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REVIEW

Antibacterial test and toxicity of plant seed extracts: a review

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Abstract

Allah has instructed all humankind to eat permissible healthy food according to His guidance as narrated in Al-Quran. Following Maqasid as-Shari'ah, it is a Darruriyyah to ensure the safety of food as an act of protecting life. Therefore, the application of antibacterial agent on food aims to improve the consumers' health and control the herd immunity. Plant seed is a newly emerged alternative as a source of antibacterial agents. Commonly discarded, the utilisation of plant seed will not only reduce environmental waste issue but also benefited economically to many industries. This article was aimed to review the current literature on antibacterial test and toxicity of various plant seed extracts that potentially contributed to the efficacy of the seed. This review presents a comprehensive analysis of the antibacterial activity assay of the plants' seeds. It covers endpoint and descriptive methods, plant seed toxicity and application of the seed extract in food industries. This review was conducted using the various science-based platform such as Science Direct, Google Scholar, Emerald etc. The keywords 'antibacterial', 'antibacterial seed', 'seed extract' and 'toxicity seed' was used in this search.

1. Introduction

Halal and toyyiban consists of two elements; Halal means lawful and permissible (Syariah compliant) and toyyiban means wholesomeness (healthy, safe, nutritious, quality, hygienic preparation and other goodness-related terms). While food consumed should not only be Halal, toyyiban and free from food pathogens (Alqudsi, 2014), other consumers' products should imply the same principles too. According to surah of Al-Quran, the Al-Baqarah and An-Nahl, Allah has instructed not only His believers but also all the humankind to eat toyyiban food. For the non-believers, eating Halal is no harm at all.

"O you People! Eat of what is on earth, Halal and pure, and do not follow the footsteps of the Satan; Indeed for he is to you an open enemy" (Al-Baqarah 2:168)

"O you who believe! Eat of the good things that We have provided for you and be grateful to Allah if it is Him that you worship" (Al-Baqarah 2:172)

"So eat of the lawful (Halal) and good food (pure wholesome, halal cattle slaughtered according to Islamic method) which Allah has provided for you. And be grateful for the Graces of Allah, if it is He Whom you worship" (An-Nahl 16:114)

All Allah's guidances as narrated in Al-Quran are purposely to protect His creatures. This is known as Maqasid as-Shari'ah (Objectives of Shari'ah Law), and it covered five primary purposes: (a) Hifzu ad-Din (protection of religion); (b) Hifzu an-Nafs (protection of life); (c) Hifzu al-'Aql (protection of intelligent); (d) Hifzu an-Nasl (protection of posterity) and; (e) Hifzu al-Mal (protection of property). Although Maqasid as-Shari'ah is personal responsibility, it is a community obligation to spread awareness and ensure the practice of protections done among their members. By doing so, the whole community will benefit a peaceful living. This

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obligation is known as Fard al-Kifayah.

Over the past centuries, there were emerging of various diseases related to foodborne pathogens, and many of them are fatal. Due to the necessity of protecting life and communities, four components of food security were established and enforced in 2002 by the Food and Agriculture Organization (FAO) of the United Nations. These four components, known as the Four Pillar of Food Security, are including (1) food availability. Enough nutritious and sufficient quality of food shall be made available for people's consumptions. Productions, distributions and exchange mechanisms shall be considered to assure this component is achievable; (2) food access. This component affected by the affordability of individuals, households or communities in accessing the food, while allocations and preferences probably based on age, gender, religion or cultural norms; (3) food utilisation. People may have accessibility to food supplies, but need to consider their ability to utilise the food. Food utilisation is mainly affected by their health status, food nutritional values, food safety (contaminant and toxic-free food), and appropriate handling of food preparation and consumption; (4) food stability. However, unforeseen factors such as war, disaster, climate change, deforestation and economic crisis may lead to a loss in food stability and then famine. Hence, risk management shall be set up to determine appropriate risk measurements and solutions (Gibson, 2012).

A food additive is a substance that is introduced into food during food preparation processes including production, processing, packaging, transportation, and storage to preserve or enhance food quality. It can extend shelf life, providing flavour or scent, improving texture or for commercial attraction. Later, the Food Additives Amendment of 1958 established in the United States' Food, Drugs and Cosmetic Act 1938. The amendment stated that food additives are required to be acknowledged as Generally Recognized as Safe (GRAS) by scientific evidence from experts before they are added into the consumers' food.

Food additives have been used worldwide since centuries ago, where their natural resources and applications are depending on the locals' preference. Up until the Industrial Revolution (IR) era, natural-sourced food additives such as spices, sugar and salt were used to preserve raw and cooked food. Later during IR, chemical food additives were introduced to the consumers without prior scientific research regarding its possible implication. Since then, the administration of food synthetic preservatives among consumers had been dramatically increasing.

The addition of natural or chemical preservatives in food is carried out to diminish microorganisms (Shee *et al*., 2010). Niacin, a natural additive, for instance, successfully inhibited Gram-positive bacteria such as *Clostridium* and *Bacillus* spp. in dairy products (Hansen, 1994). Similarly, chemical preservative of benzoic acid and its salts (Qi *et al*., 2009) had been applied to inhibit the growth of various bacteria, yeasts, and moulds (Brul and Coote, 1999). Despite its efficacy, these synthetic antibacterial agents have several significant implications on its consumers, such as allergic reaction, hypersensitivity, immunity suppression and toxicity. Besides, the emergence of drug-resistance bacteria against antibiotics and food preservatives (Russell, 1991) have led to vast anxiety. These drawbacks created awareness among consumers on the importance of having natural preservatives in their food. Besides that, the intention to produce natural preservatives will also accommodate one of the four Pillars of Food Security that required food to be safe from contamination and toxicity. Hence, ensure that food utilisation is possible to the consumers.

Plants produce a range of chemical substances as a defence mechanism from the attack of various pathogenic microorganisms by inhibiting its growth or kill it. The concentration of the antibacterial substances may vary in different tissues of the same plant (Jain *et al*., 2003). Despite this, plants are the best candidates as antibacterial agents due to its abundant availability as renewable resources. Plant by-products such as its seeds, roots and peels, which commonly discarded and contributed to the environmental waste, are the alternative sources of bioactive compounds for natural antibacterial agents.

Plant seed (PS) has been recognised as one of the primary sources of antibacterial agents. Antibacterials of PS origin are found to effectively reduce or inhibit pathogenic and spoilage microorganisms and provide solutions to antibiotic resistance (Sunday *et al*., 2016). For example, Parekh and Chanda (2011) reported on the potential of *Mesua ferrea* L. seed as sources of antibacterial compounds for drugs application. Additionally, edible and nonedible seeds such as papaya (Sani *et al*., 2017), and chia seeds (Ullah *et al*., 2016) were reported, to possess antibacterial properties. These findings have led to the use of essential oils (EO), flavour compounds, phenolic compounds and isothiocyanates from PS in ensuring the quality and extending shelf-life of fresh and processed fruits and vegetables (Lucera *et al*., 2012; Davidson *et al*., 2015).

Various studies of PS (Table 1) indicated the inhibitory capacity of the seeds against pathogenic

Table 1. Antimicrobial studies on plants seeds using various extraction methods.

Source of seeds	Extraction methods	Antibacterial activity findings	Phytochemical findings	References
Ajowan	Solvent extraction by ethanol	Ajowan seed extract ethanolic inhibit activity of all tested bacteria which are *P. aeruginosa, A. lwoffii, K. pneumonia, E. aerogenes* and *S. aureus.*	Thymol	Vazirzadeh *et al.* (2013)
Annona squamosa	Solvent extraction using water, methanol, hexane, chloroform and petroleum ether.	Water, methanol and hexane extracts of seed inhibit the activity of *E. coli, V. cholera, S. enterica* serovar Typhi, *S. enterica* serovar Paratyphi, *K. pneumonia and P. mirabilis.*	TLC showed the presence of Linalool, Carvone, Eugenol, Farnesol and Geraniol. FTIR showed the presence of alkaloids, oils, tannins, phenols and flavonoids. FRAP showed water extract of seed contained the highest level of antioxidant.	Gowdhami *et al.* (2014)
	Soxhlet extraction using chloroform as solvent.	Chloroform extract of *A. squamosa* seed inhibits the activity of *E. coli, S. enterica* serovar Typhi, *K. pneumonia, P. mirabilis, S. aureus and B. subtillis.*	No information.	Vikas *et al.* (2017)
Carica papaya	Solvents extraction using hexane, petroleum ether, diethyl ether, chloroform, dichloromethane, acetone, ethanol, methanol, acetonitrile and distilled water.	Methanol extracts with the lowest MIC among other solvents exhibit good antibacterial activity on *S. enterica* serovar Enteritidis, *V. vulnificus, P. mirabilis* and *B. cereus* at 11.25 mg/mL.	GC/MS analysis of MeOH extract identified isothiocyanatomethyl benzene, 9-octadecenoic acid, hexadecanoic acid and β-sitosterol.	Sani *et al.* (2017)
Chenopodium quinoa	3 cultivars of quinoa seeds were homogenized with 80% ethanol, where solid-to-liquid ratio is 1:10 (w/v).	*C. quinoa* cultivar Giza1 possessed highest inhibitory activity against *L. monocytogenes* and *E. coli* bacteria compared to *C. quinoa* Sajama and cultivar Red Carina. The MIC of cultivar Giza1 against *L. monocytogenes* and *E. coli* were 10 and 5 mg/mL, respectively.	Cultivar Giza1 yielded the highest mean concentration of saponin, TPC, TFC and antioxidant effects among the cultivars of *C. quinoa.*	Farajzadeh *et al.* (2019)
Cucurbita pepo variant fastigata	Cold maceration by using chloroform, acetone and methanol for 24 hours.	Methanolic extract of *C. pepo var.* fastigata potently inhibited *B. subtilis* and *S. aureus*, while not against *E. coli* and *P. aeruginosa.*	There is abundant triterpenoids, phenolic compounds and tannins. Increasing concentration of methanolic extract investigated in DPPH-scavenging and hydrogen peroxide assays showed increasing antioxidant activities.	Soni and Bali (2019)
Grapefruit seed extract (DF-100, *Quinabra-Quimica*)	The purchased grapefruit seed extract (GSE) comprised of 49.49% GSE, 50% glycerin and 0.51% naringin. The extract were mixed with cinnamaldehyde (CA) and nisin. 0.05% Tween 80 were added into the mixture.	Individual MIC of grapefruit, CA and nisin were reported to be 31.25, 500 to 1000, and 250 ppm, respectively against 3 *L. monocytogenes* strains. *A* combination of 6 to 8 ppm of GSE, 15 to 20 ppm of CA, and 5 to 6 ppm of nisin, is an effective antibacterial solution against the growth of *L. monocytogenes* on lettuce and pork loin. These concentrations are lower than their individual MIC.	No information.	(Yu *et al.,* 2019)

Table 1. Antimicrobial studies on plants seeds using various extraction methods (Cont.)

Source of seeds	Extraction methods	Antibacterial activity findings	Phytochemical findings	References
Moringa oleifera	Maceration by using deionised water, chloroform, methanol, hexane and ethyl acetate.	The seed ethyl acetate extract showed higher inhibition activity than ampicillin in the DDT screening against *Proteus vulgaris, Acinetobacter baylyi* and *Pseudomonas aeruginosa.* Higher polarity extracts inhibited more types of bacteria than the lower polarity extracts. However, chloroform and ethyl acetate possessed potent inhibitory effect against *P. vulgaris.*	All extracts showed high total phenolic contents except for hexane extract. There are presence of abundant content of water-insoluble phenolics, triterpenoids and alkaloids in seed methanolic extract. While, small amount of flavonoids was detected.	Ilanko *et al.* (2019)
Paullinia cupana	Extraction with water, methanol, 35% acetone and 60% ethanol at room temperature and boiling temperature of respective solvents.	Alcoholic seed extracts strongly exhibited antimicrobial activity against *E. coli, P. fluorescens* and *B. cereus* where the size of the inhibition zone range 44 to 100 mm.	Highest content of catechins, epicatechins and epicatechins gallate were expressed by water extraction at room temperature.	(Majhenič *et al.,* 2007)
	Solvent extraction by using 70% methanol for 72 hours with constant shaking.	10 mg/mL of the seed extract was found to be the MIC that inhibited MRSA isolates.	Phenolic compounds were found to be the richest content. Other contents of tannins, alkaloids and flavonoids were quite high.	(Tayel *et al.,* 2018)
Lycium shawii	Solvent extraction by using 70% methanol for 72 hours with constant shaking.	MIC of 8 mg/mL and 10 mg/mL inhibited each of 2 MRSA isolates, respectively.	Alkaloids content was the highest phytochemical constituent, followed by tannins, total phenolics and flavonoids compared to other seeds extracts.	(Tayel *et al.,* 2018)
Solanum stramonifolium	0.01M of HCl containing 0.15M of NaCl were used to extract the seeds with ratio 1:3 (w/v).	The seed extract successfully show inhibitory activity on Gram-positive bacteria such as *S. aureus, B. subtilis, B. licheniformis, Xanthomonas* sp. and *P. aeruginosa.*	No information	(Sarnthima and Khammuang, 2012)
Sonneratia apetala (Buch.-Ham)	Solvent extraction using methanol.	Seed extract inhibited the growth of Gram-positive and Gram-negative bacteria.	High content of polyphenols, flavonoids, anthocyanins and vitamin C. Also rich in antioxidant and antidiabetic compounds.	(Hossain *et al.,* 2013)
Vitis vinifera	The seeds were first extracted with light petroleum ether for 3 hours and then methanol for 1 hour.	The sensitivity of 43 strains of MRSA were tested by gel diffusion, growth and respirometry studies. All strains successfully inhibited by seed crude extract, including those showing low inhibition zone.	Gallic acid equivalent (GE) of grape seed extract (GPSE) is 209-fold richer than the grape pulp and its pericarp extract (PPE). Meanwhile, the pyrogallol equivalent (PE) of GPSE was lower than its GE, but still higher than PPE. GPSE containing flavonoids, but anthocyanin was not found.	(Al-habib *et al.,* 2010)

bacteria (PB) such as *Escherichia coli, Salmonella enteritidis, Vibrio vulnificus, Bacillus cereus*, etc. that had imposed outbreak of foodborne disease worldwide (Wilson *et al*., 2013). The PB can change, adapt and multiply to eliminate the suppressed environment and effectiveness of drugs, thus producing multi-antibiotics resistant isolates (MARI) that cause more harm to human. The emergence of methicillin-resistant *Staphylococcus aureus* (MRSA), which is a resistant MARI against various antibiotics has led the quest of new drugs from PS. The oil of black seed (*Nigella sativa*) had proved to render a strong inhibitory effect against MRSA in raw shellfish at retail markets in Malaysia (Othman *et al*., 2018).

Additionally, most PS extracts are classified as GRAS under 21 Code of Federal Regulations (Davidson *et al*., 2015) because they have been in our diet for many years (Calo *et al.*, 2015) and have high LD_{50} values on rat or mice. The LD_{50} for phytosterols such as γ-tocopherol, campesterol, stigmasterol and β-sitosterol are 2220 mg/kg, 3000 mg/kg, 300 mg/kg, and 3000 mg/kg respectively. A study by Upadhyay et al. (2013) reported that the lowest LD_{50} for a GRAS compound is 104.7 mg/kg for *Nigella sativa L.* extract. Due to the potential of PS as antibacterial agents, this review explains the antibacterial studies of the PS.

2. Antibacterial activity assay

The studies of the antibacterial effect of plant metabolites on food-borne bacteria and spoilage microorganisms have been growing to cater to the demands of food quality and safety. However, the lack of method standardisation due to different extract polarity has become a limiting factor (Othman *et al*., 2011). The antibacterial evaluation methods fail to achieve uniformity and standardisation with the discovery of antibacterial plant metabolites from natural sources (Juneja *et al*., 2012). The addition of non-polar EO into emulsifier such as DMSO for complete solubility (Vladimir *et al*., 2011) and inactivation of some preservatives that may inhibit bacterial growth (Orth, 1993) are different from polar polyphenol extract (Osterburg *et al*., 2009). Furthermore, the approved method by the Clinical and Laboratory Standards Institute (CLSI) only applies to pure compound while the evaluation of antibacterial efficacy initiates from the crude extract. Thus, the method is subjected to modification (Das *et al*., 2010).

2.1 Preparation of sample

Each diluted crude extract is subjected to solvent removal prior to antibacterial assay. The removal of solvent can be done either by N_2 blowing until reaching a constant weight or by rotary evaporation if non-polar extractant is used. Otherwise, the freeze-drying method is available for the polar solvent (Cano-Campos *et al*., 2011). Solvent removal is critical because of its bactericidal against tested bacteria (Table 2), and omission of this step may lead to false-positive result (Torres, Pandey, and Castro, 2011). The resultant dried extracts were dissolved in Dimethyl sulfoxide (DMSO) and filtered through 0.45 µm cellulose membrane to remove the carried over particle from the previous extraction step. DMSO is frequently chosen because of its aprotic solvent (Harish Prashanth and Tharanathan,

Table 2. Inhibition zone of solvent on gram positive food pathogens

Solvent[2]	Total inhibition[1], mm						
	S. aureus	*B. cereus*	*L. monocytogenes*	*C. diphtheria*	*C. perfringens*	*S. pneumoniae*	*B. subtilis*
Hexane	Na	Na	Na	na	na	na	na
PE	Na	na	Na	na	na	na	na
DE	Na	na	Na	$1.82\pm0.43^{a}{}_{D}$	$1.59\pm0.53^{a}{}_{C}$	na	na
CHCL3	$3.62\pm0.93^{b}{}_{C}$	$6.01\pm2.95^{c}{}_{B}$	$4.97\pm1.32^{c}{}_{A}$	na	$7.69\pm1.18^{c}{}_{B}$	$7.73\pm1.77^{c}{}_{B}$	$7.31\pm0.99^{c}{}_{B}$
DCM	$1.81\pm0.76^{a}{}_{D}$	$1.58\pm0.30^{a}{}_{C}$	Na	$7.34\pm1.18^{c}{}_{B}$	$1.58\pm0.82^{a}{}_{C}$	$1.11\pm0.56^{a}{}_{C}$	$2.15\pm0.05^{a}{}_{D}$
Acetone	$2.81\pm1.0^{a}c_{D}$	$4.34\pm1.58^{c}{}_{BC}$	$3.45\pm0.79^{b}{}_{AB}$	$3.69\pm2.05^{b}{}_{CD}$	$5.49\pm0.78^{c}{}_{B}$	$5.73\pm1.64^{c}{}_{B}$	$3.87\pm1.31^{b}{}_{CD}$
EtOH	$6.65\pm0.65^{c}{}_{B}$	$6.65\pm0.63^{c}{}_{B}$	$3.43\pm0.57^{b}{}_{AB}$	$6.27\pm1.08^{c}{}_{BC}$	$6.27\pm1.76^{c}{}_{B}$	$7.09\pm1.87^{c}{}_{B}$	$6.69\pm2.93^{c}{}_{BC}$
MeOH	$1.31\pm0.28^{a}{}_{D}$	$2.03\pm1.36^{a}{}_{C}$	$2.49\pm1.50^{a}{}_{B}$	$3.73\pm0.36^{b}{}_{CD}$	$1.62\pm0.88^{a}{}_{C}$	$1.31\pm0.27^{a}{}_{C}$	$2.85\pm0.61^{a}{}_{D}$
ACN	$1.78\pm0.77^{a}{}_{D}$	$3.72\pm0.29^{b}{}_{BC}$	$1.28\pm0.61^{a}{}_{B}$	$3.01\pm0.18^{b}{}_{D}$	$1.94\pm0.78^{a}{}_{C}$	$5.33\pm0.82^{c}{}_{B}$	$6.09\pm0.47^{c}{}_{BCD}$
Water	Na	na	na	na	na	na	na
DMSO	Na	na	na	na	na	na	na
TCH	$27.83\pm0.29^{c}{}_{A}$	$15.00\pm0.50^{c}{}_{A}$	na	$12.44\pm1.61^{c}{}_{A}$	$14.78\pm0.37^{c}{}_{A}$	$11.61\pm0.85^{c}{}_{A}$	$14.35\pm0.33^{c}{}_{A}$

[1]Means with different subscript capital letter within column are significantly difference ($p<0.05$). [2]PE - petroleum ether, DE-diethyl ether, CHCL3-chloroform, DCM- dichloromethane, EtOH - ethanol, MeOH - methanol, ACN – acetonitrile, DMSO-dimethyl sulphoxide, TCH – tetracycline hydrochloride (10 mg/mL). [3]na - No antibacterial activity (inhibition zone of sample < 1 mm), [a]Slight antibacterial activity (inhibition zone of sample 1–3 mm), [b]Moderate antibacterial activity (inhibition zone of sample 3–4 mm), [c]Clear antibacterial activity (inhibition zone of sample 4–10 mm) (Rauha *et al*., 2000).

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2007), which can stabilise the emulsion of EO (Turgis *et al.*, 2012) and do not inhibit bacterial growth (Othman *et al*., 2011). Wilson *et al*. (2002) utilised DMSO in oily extract dilution. Often, for extract stabilisation purpose, the plant extract is stored under low temperature (Cseke *et al*., 2006). However, the solubility of extract in a solvent is affected by temperature causing precipitation at low temperature for the reconstituted dried extract with DMSO; thus, Kothari (2014) suggested to perform bioassay when extract reaching room temperature.

2.2 Preparation of inoculum

The inoculums are prepared by transferring a loopful of cells from the bacterial cultures into sterile tryptone soy broths (TSB). These cultures can be maintained lower than 4°C and must be subcultured every fortnight. 1 mm glass beads can be used to assist in breaking up small clumps of organisms and incubated between 4 - 16 hrs at 37±2°C to achieve inoculum containing $10^6 - 10^8$ CFU/mL (Biehle *et al*., 1994). Recommendation against inoculum concentration exceeds this value is to avoid selection of resistant mutants (CLSI, 2012). The standardized bacterial colony numbers can be achieved through: (1) the establishment of bacterial growth within 24 hrs and corresponding turbidity at 600 nm (OD_{600}) (Othman *et al*., 2011) and (2) Comparing bacterial turbidity against 0.5 McFarland standard, which is equal to $10^6 – 10^8$ colony-forming unit (CFU)/mL (Vazirzadeh *et al*., 2013) have been practised as standard methods prior to antibacterial evaluation. The former is frequently chosen due to its viable cell count, compared to the latter, which has inconsistency of turbidity value (Othman *et al*., 2011). Conversely, since the exponential growth of bacteria is achieved at $10^6 – 10^8$ CFU/mL, the establishment of a calibration curve using McFarland standards may give acceptable turbidity range and hence reduces the error of non-viable bacterial cell.

The McFarland standards are designed for estimating concentrations of gram-negative bacteria such as *E. coli*. because it has no incubation time, or it does not need any equipment to estimate bacterial numbers. Generally, the standards are labelled from 0.5 through 10 and are filled with suspensions of barium salts. Latex bead suspensions are also available, which extend the shelf life of the material. The standards may be made in the lab by preparing a 1% solution of anhydrous $BaCl_2$ and a 1% solution of H_2SO_4 through mixing them in proportions. They should be stored in the dark, in a tightly sealed container at 20 – 25°C, and should be stable for approximately six months. However, it is inappropriate for fastidious bacterial growth.

On the other hand, for OD_{600}, few critical steps should be emphasized: (1) there is a need to calibrate the serial dilution of bacterial suspensions against their CFU/mL, (2) OD_{600} and its respective CFU/mL is exclusive for individual bacterial growth, and (3) recalibrate the bacterial suspension if the spectrophotometer's lamp is replaced.

2.3 Antibacterial activity assay

There are few techniques employed in evaluating the antibacterial activity of fruit seeds extracts. These techniques are categorised as endpoint methods and descriptive methods. Vigil *et al*. (2005) concluded that both endpoint and descriptive methods should be employed for the antibacterial of potential bioactive compounds. Generally, endpoint methods include agar diffusion test (ADT), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Bacterial growth curve and time-killed curve are the descriptive methods for antibacterial assay.

2.3.1 Endpoint methods

Endpoint method in the antimicrobial assay is a determination of the final result, which a manipulated variable influenced the bacterial growth. Endpoint method emphasises on the reason why bacterial growth resulted in a certain way due to by one or more manipulated variables. For example, what are the sample concentrations that inhibited or killed the bacterial growth? What is the minimum concentration that does so? How many percentages of bacteria that are actually be killed? These questions can be solved by conducting ADT, MIC and MBC assays. Also, automated instruments are the latest trending in determining bacterial sensitivity. In these assays, a range of concentrations of the sample is tested against bacteria of interest. Generally, the test outcome has two probabilities; (1) inhibited or not inhibited in ADT or (2) positive or negative turbidity value in MIC and MBC. The value is the difference of turbidity measured before and after 24 hrs incubation, of the inoculated broth added with extracts. However, the endpoint method is a preliminary stage, and further research is recommended by conducting descriptive method to support results reliability.

2.3.1.1 Diffusion test (DT)

DT is a qualitative method to evaluate the antibacterial properties of crude or purified extract, as a single form or in combination with other antibiotics (Roccaro *et al*., 2004) against aerobic bacteria. DT consists of two methods: disc (DDT) and agar well (AWDT) techniques. Of these, the latter produces more precise data (Cattelan *et al*., 2013) and has a low possibility of rendering false negative result (Vigil *et al*.,

2005). The DDT can be performed through pour plate (PPDD) and spread plate (SPDD) techniques.

In principle, for DDT, an aliquot of 100 µL inoculum was mixed with molten soft agar (for PPDD) or spread on sterilised plate agar (for SPDD). Sterile paper discs (6 mm diameter) containing extract solution pipetted is then placed on them. For PPDD, the molten soft agar and inoculum mixture is poured on to the layer of Mueller-Hinton agar (MHA) to ensure even distribution of bacteria (Othman *et al*., 2011). The incubation was done at 37±2 ^{o}C for 24 hrs.

Likewise, AWDT involves the pipetting of extract solution into a borer-punched well in the solidified mixture of seeded bacteria and agar. The plate agars were then incubated at 37±2^{o}C for 24 hrs. The extract diffuses through agar, resulting in concentrated gradient. The degree of inhibition is indicated by a zone of growth around the disk and well, depending on the rate of diffusion of the compound and the cell growth. Therefore, the extract should not be too hydrophobic or lipophilic to avoid non-diffusion and to cater for the need for further investigation of antibacterial efficacy especially when dealing with non-polar extract (Ríos and Recio, 2005). The bacteria tested should grow rapidly and uniformly because of slow bacteria growth results in larger inhibition zone and vice versa. Bacterial growth inhibition was determined by the diameter of the inhibition zones (mm) using calliper after subtracting 6 mm of paper disc diameter. DMSO and known antibiotics were used as negative and positive controls, respectively.

The determination of the extracts inhibitory strength is subjective between researchers. It depends on the main objective and application of the research, i.e. food industry, pharmaceuticals industry and many more. Piddock (1990) termed the bacterial inhibition activities as (1) susceptible when the zone is > 30 - 35 mm in diameter, (2) intermediate with a zone of 20 - 30 mm, (3) resistance with the zone of < 15 - 20 mm, which are applied for pharmaceutical purposes. Due to acid digestion in the stomach, the pure antibiotic will be metabolised and undergo additional reaction. Other agar diffusion assay reported for plant's antibacterial activity classifications are 0 – 0.3 mm, 0.3 – 0.7 mm, and 0.7 – 1.0 mm zone of inhibition, which indicates low, medium, and high activity, respectively (Eloff, 2004).

However, the method used by Rauha *et al*. (2000) to define the susceptibility of bacteria for food is through crude extract applied against bacteria. The criteria used are: no antibacterial activity when the zone is < 1 mm, slight antibacterial activity when the zone is between 1 – 3 mm, moderate antibacterial activity when the zone is between 3 – 4 mm, clear antibacterial activity when the zone is between 4 – 10 mm, and strong antibacterial activity when the zone is > 10 mm.

2.3.1.2 Minimum inhibitory concentration and Minimum bactericidal concentration

Until recently most *in vitro* susceptibility testing was performed by DT. However, DT is appropriate only as a preliminary test and could not tell the exact concentration of extract needed to achieve a therapeutic result (Klancnik *et al*., 2010). MIC test is introduced to overcome the above limitation. The MIC can be done by agar or broth dilution methods. However, Klancnik *et al*. (2010) found that the latter was the most accurate and faster way of assessing the antibacterial effect. It also applies to investigate the inhibitory effect of low polarity extract on bacterial growth kinetic (Othman *et al*., 2011). By conducting MIC evaluation, seed extract with the lowest concentration can be identified as a potential economic antibacterial agent against pathogenic bacteria (Rub and Sasikumar, 2016).

The MIC number is the lowest concentration of drug that inhibits the growth of the pathogen, while MBC is the lowest concentration that kills more than 90% of the initial pathogen population. Thus MBC is also known as MIC_{90} (Si *et al*., 2006), that is, a dilution greater than MIC since the former's ability is limited to the inhibition of the growth of pathogens. The MIC determination can be done by measuring the turbidity. However, MBC determination shall be done by cultivating the MIC and higher concentrations of extract, together with the positive control, onto solid mediums of nutrient agar. The nutrient agars are incubated at 37±2^{o}C for 24 hrs.

Agar dilution method had been used before the broth dilution method came into the board. Extracts are prepared in two-fold dilution and mixed with agar to give a range of extract concentration, seeded with inoculum at 10^6 CFU and incubated for 24 hrs (Gutierrez *et al*., 2009). The cell is counted (Wiegand *et al*., 2008) and MIC is defined as the lowest extract concentration yielded no bacterial growth on the agar (López *et al*., 2011).

Broth dilution method is evaluated through macroscopic evaluation or turbidity measurements. Both methods entail the dilution of extract in 96-wells microplate or tube, which is known as micro- or macro-dilution. Turbidity evaluation involves the dilution of extracts into broth medium to obtain two-fold serial dilution. The serial concentrations of EO were filled into 96-well microplate with a volume of 90 µL. Each well was then inoculated with 10 µL of a pathogenic strain at a concentration of 10^6 CFU/mL. The microplate was

incubated aerobically for 24 hrs at 37°C. A sterile broth medium incubated under the same condition was used as a blank. A broth medium incubated with a target bacterium (without an antibacterial agent) was used as a positive control of growth while a mixture of sterilised broth and extract was used as a negative control.

For macroscopic evaluation, sterile discs are soaked into each well and placed onto an inoculated agar plate, where the lowest concentration that shows visible inhibition zone is considered as MIC after comparison with control disc (Iturriaga *et al*., 2012). Furthermore, a comparison between positive control and extract dilutions in the wells determines the MIC, whereby the lowest concentration with the lack of visible growth is considered as MIC (Lv *et al*., 2011). Addition of colour indicators such as resazurin (Vladimir *et al*., 2011), TTC (2,3,5-triphenyl tetrazolium chloride) or INT (2-p-iodophenyl-3-p- nitrophenyl-5-phenyl tetrazolium chloride) to indicate the viability of aerobic bacteria has helped in determining the MIC (Klancnik *et al*., 2010). Otherwise, to reduce uncertainty due to subjectivity from macroscopic evaluation, the turbidity evaluation is sought by measuring optical density at 600 nm before (T_0) and after the 24-hour incubation (T_{24}) at 37°C. The MIC is defined as the lowest concentration of antibacterial agent showing a complete growth inhibition of the tested bacterial strain. It is related to a different absorbance of zero, that is, $T_{24} - T_0 = 0$, i.e. $T_{24} = T_0$ or $T_{24} < T_0$ (Turgis *et al*., 2012).

To confirm the MIC and MBC values, an aliquot of 10 µL from a test well is collected from those tubes that did not show any growth and pipetted onto sterile agar. Control plates containing agar alone are also streaked with the respective organisms. All plates are incubated at 37°C for 24 hrs, and the concentration at which no visible growth is seen is noted as the MBC while for MIC, the lower concentration than MBC is selected (Adejuwon *et al*., 2011).

Boulekbache-Makhlouf *et al*. (2013) classified plant extracts on the basis of their MIC values: strong inhibition = MIC < 500 µg/mL; moderate inhibition = 600 µg/mL < MIC < 1500 µg/mL, and low inhibition = MIC > 1600 µg/mL. However, fractionated plant metabolites render lower MIC due to purified extract (McGaw *et al*., 2002) and elimination of antagonistic antibacterial effect from other components of the complex crude extract composition (Broniatowski *et al*., 2015).

2.3.1.3 Automated instruments

Recently, automated instruments for identification and testing susceptibility of potential antibacterial agents have been introduced. These instruments categorised as densitometer, redox meter, gradient methods and fluorometers are tabulated in Table 3. In some studies, these instruments have been compared in determining their efficiency in the antibacterial susceptibility testing (Fader *et al*., 2013). Redox meter was compared to the conventional methods as reference method and reported to have approximately 95% agreement for genus-level identification (Carroll *et al*., 2006). Snyder, Munier, and Johnson (2008) reported that fluorometer able to detect challenging isolates correctly compared to redox meter. However, these two instruments have a reasonable correlation and agreement level of more than 95%.

Although automated instruments can produce faster results, allow standardisation, a lesser time required for data gathering and analysis, yet the employment of these modern instruments may be the main factor leading to the high cost in testing antibacterial activity. Plus, the built-in systems may be too restrictive and limiting the modification of experimental designs for some scientists. While the panels and cards are pre-formatted by the manufacturer, custom-made of these components is way too expensive.

2.3.2 Descriptive methods

The concentration of extract required to inhibit pathogens is determined through MIC and MBC tests. However, these methods provide little information concerning the effects of an antibacterial that are below the MIC. Certain antibacterial extracts may still cause an increase lag phase, reduced growth rate or even initial lethality followed by growth. In food research, total inhibition of spoilage pathogens is not always required as an increased lag phase is enough to protect the consumers (Vigil *et al*., 2005). Therefore, the descriptive method is used to describe the effectiveness of MIC and MBC of the sample.

In contrast with the endpoint method, the descriptive method can be related to which responded variable is the main concern in identifying and describing graph trends in the bacterial curve. The manipulated variable is not really taken into the discussion. The finding of the descriptive method can be used to validate the final result of the endpoint method. In the antimicrobial assay, the descriptive method involves bacterial growth curve as well as the time-kill curve. It explains the observable conditions, quantitatively and/or qualitatively, of the bacteria growth and inhibition time instead of why they happened. Based on the constructed growth curve, lag phase, log phase and growth rate can be determined. Time-kill curve explains the optimum time taken for the selected sample concentration to kill bacteria of interest.

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Table 3. Automated instruments used for antibacterial susceptibility testing

Instrument	Description	Advantages	Limitations	Findings	References
Densitometer	Employed to detect growth. Turbidimetric measurement is used to determine kinetic of the cells growth before computing the data for MIC values by regression analysis. Small wells or microcuvettes, made of plastic reagent card, allow simultaneous testing for many different antibacterial agents.	The result of susceptibility and identification can be obtained as early as 2 hrs. Able to identify challenging isolates correctly. Able to reduce handling time.	No information	Interlaboratory testing involved 3 labs had proved 100% agreement level on the identification of challenging isolates.	(Jorgensen *et al.,* 2000)
Redox meter	100 of panels can be used simultaneously. A redox indicator placed in the broth measures bacterial growth.	Result can be obtained hours of incubation.	Potential for incorrect results when analysing unusual strains.	Out of 251 isolates of family *Enterobacteriaceae*, the agreement levels were 95.6% and 94.4% for the genus- and species-level identifications, respectively.	(Carroll *et al.,* 2006)
Gradient methods	Epsilometer (E-Test) is a simple quantitative method to determine the antibacterial activity and MIC by integrating disc diffusion and agar dilution. Its inert strip carries a marked, continuous concentration gradient of a predefined antibiotic. The strip may consist of more than 15 two-fold dilutions. The MIC reading is determined at the edge of the zone of inhibition as it intersects the strip.	Can be employed with anaerobic, fast growth and penicillin-resistant bacteria. Seeded agar incubated in CO_2 produced the best result for anaerobic bacteria. Result is accurate and reproducible	No information	E-Test result is accurate and reliable as its agreement with conventional antibacterial assays was about 95%.	(Baker *et al.,* 1991; Macias *et al*., 1994)
Fluorometer	Fluorophores are released before interaction with bacterial enzymes, resulting in increased fluorescence. Since, some bacteria may not release fluorophores, fluorometer has incorporated rapid, turbidimetric readers in the panels.	Results were available as early as 4 hrs of incubation.	Range of antibiotics for certain bacteria are limited.	Reading of fluorometer panels can be read accurately by assistant of automated reader. Therefore, the results can be manually adjustment	(Pykett, 1978; Staneck *et al.,* 1983*;* Fader *et al*., 2013)

2.3.2.1 Time-killed curve

The inhibition curve method is also called the time-killed curve, and it has been used to study the effect of antibacterial lower than MIC. A mixture of broth media and the extract is prepared in which the concentration of the extract was at MIC and lower than MIC values. Positive control of known antibacterial was prepared as well. The bacteria (with concentration of 10^6 CFU/mL) were added into the broth and extract mixture and incubated at 37°C for 48 hrs for the determination of the colonies at 0, 2, 4, 8, 12, 24, and 48 hrs by spread plating on MHA (Ji *et al*., 2008). Both general and specific medium can be used for the spread plate method (Kanatt *et al*., 2010).

Another approach for the time-kill curves test is by measuring the increase of turbidity using spectrophotometer. By applying the time-kill curve test, several responses are retrieved, such as stationary-phase growth level suppression, lag-phase increase, decrease in log-phase growth rate, and lethality.

2.3.2.2 Lag phase, log phase and growth rate

The microbial lag phase reflects the time required for cells to adapt to a new environment and start duplicating. The lag phase depends on many factors, and all physical or chemical conditions of the growth environment considerably extend the lag phase and increase its variability (Aguirre *et al*., 2013). More extended lag phase indicates a more prolonged time taken for a food to be spoiled by foodborne pathogens. Lag phase identification is through (a) the intercept of initial log phase and constant growth rate line (Hall *et al*., 2014) or (b) intercept of exponential growth line to the x-axis (Carey, 2009).

A log phase is an exponential growth phase, where cell division proceeds at a constant rate (Rolfe *et al*., 2012). The specific growth rate is maintained when the exponential growth rate continually increases, which are significantly affected by the presence of a high concentration of nutrients. Based on the log phase, the specific growth rate (μ) can be calculated according to Monod equation as expressed below, where, μ_{max} is maximum specific growth rate ($time^{-1}$), S is the concentration of substrate in a solution (mass/volume), and K_S is the half velocity constant (mass/volume):

$$\mu = (\mu_{max})(\frac{S}{K_S - S})$$

Lower μ indicates lower growth of foodborne pathogens and thus, reduces the time of food spoilage and extends food shelf life. As the nutrients concentration decreases, the exponential growth rate decreases and the growth rate of the cells started to be stationery.

3. Toxicity test

Extraction using organic solvent has raised an issue of toxicity for human use. Though the toxicity of residual solvents received serious concerns in pharmaceutical products (Medley *et al*., 2014), their toxicity effect on food should not be neglected. The residual methanol allowable limit is 3000 ppm (Lee and Kim, 2015). Additionally, Lee and Kim (2015) and Li *et al*. (2008) suggested that extraction involving the usage of organic solvent should undergo proper pre-treatment such as re-dissolve in water, freeze-drying or drying under reduced pressure prior to application.

An effective antibacterial should have high selective toxicity, that imposes potent concentration against pathogens, and at the same time gives minimal effect to host and possesses stable chemical and physical properties (Taylor, 2013). Thus, toxicity test should be proposed in order to meet this requirement. Often, effective antibacterial such as quinones is toxic to human (Rodriguez *et al.*, 2004).

European Food Safety Authority (EFSA) requires rigorous toxicological testing of these antibacterial agents, including metabolism and toxicokinetic, subchronic toxicity, reproductive and developmental toxicity, and genotoxicity. These batteries of tests, including in vitro and in vivo tests in animals and humans, can take years and involves enormous expenses before they can be completed to obtain approval, which makes the pursuit of antibacterial unprofitable (Davidson *et al*., 2015)

In most countries, marketing involving food with a natural antibacterial agent requires approval from regulatory agencies. Thus, natural antibacterial agents also have to undergo the toxicology test. General food safety legislation, maximum limits and other restrictions (e.g., acceptable daily intake and the no observed adverse effect level) on the use of specific additives are established based on their toxicological information. However, it is difficult to set a specific acceptable daily intake or no observed adverse effect level for natural compounds because of the variation of components among batches. In the United States, any direct food additive substance which does not have GRAS status under the Federal Food, Drug, and Cosmetic Act, is considered as not safe (marketed before October 15, 1994) under the Dietary Supplement Health and Education Act. In cases where there is no other regulatory authorisation, the safety of such ingredient/ compound must be established for premarket approval through the petition process. Thus, not only do naturally

occurring antibacterial agents, for example, clove extract, cinnamon oil, or fermented whey, from commonly consumed plant or animal products create appealing food labels for consumers, they are also less likely to be subjected to the complex regulatory requirements and approval process for use in food compared with synthetic preservatives (Davidson *et al*., 2015).

There is a dire need for toxicity study of crude and partially purified extract before a further investigation into antibacterial in food application can be undertaken. Fractionation and purification may affect the production cost (Pinazo *et al*., 2016) as well as solvent toxicity (Capello *et al*., 2007).

There is currently a tendency to call for substituting the use of laboratory animals in toxicological tests due to the high costs and the animals′ suffering as a result of the various tests. Parra *et al*. (2001) opted for *Artemia salina* because of the strong correlation coefficient between *Artemia salina* bioassay and *in vivo* test discovered while doing toxicity investigation using mice. Burci *et al*. (2018) used *Artemia salina* as preliminary screening before usage of albino mice for toxicological tests. Abedon *et al*. (2012) suggested the utilisation of wax moth larva (*Galleria Mellon Ella*) for evaluating the toxicity and efficacy of antibacterial agents. This new method is effective in reducing toxicity, and is more economical, compared with the use of rats, mice or rabbits.

LD_{50} and LC_{50} are usually used to measure the toxicity of a sample towards tested animals. LD_{50} is an abbreviation for a lethal dose, which a single dose of a sample is administered to a group of animals and kills 50% of them. The expression of the value is the weight of extract administered per 1 kg of body weight of animal tested. Meanwhile, LC_{50} is an abbreviation for the lethal concentration of a sample that kills 50% of animals tested. LC_{50} value is expressed as the concentration of extract in a volume of the carrier liquid, or part per million, per duration of hours, exposed to the animals. The toxicity measurement indicates by the lower value of LD_{50} or $LC_{50,}$ the higher the toxicity of the extract.

There are two types of toxicological tests which known as an acute toxicity and chronic toxicity tests. An acute toxicity test can be conducted by oral or dermal exposure, where the effect of the consumed dose on the body responses and internal organs can be monitored (Padilla-Camberos *et al*., 2013). According to Meyer *et al*. (1982), a tested sample is considered as toxic when LC_{50} value is at ≤ 1000 µg/mL of tested concentration.

While acute toxicity test is conducted by a single dose consumption of a sample extract prior to daily observation, chronic toxicity test required daily dosing or exposure to a tested animal within a stipulated time. Chronic effects may result after months of daily exposure (Bouzidi *et al*., 2011).

Measuring toxicity of an extract at various concentration may lead to sigmoid-curve graph because of binomial response for the dependent variable, in example for toxicity case, death or no death. The application of Probit Analysis will transform the sigmoid graph to a linear graph, and hence regression of the relationship between response and various concentration can be determined (Finney, 1952). For an experimental study with lack of sample amount or multifactor involved, LC_{50} and LD_{50} can be determined by applying Probit Analysis.

5. Conclusion

Growing demand for natural preservatives has increased the quest for antibacterial agents from plants whereby commonly discarded plant seed is an option to cater to this demand. Although various natural products have been utilised as antibacterial agents, many of them are still underutilised in food application. However, a researcher should possess overview information of available antibacterial testing method, toxicity and application of the plant seeds to control pathogens. This information will support the research by determining the suitability of test, the safety of the plant seed for consumption. Furthermore, it helps to find an antibacterial agent with higher potency against resistant bacteria and render a provision to food safety and security and Halal industry worldwide.

Conflict of Interest

There is no conflict of interest while preparing this manuscript.

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