glikosida	+	+	+
6	Steroid/triterpenoid	+	+	+

Description: SE = Fresh banyan bark; SI = Simplisia banyan bark; E = Banyan bark extract

From the table above, it is obtained from the bark of fresh banyan stems, simplisia and ethanol extracts show the presence of alkaloid compounds shown the presence of blackish-brown color deposits on the bark of fresh banyan stems with the addition of Bouchardat reagents, brown precipitates with the addition of Dragendorff reagents, and in simplisia your banyan stem bark yellowish-white precipitates with the addition of Mayer reagents, brown precipitates with the addition of Bouchardat reagents and brown precipitates with the addition of Dragendorff reagent, and in the ethanol extract of banyan bark there was a yellowish-white precipitate with the addition of Mayer reagent, brown precipitate in the addition of Bouchardat reagent, blackish brown precipitate in the addition of Dragendorff reagent [7].

Presence of flavonoid compounds is indicated by the presence of an orange color in the addition of concentrated Mg and HCl powder and in the amyl alcohol layer given, the color of the solution will be lifted or separated and then pulled by the amyl alcohol and give it an orange color, which proves that fresh banyan bark, simplisia and ethanol extract positively contain flavonoid chemical compounds [8]. Furthermore, there is a saponin compound that forms foam with a height of 1-2 cm in shaking with hot water for 10 seconds and remains stable with the addition of HCl 2N to fresh and simplisia banyan bark, while in ethanol extract of banyan stem bark foam is formed as high as 5 cm in shaking with hot water for 10 seconds and remains stable with the addition of HCl 2N, which proves that the bark of banyan stems is positive for saponins [9]. Tannin compounds

are shown by a change in the color of the blackish-green solution with the addition of FeCl3 which indicates that the bark of the banyan stem is positive for tannins[10].

Glycoside compounds are shown to have a purple ring with the addition of the Molish reaction, which means that the bark of the banyan stem contains sugar compounds, the presence of brick-red deposits in the addition of Fehling A and B shows that the bark of the banyan stem contains reducing sugar compounds [7]. Steroid/triterpenoid compounds are shown by the formation of a purplish-red color with the addition of Lieberman-Bouchard reagents, which means that banyan bark, simplisia and ethanol extracts show the presence of steroid/triterpenoid compounds [11].

3.4. Physical evaluation test of the quality of herbal hair dyes

3.4.1. Organoleptic test

Organoleptic tests according to [16] include the smell, color, and shape of herbal hair dyes produced by visual observation. From the tests carried out, the results can be seen in Table 4.

Table 4. Hair dye organoleptic test

Preparation formulation	Color	Odour	Shape
Blanko	White	Unscented	Thick
EKBB 4%	Red brick	Typical of weak banyan skin	Thick
EKBB 6%	Red brick	Typical of banyan bark is rather strong	Thick
EKBB 8%	Deep brick red	Typical of strong banyan bark	Thick

Based on the results obtained in the form of a semi-solid shape. The color of the preparation has a variety of colors, where the higher the concentration of banyan bark extract, the lighter the color of the preparation. And the aroma produced is typical of the extract, the higher the banyan peel extract used, the stronger the aroma.

3.4.2. Homogeneity test

Homogeneity test for 1 month on herbal hair dyes of banyan bark extract is homogeneous in each formula. There are no lumps or particles separated from the preparation material which indicates that the preparation is well mixed. This homogeneity test was carried out by visual observation using a glass object, with the results of observation of the herbal hair dye of banyan bark extract obtained that the formula was physically stable.

3.4.3. pH test

Acidity (pH) is an important aspect of stability evaluation testing. A good herbal hair dye has a pH that suits the scalp and hair, which is 6-7. Because if the herbal hair dye has it, if the pH is too acidic, it will cause the scalp and hair to become oily, while the pH that is too alkaline will cause the skin to become dry and irritated[17]. The results of the ph measurement can be seen in Table 5.

Table 5. pH test of herbal hair dyes

No	Dosage	pH value				
		I	II	III	IV	Average
1	Blanko	5.58	5.59	5.73	6.30	5.8
2	EKBB 4%	6.02	6.24	6.09	6.39	6.1
3	EKBB 6%	5.69	5.72	5.81	6.24	5.8
4	EKBB 8%	5.34	5.69	6.19	6.42	5.8

The pH testing on the herbal hair dye of ethanol extract of banyan bark in the formula F0 (Blank), F1 (4%), F2 (6%), F3 (8%) has an average of 5.8 - 6.1 which is still safe to use and still meets the good pH criteria in the herbal hair dye because it is in accordance with the pH of the scalp, which is 4.6 - 6.6.

3.4.5. Viscosity test

Testing the viscosity of herbal hair dyes to determine the effect of consistency of the preparation on use, so that the greater the viscosity value, the thicker the preparation. And herbal hair dyes are more comfortable and even when used.

Table 6. Results of viscosity test of herbal hair dyes

Formulation	No spindle	Rpm	Time (s)	Measurement	Viscosity (cp)
Blanko	6	60	30	28	2800
EKBB 4%	6	60	30	29	2900
EKBB 6%	6	60	30	47	4700
EKBB 8%	6	60	30	48	4800

Viscosity testing on herbal hair dyes was obtained (2800 -4800) where the larger the extract used, the thicker it was and the greater the viscosity value of the preparation obtained. So that herbal hair dyes extracted from banyan bark can be used by meeting the requirements of a good viscosity value according to (SNI 16-4399-1996) which is 2000 – 50,000 cp. And from the comparison of the journal of herbal hair dyes from rosella flower extract, it was stated that the viscosity of the herbal hair dyes obtained ranged from (11444 – 12062 cps) of herbal hair dyes [1].

3.4.6. Dosage stability test

Stability testing of preparations to determine the stability of herbal hair dyes, and stability test checks include the texture, color and aroma of the preparations. Stability test checks are carried out during 1 month of storage and are carried out at room temperature.

Table 7. Results of the stability test of herbal hair dyes

Examination	Formulation	Weekly observations			
		M1	**M2**	**M3**	**M4**
Texture	Blanko	Kl	Kl	Kl	Kl
	EKKB 4%	Kl	Kl	Kl	Kl
	EKBB 6%	Kl	Kl	Kl	Kl
	EKBB 8%	Kl	Kl	Kl	Kl
Color	Blanko	P	P	P	P
	EKKB 4%	Mb	Mb	Mb	Mb
	EKBB 6%	Mb	Mb	Mb	Mb
	EKBB 8%	Mbp	Mbp	Mbp	Mbp
Odour	Blanko	Td	Td	Td	Td
	EKKB 4%	Kkl	Kkl	Kkl	Kkl
	EKBB 6%	Kkak	Kkak	Kkak	Kkak
	EKBB 8%	Kkk	Kkk	Kkk	Kkk
	Checklists	Tb	Tb	Tb	Tb

Description : M1 = First week; M2 = Second week; M3 = Third week; M4 = Fourth week; Kl = Thick; Cr = Liquid; P = White; Mb = Red brick; Mbp = Thick brick red Td = Unscented; Kkl = Typical aroma of weak banyan peel;

Kkak = Typical aroma of rather strong banyan peel; Kkk = Typical aroma of strong banyan peel; Tb = Unchanged

From the stability test carried out on herbal hair dyes for 1 month, the preparation did not change and remained stable, both the color of the preparation form remained thick and the aroma of the preparation was typical of banyan bark, while the comparator used on the market did not experience any change in color, shape and aroma.

3.4.7. Resulting color stability test

From the results of the color stability test observations carried out, soaking gray hair with various comparisons of herbal hair dye concentrations provides color results for gray hair as shown in Table 8 below:

Table 8. The results of the color stability test produced

Preparation formula	Colors produced	Preparation formula	Colors produced
EKBB 6%		EKBB 8%	

Color test produced from the application of herbal hair dye on gray hair produced a blonde or golden color in the immersion with a concentration of 4% because the extract used was still small, while in the immersion with a concentration of 6% it produced a light brown color, the color began to thicken because the extract used was higher. And in soaking with a concentration of 8%, gray hair changes color to dark brown. Meanwhile, from the table above, the best color is taken because with the addition of higher extracts it produces a darker and more beautiful color.

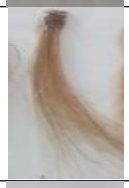
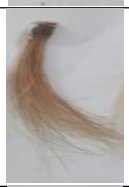
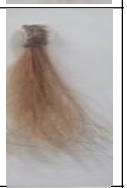
3.4.8. Color stability test against washing

Testing the color stability of herbal hair dye against washing is carried out every 2 days for 1 month. The requirement to wash permanent hair dye using shampoo is done at least 7 washes, this stability test is carried out to see if the color produced by the hair dye remains consistent or changes. Based on the color stability test on the washing of banyan bark herbal hair dye extract, the results were obtained that the four formulas have a color that remains stable up to 15 times washed with shampoo, this is because gray hair uses a mixture of natural dyes with color-producing substances. The mixture can increase the adhesion of the color to the hair so that the dye can stick more firmly to the hair shaft [1].

3.4.9. Color stability test against sunlight

Color stability testing to sunlight is performed to see the color fastness produced to the effect of sun exposure.

Table 9. Results of stability test against sunlight

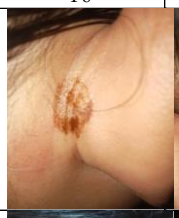
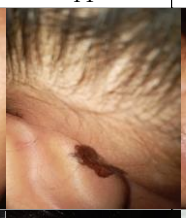
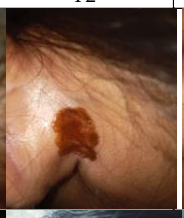
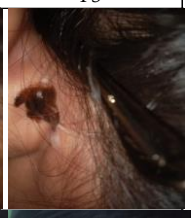
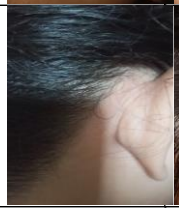
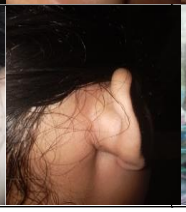
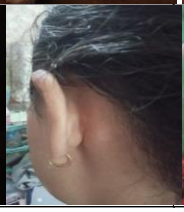
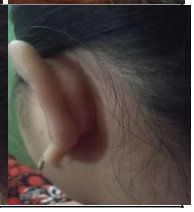
Drying to	Drying time	Color formula			
		F0	F1	F2	F3
1	0 hours				
	5 hours				
15	0 hours				
	5 hours				

The results of the stability test in sunlight carried out by direct drying in the sun showed that the color remained stable and did not change color/remained the same. This is because dyes can penetrate the cuticle and enter the hair cortex so that hair color does not change easily [1]

3.4.10. Irritation test

Irritation test was carried out to ascertain whether in the formulation of hair dye preparations banyan bark extract there was a reaction between the components so that irritant or toxic substances were formed.

Table 10. Irritation test results

No	Treatment	formula			
		F0	F1	F2	F3
1	When applying hair dye				
2	After the application of hair dye				

The results of irritation testing conducted on 6 panelists did not cause irritation such as: erythema, papules, vesicles, and edema in the tested area for 24 hours. So herbal hair dyes from banyan bark ethanol extract with various concentrations of 4%, 6% and 8% are safe to use.

3.4.11. Likeability test

The likeability test was conducted to see the formulas that were very liked by 30 panelists.

Table 11. Likeness test results

Preference test	Preparation formulation	Value range	Smallest preference value	Conclusion
Color	Blanko	3.350787 to 5.11588	3.350787 = 3	Less like
	EKBB 4%	3.899167 to 5.234166	3.899167 = 4	Like
	EKBB 6%	3.899167 to 5.234166	3.899167 = 4	Like
	EKBB 8%	4.520921 to 5.212413	4.520921 = 5	Very much like
Shape	Blanko	2.616133 to 4.250533	2.616133 = 3	Less like
	EKBB 4%	3.622174 to 3.622174	3.622174 = 4	Like
	EKBB 6%	3.751719 to 4.981615	3.751719 = 4	Like
	EKBB 8%	4.594871 to 5.205129	4.594871 = 5	Very much like
Odour	Blanko	2.596231 to 4.470436	2.596231= 3	Less like
	EKBB 4%	3.836758 to 4.963242	3.836758 = 4	Like
	EKBB 6%	3.817712 to 5.182288	3.817712 = 4	Like
	EKBB 8%	4.520921 to 5.212413	4.520921 = 5	Very much like

The results of the test conducted on 30 panelists showed that blank preparations were not preferred by the panelists in terms of color, shape and aroma, this was because blank preparations did not use EKBB so that preparations that did not have less attractive colors, aromas and shapes. While preparations with a concentration of 4% and 6% of the panelists liked the preparations both in terms of color, shape and aroma, this is because the preparations already use EKBB so that the preparations have interesting colors, aromas and shapes, while the preparations with a concentration of 8% are very liked by the panelists, this is because the preparations have a strong color, strong aroma and a more attractive shape. So that from the preference test of the 30 panelists, the concentration that was very liked was a concentration of 8% with a score of 5, which is very liked.

4. CONCLUSION

From the phytochemical screening carried out by fresh banyan bark, simplisia and its ethanol extract contain secondary metabolite compounds tannins, saponins, flavonoids, alkaloids, glycosides and steroids/triterpenoids. It can be formulated into herbal hair dyes that meet the physical quality requirements of the preparation. Then herbal hair dyes are able to cover grey hair color, by giving blonde color at a concentration of 4%, light brown color at a concentration of 6%, and dark brown color at a concentration of 8%. Herbal hair dyes are safe to use because they do not cause irritation and are very liked by panellists.

Acknowledgments: -

Conflicts of interest: Interest All authors stated that there was no conflict of interest in this study.

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Journal of
Food and Pharmaceutical Sciences

Original Article

The Evaluation of Clove oil Concentration on Physicochemical and Antimicrobial Activity in the Laponite Gel Delivery System

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Received: 18 August 2024; Revised: 22 November 2024; Accepted: 05 December 2024; Published: 31 December 2024

Abstract: Recurrent Aphthous Stomatitis (SAR) is inflammation in the oral mucosa. One of the factors that can cause SAR is bacterial and fungal infections of the oral mucosa. Clove oil is an essential oil that contains eugenol and has antimicrobial, analgesic, anti-inflammatory, and antioxidant activities. Clove oil in this study resulted from steam distillation with an eugenol content of 71.06%. Clove oil as an antimicrobial topical requires a drug delivery system that is easy and comfortable to apply. Laponite is a synthetic hydrophilic layered silicate that can hydrate and expand when water is added, is compatible with the properties of active ingredients, and is without an emulsifier. Please state the objective of this research. This research aims to determine the effect of variations in the concentration of clove oil incorporated in laponite on the gel's physicochemical properties and antimicrobial activity. Laponite with a concentration of 2.5% was developed in water and then added to clove oil with a concentration of 2%, 4% and 6%. Physicochemical properties test of oral gel preparations includes organoleptic tests, homogeneity, pH, adhesion, and spreadability tests. Antimicrobial activity test to *Streptococcus mutans* bacteria and *Candida albicans* fungi. The research showed that a gel preparation using laponite with a concentration of 2.5% was semi-solid with a clove oil odor. Clove oil, with an eugenol content of 71.06%, has potent antimicrobial activity. The higher concentration of clove oil increases the viscosity and sticky time but decreases the pH and spreadability. The adhesive time and pH of the gel are suitable for application to the oral mucosa with mouth ulcers, and 2.5% laponite can form a gel preparation that meets the gel's physicochemical properties and antimicrobial activity.

Keywords: clove oil; SAR; eugenol; antimicrobial; hydrogel

1. INTRODUCTION

Mouth ulcer or Recurrent Aphthous Stomatitis (SAR) is inflammation that occurs on the oral mucosa, such as the tongue, lips, cheeks, gums, and floor of the mouth. The prevalence of SAR on the oral mucosa is estimated to reach more than 25% of the population worldwide [1]. Based on size, the clinical form of SAR is divided into three, namely minor aphthous ulcers (90% of cases), major aphthous ulcers (8% of cases), and heptiform ulcers (2%) [2]. Most fungal infection in human is 75% caused by *Candida albicans*. About 40% of healthy adults carry this species in the oral cavity [3]. Oral

Candida species can lead to oral candidiasis and denture stomatitis [4]. Oral candidiasis, commonly known as oral thrush, appears as creamy white or yellowish, crusty, curd-like patches with cracks in the corners of the mouth, lips, tongue, palate and buccal cheeks.

Medicines for thrush circulating in the community mostly contain antibiotics and policresulen. However, inappropriate use of topical antibiotics will cause side effects such as rash, fever, chills, and urticaria [5] and policresulen can cause burning and tissue death in the mucosa mouth [6]. People usually treat SAR with drugs that contain policresulen, but this active substance had been withdrawn from market by Indonesian Drug and Food Administration (BPOM RI) since February 2018 [7]. Policresulen was reported to have side effects in 38 cases, two of them are enlargement of SAR and oral mucosal injury that causes infection, also 6 cases reported that the drugs which contain policresulen cause burn on the oral mucosa [8]. Therefore, it is necessary to develop alternative antibacterial agents that are safe and have minimal side effects, one of which is agents from natural ingredients, namely essential oils. Herbal extracts are used in dentistry for treatment of various dental disorders. The natural photochemical could offer an effective alternative to antibiotics and represent a promising approach to prevention and therapeutic strategies for various oral infections.

High eugenol concentration is founded in Clove which has antimicrobial activity for gram-negative and gram-positive bacteria, one of them is *Staphylococcus aureus*. Clove oil has been traditionally used as dental care, analgesic, and antiseptic. Eugenol was shown to have antifungal activity against *Candida albican* [9]. Eugenol of clove have strong antibacterial activity against Cariogenic (MBC 0.2-1.6μg/mL; MIC 0.1-0.8 μg/mL) and against periodontal pathogens (MIC 0.1-1.6 μg/mL) [10]. Clove oil is a group of essential oils containing the main component, eugenol, and is known to have antimicrobial, analgesic, anti-inflammatory, and antioxidant activity. Clove oil contains >70% eugenol, which can destroy proteins and react with phospholipids from cell membranes [11]. Eugenol of clove oil has been confirmed to be effective in combating some pathogenic bacteria including *Pseudomonas aeruginosa S. aureus*, *E. coli*, *Proteus mirabilis,* and *Streptococcus mutans* [12]. Crude clove oil that the use of 10 % could irritate to the skin [13]. Clove oil can be treated by administering painkillers and antiseptics, which are available in various forms, such as lozenges, sprays, mouthwash, and ointments. The gel is a semi-solid preparation used on the oral mucosa as a drug carrier material. It has high adhesion, provides a cooling effect or sensation and good drug release, and can make drug application easier [14].

Laponite® is a synthetic smectite clay that already has many important technological applications, which go beyond the conventional uses of clays in pharmaceutics and cosmetics. In biomedical applications, particularly in nanomedicine, this material holds great potential [15]. Laponite is a clay-based material composed of synthetic disk-shaped crystalline nanoparticles with highly ionic, large surface area. These characteristics enable the intercalation and dissolution of biomolecules in Laponite-based drug delivery systems. Furthermore, Laponite's innate physicochemical properties and architecture enable the development of tunable pH-responsive drug delivery systems. Laponite's coagulation capacity and cation exchangeability determine its exchange capabilities, drug encapsulation efficiency, and release profile [16].

Synthetic magnesium lithium silicate or laponite is a synthetic smectite with a structure and composition similar to the natural clay mineral hectorite in the form of a fine, dense white powder with a bulk density of around 1g/cm^3. *Laponite* is a synthetic hydrophilic layered silicate insoluble in

water but hydrates and expands when water is added. Laponite has advantages in terms of productivity, purity, and efficient availability. This makes laponite suitable for application as a filler and thickener for aqueous preparations in the industrial sector [17]. Laponite can disperse active ingredients that have both lipophilic and hydrophilic properties without additional emulsifiers. In this research, we will observe the effect of variations in the concentration of clove oil incorporated into the laponite system on the physicochemical properties of oral gel preparations. We hope to find out the best (there is no optimation step in the methods, only orientation step to get the best concentration) concentration that produces an excellent oral gel preparation and reduces clove oil's bitter and hot taste when applied to the oral mucosa area because it is incorporated into the laponite system. Laponite provides a relaxed feeling and can release active ingredients periodically for a long time, thereby reducing the frequency of drug use and patients more compliant in using medicines. In this study, the influence of the concentration of clove oil incorporated in laponite gel will evaluate its physicochemical properties and antimicrobial activity.

2. MATERIALS AND METHODS

Laponite (LABTEC, German), aquadest (repackaged by PT. Agung Jaya), citric acid (repackaged by Cipta Kimia), phenoxyethanol (repackaged by Cipta Kimia), propylene glycol (DOW), clove oil (UMKM Surya Wulan Kulonprogo), bacterial culture *Streptococcus mutans* ATCC 2517 (Thermo Scientific), fungus *Candida albicans* ATCC 14053 (Thermo Scientific), antibiotic amoxicillin, antifungal ketoconazole, PDA powder (Merck), human blood type "O" media, and BHI powder (Merck). Instrument: glassware (Pyrex, England), analytical scales (Ohaus CP214, America), stir bar (Pyrex, England), Petri dish (Pyrex, England), pH meter (Lutron-PH-207, Taiwan), magnetic stirrer (IKA C-MAG HS 7, Germany), spinbar, stopwatch, Biosafety Cabinet (BIOBASE BSC-110011A2, China.

2.1. Sample preparation

Clove leaves were steam distilled at 5 am, the oil samples were separated, and the eugenol content of clove oil was tested at LPPT Gadjah Mada University Yogyakarta using the GC method by taking 0.1 mL of the sample and diluting it using ethanol to 1.0 mL volume, then the solution was homogenized. They were using vortex. A standard eugenol solution was prepared with series concentrations, then 1.0 μL of the sample solution, and standard eugenol was injected into the GC machine, and the eugenol concentration was obtained in the Suryawulan UMKM essential oil sample.

2.2. Laponite delivery system of Clove oil

Table 1. Clove oil gel formula, incorporate in laponite delivery system

Ingredients	Concentration (%)				Function
	Base formula	Formula 1	Formula 2	Formula 3	
Clove oil	-	2.0	4.0	6.0	Active substance
Laponite	2.5	2.5	2.5	2.5	Gelling agent
Propylene glycol	0.5	0.5	0.5	0.5	Co-solvent
Citric acid	qs	qs	qs	qs	pH adjusting
Water	Add 100	Add 100	Add 100	Add 100	Solvent

Laponite is dissolved in distilled water to 100 mL, stirred using a magnetic stirrer at 450 rpm for ± 4 hours at room temperature, and placed in the refrigerator until thickened. Then, propylene glycol, and clove oil were gradually added, and the pH-adjusting citric acid solution was added (Table 1).

2.3. Clove oil Hydrogel physicochemical evaluation

Organoleptics were observe directly by the senses of the gel's shape, color, and smell. The gel is usually evident in color with a semi-solid consistency. The electrode on the Lutron-PH-207 pH meter is calibrated with buffer pH 4 and pH 7, then washed with distilled water, pressed on the calibration button, and adjusted. The electrode is then dipped into the gel and waited until stable. The gel preparation's pH must match the mouth's pH of 6.5-7.5. A total of 0.5 g of gel was placed on a glass object, another was placed on top for 1 minute, and its diameter was measured. After that, 200 g of additional load was added, and the constant diameter was measured. A total of 0.5 g of the gel preparation was placed on an object glass, attached to a rope, then covered with another object glass, given a load of 1 kg for 3 minutes, and released. A weight of 80 g is attached to the string, and while the stopwatch is turned on, the time required for the two glass objects to come off is recorded. The criteria for adhesion time of gel preparations is more than 10 seconds [15].

2.4. Antimicrobial Test

Antibacterial blood media: BHI powder was weighed 9.3 grams, dissolved in 250 ml water, and heated while homogenized using a magnetic stirrer for 15 minutes. The solution was incubated for 15 minutes; after it solidifies, weigh 5 grams of agar medium and dissolve it into 125 ml water, then heat it while stirring using a magnetic stirrer for 15 minutes; finally, add five cc of human blood type "O." The solution was incubated for 15 minutes and then poured into sterile Petri dishes [15]. *Streptococcus mutans* bacteria, available in BHI-B media, are put into an anaerobic jar and incubated at 37 ºC for 24 hours. Make seven holes in the agar blood media using a tip punch (7 mm), then fill each hole with clove oil as an oil control, the antibiotic amoxicillin as an antibacterial control, water as negative control, base formula as base control, and F1, F2, and F3 as test samples. The experiment was repeated three times, then incubated for 3x24 hours at 30°C [15].

Mueler Hinton Agar media weighed 3.9 grams, was dissolved in 100 mL of water, then sterilized in an autoclave at 121°C for 15 minutes. Fungal cultivation: The Candida albicans fungus suspension was then suspended with 10 mL of physiological NaCl, then mixed and adjusted to the turbidity to be the same as the Mc Farland solution [16]. Make seven holes in the MHA media using a tip punch (7 mm), then fill each hole with clove oil as an oil control, the ketoconazole as an antifungal control, water as negative control, base formula as base control, and F1, F2, and F3 as test samples. The experiment was repeated three times, then incubated for 3x24 hours at 30°C [15].

2.5. Statistical Analysis

The data obtained from the test results is then compared with parameters from several sources or libraries. The data obtained was then analyzed statistically using IBM SPSS statistics 25 software, which uses the One-Way Anova method if the sig. <0.05. The results of the statistical tests have not yet been explained in the results and discussion section.

3. RESULTS AND DISCUSSION

Active substance content test in clove oil as a quality parameter that the clove oil from steam distillation of UMKM Suryawulan Kulonprogo meets the quality requirements for eugenol content, which has antibacterial and antifungal activity using the gas chromatography method. Eugenol content testing at the Integrated Research and Testing Laboratory (LPPT Gadjah Mada University, Yogyakarta) showed that the eugenol content in clove oil was 71.6%. The main component of clove oil is eugenol, which is 70 – 80% [20]. The eugenol as the major constituent of clove oil, followed by

eugenol acetate. The mass spectral data for eugenol acetate showed the molecular ion peak at 206 corresponding to the relative molecular mass of eugenol acetate, the peak at 164. Eugenol is a phenolic compound (Figure 1). Phenols are known to have antiseptic properties, which is consistent with the antimicrobial data obtained for these compounds. Generally, eugenol (4-allyl-2-methoxyphenol) accounts for 60%–90% of the total composition of clove oil and is the source of the antiseptic property of clove oil [12]. Clove oil was mainly composed of three terpenic compounds in which eugenol was the most abundant, accounting for 78.55%, followed by caryophyllene (15.75%) and humulene (4.28%). Meanwhile, two minor constituents, methyleugenol (0.27%) and epizonarene (0.92%) [13]. So, the eugenol content in clove oil producted by UMKM Suryawulan Kulonprogo, Indonesia meets the requirements for eugenol content.

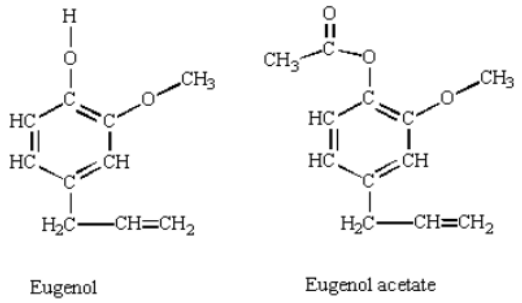


Figure 1. Chemical structures of the components of clove oil [x]

Table 2. Antimicrobial activity of crude clove oil producted by UMKM Suryowulan Yogyakarta compare with clove oil brand product

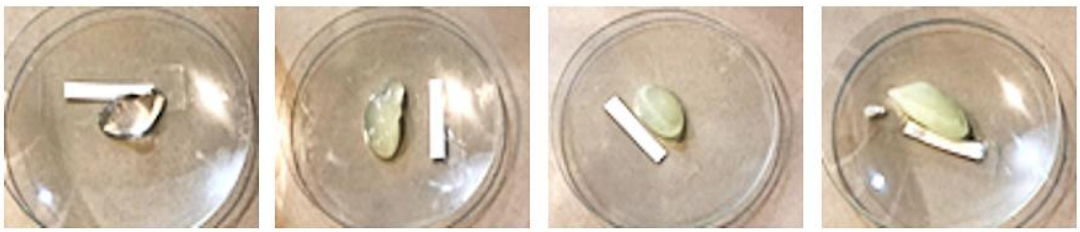
Microbes Species	Inhibitor Zone Diameter (mm)			
	Clove oil UMKM	**Clove oil Brand**	**Control Antibiotics/anti-fungal**	**Control Negatif**
Escherichia coli	10.30	9.70	27.90	0.00
Staphylococcus aureus	29.30	23.40	23.70	0.00
Candida albicans	32.90	24.60	30.40	0.00

Based on the results of antimicrobial testing (Table 2), clove oil produced by UMKM Suryowulan Yogyakarta has better antimicrobial activity than clove oil sold on the market under leading trademarks. *Staphylococcus aureus* was found to be the most sensitive to clove extract with an inhibition zone diameter (IZD) of 29.30 mm and 23.40, respectively. The modes of action by which microorganisms are inhibited by essential oil and their chemical compounds seem to involve different mechanisms. It has been hypothesized that the inhibition involves phenolic compounds, because these compounds sensitize the phospholipid bilayer of the microbial cytoplasmic membrane causing increased permeability, unavailability of vital intracellular constituents [18].

3.1. Physicochemical Properties

The previous orientation of clove oil concentrations in 0.5%, 1%, and 2%. However, there is no change in the physical properties of the hydrogel, and it does not have antibacterial and antifungal activity, so we increased the concentration to 2%, 4%, and 6%. Based on previous research, the lowest concentration was 2.5%, so we used a concentration of 2% as the lowest dose. Research by Gupta et al., 2009 [19] indicated that 5% clove oil had a diameter of inhibition of 18 mm to *S. aureus*. In this

research, we want to know If the 4-6% concentration of clove oil is higher, lower, or equal to the previous research by Gupta. The oral gel preparation has a semisolid consistency and a characteristic clove oil aroma. The aroma becomes stronger, and the color becomes cloudier as the concentration of oil used increases (Figure 2). The base formula of laponite gel is clear and odorless. The clove oil hydrogel was pale yellow in F1, F2, and F3.



(a) (b) (c) (d)

Figure 2. Citronella oil laponite hydrogel using various concentration of clove oil where (**a**) base formula of clove oil; (**b**) 2% of clove oil hydrogel; (c) 4% of clove oil hydrogel; (d) 6% of clove oil hydrogel. The hydrogel appearance becomes cloudy with increasing concentration of clove oil

The viscosity of the gel preparation is influenced by several factors, namely the type and concentration of the gelling agent, the mixing time, and material incompatibility [11]. The pH value test aims to see if the pH of the gel preparation is suitable and can be accepted by the skin and oral mucous membranes. The higher the concentration of clove oil, the greater the pH value. The pH measurement results were obtained from 6.6-7.7 (Table 3). The pH value of laponite based on the MSDS has a basic pH of 9.8, while clove oil has an acidic pH of 4. However, the pH of the resulting clove oil gel preparation has been added to a citric acid solution, a pH-adjusting solution. In the Laponite MSDS, citric acid is the recommended agent for lowering pH. The citric acid solution is added little by little according to the expected pH value. The results of the homogeneity test using the Levene test are seen based on the mean being 0.178, which shows that the data is homogeneous because it has a significant value≥ 0.05. The ANOVA test results show a Sig value. 0.000 shows that the data is significant because it has a Sig value. <0.005. The results of this analysis show that there are differences. The concentration of clove oil has a significant effect on the pH value of the gel preparation.

The wider the distribution capacity of the preparation, the better the distribution of the active substance of the preparation. The increase in the diameter of the spreadability will be inversely proportional to the increase in the viscosity of the preparation [2]. This is based on research [18] which states that increasing the concentration of laponite will cause the gel to become viscous, where the consistency of the gel will become thicker. The viscosity will be greater, causing the spreadability of the gel to decrease. The results of the homogeneity test using the Levene test are seen based on the mean being 0.182, which shows that the data is homogeneous because it has a significant value≥ 0.05. The ANOVA test results show a Sig value. 0.000 shows that the data is significant because it has a Sig value. <0.005. The results of this analysis show that there are differences. The concentration of clove oil has a significant effect on the value spreadability of the gel preparation.

The sticking power of a gel is directly proportional to viscosity. The higher the viscosity, the higher the sticking power. The ability of the gel to stick will influence the therapeutic effect. The longer the gel can stick to the skin, and the skin absorbs the more active substances, the longer the gel can have a therapeutic effect and the more effective its use will be. If it is too weak, the therapeutic

effect will not be achieved [23]. The standard adhesion time for an excellent topical preparation is 4.0 seconds [24]. The higher the concentration of laponite in the gel preparation, the greater the spreading power, thereby reducing the adhesive power. The results of the homogeneity test using the Levene test are seen based on the mean being 0.836, which shows that the data is homogeneous because it has a significant value≥ 0.05. The ANOVA test results show a Sig value. 0.000 shows that the data is significant because it has a Sig value. <0.005. The results of this analysis show that there are differences. The concentration of clove oil has a significant effect on the value adhesion of the gel preparation.

Viscosity testing aims to determine the viscosity of flowing liquid; the higher the viscosity value, the higher the viscosity of the preparation [25]. The gel viscosity results meet the viscosity standards for good gel preparations, namely the 3000-50,000 cps (SNI, 1996). Viscosity can be influenced by temperature, electrolyte, and pH level. The results of the homogeneity test using the Levene test are seen based on the mean being 0.021, which shows that the data is homogeneous because it has a significant value≥ 0.05. The ANOVA test results show a Sig value. 0.000 shows that the data is significant because it has a Sig value. <0.005. The results of this analysis show that there are differences. The concentration of clove oil has a significant effect on the viscosity value of the gel preparation.

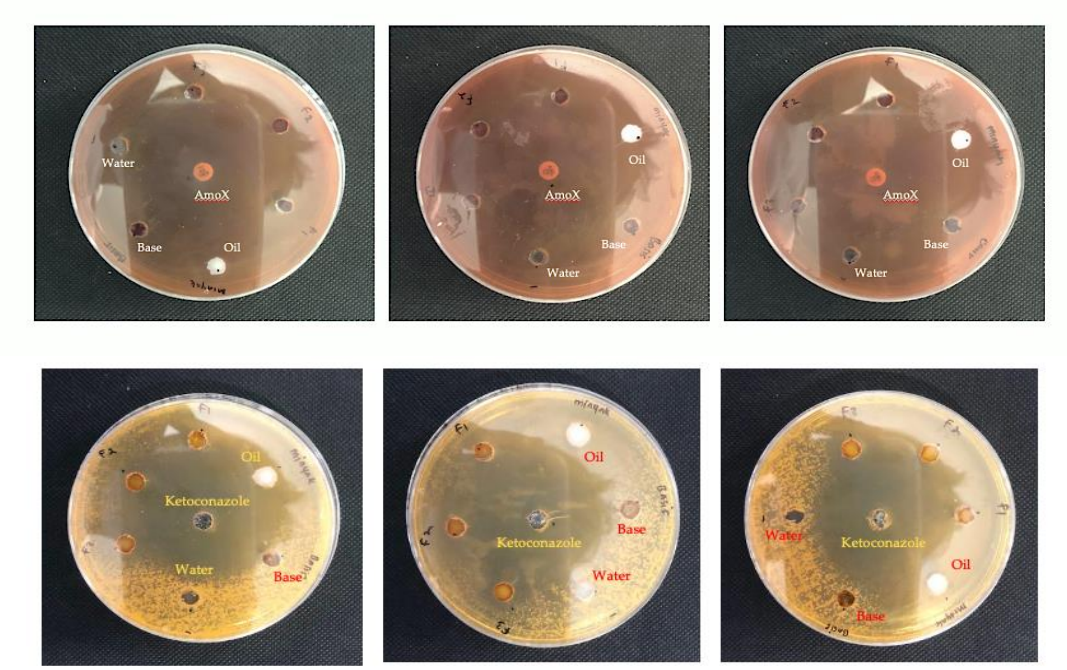


Figure 3. The results of diameter inhibitor zone of antibacterial activity to *Streptococcus mutans* and antifungal activity to *Candida albicans*.

Table 3. The physicochemical test result of clove oil hydrogel using various concentration of clove oil

Formulas	Physicochemical properties			
	pH	Viscosity (cps)	Spreadability (cm)	Sticky time (s)
Base formula	7.70±0.03	1541±6.92	5.00±0.15	0.91±0.02
F1	7.50±0.07	3420±7.53	5.72±0.10	0.98±0.06
F2	6.80±0.01	4617±5.50	5.60±0.15	1.03±0.07
F3	6.60±0.04	6389±6.35	5.41±0.10	1.25±0.04

Table 4. The physicochemical test result of clove oil hydrogel using various concentration of clove oil

Formulas	Diameter of Inhibitor Zone (mm)	
	Antibacterial	**Antifungal**
Base formula	0.00±0.00	0.00±0.00
F1	13.84±0.10	13.35±1.03
F2	18.96±0.11	14.08±0.50
F3	19.05±0.05	14.07±0.35
Citronella oil	22.47±0.10	17.06±1.00
Antibiotics agent	36.80±0.00	-
Antifungal agent	-	14.72±0.00
Water	0.00±0.00	0.00±0.00

Based on the classification of antimicrobial inhibitory categories, both antibacterial and antifungal, an inhibitory zone diameter of >20 mm is a very strong inhibitory; an inhibition zone diameter of 10 mm-20 mm is categorized as strong; an inhibition zone diameter of 5 mm-10 mm is categorized as medium; and the diameter of the inhibition zone is <5 mm, it is categorized as weak. The results of the antimicrobial activity test, both antibacterial and antifungal, in Table 4 show that the diameter of the inhibition zone is largest in F3 with a strong inhibition category, whereas. This shows that the greater the concentration of clove oil used, the greater the inhibitory power, which is indicated by the greater the diameter of the inhibition zone. The results of the antimicrobial activity test showed that clove flower essential oil in the oral gel preparation had inhibitory power against the growth of *Streptococcus mutans* bacteria and the fungus *Candida albicans* [26].

In this study, eugenol was confirmed as the main component of 78% clove oil. Eugenol of clove oil showed obviously antibacterial activities against *S. aureus* and *C.albicans*. The antibacterial mechanism of eugenol against *S. aureus* was probably related to the damage of cell wall and membrane, the inhibition on biofilm formation, the oxidative stress-mediated apoptosis and the disruption of DNA synthesis. These results indicated that clove oil and eugenol may be used as substitutes for antibiotics and synthetic antimicrobial agents [13]. Based on test results using crude clove oil sample showed that the use of 10 % crude clove oil could irritate to the skin [12]. The concentrations of *Syzygium aromaticum* oil were 2,5% (FI), 5% (FII) and 10% (FIII), respectively, variation of essential oil did not influence the irritation index of the ointment. The irritation index of the ointment was negligible [29]. Based on the previous research, where the clove oil topical preparation used various concentrations, our clove oil concentration used was under the previous research's clove oil concentration. It can be concluded that the concentration used is safe even though we did not conduct the anti-irritation test [13].

4. CONCLUSION

The research showed that a gel preparation using laponite with a concentration of 2.5% was semi-solid with a clove oil odor. Clove oil, with an eugenol content of 71.06%, has potent antimicrobial activity. The higher concentration of clove oil increases the viscosity and sticky time but decreases the pH and spreadability. The adhesive time and pH of the gel are suitable for application to the oral mucosa with mouth ulcers, and 2.5% laponite (Table 1 state 2.5% laponite) can form a gel preparation that meets the gel's physicochemical properties and antimicrobial activity. Please ensure that the conclusion aligns with the objectives stated in the background.

Funding: Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia for the funding provided to the Student Creativity Program 2023 [PKM-R].

Acknowledgments: Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia for the funding provided to the Student Creativity Program 2023 [PKM-R].

Conflicts of interest: "The authors declare no conflict of interest."

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Journal of
Food and Pharmaceutical Sciences

Original Article

Antibacterial Potential of *Kaempferia parviflora* Rhizome Extract against *Staphylococcus aureus* ATCC 25923

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Received: 30 November 2024; Revised: 18 December 2024; Accepted: 20 December 2024; Published: 31 December 2024

Abstract: Bacteria are one of the organisms that cause infectious diseases. One of the bacteria that can cause infection is *Staphylococcus aureus*. Treatment of bacterial infections is by using antibiotics, but using the antibiotics for a long term can cause resistance. It is necessary to look for alternative compounds that can inhibit the growth of *Staphylococcus aureus*. In the bioprospecting process of medicinal plants, it is not only necessary to rely on empirical information but also to have scientific evidence proven through scientific testing. One of the plants with potential as a medicine is black ginger (*Kaempferia parviflora*). The aim of this study is to determine the effectiveness of *Kaempferia parviflora* rhizome in inhibiting the growth of *Staphylococcus aureus* bacteria. The concentrations of the *Kaempferia parviflora* extract tested were 25%, 50%, 75%, and 100%. Based on the results, it can be concluded that the ethanol extract of *Kaempferia parviflora* rhizome has antibacterial activity against the growth of *Staphylococcus aureus* ATCC 25923 at concentrations of 25%, 50%, 75%, and 100%, with strong antibacterial inhibition. This study is expected to contribute to the field of health in general and pharmacy in particular by developing the potential of Indonesian medicinal plants. The findings of this study will be directed toward the development of formulations with antibacterial activity.

Keywords: Bioprospecting, Antibacterial, Black Ginger, *Kaempferia parviflora*, *Staphylococcus aureus*

1. INTRODUCTION

Bacteria are one of the organisms that cause infectious diseases. One of the bacteria that can cause infection is *Staphylococcus aureus*. Treatment of bacterial infections is by using antibiotics, but using the antibiotics for a long term can cause resistance. It is necessary to look for alternative compounds that can inhibit the growth of *Staphylococcus aureus*. In the bioprospecting process of medicinal plants, it is not only necessary to rely on empirical information but also to have scientific evidence proven through scientific testing.

It is necessary to optimize the medicinal properties of these plants. Scientific evidence regarding the therapeutic properties of medicinal plants is essential as raw material for the development of innovative products, both as modern medicine and cosmetics. Given the importance of scientific information on the active compounds contained in medicinal plants, bioprospecting research is needed to facilitate the selection of plants as raw materials for pharmaceutical products. This bioprospecting activity focuses on exploring the medicinal properties of biological resources as raw

materials for pharmaceutical products, ultimately leading to the commercialization of health innovation products [11].

The initial step in the bioprospecting activities of this research involves testing the efficacy or medicinal potential of plant parts that have the potential to act as antibacterial agents. One such plant part is the rhizome of *Kaempferia parviflora*. The selection of *Kaempferia parviflora* rhizome for testing its antibacterial potential is based on literature studies showing that ginger plants contain flavonoids, terpenoids, essential oils, and phenols, which have the potential to inhibit the growth of several pathogenic bacteria that can cause diseases in humans. This ability has been demonstrated in studies showing that *Kaempferia parviflora* extract has antibacterial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Cutibacterium acnes* [1]. Other studies have also shown antibacterial activity of *Kaempferia parviflora* extract against *Cronobacter* spp. and *Enterohemorrhagic Escherichia coli* (EHEC) [2]. In this study, the *Kaempferia parviflora* rhizome is tested on *Staphylococcus aureus*. *Staphylococcus aureus* is a bacterium that can cause infections, particularly of the skin. Additionally, the 2022 Global Burden of Disease publication reports that *Staphylococcus aureus* is a leading cause of death in 135 countries worldwide, especially the Methicillin-resistant *Staphylococcus aureus* (MRSA) strain, which is difficult to treat with antibiotics [3].

Based on the above discussion, the author will conduct research on the bioprospecting of ethanol extract from *Kaempferia parviflora* rhizomes as an antibacterial agent against *Staphylococcus aureus*. The aim of this study is to investigate the bioprospecting of secondary metabolite compounds and the antibacterial effects of *Kaempferia parviflora* rhizome extract on the growth of *Staphylococcus aureus*. This research is expected to provide information on the antibacterial effectiveness of ethanol extract from *Kaempferia parviflora* rhizomes against *Staphylococcus aureus*. The solvent used for extraction is 70% ethanol, because it's characteristic, it dissolve polar compounds such as flavonoids that contain in *Kaempferia parviflora* rhizomes that has antibacterial activity.

2. MATERIALS AND METHODS

2.1. Place and time of research

This research was conducted at the Biology Pharmacy Laboratory of Universitas Jenderal Ahmad Yani Yogyakarta.

2.2. Tools and materials

The materials used include the *Kaempferia parviflora* Rhizome, ethanol 70%, DMSO, *Staphylococcus aureus*, Nutrient Agar (NA) media, Mueller Hinton Agar (MHA), paper disc blank, chloramphenicol paper disc. The tools used are Class II BSC, autoclaves, petri dishes, micropipette (Eppendorf), analytical balance (Ohaus), and other glasses.

2.3. Sampling and Plant Determination

Kaempferia parviflora Rhizome were obtained from Sleman, Yogyakarta. The determination of samples was carried out at the Biology Learning Laboratory, Ahmad Dahlan University.

2.4. Preparation and sample extraction of Kaempferia parviflora Rhizome

Kaempferia parviflora Rhizome were washed, then chopped into small pieces. After that, its dried in an oven at 40^{o}C and reduced in particle size using a grinder and 35-mesh sieves. The extraction method used in the research is the maceration method. The maceration method in this study used 70% ethanol solvent ratio 1:10 for 48 hours [4].

2.5. Sterilization

The tools were sterilized in an oven at a temperature of 171 °C for 1 hour. The materials used were sterilized by autoclave at a temperature of 121 °C for 15 minutes [5]. Nutrient agar (NA) media was dissolved in an aqueous solution and homogenized with a stirrer at a temperature of 100 °C until boiling. The NA was sterilized by an autoclave at 121 °C for 15 minutes. Continuously, the NA was cultured with *Staphylococcus aureus.*

2.6. Inoculation of Staphylococcus aureus

In the Bio Safety Cabinet (BSC), *Staphylococcus aureus* were inoculated by the spread plate method on NA media and then incubated for 24 hours at 37 °C. *Staphylococcus aureus* were suspended in a tube containing 10 mL of a 0.9% NaCl solution. Then McFarland standard 0.5 measured turbidity with a turbidimeter.

2.7. Antibacterial Activity Test by Disc Diffusion Method

The serial concentrations of *Kaempferia parviflora* Rhizome extract were dissolved in DMSO 10%. The blank paper disc was soaked in samples for 10 minutes. 100 µL of bacterial suspension was inserted into a petri dish containing *Muller Hinton Agar* media. The petri dish media was divided into two parts, such as control and samples. As a positive control, paper discs containing chloramphenicol 1%. Blank paper discs contained DMSO 10% as negative controls. After that, the petri dish was incubated at 37 °C for 24 hours, and the clear zone formed was measured by their zone inhibition diameter. This test was repeated three times modified by [6].

3. RESULTS AND DISCUSSION

3.1. Extraction of Kaempferia parviflora Rhizome

Plant determination carried out at the Biology Learning Laboratory, Faculty of Applied Science and Technology, Ahmad Dahlan University, Yogyakarta, showed that the plant used was *Kaempferia parviflora* Wall. ex Baker. The extraction process uses a maceration technique with 70% ethanol solvent with a maceration time of 48 hours with a weight ratio of simplicia: solvent of 1:1. The maceration method chosen was because this method is good for active compounds that are not resistant to heating and simple in the processing process. The extraction results can be seen in Table 1.

Table 1. Results of Black Ginger Rhizome Extraction (*Kaempferia parviflora*)

Replication	Simplicia (g)	Extract (g)	Yield (%)
1	200	69.68	34.84
2	200	59.88	29.94
3	200	64.50	32.25

Maceration is the extraction method used in this study, aimed at extracting flavonoids from *Kaempferia parviflora* rhizome. The principle of extraction using the maceration method involves soaking powdered plant material (simplicia) in an appropriate solvent. The solvent used for maceration is 70% ethanol. Ethanol 70% is polar, allowing it to dissolve polar compounds such as flavonoids. In addition, the 70% ethanol solvent contains 30% water, which is expected to wet the plant material, allowing the extracting agent to penetrate the cell walls of the plant material [7]. The yield data from the extraction process shows that the yield from three repetitions of the extraction was greater than 25%. This result meets the standards set by the Indonesian Herbal Pharmacopoeia,

which requires that the yield percentage should not be less than 10%. The yield percentage indicates the amount of compounds contained in the *Kaempferia parviflora* rhizome simplicia that can be extracted using the 70% ethanol solvent.

3.2. Qualitative analysis

3.2.1. Phytochemical screening

Phytochemical screening is carried out using test tubes by adding various reagents. The results of phytochemical screening showed that *Kaempferia parviflora* Rhizome contains Flavoind, Phenolic, Tannin, Alkaloids, and Steroids.

Table 2. Phytochemical Screening Results

No.	Active Compounds	Screening Result
1.	Flavonoid	+
2.	Phenolic	+
3.	Saponin	-
4.	Tannin	+
5.	Alkaloids	+
6.	Steroids	+

Notes: (+) Positive; (-) Negative

3.3. Antibacterial activity using disk diffusion method

Antibacterial activity was evaluated the inhibition zone in various concentration of *Kaempferia parviflora* Rhizome extract. The reference antibiotic in this test is 1% chloramphenicol. Chloramphenicol is a broad-spectrum antimicrobial agent with a bacteriostatic effect against both gram-positive and gram-negative bacteria. In this study, 10% DMSO is used as the solvent for the extract to be tested. The 10% DMSO solution was chosen because it does not have antibacterial activity that could affect the test results, ensuring that the inhibition zones observed are solely due to the ethanol extract of *Kaempferia parviflora* rhizome and not influenced by the solvent. The test results show that the ethanol extract of *Kaempferia parviflora* rhizome can inhibit the growth of *Staphylococcus aureus* at all concentrations tested. The diameter of the inhibition zones of the ethanol extract of *Kaempferia parviflora* rhizome can be seen in Table 3.

Table 3. Inhibition Zone Diameter (mm) Evaluated Antibacterial Activity

Treatments	Concentration (%)	Replication (mm)			Mean ±SD	Interpretation
		I	II	III		
Kaempferia parviflora Rhizome extract	100	13.6	13.9	12.6	13.37 ±0.68	Strong
	75	12.0	15.6	8.8	12.13±3.40	Strong
	50	12.9	13.0	10.3	12.07 ±1.53	Strong
	25	10.2	9.0	11.8	10.33±1.40	Strong
Chloramphenicol	1	34.2	36.3	34.6	35.03±1.12	Powerful
DMSO	10	0	0	0	0.00 ±0.00	None

Based on **Table 3**, the results of the inhibition zones show that the concentrations of 25%, 50%, 75%, and 100% exhibit a strong inhibitory effect. The results indicate that the higher the concentration

used, the better the ability to inhibit the growth of *Staphylococcus aureus*. This can be seen from the increasing size of the inhibition zones. These findings are also supported by a study by Husnia [8], which states that the higher the concentration of the extract tested, the greater the active compounds present, resulting in larger inhibition zones.

The ability of the ethanol extract of *Kaempferia parviflora* rhizome pounds that act as antibacterial agents contained in the rhizome. Based on phytochemical testing, the ethanol extract of *Kaempferia parviflora* rhizome contains flavonoids, phenolics, alkaloids, tannins, and steroids. Flavonoid compounds act as antibacterial agents by forming complex compounds with extracellular proteins, leading to damage to the bacterial cell membrane, which results in cell lysis [9]. Another compound with antibacterial activity is tannin. Tannins work by inactivating bacterial enzymes and disrupting the protein pathways within the cells, leading to cell death[10]The antibacterial mechanisms of each secondary metabolite compound in the ethanol extract of *Kaempferia parviflora* rhizome can inhibit the growth of *Staphylococcus aureus*.

3.6. Research limitations

The limitation of this study was that the raw material of *Kaempferia parviflora* Rhizome extract might not be easily available in several areas.

4. CONCLUSION

Based on the research results, it can be concluded that the ethanol extract of *Kaempferia parviflora* rhizome has antibacterial activity against the growth of *Staphylococcus aureus* ATCC 25923 with strong antibacterial inhibition category with sequentially the best inhibition zone from 100%, 75%, 50% and 25%.

Funding: This research was financed by Lembaga Penelitian dan Pengabdian Masyarakat (LPPM) Unjaya

Acknowledgments: We would like to express our gratitude to Bachelor Pharmacy, the Faculty of Health, Jenderal Achmad Yani University and Lembaga Penelitian dan Pengabdian Masyarakat (LPPM) Unjaya.

Conflicts of interest: The authors declare no conflict of interest.

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Journal of
Food and Pharmaceutical Sciences

Review Article

Porcine-Derived Ingredients in Cosmetic Products and its Halal Authentication Method within Complex Matrices

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Received: 17 November 2024; Revised: 18 December 2024; Accepted: 20 December 2024; Published: 31 December 2024

Abstract: Nowadays cosmetics are an important commodity and the market for halal cosmetics is seeing growth. Cosmetics that contain porcine-derived ingredients are typically the source of halal problems. Gelatin and collagen are porcine derivatives that are extensively used in cosmetics. Hence, verifying the presence of porcine derivatives in cosmetics by developing analytical methods is critical. Despite this urgency, determining porcine-derived components in cosmetics is challenging, since cosmetics are quite complex with variable matrix forms. Moreover, to the best of our knowledge, there are only a few papers on developing porcine derivatives analysis in cosmetic items. This mini-review objective is to depict the current understanding of determining porcine collagen and gelatin in cosmetic matrixes. The findings revealed that the LC-MS/MS method is superior for determining gelatin and collagen sources in complex matrixes due to its sensitivity and accuracy. PCR and ELISA methods have challenges with the marker degradation problem since the derivatives undergo extensive processing conditions, thus lowering the methods' specificity and sensitivity, especially in complex matrixes. The SDS-PAGE method applications are limited and the method is suitable for a relatively simple matrix. This review highlights findings that support future advancements in cosmetic analysis for halal authentication.

Keywords: Halal Authentication; Cosmetic Matrixes; Porcine; Analytical Challenges

1. INTRODUCTION

Cosmetics are essential products in modern life due to increasingly high beauty standards [1]. They promote well-being, maintain health, improve beauty, and boost self-esteem. The cosmetics sector has had substantial growth and is predicted to continue to grow in the years to come. According to the cosmetic market analysis, the size of the worldwide cosmetics business was assessed at USD 374.18 billion in 2023 and is expected to increase from USD 393.75 billion in 2024 to USD 758.05 billion by 2032 [2]. Additionally, this notable improvement in the cosmetics industry as a whole was followed by the expansion of the global halal cosmetics market, which was estimated to be worth USD 42.39 billion in 2023 and is projected to reach USD 47.76 billion and USD 115.03 billion in 2024 and 2032, respectively, as the Muslim population has a big market size with about 2.4 billion people globally. These increases are driven by Muslims' growing receptivity to more Islamic teachings and the general public's growing understanding of the advantages of cosmetics. As a result,

consumers' desire for transparency regarding the components in cosmetics has increased, particularly concerning the halal material authenticity of ingredients used [3], [4], [5], [6]. Moreover, halal issues regarding cosmetic products are typically due to the source of animal-derived cosmetic ingredients. This issue emerges since some widely used ingredients in cosmetics use non-halal animals, such as pigs, as their main sources. For instance, in the gelatin industry, pig skin is the main source, accounting for about 46% of global gelatin production. Bovine hides come in second at 29.4%, while both pig and cow bones come in third at 23.1% [7].

Despite the urgency of determining animal-derived sources in cosmetic products, there are a number of obstacles. Firstly, cosmetic products are highly complex with variable matrix forms, consisting of numerous and diverse substances with their particular properties, making cosmetics a challenging matrix to analyze [35],[36]. Furthermore, due to the near compositional similarities across various sources, it is highly challenging to confirm the origin of animal protein compounds like collagen and gelatine for halal purposes and to identify any adulteration. Moreover, the degradation of protein and DNA biomarkers might happen since the production process of gelatine and collagen engages high temperatures and pressures. Further cosmetics production process involving complex processing conditions couples the matrix factor difficulties [10], [11], [12].

Therefore, the development of analytical methods performing specific, sensitive, and reliable results is highly desirable. Several analytical techniques have been developed to identify components generated from porcine in complex matrixes, including cosmetics. These analytical methods are liquid chromatography-mass spectrometry tandem mass spectrometry or LC-MS/MS [13], [14], polymerase chain reaction [15], [16], enzyme-linked immunosorbent assay (ELISA) [17], [18], [19], [20], and SDS-PAGE with or without the combination of nucleic-acid based method for less complex matrixes such as gelatine capsule shell [21], [22]. Nonetheless, to the best of our knowledge, there are only a limited number of publications that focus on the development of porcine derivatives analysis in cosmetic products, differing from highly processed food that is more extensively studied. This mini-review discusses the presence of animal-derived ingredients in cosmetics and focuses on the analytical methods to determine the presence of porcine-derived ingredients determination in cosmetic products, especially gelatin and collagen ingredients. The review also highlights some key sample preparation related to the method. This review objective is to depict the method's ability to discriminate animal protein sources from various origins in complex cosmetic matrix samples thus facilitating further development toward cosmetic analysis in the context of halal authenticity.

2. MATERIALS AND METHODS

The literature search was carried out using Scopus, PubMed, ScienceDirect, Google Scholar, and Google Search to find English-language publications that mainly discussed different cosmetics ingredients or the development of certain analytical techniques in determining the sources of gelatin or collagen within various forms of cosmetic matrixes. The methods chosen were the renowned and widely used methods that have good specificity, sensitivity, and reliability for animal source determination in complex matrices.

Several reports on the determination of porcine-derived gelatin or collagen using other complex matrix samples besides cosmetics were also used, if from the literature search, there are no reports of the application in cosmetic matrixes, yet the method is known to be potential. Despite that, these reports were already performed using complex matrix samples, for instance highly processed food,

and could depict the method's ability to discriminate animal protein sources from various origins in complex cosmetic matrix samples. The discussion also involves advantages, challenges, and limitations regarding the respective method.

3. RESULTS AND DISCUSSION

3.1. Non-halal animal-derived ingredients in cosmetics

Porcine is one of the restricted animal sources used in cosmetic products. Porcine-derived ingredients, such as lard, glycerin, fatty acids, gelatin, and collagen, are commonly used in cosmetic products. These ingredients play many roles in the production of cosmetic products. Lard is frequently used in lipid-based cosmetic products including creams, lotion, and lipstick, whereas glycerin contributes as a humectant, denaturant, skin protectant, and lowering viscosity agent [23], [24]. Fatty acids and their derivatives are primarily used as surface active agents in cosmetic cleansers and as components of the emulsion stabilization system to lower interfacial surface tension [25]. Gelatin and collagen themselves have a variety of purposes in cosmetics, whether as active agents to provide skin protection and nutrition or as supporting components to meet specific formulation needs such as gelling and viscosity-enhancing agents, that present in various forms of cosmetic products [26], [27], [28].

Gelatin is made from the source protein collagen. Gelatin is a heterogenous blend of peptides that is produced by partially breaking polypeptide connections and eliminating cross-links between polypeptide chains. Collagen itself is the most prevalent structural protein in both vertebrates and invertebrates and composes about 30% of an animal's overall proteins [26]. Collagen is present in animal skin, tendons, cartilaginous tissues, and mammalian bones such as pigs, cows, donkeys, and horses. Furthermore, it is present in fowl including chickens, ducks, wilds, and turkeys as well as aquatic animals like tuna, sea cucumber, and jellyfish [29]. Gelatins and collagen found in the market are primarily derived from pigs as they are economical and also have superior physical and functional properties compared to other sources [30], [31], [32].

Different ideas for innovative cosmetics have emerged. The use of oral supplements to improve appearance is known as nutricosmetics. They are frequently called "oral cosmetics," "beauty pills," or "beauty from within." [33], [34], [35]. In the last few years, a variety of skincare products have used gelatin and collagen as excipients or as advantageous ingredients for beauty and appearance. Gelatin's special physicochemical properties, such as foaming, stabilizing, thickening, gelling, emulsifying, and binding, have led to its widespread application in pharmaceuticals and cosmetics [28]. Soft gelatin capsule is the most commonly utilized pharmaceutical form, behind tablets. Numerous dietary supplements are frequently encapsulated in gelatin capsules [21], [36]. Additionally, a wide range of cosmetic goods, such as bubbles, face creams, body lotions, shampoos, hair sprays, sunscreens, and bath salts, use gelatin as a gelling agent [37]. Furthermore, gelatin has also been utilized to protect the skin from UV radiation damage. The balanced lipids of the skin are maintained by gelatin through its antioxidant properties, which help to correct the damage to the skin's structure [27], [38]. The antioxidant enzymes that contribute to building up the body's defenses against oxidative stress are diminished when exposed to ultraviolet light. The study claims that employing gelatin hydrolysates will greatly boost the antioxidant enzymes' activity [27].

Collagen has been identified as a possible treatment for wrinkles and the aging process. One of the main indicators of the decline in skin quality with age is a reduction in collagen and elastin fibers,

which results in elasticity loss and wrinkle formation [26], [39]. Aging also causes the skin to produce less glycosaminoglycan (like hyaluronic acid), which makes the skin dry and loses its integrity since hyaluronic acid is the main molecule involved in skin moisture because of its unique capacity to hold water molecules [40], [41], [42]. A systematic review and meta-analysis were carried out by Pu et al. (2023) [43] to assess the impact of collagen supplements on skin aging. 26 RCTs were examined to assess how oral collagen supplements affected skin elasticity and hydration, two characteristics that characterize skin aging. Their study found that oral collagen supplements increased the moisture and elasticity of the skin, with noticeable improvements after eight weeks or longer of collagen supplementation. Another study by Sanz et al. (2016) [44] examining the influence of applied topically collagen on the process of aging found that approximately 75% of women treated with an applied topical product containing collagen proved anti-wrinkling effects and significant increases in the dermal density and elasticity of their skin when compared to those in the control group. In a similar vein, the study by Maia Campos et al. (2019) [45] revealed that healthy female participants who applied a topical product showed a substantial increase in skin elasticity and moisture.

Concomitantly with the advancement of technology, animal-derived-containing products have been extensively developed and are currently being utilized in the cosmetic and pharmaceutical industries. Consequently, the halal status of these products may be questioned. The acceptability of cosmetics made with animal-derived components usually depends on the type of animal from which the components are produced to meet religious restrictions. Halal itself denotes permissible things that are lawful by Islamic law, whereas non-halal or haram refers to those that are forbidden [6]. Generally speaking, a product is considered non-halal under Muslim law if it contains elements obtained from pigs or other animals that are not slaughtered in accordance with Islamic law [31], [46]. Muslims are severely forbidden from using or consuming any products that include non-halal components, even in trace amounts because halal products have a zero-tolerance policy [47]. Additionally, halal takes into account the entire process, from product manufacturing to distribution, rather than just the contents. In general, according to The Indonesia Council of Ulama and Malaysia Standard of Halal Pharmaceuticals, halal products need to adhere to a number of key principles, which are:

a. It should not include any animal parts or products that are not halal or that have not been killed in accordance with Islamic law.
b. Absent najs in accordance with Islamic law.
c. Demonstrate safety and effectiveness for humans with the recommended dosage, quality, and hygiene.
d. In accordance with Islamic law, was not made, processed, or prepared using machinery contaminated by najs. Halal requirements and Islamic law should be followed throughout the entire production process.
e. Don't include any human parts or derivatives, as this is prohibited by Islamic law.
f. Halal products are physically separated from any other products that do not meet the requirements listed in items a, b, c, d, e, or any other items that have been declared non-halal and najs by Islamic law during all processes, including preparation, processing, handling, packaging, storing, and distribution. Any potential cross-contamination must be prevented [6], [48], [49].

Halal certification can be viewed as an added benefit since the main criteria are the effectiveness, safety, and quality of all products [6]. Because of this, halal products are starting to become the new norm for safety and quality control, even among non-Muslim customers [24], [50]. The halal certification requires a proof document that shows the ingredients used or the final product does not contain any non-halal animal-derived ingredients. Thus, the analytical methods to determine this target analysis are highly in demand.

3.2. Analytical method for animal-derived identification in cosmetics

Various analytical techniques have been developed to identify components derived from porcine in complex matrixes, including cosmetics. These methods have different approaches and principles in determining the sources of gelatin and collagen with respective benefits and limitations. Liquid chromatography-mass spectrometry tandem mass spectrometry (LC-MS/MS) using specific peptide analysis for identification, polymerase chain reaction (PCR) exploring specific markers of the DNA, ELISA is dependent on antigen-antibody interaction, and SDS-PAGE relies on the molecular weight of protein. Table 1 compiles the benefits and limitations of respective analytical methods. Further respective descriptions of the methods are discussed below.

Table 1. List of some analytical methods for identifying porcine-derived components in complex matrixes along with each method's advantages and disadvantages

Methods	Advantages	Disadvantages	References
LC-MS/MS	• Precise mass measurement, superior sensitivity, and good resolution enable accurate identification compared to other techniques • Suitable for identifying complex and highly processed matrixes	• More expensive compared to other techniques • Time-consuming • Requiring specialized knowledge	[51], [54], [55]
PCR	• Easy to perform and frequently used for species identification • More affordable than the LC-MS/MS method	• Less sensitive and specific than LC-MS/MS especially in complex and highly processed matrices like cosmetics. • The quality of DNA isolates and DNA degradation can have an impact on the test result • The PCR approach is unable to identify the degree of contamination in a gelatin sample because DNA detection is not directly correlated with the amount of protein or peptide present in a sample.	[13], [54], [56], [57]
ELISA	• Easy to perform • Using low-cost reagents, therefore more affordable to perform than the LC-MS/MS method • Offer benefits over methods based on nucleic acid as proteins may have a primary structure that is more stable than DNA	• Less sensitive and specific than LC-MS/MS especially in complex and highly processed matrices like cosmetics • Heat processing may alter the original structure of the antigen's epitopes, making it more difficult for the antigen to be recognized by its particular antibody.	[13], [58], [59], [60], [61], [62]

continued Table 1..		• If certain antibodies are not readily available, it could take months to produce them	
SDS-PAGE	• Easy to perform • Inexpensive method	• Only suitable for relatively simple matrix	[21]

3.2.1. LC-MS/MS

In recent years, the liquid chromatography-mass spectrometry tandem mass spectrometry (LC-MS/MS) method using specific peptide analysis for identifying the animal sources of gelatin has garnered significant attention for development. Due to variations in amino acid sequence, mass spectrometry enables the identification of the animal sources of gelatin accurately [51]. As previously mentioned, collagen partially hydrolyzes to produce gelatin, with type I collagen being the most prevalent collagen in connective tissue among all 28 collagen types [52]. Because hydrolyzed type I collagen is the most common type of collagen, gelatins may contain peptides from this type of collagen. According to sequence alignment findings, the type I collagens of pig and cow, which are two of the most widely available gelatin sources, exhibit high similarity with 99% sequence similarity. Nonetheless, a small number of amino acid variations exist, allowing it possible to distinguish between the gelatin origin of porcine and bovine based on peptides derived from type I collagen [12], [53]. Table 2 tabulated several potential marker peptides for distinguishing between bovine and porcine gelatin.

Table 2. Several potential marker peptides to differentiate between porcine and bovine gelatin

Potential marker peptides	References
GRPGPPGPAGAR GEPGPTGVQGPPGPAGEEGK GSPGPAGPK QGPSGPSGER DLEVDTXLK GPNGEVGSAGPPGPPGLR GFPGSPGNVGPAGK GAPGPDGNNGAQGPPGPQGVQGGK GIPGEFGLPGPAGPR IGPPGPSGISGPPGPPGPAGK TGETGASGPPGFAGEK GPPGAVGNPGVNGAPGEAGR	[12]
GPPGESGAAGPAGPIGSR GSPGADGPAGAPGTPGPQGIAGQR	[63]
GEPGPAGSVGPAGAVGPR GPTGPAGVR GETGPAGPAGPVGPVGAR	[12], [63]

The determination of gelatines from various products through LC-MS/MS is conducted by extraction of gelatin from matrix samples, after which the extracted gelatin undergoes digestion with proteases like trypsin. The resulting peptides then are separated using liquid chromatography and subsequently sequenced through various databases available as part of the instrument to analyze the animal source of gelatin [54]. The comprehensive sample preparation procedure for gelatin extraction from cosmetic samples is presented in Figure 1.

In the sample preparation protocols for gelatin extraction (Figure 1), hexane is added to the sample to separate lipid contaminants which are commonly present in certain types of samples, such as cosmetics [13], [64]. Additionally, to examine the gelatin in the capsule shell, the sample is first washed with milli-Q water in order to remove the remaining residual contents of the capsule. The capsule shell is then dried, cut, and gone through further sample preparation [54], [60]. Unlike bottom-up proteomics protocols within meat samples which need reduction and alkylation steps, in order to break disulfide bonds and alkylate cysteine groups to prevent them from forming undesirable new disulfide bonds, in the gelatin-containing samples analysis, gelatin does not require reduction and alkylation steps, since gelatin proteins are already hydrolyzed during the production process [64], [65]. Subsequently, the protein digestion stage is performed by using trypsin enzyme in an ammonium bicarbonate solution at 37 - 40° C for 12 – 24 h [13], [14], [66], [67]. Various digestion methods have been developed to overcome this time-consuming conventional digestion process including the employment of ultrasound and microwave digestion methods thus speeding up the process from an overnight digestion to just a matter of minutes [68], [69].

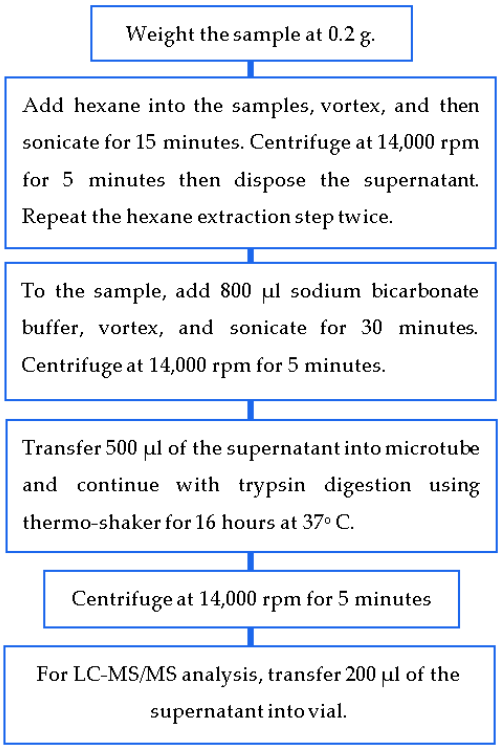

Figure 1. Sample preparation procedures for gelatin extraction from cosmetic samples using conventional digestion method [13]

Figure 2 illustrates the novel digestion approach procedures. However, these protocols need further optimization as the study demonstrated that compared to microwave and ultrasonic digestion, conventional digestion produced a higher detection rate [69]. Chia et al. (2020) [13] combined a UHPLC-fast separation with an SRM-based targeted method to develop a testing protocol on the utilization of triple quadrupole LC-MS/MS technology for detecting the presence of porcine gelatin in cosmetic and food confectionary samples. In order to evaluate the robustness sensitivity, and accuracy of the SRM-based LC-MS/MS method for identifying porcine gelatin, a number of tests were carried out using standard reference gelatin material as well as actual cosmetic and food samples. Firstly, the developed method was used to examine reference gelatin standards

derived from bovine and porcine sources. The results showed that no porcine markers were found in the bovine gelatin standard, while all 11 porcine peptide markers, that had been discovered in their previous study, were successfully identified in the porcine gelatin standard. To determine the positive identification of porcine gelatin, all SRM transition peaks must be correctly aligned, retention times must be matched, and the correct ion ratio between different ions for the same peptide marker. This application of the three levels of identification criteria guarantees reliable and accurate results, even at low detection limits.

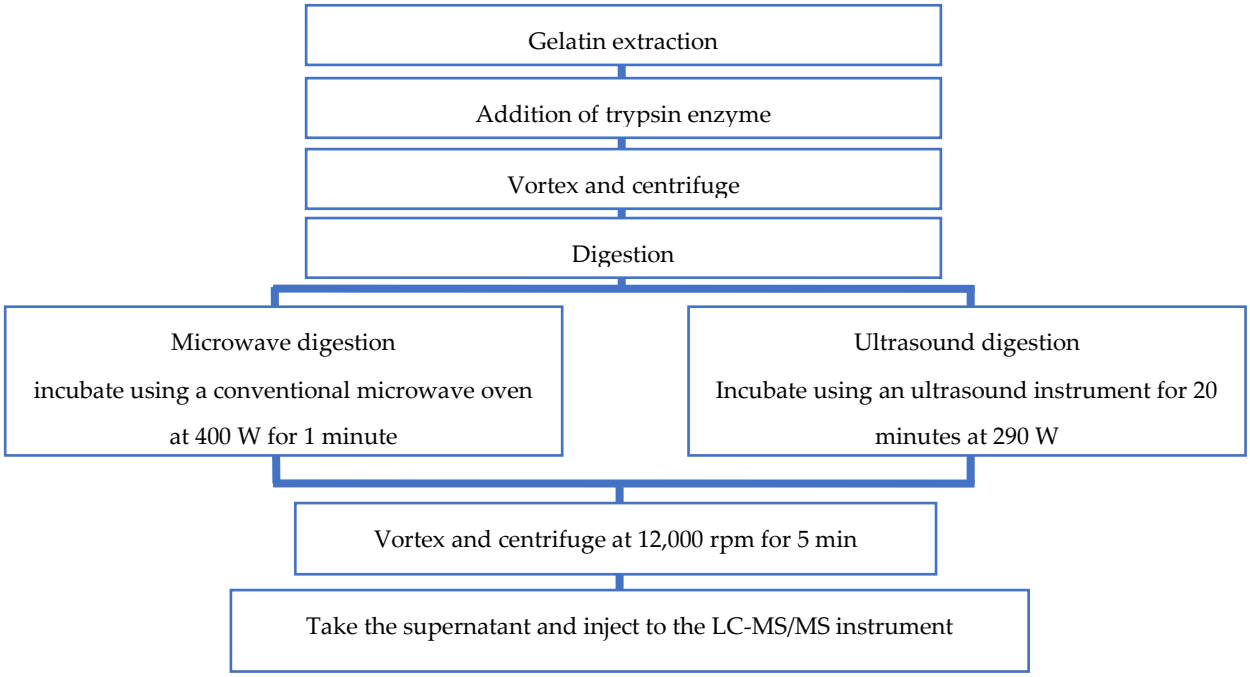

Figure 2. Microwave and ultrasound digestion protocols [69], [70], [71]

The method's performance under different matrix conditions was assessed by spiking porcine gelatin standard in hair moisturizer cream and facial gel samples to mimic different contamination levels (spiked solution concentrations of 0.01 %, 0.05 %, 0.1 %, 0.5 %, 1.0 %, and 5.0 %). As a result, even at the lowest concentration of 0.01% in the spiked sample, all 11 porcine peptide markers were detected. The method achieved a remarkable linear calibration curve, with an r^2 value exceeding 0.99 and the inter-day analysis of the samples demonstrated good reproducibility. Furthermore, a comparative study between the SRM-based LC-MS/MS method and the ELISA method was conducted on 27 different commercially available food and cosmetic products containing gelatin with the goal of examining the developed method's accuracy. For the majority of samples containing gelatin, the results indicated a positive correlation between the LC-MS/MS and ELISA methods. However, two cosmetic samples showed inconsistent results using the ELISA method and were confirmed to be negative for porcine peptides using the LC-MS/MS method. The LC-MS/MS method's feasibility for analyzing porcine gelatin in such challenging various cosmetic and food matrixes was confirmed by the experiment. According to the experiment's findings, LC-MS/MS demonstrated a sensitive, robust, and reliable method for identifying porcine derivatives in cosmetic and food products [13].

The presence of Hyp may influence the judgment findings of amino acid sequence, potentially resulting in inaccurate animal origin identification [54]. For instance, in bovine COL1A2 with the

marker peptide sequence $G^{308}AAGLP^{313}GVAGAPGLPGPR^{325}$, whereas the sequence $G^{310}AACLL^{315}GVAGAPGLPGPR^{327}$ is present in porcine COL1A2. In the process of determining the peptide sequence, if Pro313 hydroxylation (+16 mass) occurred, it could be mistaken with a similar mass of amino acids, such as Leu and Ile [51], [53]. Therefore, Jumhawan et al. (2019) [14] developed an LC-MS/MS method using the multiple reaction monitoring (MRM) method for the identification of porcine and bovine gelatins in pharmaceutical, cosmetic, and food products. With MRM, which is a modified form of SRM, several SRM transitions for the same or distinct analytes can be tracked in a single mass spectrometry run. MRM can recognize post-translational modifications (PTMs) and differentiate between very similar protein forms by monitoring several transitions in a single run. For protein identification in complex matrixes, MRM is therefore preferred [72]. Jumhawan et al. (2019) [14] study performed in silico MRM transitions prediction of bovine and porcine peptides using Skyline software. Skyline software can be used to identify the location and amount of proline hydroxylation in order to obtain all MRM transitions and to identify the variations in peptides caused by the hydroxylation of proline [54]. Table 3 shows the prediction of bovine and porcine marker peptides performed in the study. The prediction test indicates that the precursor and product ion mass-to-charge ratios (m/z) of porcine and bovine peptides are identical. Peptide sequences from porcine (GPPGSAGAPGK) and bovine (GPPGSAGSPGK) gelatins exhibit slight discrepancies, differ only by a single amino acid (the 8th amino acid), and unpredicted proline hydroxylation to gelatin occurrence may result in peptides with the same mass and identical MRM transitions for both species.

Table 3. In silico MRM transitions prediction of bovine and porcine marker peptides using Skyline software. Bold, underlined proline (P) signifies proline hydroxylation [14]

Peptide Marker	Sequence	Precursor (m/z)	Product (m/z)	
Bovine Specific Marker	GPPGSAGSPGK	456.2327++	y10	854.4367+
			y9	757.3839+
			y3	301.1870+
Porcine Specific Marker	G**P**PGSAGAPGK	456.2327++	y10	854.4367+
			y9	741.3890+
			y3	301.1870+

Moreover, Jumhawan et al. (2019) [14] study performed a method validation. Following sequence verification, 8 and 9 peptides were discovered to be specific for porcine and bovine gelatine, respectively. Every peptide marker performed good repeatability (RSD < 15%). The developed method can successfully identify adulteration levels in spiked bovine and porcine gelatin matrixes as low as 0.1%. Additionally, the method has shown success in determining porcine and bovine gelatin in the test of commercial goods (encompassing pharmaceutical capsules, personal care, and food products). In conclusion, the developed method provides a reliable strategy to identify and specify animal-based gelatins that enforce halal testing [14]. Overall, the LC-MS/MS method offers excellent resolution, high sensitivity in complex matrixes, and accurate mass measurement. Despite that, this method has several drawbacks such as not being widely available in laboratories, being time-consuming, high cost, and need for expertise [51], [54], [55].

3.2.2. Polymerase Chain Reaction

Because of its sensitivity and selectivity, the polymerase chain reaction (PCR) method is frequently used to identify species by identifying animal DNA present in the sample [54]. Since DNA

is unique and species-specific, enabling accurate identification of porcine-derived content, thus PCR is one of the established methods for identifying porcine-derived in different products. The use of PCR to identify the source of gelatin in powder and capsule shells has been the subject of numerous studies, whereas the use of PCR to determine gelatin and collagen in more complex cosmetic matrix samples has not yet been extensively performed. Table 4 compiled the use of PCR methods along with reported porcine primers for the analysis of gelatin in capsule shells.

Table 4. Several applications of PCR methods and primers for gelatin analysis in capsule shell samples

PCR Method	Applications	Porcine Primers	References
Conventional PCR	Identification of bovine and porcine gelatin in gelatin powders and gelatin capsule shells	Target gene: Cyt b 5′-GCCTAAATCTCCCCTCAATGGTA-3′ 5′-ATGAAAGAGGCAAATAGATTTTCG-3′	[70]
Real-time PCR	The identification of gelatin powders and capsule shells of bovine and porcine origin	Target gene: Cyt b 5′-CAACCTTGACTAGAGAGTAAAACC-3′ 5′-GGTATTGGGCTAGGAGTTGTT-3′	[71]
Multiplex PCR-RLFP	Differentiation of the gelatin sources of bovine, porcine, and fish in capsules	Target gene: Cyt b 5′-GGTAGTGACGAAAAATAACAATACAGGAC-3′ 5′-ATACGCTATTGGAGCTGGAATTACC-3′	[72]
Duplex PCR	Identification of bovine and porcine DNA in gelatin capsules	Target gene Cyt b 5′-ATGAAACATTGGAGTAGTCCTACTATTTACC-3′ 5′-CTACGAGGTCTGTTCCGATATAAGG-3′ Target gene: Cyt b 5′-GCCTAAATCTCCCCTCAATGGTA-3′ 5′-ATGAAAGAGGCAAATAGATTTTCG-3′	[73]

Zabidi et al. (2020) [16] developed methods for detecting porcine DNA in collagen cream cosmetic products by using conventional PCR with species-specific primer and electrophoresis gel. The study used a set of porcine-specific primers of 12SFW (5′-CCACCTAGAGGAGCCTGTTCT(AG)TAAT-3′) and 12SP (5′-GTTACGACTTGTCTCTTCGTGCA-3′) with the amplicon length of 387 bp. The study performed an optimization of primer annealing temperature by gradient the annealing step at 40 – 70^{o} C. Subsequently, PCR products were analyzed using electrophoresis gel in 1.0% (w/v) agarose gel. According to electrophoresis analysis, the annealing temperature of 44.4^{o} C exhibited the highest band intensity. Therefore, 44.4^{o} C was determined as the optimum annealing temperature and will be used for subsequent PCR assays. The analysis in cream sample products showed the presence of PCR amplicon, around 387 bp, in raw pork (positive control), piggy collagen cream, and hand cream which contains collagen from an unknown source. The study demonstrated that even though the cosmetic samples generally contained very small amounts of highly degraded porcine DNA, the developed method can detect and amplify the DNA.

Even though PCR is considered to be a selective and specific technique, the quality of the DNA used can affect the results [56]. Several components that may be found in cosmetic products including alcohol, metal ions, fats, pectin, detergents, and polysaccharides may interfere with PCR [73], [74], [75]. As a result, the DNA extraction method that is used should minimize these PCR inhibitors that

might be present in the samples [76]. There is a limited number of studies that have studied the efficiency of DNA extraction kits used in cosmetic sample preparation. Kim et al. (2018) [15] designed a real-time PCR method to identify porcine DNA in halal cosmetics and measured the real-time PCR detection limit to compare the effectiveness of different DNA extraction techniques. Figure 3 illustrates the workflows of RT-PCR to identify the presence of porcine DNA in cosmetics containing gelatin. A set of primers Sus NDH5 was developed in order to identify pig DNA in cosmetics (F: GCC TCA CTC ACA TTA ACC ACA CT and R: AGG GGA CTA GGC TGA GAG TGA A) with the amplicon size of 139 bp. Taqman probe (GGC GTA GGA TAY CCT CGT TTT TAC GT) was used in this experiment, labeled at the 5' and 3' ends with 6-carboxyfluorescein and black hole quencher 1, respectively. The specificity of the NDH5 primer was investigated by testing it with four bacterial strains nine vegetables, and eight different kinds of meat. The specificity result showed that, with the exception of the pig, no florescent signal was observed in non-target samples even after 40 cycles, proving the developed method is specific and facilitates the detection of porcine components in cosmetics. Additionally, tests conducted on 15 beauty goods revealed that none of the 15 samples examined using the developed rt-PCR contained any traces of pig DNA.

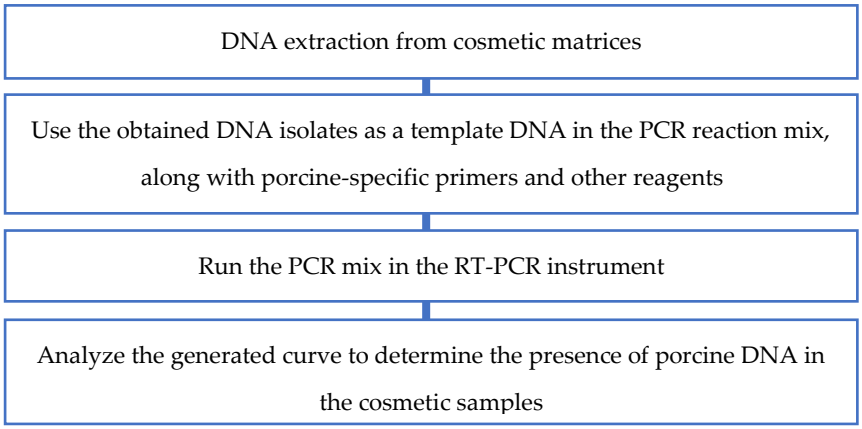

Figure 3. RT-PCR workflows of the source identification of gelatin in cosmetic matrices sample

Kim et al. (2018) [15] also conducted a test to examine how DNA extraction techniques affected real-time PCR amplification. Initially, cosmetic samples that will be spiked were confirmed with the developed method to detect the absence of porcine DNA. The confirmed cosmetic samples were then purposefully spiked with serially diluted porcine template DNA, then the DNA was extracted employing six distinct DNA extraction procedures (CTAB; Power Prep™ DNA extraction kit; QIAamp DNA stool mini kit; Wizard genomic DNA purification kit; TIANamp genomic DNA Kit; Nucleo spin food kit). Porcine DNA taken from the modified cosmetic samples was subjected to real-time PCR in order to investigate the trial's limit of detection. This LOD is then utilized to depict the performance of respective DNA extraction techniques. According to the findings, the rt-PCR method could identify the template DNA as low as 2.28×10^0 copies for the liquid-type mask pack using the Power PrepTM DNA extraction kit (Ct value of 38.51) and TIANamp Genomic DNA kit (Ct value of 38.71), which provide rt-PCR sensitivity 10–100 times higher than alternative extraction techniques. The rt-PCR limit of detection for the powder-type mask is 2.28×10^1 copies using the QIAamp DNA stool mini kit (Ct value of 37.63) and Power Prep™ DNA extraction kit (Ct value of 39.07). For both

kits, the rt-PCR limit of detection is 10–100 times greater than that of the alternative extraction techniques. Moreover, rt-PCR showed a limit of detection of 2.28 x 10^0 copies (Ct value of 39.07) when using the Power PrepTM DNA extraction kit on cream samples. This was 100–1000 times higher than the other extraction kits. Essential to note that hexane or chloroform treatment during the DNA extraction process results in fewer PCR inhibitors in cosmetic samples with high lipid content [77]. Hence, the Power Prep™ DNA extraction kit, which employs chloroform to extract DNA, may perform better yield in rt-PCR analysis. Therefore, this type of extraction kit can become a preferable option for isolating DNA within the gelatin component in cosmetic matrix samples. Additionally, Gina et al. (2024) [78] evaluated the effectiveness of two commercial DNA extraction kits to isolate DNA from gelatin powder, namely the DNeasy Mericon Food Kit (DM kit) and the Processed Food DNA Extraction (PF kit). DM kit was selected because it has been utilized to extract DNA from gelatin powder matrixes and drug capsules in several studies, whereas PF kit even though was developed for processed food products and has not been specifically reported for gelatin extraction, it may show effective in isolating DNA from gelatin matrixes. Nevertheless, the result showed that the PF kit produced a much higher DNA concentration than the DM kit, with 34.03 µL and 4.25 ng/µL, respectively. PF kit also gave better DNA purity levels in A260/280 and A260/230 ratios.

Unfortunately, because the experiment was conducted on cosmetic samples that had been spiked with serially diluted porcine DNA from meat samples, Kim et al. (2018) [15] experiment did not capture the comparison of the various extraction methods in terms of their performance to extract DNA from actual gelatin contained in cosmetics. Moreover, Gina et al.'s (2024) [78] experiment compared DNA extraction kits using gelatin powder rather than more complex cosmetic matrixes. Nonetheless, since the performance comparison of extraction methods on gelatin matrixes and cosmetics was limited to a small number of experiments, both studies provide an essential overview of DNA extraction methods' performance in highly complex samples and gelatin matrixes as well as depict the methods' potential ability to isolate DNA from gelatin contained in cosmetics. Additionally, both papers provide extraction kit choices that give favorable DNA isolate compared to other kits. Further experiment is needed to explore and compare how efficiently DNA extraction methods extract DNA from actual gelatin contained in cosmetics.

PCR method has several challenges as employing PCR to identify the origin of gelatin species has been proven to be quite challenging [13], [57]. DNA degradation is thought to be the primary factor influencing PCR success. This is a result of the high temperature and pressure involved during the processing of collagen into gelatin, leading to the almost complete destruction of any DNA present or DNA degradation into small fragments [10], [11], [13]. Furthermore, as the amount of protein or peptide in a sample is not directly correlated with the presence of DNA, the PCR method cannot be used to assess the degree of contamination in a gelatin sample [13].

3.3. ELISA

The other widely used technique is based on proteins and is known as ELISA (enzyme-linked immunosorbent assay). The basis of the ELISA analysis is the identification of specific antigens to proteins of the targeted species which is enabled by antigen-antibody interaction [13], [54]. Due to its sensitivity, specificity, and accuracy in identifying antigenic proteins, the ELISA method has been employed as a means of authenticity verification [58], [59]. The primary structure of proteins may be more stable than that of DNA, which can degrade under complicated processing circumstances and affect the identification result of the DNA-based gelatin authentication method [62]. Additionally,

the ELISA approach is a good alternative to other methods since it is simple to use, inexpensive reagents are available, and it can screen or quantify plenty of samples for the presence of target analytes [61]. Several studies have employed the ELISA method to detect gelatin in both raw and processed samples. Compared to several other formats of ELISA, indirect ELISA has been widely used for the determination of gelatin sources [18], [19], [20]. Figure 4 illustrates the indirect ELISA workflows for gelatin source differentiation. In indirect ELISA, a primary antibody that binds to the target protein is combined with a secondary antibody that is specific to the primary antibody. Before adding a primary antibody that will bind to the samples, the gelatin samples (antigen) on the microplate surface should be immobilized. Secondary antibodies that have been conjugate-labeled are then added to the primary antibodies in order to bind to them [79], [80].

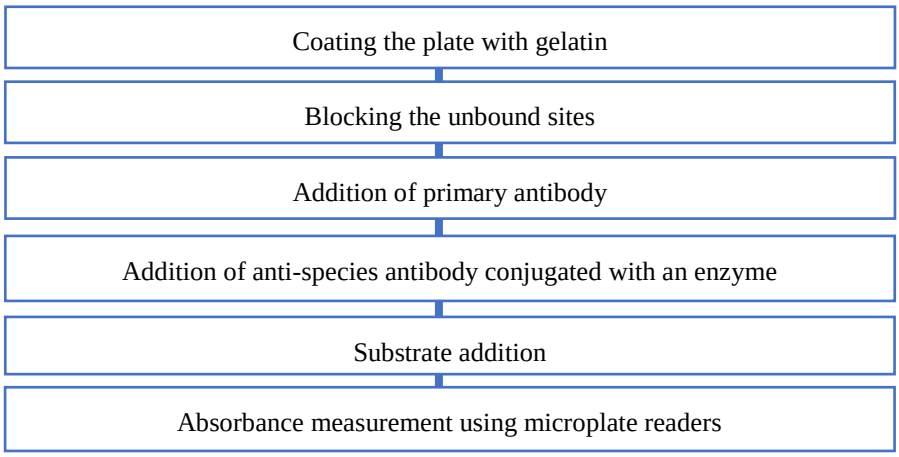

Figure 4. Indirect ELISA workflows for gelatin source differentiation

Using polyclonal anti-peptide antibodies, Venien & Levieux (2005) [20] created indirect ELISA formats to distinguish between raw bovine and porcine gelatines which can illustrate the ELISA method's ability to characterize the species origin of the gelatin. The two suggested sequences of bovine collagen, Peptide 1 (Glu-Phe-Asp-Ala-Lys-Gly-Gly-Gly-Pro-Gly) and Peptide 2 (Gly-Pro-Ala-Gly-Ala-Pro-Gly-Pro-Pro-Gly), were synthesized and used as the immunogens. Additionally, gelatins were also immunized to the rabbit, but because gelatin has very little immunogenicity, the molecule should undergo chemical alteration in order to produce antibodies in rabbits. Thus, tyrosylation was applied to the gelatins [81]. Then, at monthly intervals, rabbits (four rabbits for each antigen) received threefold immunization. Following each booster injection, the animals were bled seven to nine days later, and the sera were examined using indirect and competitive indirect ELISA to determine the antibody activity and specificity. The results showed that when used as coating antigens, the indirect ELISA performed using anti-peptide 2 antiserum enabled unambiguous discrimination among all of the bovine and porcine gelatines. On the other hand, when tested against collagen and gelatin, the anti-peptide 1 antibodies showed a low degree of reactivity. Furthermore, there was no discernible difference in the reactivity of bovine and porcine gelatins when utilizing a rabbit antiserum against bovine tyrosylated gelatins. This indirect ELISA could be used as a simple and rapid identification essay. Nevertheless, this format assay is unsuitable for identifying small quantities of bovine gelatin within porcine gelatin, as the results obtained from testing mixtures of bovine and porcine gelatin were relatively unsatisfactory. In contrast, the indirect competitive ELISA format that was designed exhibited a high sensitivity of 2 to 4 parts per 1000 for detecting bovine gelatines in pig gelatines that were bought from suppliers of laboratory chemicals. Additionally,

when industrial batches were examined, the sensitivity was 8 parts per 100 bovine gelatines in porcine gelatines.

Even though ELISA has several advantages, there are a number of obstacles to overcome. For instance, heat processing that denatures a target antigen may alter the original structure of its epitopes, making it more difficult for the antigen to be recognized by its particular antibody. According to a study by Doi et al. (2009), which used pAbs from rabbits (pAb1 and pAb2) and goats (pAb3) that were previously immunized using bovine gelatin as immunogen, the developed sandwich ELISAs reacted with porcine gelatin with a same or higher degree than that with bovine gelatin because the structures of the two gelatins are thought to be similar or their epitope areas are very similar. The developed method also has a limited specificity as it showed a low cross-reactivity to raw pork and cross-reacted with heated pork meat, goat, venison, boar, and rabbit meats. The high cross-reactivity with heated foods was most likely caused by the denatured collagen in meats and subsequently recognized by the antibodies used in this study.

Several approaches have been put out to get around the limitations. Among these is the discovery of thermostable proteins coupled with the production of particular antibodies directed against them. Since these thermostable proteins are present in both raw and highly processed samples, they can be utilized as target antigens for antibodies in the ELISA [82], [83], [84]. Additionally, the use of polyclonal antibodies is recommended over monoclonal antibodies for the identification of denatured proteins, especially in processed samples, due to their wider recognition of various epitopes and increased tolerance to minute changes [85]. For example, some processing methods may change or affect the accessibility of crucial epitopes, which could impair the immunoassay's capacity to identify antibodies [86], [87].

As far as we know, not much study has been performed to date examining the ability of ELISA to verify the origins of porcine gelatin in complex cosmetic samples. However, the ELISA approach has been performed for the detection and quantification of gelatines in relatively simple cosmetic matrixes, such as capsule shells, and various food matrix samples. Table 5 compiles several ELISA formats for the identification of gelatin in various matrixes. Therefore, these studies could have depicted the ability of the ELISA method to differentiate gelatin sources from various origins in complex cosmetic matrix samples. Overall, the use of the ELISA method for gelatin source identification is achievable although it is quite challenging.

Table 5. Some ELISA methods applied for the identification of gelatines in various matrix samples

ELISA Formats	Application	Remarks and Comments	References
Indirect Competitive ELISA	Determination of mammalian gelatin in capsules	All of the developed pAbs (pAb1 & pAb2), using porcine collagen sequences as the immunogens, showed cross-reactivity with more than 58% and 76% towards bovine gelatin from skin and bone, respectively. pAb1 showed cross-reactivity for more than 64% and 20% towards fish and chicken gelatins, respectively, while pAb2 exhibited <1% of cross-reactivity to both fish and chicken gelatin. Unfortunately, the study has limitations in the context of differentiating gelatin sources from porcine and bovine since all of the developed pAbs have cross-reactivity toward bovine, although pAb2 can discriminate mammalian from fish and chicken sources.	[88]

continued Table 5…

Competitive Indirect ELISA	Determination of porcine gelatin in edible bird's nest	Polyclonal antibodies (pAbs) from rabbits immunized with collagen amino acid sequences specific to pig species (pAb1, pAb2, and pAb3) were used to develop ELISA techniques. The results showed that pAb3 has superior selectivity among all of the developed pAbs. Moreover, pAb3 is sufficient for EBNs authentication from gelatin adulterants as pAb3 could identify porcine, bovine, and fish gelatines in EBN matrixes. However, the developed methods were not appropriate for detecting porcine gelatin for halal authentication since each pAb exhibited cross-reactivity with fish and bovine gelatines.	[18].
Competitive indirect ELISA	Determination of gelatin in confectionery products	The ELISA method is developed using porcine collagen immunogen to produce polyclonal antibodies. ELISA method performed low-cross reaction to fish and chicken gelatins but cross-reaction with bovine gelatin. The developed method is not appropriate for the identification of the presence of porcine gelatin as the developed pAbs showed cross-reactivity with bovine gelatin.	[19]
Sandwich ELISA	Determination of bovine and porcine gelatin in processed food	Novel sandwich ELISA methods were developed by using pAbs from rabbits (pAb1 and pAb2) and goats (pAb3) against bovine gelatin as an immunogen. Two sandwich ELISAs were performed by using pAb1-pAb2 and pAb3-pAb3. The cross-reactivity profiles of the two ELISAs were different. Both ELISAs showed positive responses toward boiled meat samples, although pAb3-pAb3 ELISA did not produce a positive response with boiled chicken. Furthermore, pAb3-pAb3 ELISA performed less cross-reactivity with boiled squid and various seafood and also produced weaker cross-reactivity with cooked meat. No negative nor false positives (except for gelatinized heated meat) were produced by the pAb3-pAb3 ELISA method. However, these developed methods mainly explored the ELISA's potency to differentiate bovine and porcine gelatin from fish gelatin in processed samples, as the results showed that both developed ELISA methods reacted with bovine and porcine gelatin. Therefore, the method has its limitations in the facet of halal authentication application.	[17]

3.3.1. Polypeptide Molecular Weight

Analytical techniques for the identification of the animal origin of gelatin are complex and expensive. Therefore, the development of simpler and less expensive methods to differentiate bovine and porcine gelatins is needed, especially in samples that do not have numerous and various components such as gelatin capsule shells in nutricosmetic or "oral cosmetic" products. Polyacrylamide gel electrophoresis (PAGE) is the most widely utilized technique for separating and assessing the molecular weight of proteins and peptides since it is simple to use and efficient [21]. Yap & Gam (2019) [22] developed a simple gel electrophoresis method to differentiate between gelatin capsules from cows and pigs using ammonium sulfate precipitation. In gelatin capsule identification, the sample preparation steps are relatively simple. Firstly, the content of the capsule is removed, then the capsule is cut and cleaned using blotter paper. Subsequently, the cut gelatin

capsule is weighed and dissolved. The result of the gel electrophoresis experiment showed the respective bands characteristic of porcine and bovine gelatins. Porcine gelatin has two bands at 110 kDa and 125 kDa, while bovine gelatin has two bands at greater molecular weights (125 kDa and 140 kDa). Through this band profile differences, bovine and porcine gelatin could be easily differentiated. A blind evaluation of the developed approach was also conducted by an authorized pharmaceutical organization, which used the developed gel electrophoresis method to examine 13 samples in total (8 capsule shells and 5 over-the-counter final products). The findings of the blind test demonstrated that the developed method could verify the source of gelatin as stated by the manufacturer for each sample.

The origin of gelatin can be identified by combining protein-based methods with nucleic acid-based methods. Malik et al. (2016) [21] demonstrated the use of these two approaches complement one another and aid in determining the origin of gelatin. They used polymerase chain reaction and electrophoresis to simultaneously identify and confirm the type of gelatin present in capsule shells. The results showed that pig gelatin had a broader molecular weight variation than bovine gelatin because, at optimal SDS-PAGE conditions, more bands were observed in porcine gelatin. Additionally, the results of the densitometry revealed that the densitometry profile of pig gelatin had 12 peaks, but the profile of bovine gelatin had just 4 major peaks. The densitometry profile analysis revealed that the porcine profile had peaks at < 100 kDa, whereas the bovine profile did not. Consequently, it is possible to distinguish between bovine and porcine gelatin using these peaks. Another specific difference was observed at the peak of 115 kDa of the porcine gelatin, whereas bovine gelatin does not have this peak.

Moreover, Malik et al. (2016) [21] employed principal component analysis (PCA) in conjunction with SDS-PAGE-densitometry to detect and confirm four gelatin capsule samples. PCA is a multivariate analysis that can find the connections between the samples and variables, capable of identifying patterns, grouping, similarities, and differences within the input data [89]. The result revealed that sample 1, was near the porcine gelatin reference and can be confirmed to contain porcine gelatin as labeled by producer. In contrast, samples 2 & 3, which are capsules containing gelatin from unidentified origins, were closer to the reference cow and were therefore likely to include bovine gelatin. Sample 4 exhibited a poor band pattern, and thus was unable to identify by SDS-PAGE-densitometry. Then, using universal oligonucleotide primers [forward (cyt b1): 5'-CCA TCC AAC ATC TCA GCA TGA AA-3' and reverse (cyt b2): 5'-GCC CCT CAG AAT GAT ATT TGT CCT CA-3'] and *BsaJI* as a restriction enzyme, a nucleic acid-based analysis (PCR-RLFP) was carried out to amplify the genomic DNAs of porcine and bovine. *BsaJ*I cut the 360 bp of target amplicon into two fragments of 228 and 131 bp for *Sus scrofa* and 316 and 44 bp fragments for *Bos taurus*. Using the nucleic acid-based method, it can be concluded that sample 1 was verified to contain porcine gelatin as labeled by the manufacturer as well as by the SDS-PAGE-densitometry coupled PCA result. Furthermore, the presence of bovine gelatin in samples 2 and 3—the same as in the protein-based method—was confirmed. After all, even while simultaneous approaches seem like a useful complement, it's crucial to maintain the simultaneous techniques at a low cost and have simple protocols.

4. CONCLUSION

In recent years, there has been a rise in the demand for cosmetics. Modern life has driven people to enhance their appearance. Along with such a phenomenon, customers are becoming more aware of embracing religious teaching, such as using halal products since the use of halal goods is highly advocated in Islam. Prohibited sources of animal-derived ingredients, such as porcine species mainly cause halal issues toward cosmetic products. Porcine-derived ingredients, such as gelatin and collagen, are extensively used in various forms of cosmetic products, whether as excipients or as beneficial components towards beauty and appearance since these ingredients offer economic advantages and superior properties among other animal protein sources. These advantages may result in the potency of adulteration practices and become a problem for the Muslim population since halal products have a zero-tolerance policy, meaning that haram ingredients are prohibited even in minuscule quantities. Therefore, the development of analytical methods performing specific, sensitive, and reliable results is highly desirable in order to support halal authentication regulations.

Various techniques have been performed for gelatin and collagen analysis in highly complex matrixes including liquid chromatography-tandem mass spectrometry (LC-MS/MS), polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), and polypeptide molecular weight such as SDS-PAGE. These methods have different approaches and principles in determining the sources of gelatin and collagen, with respective benefits and limitations. Various forms of cosmetic products are present nowadays and only a limited number of experiments were conducted in examining porcine protein in cosmetic matrix samples. Therefore, further exploration, development, and optimization of the established method are needed. Further optimization in LC-MS/MS workflows such as the optimization of non-conventional digestion methods is needed to overcome the lengthy preparation problem in the LC-MS/MS method. The exploration of the DNA extraction method to isolate the DNA within actual gelatin contained in the cosmetic sample is also critical, to demonstrate the performance of the method in real-life samples in favor of establishing simple protocols and reducing time-consuming analysis. Further research on the ELISA capability to determine gelatin source identification in cosmetic matrix samples is also needed to give further understanding of the method's capability as an alternative technique for the expensive LC/MS-MS method since as far as we know there is no publication specifically examining gelatin sources from the cosmetic matrix samples using ELISA.

Funding: This research received no external funding

Acknowledgments: The author would like to acknowledge the support from Institute of Halal Industry and Systems (IHIS) Gadjah Mada University.

Conflicts of interest: The authors declare no conflict of interest.

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Journal of
Food and Pharmaceutical Sciences

Original Article

The Effect of Variation in Solvent Concentration on Caffeine Content in Green Arabica Coffee Bean Extract (*Coffea arabica*) using UV-Vis Spectrophotometry

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Received: 30 November 2024; Revised: 20 December 2024; Accepted: 25 December 2024; Published: 31 December 2024

Abstract: Coffee is one type of beverage that is widely favoured by the community, Arabica coffee plants are a variant that grows a lot in Indonesia. Coffee beans contain various chemical compounds, one of which is caffeine. This study aims to determine the caffeine content with different solvent concentrations. Green Arabica coffee beans (*Coffea arabica*) were extracted using the maceration method. Ethanol extract 70% and ethanol extract 96% of green Arabica coffee beans were then fractionated in liquid-liquid extraction of chloroform and water phases. Then the analysis and determination of caffeine content were carried out with a UV-Vis Spectrophotometer. Qualitative tests were carried out using the Parry reagent color test showing positive results with a change in color to green. The results of the study in the form of a % caffeine content value to the weight of the extract sample, showed that in the 70% ethanol extract of green Arabica coffee beans contained a caffeine content of 10.115 ± 0.06% and the 96% ethanol extract of green Arabica coffee beans was 12.760 ± 0.580%. The results of the analysis showed that the amount of caffeine content had a significant difference with a p-value <0.05. So it can be concluded that the optimum solvent for extracting caffeine from green Arabica coffee bean extract is 96% solvent.

Keywords: Arabica Green Coffee Beans; Caffeine; Maseration; UV-Vis Spectrophotometer.

1. INTRODUCTION

Arabica coffee (*Coffea arabica*) is a type of coffee that is often planted and popular in global trade. The characteristics of Arabica coffee are small leaves, thin crowns, few ketai, and small flowers. Arabica coffee beans are different from other coffee beans. The shape is rather long with a convex surface but not too high. The color of the beans is lighter and the tips are bright. The center of the beans has a flat and bent groove [1]. Coffee contains high phenolic compounds such as caffeine, chlorogenic acid, caffeic acid, ferulic acid, pyrogallic acid, and trigonelline which are often used in diet programs. In addition, there are also other bioactive compounds such as polyphenols, flavonoids, melanoidins, trigonelline, various minerals, and carbohydrates that function as antiproliferatives, antimicrobials, anti-inflammatories and antioxidants [2]. Caffeine has clinically useful pharmacological effects, such as stimulating the central nervous system by relieving feelings of fatigue, hunger, and drowsiness[3]. Green Arabica coffee beans are chosen because they have a

better caffeine and chlorogenic acid content than roasted Arabica coffee. Caffeine and chlorogenic acid can act as photoprotectors so they can protect against UV-B radiation [4].

The amount of compounds contained in coffee beans depends on the solvent used[5]. Caffeine solubility is very low in water, slightly in ethyl acetate, pyrimidine, pyrrole, acetone, and very high in petroleum ether, ether, benzene and chloroform. In FI Edition V, 2015 it is stated that caffeine is soluble in 1:1 parts of hot water, 1:7 in chloroform and 1:130 in ethanol. The analytical method used in determining caffeine levels is UV Spectrophotometry. Caffeine is a compound that has chromophore and auxochrome groups, so it can be analyzed using UV Spectrophotometry [6]. The purpose of this study was to determine the acquisition of caffeine compounds in 96% ethanol extract of green Arabica coffee beans and 70% ethanol extract of green Arabica coffee beans using UV Spectrophotometry.

2. MATERIALS AND METHODS

2.1. Materials and tools

The tools used in this study were analytical scales, porcelain cups, funnels, Erlenmeyer flasks, beakers, measuring cups, sieves, grinders, measuring flasks, micropipettes, sonicators, hotplate stirrers, UV-Vis Spectrophotometry. The samples used were green Arabica coffee beans (*Coffea arabica*) taken from coffee plantations in the Genting Village area, Semarang, Central Java. The materials used in this study were 70% ethanol, 96% ethanol, and chloroform (Merck). caffeine standard, filter paper, $CaCO_3$.

2.2. Method

2.2.1. Sample Preparation

Arabica green coffee beans were taken from Genting Village, Semarang, Central Java. The clean samples were dried again using an oven at a temperature of 50^0C then ground and sieved with a sieve with a mesh number of 30.

2.2.2. Extract Preparation

The sieved powder was weighed as much as 300 g to then be extracted by maceration using 70% ethanol and 96% ethanol as much as 1200 ml each. The macerate was left for 24 hours then the macerate was re-macerated twice using 70% ethanol and 96% ethanol each as much as 600 ml for 24 hours with occasional stirring. The total macerate was filtered with a Buchner funnel to separate it from impurities or other solids, then evaporated with a rotary evaporator at a temperature of 50°C to remove the solvent. The extract results after being thickened are evaporated again with a water bath to produce a thick extract that can be weighed and its yield value calculated [7].

$$\textbf{\% Yield} = \frac{\text{Weight of thick extract}}{\text{Weight of Simplisia}} \times 100\%$$

2.2.3. Liquid-Liquid Extraction

The separation of caffeine from Arabica green coffee extract was carried out using the extraction method. Green coffee extract was dissolved in 100 ml of distilled water and then stirred using a magnetic stirrer for 1 hour accompanied by heating to a temperature of 70°C. Then 250 mg of $CaCO_3$ was added and the solution was filtered to separate the solids from the extract powder. A total of 25 mL of chloroform was put into a separating funnel, then shaken, and two layers were formed, take the chloroform layer and evaporate to dryness. After the chloroform was dry, the caffeine was

dissolved in distilled water up to 100 mL to be used for identification and determination of caffeine levels .

2.2.4. Qualitative Caffeine Test

Parry's reagent was made by reacting $Co(NO_3)_2$ with methanol p.a. The Parry test was carried out by taking 1 ml of the preparation sample and then adding Parry's reagent and dilute ammonia. A positive solution contains caffeine if the solution changes color to dark blue/green [8].

2.2.5. Quantitative Caffeine Test

a. Making Caffeine Standard Solution

Caffeine standards were weighed as much as 10.0 mg each then dissolved with distilled water up to 10.0 ml at a temperature of ±70^{0}C to obtain a stock solution with a concentration of 1 mg/ml and sonicated to dissolve completely [7].

b. Making Standard Curve

Stock solutions were taken as much as 60, 80, 100, 120, 140, 160 µl then dissolved with distilled water up to 10.0 ml to obtain a series of standard solutions with concentrations of 6, 8, 10, 12, 14, 16 µg/ml. Then the absorbance value was read according to the maximum lambda of each standard and the linear regression equation was calculated [1],[8].

c. Determination of Maximum Wavelength

Each standard solution of caffeine and chlorogenic acid was taken as much as 80 µl, put into a 10.0 mL measuring flask (concentration 8 µg/ml), then added with distilled water to the boundary line and homogenized. The solution was measured for maximum absorbance in the wavelength range of 200-300 nm [11].

d. Determination of Content

The caffeine sample that had been separated using liquid-liquid extraction was read for its absorbance using UV-Vis spectrophotometry at the maximum wavelength of caffeine and chlorogenic acid obtained in the previous process. Then the absorbance results and spectral diagram images were obtained for each fraction. And then the results obtained were analyzed using SPSS.

3. RESULTS AND DISCUSSION

3.1. Extraction

The initial step of this research is that Arabica green coffee beans obtained from Genting Village, Central Java are re-dried to reduce the water content so that it can prevent rotting, and growth of fungi and mold in the sample. The filtering stage with a mesh fineness of 30 is intended so that the powder size is uniform and the extraction process takes place optimally. This is because the smaller the powder size, the wider the contact surface of the sample with the solvent so that the compounds contained in the simplicial will be more extracted. Maceration is the simplest extraction method that is most widely used by soaking the powder in a suitable solvent in a closed container at room temperature. The choice of this method is based on the presence of compounds that are not heat-resistant or thermolabile [12]. Ethanol is a universal solvent that can dissolve various compounds, mold and germs are difficult to grow in ethanol 20% and above, and to evaporate the solvent requires a relatively faster time. The yield percentage is calculated with the aim of knowing the compound content contained in the extract and that the influence of the concentration of the

solvent used will affect the yield% results [13]. The yield obtained from the study results can be seen in Table 1. The yield of 96% ethanol extract is greater than 70% ethanol extract.

Table 1. Results of Arabica Green Coffee Bean Extraction Yield

Sample	% Yield
Ethanol Extract 70 %	11.15
Ethanol Extract 96 %	24.48

The thick extract obtained in this study was then re-extracted using the liquid-liquid method, namely the separation of compound components by dissolving the sample with another solvent. This extraction produces two immiscible phases, namely the water phase and the organic phase [14]. Some components will dissolve in the water phase and some will dissolve in the organic phase, the separation is according to the level of polarity with a fixed concentration ratio [15]. The organic solvent used is chloroform because caffeine has high solubility in chloroform [16]. The separation process occurs according to the level of polarity with a fixed concentration ratio [15]. So that later some of the compound components in the sample will dissolve in the water phase and some will dissolve in the chloroform phase. The function of adding $CaCO_3$ powder here is to break the bond between caffeine and other compounds in the sample, so that caffeine will be in a free base state. Caffeine in the form of a free base will be bound by chloroform. The chloroform layer is at the bottom because the specific gravity of chloroform is greater than water. Liquid-liquid extraction is repeated with the addition of new chloroform solution which aims to maximize the caffeine that can be extracted in chloroform.

3.2. Identification of Caffeine in Arabica Green Coffee Bean Extract

The qualitative identification results of Arabica coffee bean extract showed positive caffeine content as indicated by a significant color change using the Parry method. In this method, if several substances are dissolved in alcohol, then Parry's reagent and dilute ammonia are added, a positive result for caffeine content in the sample will show a change in the color of the solution to dark blue/green. The mechanism of the formation green and moss green colors produced come from the reaction between Parry's Reagent (which contains cobalt ions (Co) with a charge of +2) which binds the nitrogen group in the caffeine compound. Parry's Reagent is made by reacting Cobalt nitrate $[Co(NO_3)_2]$ with methanol. This reaction forms a green complex compound [17]. The more caffeine content, the more concentrated the green color like moss green [1]. The results of the identification test can be seen in Table 2.

Table 2. Results of Caffeine Identification using the Parry Method

Sample	Change		Result
	Before	After	
Ethanol Extract 70 %	Clear	Green	+
Ethanol Extract 96 %	Clear	Moss Green	+

Description: (+) = Contains Caffeine, (-) = Does Not Contains Caffeine

3.3. Caffeine Content in Arabica Green Coffee Beans

Determination of caffeine levels in green Arabica coffee beans was carried out using the UV spectrophotometry method. The UV spectrophotometry method was chosen because this method is a relatively fast, inexpensive, and easy to perform method. Determination of the maximum absorbance wavelength of caffeine with a wavelength range (λ) of 200-300 nm, the results of the measurements obtained a wavelength of 273 nm which is not much different from the theoretical wavelength, namely the caffeine wavelength theory ranges from 272-276 nm [18]. The results of measuring the standard caffeine absorbance value can be seen in Figure 1.

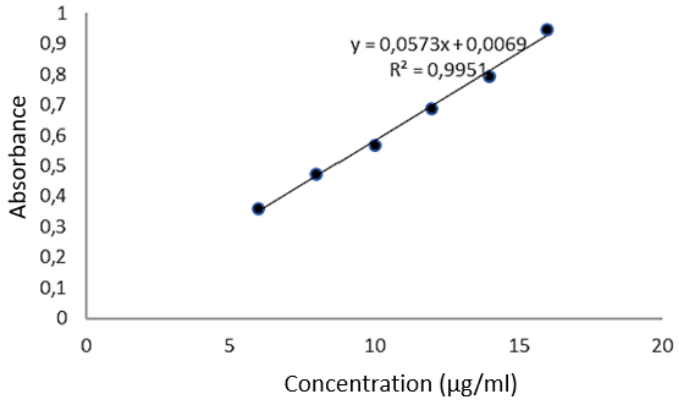

Figure 1. Caffeine standard curve

Caffeine is an alkaloid compound that is semi-polar or weakly polar, where there are two carbonyl groups together with a lone pair of electrons on the nitrogen atom increasing the polarity of the molecule. So caffeine will dissolve in more polar organic solvents. Caffeine which is polar will dissolve in solvents that are also polar or semi-polar according to the solubility principle "like dissolves like" that a substance will dissolve in a similar solvent [19]. Caffeine levels can be influenced by several factors, namely, the high concentration of solvents in dissolving caffeine components and the extraction temperature used. In this study, caffeine was highly soluble in ethanol solvent concentrations of 96% compared to ethanol solvent concentrations of 70%. Caffeine is an alkaloid that is non-polar so it tends to soluble in semipolar and non-polar solvents [20]. The results of determining caffeine levels can be seen in Table 3.

Table 3. Results of Caffeine Content of Arabica Green Coffee Beans

Sample	Result (Average± SD)
Ethanol Extract 70 %	10.115 ± 0.06 %
Ethanol Extract 96 %	12.760 ± 0.580 %

Further data were analyzed using SPSS showing that the significance value of $p<0.05$, which means that there is a significant difference in caffeine levels in 70% ethanol extract and 96% ethanol extract of green Arabica coffee beans.

4. CONCLUSION

Based on the research results obtained to determine the caffeine content in Arabica Green Coffee Beans (*Coffea arabica*), it can be concluded that the solvent that produces the highest caffeine content is 96% ethanol with a yield of 24.48% and a caffeine content of 12.760 ± 0.580%.

Acknowledgments: The author would like to thank Jenderal Achmad Yani University Yogyakarta for the support of facilities and infrastructure so that this research can run smoothly.

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Journal of
Food and Pharmaceutical Sciences

Original Article

Detection of Garlic Powder Adulteration Using FTIR Spectroscopy and Chemometrics: A Case Study in an Indonesia Marketplace

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Received: 16 May 2024; Revised: 06 August 2024; Accepted: 24 August 2024; Published: 31 December 2024

Abstract: Garlic is a plant with numerous benefits, including its use as a culinary ingredient and for medicinal purposes. However, the rising demand does not align with the availability of garlic in Indonesia, resulting in high garlic prices. Excessively high prices may potentially lead to garlic powder adulteration. The objective of this study is to determine whether there are garlic powder products adulterated with maltodextrin in the Marketplace. A total of 10 garlic powder samples were purchased on Marketplace. The study utilized a combination of FTIR spectroscopy with chemometrics. The chemometrics employed in this study were OPLS-DA (Orthogonal Partial Least Squares Discriminant Analysis) for sample grouping, as well as PLS (Partial Least Squares) for multivariate calibration. The findings revealed that OPLS-DA could effectively group the samples. Additionally, the best multivariate calibration model (PLS) was obtained at the wavenumber range of 679-440 cm^{-1} with a calibration R^2 value of 0.9981, RMSEC value of 1.11, validation R^2 value of 0.9926, and RMSEP value of 2.12. Based on the research results, it was found that three products were not pure garlic powder, with details indicating two products containing maltodextrin as an adulterant and one product containing a non-maltodextrin adulterant.

Keywords: Garlic powder; maltodextrin; FTIR; OPLS-DA; PLS

1. INTRODUCTION

Garlic (Allium sativum) is a horticultural plant commonly used as a primary seasoning in various forms such as fresh bulbs, dried powder, fried garlic, or garlic oil. The addition of garlic to dishes provides a unique flavor that cannot be replaced by other spices. The aroma and taste are attributed to the presence of allicin compound, which is the primary secondary metabolite in garlic. When garlic is damaged, it releases the enzyme alliinase to convert alliin into allicin. Apart from its culinary use, garlic is also utilized for medicinal purposes. Garlic is believed to have antimicrobial, antihypertensive, antioxidant, antitumor, and anticoagulant activities [1].

Garlic is known to have numerous benefits, making it an important commodity that increases consumption demand by 1.38% annually. The estimated consumption demand for garlic in 2023 reached 517.930 tons, increasing to 526.770 tons in 2024. However, the available amount of garlic in Indonesia cannot meet the projected consumption demand. From 2020 to 2024, Indonesia will

experience a significant decrease in garlic availability. In 2023, Indonesia experienced a garlic availability deficit of 405.200 tons, which increased to 411.440 tons the following year [2]. Based on this data, it can be observed that the garlic balance in Indonesia is unstable, indicating an imbalance between garlic availability and demand.

According to data from the Ministry of Trade's Market and Basic Needs Monitoring System (SP2KP), this phenomenon has led to a 6.99% increase in garlic prices from July 2023 to August 2023. Therefore, garlic is dubbed as "white diamonds" due to its easy saleability and high economic value resulting from continuous increasing demand [3]. High garlic prices can increase the likelihood of fraud, with one potential fraud being the adulteration of garlic powder. Garlic powder is obtained from garlic that has undergone drying and grinding processes. Adulteration of garlic powder is aimed at minimizing production costs with greater profits. This practice is known as adulteration, involving the addition or substitution of cheaper ingredients than the main raw materials [4]. Garlic powder can be adulterated with various substances such as maltodextrin, sodium caseinate, talc, cornstarch, corn flour (maizena), peanut powder, potato starch, and rice flour [5].

The FDA's Forensic Chemistry Center in the United States has found instances of garlic powder products being adulterated, where only one ingredient, garlic (pure garlic), is listed. However, the product actually contained 70% maltodextrin [6]. Additionally, research by Patarroyo-Leon discovered 15 samples of counterfeit garlic powder commercially available, mixed with cornstarch without labelling the addition of cornstarch as an additional ingredient [7]. Furthermore, in 2017, several food companies in the USA and Australia found peanut contamination in garlic powder [8]. Given these issues, there is a need for methods to detect food adulteration.

FTIR has been chosen as a popular methodology for detecting food fraud [9]. FTIR is an instrument used to analyze compounds in a product [10]. The basic principle of FTIR involves the interaction between a sample (material) and electromagnetic radiation. FTIR is commonly used in the pharmaceutical, environmental, and food industries [11]. In food fraud, FTIR is often used for authentication of herbal products, agricultural products, vegetable oils, and milk [9]. The advantages of FTIR instruments include the use of small sample sizes, simple sample preparation, minimal use of solvents and chemical reagents, no special treatment required for samples, non-destructive analysis, rapid analysis, and greater sensitivity [12]. Combining FT-IR spectroscopy with chemometrics is a good method choice for food authentication [13].

Chemometrics is a branch of statistics, mathematics, and formal logic used to obtain chemical information from data. Chemometrics is often used for analysis in pharmaceuticals, forensic science, environmental science, agriculture, and food authentication [14]. In food authentication, chemometrics has been used in several studies to detect fraud in oils, honey, dairy products, and meat [5]. There are two common chemometric methods used for food analysis: partial least squares (PLS) for calibration model creation and principal component analysis (PCA) for sample classification [13].

Previous research by Galvin-King successfully detected adulteration of garlic powder samples using FTIR and NIR spectroscopy [5]. Additionally, research by Lohumi also successfully detected cornstarch adulteration in garlic powder using FTIR spectroscopy combined with partial least squares regression (PLSR) [15]. Both samples and studies were conducted abroad. In this regard, there is currently no research on the authenticity testing of garlic powder in Indonesia. Therefore, there is a need to detect garlic powder adulteration in Indonesia, especially those sold in marketplaces

like Shopee. Shopee chosen as an online shopping platform because it has a high level of visitors the highest compared to other platforms, namely 241.6 million visitors in October until December 2023 [16]. This is proven by the complete availability of goods and features on Shopee, making it easier for customers to purchase goods [17].

2. MATERIALS AND METHODS

2.1. Sample

In this study, garlic powder samples sold on the Shopee marketplace, particularly in Surabaya, were utilized. After conducting a search using the keyword "garlic powder" in the Shopee application, 19 garlic powder sellers were found in the Surabaya area, offering products under different brands. From these 19 products, samples were selected based on specific criteria.

According to the inclusion and exclusion criteria, 10 garlic powder products meeting the criteria were obtained. Therefore, this study utilized 10 samples of garlic powder that were not registered with BPOM and not certified by the Indonesian Ministry of Agriculture, purchased from Shopee in the Surabaya area. Subsequently, detection related to the presence of maltodextrin in counterfeit garlic powder will be conducted on these 10 samples.

Pure garlic is needed as a comparison to garlic powder purchased at Shopee. Pure garlic was obtained from Blimbing Market, Lowokwaru District, Malang City. Next, the merger and integration was carried out at the Batu Herbal Materia Medica Laboratory. Subsequently, identification and pulverization were carried out at the Herbal Materia Medica Laboratory, Batu.

2.2 Materials

Maltodextrin is used as an adulterant in garlic powder mixtures. Acetone (EMSURE®) is used to clean ATR crystals in FTIR instruments.

2.3 Instrumentation

The equipment used in this study included a set of glassware (PYREX®, Arizona), an analytical balance (Ohaus Pioneer®, China), FTIR spectroscopy (Shimadzu®, Japan), and Chemometrics software (TQ Analyst®, Norway and SIMCA software (Umetrics, Umeå Sweden)).

2.4 Methods

2.4.1. Sample Preparation

Garlic bulbs were sorted, peeled, and washed with running water. Then the garlic bulbs were sliced into smaller and thinner pieces. The sliced garlic was dried using an oven at 60°C for 15 hours. The dried garlic slices were ground using a blender until garlic powder was formed. The garlic powder was sieved using a 60-mesh sieve. In addition, a mixture of pure garlic powder and maltodextrin was made with a concentration of 0-50%w/w to create a calibration model for PLS.

2.4.2. FT-IR Spectral Acquisition

The FTIR instrument used is the Qatar-S Single Bounce Diamond ATR (Shimadzu, Japan) with attenuated total reflectance (ATR) sample handling, the sample area is cleaned using acetone, the basic spectrum (background) is scanned before taking measurements on the sample, the sample to be scanned is prepared, the sample is placed under the crystal ATR, the sample was measured in 32 scans and at a resolving power (resolution) of 16 cm^{-1}, scans were carried out at a wavelength of 4000-650 cm^{-1} and replicated 2 times after being recorded in the form of absorbance, after scanning, the ATR was cleaned using acetone, then dry with a tissue.

2.4.3. Data Processing

The data was processed using SIMCA software (Umetrics, Umeå Sweden) for OPLS-DA, and then the data was processed using TQ Analyst software (ThermoScientific, USA) for PLS. Data is entered into the software (numerical and nominal data), optimization is carried out, and a score plot is obtained which describes grouping OPLS-DA and R^2 in PLS.

2.4.4 Validation Process

OPLS-DA validation required 15 samples of maltodextrin and 15 samples of control garlic powder. OPLS-DA validation was carried out by taking 1/3 samples (5 samples) for validation and 2/3 samples (10 samples) for calibration. Validation of the PLS calibration model was carried out using the Leave One Out cross validation technique. Leave One Out cross validation is carried out by discarding one sample and using the remaining samples to form a new model. Optimization is carried out to discard a number of samples until the best model is found. This selection is based on the R2 value which is closest to 1; RMSEC <2.4; and RMSEP <2.79.

3. RESULTS AND DISCUSSION

3.1. Spectral Interpretation

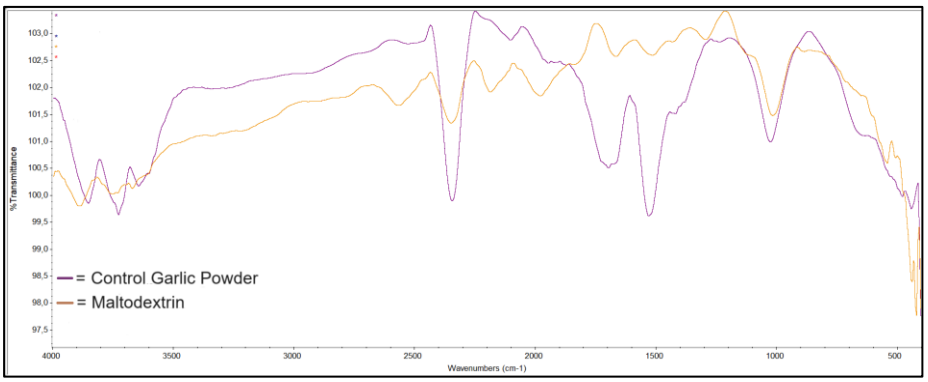

Figure 1. FTIR Spectra of Control Garlic Powder and Maltodextrin

Table 1. FTIR Interpretation Results of Control Garlic Powder

Functional Groups	Wavenumber (cm^{-1})	Control Garlic Powder		References
		Wavenumber (cm^{-1})	Functional Group Description	
O-H	3650-3600	3616.71	Stretching vibration	[18]
C=O	1830-1650	1687.80	Stretching vibration	[19]
C=C	1680-1650	1687.80	Stretching vibration	[18]
N-H	1640-1550	1521.32	Bending vibration of primary amine	[18]
CH_2 (alkane)	1465	1521.32	Cutout bending vibration	[18]
C-N	1350-1000	1021.87	Bending vibration of primary amine	[18]
$=CH_2$	900-600	665.93	Bending vibration out of plane	[18]
C-C	Around 620.72	665.93	Stretching vibration	[20]
C-S	700-600	665.93	Stretching vibration	[19]

The control garlic powder and maltodextrin as adulterants were tested using FTIR spectroscopy. The scanning process was replicated three times in the wavenumber range of 4000-650 cm^{-1}. The spectrum of the measurement results depicts characteristic functional groups in each compound. The FTIR spectra were interpreted by observing and comparing the position, intensity, and shape of the absorptions with reference tables of characteristic functional groups.

Table 1 shows that garlic powder has typical absorption in the O-H, C=O, C-N, N-H, C=C, C-C, and C-S groups. Based on the spectral readings of the garlic powder, absorption was observed at the wavenumber of 3616.71 cm^{-1}. This absorption occurred due to stretching vibration of the O-H group

with moderate intensity, indicating the presence of carbohydrates and amino acids. The presence of hydrogen bonds caused the O-H absorption peak to appear broadened. At the wavenumber of 1687.80 cm^{-1}, stretching vibration of the C=O group was observed, indicating the presence of protein components. At the wavenumber of 1521.32 cm^{-1}, bending vibration of the N-H group with moderate intensity was observed, indicating the presence of protein components. Bending vibration can cause changes in bond angles, resulting in bent bonds. At the wavenumber of 665.93 cm^{-1}, stretching vibration of the C-S group was observed, indicating the presence of sulfur components.

Table 2. FTIR Interpretation Results of Maltodextrin

Functional Groups	Wavenumber (cm^{-1})	Control Garlic Powder		References
		Wavenumber (cm^{-1})	Functional Group Description	
O-H	3650-3600	3622.45	Stretching vibration	[18]
C-H *sp3*	1450	1518.09	Bending vibration	[18]
C-O	1300-1000	1016.12	Stretching vibration	[18]
C-C	Around 620.72	617.68	Stretching vibration	[20]

Table 2 shows that maltodextrin has typical absorption in the O-H, C-H *sp3*, C-O, and C-C groups. Based on the results of the maltodextrin spectrum readings obtained there is absorption at the wave 3622.45 cm^{-1} which is describes the stretching vibration of the O-H group. There is a bond hydrogen causes the peak to appear broadened. On numbers wave 1016.12 cm^{-1} stretching vibration occurs which describes the existence of C-O group. At a wavenumber of 1518.09 cm^{-1} bending vibration occurs from the C-H *sp3* group. Stretching vibrations can cause changes bond length. A comparison of the two spectra revealed differences in the constituent groups between garlic powder and maltodextrin.

3.2 Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA)

3.2.1. Result of OPLS-DA Control Garlic Powder and Maltodextrin

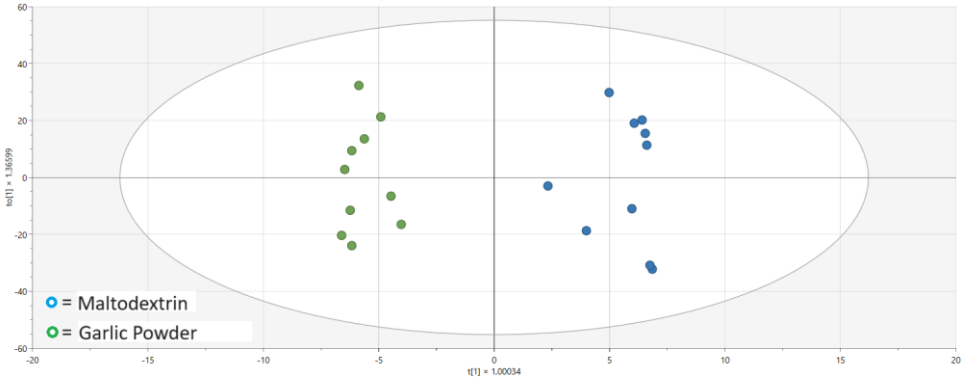

Figure 2. Score Plot Control Garlic Powder with Maltodextrin

OPLS-DA is a supervised clustering method. In this case, grouping is done by deleting the information in each group. Then, an analysis is carried out to find out whether groups whose information has been removed can gather with other groups that have similar characteristics. Based on Figure 3, it is found that OPLS-DA can group samples well. In the OPLS-DA score plot results, it was found that garlic powder and maltodextrin were well separated into two groups. The control

garlic powder group was in the left quadrant, while the maltodextrin group was in the right quadrant. In this case, both have different characteristics so they are in different groups.

3.2.2. OPLS-DA Validation

Validation is carried out to ensure that OPLS-DA can group samples well. Validation is carried out by taking 1/3 samples for validation and 2/3 samples for calibration. A total of 1/3 of the samples used for validation had their information removed (group of origin). Then, an analysis is carried out to see whether the sample without information can join according to the original group that was previously known. Based on the Table 3, it is found that the validation results show a truth level of 100%, which means that 1/3 of the samples whose information has been removed can be gathered according to the group of origin. Therefore, it can be said that OPLS-DA can group samples well.

Table 3. OPLS-DA Validation of Control Garlic Powder and Maltodextrin)

Design	Calibration		Validation		Total
	Number of samples	Correct	Number of samples	Correct	
Control Garlic Powder	10	100%	5	100%	15
Maltodextrin	10	100%	5	100%	15
Total	20	100%	10	100%	30

3.2.3. Garlic Powder Sample Prediction Using OPLS-DA

OPLS-DA which has successfully grouped samples is then used to group 10 samples purchased on Shopee. This stage was carried out to determine whether garlic powder samples 1-10 tended to group in the pure garlic powder group or the adulterant group. The large distance between samples indicates the similarity of the samples. The closer the points between samples indicate the higher the similarity, conversely, the farther the points between samples, the lower the level of similarity between the samples. In Table 4, it is found that there are three samples that do not pure, namely samples 2, 5, and 6. When grouping is carried out using OPLS-DA, the three samples converge on maltodextrin group. As for the pure sample of garlic powder are samples 1, 3, 4, 7, 8, 9, and 10. When carried out grouping using OPLS-DA, the three samples gather in the group of pure garlic powder so can be said to be pure garlic powder without mixture.

Table 4. Prediction of Garlic Powder Samples with OPLS-DA

Sample Number	Sample Total	Garlic Powder	Maltodextrin	Conclusion
1	3	3	0	Pure garlic
2	3	0	3	Impure
3	3	3	0	Pure garlic
4	3	3	0	Impure
5	3	0	3	Impure
6	3	0	3	Pure garlic
7	3	3	0	Pure garlic
8	3	3	0	Pure garlic
9	3	3	0	Pure garlic
10	3	3	0	Pure garlic

3.3 Partial Least Square (PLS)

3.3.1. PLS Wavenumber Optimization

Table 5. Wavenumber Optimization Results for PLS

Wavenumber (cm^{-1})	Calibration		Validation	
	R^2	RMSEC	R^2	RMSEP
4000-400	0.9799	3.76	0.9715	5.11
2800-1800	0.8838	8.42	0.7164	14.3
2390-2000	0.9077	7.55	0.7741	12.5
1930-1700	0.8466	9.57	0.8071	9.87
1560-1500	0.9150	7.26	0.9113	7.22
1200-600	0.9166	7.19	0.7519	11.1
1133-1000	0.9500	5.62	0.9539	5.26
679-440	**0.9981**	**1.11**	**0.9926**	**2.12**

Multivariate calibration using PLS is carried out by first selecting the most optimal wavenumber which has an R^2 value >0.99 and the smallest RMSEC (Root Mean Square Error Calibration) and RMSEP (Root Mean Square Error Prediction) values. Based on Table 5, it is found that the best wavenumbers are in the area 679-440 cm^{-1} with a calibration R^2 value of 0.9981; the RMSEC value of 1.11; the validation R^2 value of 0.9926; and the RMSEP value is 2.12. The values obtained are by the criteria so it can be said that the model used has good linearity, that is, it has a strong relationship or correlation between predicted and actual values. The optimum wave number was then used to process PLS data on control garlic powder, garlic powder samples 1-10, and a mixture of garlic powder with maltodextrin at a concentration of 0-50%w/w.

3.3.2. PLS Validation

The selected calibration model is tested to test the level of truth using cross validation. The cross-validation technique used is Leave One Out. Validation is carried out using the optimized wavenumber, which is 679-440 cm^{-1}. This validation technique is carried out by removing one of the calibration samples (for example, removing sample data with a concentration of 20%), then the remaining samples are used to create a new model [21-23] . Figure 4 that the results of the Leave One Out cross-validation on the selected model can provide a good validation R^2 value. After validation, the selected model can be used to predict maltodextrin levels in samples 2, 5, and 6.

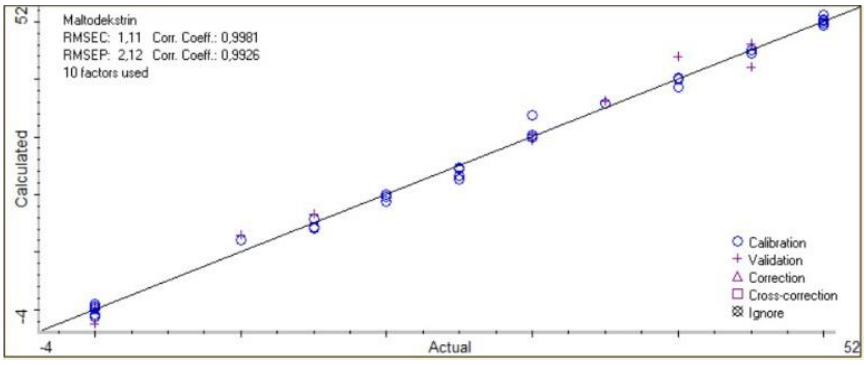

Figure 3. PLS Validation of Mixed Samples between Garlic Powder and Maltodextrin

3.3.3. Garlic Powder Sample Prediction with PLS

Garlic powder samples 1-10 were predicted to find the presence of maltodextrin as an adulterant in garlic powder sold in the Shopee. In Table 6, it can be seen that in the 5th sample there was an adulterant in the form of maltodextrin amounting to 14.96%, and in the 6th sample there was an adulterant in the form of maltodextrin amounting to 10.23%, and in the 2nd sample there was an adulterant but it did not come from maltodextrin. Therefore, it can be said that the combination of FTIR spectroscopy and chemometrics in this research has been successful and can carry out adulteration analysis in garlic powder.

Table 6. Prediction Results for Garlic Powder Samples with PLS

Sample Name	1 %(b/b)	2 %(b/b)	3 %(b/b)	Average %(w/w)	Conclusion
2nd sample	-4.03	-2.37	-1.93	-2.78 ± 1.11	The adulterant is not from maltodextrin
5th sample	4.46	22.75	17.67	14.96± 9.44	Added maltodextrin
6th sample	14.15	8.94	7.60	10.23± 3.46	Added maltodextrin

4. CONCLUSION

Based on the conducted research, the developed FTIR spectroscopy and chemometrics method can detect the adulteration of garlic powder. In sample grouping, it was found that OPLS-DA could classify samples based on their characteristic similarities, whereas PCA could not effectively classify the samples. The combination of FTIR with chemometrics can form a good model for multivariate calibration. Based on the optimization results at the optimal wavenumber range of 679-440 cm^{-1}, the calibration R^2 value obtained was 0.9981, the RMSEC value was 1.11, the validation R^2 value was 0.9926, and the RMSEP value was 2.12. There are three counterfeit garlic powder products sold on Shopee in the Surabaya area. Based on data analysis using OPLS-DA, it was found that samples 1, 3, 4, 7, 8, 9, and 10 are pure garlic powder, while samples 2, 5, and 6 are not pure garlic powder. According to the PLS data analysis, it was found that samples 5 and 6 are adulterated with maltodextrin, while sample 2 is adulterated with a substance other than maltodextrin.

Funding: This research received no external funding

Acknowledgments: -

Conflicts of interest: The authors declare no conflict of interest.

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