Chempublish Journal
Journal Homepage: online-journal.unja.ac.id/chp
Department of Chemistry
Faculty of Science and Technology
Universitas Jambi
ISSN (Online): ISSN 2503-4588
No. 23/E/KPT/2019

Article

Green Synthesis of Ag/Chitosan Nanoparticles using Avocado Leaves Bioreductor (*Persea americana* Mill.) as a Nitrite Colorimetry Detector

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Abstract

Urinary tract infection (UTI) is one of the most common bacterial infections, characterized by the proliferation of microorganisms within the human urinary tract. Nitrite serves as an important diagnostic indicator of UTI because it is produced through the bacterial reduction of nitrate. Various analytical techniques—including spectroscopy, electrochemistry, chemiluminescence, chromatography, capillary electrophoresis, and flow injection analysis—have been employed to detect nitrite. However, these methods generally require lengthy procedures involving bacterial incubation and extensive sample preparation; therefore, they are less suitable for rapid screening. This limitation highlights the need for a fast, reliable, and cost-effective detection approach. Colorimetric sensors, particularly those based on nanoparticles, offer a promising alternative due to their rapid response, low cost, and ability to provide visually observable results. In this context, the present study aims to synthesize silver nanoparticles (AgNPs) using avocado leaf extract (*Persea americana* Mill.) as a natural bio-reductant and chitosan as a stabilizing agent, thereby reducing reliance on hazardous and environmentally unfriendly inorganic chemicals. The synthesized AgNPs were characterized using UV–Vis spectroscopy, Fourier Transform Infrared (FT-IR) spectroscopy, and Particle Size Analyzer (PSA) measurements. The synthesis was optimized by varying the bioreductant volume, reaction time, and chitosan concentration. The optimal synthesis time was determined to be 4 h, yielding a surface plasmon resonance (SPR) peak at 428 nm with an absorbance of 2.112 and nanoparticle size within the desirable range. Furthermore, a chitosan concentration of 2.5% produced the most stable nanoparticles, indicated by an SPR peak at 435 nm and an absorbance of 1.341. The resulting AgNPs/chitosan system functioned effectively as a colorimetric nitrite sensor, exhibiting a visible color change to purple, with a limit of detection (LOD) of 0.1303 µM and a limit of quantification (LOQ) of 0.4345 µM. All measurements were conducted in triplicate to ensure reproducibility.

Keywords: *Ag Nanoparticles; Avocado Leaves; Colorimetry; Nitrite.*

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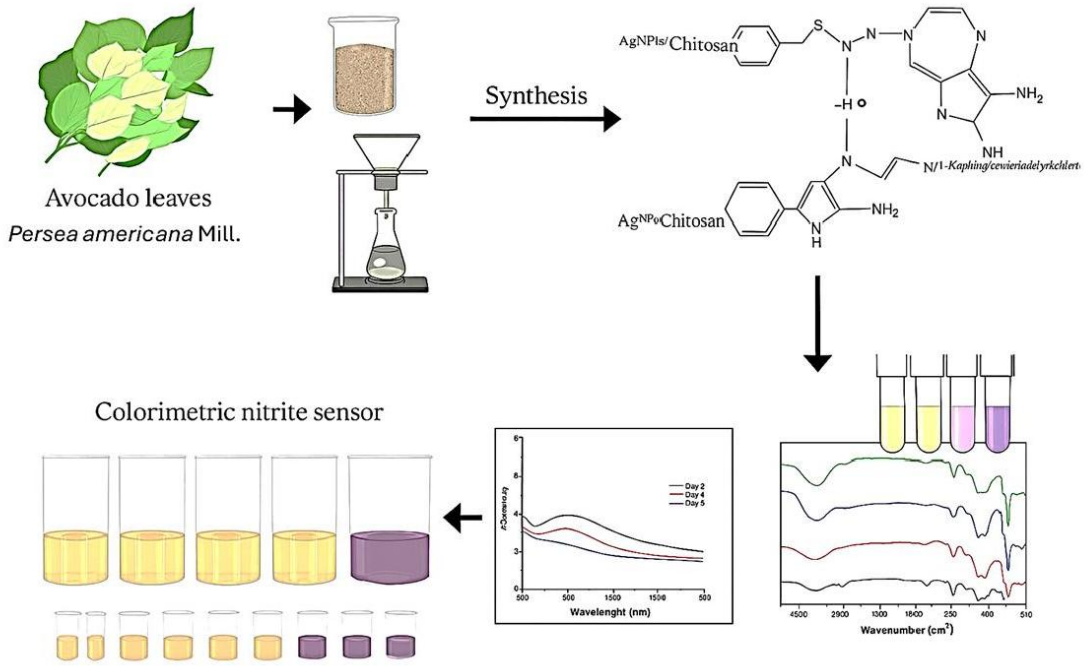
DOI: https://doi.org/10.22437/chp.v9i2.46583
Received July 04th 2025; **Accepted** October 08th 2025; **Available online** November 25th 2025

Graphical Abstract



Introduction

Health problems in the world are increasing along with the development of disease [1]. Infectious diseases are risky, especially in developing countries [2]. One of the most common infections is Urinary Tract Infection (UTI), which is a disease that can attack women and men of all ages. Urinary tract infection is a common contagion among men and women but the incidence is quite high among women due to their physiology [3]. Urinary tract infection (UTI) is an infection caused by the growth of microorganisms and bacteriuria in the urinary tract. This bacteriuria includes *Escherichia coli, Klebsiella sp, Proteus sp, Providensiac*, *P. aeruginosa*, *Acinetobacter*, and *Enterococu faecali*, but 90% is caused by Escherichia coli [4]. Currently, general techniques for diagnosing urinary tract infections (UTI) tend to take quite a long time, so they are not suitable for rapid screening because they require adequate bacterial incubation time and sample preparation [5]. There is a need for a rapid screening method to diagnose UTIs based on a nitrite colorimetric sensor. Nitrite is an indicator of UTI. Under normal circumstances, nitrites are not found in urine, but the presence of bacteria can convert nitrates in urine into nitrites, which indicates the presence of bacteria and diagnoses UTI [6-7]. The nitrate reductase enzyme is useful for reducing nitrate to nitrite. In the urinary tract, pathogenic bacteria contain nitrate enzymes that can reduce nitrates to nitrites [8]. Nitrate reductase that occurs in leaves and roots comes from photosynthesis and respiration, which causes the transfer of electrons. In the nitrate reductase enzyme, nitrate is converted into nitrite, nitrate (NO_3) will be reduced to nitrite (NO_2) [9].

Several analytical methods used to determine nitrite include colorimetric methods, spectrophotometric methods, chromatographic methods, and electroanalytical methods. Urine examination as an indicator of health can be done macroscopically, microscopically, and

chemically [9-10]. Macroscopic examination of urine consists of assessing color, clarity, odor, specific gravity, and pH. Microscopic examination to see the presence of urine sediment such as erythrocytes, leukocytes, epithelial cells, crystals, cylinders, bacteria, fungi, parasites, and spermatozoa. To detect NO_2 sensitively and selectively, sophisticated instruments and highly trained operators are needed. Colorimetric detection is attractive because of its simplicity and low cost and can be used for on-site visual analysis. NO_2 can also be detected indirectly in a colorimetric assay by utilizing a highly selective diazo reaction [11].

Silver nanoparticles are one of the most researched nanoparticles and their most common application is the use of silver nanoparticles as antibacterial, antimicrobial, anti-inflammatory, anti-angiogenesis, anti-fungal, antiviral, and anti-platelet activity [12]. Currently, various types of nanoparticles have been synthesized, such as gold, silver, iron, zinc, and metal oxide nanoparticles [13]. Silver nanoparticles (NPP) have advantages compared to gold nanoparticles because the optical properties of NPP are better [14], so they can be used as a detector and also as a coloring indicator (colorimetry). In addition, silver nanoparticles have been widely used in clothing, footwear, paint, bandages, household appliances, cosmetics, and plastics because they have antibacterial properties [15].

Silver nanoparticles can be synthesized by physical, chemical, and biological methods. Although physical and chemical methods produce pure particles, they are expensive and not environmentally friendly. Recently, silver nanoparticle synthesis techniques have been developed that are simpler, cost-effective, efficient, and environmentally friendly, one of which is using plant extracts. This plant extract contains alkaloids, tannins, steroids, phenolics, saponins, and flavonoids, which basically can reduce silver ions into silver atoms and form silver nanoparticles [16]. Currently, there is a new method, namely the biosynthesis of plant- based nanoparticles as a bioreductant. The use of plant organic compounds for nanoparticle synthesis is an environmentally friendly and simpler method. Apart from that, the types of plants that contain this reducing agent are quite abundant and easy to obtain in Indonesia [16].

Several studies have succeeded in synthesizing silver nanoparticles using plant extracts, such as using bamboo leaf extract as a reducer of silver ions from the $AgNO_3$ compound to become silver nanoparticles at a temperature of 659C which obtained less than 100 nm [17]. Synthesis of silver nanoparticles using tapak dara leaf extract, with an average nanoparticle size of 35-55 nm in random cubic shape [18]. Synthesis of silver nanoparticles using extracts *Lantara camara* leaf produced nanoparticles with an average size 1.6 to 25 nm [19]. Synthesis of silver nanoparticles using strawberry leaf extract obtained spherical silver nanoparticles with a size of 9-15 nm [20]. Synthesis of silver nanoparticles using methanol extract from green tea leaves obtained a size of 157.8 nm [21]. Bioactive compounds contained in plants such as antioxidant compounds and certain secondary metabolite compounds, such as the group of terpenoid and flavonoid compounds which are thought to play a role in the metal ion reduction process [22]. Various types of plant groups contain secondary metabolites as written above, one of which is the avocado plant.

The secondary metabolite content in avocado leaves includes saponins, alkaloids, flavonoids, and tannins [23]. Previous research stated that the flavonoid content contained in avocado leaves has antifungal, antiviral, and antibacterial activity [24].

Avocado leaves have high antioxidant activity compared to other parts of this plant (fruit skin, flesh, and fat). From the results of the FTIR spectrum on avocado leaves, strong peaks appeared at 3300-3284 cm^{-1} and 1671- 1611 cm^{-1}, which indicates the presence of polyphenolic compounds (flavonoids) [25].

Silver nanoparticles have low stability and can form aggregations so that the size becomes non-uniform and large. Therefore, a capping agent is needed to prevent the formation of aggregates between the surfaces of silver nanoparticles. The stabilization of silver nanoparticles can be enhanced by polymers. One polymer that can be used is chitosan [26]. Chitosan has a - NH_2 group which can interact with the surface of silver nanoparticles.

Further research on green synthesis of silver nanoparticles (Ag/Chitosan) using avocado leaves as a reducing agent for colorimetric detection of nitrite has not been carried out. However, based on previous reported that stabilized Chitosan AgNPs are believed to be AgNPs composites, which are simple, strong, and cheap, and have great potential for application in sensitive low-level NO_2^- detection. and low [27]. This research aims to synthesize Ag/Chitosan Nanoparticles using Avocado Leaves bioreductor (*Persea americana* Mill.) as a Colorimetric Nitrite Detector for urine.

Materials and Methods

Materials

Avocado Leaves were collected from Muaro Jambi Regency, Jambi Province, South Sumatera, Indonesia. Laboratory grade Silver Nitrate ($AgNO_3$), Sodium nitrate ($NaNO_3$), Sodium nitrite ($NaNO_2$), Ethanol, Distilled water, Hydrochloric acid (HCl) 2N, 4-Aminothiophenol (4-ATP), chitosan, pH 9 buffer, Ammonia (NH_3), Drangendorff's reagent, Mg powder, Ferric chloride ($FeCl_3$), Liebermann's reagent- Burchard, Naphthyl ethylenediamine, Nitric acid (HNO_3), Phosporic acid (H_3PO_4), Sulfuric acid (H_2SO_4) and sodium carbonate (Na_2CO_3) were purchased from Merck Sigma-Aldrich Reagent Pte, Singapore.

Preparation of Ethanol Extract

Extraction method refers to the research by [20]. 50 grams of avocado leaves were washed cleaned and dried by airing at room temperature. The dried leaves are weighed again to determine the final water content. After that, cut it into small pieces and then puree it with a blender. Avocado leaf extraction is carried out using the maceration method. Fifty (50) grams of dry simpilicia powder was soaked with 500 mL of ethanol solvent (ratio 1:10 w/v). The maceration process lasts for 2 days. The mixture is soaked for the first 6 hours, stirring occasionally, then left for 18 hours. The macerate is separated with filter paper. Next, it is evaporated at a temperature below 40℃ and evaporated until a thick extract is obtained. The extract obtained was then characterized using a UV-Vis and FT-IR spectrophotometer which will be used for the next process.

Phytochemical Analysis.

Phytochemical analysis method refers to the research by [16]. Phytochemical screening was carried out; a) Alkaloid Test: A total of 1 mL of the sample was dissolved in several drops of 2N sulfuric acid, and then tested with Dragendorff's reagent, Meyer's reagent and Wagner's reagent. The test result is declared positive if the Dragendorff reagent forms a red-orange precipitate, in the Meyer reagent a yellowish-white precipitate forms, and in the Wagner reagent a brown precipitate forms.b) Flavonoid Test: A few

drops of concentrated HCl were added to several samples and then Mg powder was added. The positive results of the HCl reagent and Mg powder were indicated by the formation of foam and the color change of the solution to orange. c) Phenolic Test: Several samples were added with $FeCl_3$ and homogenized. Positive results from the $FeCl_3$ reagent are indicated by the formation of a blackish-purple color. d) Triterpenoid/Steroid Test: Several samples were added with the Liebermann-Burchad reagent. If a blue/green color forms, it indicates the presence of steroids. If a purple or orange color forms, it indicates the presence of triterpenoids. e) Saponin Test: Saponins can be detected by the foam test in hot water. Stable foam that can last a long time and does not disappear when adding 1 drop of 2N HCl indicates the presence of saponin.

[A] [B] [C]

Figure 1. Simplicia Preparation [A] avocado leaves; [B] dried avocado leaves; [C] avocado leaves simplicia powder.

[A] [B] [C]

Figure 2. Maceration Process: [A] Simplicia is added; [B] Ethanol is added; [C] the resulting maceration extract is filtered

Nanocomposite Biosynthesis

Synthesis time optimization. 0.01 M $AgNO_3$ into a glass beaker, add 5 mL of 0.5% avocado leaf extract, add NH_3 solution until pH 9, then stir using a magnetic stirrer with varying times of 1, 2, 4, and 6 hours. The solution was centrifuged at 3500 rpm for 10 minutes, and the solution was taken for analysis using a UV-Vis spectrophotometer and FTIR (Fabiani et al., 2018). The resulting colloid was dried using a freeze-dryer. The silver nanoparticles obtained were characterized using PSA spectrophotometer.

Chitosan Concentration. Chitosan was dissolved in 1% acetic acid with a concentration of 1.5, 2, 2.5% (w/v). Put 0.01 M $AgNO_3$ into a glass beaker, add 5 mL of 0.5% avocado leaf extract, add NH_3 solution until pH 9, then stir using a magnetic stirrer for the optimal time obtained in the previous

process. A total of 2 mL of chitosan was added to the synthesized solution. NH_3 was added until it reached pH 9. The solution was centrifuged at 3500 rpm for 10 minutes, and the solution was taken for analysis by UV-Vis and FTIR spectrophotometer; the resulting colloid was dried using a freeze dryer. The optimal results of adding chitosan were observed within 0, 3, and 6 days to see the stability of silver nanoparticles [28]. Ag/Chitosan nanoparticle colloids produced under optimal conditions were characterized using particle size analyzer.

Nitrite Colorimetric Sensor

AgNPs/Carrageenan was added with 1M H_2SO_4 until pH 3, then the AgNPs/carrageenan solution was mixed with 10 mM 4-ATP at a ratio of 8:1 (v/v). A total of 0.5 ml of nitrite with a concentration of 0; 0.5; 1; 1.5; 2 and 2.5 µM were added with 0.6 ml of 1M H_2SO_4 to create an acidic atmosphere. Then 1 ml of 4-ATP-AgNPs/Carrageenan was added. Then 1 ml of 1 M CH_3COOH was added and 0.5 ml of 20 mM NED was added [29]. The solution was left for 5 minutes at room temperature and the color changes were recorded using a camera. A colorimetric selectivity test was carried out with the addition of NO_3^-, PO_4^-, SO_4^-, CO_3^-, and Cl^- ions using a method that has been carried out on nitrite [30].

Method Validation

Method validation was carried out for selectivity tests, linearity tests, LOD (Limit of Detection), and LOQ (Limit of Quantity) tests. The absorbance value of the test solution was measured using a UV-Vis spectrophotometer to obtain a calibration curve and r value. Meanwhile, determining the LOD and LOQ values is carried out statistically through linear regression and calibration curves. The LOD can be calculated using equation (1) and LOQ using equation (2).

$$LOD = 3\times \frac{SD}{b} \quad (1)$$

$$LOQ = 10\times \frac{SD}{b} \quad (2)$$

SD: standard deviation of absorbance values from measurement results; b: slope of the calibration curve equation

Result and Discussion

Preparation of Ethanolic Extract

Avocado leaves are used as an organic compound in the green synthesis process. Then, it is dried by air-drying. After drying, the avocado leaves are then separated between the leaf veins and the leaf stalks. Once separated, the avocado leaves are blended until they become simple (Figure 1). Avocado leaves simplicia powder is stored in a clean container and protected from light to avoid damage and loss of quality. The maceration method is used to make avocado leaf extract. The maceration method is a method commonly used to extract active compounds from natural materials by soaking the material in a solvent that is suitable for the active compound to be taken. This method is used because it is more straightforward, relatively easy, and cheap. It can be carried out on a large scale without going through a heating process, thereby minimizing the possibility of damage to the chemical compound components in the sample. Process of making the extract can be seen in Figure 2.

Simplicia maceration was carried out for 2 x 24 h using ethanol (96%), stirring in a closed place and calculated at 6 h, 18 h and 48 h of maceration time. The longer the extraction time, the greater the amount of material extracted because the opportunity for contact between the material and the

solvent is more significant. Maceration time that exceeds the optimum time will cause the extracted components to decrease. Maceration times that exceed the optimum time will damage the dissolved substances in the material and have the potential to increase the process of loss of compounds in the extraction solution due to evaporation. Ethanol's polarity value allows compounds that dissolve in polar solvents to attract each other through the formation of hydrogen bonds and dipole-dipole interactions between the ethanol hydroxyl group and the polar groups in the compound. The extract was evaporated using a Rotary evaporator at a speed of 32 rpm and a temperature of 50ºC.

Phytochemical Profiling.

The phytochemical test was carried out as a preliminary test to screen the compounds contained in avocado leaf extract. Based on the results of the phytochemical screening carried out, the secondary metabolite compounds contained in avocado leaf extract can be seen in Table 1. The positive results obtained in this phytochemical test indicate the presence of flavonoid, phenolic, steroid and saponin compounds in avocado leaf extract. Flavonoid compounds are the largest group of phenolic compounds found in nature. These compounds are red, purple and blue dyes, as well as yellow dyes found in plants. One of the secondary metabolite contents that can be derived is flavonoid compounds. Flavonoid compounds have hydroxyl (-OH) and carbonyl (-CO) functional groups as stabilizers and form a two-layer silver electrical layer. The size of silver nanoparticles will be smaller if the concentration of the stabilizing compound is higher.

Table 1. Phytochemical Screening of Avocado Leaves Extract

Compound Indetification	Reaction	Result	
		Avocado Leaves extract	Reference*
Alkaloids	Meyer's reagent	-	-
Flavonoids	Concentrated HCl + Mg powder	+	+
Phenolic	$FeCl_3$	+	+
Steroids	Liebermann-Burchad's reagent	+	+
Terpenoids	Liebermann-Burchad's reagent	-	+
Saponins	Hot water + 2N HCl	+	+

Information: (+) There are groups of compounds/ (-) There are no compound classes

Synthesis Time-Dependent Optimization

Time optimization is carried out to determine the optimal time for the synthesis to be carried out. At this stage, the avocado leaf extract is used as a bioreductant to make nanoparticles. Then, functions are added to provide an alkaline atmosphere. Ag^+ ions interact more effectively with the phytochemicals in avocado leaf extract at higher pH conditions (>pH-7). Silver nanoparticles can form in alkaline environmental conditions. Alkaline pH causes the functional groups of the extract to be deprotonated so that its ability to chelate metals is stronger, as previous research reported that alkaline pH mediates the rapid synthesis of AgNPs, where immediately after adding NH_3 as a function of the pH, it becomes more alkaline causing an accelerated reduction in the mixture of Ag

and extract. This is caused by changes in the electrical charge of the biomolecules in the reducer to become more reactive [31]. This is also proven by research conducted by et al.; the synthesis was carried out under alkaline conditions (pH 9), which has been optimized for the synthesis of SC bark at room temperature. The synthesis of nanoparticles obtained with an absorption peak of 420 nm at pH 9 resulted in the formation of more extensive nanoparticles.
The color change to yellow indicates that Ag nanoparticles have been formed. This color change indicates that the reduction reaction has taken place and silver nanoparticles have been formed. The formation of AgNPs is characterized by a change in the color of the solution to yellow as time increases. Indications of the formation of nanoparticles with color changes were proven using a UV-Vis spectrophotometer [19]. Then, the synthesis results were centrifuged at 3500 rpm for 45 minutes, and the filtrate was taken for analysis, and it could settle so that in the UV-Vis spectrophotometer analysis, there were no impurities that interfered with the reading.

Table 2. UV-Vis Spectrophotometry Results of Ag Chitosan Nanoparticles

No.	Chitosan Concentration (%)	SPR *(Surface Plasmone Responance)*	Absorbance
1.	1.5	414	2.028
2.	2.0	440	0.753
3.	2.5	435	1.341

Table 3. UV-Vis Spectrum Characterization of the Stability of Ag/Chitosan Nanoparticles

No.	Storage Time (Days)	SPR *(Surface Plasmone Responance)* (nm)	Absorbance
1.	0	435	1.41
2.	3	427	1.290
3.	6	430	1.249

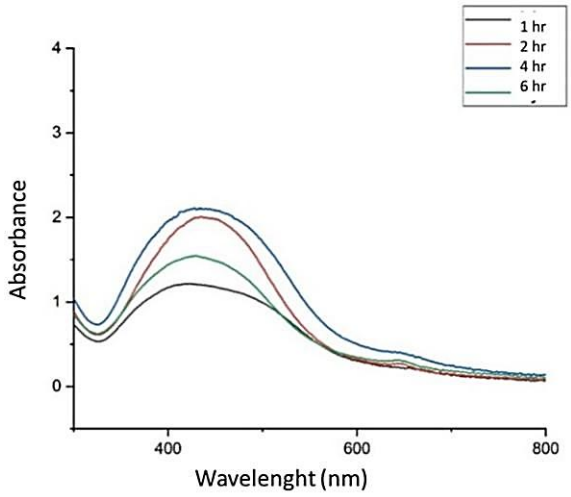


Figure 3. UV-Vis Spectrophotometric Characterization of Ag Nanoparticles with Various Time of Synthesis

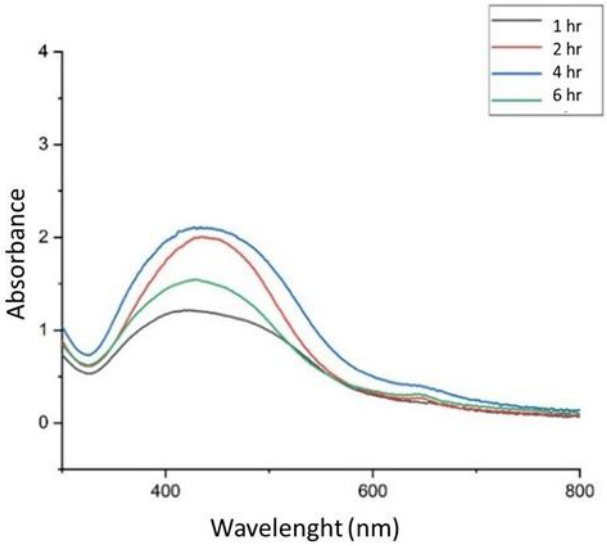


Figure 4. UV-Vis Spectrophotometry Results for Chitosan Concentration Optimization

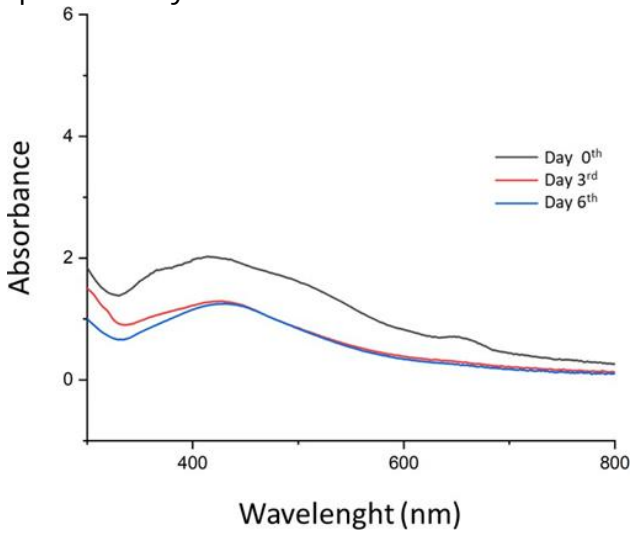


Figure 5. Results of UV-Vis Spectrophotometric Characterization of the Stability of Ag/Chitosan Nanoparticles

The optimal time obtained was obtained at a synthesis time of 4 h, as shown by the highest absorption peak at a synthesis time of 4 hr with the highest absorbance (Figure 3). As seen in Table 3, the absorbance value was high at 4 hr. Ag nanoparticles can be seen from the characteristic absorption characteristic of silver nanoparticles with a wavelength of 400-450 nm [18]; apart from that, the number of silver nanoparticles formed can be determined from the absorbance value. The higher the absorbance value, the higher the nanoparticles formed. Based on the results of UV-Vis characterization in Figure 4, the absorption peak time increases, and the absorbance increases. However, the variation in synthesis time of 6 h decreased, which seems influenced by the length of stirring, which causes the nanoparticles to re-aggregate into clusters

Based on the results of the PSA characterization test, the nanoparticle size obtained was 129 nm. It can be said to be nano if the particle size is 1-100 nm. However, some literature states that the size of nanoparticles ranges from 1-1000 nm. Nanoparticles are particles with a size of 10-1000 nm. The size of the nanoparticles can

be influenced by the stability of the solution. Apart from that, agglomeration is the main cause of increasing nanoparticle size. Where the agglomeration event can be characterized by a change in the color of the solution to ash and the absorbance of the solution increases or decreases, which means the AgNPs are unstable.

Optimization of Chitosan Concentration.

AgNO3, which was reacted with the bioreactor, experienced a color change from clear greenish to brownish yellow. This change indicates that silver nanoparticles are starting to form. Apart from color changes, the characteristic absorption peaks of the nanoparticles can also be seen [29]. As seen in Figure 4, all variations in concentration have characteristic absorption peaks of nanoparticles. 1.5% chitosan has the lowest absorption peak of 414 nm and absorbance of 2.028. 2% chitosan has an absorption peak of 440 nm and an absorbance of 0.753 (Table 4). Chitosan 2.5% has an absorption peak of 435 nm with an absorbance of 1.341. The higher the absorbance, the smaller the resulting nanoparticles, and the higher the absorption peak obtained, the more nanoparticles will be obtained. The stability of AgNP colloidal solutions based on time can also be analyzed based on changes in absorption peaks [32]. The shift in the absorption peak to a larger wavelength indicates that the stability of silver nanoparticles is less, and they tend to experience agglomeration [28]. The results of determining the stability of the synthesized Ag/Chitosan Nanoparticles are shown in Figure 5. Stability was measured on days 0, 3, and 6 after synthesis.

The stability of Ag/Chitosan nanoparticles can be measured using UV-Vis Spectrophotometry and can be seen from their color. The higher the absorbance, the more the nanoparticles agglomerate. If the color of the solution changes from brownish-yellow to transparent, the formation of nanoparticles in the solution stops. Based on Table 2, the results of 1% chitosan have the lowest absorption peak of 414 nm and absorbance of 2.028. 2% chitosan has an absorption peak of 440 nm and an absorbance of 0.753. Chitosan 2.5% has an absorption peak of 435 nm with an absorbance of 1.341.

The results of measuring the stability of Ag/Chitosan nanoparticles in Figure 6 show that the length of storage affects the stability of silver nanoparticles. Increasing storage time shows the formation of particles with larger sizes. The absorbance value continues to increase as the absorption peak shifts to a larger wavelength, indicating that the number of silver nanoparticles formed increases with the length of contact time. Characterize Fourier Transform Infra-Red (FTIR), which aims to show the functional groups contained in the sample. The occurrence of the oxidation process as a result of the reduction process of silver nanoparticles will result in a shift in the wave number, which indicates that there has been an interaction between the functional groups and the silver nanoparticles [33]. Specific groups in the IR spectrum are presented in Table 4. Detected functional groups will appear in the presence of peaks in the FT-IR spectrum. Each functional group has a different wave number according to the functional group's ability to absorb infrared energy.

From Figure 6 it can be seen that the FTIR graph looks the same at each intensity, showing a wide and strong band in the O-H group at the wave number frequency cm-1 which is typical of the alcohol functional group. A reduced frequency value indicates a downward shift in intensity. The decreasing O-H and C-O-C groups are due to contributing ions to reduce Ag+ to Ag0.

Hydroxide ions are needed to accelerate the Ag+ ion reduction process in the synthesis of silver nanoparticles. The possible mechanism between biomolecules and bioreduction influences the reduction process of Ag+ ions undergoing stabilization into AgNPs. The functional groups experienced a shift in their absorption peaks from the data obtained. A wave number shift shows an interaction between Ag nanoparticles and secondary metabolite compounds contained in the bioreduction, indicating the presence of metabolite compounds that react Ag+ ions into Ag0 [34].

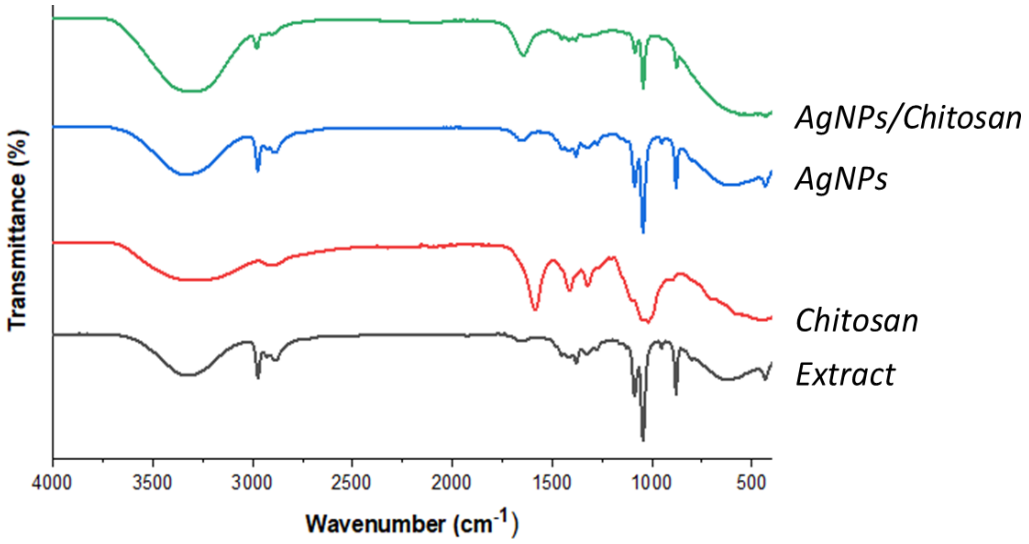


Figure 6. FTIR characterization results

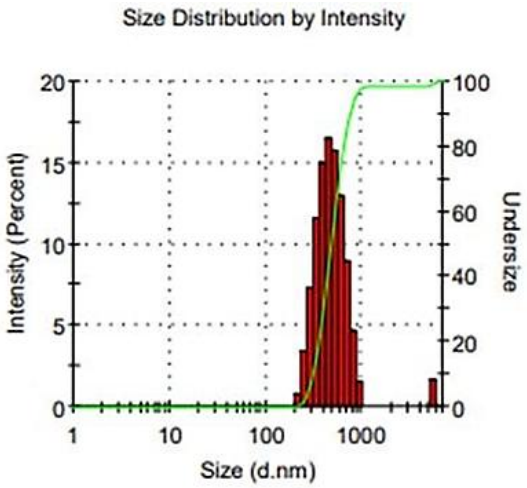


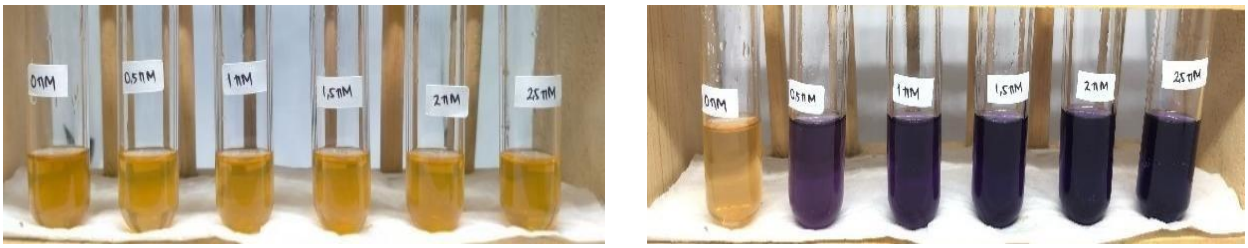
Figure 7. Particle size of AgNPs/Chitosan

The size of the nanoparticles can be influenced by several factors, such as solution temperature, pH, stirring speed, reductant, and reaction time. Temperature variations were carried out during the synthesis of Ag/Chitosan nanoparticles. This results in the formation rate of silver nanoparticles taking longer to produce more nanoparticles [28]. The slightly clumped chitosan affects the size of the nanoparticles. It coats the nanoparticles thicker. This event is supported by the results of particle size characterization using the Particle Size Analyzer (PSA). Characterization of Ag/Chitosan nanoparticles with PSA aims to determine the size distribution and uniformity of the particles. The results of the size analysis of Ag/Chitosan Nanoparticles with the optimal concentration of chitosan can be seen in Figure 7. Based on Figure 7, the size of AgNPs/Chitosan is 479.3 nm.

Nitrite Colorimetric Sensor

Detection preparation begins by reacting 8 ml of AgNPs/Chitosan with 4-ATP added with H_2SO_4 so that the solution is in an acidic condition. Then, a standard $NaNO_2$ solution was prepared with a concentration of 0 µM, 0.5 µM, 1 µM, 1.5 µM, 2 µM and 2.5 µM,

which was made from a stock solution with a concentration of 50 µM. At each nitrite concentration, sulfuric acid was added, and AgNPs/Chitosan-4ATP was added. The solution changed color to yellowish following the color of AgNPs/Chitosan-4ATP. Then acetic acid is a weak acid. Then, the addition of NED functions as a coupling agent. Where diazonium salts are unstable in water due to their tendency to lose diazonium groups, this can be stabilized through the formation of azo dyes, which cause bathochromic shifts in the SPR (Surface Plasmon Response) absorption of AgNP/chitosan bands as a result of charge transfer interactions on the nanoparticle surface. Apart from that, the addition of NED aims to lengthen the conjugated double bonds, which are based on the diazotation reaction of aromatic primary amine compounds coupled with naphthylethylenediamine. The presence of nitrite will produce a reddish-purple compound, which can be measured using a spectrophotometer, where the number of moles of nitrite reacting is the same as the number of azo compounds produced by the reaction (Sinaga et al., 2013). The response time to reach 90% signal change was less than 5 minutes. Calibration curves were obtained from triplicate measurements to ensure reproducibility.



[A] **[B]**

Figure 8. Coloring changes; [A] Addition of AgNPS/Chitosa; [B] after addition of NED

The working principle of the colorimetric sensor is based on changing the color of the silver nanoparticle reagent and NED from yellow to purple. Nitrite is reacted with H_2SO_4 to provide an acidic atmosphere because the diazotization-coupling process can take place in acidic conditions. Because at higher pH conditions, the diazotization-coupling reaction does not run perfectly due to the lack of acid [35]. Nitrite reacts with AgNPs/chitosan-4ATP to produce diazonium salt. Under acidic conditions, 4-ATP is converted into the cationic conjugate acid form in the presence of nitrite and acid, and aromatic amines change to diazonium salts [30]. Figure 8 shows that when given NED, nitrite can be detected. The higher the nitrite concentration, the more concentrated it is, and the color of the resulting solution.

Nitrite reacts with 4-ATP−AgNPs to produce NED-coupled diazonium salts to form nanoparticle-modified silver chromophores, as shown in Figure 9. Under acidic conditions, the amine groups of 4-aminothiophenol- modified AgNPs are converted into cationic groups. A conjugate acid (ammonium) forms, which stabilizes the nanoparticles by electrostatic repulsion.

Nitrites and acid minerals, primary aromatic amines, turn into diazonium salts. Although diazonium salts are unstable in aqueous solutions due to their tendency to lose diazonium groups, they are stabilized through the formation of azo-dyes with the addition of NED, causing a bathochromic shift in the local SPR absorption band of AgNPs as a result of charge transfer interactions at the nanoparticle surface.

Surface complexation between organic molecules having active functional groups and AgNPs is reported to play an important role in both the stabilization and formation of new chromophores of nanoparticles. The proposed method (4-ATP−AgNP+NED) was proven to be superior to the application of the (4-ATP+NED) binary combination without the involvement of AgNPs due to its much better sensitivity and linearity concerning nitramine concentration.

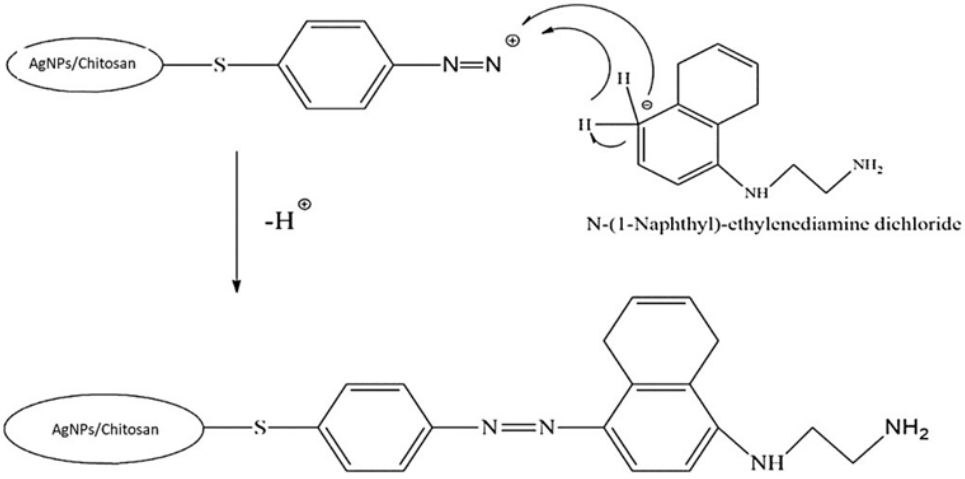


Figure 9. Reaction mechanism for NO^- detection with Ag/Chitosan Nanoparticles + 4-ATP and NED

Validation Method

The selectivity test was carried out using NO_3^-, PO_4^-, SO_4^{2-}, CO_3^{2-}, Cl^- and NO_2^-, 2.5 µM ions as a comparison. This selectivity test was carried out based on colorimetry. Where color changes are the main observation. If the sample does not change color, it can be said that the sample cannot detect anything other than nitrite. In observing the selectivity test using colorimetry, 4-ATP was first reacted with Chitosan AgNPs at pH 2 to form a complex matrix. NO_3^-, PO_4^-, SO_4^{2-}, CO_3^-, Cl^- and NO_2^-, 2.5 µM ions were added with sulfuric acid to form an acidic atmosphere. The addition of complex matrix and acetic acid was carried out sequentially. Then the addition of NED is used to form a diazonium reaction.

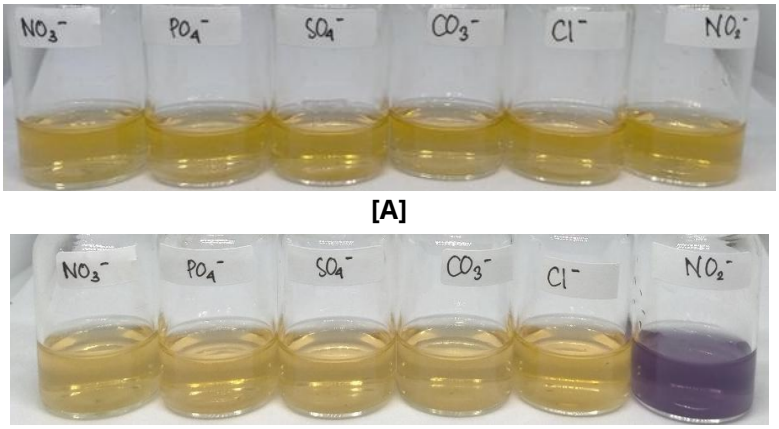


Figure 10. NO_3^-, PO_4^-, SO_4^-, CO_3^-, Cl^- and NO_2^- ion selectivity test (a). Before the addition of NED (b). After adding NED

Based on the observations made, it can be seen in Figure 10. Before adding NED, the sample containing ions will follow the color of the complex matrix. When NED was added to the sample containing NO_2^- ions, the color changed to purple, while the NO_3^-, PO_4^-, SO_4^-, CO_3^-, and Cl^- ions did not change color. Based on the selectivity test, it can be stated that the analytical method is selective for nitrite. The selectivity test is carried out based on colorimetry or color observation.

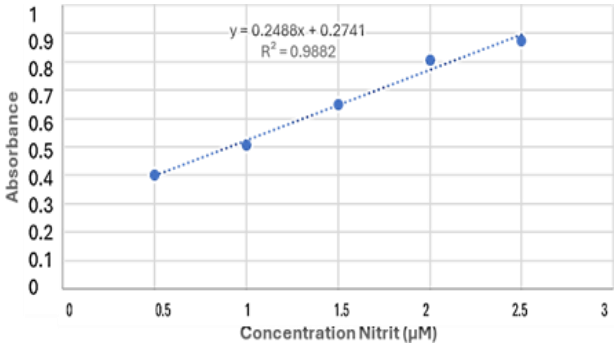


Figure 11. Nitrite calibration curve

Determination of linearity is determined to determine the ability of an analytical method to obtain results that are appropriate to the analyte concentration in the sample. This measurement is to obtain the equation of the regression line from the calibration curve created. The linearity test is expressed as a correlation coefficient (r). The following is the calibration curve for a standard nitrite solution using AgNP/chitosan. Based on statistical calculations, it can be seen in Figure 10 that the regression equation y = 0.2488x + 0.2741 is obtained with a regression value of R2 = 0.9882. The requirement for the correlation coefficient (r) value is that it must be greater than 0.99 according to SNI. The r value obtained is 0.994 so it can be said that the linearity data is declared valid and there is a correlation between concentration and intensity

Table 5. LOD and LOQ Test Results

Concentration (µM) **(x)**	**Absorbance (y)**	**(Ŷ)**	**(Y-Ŷ)**	**(Y-Ŷ)²**
0.5	0.401	0.3985	0.0025	0.000006
1	0.507	0.5229	-0.0159	0.000253
1.5	0.651	0.6473	0.0037	0.000014
2	0.805	0.7717	0.0333	0.001109
2.5	0.874	0.8961	-0.0221	0.000488
			Total	0.0019
			Sy(Y/X)²	0.0001
			Sy(Y/X)	0.0108
			LOD	0.1304
			LOQ	0.4345

LOD is the smallest amount of analyte in a sample that can be detected which still provides a significant response compared to a blank. LOQ is an analytical parameter that is defined as the smallest amount of analyte in a sample that can still meet the precision and accuracy criteria. Detection and quantitation limits can be calculated statistically via a linear regression line from the calibration curve. The research results show that the developed colorimetric sensor can detect nitrite in silver nanoparticles in the concentration range of 0.5-2.5 µM and produces detection limits and quantitation limits of 0.130 µM and 0.434 µM respectively (Table 5). This value shows the amount of analyte that can still be measured by AgNPs/chitosan. The nitrite concentration in

the analyte can be detected if the concentration is greater than the detection limit, namely 0.1304 µM. If the analyte concentration is less than 0.1304 µM then AgNPs/chitosan cannot detect the analyte.

Conclusions

Based on the research that has been carried out, the following conclusions can be obtained that the optimal time for the synthesis of silver nanoparticles using avocado leaf bioreduction is 4 h with an SPR (Surface Plasmon Response) value of 428 nm and an absorbance value of 2.112 with a nanoparticle size of 129 nm. The addition of chitosan can affect the stability of chitosan. Chitosan has amine (-NH2) and hydroxyl (-OH) groups so chitosan can act as a capping agent for silver nanoparticles using avocado leaf reductants. The limit of detection (LOD) and quantitation (LOQ) values of Ag/Chitosan nanoparticles as a colorimetric nitrite detector respectively obtained LOD values of 0.01303 µM and LOQ 0.0434 µM. Limitations and Future Work: While PSA was used to measure particle size, more accurate characterization such as SEM/TEM, DLS (hydrodynamic diameter, zeta potential), and XRD (crystallinity) will be included in future studies. The current sensor was single-use; regeneration and reusability will be addressed in ongoing work. Biosafety was considered, and the small amount of AgNPs used poses minimal risks when disposed according to laboratory standards.

Acknowledgement

This research is supported by the University of Riau, Chemical Engineering Department. We extend our sincere appreciation to the University of Riau, Chemical Engineering Department for providing facilities, resources, and technical assistance essential to the completion of this study. We would like to express our special thanks are also extended to Muhammad Ghazy Fernandes for preparing and providing the coating samples used in this study. This research would not have been successfully completed without the collective effort of all involved.

Author Contributions

All authors have read and agreed to the published version of the manuscript. Conceptualization: RDP., NCAS., and ILT; research design, RDP., NCAS., and ILT; methodology: RDP., NL., ILT., and NN. Validation: RDP., ILT., NN. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Ethical Standards

This article does not contain any studies involving human or animal subjects.

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