Antifungal Analysis of Papaya Seed Extracts and Biosynthesized Silver Nanoparticles

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Abstract
The purpose of research is to investigate the antifungal potential of papaya seed extract and the biosynthesis of silver nanoparticles by C. papaya extract. Here we report 14 secondary metabolites from C. papaya seed extract detected by gas chromatography-mass spectroscopy. Bioactive components were majority classified as fatty acid-methyl ester, heterocyclic amides, and phenolic compounds. The proposed mechanisms of those metabolites on inducing antifungal activity were comprehensively studied. We found that the n-hexane fraction was the solely fraction to produce the silver nanoparticles. Scanning electron images presented the aggregation and evenly distribution of spherical silver nanoparticles. The XRD exhibited the crystallization of the bio-organic synthetic phase based on the specific spectrum of 2-theta at 38.9 degree with miller index [1,1,1]. The particle size analyzer also confirmed the nanoscale of synthesized materials as in the average size of 92.1 nm (Z) and 0.406 (PI). Antifungal effects were examined by disc diffusion method upon each fraction with varying concentrations of 25%, 50%, 75%, and 100% (w/w) against C. albicans. ANOVA analysis showed no significant difference among all fractions tested (p > 0.25). The antifungal activity was categorized as a moderate effect with the mean of inhibition zone ranging from 6.1 to 6.8 mm. However, the potential of papaya seed extract is relatively better than the papaya leaves extract, as previously reported. We suggest further studies on the molecular docking of the secondary metabolites against nanoparticle, and specific biomolecular analysis according to the mechanism of action.

Keywords
Antifungal, Biosynthesis, Silver Nanoparticle, n-Hexane, C. albicans

1. INTRODUCTION
Fungal infectious disease such as vulvovaginal candidiasis was often caused by C. albicans (Rathod and Bufler, 2014). In some cases, C. albicans is also found at diaper dermatitis or diaper rash in infants and children (Carr et al., 2020; Infanti et al., 2020). The existence of antifungal resistance encourages the development of new effective antifungal drugs, minimum side effects, and inexpensive ones (Farahyar et al., 2020). The papaya plant (C. papaya) has been efficacious in traditional treatment for many years. The seed and leave extract of C. papaya using methanol, and aqueous solvents have been reported for its antibacterial activity (Peter et al., 2014). The properties of papaya seed oil have been studied previously by the extraction with petroleum ether and aqueous enzymatic methods (Puangri et al., 2005). A study reported that the content of 2,3,4-trihydroxytoluene in methanolic extract of the papaya seeds had shown antifungal effects on A. flavus, C. albicans, and P. citrinum (Singh and Ali, 2011). Although a new study by Siagian et al. (2021) showed that papaya Bangkok (C. papaya L. var. Bangkok) seed extract has no antifungal activity, unfortunately, this study had no report on the phytochemical compound of the methanol extract. In response to the negative result of an investigation by Siagian et al. (2021), we are supposed to scrutinize the absence of the extraction methods that may degrade the bioactive components so that antifungal activity was undetected (Siagian et al., 2021). The bioactive components play essential roles in the antifungal effect, e.g., isolated flavonoids from Citrus species against C. albicans (Salas et al., 2011). The antifungal effects, mechanism of action, and its combination with pharmaceutical drugs have been resumed by Al Aboody and Mickymaray (2020).

As the most prominent research for decades, nanotechnology has rapidly developed and used in the medical applications (Devanesan et al., 2021; Sintubin et al., 2012), i.e., silver nanoparticles (AgNPs) of papaya seed extract evaluated as anti-cancer in human prostate in-vitro (Singh et al., 2021),
antibacterial effect (Al-Otibi et al., 2021; Dewi et al., 2019; Kalwar and Shan, 2018), and antifungal activity (Kim et al., 2009; Krishnaraj et al., 2012).

However, we still find a research gap on the antifungal effect of C. papaya seed extracts and non-polar extract as a natural bio-reduction for AgNPs synthesis. Several pieces of research have been reported on the AgNPs biosynthesis by using C. papaya peel extract (Kokila et al., 2016) papaya seed ethanol extract (Yani and Putri, 2019). The current literature review on the phytochemical aspects, pharmacological insights, nanoparticle synthesis, commercial production, and utilization of C. papaya waste have been resumed by Sharma et al. (2020). The most recent study on the phytochemical screening and antimicrobial activities of methanolic and aqueous leaf extracts of C. papaya was reported by Callixte et al. (2020). To the best of our knowledge, the comprehensive evaluation of bioactive compounds attributed to antifungal effect altogether with the use of the papaya seed extract to fabricate the AgNPs in the non-polar fraction is still not found. Therefore, this research is aimed to contribute a more comprehensive analysis of the papaya seed metabolites, AgNPs bio-synthesis, and their antifungal potential.

2. EXPERIMENTAL SECTION

2.1 Materials
The papaya seeds obtained from the MMTC Market, Deli Serdang Regency, North Sumatra Province. Then seeds were washed and dried. Extraction solvents used ethanol 96%, ethyl acetate, and n-hexane. The antifungal activity used C. albicans, Heart Infusion Broth, and Sabouraud Dextrose Agar (SDA) media. Lab instruments are electrothermal heating mantle (HTM-300-150), soxhlet extractor (Iwaki), oven (Memmert), microwave (LG), analytical balance (Denver Instruments), 50 mesh sieve (SS 304 Class-A), magnetic stirrer (Daihan LabTech), Gas Chromatography-Mass Spectrometry (GC-MS) Thermo Scientific Trace 1310, Scanning Electron Microscopy (SEM) ZEISS EVO® MA 10, X-Ray Diffraction (XRD) Rigaku MiniFlex, and Particle Size Analyzer (PSA) Horiba Scientific SZ-100.

2.2 Methods
Papaya seeds (1.7 kg) were dried at 70°C then macerated with 10 L solvents for three days with ethanol, ethyl acetate, water, and n-hexane simultaneously with stirring one time per day. Crude extract obtained 48.192 g (2.83%) after being treated with a rotary evaporator and followed by fractionation with water, ethanol, n-hexane, and ethyl acetate. Papaya seed extract 5 g dissolved in 80 mL with water at 70°C and placed into a separating funnel. The graded fraction of the n-hexane, ethyl acetate, and ethanol was repeated three times. Then the secondary metabolites of all fractions were analyzed by GC-MS (Buckley, 1966; Suman et al., 2016). The AgNPs were synthesized by mixing 10 mL of AgNO₃ 1 mmol solution and 200 µL of each fraction of papaya seed extracts. The mixture was stirred for about 30 min until homogeneous. The formation of AgNPs was observed by SEM, XRD, and PSA (Banala et al., 2015; Devanesan et al., 2021; Lohrasbi et al., 2019). For SEM analysis, the silver samples were centrifuged at 10,000 rpm for 30 min and the pellet was redispersed in 10 mL ethanol and washed 3 times with sterile distilled water. The pellet was dried in an oven and thin films of dried samples (10 mg/mL) were prepared. XRD analysis was conducted in 2-theta region at 40 kV and a current of 30 mA with Cu K-alpha radiation. Particle size distribution was obtained using ImageJ software version 1.47v.

The Sabouraud Dextrose Agar (SDA) media was poured aseptically and solidified to evaluate the antifungal activity. The cotton swab was used to scratch the C. albicans suspension on the surface of the SDA media. The disc was divided into four quadrants for 25%, 50%, 75%, and 100% of samples variation and label given outside the petri dish. A paper disc with AgNPs was placed on the marked part then incubated for 24h. The clear zone was measured on the paper disc (Davis and Stout, 1971). Statistical analysis uses Kolmogorov-Smirnov to check the normality of data and the Levene method to check homogeneity. The one-way ANOVA was applied to evaluate the significance of each sample.

3. RESULTS AND DISCUSSION

3.1 Secondary Metabolites Analysis
To detect the metabolite compounds in the papaya seed extract, we have analyzed each fraction; ethanol, ethyl acetate, and n-hexane by GC-MS, as shown in Figure 1.

Based on Figure 1, most metabolites compounds were almost detected in the ethanol and ethyl acetate fractions. These results indicate that most metabolites vary from polar to semi-polar even though some fatty acids are found in the non-polar solvent in small quantities. The major compounds in the EtOH fraction are benzyl nitrile, hexadecenoic acid, methyl ester and octadecenoic acid, methyl ester. Despite their small presence, the eugenol (Abdou et al., 2021) and acetamide (Thakral and Singh, 2019) compounds are widely known as antimicrobial. An isoeugenol showed its potential inhibitor for 14-α-demethylase and delta-14-sterol reductase that interfered with the biosynthesis of fungal cell membrane (Medeiros et al., 2020). Another study found that eugenol's antifungal activity is related to membrane binding and permeability changes, which leads to plasma membrane destabilization and disruption (Wang et al., 2010). Moreover, lipophilic acetamide derivatives have been reported as an antimicrobial agent via EGFR protein kinase enzyme and stable interactions were similar to the co-crystallized reference ligand (Ahmed et al., 2018). Benzylamine as a variety of heterocyclic amides have been reported as excellent therapeutic efficacy and remarkable application to treat various mycoses topically (Thakral and Singh, 2019). Because of the molecule’s lipophilic character, its effectiveness could be correlated to the interactions with phospholipids in cell membranes and permeabilization of the fungal cell wall (Mingeot-Leclercq et al., 2001) as well as the direct membrane-
damaging effect of butenane, a benzylamine derivative, which play a significant role on its anticandidal activity (Iwatani et al., 1993). Fatty acid methyl esters (FAME) group has been reported for their antifungal activity against isolates of Paracoccidioides sp. (Pinto et al., 2017). As one of FAME’s compounds, Benzylcarbamic acid-methyl ester may also contribute to the antifungal effect (Raghunath and Viswanathan, 2014). Another FAME compound from S. portulacastrum reported the fatty acid composition and antimicrobial activity against human pathogenic microorganisms (Chandrasekaran et al., 2011).

Phenol, Thiocyanic acids, and Heneicosane were dominantly found in the EtOAc fraction. Phenol is active against many micro-organisms, including some fungi and viruses. Phenol may cause a change in cell surface hydrophobicity and charge, resulting in cytoplasmic content leakage (Teodoro et al., 2015). Carvacrol and other terpenoid phenols, such as carvacrol, have antifungal activity against a variety of infections, including C. albicans (Rao et al., 2010). Caffeic acid derivatives may interfere with the 1,3-glucan synthase, which could have a deleterious effect on the C. albicans cell wall. According to a literature study on the antifungal activity of herbal extracts C. albicans, phenolic components such gallic acid, thymol, catechin, and other polyphenols like tannins, terpenoids, and saponins, which were the primary component, are linked to the antifungal action. The incorporation of nanoparticles is also predicted to improve the antifungal properties of natural compounds (Hsu et al., 2021). The presence of thiocyanic acids may be attributed to antifungal activity.

In contrast, under a transmission electron microscope, C. papaya seed extract against A. flavus revealed abnormal cell shape and destruction of the fungus cell wall. Still, the detail of its mechanism was unexplained (Abdel El-Zaheer, 2014). The most recent study on the antifungal activity of Raphanus raphanistrum extracts against Fusarium and Pythiaceous reported that thiocyanic acid-ethyl ester was responsible for the antifungal activities (Mannai et al., 2021). In this report, Heneicosane has the highest intensity in the EtOAc fraction, related to the antifungal activity (Chandrasekaran et al., 2011). Other studies reported Heneicosane and further FAME’s compounds from C. papaya seed extract by subjecting heat treatment to the potent antibacterial effect (Amid and Amid, 2021), and Pinus knot wood extracts showed antimicrobial activity in its FAME’s content (Välimaa et al., 2007).

In this study, FAME’s compounds were diversely found in the EtOH, EtOAc, and n-hexane fraction, named as do-decanoic acid, methyl ester; undecanoic acid, methyl ester; tetradecanoic acid, methyl ester; hexadecenoic acid, methyl/ethyl ester; octadecenoic acid, methyl ester; heneicosane; tricosanoic acid, methyl ester; and so forth.
Table 1. Fourteen Bioactive Compounds Detected by GC-MC of Papaya Seed Extract Found in Our Study and Subjected to Explore Their Antifungal Mechanisms Based on Literature

| No | Bioactive Compounds                  | EtOH fraction | EtOAc fraction | n-hexane fraction | Antifungal effect and proposed mechanisms (Ref.) |
|----|-------------------------------------|---------------|---------------|------------------|-------------------------------------------------|
| 1  | Benzylamine                          | +             | ++            | -                | (Iwatani et al., 1993; Mingeot-Leclercq et al., 2001; Thakral and Singh, 2019) |
| 2  | Benzylcarbamic acid, methyl ester*    | +             | -             | -                | (Chandrasekaran et al., 2011; Pinto et al., 2017) |
| 3  | Phenol                               | -             | ++            | -                | (Hsu et al., 2021; Ma et al., 2010; Rao et al., 2010; Teodoro et al., 2015) |
| 4  | Thiocyanic acid, phenylmethyl ester* | +             | ++            | -                | (Abd El-Zaher, 2014; Mamai et al., 2021) |
| 5  | Acetamide                            | +             | +             | -                | (Ahmed et al., 2018; Medeiros et al., 2020; Thakral and Singh, 2019) |
| 6  | Eugenol                              | +             | -             | -                | (Abdou et al., 2021; Wang et al., 2010) |
| 7  | Dodecanoic acid, methyl ester*       | +             | +             | +                | (Lima et al., 2011; Rafiq et al., 2021) |
| 8  | Undecanoic acid, methyl ester*       | -             | -             | +                | (Lima et al., 2011; Rafiq et al., 2021; Rossi et al., 2021) |
| 9  | Tetradecanoic acid, methyl ester*    | +             | -             | +                | (Lima et al., 2011; Rafiq et al., 2021) |
| 10 | Hexadecenoic acid, methyl/ethyl ester* | ++         | +             | +                | (Alkooranee et al., 2020; Lima et al., 2011; Rafiq et al., 2021) |
| 11 | Octadecenoic acid, methyl ester*     | ++            | -             | -                | (Alkooranee et al., 2020; Lima et al., 2011; Rafiq et al., 2021) |
| 12 | Heneicosane*                         | -             | +++           | -                | (Amid and Amid, 2021; Chandrasekaran et al., 2011; Välimaa et al., 2007) |
| 13 | Tricosanoic acid, methyl ester*      | +             | -             | -                | (Lima et al., 2011; Rafiq et al., 2021; Perveen et al., 2021; Rafiq et al., 2021) |
| 14 | Eicosanoic acid*                     | -             | +             | -                | (Lima et al., 2011; Rafiq et al., 2021; Perveen et al., 2021; Rafiq et al., 2021) |

The (+) presence and (-) absence of compound were shown based on peak area quantitatively. *FAME's compounds.

Acid, methyl ester; and eicosanoic acid even though FAME were easily found in non-polar solvents due to its solubility characteristics. A study reported that fatty acid methyl esters (FAME) were found inhibit 12 clinical strains of the pathogenic fungus 

3.2 Biosynthesized Silver Nanoparticles

The n-hexane fraction was the solely fraction for the biosynthesis of AgNPs; this result may be due to the nucleation process of nanoparticles induced by metabolites content which play the role as capping agents and as natural reductor for the formation of Ag⁺ ions into Ag₀ (Zulaicha et al., 2021). Some studies also reported that the synthesis of nanoparticles depends on pH, temperature, extract concentration, metal salt concentration, and reaction time factors (Mittal et al., 2013; Shah et al., 2015).

The SEM images analysis of synthesized AgNPs in Figure 2(a) showed a spherical particles at a scale around 100-200 nm, indicating a high density of AgNPs. At the same
Table 2. Antifungal Activities on C. albicans

| Fungi species | Sample      | Concentration (%) | Inhibition zone (mm) | Mean | SD  | p-value |
|---------------|-------------|-------------------|----------------------|------|-----|---------|
|               |             |                   | u1       | u2       | u3       |         |
| C. albicans   | Water Fraction | 25                | 6.2     | 6.1     | 6.3     | 6.20   | 0.017   | p > 0.05 |
|               |             | 50                | 6.2     | 6.3     | 6.2     | 6.23   | 0.183   | p > 0.05 |
|               |             | 75                | 6.2     | 6.2     | 6.2     | 6.20   | 0.047   | p > 0.05 |
|               |             | 100               | 6.2     | 6.1     | 6.4     | 6.23   | 0.183   | p > 0.05 |
|               | EtOH Fraction | 25                | 6.3     | 6.3     | 6.3     | 6.30   | 0.183   | p > 0.05 |
|               |             | 75                | 6.5     | 6.4     | 6.6     | 6.50   | 0.335   | p > 0.05 |
|               |             | 100               | 6.7     | 6.5     | 6.7     | 6.63   | 0.335   | p > 0.05 |
|               | EtOAc Fraction | 25                | 6.1     | 6.2     | 6.1     | 6.13   | 0.047   | p > 0.05 |
|               |             | 50                | 6.2     | 6.1     | 6.2     | 6.16   | 0.335   | p > 0.05 |
|               |             | 75                | 6.1     | 6.1     | 6.1     | 6.10   | 0.047   | p > 0.05 |
|               |             | 100               | 7.9     | 6.3     | 6.2     | 6.80   | 0.047   | p > 0.05 |
|               | n-hexane/AgNPs | 25                | 6.1     | 6.2     | 6.1     | 6.13   | 0.335   | p > 0.05 |
|               |             | 50                | 6.1     | 6.1     | 6.1     | 6.10   | 0.335   | p > 0.05 |
|               |             | 75                | 6.1     | 6.2     | 6.1     | 6.20   | 0.335   | p > 0.05 |
|               |             | 100               | 6.1     | 6.3     | 6.2     | 6.20   | 0.335   | p > 0.05 |

Figure 2. SEM Images of Synthesized AgNPs showed that The Nanoscale of Spherical Particles Tends to be Aggregated (a. 30k and b. 50k magnifications)

time, Figure 2(b) showed that AgNPs were distributed and agglomerated, which might be due to a high concentration of plant extracts (Balavijayalakshmi and Ramalakshmi, 2017). Biosynthesis of AgNPs from M. charantia fruit extract showed a spherical morphology (Jha and Shimpi, 2018). This study also reported flavonoid content as a reducer agent and protein stabilizer during the catalytic process. Furthermore, another study has suggested using surfactants to enhance the stability and antimicrobial capabilities of spherical AgNPs (Mahmood et al., 2020).

The particle size distribution analysis (PSA), as shown in Figure 3, showed the average size of materials is about 92.1 nm (Z) and 0.406 (PI). PSA result confirmed the heterogenous and wide range of nanoscale distribution of synthesized AgNPs, as previously reported by Surura et al. (2021).

Figure 3. Particle Size Distribution Analysis of Monodispersed AgNPs with Cumulant Operations of Z = 92.1 nm and PI = 0.406

The XRD instrument was used to study the crystallography analysis of AgNPs showed in Figure 4.

The XRD pattern showed five intense peaks in the 2θ(deg) at 23.09(15), 38.90(5), 42.58(8), 64.18(8), and 65.92(2) indicated that crystalline silver nanoparticles produced by conversion of Ag⁺ to Ag⁰. The phase formation of AgNPs was confirmed with
XRD analysis. However, the biosynthesized AgNPs contains of some organic components obtained from papaya seed extract as the reducing agent. As a result, the XRD analysis exhibits a broad amorphous background peak and covers most of the characteristic peaks of Ag based on the PDF file #04-0783. Fortunately, the highest peak of AgNPs is appeared at 2 theta = 38.9 degree, indicating the AgNO\textsubscript{3} has been reduced to AgNPs during the biosynthesis process. These results conform to the bioorganic phase crystallization of AgNPs proposed by previous studies (Banala et al., 2015; Devanesan et al., 2021; Krishnaraj et al., 2012; Singh et al., 2021).

**Figure 4.** XRD Pattern of AgNPs Synthesized with n-hexane As Bio-reduction

### 3.3 Antifungal Activity

All fractions exhibited their most inhibitory effect at the concentration of 100%. ANOVA analysis showed no significant difference among all samples tested, whereas \( p\text{-value} > 0.25 \), and the antifungal activity ranged from 6.1 to 6.8 mm, categorized as moderate effect (Davis and Stout, 1971). The antifungal inhibition zone has four criteria based on the diameter size of the clear zone, as defined that very strong (> 20 mm), strong (10-20 mm), moderate (5-10 mm), and weak (< 5 mm).

Studies on the ethanolic seed extract of *C. papaya* described the moderate level might due to the presence of the alkaloids, flavonoids, steroids, polyphenols, tannins, and saponins as antimicrobial agents (Yani and Putri, 2019).

The non-significant difference of antifungal activity between samples has been suspected by the high temperature exposed to thermolabile extract components; this might bring the degradation of components (Dewi et al., 2019), while another reason of similar resistance triggered by AgNPs (Panáček et al., 2018).

The intermediate effect of papaya seed extracts in all samples is considerably less effective than commercial antifungal Fluconazole with inhibition zone > 20 mm (Kirkpatrick et al., 1998). However, these results are relatively better than the papaya leaves extract by ciprofloxacin (Callixte et al., 2020). In contrast to the combined effect of the AgNPs synthesized in polar solvents could enhance the antifungal activity as reported by previous studies (Hsu et al., 2021), it is interesting to have more investigation on the non-polar extract as bioreduction agents according to the antifungal effectiveness. Based on Table 2, we also conclude that there was no significant effect of variation of concentration of each sample tested (\( p > 0.05 \)).

The complete pictures of disc diffusion methods on the inhibition zone are shown in Figure 5.

The morphological observation of the antifungal effect showed in Figure 6.

### 3.4 Antifungal Mechanisms

Research by Kim et al. (2009) described that silver nanoparticles disrupt the structure of cell membranes and destroy the membrane integrity, thereby disrupting the membrane potential. Multiple ion gradients across the cytoplasmic membrane help fungal cells maintain their membrane potential. For fungal viability and the release of glucose and trehalose, appropriate preservation of intracellular components is required. Trehalose can protect proteins and biological membranes from inactivation or denaturation caused by various stress conditions, including oxidative stress, drying, dehydration, heat, cold, and toxic agents (Alvarez-Peral et al., 2002; Elbein et al., 2003).

Analysis of the release of glucose and trehalose during exposure to AgNPs succeeded in breaking the membrane permeability barrier, this possibly due to disruption of the lipid membrane bilayer. By the TEM analysis, Kim et al. (2009) demonstrated...
the interaction between AgNPs and membrane structure in C. albicans cells; there was a significant change in the membrane during exposure to AgNPs, which was recognized by cell surface deformation and cell death. A flow cytometric examination of the cell cycle revealed that AgNPs resist the cell cycle in the G2/M phase of C. albicans, which helps to explain the physiological alterations in fungal cells caused by AgNPs. These data suggest that AgNPs could inhibit several cellular processes involved in average growth.

4. CONCLUSIONS

In this report, the n-hexane, ethyl acetate, water, and ethanol fractions were used to extract papaya seed. Although the AgNPs were created entirely with n-hexane, the all samples exhibit moderate effects as antifungal agents. These results are relatively better than the papaya leaves extract by ciprofloxacin, as previously reported by Callixte et al. (2020). Abundant compositions of FAME’s compound, phenolics, and heterocyclic-amides might be influenced the antifungal activity. However, there was no apparent differences between all fractions, so the presence of AgNPs caused no substantial improvement of antifungal activity. The main concern for future research is the specific interaction between biosynthesized nanoparticles with the complexities of secondary metabolites and biomolecular aspects.

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