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IN-VITRO EVALUATION OF THE ANTIBACTERIAL ACTIVITY OF RED BEET PEELS AND DATE PITS ON SELECTED BACTERIAL STRAINS

SALMA EMAD AHMED

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BY
SALMA EMAD AHMED

A project submitted as a partial fulfilment of the requirement for the degree of Bachelor of Science (Nutrition and Community Health) from the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia

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APPROVAL

This project entitled "*In-vitro* evaluation of the antibacterial activity of red beet peels and date pits on selected bacterial strains" was prepared by Salma Emad Ahmed (195062) and submitted to the Faculty of Medicine and Health Sciences as a partial fulfilment of the requirement for the degree of Bachelor of Science (Nutrition and Community Health) from the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia.

Received and examined by:

(DR. SITI RAIHANAH BINTI SHAFIE)

Faculty of Medicine and Health Sciences

Universiti Putra Malaysia

(Supervisor)

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DECLARATION

I hereby declare that this thesis is based on my original work except for quotations and citations, duly acknowledged.

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SALMA EMAD AHMED

Matric Number: 195062

Bachelor of Science in Nutrition and Community Health

Department of Nutrition

Faculty of Medicine and Health Sciences

Universiti Putra Malaysia

(Student)

Date: 17/9/2021

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LIST OF ABBREVIATIONS

WHO	World Health Organization
FAO	Food and Agricultura Organization
MRSA	Methicillin-resistant S. *aureus*
GM	Gentamicin
RBP	Red beet peels
DP	Date pits
DMSO	Dimethyl Sulfoxide
CLSI	Clinical and Laboratory Standards Institute

ABSTRACT

Microbial contamination poses a global challenge in the food industry. Thus, chemical preservatives are added to extend the shelf life. However, chemical preservatives were found to possess side effects on health when added with exceeded doses or when the containing food is consumed excessively. Red beet peels (RBP) and date pits (DP) are natural by-products rich with quality and quantity bioactive compounds. In this study they were evaluated for their antibacterial activity. By using the sonication bath, different solvent extractions were applied. RBP was extracted with 50% aqueous ethanol + 0.5% acetic acid, and 50% aqueous methanol + 0.1% formic acid. Whereas DP was extracted by 50% aqueous ethanol, methanol, and ethanol. The resultant extracts were administered separately and as an antibiotic synergize (gentamicin) on *Salmonella typhimorium, Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* using the agar disc diffusion method. There was a significant difference ($p<0.05$) among certain solvent extractions for both plant samples; however, it was inconsistent with all strains. Generally, levels of susceptibility of the plant extract ranged from weak to moderate due to several possible factors. RBP is suggested to have synergistic activity when combined with the aminoglycoside class of antibiotics compared to the application of the antibiotic only. Aminoglycosides function by inhibiting protein synthesis. Whereas RBP phytochemicals role is suggested to increase cell wall permeability, thus reducing higher antibiotic concentrations. DP is suggested for having antagonistic activity with *Escherichia coli, Salmonella tyhpimurium,* and *Pseudomonas aeruginosa*. Antagonism could occur when bacteriostatic drugs reach to the infection site before bactericidal drugs. Another possible factor is the interaction of extracts and antibiotic chemical compounds. In general, more comprehensive methodological approaches are needed in future work to drive an in-depth understanding of plants–antibiotics and plants-microbial mechanisms of action.

Abstrak

Pencemaran mikrob menimbulkan cabaran global dalam industri makanan. Oleh itu, bahan pengawet kimia ditambahkan untuk memanjangkan jangka hayat makanan. Namun, pengawet kimia telah didapati mengandungi kesan sampingan terhadap kesihatan sekiranya kelebihan dos ataupun kelebihan pengambilan makanan yang mengandunginya. Kulit ubi bit merah (RBP) dan biji kurma (DP) merupakan produk sampingan semula jadi yang kaya dengan sebatian bioaktif yang berkualiti dan berkuantiti. Dalam kajian ini, aktiviti antibakteria sampel telah dinilai. Pengekstrakan pelarut yang berbeza telah digunakan dalam proses sonikasi. RBP telah diekstrak dengan 50% etanol akues + 0.5% asid asetik dan 50% metanol akues + 0.1% asid formik manakala DP telah diekstrak dengan 50% etanol, metanol, dan etanol akues. Ekstrak yang dihasilkan telah diberikan kepada *Salmonella typhimorium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* dan gentamicin sebagai sinergi antibiotik dengan menggunakan kaedah penyebaran agar secara berasingan. Terdapat perbezaan yang signifikan ($p < 0.05$) antara pengekstrakan pelarut tertentu untuk kedua-dua sampel; namun, ia tidak konsisten antara kesemua strain. Secara amnya, kerentanan ekstrak sampel bertahap dari lemah hingga sederhana berkemungkinan disebabkan beberapa faktor. RBP disarankan mempunyai aktiviti sinergi apabila digabungkan dengan antibiotik kelas aminoglikosida berbanding dengan penggunaan antibiotik saja. Aminoglikosida berfungsi sebagai penghalang sintesis protein. Fitokimia RBP berperanan untuk meningkatkan kebolehtelapan dinding sel, oleh itu, ia dapat mengurangkan kepekatan antibiotik secara lebih tinggi. DP dapat melakukan aktiviti antagonis dengan *Escherichia coli*, *Salmonella tyhpimurium*, dan *Pseudomonas aeruginosa*. Antagonisme boleh berlaku sebelum ubat bakteriostatik sampai ke tempat jangkitan. Faktor lain yang mungkin menyebabkan ketidakkonsistenan keputusan ialah interaksi antara ekstrak dan sebatian kimia antibiotik.. Secara umum, pendekatan metodologi yang lebih komprehensif diperlukan dimasa hadapan bagi memahami dengan lebih mendalam mengenai mekanisma tindakan tumbuhan-antibiotik dan tindakan mikrob-tumbuhan.

CHAPTER 1

INTRODUCTION

1.1 Background

With the developed awareness of consumers towards healthy dietary options, the food industry anticipates natural preservatives as an alternative to synthetic ones (Gyawali & Ibrahim, 2014). Preservation is a vital step during manufacturing to eliminate microbial and other hazardous contamination (Silva et al., 2018). The preservative agents mainly function within two aspects of food preservation: inhibiting oxidative damage and restricting microbial growth. Without control over those factors, enzymatic browning, nutrient loss, odd flavors, and food poisoning will occur (Han et al., 2018). Food poisoning is the fifth cause of hospitalization in Malaysia (Ministry of Health Malaysia, 2018). While worldwide, it is estimated that around 600 million people get food poisoning yearly, with 30% from children (World Health Organization, 2020). As a result, 110 billion US dollars is expended globally on medical and productivity outcomes of food poisoning and infections. Bacteria can contaminate numerous food types, including infant formula, infant rice cereal, cooked rice, dried milk products, dehydrated potato products, eggs, meat, spices, pasta, and noodles (Kumariya et al., 2019).

Moreover, bacterial developed resistance towards antimicrobial agents is a topic of concern. Methicillin-resistant staphylococcus aureus (MRSA) was first observed in 1961, 19 years after penicillin mass production (Clemente et al., 2015). Hence, researchers

actively work to find alternative substitutes which can aid in reversing antibiotic resistance (Khameneh et al., 2019). The plants are a rich source of natural compounds that has the potential to inhibit bacterial growth. These compounds are known as phytochemicals. It interrupts bacterial virulence either by disturbing the resisting mechanisms of bacteria's vital structure and function (Franco et al., 2019). Plant active compounds can solely work as antibacterial agents and synergize with traditional antibiotics (Ayaz et al., 2019). Red beets were found to be active against different bacterial strains (Kumar & Brooks, 2018). It is suggested that betalains are the underlying antimicrobial compound, yet to be proven by research (Kumar & Brooks, 2018). RBP was found to have the highest content of betalains compounds (Sawicki et al., 2016). Also, it has a considerable quantity of phenolic and flavonoid compounds. However, peels are considered a plant by-product not fully utilized (Vodnar et al., 2017). Similarly, DP contains essential oils, carotenoids, and flavonoids; thus, it is considered a functional food. It was active against multiple bacterial strains such as *Escherichia coli* and *Pseudomonas aeruginosa* (Maqsood et al., 2020).

1.2 Problem Statement

Microbial contamination poses a global challenge in the food industry (Silva et al., 2018). It is estimated that nearly 1 in 10 people worldwide fall sick due to food poisoning (WHO, 2020). Food contamination results from bacterial growth in food. Thus, chemical preservatives are added to extend the shelf life (Franco et al., 2019). However, chemical preservatives were found to possess side effects on health when added with exceeded doses or when the containing food is consumed excessively (Franco et al., 2019). For instance, excessive consumption of sulfites can cause thiamine degradation, hypersensitivity, and respiratory problems (Franco et al., 2019).

Furthermore, microbial resistance to antibacterial agents is a current severe challenge that hinders nutraceutical and food industries (Khameneh et al., 2019). Due to bacterial resistance evolution, the mechanism of current antibacterial drugs' action became weak or even ineffective (Baym et al., 2016; Morrison & Zembower, 2014). Therefore, the current strategies target on exploring new agents with antibacterial potency (Khameneh et al., 2019). Besides, the agricultural industry produces annually myriad tons of fruit and vegetable by-products (Gowe, 2015). This contributes to rising methane emissions. It is estimated that it accounts for 30% of waste for one fruit or vegetable and, if processed, can reach up to 75% of waste (Gowe, 2015). Thus, it increases the environmental load and opposes the Sustainable Development Goals target (goal 13: climate action). Moreover, multiple studies that investigated the antibacterial potency of RBP and DP on gram-negative and gram-positive bacteria were done. However, they showed mixed findings due to differences in extraction methods and the administered dosages (Table 2.1 & Table 2.2).

1.3 Significance of the Study

Pits, peels, and other plant waste parts are usually used in animal feed or wasted. Several studies discovered the nutritional value of plant wastes, and some revealed its richness with bioactive compounds that may even outweigh the plant flesh in certain species. RBP was found higher in betalains and other polyphenols than in the flesh (Sawicki et al., 2016). Furthermore, DP was found to be rich in essential oils and fat-soluble bioactive compounds. The potential incorporation of these compounds in food preservation is suggested as a promising strategy due to their antibacterial activity. However, due to the application of different extraction methods, the studies examining

the antimicrobial effect of RBP and DP had mixed findings (Table 2.1 and Table 2.2). Therefore, this study aims to address the antibacterial activity of each of these plant by-products. Also, it seeks to administer different solvent extractions to confirm previous studies' findings. Moreover, due to the bacterial resistance challenge to the commonly used antibiotics and the potential adverse side effects of certain preservatives, there is a demand for alternative antimicrobial compounds that aid the pharmaceutical and food industries in overcoming these challenges. This strategy can help to minimize antibiotics toxicity, as lower concentrations are applied when accompanied by plant active compounds. It can increase the sensitivity to multi-drug resistance bacteria. Therefore, this study will evaluate the synergistic activity of RBP with GM and DP with GM. In general, this study is suggesting a cost-effective food preservative and antibiotic enhancer from plant wastes. This objective works in favor of environmental sustainability.

1.4 Objectives

1.4.1 General objective

To evaluate the antibacterial activity of RBP and DP extracts against selected bacterial strains.

1.4.2 Specific objectives

1. To determine and compare the antibacterial activity of different solvent extractions of red beet peels (RBP) on *Salmonella typhi, Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus* bacteria strains.
2. To determine and compare the antibacterial activity of different solvent extraction of date pit (DP) on *Salmonella typhi, Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus* bacteria strains.
3. To determine and compare the 1:1 synergistic plant-antibiotic activity of RBP extract incorporated with gentamicin (GM) effect against *Salmonella typhi, Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus* bacteria strains.
4. To determine and compare the 1:1 synergistic plant-antibiotic effect of DP extract incorporated with GM effect against *Salmonella typhi, Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus* bacteria strains.

1.5 Hypothesis

1. There is an antibacterial activity of RBP extracts against *Salmonella typhi, Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus* bacteria strains.
2. There is an antibacterial activity of DP extracts against *Salmonella typhi, Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus* bacteria strains.
3. There is a plant-antibiotic synergistic effect when RBP extracts are incorporated with GM against *Salmonella typhi, Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus* bacteria strains.
4. There is a plant-antibiotic synergistic effect when DP extracts are incorporated with GM against *Salmonella typhi, Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus* bacteria strains.

CHAPTER 2

LITERATURE REVIEW

2.1 Red beets

Red beet is an occasionally annual crop known scientifically as *Beta vulgaris L.* used for its edible roots and leaves (Neelwarne, 2012). Different varieties of red beets have different colors that vary from yellow to red. In 2010, more than 45 million tons of vegetables were produced in European countries, including red beet as one of the major cultivated vegetable crops (European Commission, 2010). Consumers use red beet dominantly in salads, pickles, juices, and cook (Singh & Hathan, 2014). It provides a natural colorant used in pharmaceutical, cosmetics, and food industries, giving this plant a commercial significance (Neelwarne, 2012; Rasheed et al., 2020). The compounds behind this strong, nontoxic pigment are known as betalains (Kumar & Brooks, 2018). Betalains are nitrogenous and water-soluble compounds (Kumar & Brooks, 2018). These compounds are approved by the Food and Drug Administration (FDA) and European Union (EU) to be used as food colorants. Many studies have recently examined bioactive beetroot compounds potential in inhibiting inflammation, microbial growth, tumor development, and cytotoxicity risks (Clifford et al., 2015; El Gamal et al., 2014). Some studies investigated the antimicrobial capacity of RBP and pomace (all red beet wastes) (Table 1). However, inconsistent findings were obtained due to differences in varieties, cultivation regions, food storage and processing, extraction methods, and dosage concentrations. For instance, *Escherichia coli*, the commonly studied species among those antimicrobial studies, showed a range of 8mm to 26mm of inhibition zone. These microorganisms are suggested to be

suppressed due to betalains and polyphenol compounds in red beets (Vulić et al., 2013). Polyphenols can act as an antimicrobial compound and retard microbial growth, thus slowing down the food spoilage process (Gyawali & Ibrahim, 2014). However, the responsible compounds for microbial retardation are not yet identified in research (Kumar & Brooks, 2018). Besides, antioxidants are highly present in this fruit. They work as scavengers for free radicals that cause nutrient degradation and enzymatic browning in food; thus, due to these properties, it is considered a functional food; therefore, these plant extractions can be incorporated in innovative active food packaging (Kumar & Brooks, 2018).

Red beets have significant amounts of bio-active compounds such as B-vitamins, betalains, carotenoids, phenols, sugars, and fibers (Kale RG, Sawate AR, Kshirsagar RB, 2018). Betalains were found to be at the highest level in beetroots compared to other plants (Fincan et al., 2004). It ranges from 546 to 1124.5 μg/g in different varieties of beetroot fruit. Betalains are the main bioactive compound present in red beets. It is composites of betacyanins, the red-violet pigment, and betaxanthins, the yellow-orange pigment (Kumar & Brooks, 2018). Betacyanins are the main compounds representing betalains in red beets, while betaxanthins were detected in trace amounts (Sawicki et al., 2016). Red beet fruit morphology is consists of layers/rings that differ in their bioactive compound values, such as betalains. In a study divided the red beet parts into seven rings, including the peels, it was found that 26.63%, 18.48%, 19.02%, 13.59%, 10.87%, 7.07%, 4.35% of betalains present in peels, ring 1, ring 2, ring 3, ring4, ring 5, ring 6 (the most inner ring/layer), respectively (Sawicki et al., 2016; Slatnar et al., 2015). Interestingly, the same study detected a significant correlation between betalains and antioxidant capacity (AC) in

different parts of red beets, as AC was at the highest quantity in the peels. This is due to the aging of the outer layer and frequent sunlight exposure (Sawicki et al., 2016). Betalains are stable compounds and pH ranging from 3 to 7 (Sawicki et al., 2016). Further, total phenolic contents (TPC) in red beet varieties were ranged from 1326 – 2572 µg of GAEs/ g (Isabelle et al., 2010; Moussa-Ayoub et al., 2011; Vulić et al., 2013). Moreover, the TPC varied in different tissue parts of red beets. A total of 15.5 mg/g GAE was found in peels, which is considered the highest compared to 11.4 and 4.2 mg/g GAE for the crown and flesh, respectively (Kujala et al., 2000). Besides, a study compared the TPC of fresh and thermally processed (80 ºC) red beet pomace found that the fresh sample had higher TFC (14.1 mg/100g GAE) as compared to the thermally processed sample (5.5 mg/100g GAE) (Vodnar et al., 2017). However, from the same study, betacyanins were slightly higher in the thermally processed sample (3.952mg/100g DW) than the fresh sample (3.866 mg/100g DW). Therefore, this study suggested a positive correlation between betacyanin content and antioxidants activity as it was found that antioxidant activity is higher in thermally processed red beet pomace (46%) as compared to fresh red beet pomace (40%) (Vodnar et al., 2017). Furthermore, the total flavonoid content (TFC) ranged from 0.34 to 260 mg rutin equivalents per 100g of red beet fruit (Guldiken et al., 2016; Tumbas et al., 2016). A total of 2.3 mg QE/ 100 g DW and 6 mg QE/ 100g DW of TFC was found in fresh and thermally processed (80ºC) pomace samples (Vodnar et al., 2017). In raw red beet, vitamin C content ranged from 9.5g – 66g per 100g, represented as ascorbic acid (Paciulli et al., 2016; Shyamala & Jamuna, 2010). In another study investigating the nutritional values in different formulations of beetroots (Cooked, juice, powder, chips), 0.51 mg/g of TPC was found in beet's

powder (BP), the lowest content as compared to other formulations. The total antioxidant potential was 95.31% in BP which is higher than the juice and the cooked beets. For the sugar analysis, the sugar content was higher in juice > chips > powder > cooked beets. A total of 444.05 mg/g was found in the powder beets, and it was mainly sucrose. Lastly, the organic acid was significantly higher in powder > chips > juice and cooked beet. 47.93 mg/g was obtained from the powder formulation (Vasconcellos et al., 2016). In addition, through innovative extracting techniques, the dietary fibre, protein and minerals were analysed in RBP as their quantity was 33.6%, 18.3% and 41.86 mg/g respectively (Šeremet et al., 2020).

In general, the literature investigating the phytochemical compounds in red beets is well established. Besides, several studies investigated the antimicrobial capacity of red beet. However, there is scarce information that leads to which phenolic compounds are responsible for bacterial suspension activity. Moreover, although RBP is more likely to be the most affluent part of the plant with phytochemicals, few studies had examined its antimicrobial potential with mixed findings and limited bacterial strain coverage. Also, the peels were not studied solely in most of the studies. However, it was incorporated as part of the whole plant wastes.

2.2 Date fruits

The date is the fruit of date palm scientifically known as *Phoenix dactylifera L.* that generally grows in arid and semi-arid lands such as the Middle Eastern countries (Maqsood et al., 2020). In 2018, it was estimated that more than 8.5 million tonnes of date crops were produced (FAO, 2018). By considering the fruit's variation, the pits account for approximately 10% to 15% of the whole fruit weight (Ghnimi et al., 2017). DP is considered a primary by-product that is either wasted or used as animal

feed (Amany et al., 2012; Baliga et al., 2011). However, different studies had examined its potential nutritional values, which can be utilized in the nutraceutical and food industries (Sirisena et al., 2015). The date fruit undergoes five stages that identify its ripeness degree. Along ripening stages, the nutritional and bioactive quality and quantity will vary for the same fruit variety (Amira et al., 2012). These stages are Hababouk, Kimri, Khalal, Rutab, and Tamar (Sirisena et al., 2015). In most of the varieties analyzed, total phenolic content and total flavonoid content were at their highest presence at the Rutab stage (Amira et al., 2012). In addition, multiple studies analyzed the phytochemicals capacity in DP. For instance, in a study investigating 18 types of seeds obtained from different date varieties cultivated in the United Arab Emirates, carotenoid content ranged from 1.46 to 3.53 mg/ 100 g of seeds oil (Habib & Ibrahim, 2009). However, another study analyzed fully ripened date seeds obtained from Tunisian cultivation, 5.51 mg of carotenoids was obtained per 100 g of the seed oil. Also, significant values of tocopherols and tocotrienols were found in Tunisian date seed cultivation were 34.01 mg/100g, 10.30 mg/100g, 4.63 mg/100g for α-tocotrienol, ϒ-tocopherol and ϒ-tocotrienol respectively (Nehdi et al., 2010).

Phytosterols were found in date fruit and particularly in the pits (Maqsood et al., 2020). In addition, date seeds were found to be the highest in polyphenols if compared to date fruit, nut seed, grapes, flaxseed (Liang et al., 2012; Pérez-Jiménez et al., 2010; Vayalil, 2012). Flavonoids were representing nearly 99% of polyphenols in date seeds. The flavonoid compound was identified, and their values were 46.8 g/kg for epicatechin and 3.38 619 g/kg for catechin (Habib et al., 2014). In another study, a range of 1224 to 1844 mg/100 g of flavonoids was obtained from Moroccan

date seed varieties (Bouhlali et al., 2017). A total of 5342 mg GAE/100 g and 1844 mg/100g GAE for total phenolic content and total flavonoid content were reported from the Bousthammi date seed variety (Bouhlali et al., 2017). The high abundance of polyphenols in date pits is possibly the reason behind the high antioxidant potential. A study conducted on seven Algerian date cultivation founds a significant correlation between total phenolic content and antioxidant activity (Messaoudi et al., 2013). In addition, potassium and phosphorus ranged from 204.3 to 300 mg/100g and 68.3 to124 mg/100g, respectively (Metoui et al., 2019). Notable amounts of minerals were detected in date seed, such as Cr, Al, Sr, Ni, Ba, Pb, As, Cd, and V (Habib & Ibrahim, 2009). The dietary fiber content identified in date seeds ranged from 67% to 80% (Al-Farsi & Lee, 2008; Habib & Ibrahim, 2009).

2.3 Bacteria properties

Bacteria are prokaryotic organisms characterized by being unicellular. They are present everywhere, on surfaces, soil, human microbiota, etc. Their structure comprises cell walls, plasma membrane, ribosomes, replication, transcription and translation enzymes, DNA, and some have an outer membrane (gram-negative). Bacteria are categorized into two major groups in terms of morphology, known as gram-positive and gram-negative bacteria. The latter has an extra outer layer membrane that enhances its resistance against antibacterial agents (Zhivich, 2017). *Enterobacteriaceae* is a bacterial family that incorporates several common species, for instance, *Escherichia coli.* It is an aerobic gram-negative species that grow optimally at 37ºC. Although E. *coli* usually confine the human's gut peacefully, when gastrointestinal walls are breached, even nonpathogenic E. *coli* can infect individuals (Page & Liles, 2013). Further, pathogenic strains such as E. *coli* 0157:H7 can cause

fatal infections to humans, particularly immunocompromised people (Page & Liles, 2013). Another gram-negative aerobic species known as *Pseudomonas aeruginosa* is mainly a nosocomial pathogen with a higher frequency in causing infections. According to the Centers of Disease Control and Prevention (CDC), 14% of pneumonia, 7% of urinary tract infections, 8% of surgical site infections, and 9% of overall conditions are caused by this species (Gales et al., 2001). This species contributes to several ecological concerns, including plants and animals, due to its relative resistance and limited nutritional requirements (Gales et al., 2001). Further, *Salmonella typhimorium* is another aerobic gram-negative pathogenic bacteria isolated commonly from the intestinal lumen, responsible for gastroenteritis incidences in humans and other mammals (Kapperud et al., 1990). An example of gram-positive bacteria is *Staphylococcus aureus* species isolated commonly from human skin flora. The pathogenicity manifests during injuries where open wounds enable infections that might reach the bone and respiratory tracts (Bagdonas et al., 2003).

2.4 Bacterial resistance

One of the biggest challenges facing food and drug industries is the bacterial resistance to traditional antibiotics (Khameneh et al., 2019). The antibiotics are either became less effective or not effective at all with specific bacterial species (Baym et al., 2016; Morrison & Zembower, 2014). Bacterial resistance can occur in two ways. First, certain bacterial species might have an inherent resistance towards certain antibacterial agent classes. Second, it can occur through an evolutionary mechanism along with many subsequent generations, as the species can be initially susceptible to the antibacterial agents. Along the time, it mutates genetically to become more

resistant (Khameneh et al., 2019). The bacterial resistance mechanisms can occur through structural modification of porins, destroying the antibacterial agents, modifying antibiotics, and altering the target and the efflux pump (Khameneh et al., 2019). An efflux pump is a system that exports the antimicrobial agent before adequate concentration can diffuse through the bacterial wall. Thus, the agent cannot perform its mechanism efficiently (Khameneh et al., 2019).

2.5 The antimicrobial potential and mechanisms of plant-derived compounds

Like other creatures, plants have defensive tools that continuously adapt to external damaging factors by producing their defending metabolites (Ncube et al., 2012). Indeed, not only do plants have this impressive potential, other creatures such as animals, algae, insects, and fungi can produce protective metabolites that suit the surrounding external threatening (Gyawali & Ibrahim, 2014). Those metabolites are mainly polyphenols, alkaloids, alcohol, aldehyde, acidic, and terpenoid-containing compounds (Kawacka et al., 2021; Khameneh et al., 2019). Among multiple compounds extracted from plants, phenolics demonstrated a wide variation in their chemical composition, which behaved with different bacterial inhibition mechanisms (Stojković et al., 2013). It is suggested that the hydroxyl group in phenolic compound disrupts the cell membrane of the bacteria, which causes cell leakage then death (Xue et al., 2013). It is also suggested that the antimicrobial role of these compounds is influenced by the hydroxyl position and the number of double bonds in the chemical structure (Gochev et al., 2010; Gyawali & Ibrahim, 2014). The hydroxyl group binds to the enzyme active sites and disturbs the bacterial cellular metabolism (Stojković et al., 2013). In addition, the presence of hydroxyl group in phenolic compounds considers them as an antioxidant as well. Therefore, it creates a lowered redox

medium that may inhibit bacterial growth (Gyawali & Ibrahim, 2014; Stojković et al., 2013).

The incorporation of plant bioactive compounds with antibiotics is a new strategy that helps in getting a more substantial effect. This concept is known as synergism. For the antimicrobial activity, the combination of those compounds enhances interaction with the bacteria's target site. In other words, having more antimicrobial compounds can have multiple antimicrobial mechanisms of action that enhance and amplify their antimicrobial effect and reduce the ability of bacterial resistance (Table 2.3). For example, gallic acid, ferulic acid, quercetin, myricetin, and curcumin were found to play different roles of action. They decrease NorA efflux pump, damage bacterial membrane, and reduce the minimum inhibitory concentrations (MICs) (RD et al., 2005; Saavedra et al., 2010; Sanhueza et al., 2017). Moreover, synergism allows for using a low dosage of each compound, which reduces the toxicity risk compared to the application of individual agents (Ayaz et al., 2019).

2.6 Antimicrobial properties of red beets and dates

Due to the considerable quality and quantity of phytochemicals discovered in many plants, these plants' application in preservation and shelf life extension is an emerging upcoming strategy (Gyawali & Ibrahim 2014). Indeed, plants are exposed to biotic and abiotic stressors. The bacterial infection is an abiotic stressor that plants have naturally developed their resisting mechanism to overcome. This mechanism is the synthesis of antibacterial metabolites (Samad et al., 2016).

Moreover, multiple studies examined the association between RBP and pomace and their antibacterial potential (Table 2.1). Different solvents extraction methods were applied, such as acidified aqueous methanol and 50% aqueous ethanol with 0.5%

acetic acid (Guldiken et al., 2016; Vulić et al., 2013). Findings showed a range zone of inhibition in gram-positive bacteria such as *Bacillus anthracis* (7-9mm), *Bacillus cereus* (20.3mm), *Bacillus subtilis* (10mm), *Listeria monocytogenes* (8mm), *Micrococcus luteus* (<2mm), *Staphylococcus aureus* (1-20mm), *Staphylococcus equorum* (20.3mm), *Streptococcus pyogenes* (7-11mm), *Staphylococcus saprophyticus* (20mm), and *Staphylococcus sciuri* (20mm) (Koochak et al., 2010; Velićanski et al., 2011; Vulić et al., 2013). Furthermore, gram-negative bacteria were less affected by red beets extracts than gram-positive due to their complex cell wall. *Escherichia coli* (1-13.3mm), *Citrobacter freundii* (20.3mm), *Citrobacter youngae* (10.6mm), *Enterobacter cloacae* (12mm), *Pseudomonas aeruginosa* (13.3mm), *Salmonella typhimurium* (25mm), *Klebsiellla pneumoniae* (7-8mm) and *Vibrio parahaemolyticus* (2-5mm) (Boo et al., 2012; Koochak et al., 2010; Rauha et al., 2000; Velićanski et al., 2011; Vulić et al., 2013).

Few studies had examined the potential role of DP as a natural antimicrobial agent (Table 2.2). DP exhibited antimicrobial effect against different gram-positive (G+) and gram-negative (G-) bacterial strains. From the gram-positive, those zones of inhibition were detected for each strain. *Staphylococcus aureus* (8-17.5 mm), *Bacillus cereus* (1.3–13.5mm), *Bacillus subtilis* (11.5-19mm), *Enterococcus faecalis* (5-18mm), *listeria monocytogenes* (12-20mm), *Staphylococcus saprophyticus* (8.5–13mm) and *Micrococcus luteus* (12-13mm). While for the gram negative bacteria, those are the strains that were addressed with their zone of inhibiton: *Escherichia coli* (1-18.2 mm), *Klebsiella pneumoniae* (12-16 mm), *Salmonella enterica* (9-20mm), *Serratia marcescens* (5.5mm), and *Pseudomonas aeruginosa* (8-13.5mm) (Javed et al., 2013; Kahkashan et al., 2012; Metoui et al., 2019; Sooad Al-daihan, 2012).

Further, the antimicrobial activity of date seed was analyzed in multiple studies. Different extraction methodologies were applied on different date varieties. Thus, the results obtained varied among the studies (Javed et al., 2013; Kahkashan et al., 2012; Metoui et al., 2019; Sooad Al-daihan, 2012). From the gram-negative strains, those zone of inhibition ranges obtained for each: *Escherichia coli* (5.3–22.9mm), *Pseudomonas aeruginosa* (9-23mm), *Shigella. flexeneri* (6.3-19.3mm), *Salmonella paratyphi* (14-17mm), *Salmonella typhymurium* (5.8-20mm), and *Klebsiella pneumoniae* (10-13mm). From the gram-positive strains, those zone of inhibition ranges were obtained for each: *Staphylococcus aureus (*6.2-25mm*), Enterococcus faecalis (*18-23mm*), Streptococcus pyogenes (*8-32mm*), Bacillus subtilis (*6.3-23.7mm*), Staphylococcus epidermis* (6.2-20.5mm).

2.7 Synthetic preservatives

Synthetic preservatives are used widely to maintain food quality and extend shelf life (Franco et al., 2019). Different agents are used in preservation. However, some of these preservatives have drawbacks, such as sulfites and nitrites. They are added to various food staffs to act as antimicrobe, antioxidant, control enzymatic and non-enzymatic browning activity, improve food appearance, and inhibit discoloration (Lamas et al., 2016). However, these agents are not undoubtedly safe for consumption; they increase mutagenicity and cytotoxicity and reduce foods' nutritional value (e.g. thiamine degradation)(Franco et al., 2019). Children's hyperactivity is linked with ingestion of dyes and synthetic preservatives (McCann et al., 2007; Payne et al., 2005). Moreover, Sodium nitrite can possess a carcinogenic effect in the presence of amines in the human body; it transforms into nitrosamine, which is the carcinogenic form (Mozaffarian & Wu, 2011).

2.8 Plant preparation and extraction methods

The extraction methods applied to the plants are vital to determining the quality and quantity of yield extract. The pre-extraction step includes the use of dry or fresh samples. A fresh than a dried sample (Nn, 2015). However, this characteristic varies from one plant to another. Powdering the plant to a size of 0.5mm or smaller is ideal for plant extraction. The plant particles are highly exposed to the extracting solvent (Methods Optimization in Accelerated Solvent Extraction, 2013). This size can be achieved by mills, conventional mortar, and blender (Nn, 2015). The drying method is another critical step before the pre-extraction step. Air drying takes up to months or even years, depending on the plant part and type. It helps to preserve chemical compounds that are sensitive to high temperatures. Microwave drying applies electromagnetic radiation. It is a fast method but might cause the degradation of certain phytochemicals. Oven drying is the application of heat to remove the moisture of the sample. It is commonly used as it is easy and fast. However, certain antioxidants might be degraded upon certain levels of heat exposure. Freeze-drying usually is used for heat-sensitive compounds as it preserves most od phytochemicals. However, this method is tedious and expensive (Nn, 2015). Extraction is the isolation of active compounds known as phytochemicals from plants to achieve better functional application. Different solvent and extraction devices are used. The extraction devices are mainly categorized under classical and advanced extraction methods (Table 2.4). These methods have advantages and disadvantages that outweigh each of them on another. The selection of a particular method relies on multiple factors, including the type of plants and the type of compounds to be extracted. Organic solvents or their aqueous formulations mainly extract phytochemicals. However, it is not clearly identified which solvent can give the

highest polyphenol extraction yields. For instance, lychee produced a higher extraction yield when extracted with acetone. On the other hand, walnut was better removed by water. Interestingly, recent studies found that aqueous-organic solvents can give better extraction outcomes than absolute organic solvents. Although the extraction solvents are not decidedly adhering to specific solvents, polyphenols are more soluble with polar compounds such as water, methanol, and ethanol (Aires, 2017). Indeed, the reason behind having variation for polyphenols extraction solvents is that polyphenols have diverse structures. For example, it can be conjugated with acids, alkyl groups, sugars, and multiple hydroxyl groups at different chemical positions; thus, it can interfere with the extraction process. Various techniques are used in polyphenols extraction, and researchers have categorized them as classical and advanced methods (Aires, 2017). The classical methods are maceration, percolation, and Soxhlet extraction, while the advanced extraction methods are microwave-assisted extraction, supercritical CO_2 extraction, enzyme-assisted extraction, ultrasound-assisted extraction, pressurized fluid extraction, and combined approaches of any of those techniques (Aires, 2017).

RBP extraction materials varied in different studies. Red beet pomace was dissolved in distilled water to a 100 mg /ml concentration, then 100µl of the extraction was added to the bacterial samples (Vulić et al., 2013). In addition, red beet flesh was freeze-dried, ground then dissolved in distilled water before the application of 30µl on the bacterial sample (Boo et al., 2012). In another study, 1 g of the red beet aerial part powder was dissolved in 10ml 80% ethanol and centrifuged for 15 min. Then the supernatant was harvested. Those concentrations were applied on the bacterial samples: 5%, 10%, 20%, and 40% (Koochak et al., 2010). Moreover, beetroot pomace extraction was applied with the addition of 50% ethanol and 0.5% acetic acid. Then, under reduced pressure, it was

left to dry. 15 µl, 50µl, and 100µl of the extract were applied to the samples (Velićanski et al., 2011). The most effective method among those studies was Velićanski et al. (2011) and Vulić et al. (2013) methods that showed higher inhibition zones when 100µl of either ethanol and acetic acid extraction or distilled water extraction was added. The polarity index of methanol and acetone is (5.1). It is reported that polyphenols are extracted better through these compounds (Samad et al., 2016).

DP extraction methods varied along with the literature. For instance, DP powder was soaked in 100 ml distilled water, acetone, and methanol separately, then filtrated, then oven-dried dissolved in 2 ml distilled water (Kahkashan et al., 2012). In another method, 10g of the date seed powder was added to 100 ml distilled water, then boiled for 2 hours on low flame heat. Later, this mixture was filtered and centrifuged at 5000g for 10 minutes. The formed supernatant was collected repeatedly every 2 hours to end up with a 25 ml concentrated solution. Lastly, it was autoclaved at 121°C and 15 Ibs pressure and kept in the fridge at 4°C. While for methanol and acetone, the same amount of pits powder was added to 100 ml of methanol and acetone for each of them. It was kept in a rotary shaker at 190 to 220 rpm for 24 hours. 25% of the original solvent volume was taken after supernatant collection and solvent evaporation for 2-3 days at room temperature (Sooad Al-daihan, 2012). Lastly, 50 g of the DP powder was extracted with 100 ml of methanol, and then extraction was done using the Soxhlet apparatus for 36 hours. Later, it was filtered, then vacuum evaporated at 40°C. This extract was freeze-dried (lyophilized) in the form of powder at -55 celsius in vacuumed conditions (Javed et al., 2013). Among mentioned methodologies, Javed et al. (2013) method showed the highest zone of inhibition when 10µl of the pits powder-methanol extraction was added.

Table 2.1: The antibacterial activity of RBP and pomace extracts against certain gram-positive and gram-negative bacteria

Group	Tested strains	Inhibition zone (mm)									
		(John et al., 2017)[2]						(Vulić et al., 2013)[1]	(Maqbool et al., 2020)[2]	(Canadanovic et al., 2011)[1]	(Narender et al., 2017)[2]
		Methanol			Acetone			50% Ethanol aqueous (100mg/ml)	4.38mg/ml		
		50µl	75 µl	100 µl	50µl	75 µl	100 µl	100 µl	100µl	15µl	
G-	*Escherichia coli*	15	16	18	11	14	17	13.33±0.58	14.7	8.0	26.0
	Pseudomonas aeruginosa		Not tested			Not tested		13.33±0.58	-	-	Not tested
	Citrobacter freundi		Not tested			Not tested		20.33±0.58	Not tested	Not tested	Not tested
	Enterobacter cloacae		Not tested			Not tested		12.0±0.0	Not tested	Not tested	Not tested
	Salmonella typhymurium		Not tested			Not tested		25.0±1.0	12.6	Not tested	Not tested
	Klebsiella pneumoniae	14	17	22	11	12	14	Not tested	Not tested	Not tested	Not tested
	Aeromonas hydrophila		Not tested			Not tested		Not tested	-	Not tested	Not tested
	Vibrio cholera		Not tested			Not tested		Not tested	-	Not tested	Not tested
	Shigella flexneri	-	-	12	-	-	11	Not tested	Not tested	Not tested	Not tested

Table 2.1: The antibacterial activity of RBP and pomace extracts against certain gram-positive and gram-negative bacteria (continued)

	Staphylococcus aureus	-	10	12	-	-	-	20.0±1.0	13.2	8.0	25.0
	Bacillus cereus	Not tested			Not tested			20.33±0.58	Not tested	10.3	Not tested
	Bacillus spp.	Not tested			Not tested			-	Not tested	Not tested	Not tested
	Enterococcus faecalis	Not tested			Not tested			-	Not tested	Not tested	Not tested
	Listeria monocytogenes	Not tested			Not tested			-	Not tested	Not tested	Not tested
G+	*Streptococcus pyogenes*	Not tested			Not tested			Not tested	Not tested	Not tested	Not tested
	Micrococcus luteus	Not tested			Not tested			Not tested	Not tested	Not tested	Not tested
	Staphylococcus saprophyticus	Not tested			Not tested			20.0±0.0	Not tested	Not tested	Not tested
	Staphylococcus equorum	Not tested			Not tested			20.33±0.58	Not tested	Not tested	Not tested
	Staphylococcus sciuri	Not tested			Not tested			12.33±0.58	Not tested	Not tested	Not tested
	Lacto bacillus	Not tested			Not tested			Not tested	Not tested	Not tested	26
	Proteus vulgaris	Not tested			Not tested			Not tested	Not tested	Not tested	18

1: is the study conducted on red beet pomace
2: is the study conducted on RBP

Table 2.2: The antibacterial activity of DP extracts against certain gram-positive and gram-negative bacteria

Group	Tested strains	Inhibition zone (mm)												
		(Sooad Al-daihan, 2012) (30 µg/disc)			(Javed et al., 2013) (500µg/disc)				(Kahkashan Perveen, 2012) (50µl/disc)			(Metoui et al., 2019) (7mg/disc)		
		Aqueous	Methanol	Acetone	Methanol				Aqueous	Methanol	Acetone	Methanol	Acetone	Aqueous
		-	-	-	1µl	2.5 µl	5 µl	10 µl	-	-	-	-	-	-
G-	*Escherichia coli*	9.0	11.5	11.0	9.0	11.0	16.0	18.0	5.3	21.0	18.7	22.9	18.9	5.7
	Pseudomonas aeruginosa	9.0	11.5	11.0	19.0	21.0	23.0	23.0	-	19.7	19.0	Not tested		
	Shigella. flexeneri	Not tested			Not tested				6.3	19.3	18.3	Not tested		
	Salmonella paratyphi	Not tested			14.0	16.0	16.0	17.0	Not tested			Not tested		
	Salmonella typhymurium	Not tested			12.0	15.0	18.0	20.0	Not tested			18.3	16.5	5.8
	Klebsiella pneumoniae	Not tested			10.0	13.0	13.0	13.0	Not tested			Not tested		

Table 2.2: The antibacterial activity of DP extracts against certain gram-positive and gram-negative bacteria (continued)

G$^+$	*Staphylococcus aureus*	12.5±0.55	16.0±0.0	20.0±1.0	8	11	13	15	Not tested	20.0±1.0
	Staphylococcus epidermidis	-	-	13.33±0.58	12	15	23	20	Not tested	Not tested
	Staphylococcus cohni spp. cohni	-	-	-	Not tested				Not tested	Not tested
	Bacillus cereus	10.67±1.03	17.0±1.0	20.33±0.58	-	-	-	8	Not tested	20.33±0.58
	Bacillus spp.	-	-	-	Not tested				Not tested	-
	Enterococcus faecalis	-	-	-	Not tested				Not tested	-
	Listeria monocytogenes	-	-	-	-	-	-	8	Not tested	-
	Streptococcus pyogenes	Not tested			-	7	8	11	Not tested	Not tested
	Bacillus anthracis	Not tested			-	7	9	9	Not tested	Not tested
	Bacillus subtilis	Not tested			Not tested				>10	Not tested
	Micrococcus luteus	Not tested			Not tested				<2	Not tested
	Staphylococcus saprophyticus	Not tested			Not tested				Not tested	20.0±0.0
	Staphylococcus equorum	Not tested			Not tested				Not tested	20.33±0.58
	Staphylococcus sciuri	Not tested			Not tested				Not tested	12.33±0.58

Table 2.3: The antibiotic-phytochemical synergism activity

Plant extracts/Phytochemicals	Combination with Compound/antibiotic	Pathogens	Mechanism of action	Ref.
Gallic acid, ferulic acid, chlorogenic acid, allylisothiocyanate, and 2-phenyl-ethyl isothiocyanate	Streptomycin	Gram-negative bacteria	-	(Saavedra et al., 2010)
Quercetin, gallic acid, protocatechuic acid and luteolin)	β-Lactam, Quinolone, Fluoroquinolone, Tetracycline, Amphenicol, Amphenicol	S.aureus, E. coli Mycobacterium	-	(Sanhueza et al., 2017)
Myricetin	Cefoxitin, Amoxicillin-clavulanate, Ampicillin-sulbactam	ESBL expressing K. pneumoniae and MSSA	Decrease NorA efflux pump	(RD et al., 2005)
Curcumin	Norfloxacin, Oxacillin, Cipofloxacin, Ampicillin, Ceftriaxone, Cefepime, Gentamicin, Amikacin, Imipenim, Meropenem, Fusidic acid, Penicillin, Erythromycin, Clindamycin, Tetracycline, Vancomycin	MRSA Biofilm forming Gram positive and Gram-negative strains	Damage bacterial membrane and decrease MICs.	(Kali et al., 2016)

Table 2.4: Plant extraction methods.

Extraction method	Description	Advantages	Disadvantages
Classical Extraction Methods			
Maceration (Olejar et al., 2015)	In-room temperature, the powdered sample is dissolved with the solvent in a closed container and frequently agitated. Then, to obtain the solid extraction, filtration can be applied.	• Feasible	• Time-consuming, and require a large amount of solvent
Percolation (Vongsak et al., 2013)	The solvent and the powder sample are dissolved in a closed container (cylindrical shape). In dropwise movement from top to bottom, the solvent is discharged.	• Feasible	• Time-consuming, require large amount of solvent,
Soxhlet extraction (Azmir et al., 2013)	The sample is placed in the extraction chamber. Below is the collecting flask that collects the solvent with the extraction. On the top is the reflux condenser. This system will be heated, which causes solvent condensation. This reflux occurs repeatedly, and the liquid will be collected in the collecting flask.	• Less time and solvent consumption	• It needs to be handled carefully as the raised temperature can affect the thermolabile polyphenols

Table 2.4: Plant extraction methods (continued).

Advanced Extraction Methods			
Microwave assisted extraction (MAE) (Pinela et al., 2016)	The energy of microwave radiation heats the solvent. The hydrogen bonds disrupt which induces diffusion of solvent into sample matrix.	• Less time and solvent consumption • Higher yields of polyphenols	• Useful in monomeric (short chains) polyphenols extraction such as phenolic acids, quacertin and iso-flavine.
Supercritical CO_2 extraction (SC-CO_2) (De Zordi et al., 2014).	It is a substance that combines gas and liquid characteristics at its critical point of pressure and temperature. It works by adjusting its critical point.	• Good solvent for non-polar analytes. • CO_2 is available with low cost and has low toxicity • High yield extraction	• The initial cost of the equipment is very high
Ultrasound assisted extraction (UAE) (Makris, 2016).	It involves the use of ultrasound with a range of 20 to 2000 kHz. The acoustic cavitation mechanic effect from the ultrasound increases the surface contact between solvents and samples and the permeability of cell walls. Thus, it aids in mass transport into cell walls and the release of active compounds	• Less time and solvent consumption	• The use of ultrasound energy more than 20kHz may affect phytochemicals and induce free radical formation
Pressurized Liquid Extraction (PLE)/ Accelerated solvent extraction (ASE) (Azmir et al., 2013).	High pressure (3.3-20 MPa) and high temperature (40-200ºC) are applied	• Less time and solvent consumption • Enables the use of water as solvent	• Low selectivity towards the analytes during extraction • High costs due to instrumentation high requirements

CHAPTER 3

METHODOLOGY

3.1 Sample collection

Red beets and date fruits were purchased from the local market and Arab shop, respectively, in Serdang. Date fruit product is imported from Saudi Arabia, while red beet is Malaysian cultivation.

A convenient (non-probability) sampling method is adopted where the available date fruit and red beets were bought. A total of 2.3 kg of red beet and 1 kg dates were purchased and homogenized. Then, red beets were washed with distilled water then the peels were extracted with a sharp knife. DP was manually removed and thoroughly washed with distilled water (Fig. 3.1). Both samples were oven-dried by laboratory oven (Memmert Universal, Schwabach, Germany) at 35ºC for three days, with proper ventilation to avoid moisture effects (Fig. 3.2 & Fig. 3.3). RBP was powdered using the electric blender, while DP was grounded using a 1mm microfine grinder (IKA Werke GmbH & Co. KG, Breisgau, Germany) (Perveen et al., 2012; Saleem & Saeed, 2020).

Figure 3.1. Manually Extracted Samples

Figure 3.2. DP After Drying

Figure 3.3. RBP After Drying

3.2 Chemicals and materials used

Methanol, ethanol, formic acid, acetic acid, dimethyl sulfoxide, agar nutrient, petri plates, and cotton swabs.

3.3 Preparation of RBP and DP extracts

A total of 4g of powdered RBP sample was dissolved in 40 ml of each 50% aqueous ethanol + 0.05% acetic acid solvent and 50% aqueous methanol + 0.1% formic acid solvent (Guldiken et al., 2016; Vulić et al., 2013). A total of 4g of powdered DP sample was dissolved in 40 ml of each ethanol, methanol, and 50% aqueous ethanol solvents (Javed et al., 2013; Metoui et al., 2019; Sooad Al-daihan, 2012). Then, all samples were sonicated for 15 minutes by PowerSonic 405 (Hwashin Technology, Seoul, Korea) (Fig. 3.4) (Sawicki et al., 2016). Later, the samples were centrifuged for 10 minutes (Hettich Zentrifugen, Westphalia, Germany) (Fig. 3.5) and filtered by 125mm qualitative filter paper (Fig. 3.6). The extracts were collected, and this entire procedure was done in 3 times repetition using the same plant sample to obtain the highest extraction yield of each plant sample (Sawicki et al., 2016). The final extract of each procedure was combined altogether. Later, they were vacuum evaporated at 40°C and 4000 rpm using the rotary evaporator (Buchi, Heerbrugg, Switzerland) to evaporate the organic portion of the solvent. The aqueous portion was separated using the freeze dryer. This whole procedure was conducted in triplicate. The sample extracts were stored at 4°C for later use. (Javed et al., 2013; Saleem & Saeed, 2020).

The following equation used to calculate yield extract %:

$$\% \text{ yield extract} = (\text{Extract weight} \div \text{dry weight powder}) \times 100$$

Later, 500mg/ml of each of the stocks was dissolved in 50% aqueous DMSO for plant antibacterial activity tests (Sohaimy et al., 2015). Whereas, for plant-antibiotic combination, 1g/ml of GM and 1g/ml of each extract were combined and dissolved in 50% DMSO (Ncube et al., 2012). All solutions were vortexed to dissolve solutes (the extract) with solvents (DMSO) properly.

Figure 3.4. Sample After Ultra-Sonic Bath Extraction

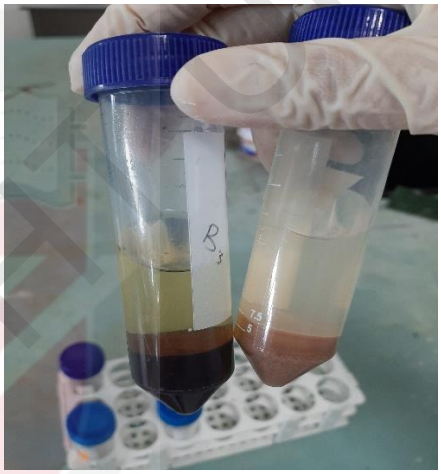

Figure 3.5. Centrifuged Sample

Figure 3.6. Sample Filtration

3.4 Microorganisms and culture

The selected gram-negative bacteria were *Salmonella typhi, Pseudomonas aeruginosa,* and *Escherichia coli,* while the selected gram-positive bacteria was *Staphylococcus aureus.* The selected bacteria are commonly known for causing food poisoning and food spoilage.

3.5 Antimicrobial studies of the extracts

The Agar disc diffusion method was used for antimicrobial activity evaluation. The plant extracts were tested against each of the selected bacterial strains. Besides, positive (GM) and negative (50% DMSO) controls were administered as well in each plate. Preparation of all bacterial strains was performed according to the Clinical and Laboratory Standards Institute (2020). According to Kirby-Bauer Disk Diffusion Susceptibility Test Protocol, a proximate of 25ml of autoclaved agar nutrient was poured at 40-45 °C and left to solidify in each plate. A total of 10^8–10^9 CFU/ml of each bacterial strain were mixed with 10 ml autoclaved normal saline. To ensure 0.5 turbidity, the sample was compared with McFarland Standard. Using micropipette, 100µl of bacterial suspension was inoculated in their respective Petri plate and then using cotton swabs, it was swabbed streaked to ensure even distribution of bacteria (Lawn technique). Inoculated discs were placed in each petri dish and seeded with 20µl of each dissolved extract of RBP and DP and positive and negative controls as well. The same procedure was administered for antibiotic–plant extract combinations mentioned earlier (Fig. 3.8). Later, these samples were cultured for 24 hours at 37 °C. The antimicrobial activity was interpreted by measuring the diameter (mm) of the clear inhibition zone (Clinical and Laboratory Standards Institute, 2020). Note that this whole procedure was conducted in triplicates.

Sample is considered susceptible if their inhibition zone is ≥ 15mm, intermediate if 13-14mm, and resistant if ≤ 12mm.

3.6 Statistical analysis

IBM SPSS Statistics 25 software was used for analyzing the data. The descriptive analysis was carried out to present the data as mean and standard deviation (±SD). The normality test was carried to explore the quantitative data for normality (skewness and kurtosis test). ANOVA (with Tukey and post hoc tests) and independent sample t-test were run to estimate the significant difference and range of distribution. $P<0.05$ was considered significant.

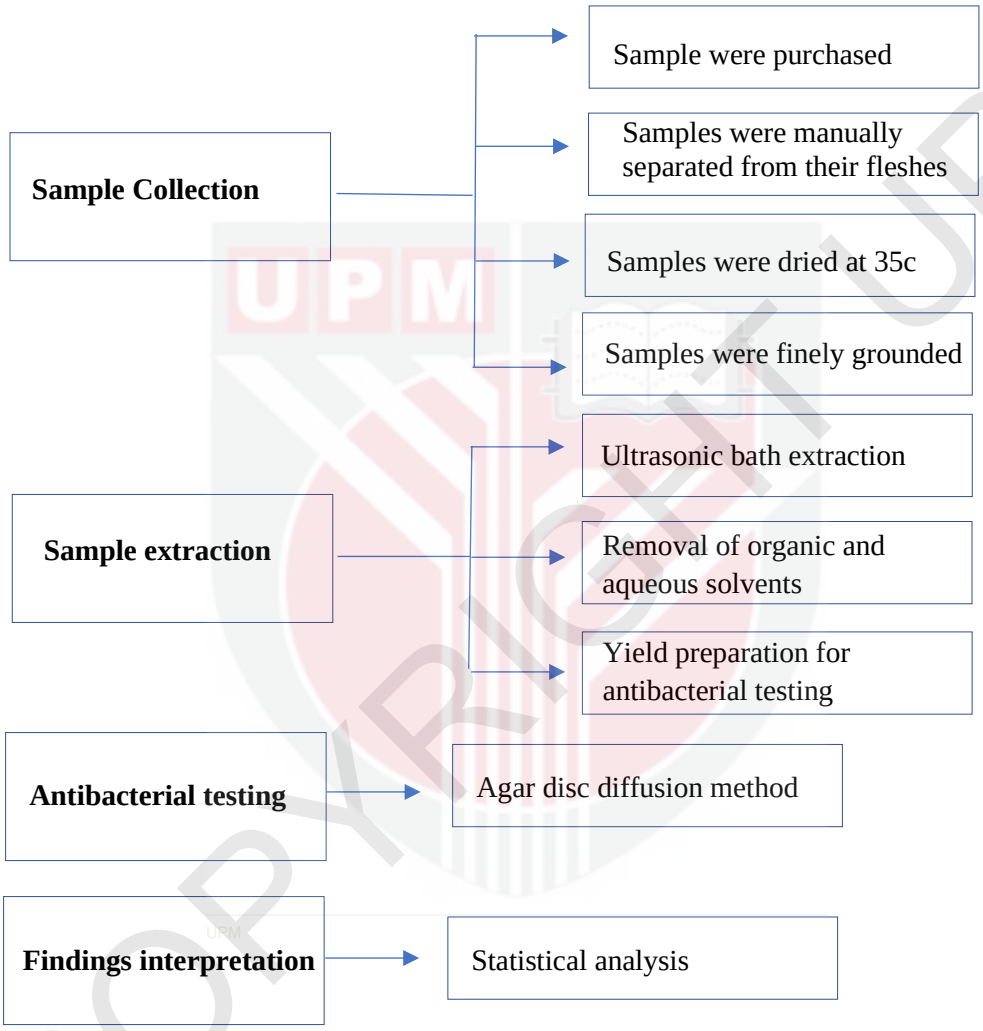

Figure 3.7: Methodology flow

CHAPTER 4

RESULTS

4.1 Total yield of RBP and DP extractions

RBP and DP were extracted with different solvents. A significant difference was obtained from all yields ($p<0.05$). For RBP, the 50% aqueous ethanol + 0.05% acetic acid extraction produced the highest yield proportion (51.30% ± 2.95) compared to 50% aqueous methanol + 0.1% formic acid extraction (37.90% ± 1.35). For DP, ethanol extraction had the highest yield (27.23%±2.11), followed by methanol extraction (22.70% ±0.75), and lastly, 50% aqueous ethanol (15.00% ± 0.00) (Table 4.1.1 & Table 4.1.2).

Table 4.1.1: Total yield proportion of different solvent extraction of RBP (Mean± SD)

	Total yield (%)	
	50% aqueous ethanol + 0.5% acetic acid	50% aqueous methanol + 0.1% formic acid
RBP	51.30 ± 2.95^{a}	37.90 ± 1.35^{b}

Means that do not share a subscript letter are significantly different.

Table 4.1.2: Total yield proportion of different solvent extraction of DP (Mean± SD)

	Total yield (%)		
	50% aqueous ethanol	Ethanol	Methanol
DP	15.00 ± 0.00^{c}	27.23 ± 2.11^{a}	22.70 ± 0.75^{b}

Means that do not share a subscript letter are significantly different.

4.2 Antibacterial activity of RBP and RBP-GM combination

Different solvent extracts of RBP were tested for their antibacterial activity against selected gram-positive and gram-negative bacterial strains. There was a significant difference between both extracts inhibition zones on *Salmonella typhi* and *pseudomonas aeruginosa* ($p<0.05$). Further, by referring to *the* Clinical and Laboratory Standards Institute (CLSI), the mean zones of inhibition were relatively weak for both extracts on all tested strains except *pseudomonas aeruginosa* that had a mild susceptibility with 50% aqueous ethanol + 0.5% acetic acid extract (Table 4.2.1) (Fig. 3.8.1, 3.8.2, 3.8.3, and 3.8.4). The synergistic activity of 1:1 RBP with GM (20mg/disc) was evaluated for their antibacterial capacity. The 50% aqueous methanol + formic acid extraction had the highest susceptibility activity; the mean zones of inhibition was relatively strong for *Salmonella typhi* (18.66±0.57*), Escherichia coli* (19.00±1.00*), Pseudomonas aeruginosa* (18.33±0.57), and *Staphylococcus aureus* (18.00±0.50) bacteria strains. A significant difference between both solvents extracts was observed on all strains ($p<0.05$) except for *Salmonella typhi* (Table 4.2.2) (Fig. 3.9).

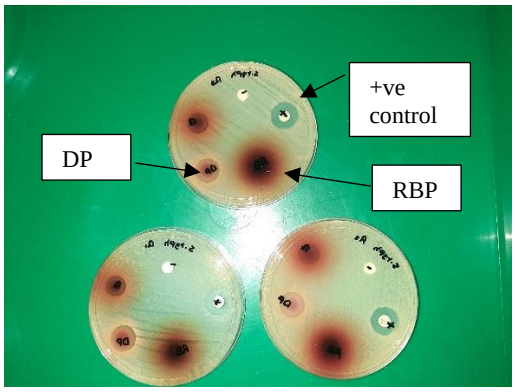

Figure 3.8.1. Antibacterial Activity Done on *Salmonella typhimorium*

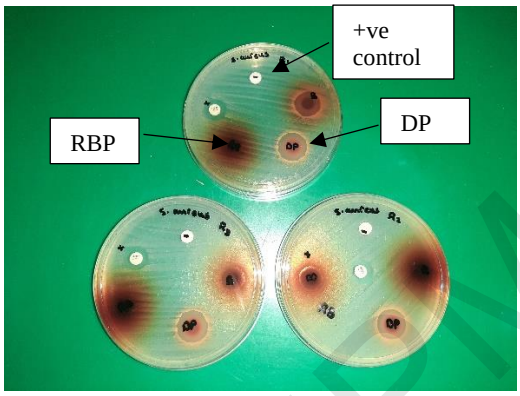

Figure 3.8.2. Antibacterial activity Done on *Staphylococcus aureus*

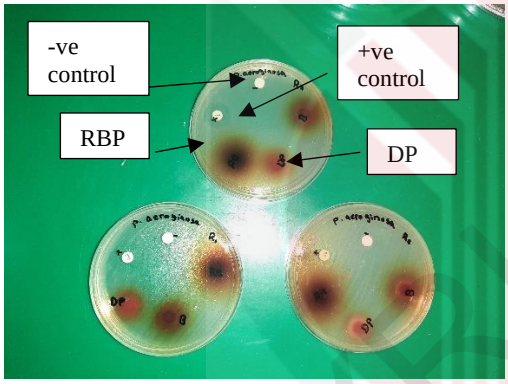

Figure 3.8.3. Antibacterial activity Done on *Pseudomonas aeruginosa*

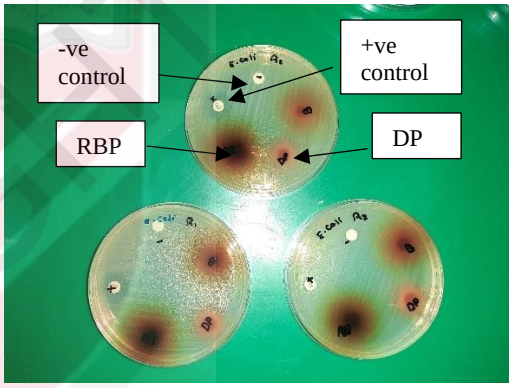

Figure 3.8.4. Antibacterial activity Done on *Escherichia coli*

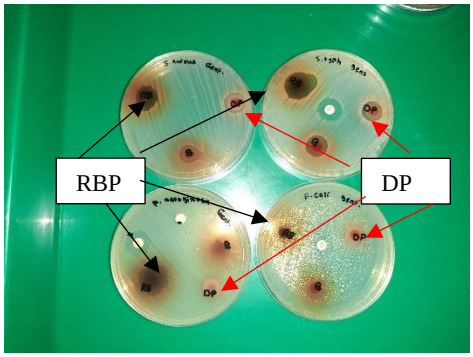

Figure 3.9. Plant-antibiotic Synergistic Activity on All Selected Strains

Table 4.2.1: Effect of different solvent extractions of RBP on bacterial strains (Mean ±SD)

Test organisms		Different solvent extracts (500mg/ml)			
		50% aqueous methanol + 0.1% formic acid	50% aqueous ethanol + 0.5% acetic acid	Positive control (GM)	Negative control (50% DMSO)
		Zone of inhibition (mm)			
G-	*Escherichia coli*	6.00±0.00^{b}	6.00±0.00^{b}	14.66±0.57^{a}	0.00±0.00^{c}
	Salmonella typhimorium	8.66±0.98^{b}	6.00±0.00^{c}	15.00±0.00^{a}	0.00±0.00^{d}
	Pseudomonas aeruginosa	6.00±0.00^{c}	11.33±0.60^{b}	15.00^{a}±0.00^{a}	0.00±0.00^{d}
G+	*Staphylococcus aureus*	6.00±0.00^{b}	6.00±0.00^{b}	12.66±0.57^{a}	0.00±0.00^{c}

Means that do not share a letter are significantly different ($p<0.05$).
Susceptible if their inhibition zone is ≥ 15mm, intermediate if 13-14mm, and resistant if ≤ 12mm.

Table 4.2.2: Synergistic effect of different solvent extraction of RBP combined with GM (1:1) on selected bacterial strains (Mean ±SD)

Test organisms		Different solvent extracts + GM*			
		50% aqueous methanol + 0.1% formic acid	50% aqueous ethanol + 0.5% acetic acid	Positive control (GM)	Negative control (50% DMSO)
		Zone of inhibition (mm)			
G-	*Escherichia coli*	19.00^{a}±1.00	16.33^{b}±0.28	14.66^{c}±0.57	0.00±0.00^{d}
	Salmonella typhimorium	18.66^{a}±0.57	17.83^{a}±0.28	15.00^{b}±0.00	0.00±0.00^{c}
	Pseudomonas aeruginosa	18.33^{a}±0.57	17.00^{b}±0.50	15.00^{c}±0.00	0.00±0.00^{d}
G+	*Staphylococcus aureus*	18.00^{a}±0.50	11.66^{b}±0.57	12.66^{b}±0.57	0.00±0.00^{c}

*In each disc, 20µl of 1:1 of GM and the respective solvent extract with a concentration of (1000mg/ml)
Means that do not share a letter are significantly different ($p<0.05$).
Susceptible if their inhibition zone is ≥ 15mm, intermediate if 13-14mm, and resistant if ≤ 12mm.

4.3 Antibacterial activity of DP and GM-DP combination

Further, the DP ethanolic extract had the highest antibacterial activity compared to other extracts except for *Escherichia coli*, with a significant difference observed on *Salmonella typhi* and *Staphylococcus aureus* particularly ($p<0.05$). The mean zones of inhibition (mm) resulted from ethanol extraction were relatively moderate for *Salmonella typhi* (13.25±0.88) and *Staphylococcus aureus* (14.41±1.46), whereas a weak antibacterial activity obtained by all extracts application for *Escherichia coli* and *Pseudomonas aeruginosa* there was (Table 4.3.1).

Further, the synergistic activity of 1:1 DP with GM (20mg/disc) was evaluated for their antibacterial capacity. A significant difference between extracts was observed on *Pseudomonas aeruginosa* only ($p<0.05$). Also, a slightly better antibacterial activity was observed from 50% aqueous ethanol extract on *Staphylococcus aureus* (14.16± 0.28) as compared to GM (12.66± 0.57) (Table 4.3.1 & Table 4.3.2). However, antagonistic activity was observed for gram-negative bacteria strain *Salmonella typhi*, *Escherichia coli*, and *Pseudomonas aeruginosa* regardless of the type of extract applied.

Table 4.3.1: Effect different solvent extraction of DP on bacterial strains (Mean ±SD)

Test organisms		Different solvent extracts (500mg/ml)				
		50% aqueous ethanol	Methanol	Ethanol	Positive control (GM)	Negative control (50% DMSO)
		Zone of inhibition (mm)				
G-	*Escherichia coli*	6.00±0.00^{c}	7.66±0.40^{b}	6.00±0.00^{c}	14.66±0.57^{a}	0.00±0.00^{d}
	Salmonella typhimorium	11.50±0.50^{c}	11.08±0.37^{c}	14.00±0.00^{b}	15.00±0.00^{a}	0.00±0.00^{d}
	Pseudomonas aeruginosa	6.00±0.00^{b}	6.00±0.00^{b}	6.50±0.83^{b}	15.00±0.00^{a}	0.00±0.00^{c}
G+	*Staphylococcus aureus*	12.83±0.28^{b}	6.00±0.00^{c}	15.67±0.76^{a}	12.66±0.57^{b}	0.00±0.00^{d}

Means that do not share a letter are significantly different ($p<0.05$).
Susceptible if their inhibition zone is ≥ 15mm, intermediate if 13-14mm, and resistant if ≤ 12mm.

Table 4.3.2: Antibacterial activity of different solvent extractions of DP combined with GM (1:1) on bacterial strains (Mean ±SD)

Test organisms		Different solvent extracts + GM*				
		50% aqueous ethanol	Methanol	Ethanol	Positive control (GM)	Negative control (50% DMSO)
		Zone of inhibition (mm)				
G-	*Escherichia coli*	6.00±0.00^{b}	6.00±0.00^{b}	6.00±0.00^{b}	14.66±0.57^{a}	0.00±0.00^{c}
	Salmonella typhimorium	13.50±0.50^{b}	12.33±0.28^{c}	13.83±0.28^{b}	15.00±0.00^{a}	0.00±0.00^{d}
	Pseudomonas aeruginosa	6.00±0.00^{d}	9.00±0.00^{b}	8.16^{c}±0.28^{c}	15.00±0.00^{a}	0.00±0.00^{e}
G+	*Staphylococcus aureus*	14.16±0.28	11.33^{c}±0.28	11.33^{c}±0.28	12.66^{b}±0.57	0.00±0.00^{d}

*In each disc, 20µl of 1:1 of GM and the respective solvent extract with a concentration of (1000mg/ml)
Means that do not share a letter are significantly different ($p<0.05$).
Susceptible if their inhibition zone is ≥ 15mm, intermediate if 13-14mm, and resistant if ≤ 12mm.

CHAPTER 5

DISCUSSION

5.1 RBP and DP antibacterial activity

Bacterial contamination is one of the serious challenges that face food producers. It contributes to food poisoning, spoilage, waste, and loss. This fact compromises economic as well as public health sectors. Therefore, incorporating natural antimicrobial compounds obtained from plants is a promising strategy to combat bacterial pathogenicity and extend food's shelf-life (Gyawali & Ibrahim, 2014).

Red beets were long used in folk medicine due to the availability of high antioxidants associated with curing infectious diseases through their antimicrobial activity (Kumar & Brooks, 2018). The peels had the highest polyphenols availability compared to the inner flesh of red beet fruit (Sawicki et al., 2016). However, during red beet processing for juice production, peels are part of the total pomace which is usually wasted (Canadanovic et al., 2011). The current study has investigated the antibacterial activity of different solvent extracts of RBP (Table 4.2.1). By referring to the standard protocol provided by the Clinical and Laboratory Standards Institute (CLSI), for all examined bacterial strains, bacteria will be considered susceptible if their inhibition zone is ≥ 15mm, intermediate if 13-14mm, and resistant if ≤ 12mm. All bacterial strains were resistant to RBP extracts. These findings are similar to Velićanski et al. (2011) for S. *typhi*, E. *coli*, and P. *aeruginosa*, who administered a 1500µg in disc diffusion method. However, the current study applied 10,000µg in 20µl/ disc

volume. This concentration was administered in previous research in a volume of 100µl through the agar well diffusion method instead of the agar disc diffusion method adapted in the current study. Their findings show intermediate to strong inhibitory activity: E. *coli* (13.33mm), S. *typhi* (25.0mm), P. *aeruginosa* (13.33mm), and S. *aureus* (20.0mm) (Vulić et al., 2013). In another words, the study that administered a similar antibacterial concentration had contradictory findings (inhibitory activity) to the current findings (Velićanski et al., 2011). While the study that administered a lower concentration had a similar findings to this study findings (Vulić et al., 2013). The agar well diffusion method enables higher volume administration, thus, facilitate extract solubility and diffusibility. However, the drawback of this method is the possibility of extract leakage, which may lead to inaccurate findings. Further, the variation in findings could be attributed to the availability of active compounds in different varieties and regions of cultivation. Different environmental exposures impose different coping mechanisms from one variety to another, leading to variation in bioactive availability and functioning (Samad et al., 2016). Also, strains examined in this study were wild strains that have no phenotypically known susceptibility. Different bacterial strains and sources of isolates can give rise to different responses to antimicrobial agents, although they are within a similar species (Clinical and Laboratory Standards Institute, 2020).

In addition, date fruit was long known for high polyphenol availability (Maqsood et al., 2020). It is rich with phytochemicals, even their pits. DP represents 10% of the fruit on average and is usually wasted or fed to the animals (Chandrasekaran & Bahkali, 2013). DP was examined in the current study for its antibacterial activity (Table 4.3.1). The ethanolic extract had moderate outcomes on S. *typhi*, and S. *aureus* strains compared to methanolic

extract that had weak-moderate effects. However, none of the present findings from DP extracts had any influence on E. *coli* and P. *aeruginosa.* In previous studies, methanolic extraction possessed strong antibacterial activity as compared to aqueous and acetone extraction (Javed et al., 2013; Kahkashan et al., 2012; Metoui et al., 2019; Sooad Al-daihan, 2012). However, ethanolic extraction was not examined on DP in previous literature. Inconclusive findings could be subjected to multiple factors. First, the date varieties investigated before were different from the date variety administered in this study (Sukkary); thus, differences in bioactive compounds availability might give different antibacterial outcomes. For instance, a total of 62.50 mg GAE/100 g of total phenolic content was found for the Sukkary variety (Siddeeg et al., 2019), whereas for the Deglet Nour variety, it had 10.06g GAE/100g (Metoui et al., 2019). Hence, variation in date location, environmental conditions, ripening stage, food storage and processing, and plant variety demonstrate different antioxidants availability and functionality (Maqsood et al., 2020; Metoui et al., 2019). Further, the dosage administered in the current study was 10,000µg/disc in a volume of 20µl as compared to Javed et al. (2013) study who applied 500µg/disc in a volume of 10µl. Although his dosage was far lesser than the dosage administered by the current research, he obtained considerable antibacterial activity through his methanolic extraction compared to the ethanolic extraction of the present findings; S. *aureus* had 25mm compared to 14.4mm, S. *typhi* 20mm compared to 13.2mm, E. *coli* 18mm compared to 7.6mm, and P. *aeruginosa* 23mm compared to 6mm. Moreover, the current study examined wild bacterial strains with unknown susceptibility compared to American Type Culture Collection (ATCC) standard strains; thus, this might elucidate the reason behind the partial resistance observed. Further, the extraction

method administered in the current study (sonication) might be another attributing factor. Javed et al. (2013) applied Soxhlet extraction, known for the high-temperature application depending on the boiling point of each solvent. Although high temperature might degrade thermolabile compounds, it can aid in extracting more stabilized compounds that might be the main contributor to antibacterial activity. Therefore, the antibacterial activity of DP requires further future investigations.

5.2 RBP and DP synergistic/antagonistic activity with GM

Incorporating plant's active compounds with commonly used antibiotics is a promising mechanism that can alter bacterial resistance. Such an approach can minimize antibiotics toxicity, as lower concentrations are applied when accompanied by plant active compounds. It can increase the sensitivity to multi-drug resistance bacteria (Ayaz et al., 2019). In the current study, different RBP extracts combined with GM mostly had better synergistic activity at different levels (>15 mm inhibition zone) than GM only, which had strong-intermediate activity (≤15 mm inhibition zone). All bacterial strains tested were susceptible to RBP-GM combinations with different susceptibility levels depending on the type of solvent extraction. Among all tested strains, 50% aqueous methanol + 0.1% formic acid extraction had higher inhibition zone values as compared to 50% aqueous ethanol + 0.5% acetic acid (Table 4.2.2). In fact, this area in research is firstly addressed for RBP combination with GM in the current study. Therefore, no comparison with previous literature is available. Further, since the dosage criteria applied are similar to the formerly mentioned approach, the disc diffusion method might impair the diffusibility of antibiotic-extract combination. Hence, there is a possibility of having more stronger synergism with a similar dosage but higher volume using the well

diffusion method. The phenolic compounds that might synergize with antibiotics in RBP are gallic acid, thymol, epicatechin, syringic acid, p-coumaric, and caffeic acid (Ayaz et al., 2019; Saavedra et al., 2010) (Table 2.3). These compounds increase gram-negative outer membrane permeability by disintegrating the lipopolysaccharide layer; thus, higher concentrations of antibiotics can diffuse into bacteria. Antibiotics function in different mechanisms according to their classes (Khameneh et al., 2019). For example, aminoglycosides (eg. GM) functions by disrupting protein synthesis. Therefore, approaching plant-antibiotic synergism requires a profound understanding of the underlying mechanisms of action. The molecular docking simulation method can explain the intermolecular interactions of antibacterial agents among each other as well as at their target sites (Maia et al., 2018). Moreover, the antibiotic- DP combination was addressed for its antibacterial activity (Table 4.3.2). Mixed findings were observed from DP; it antagonized GM for all gram-negative tested strains, whereas for *Staphylococcus aureus* (gram-positive), 50%aqueous ethanol extract - GM combination possessed slightly higher susceptibility (14mm) compared to GM (12.6mm). This could be attributed to the differences between gram-negative and gram-positive structural configurations. The former has an additional outer layer membrane which supports its defencing capacity. However, if more gram-positive strains were examined, this would help to infer the relationship better. Further, antagonism could occur when bacteriostatic drugs reach to the infection site before bactericidal drugs (Levinson & Jawetz, 2002). Another possible factor is the interaction of extracts and antibiotic chemical compounds, which can drive these mechanisms: First, the competition for similar active site can inhibit both antibacterial agents.

Second, the direct interaction of both agents might change the chemical configuration of one or both of them; thus, alter their antibacterial potency (Mandalari et al., 2010).

5.3 Conclusion

RBP and DP were tested for their antibacterial activity and their synergistic/antagonistic activity against selected bacteria strains. Generally, levels of susceptibility ranged from weak to moderate due to several possible factors. RBP is suggested to have synergistic activity with the aminoglycoside class of antibiotics as they function by inhibiting protein synthesis, whereas RBP phytochemicals role is suggested to be by increasing cell wall permeability; thus, diffusion of higher antibiotic concentrations. In addition, DP is recommended for having antagonistic activity with certain gram-negative species. In general, these two plants can be incorporated in food preservation; however, adopting more comprehensive methodological approaches is needed in future work to drive in-depth understanding of plant–antibiotic and plant-microbe mechanisms of action.

5.4 Limitations and recommendations

This study was subjected to limitations. First, limited tested organisms, particularly in Gram-positive bacteria, which obstructed from having antibacterial representing findings. The present study did not test the minimum inhibitory concentration (MIC); thus, concluding that certain tested species are resistant to specific extract might not be correct, as bacteria might be susceptible to higher or lower dosages. Further, adopting a disc diffusion technique with a higher concentration of antibacterial agents might restrict their diffusibility into the surrounding media; thus, weak to moderate susceptibility was observed. In addition, examining wild strains with unknown resistance mechanisms might be another restricting

factor. Also, mixed antibacterial findings on different strains were obtained from the different solvent extracts applied; therefore, no superiority of specific solvent extract on another solvent can be concluded since the trend of susciptiblity levels is not consistent with different solvent yields. Hence, it is recommended to involve a wide range of bacterial strains in future work, including ATCC reference standard strains and wild strains to compare their susceptibility and better infer associations. Further, testing MIC will guide in knowing the lowest antibacterial concentration that can drive susceptibility. Administering agar well diffusion method is better when high dosages and volumes are needed. Also, since antibiotics have different mechanisms of action, the synergistic/antagonistic activity of specific plants can vary from one antibiotic to another; therefore, molecular interaction is needed to understand the details of interactions.

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