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Evaluation of Antioxidant and Antibacterial Activities of Bubble Belly Massage Oil and their Crude Ingredients

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ABSTRACT

Bubble Belly massage oil is popular among Malaysians since its commercialization in 2018. The massage oil contains lemon oil, vitamin E oil, aloe vera oil, eucalyptus oil, ginger oil, black pepper, fenugreek, *Caesalpinia sappan*, *Usnea barbata*, and *Helicteres isora*. The massage oil is believed to reduce weight, cellulite, menstrual pain, body ache, and scar appearances. The study evaluated oil and its crude ingredients for antioxidant activity using DPPH and ABTS assays, antibacterial activity was evaluated by using disc diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) assays. The crude ingredients soaked in the massage oil were dried and underwent aqueous extraction. Phenols, tannins, and quinones were detected qualitatively in the samples. Highest DPPH and ABTS radical scavenging of 73.1% at 0.78% (v/v), and 98.2% at 12.5% (v/v), respectively were shown by the oil. Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Bacillus cereus*, *Klebsiella pneumonia*, and *Enterococcus faecalis* were susceptible to the oil at 100% (v/v) with a zone of inhibition of 15.0 mm, 14.0 mm, 12.0 mm, 9.0 mm, and 14.0 mm, respectively. All the tested bacteria were resistant to the crude ingredients. The MIC values against *B. cereus*, MRSA, *K. pneumonia*, and *E. coli* treated with oil were in the range of 0.39 to 0.78% (v/v). Both the crude ingredients and oil showed MBC values of 12.5 mg/mL and 0.39% against *B. cereus* and MRSA, respectively. In a nutshell, the massage oil showed significant inhibitory and radical scavenging activities and thus is potential as an antibacterial and antioxidant agent.

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1 Introduction

Natural products have become a key focus of inquiry for therapeutic agents and drug discovery research. Natural therapeutic compounds are derived from numerous sources such as microbes, minerals, plants, and animals. However, plants have emerged as the most prominent source of natural medicine attributed to their structural and chemical diversity of secondary metabolites. Secondary metabolites such as phenolic, alkaloids, and terpenoids contribute significantly to a plant's survival and ability to thrive in a harsh environment (Bernhoft 2010). Many oil-based drugs isolated from plants possess antibacterial and anticancer properties. For example, camphene isolated from *Piper nigrum*, carvacrol from the essential oil of oregano and thyme, and vapor of *Litsea cubeba* seed oil were reported to inhibit bacteria (Blowman et al. 2018).

Bubble belly massage oil is a commercialized product introduced in Malaysia in early 2018 and since then, this product has become popular among women and men. Traditionally, massage oil is applied to the skin to reduce belly fat, improve circulation, reduce constipation, act as a moisturizer, lighten black marks, and tighten the skin. The massage oil is also believed to reduce cellulite, menstrual pain, body and joint pains, and scars (Natalie 2017; Debra 2019; Deepali 2019; Debra 2020; Go Outdoor 2021). The oil contains various herbs namely lemon oil, vitamin E oil, aloe vera oil, eucalyptus oil, ginger oil, black pepper, fenugreek, *C. sappan*, *U. barbata*, and *H. isora*. The oil can be applied daily to the skin and according to end users, the oil is effective in reducing pain and scar appearances (New Directions Aromatic Inc 2018; Go Outdoor 2021). The herbs found in the massage oil are reported to exhibit medicinal values such as antibacterial, anti-inflammatory, antiviral, antidiabetic activities, treating skin diseases and stomach aches (Nirmal and Anil 2014; Kalunta 2017; Takooree et al. 2019; Akhlaghi and Najafpou 2021; Pandey et al. 2021; Hwang et al. 2021; Sepahvand et al. 2021; Luo et al. 2021; Almatrodi et al. 2021). The oil can reduce anxiety and depression symptoms, relieve morning sickness, prevent acne breakouts, inhibit fungal growth, anti-aging, and remove stretch marks (Jugreet et al. 2020).

World Health Organization (2021) stated that infectious diseases are caused by pathogenic microorganisms such as bacteria, viruses, parasites, or fungi and can be spread directly or indirectly from one person to another. Infectious diseases caused by multidrug-resistant (MDR) bacteria have become a public health concern all over the world. These bacteria drastically reduced the efficacy of antibiotics, consequently, increasing the therapeutic failure and mortality rate. It was estimated that every year, around 25,000 patients die due to infections with MDR bacteria (Fankam et al. 2017). Scientists are searching for new antimicrobial substances especially from plant extracts and natural products due to their potential source of novel antibiotic prototypes (Dzotam et al. 2016). The essential oils of cinnamon, oregano, and thyme individually showed strong antimicrobial activities with MIC ≥ 0.125 µL/mL and MBC ≥ 0.25 µL/mL (Mith et al. 2014).

With the rise in multidrug-resistant bacteria and mortality rate, the present study was intended to reveal the potential of massage oil and crude extract as antibacterial agents. To date, no study was reported on the biological properties of the bubble belly massage oil conducted *in vitro* and *in vivo*, hence this study aimed to evaluate the effect of massage oil and its crude ingredients for antioxidant and antibacterial activities.

2 Materials and Methods

2.1 Aqueous extraction and lyophilization

Bubble belly massage oil was purchased from a few Indian shops located at Perak and Seremban, Malaysia. The crude ingredients of the massage oil namely black pepper, fenugreek, *C. sappan*, *U. barbata*, and *H. isora* were dried in an oven at 50°C. The completely dried ingredients were powdered and boiled using deionized water for 20 minutes thrice to obtain a higher aqueous extract yield. The filtrates were kept at -20°C overnight and lyophilized using a freeze-dryer. A stock concentration of 100 mg/mL of the crude aqueous extract was prepared using either distilled water or dimethyl sulfoxide (DMSO) based on the bioassays.

2.2 Phytochemical analysis

Both the crude aqueous extract (100 mg/mL, w/v) and oil (100%, v/v) were tested qualitatively for the presence of phenols, saponins tannins, quinones, flavonoids, and coumarins as described by Anjali and Sheetal (2013) with minor modifications, especially on the volume of the samples. The formation of color, froth, or layers were observed and compared with respective standards for the detection of classes of secondary metabolites.

2.3 Antioxidant assays

2.3.1 DPPH assay

The crude aqueous extract and oil were evaluated for antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay based on Chen et al. (2012) with minor modifications in terms of the volume of the samples. The samples were serially diluted (two-fold) at various concentrations ranging from 0.02 to 100 mg/mL (mg/mL or % v/v) in a 96-well, along with standards (ascorbic acid and α-tocopherol), and negative control (DMSO). DPPH reagent (0.2 mM) was added to each well and incubated in dark for 20 minutes at room temperature. The absorbance was measured using a microplate reader at 517 nm. The percentage of radical scavenging activity was calculated based on equation [1] (Shekhar

and Anju 2014). The values are expressed as the means of triplicate analyses.

DPPH radical scavenging activity = $[(A_{NC} - A_S) / (A_{NC})] \times 100\%$ [1]

Where A_{NC} is absorbance of negative control and A_S is absorbance of samples.

2.3.2 ABTS assay

The crude aqueous extract and oil were evaluated for 2,2′ -azino-bis-3-ethylbenzthiazoline-6 sulphonic acid (ABTS) radical scavenging activity based on Nilima and Hande (2011) with minor modifications. The crude aqueous extract (100 mg/mL) and oil (100%, v/v) were serially diluted at various concentrations in a 96-well along with standards (quercetin, α-tocopherol), and negative control (DMSO). ABTS reagent (7 mM) was added to each well and incubated in dark for 10 minutes at room temperature. The absorbance was measured using a microplate reader at 734 nm and the percentage of ABTS scavenging activity was calculated based on equation [2] (Nilima and Hande 2011). The values are expressed as the means of triplicate analyses.

ABTS radical scavenging activity = $[(A_{NC-} A_S) / (A_{NC})] \times 100\%$ [2]

Where A_{NC} is absorbance of negative control and A_S is absorbance of samples.

2.4 Antibacterial assays

Both the crude aqueous extract and oil were evaluated for antibacterial activities using disc diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) assays on *B. cereus* (ATCC11778), *E. faecalis* (ATCC29212), *E. coli* (ATCC35218), *K. pneumonia* (ATCC13883), and methicillin-resistant *S. aureus* (MRSA)(ATCC33591). The bacterial concentration of 1×10^6 CFU/mL was used in the bioassays. These bacteria were adjusted to a 0.5 McFarland standard by measuring absorbance at 625 nm. The absorbance of 0.08 to 0.1 is equivalent to 0.5 McFarland standard proportional to 10^8 CFU/mL (Carvalho et al. 2021).

2.4.1 Disc diffusion assay

Gram-positive and Gram-negative bacteria at a concentration of 1×10^6 CFU/mL were streaked onto MH agar plates in disc diffusion assay. Approximately 20 µL of various concentrations of massage oil (12.5 to 100.0%), crude aqueous extract (0.02 to 100 mg/mL), negative control (10% DMSO), and positive control (streptomycin sulfate) were impregnated onto a sterile, blank disc of 6 mm in diameter. The air-dried discs were placed on agar and incubated at 37°C in an incubator for 18 to 24 hours. The zone of inhibition was measured and recorded (Horvath et al. 2016). The assay was repeated thrice and the antibacterial activity was expressed as the mean diameter zone of inhibition (mm).

2.4.2 MIC and MBC assays

The concentration of 1×10^6 CFU/mL was used in the MIC assay using broth dilution method as described by Eloff (1998) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Crude aqueous extract (0.02 to 100 mg/mL), oil (12.5 to 100.0%), negative control (10% DMSO) and positive control (streptomycin sulfate) were two-fold diluted in a 96-well plate, followed by the addition of adjusted bacteria suspension. The plate was incubated at 37°C for 18 to 24 hours. INT dye (0.4 mg/mL) was added and incubated for 20 minutes, followed by an absorbance reading at 600 nm. Based on the color observation, pink color indicates the viable cells and yellow indicates the non-viable cells. The percentage of cell viability was calculated based on equation [3] (Eloff 1998).

Percentage of cell viability = $[A_S / A_{NC}] \times 100$ [3]

Where A_{NC} is absorbance of the negative control and A_S is absorbance of the samples

Sample well with no color changes and lower absorbance were subjected to MBC by streaking on MH agar aseptically. The agar was incubated at 37°C for 18 to 24 hours and the number of colonies in the sample was compared with positive and negative controls. The lowest concentration that inhibited the most colony formation on agar was recorded as MBC (Eloff 1998).

2.5 Data analysis

Both antioxidant and antibacterial assays were repeated thrice and the data were reported as mean±standard deviation. EC_{50} values were determined using GraphPad prism (version 9.3.1). Levels of significance for the bioassays were compared between treatments and standards using a student t-test and $P < 0.05$ was considered statistically significant unless otherwise specified.

3 Results and Discussion

3.1 Phytochemical analysis

The presence of phenols, saponins, tannins, and quinones was detected from the crude aqueous extract, while only phenols, tannins, and quinones were detected in the oil. In addition, flavonoids and coumarins were not detected in both oil and crude aqueous extract (Table 1). Even though essential oils found in the bubble belly massage oil separately are rich with flavonoids and coumarins as reported previously (Mariappan et al. 2014; Sruthi

Table 1 Determination of classes of secondary metabolites in qualitative phytochemical analysis

Class of secondary metabolites	Crude extract (mg/mL)	Massage oil (%, v/v)
Phenols	+	+
Saponins	+	-
Tannins	+	+
Quinones	+	+
Flavonoids	-	-
Coumarins	-	-

+ Presence, - Absent

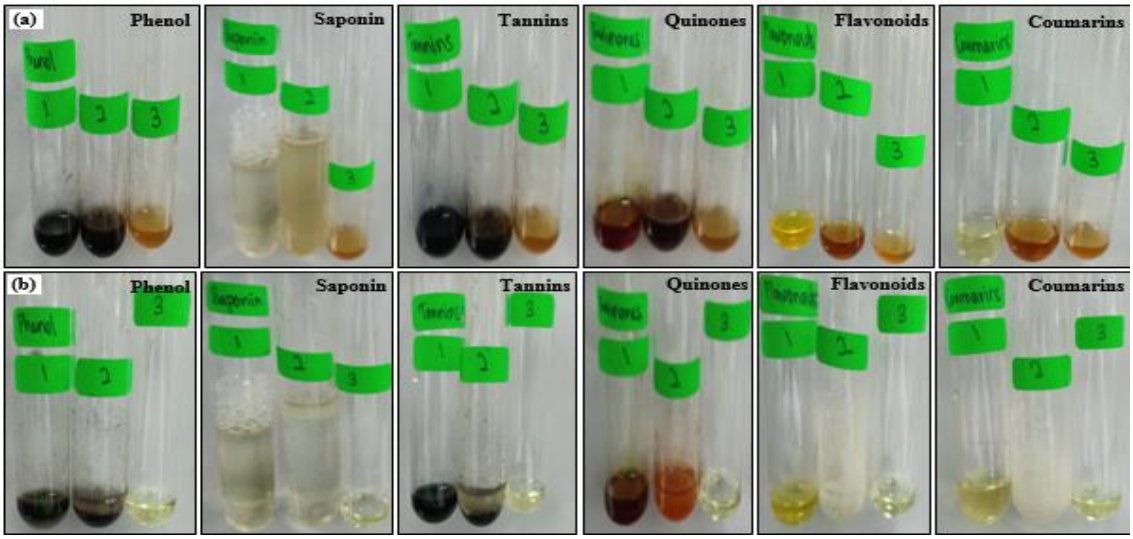

Figure 1 Qualitative observation of the classes of secondary metabolites as compared to standard in crude aqueous extract (a) and massage oil (b); (1) Standard, (2) Samples and test reagent, (3) Samples only.

and Zachariah 2017; Akhlaghi and Najafpou 2021; Manjunath and Mahurkar 2021), however, these two metabolites were not detected qualitatively in the study. The detected classes of secondary metabolites consist primarily of polar functional groups. Functional groups such as hydroxyl, carbonyl, and amines are found to enhance solubility, whilst retaining lipophilic character in both ionized and non-ionized forms by binding with its target through specific hydrogen bonding and/or formation of salt bridges (Dai and Mumper 2010; Nicholas and Graham 2019).

Flavonoids and coumarins were not detected in the study possibly due to the extraction method, which is aqueous extraction. The choice of solvent is critical in extraction because it will influence the quantity and bioactive compounds composition of the crude extract (Ncube et al. 2008). Factors to be considered when selecting an extraction solvent include polarity of the target compound of interest, low toxicity, low boiling point or high volatility for ease of removal, inability to cause structural or functional alteration to the extracted compound, and rapid diffusivity (Ncube et al. 2008; Abubakar and Haque 2020). The best solvent used to isolate flavonoids and coumarins is methanol, as water has lesser extraction efficiency. This result is in agreement with a study by Truong et al. (2019) which stated the highest levels of flavonoids were observed in methanolic extract due to higher solubility of these compounds in methanol than in the other solvents such as distilled water. In addition, quantitative analysis using liquid chromatography (LC) coupled with mass spectrometry (MS) can be used to identify the components, instead of qualitative determination.

Methanol, ethanol, acetone, ethyl acetate, and their combinations have been used for the extraction of phenolics from plants (Xu and Chang 2007). For example, methanol is more efficient in isolating polyphenols, while flavanols with aqueous acetone. This solvent can degrade cell membranes and simultaneously dissolves the secondary metabolites and stabilizes them (Metivar et al. 1980; Shi et al. 2005; Nirmal and Anil 2014). Water extraction is suitable for extracting heat-stable compounds and usually results in the isolation of more oil-soluble compounds (Azwanida 2015; Zhang et al. 2018). Figure 1 shows the presence and absence of the classes of secondary metabolites in both samples.

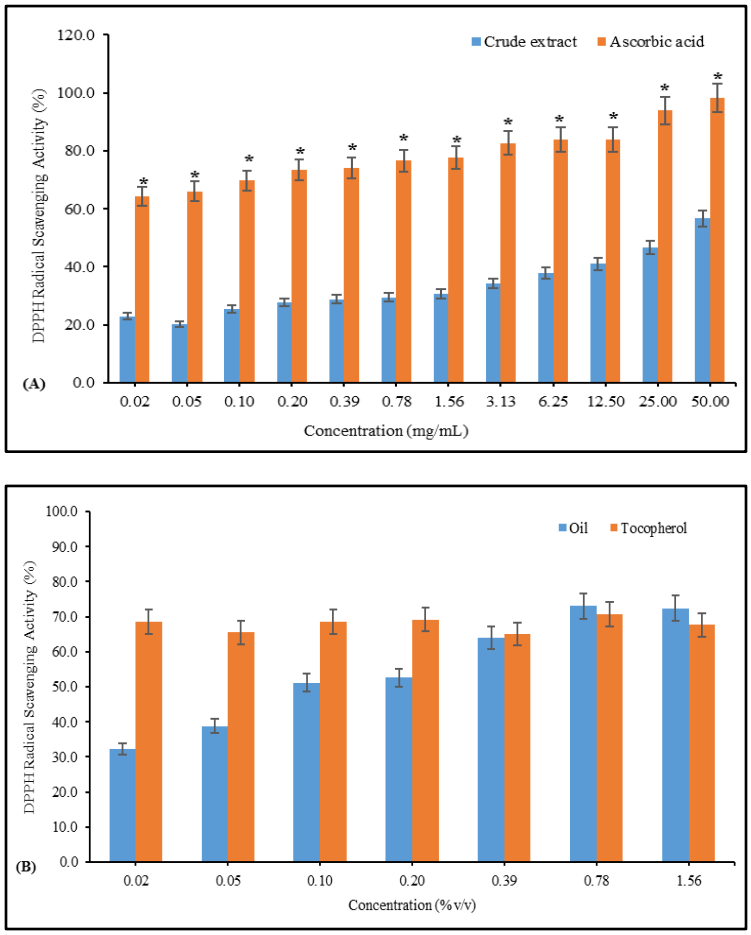

Figure 2 DPPH radical scavenging activity of crude aqueous extract (A) and massage oil (B) at various concentrations. The points represent means ± standard deviation (n=3). * Indicates P < 0.05, student t-test.

Table 2 Effective concentration at 50% (EC_{50}) showed by samples in both antioxidant assays.

Sample	EC_{50} values (mg/mL or %, v/v)	
	DPPH assay	ABTS assay
Crude extract	0.288 ± 0.030	0.221 ± 0.020
Massage oil	0.026 ± 0.019	0.117 ± 0.020
Ascorbic acid	0.003 ± 0.010	*not tested*
α-tocopherol	14.900 ± 0.080	40.620 ± 0.010
Quercetin	*not tested*	377.700 ± 0.010

3.2 Antioxidant activities

The antioxidant capabilities of the samples were determined using ABTS and DPPH radical scavenging activity based on their respective mechanism of action. DPPH radical forms a purple solution and will be reduced upon reacting with an antioxidant that can donate a hydrogen atom forming yellow colored diphenylpicrylhydrazine (Marrufo et al. 2013; Albayrak and Aksoy 2013). In comparison, ABTS will be oxidized to its radical cation ABTS+, which turns blue when reacted with potassium persulfate. The radical scavenging capacity of antioxidants is determined when the blue ABTS+ is converted back to the colorless ABTS (Ammar et al. 2012).

Both the crude aqueous extract and oil showed increasing trends in the DPPH and ABTS radical scavenging activities as the concentration increases from 0.02 to 1.56 mg/mL. The radical scavenging activity displayed a concentration-dependent manner as

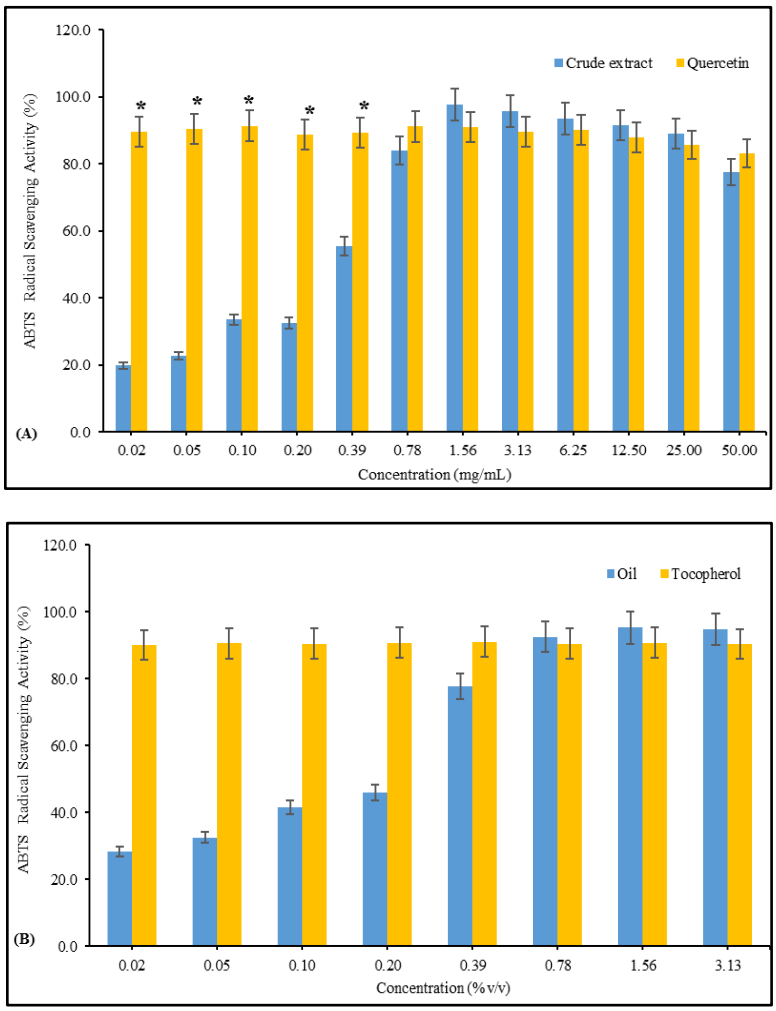

Figure 3 ABTS radical scavenging activity of crude aqueous extract (A) and massage oil (B) at various concentrations. The points represent means ± standard deviation (n=3). * Indicates P < 0.05, student t-test.

shown in Figures 2 and 3. However, it was observed that the oil exhibited higher radical scavenging activity in DPPH (73.1%) and ABTS (98.2%) as compared to crude aqueous extract. Ascorbic acid, α-tocopherol, and quercetin displayed higher radical capacity in the range of 75.0 to 99.0% in both DPPH and ABTS assays. DPPH radical scavenging activity of the crude aqueous extract and ascorbic acid were significantly different ($p<0.05$) based on the student t-test. In contrast, the radical scavenging activity of the oil was comparably good as α-tocopherol, a well-known antioxidant agent. The same trend was observed in the ABTS assay. The effectiveness of the oil and crude aqueous extract was determined using EC_{50} values. The lowest EC_{50} value of 0.026 ± 0.019% (v/v) and 0.117 ± 0.020% (v/v) was exhibited by oil in DPPH and ABTS assays, respectively as shown in Table 2.

The differences exerted by both assays could be attributed to the stereochemical structures of ABTS $^{+\cdot}$ and DPPH radicals, sample solubility, polarity, and the metal-chelating capacity of antioxidants (Shalaby and Shanab 2013). Generally, lemon oil, vitamin E oil, aloe vera oil, eucalyptus oil, and ginger oil separately were reported as good antioxidant agents (Zoran et al. 2022; Oussame et al. 2022; Chatarina et al. 2022). Previous studies on Eucalyptus essential oil showed 79.55 ± 0.82% (v/v) of DPPH radical scavenging activity (Arun et al. 2010), while lemon essential oil showed a reduction of lipid peroxidation levels and nitrile content, increased reduced glutathione levels, superoxide dismutase, catalase and glutathione peroxidase activities in mouse hippocampus model (Campêlo et al. 2011). In addition, oils isolated from a few *Eucalyptus* species had an antioxidant effect with an IC_{50} value of 4.21 ± 0.35 mg/mL for DPPH free radical scavenging due to the presence of the phenolic terpenoids, thymol, and carvacrol (Sapit et al. 2022). Hence, the massage oil showed promising antioxidant activity possibly due to the synergistic effect among the detected phenols, tannins, and quinones as a free scavenger to destroy radical molecules.

3.3 Antibacterial activities

The antibacterial properties of the crude aqueous extract and oil were performed using disc diffusion, MIC, and MBC assays. In the disc diffusion assay, it was noted that the crude aqueous extract did not inhibit all the test bacteria, while the oil inhibited all the bacteria at higher concentrations. Disc diffusion is a simple method that does not require special tools and the results can be easily interpreted as susceptible, intermediate, or resistant. The present study showed that the disc diffusion assay may not be a reliable tool for susceptibility testing due to some factors such as the absorption of secondary metabolites onto the disc and manual preparation of the disc which might introduce some errors. Since the crude aqueous extract was detected with many phytochemical constituents, perhaps these components were not able to penetrate completely onto the agar and leading to no inhibition. However, the broth microdilution method showed inhibition due to its nature as a quantitative measurement (Alizade et al. 2016).

Table 3 and Figure 4 show the diameter zone of inhibition observed in MRSA and *E. coli*, in which these bacteria displayed the highest zone of 15.0 mm and 14.0 mm, respectively at 100% (v/v). Treatment of streptomycin sulfate (25 µg/mL) to MRSA showed the largest inhibition zone of 26.0 mm indicating susceptibility. Reducing trends were observed at various concentrations of MRSA and *E. coli* indicating the death of the cells upon treatment with oil (Figure 5).

Table 3 Zone of inhibition of the respective samples and streptomycin sulfate against test microorganisms.

Sample	Diameter zone of inhibition (mm)				
	B.cereus	*E.faecalis*	*K.pneumonia*	*E.coli*	MRSA
Crude aqueous extract (mg/mL)					
12.5	-	-	-	-	-
25.0	-	-	-	-	-
50.0	-	-	-	-	-
100.0	-	-	-	-	-
Massage oil (%, v/v)					
12.5	-	-	-	-	-
25.0	-	-	-	7.0	7.0
50.0	10.0	11.0	7.0	11.0	10.0
100.0	12.0	14.0	9.0	14.0	15.0
Streptomycin sulfate (25 µg/mL)	19.0	14.0	19.0	15.0	26.0

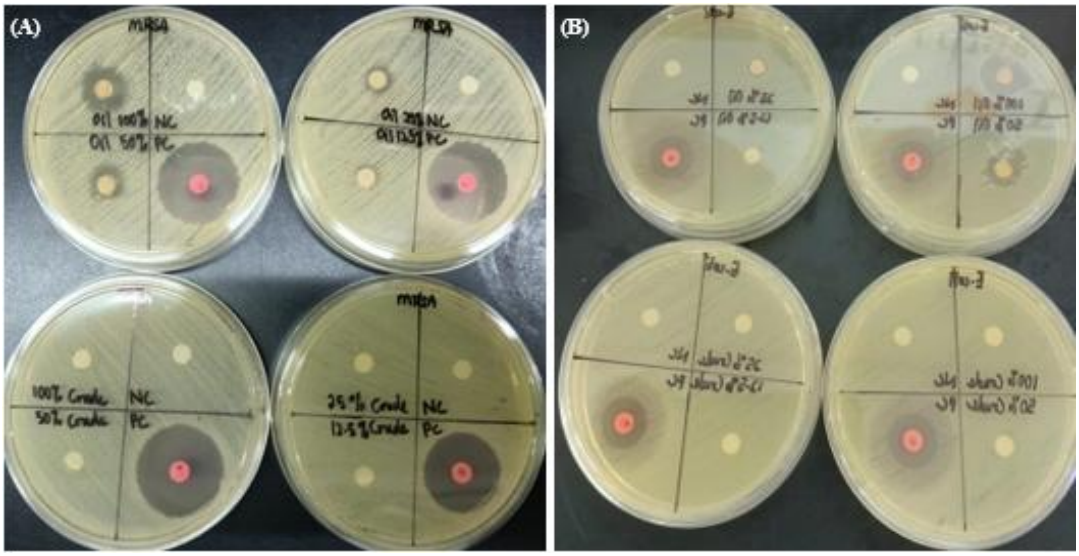

Figure 4 Diameter zone of inhibition observed using both crude aqueous extract and massage oil against (A) MRSA, (B) *E. coli* (NC = negative control, PC = positive control).

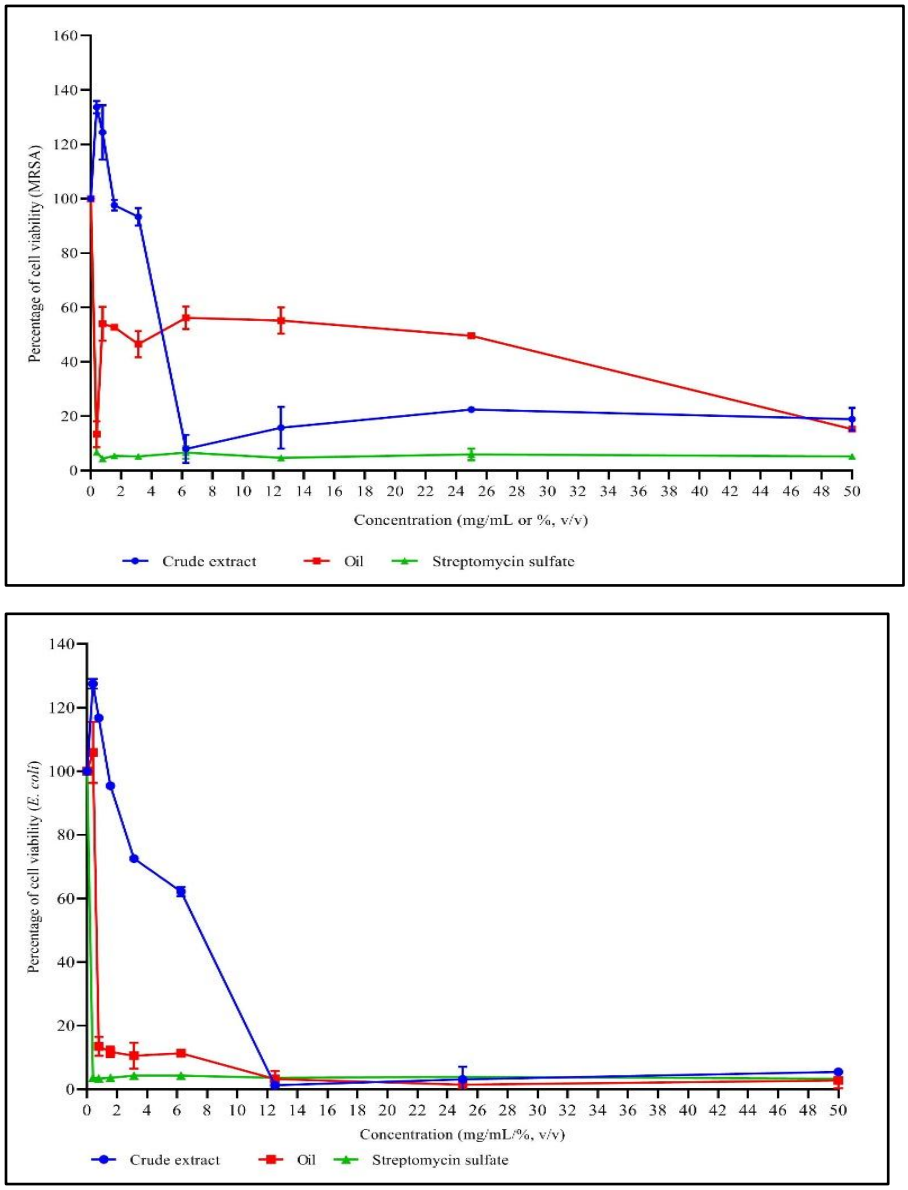

Figure 5 Percentage cell viability of MRSA and *E. coli* upon treatment with various concentrations of samples for 18 to 24 hours

Table 4 Minimum inhibition concentration values of the samples and streptomycin sulfate against test microorganisms.

Bacteria	Minimum inhibition concentration (MIC)		
	Crude aqueous extract (mg/mL)	Massage oil (%, v/v)	Streptomycin sulfate (mg/mL)
Gram-positive			
B. cereus	12.5	0.39	0.39
E. faecalis	50.0	1.56	0.39
MRSA	12.5	0.39	0.39
Gram-negative			
K. pneumonia	12.5	0.78	0.39
E. coli	12.5	0.78	0.39

Table 5 Minimum bactericidal concentration values of samples and streptomycin sulfate against test microorganisms.

Bacteria	Minimum bactericidal concentration (MBC)		
	Crude aqueous extract (mg/mL)	Massage oil (%, v/v)	Streptomycin sulfate (mg/mL)
Gram-positive			
B. cereus	12.5	0.39	0.39
E. faecalis	> 50.0	3.13	0.39
MRSA	12.5	0.39	0.39
Gram-negative			
K. pneumonia	25.0	0.78	0.39
E. coli	25.0	0.78	0.39

*MBC value > 50.0 mg/mL indicates possible activity using higher concentration of samples.

In MIC and MBC assays, both the crude aqueous extract and oil showed inhibition upon all the bacteria. However, once again oil displayed MIC and MBC values. MIC and MBC values of 0.39% (v/v) was observed on *B. cereus* and MRSA, respectively, and compared with streptomycin sulfate. Both Gram-positive bacteria were killed efficiently upon treatment with the oil. The MIC and MBC values are shown in Tables 4 and 5. *K. pneumonia* and *E. coli* showed bacteriostatic and bactericidal at a concentration of 0.78% respectively upon treatment with the massage oil. However, *E. faecalis* showed MIC of 50 mg/mL and 1.56% upon treatment with the crude aqueous extract and oil, respectively and this was the highest inhibitory concentration needed to inhibit their growth.

The possible explanation for the antibacterial activity of the oil could be due to the interaction between the secondary metabolites and bacterial cells that may lead to inhibition of enzyme activity, disruption of cell wall synthesis, plasma membrane interference, prevention of protein synthesis, and inhibition of DNA synthesis (Nicholas and Graham 2019). Gram-negative organisms tend to be less susceptible compared to Gram-positive due to the presence of an outer membrane and hydrophilic periplasmic space. This feature will possibly influence the penetration and the fate of compounds or secondary metabolites. In addition, secondary metabolites with broad spectrum activities and polarities can penetrate in both Gram types, probably passing through the biological membrane space by hydrophilic groups. Thus, the oil can kill the bacteria due to the penetration of the secondary metabolites into the biological membrane and affects the cellular processes of the target cells synergistically as described by Nicholas and Graham (2019).

Conclusion

The present study showed that massage oil can exhibit significant antioxidant and antibacterial activities as compared to crude aqueous extract. Thus, a mixture of oils found in the bubble belly massage are potential antibacterial and antioxidant agents and further investigation on other biological activities such as anticancer, antifungal, animal study, mechanistic study on the chemical modification of the functional groups, structure-activity relationship, and identification of putative molecular target will be useful in the development of the compounds as potential drugs.

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Conflicts of Interest

The authors declare no conflict of interest.

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