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Characterization of Phytochemical Compounds and Antimicrobial Activity of Crude Alkaloid from Papaya (*Carica papaya* L. var. Eksotika) Leaf Extract

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ABSTRACT

The papaya plant yields a lot of by-products after its fruit is harvested. Papaya leaves contain a bio-active phytochemical that can serve as a potential source of natural antibacterial agents. Phytochemical screening has discovered that papaya leaf extract contains compounds such as alkaloids, tannins and saponins. Crude alkaloid detection by Dragendorff's test was positive. Chemical structure characterisation of extracted crude alkaloids revealed by ^{1}H-NMR spectrum testing was partial, as the spectrum identified only the chemical structure corresponding to aliphatic carbon compounds. Crude alkaloid dissolved in organic solvent dichloromethane showed no antifungal properties towards *Candida albicans*. Antibacterial properties of crude alkaloid dissolved in dichloromethane were selective on both Gram positive and negative bacteria. Kirby Bauer test results showed negative inhibition for both *Staphylococcus aureus* (Gram positive) and *Salmonella typhi* (Gram negative) but inhibition was positive for antibiotic resistant bacteria, MRSA (Gram positive) and *Escherichia coli* (Gram negative) bacteria. Inhibition efficacy increased with the amount of crude alkaloids used. The present study supports the use of papaya by-products as an alternative natural antibiotic for both the community and healthcare personnel.

Keywords: Alkaloid, *Carica papaya* leaves, dichloromethane, ^{1}H-NMR, antibacterial and antifungal properties

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INTRODUCTION

The leaves of the *Carica papaya* plant, locally known as 'betik' in Malaysia have been widely utilised as traditional medicine for centuries in this country. The leaf extract is used traditionally to treat intestinal worm infection, gastric pain, fever and

amoebic dysentery as genito urinary ailment, to relieve symptoms of asthma, as a vermifuge (Teixeira et al., 2007). The leaves of *C. papaya* contain many bio-active phytochemical compounds in different concentrations such as steroidal saponins, flavonols, tannins, phenols and alkaloids as well as organic acids and unsaturated sterols (Head & Lauter, 1956). Phytochemicals are categorised as secondary metabolites, which are organic compounds that are not involved directly in normal growth, development or reproduction of an organism. These phytochemical compounds might independently or synergistically interact to react against pathogens active in the plant.

Alkaloids are naturally occurring chemical compounds and can be found in most plant leaves. Alkaloids in the form of carpaine and carpasemine are reported to be found abundantly in *C. papaya* leaves (Burdick, 1971; Chávez-Quintal, González-Flores, Rodríguez-Buenfil, & Gallegos-Tintoré, 2011). The function of these alkaloid forms vary. For instance, carpaine present in papaya leaves native to Indonesia and Central and South America was believed to be harmful (Head & Lauter, 1956). In other reports, carpaine present in *C. papaya* leaves in general was found not to be microbicidal (Nkuo-Akenji, Ndip, McThomas, & Fru, 2001; Dawkins, Hewitt, Wint, Obiefuna, & Wint, 2003; Leite, Nardi, Nicoli, Chartone-Souza, & Nascimento, 2005; Nayak, Pereira, & Maharaj, 2007). Moreover, many studies have reported that antimicrobial and antifungal properties of papaya leaf extract were dependable on the type of solvent being used. For instance, papaya leaf extract dissolved in alcohol-based solvents such as ethanol and methanol has been known to show positive antibacterial and antifungal properties. However, papaya leaf extract dissolved in hot water was unable to inhibit microbe and fungi activity (Baskaran, Bai, Velu, & Kumaran, 2012).

The papaya plant yields a lot of by-products after its fruit is harvested. These by-products include the stem and leaves, which are usually disposed of in open areas and left to rot. Eventually, phytopathogens will inhabit and grow on the waste that will subsequently cause ecological problems and health risks for human (Thomas et al., 2009). A potential solution to this problem of papaya waste is to extract the bioactive compounds and exploit their usage. To the best of our knowledge, comprehensive information on the efficacy of crude alkaloids dissolved in organic solvents such as dichloromethane on bacteria and fungi has not been elucidated so far. Hence, this study was carried out a) to profile phytochemical components of papaya leaf extract and b) to test the antimicrobial and antifungal efficacy of crude alkaloids dissolved in dichloromethane against selected strains of bacteria and fungi.

MATERIALS AND METHOD

Plant Sample Collection

Fresh leaves of papaya (*Carica papaya* L. var. Eksotika) were randomly sampled from Merlimau, Malacca, Malaysia. All the

leaf samples were dried in an incubator at 55°C and finely crushed with a mortar and pestle prior to use.

Extracts and Phytochemicals Screening to Identify Active Compounds

Phytochemical tests for alkaloids, flavonoids, saponins and tannins were carried out according to protocol described by Harborne (1973) with some modification. Initial detection of alkaloids was tested by moistening approximately 20 g of dried leaves with 10% ammonia solution. On two separate occasions, the crushed leaves were soaked completely with dichloromethane or methanol until a uniform pulp was formed. The leaf mixture soaked in dichloromethane or methanol was heated for 30 min followed by normal filtration. Hydrochloric acid (5%) was added to the collected filtrate and the mixture was tested with 2 to 3 drops of Mayer's reagent. A positive reading for alkaloids is indicated by the appearance of a turbid or milky solution.

Flavonoids were tested for by soaking 20 g of crushed dried leaves with dichloromethane or methanol on two separate occasions and filtered. Next, 2 to 3 drops of concentrated hydrochloric acid was added together with a strip of magnesium ribbon into the filtrate. The appearance of the colours red or blue green after a few seconds was considered a positive reading.

Saponins were tested for using the froth test. Briefly, approximately 20 g of crushed dried leaves was soaked in either dichloromethane or methanol on two separate occasions and were filtered. Next, 3 ml of boiled distilled water was added to the filtrate and the mixture was shaken vigorously. The mixture was left at room temperature for 30 min. The formation of froth was considered a positive reading.

Tannins were determined by dissolving 20 g of crushed dried leaves soaked in either dichloromethane or methanol in potassium hydroxide (10%). The formation of a white precipitation indicated the presence of tannins.

Extraction of Crude Alkaloids

Extraction of alkaloids was carried out according to protocols described by Head and Lauter (1956) with modification. The leaves of *C. papaya* were dried and finely ground, then de-fattened, which involved soaking in hexane for 3 days at room temperature in a Soxhlet extractor and eventually being filtered. The filtrate was dried at room temperature for 24 h and was subsequently moistened by ammonium (25%). Next, dichloromethane was added and the mixture was left at room temperature for 17 h and filtered. The filtrate was concentrated to approximately 500 ml in a rotary evaporator. The solution was filtered and 10 ml of hydrochloric acid (5%) was added to the filtrate. The mixture was left to settle until two layers of solution were formed. The top layer of solution was removed and made basic by adding ammonia (10%) continuously until pH 11 was achieved. Next, 50 ml of dichloromethane was added to the solution and it was left to settle. The bottom

aqueous layer was removed, rinsed with water and precipitated using anhydrous sodium sulfate. Subsequently, the mixture was filtered and the collected filtrate was air-dried to obtain crude alkaloids.

An alkaloid stock solution was prepared by dissolving 50 g of crude alkaloids in 10 ml of dichloromethane. Finally, the stock solution was diluted with dichloromethane into a series of different amounts of solution i.e. 10, 20 and 30 mg/ml. The different amounts of crude alkaloid were achieved by performing a serial dilution from the stock solution. Intermediary dilution was carried out if the amount of stock was too small to be pipetted.

Identification of Crude Alkaloids

The crude alkaloid extract was assayed using Dragendroff's reagent. Briefly, the crude alkaloid was first separated using the thin liquid chromatography (TLC) method, where it was soaked in dichloromethane and then the alkaloids were identified by spraying the TLC plate with Dragendorff's reagent. The presence of orange spots was considered a positive reading.

Nuclear Magnetic Resonance Spectrometry ^{1}H-NMR

An amount of 3 mg of crude alkaloids was dissolved in deuterated chloroform ($CDCl_3$) and a ^{1}H-NMR spectrum was taken on a nuclear magnetic resonance (JOEL 500 MHz JNM-FX-100) spectrometer with tetramethylsilane (TMS) as the internal standard. The chemical shift, δ, was expressed in parts per million (ppm).

In Vitro Antimicrobial and Antifungal Assay

Several bacterial strains (two Gram negative, one Gram positive and one antibiotic resistant Gram positive) and a fungal strain were obtained from the Microbiology Laboratory, Universiti Pendidikan Sultan Idris (UPSI). The strains used were: *Escherichia coli, Salmonella typhi, Staphylococcus aureus, Methicillin-resistant Staphylococcus aureus* (MRSA) and *Candida albicans*. The strains were kept in LB agar and the cultures were stored at 37°C in an incubator.

Bacterial and fungal growth inhibition was carried out using the standard Kirby Bauer test on crude alkaloids only. The zone of inhibition was measured using a ruler in millimetres in three replicates per set-up. The results were expressed as the average zone of inhibition diameter of different alkaloid amounts (10, 20 and 30 mg/ml) and dichloromethane solvent was used as a negative control for the different bacterial and fungal strains.

RESULTS

Phytochemical Screening

Phytochemical screening detected the presence of alkaloids in the leaf extract soaked in dichloromethane only while the test for saponins and tannins was positive only for extracts soaked in methanol. Flavonoids were not detected in the leaf extract for both the dichloromethane and methanol treatment (Table 1). On the other hand, identification of crude alkaloids using Dragendroff's reagent was positive.

Table 1
Qualitative identification of phytochemical compounds of papaya leaves soaked in dichloromethane and methanol

By-product	Solvent	Alkaloids	Flavonoids	Saponins	Tannins
Leaves	Dichloromethane	+	-	-	-
	Methanol	-	-	+	+

Positive (+), negative (-)

Alkaloid Characterisation via Nuclear Magnetic Resonance Spectrometry ^{1}H-NMR

The ^{1}H-NMR spectrum revealed the following peaks for extracted crude alkaloids: δ ^{1}H (500 MHz, $CDCl_3$): 1.0 – 2.0, as shown in Figure 1. The ^{1}H-NMR spectrum of extracted alkaloids corresponded with aliphatic carbon compounds. The presence of halogens, aromatics, carboxyl and aldehyde functional groups revealed by ^{1}H-NMR spectrum was highly unlikely.

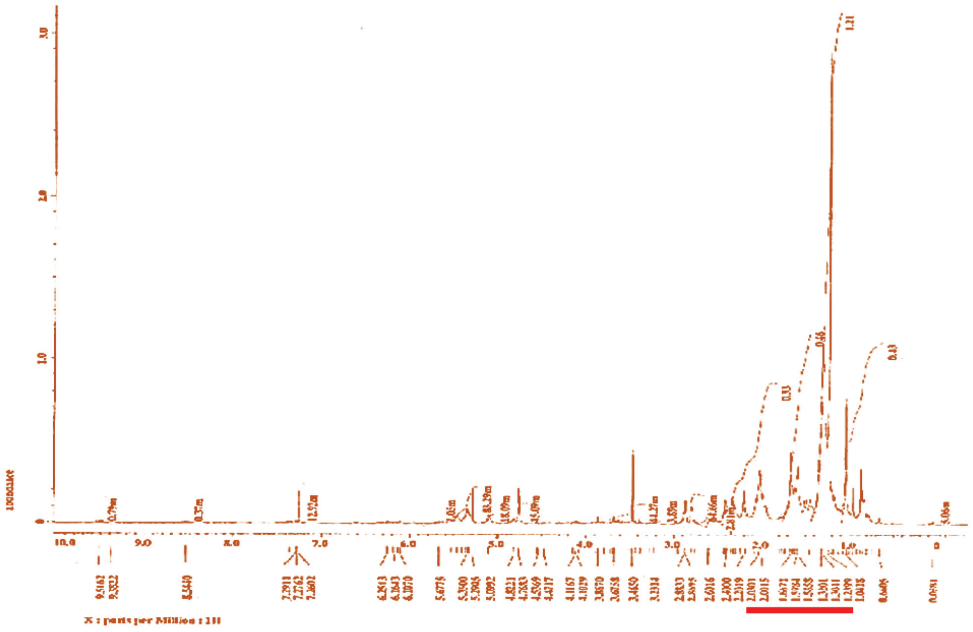

Figure 1. The ^{1}H-NMR spectrum of crude alkaloid extract from papaya leaves. The peak of chemical shift range, δ ^{1}H (500 MHz, $CDCl_3$), 1.0 – 2.0, is highlighted by the red line

In Vitro Antimicrobial and Antifungal Property

The antifungal assay results obtained in this study showed no inhibiting properties of alkaloids towards the only fungal strain tested, *C. albicans.* Interestingly, antimicrobial assay inhibition of alkaloids towards strains of Gram positive and Gram negative bacteria was selective. Inhibition was negative for both *S. aureus* (Gram positive) and *S. typhi* (Gram negative). However, the Gram positive antibiotic resistant bacteria, MRSA, and the Gram negative bacteria, *E. coli*, were susceptible to alkaloid treatment (Table 2).

Inhibition efficacy as measured by the zone of inhibition diameter showed increment in the diameter when the amount of alkaloid increased. In other words, the zone of inhibition increased with the amount of alkaloids used (Table 2).

Table 2
Mean and standard deviation (*means ± STDEV*) *of inhibition zone on fungal and bacterial strains*

Amount (mg/ml)	Zone of Inhibition Diameter (mm)				
	Candida albicans	*Escherichia coli*	*Salmonella typhi*	*Staphylococcus aureus*	*Methicillin-Resistant Staphylococcus aureus* (MRSA)
Control	-	-	-	-	-
10	-	6.2 ± 0.06	-	-	7.1 ± 0.11
20	-	6.7 ± 0.06	-	-	8.0 ± 0.11
30	-	7.2 ± 0.10	-	-	9.3 ± 0.20

No inhibition (-)

DISCUSSION

Alkaloids are a secondary metabolite compound that are found abundantly in the leaves of plants. The presence of alkaloids is believed to serve as a plant natural's defence system against plant pathogens (Oliva, Meepagala, & Wedge, 2003) and as a repellent to herbivores. Besides that, humans have been using secondary metabolites from plants as medicine, flavouring and recreational drugs for centuries. However, several reports have shown that alkaloid ingestion by humans resulted in physiological defects such as inhibition of enzymes, neurotransmission interference and loss of coordination and caused hallucinations, convulsions, vomiting and even death (Agosta, 1997; Karban & Baldwin, 1997; Bidlack, Omaye, Meskin, & Topham, 2000; Rosenthal & Berenbaum, 2012).

The function of alkaloids in papaya leaf cannot be overlooked as many researchers have identified alkaloids as having antibacterial and antifungal properties (Chávez-Quintal et al., 2011;

Krishna, Paridhavi, & Patel, 2008). For instance, Krishna et al. (2008) reported that herbal formulation of papaya leaves alone showed antibacterial activity towards *S. typhi*. However, our study discovered that crude alkaloid extract did not show any antibacterial inhibition towards *S. typhi*. This might have been due to the different types of solvent used in the two studies. Perhaps, antibacterial properties of papaya leaves are less effective when dissolved in dichloromethane rather than a herbal formulation. On the other hand, Chávez-Quintal et al. (2011) discovered that leaf extracts dissolved in an organic solvent was a better antifungal inhibitor compared to when dissolved in an aqueous solvent. However, in our study, no antifungal inhibition of *C. albicans* was detected when the alkaloid leaf extract was dissolved in an organic solvent, dichloromethane. Perhaps, alkaloids from the leaves alone are insufficient to inhibit fungal growth. Possibly, fungal inhibition might have been effective on *C. albicans* if the treatment had involved alkaloids present together with other phytochemical compounds. Indeed, studies by Giordani, Gachon, Moulin-Traffort and Regli (1997) had shown that papaya latex together with Fluconazole, an antifungal medication, had synergistic action against *C. albicans* inhibition.

All the three different amounts of crude alkaloid extract used during antibacterial treatment was effective as MRSA and *E.coli* inhibitors. Furthermore, this study also showed a significant increment in inhibition efficacy with the amount of alkaloids used. The Gram positive bacteria, MRSA, was believed to have been more prone towards treatment using antibiotics due to its lack of an outer membrane that could impede entry of drugs. On the other hand, Gram negative bacteria such as *E. coli* was expected to be more resistant towards antibiotics because of the presence of an outer membrane in its cell wall, a common feature in Gram-negative bacteria. However, in this study crude alkaloid extract from papaya leaves showed positive inhibition towards *E. coli*. Further to this, empirical results from other researchers had likewise shown that papaya seed and pulp extract was bacteriostatic against several enteropathogens including *E. coli* (Osato, Santiago, Remo, Cuadra, & Mori, 1993). MRSA is very often associated with healthcare due to the prevalence of MRSA-caused illness among health caregivers. This strain of bacteria, once prone to methicillin treatment, developed resistance by modifying penicillin-binding protein on its cell wall (Van Bambeke, Mingeot-Leclercq, Struelens, & Tulkens, 2008). The present study, which showed positive inhibition of MRSA growth by crude alkaloid extract, proved the potential of crude alkaloid extract as an alternative natural antibiotic. At this stage, *in vivo* experiments need to be undertaken simultaneously to test consistency between *in vivo* and *in vitro* results. *In vivo* conditions can be complex and might cause susceptibility of treatment, causing results to differ from those observed in *in vitro* conditions (Ekanem, Obiekezie, Kloas, & Knopf, 2004).

The ^{1}H-NMR spectrum on crude alkaloid extract obtained in this study revealed that δ ^{1}H (500 MHz, $CDCl_3$) was 1.0-2.0, corresponding with aliphatic carbon compounds. Conventionally, many researchers have associated alkaloids present in papaya with carpaine (Burdick, 1971; Fhaizal et al., 2014), but in this study, the exact chemical structure classification based on the ^{1}H-NMR spectrum results obtained was only partial and imprecise, making it difficult to make any conclusive predictions. In fact, Govindachari (2002) and Tang (1979) revealed that carpaine from papaya leaves inadvertently contained other impurities such as carpane, carpamic acid and dehydrocarpaine 1 and 2, making chemical structure identification difficult.

Papaya by-products such as leaves contain bio-active phytochemicals that can serve as a potential source of natural antibacterial agents. This study showed that papaya leaf extract contains secondary metabolites such as saponins, tannins and alkaloids. The crude alkaloid extract soaked in dichloromethane exhibited selective antibacterial properties towards Gram-positive (MRSA) and Gram-negative (*E. coli*) bacteria. The present study supports the potential use of papaya by-products as alternative natural antibiotics for both the community and healthcare personnel.

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