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Antibacterial activity of fractions from papaya seeds (*Carica papaya* L.) extract against *Escherichia coli* and *Salmonella typhi* and the contributing compounds

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ABSTRACT

Papaya (*Carica papaya* L.) seed has been reported to have antibacterial activity against *Escherichia coli* and *Salmonella typhi*. It contains compounds like alkaloids, flavonoids, saponins, and terpenoids. This study aimed to determine the antibacterial activity of fractions from the ethanolic extract of papaya seed against *Escherichia coli* and *Salmonella typhi* and identify the specific compounds that contribute to their efficacy. Papaya seeds were extracted through maceration with 70% ethanol solvent, and the extracts were subjected to fractionation with n-hexane, ethyl acetate, and water solvents and followed by a diffusion method to examine the antibacterial activity of both extracts and fractions. Fractions with a series of concentrations were used, namely 10-50% (n-hexane and ethyl acetate fractions) and 100-500% (water fraction). The compounds of the ethanolic extract and its fractions were identified with phytochemical screening. The results showed that the n-hexane and water fractions exhibited antibacterial activity against *Escherichia coli* when applied at a minimum of 10% and 100% concentration, respectively, but not against *Salmonella typhi*. Meanwhile, starting from 10% concentration, the ethyl acetate fraction was able to prevent the growth of both *Escherichia coli* and *Salmonella typhi*. The compound detection tests revealed that the n-hexane fraction contained alkaloids and terpenoids, the ethyl acetate fraction had alkaloids, flavonoids, and saponins, and the water fraction comprised flavonoids and saponins.

Keywords: *Carica papaya* L. seeds, fractions, *Escherichia coli*, *Salmonella typhi*

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INTRODUCTION

The high incidence rate of infections in Indonesia, which claims 3 million victims per year (Tjaniadi *et al*., 2003), encourages the development of a treatment that can solve this problem. The most common infections among the population include diarrhea and typhus. Both diseases are caused by, for instance, *Escherichia coli* and *Salmonella typhi* (Jawetz *et al*., 1995). Since the use of synthetic antibiotics may lead to resistance problems, the discovery of medicines derived from natural ingredients is encouraged (Radji, 2005). One of the potential herbal remedies to treat diarrhea, colds, indigestion, and skin diseases is papaya seed. The ethanolic extract of papaya seed has been proved to exhibit antibacterial activity against *Escherichia coli* and *Salmonella typhi* and contain alkaloids, flavonoids, saponins, and tannins (Warisno, 2003). Singh and Ali (2011) confirm that the ethanolic extract of papaya seed can treat diarrhea. The antibacterial potential of papaya seeds needs to be further subjected to fractionation to find out which compounds are believed to play a role in this activity according to their polarity.

MATERIALS AND METHODS

Materials

The white papaya seeds were taken from papaya plants aged 4-5 months and cultivated in Gunungpati Semarang. Ethanol 70%, n-hexane, ethyl acetate, Aqua Dest, *Escherichia coli*, and *Salmonella typhi* were obtained from the Microbiology Laboratory of dr. Karyadi General Hospital, Semarang. The other materials were chloramphenicol, nutrient agar medium, dimethylsulfoxide, 2N HCl, 2% HCl, Mg powder, Dragendorff's reagent, Mayer's reagent, and Liebermann-Burchard reagent. Meanwhile, the equipment used in this research included a rotary evaporator (Heidolph), Laminar Air Flow (AIRTECH), micropipette (Gilson), and an incubator (BINDER).

Methods

Plant Determination

The plant was identified at the Ecology-Biosystematic Laboratory organized by the Department of Biology, Faculty of Mathematics and Natural Sciences, Diponegoro University, Semarang. This process aimed to ensure that the material used in this research was the seeds of papaya plants (*Carica papaya* L.).

Preparation of fractions from the ethanolic extract of papaya seeds

One kg of papaya seed powder was extracted by maceration using 70% ethanol solvent for five (5) days. The ratio of powder to solvent was 1:10. The papaya seed extract was fractionated using three different solvents, namely n-hexane, ethyl acetate, and Aqua Dest, and this process yielded n-hexane fraction, ethyl acetate fraction, and water fraction. The extracts and fractions were thickened with a rotary evaporator.

Identification of the antibacterial activity of the fractions

The antibacterial activities against *Escherichia coli* (ATCC 25992) and *Salmonella typhi* (ATCC 1408) were performed by adjusting the turbidity of each bacterial suspension to that of a 0.5 McFarland standard. A total of 200 µl of the bacterial suspension was poured into 15 ml of warm nutrient medium aseptically, then homogenized. Blank disks were placed in the Petri—on the surface of the nutrient agar medium containing bacteria, and dripped with n-hexane and ethyl acetate fraction with the concentrations of 10, 20, 30, 40 and 50% and the water fraction with the concentrations of 100, 200, 300, 400, and 500%; each drop amounted to 10 µg. The positive controls used 30 µg-chloramphenicol disks, while the negative control used DMSO solution. Petri dishes containing the prepared media and fractions were incubated at 37°C for 24 hours (Depkes RI, 1991). The diameter of the inhibition zones formed in this test was measured in millimeters.

Identification of compounds

Alkaloids

Each of the three fractions was added with 2% HCl. The solution was then divided and poured into two tubes. The first tube was added with 2-3 drops of Dragendorff's reagent, whereas the second one with 2-3 drops of Mayer's reagent. The appearances of orange precipitate in the first tube and white deposits in the second tube indicate the presence of alkaloids (Harborne, 1987).

Flavonoids

Each of the three fractions was added with ethanol, 0.5 g of Mg powder, and 2 ml of 2N HCl. The solution was allowed to rest for one minute before the addition of 2 ml of concentrated HCl. When the color sample turns into orange or purple, it shows the presence of flavonoids (Harborne, 1987).

Terpenoids

For each fraction: 1 ml of the fraction was poured into a test tube, then added with the Liebermann-Burchard test reactor. The formation of blue or green color indicates the presence of steroids, while purple or orange color signifies triterpenoid content (Harborne, 1987).

Saponins

For each fraction: 1 ml of the fraction solution was poured into a test tube. The tube was then shaken for 1-2 minutes. The formation of foam or froth that persists for at least five minutes signifies the presence of saponins (Harborne, 1987).

Identification with thin layer chromatography:

Alkaloids (Harborne, 1987)

Stationary phase	: Silica gel GF 254 nm
Mobile phase	: A mixture of chloroform and methanol (7:3)
Reagent	: Dragendorff's

Flavonoids (Markham, 1988)

Stationary phase	: Cellulose
Mobile phase	: A mixture of n-butanol, glacial acetic acid, and water (4:1:5)
Reagent	: Ammonia vapor

Data Analysis

The fractions of the ethanolic extract of papaya seeds were tested for their antibacterial activity using the disk diffusion method. The diameter of the inhibition zones (DDH) formed in this method was measured with calipers.

Results and Discussion

The plant determination proved that the plant used in the research was *Carica papaya* L. The white papaya seeds were first washed to remove the dirt and then oven-dried at 50° C. Drying aims to produce *simplicia* that is not easily damaged and overgrown with mold (BPOM RI, 2013). *Simplicia* is unprocessed natural ingredients used for medicines that, unless stated otherwise, are generally available in the form of dried specimens. Papaya seeds contain 6.5% water, and the requirement for medicinal plants is <10%. This moisture content was measured to prevent mold growth and decrease enzymatic reactions; all of which can reduce the risk of damages to the *simplicia* (Gunawan and Mulyani, 2004). The oven-drying yielded 13.1% *simplicia*.

The research employed extraction by maceration to avoid damages to compounds that are not resistant to heat (Voight, 1994). This procedure used 70% ethanol solvent because it has high extracting power and is relatively non-toxic compared to methanol (Tiwari *et al*., 2011). Also, since it has two polar hydroxyl groups and non-polar alkyl groups, it is believed to be able to take out the compounds from the papaya seeds (Taufiq *et al*., 2015). This process yielded 23.7% products of a viscous blackish brown extract with peculiar bean smell.

The thick papaya seed extract was then subjected to fractionation. This procedure separated the secondary metabolite compounds in the extracts according to their polarity. Fractionation proceeds from nonpolar to polar solvents to acquire specific polar and nonpolar compounds with the same polarity as the solvents (Harborne, 1987). Therefore, in this research, the fractionation was carried out from n-hexane to ethyl acetate then water solvents. The yields of the n-hexane, ethyl acetate, and water fractions were 4.92%, 5.1%, and 87.07%, respectively.

The phytochemical test results of these fractions are presented in Table I. This table shows that the n-hexane fraction of papaya seed extract contains alkaloids and terpenoids. The alkaloid detection test involved the addition of Mayer's and Dragendorff's reagent. The positive result shows precipitate because metal ions in nitrogen atoms that have free electron pairs are replaced and, thereby, form coordinate covalent bonds with metal ions (McMurry and Fay, 2004). Meanwhile, the chemical reaction of the terpenoid detection test involves the condensation of H_2O and the incorporation of carbocation that induce electrophilic addition, followed by hydrogen release. The hydrogen group and its electrons are removed so that the extension of the conjugation indicates the presence of a brown ring (Siadi, 2012).

Table I. Analysis of phytochemical fractions from the ethanolic extract of papaya seeds

Fractions	Phytochemicals		Results
n-hexane	Alkaloids	White precipitate Orange precipitate	+
	Flavonoids	Yellow	-
	Terpenoids	Red yellow	+
	Saponins	No froth or foam	-
ethyl acetate	Alkaloids	White precipitate Red to orange precipitate	+
	Flavonoids	Red to orange	+
	Saponins	No froth or foam	-
water	Flavonoids	Orange	+
	Saponins	Dark brown with persistent froth or foam for 5 minutes	+

Note :
(+) = positive indication of compound
(-) = negative indication of compound

The ethyl acetate fraction of the ethanolic extract of papaya seed contains flavonoid and alkaloid compounds, while the water fraction contains flavonoids and saponins. The flavonoid content was identified through the addition of Mg powder and HCl. The addition of concentrated HCl hydrolyzes flavonoid into its aglycone (Markham, 1988). In the saponin test, the occurrence of foam was observed. This foam or froth is generated by the combination of its constituent compound, i.e., nonpolar sapogenin chain, and the water-soluble polar side chain (Oleszek, 2002).

The compounds were further analyzed with TLC analysis. The results of the alkaloid detection test on the ethanolic extract of papaya seeds and its n-hexane and ethyl acetate fractions are presented in Figure 1. The figure shows a stronger color intensity of orange patches with a yellow background (Harborne, 1987). This visualization occurred after the plates were sprayed with Dragendorff's reagent. Meanwhile, the examination of flavonoid content in the ethyl acetate fraction (Figure 2) and the water fraction (Figure 3) showed positive results. Spottings on the TLC plates was compared with quercetin. Fractions that contain flavonoids turn yellow under visible light after exposure to ammonia vapor.

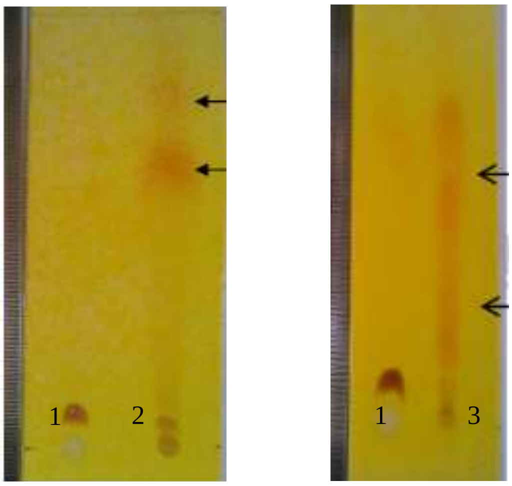


Figure 1. The alkaloid detection test results of (1) the ethanolic extract of papaya seed, (2) the ethyl acetate fraction of the ethanolic extract of papaya seed and (3) the n-hexane fraction of the ethanolic extract of papaya seed

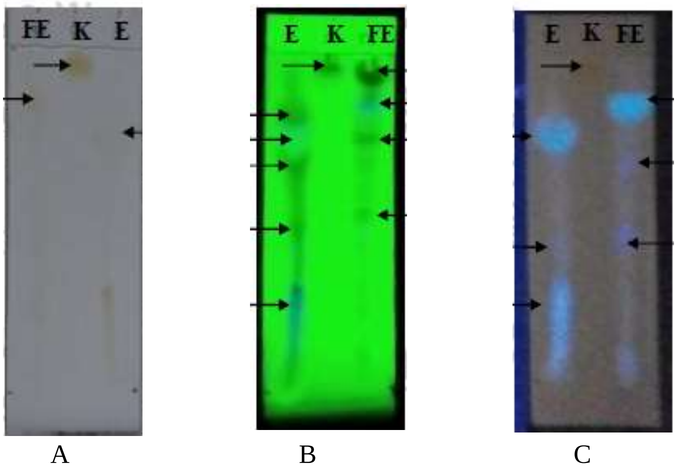
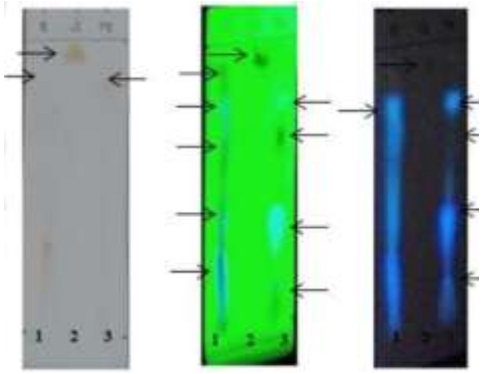


Figure 2. The flavonoid detection test results under (A) visible light, (B) UV254 nm, and (C) UV366 nm. (E) the ethanolic extract of papaya seed, (K) quercetin, and (FE) the ethyl acetate fraction of the ethanolic extract of papaya seed



A B C

Figure 3. The flavonoid detection test results under (A) visible light, (B) UV254 nm, and (C) UV366 nm. (1) the ethanolic extract of papaya seed, (2) quercetin, (3) the water fraction of the ethanolic extract of papaya seed

Taufiq (2015) states that the ethanolic extract of papaya can inhibit the growth of *Escherichia coli* and *Salmonella typhi*. Meanwhile, this study applied further analysis to the ethanolic extract, i.e., by fractionating it using three solvents with different polarity. The results of the antibacterial activity test of the fractions produced in this process are summarized in Table II. Each fraction can inhibit the growth of *Escherichia coli*, while only the ethyl acetate fraction has the growth-inhibiting properties against *Salmonella typhi*. The inhibitory activity is related to the active compounds in each fraction. Alkaloids can interfere with peptidoglycan and inhibit topoisomerase enzymes in bacteria (Campbell *et al*., 2010). As for flavonoids, these compounds inhibit bacterial growth by decreasing the permeability of the cell membrane due to the complex bonding of proteins and membranes (Pelczar and Chan., 1988). The inhibitory ability of terpenoids is suspected to involve a reaction that forms a strong polymer bond between terpenoids and the outermost membrane of the bacterial cell wall, leading to the infection of porins (Hernández *et al*., 2000). Meanwhile, saponins are thought to cause leakage of proteins and enzymes in cells (Zablotowicz *et al*., 1996).

Table II. The antibacterial activities exhibited by the fractions of the ethanolic extract of papaya seeds (three replications)

Test Objects	Concentrations (%)	The diameter of the inhibition zones (DDH; mean±SD in mm)	
		Escherichia coli	*Salmonella typhi*
n-hexane fraction	10	9.42±0.52	0.0
	20	11.42±1.18	0.0
	30	11.98±1.58	0.0
	40	10.33±0.29	0.0
	50	9.47±0.55	0.0
Ethyl acetate fraction	10	13.11±0.05	6.27±0.25
	20	13.54±0.07	6.95±0.96
	30	14.13±0.89	7.35±0.98
	40	14.80±0.84	7.50±0.88
	50	13.58±1.51	6.84±0.57
Water fraction	100	6.50±0.25	0.0
	200	7.16±0.72	0.0
	300	7.58±0.8	0.0
	400	8.33±0.52	0.0
	500	9.00±0.66	0.0
Chloramphenicol	30 µg/disk	17.78±0.14	31.32±1.24

The inhibitory regions of *Escherichia coli* bacteria caused by the application of 10% n-hexane fraction created an inhibition zone with a diameter of (DDH) 9.42±0.52 mm, whereas at the same concentration the ethyl acetate fraction produced DDH= 13.11±0.05 mm. The water fraction exhibited inhibitory activity at 100% concentration, forming inhibition zones with DDH= 6.50±0.25 mm. The ethyl acetate fraction only induced the antibacterial activity against *Salmonella typhi*, and the lowest concentration (10%) created the smallest DDH (6.27±0.25 mm). On the contrary, all three fractions expressed antibacterial responses against *Escherichia coli* and *Salmonella typhi*. Considering the inhibiting concentrations and the resultant DDH, the ethyl acetate fraction is considered as a fraction of the ethanolic extract of papaya seeds that generates the best activity.

The ethyl acetate fraction has been proven to exhibit antibacterial activity against *Salmonella typhi* due to its flavonoid and alkaloid contents. Such inhibitory ability is suspected to be the result of flavonoid that occurs as aglycones in the ethyl acetate fraction. The water fraction also contains flavonoids, possibly glycosides. Isoflavones, flavanones, and flavones are flavonoid derivatives that tend to dissolve in ether, ethyl acetate, chloroform, and n-butanol solvents, whereas glycosides tend to dissolve in polar solvents like water (Markham, 1988). Nevertheless, the identification of compounds that play an active role in inhibiting the growth of *Salmonella typhi* requires further analysis.

CONCLUSION

The n-hexane, ethyl acetate, and water fractions of the ethanolic extract of papaya seeds can inhibit the growth of *Escherichia coli*, but only the ethyl acetate fraction can prevent the growth of *Salmonella typhi*. The n-hexane fraction contains alkaloids and terpenoids, the ethyl acetate fraction comprises alkaloids, flavonoids, and saponins, and the water fraction has flavonoids and saponins.

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