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Antibacterial Properties of Ocimum Spp. (Ocimum Basilicum L. and Ocimum Basilicum Var. Purpurascens) Against Selected Bacteria

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Abstract: Basil is known as 'Herbe Royale' which has potential as a source of antibacterial compounds which emphasizing in the importance of humans' health and food manufacturing in the future. This study evaluated the antibacterial activities of the methanol extracts of two Ocimum spp. which include Ocimum basilicum L. and Ocimum basilicum var. purpurascens. These methanol extracts were tested against four pathogenic bacteria; Bacillus cereus, Staphylococcus aureus, Escherichia coli and Salmonella typhimurium. Significant antibacterial activity was shown for these tested crude extracts in agar disc diffusion, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests. The methanol extracts of these basil species were most effective against Grampositive bacteria and least effective against E. coli which is the Gram-negative bacteria in agar disc diffusion method. The sweet basil showed highest diameter inhibition zone with 16.33 ± 1.53 mm against *B. cereus* while the red rubin basil exhibited 11.33 ± 1.15 mm against S. aureus. Minimum inhibitory concentration and minimum bactericidal concentration were assessed by microdilution method and the results showed that the extracts of O. basilicum L. and O. basilicum var. purpurascens strongly inhibited the growth of all the tested pathogens, especially the Gram-positive strains, whereas the moderate inhibition activity against Gramnegative strains. The result may suggest that, these two basil extracts possess compound with good antibacterial properties that can be used as antibacterial agents in the search for new drugs.

Keywords: Ocimum basilicum L., Ocimum basilicum var. purpurascens, Antibacterial activity, Minimum inhibitory concentration, Minimum bactericidal concentration.

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INTRODUCTION

Ocimum basilicum L. is one of the herbal and annual aromatic plants, globally cultivated which native to Southeast Asia particularly tropical and warm temperature region which possess significant economic value. The genus of Ocimum is from Lamiaceae family and commonly known as basil (Joshi, 2014). The basil is considered to be a source of high antibacterial and antioxidant activities (Kaya et al., 2008) and some of the compounds are found to be effective as fungistatic and insectidal (Varga et al., 2017). Some parts of basil plant such as flowers and leaves are used as galactogogue, stomachic, carminative and antispasmodic medicinal plant in folk medicine. Due to presence of active phytocompounds such as polyphenols and flavonoids contents, the profound medical effects of this herb may be attributed to its pharmaceutical potential. This herb is among of the

medicinal plants that is a source of antimicrobial agents and source of many potent and all-powerful medicines. The interest towards the application of natural medicine leads to the study which focusing on newer antibacterial compounds from medicinal plant. The present work aimed at evaluating the content and constituents of essential oil extracted from these basil varieties and its antibacterial properties. Therefore, this research was carried out to evaluate the antibacterial properties of two *Ocimum spp.* (*Ocimum basilicum L.* and *Ocimum basilicum var. purpurascesns*) methanol extracts against selected bacteria.

LITERATURE REVIEW

Ocimum basilicum L.

Ocimum basilicum L.is commonly known as a Lamiaceae family member (Varga *et al.*, 2017) which includes about 200 species in various botanic varieties and forms (Joshi, 2014). *Ocimum* genus is considered as the largest genera in Lamiaceae family (Piras *et al.*, 2018). These *Ocimum* plant is a type of an annual spicy herbs, indigenous to India and commonly known as basil. Several species of *Ocimum* well been known use as medicinal and aromatic uses and are cultivated in India. Sweet basil is native to India and tropical Asia and is now wild in tropical and subtropical regions like Central Africa and South East Asia (Pushpangadan and George, 2012) and cultivated frequently for production of essential oil in several country of East Asia, Europe, America and Australia (Pandey *et al.*, 2014).

Ocimum basilicum var. purpurascens

Ocimum basilicum var. purpurascens is commonly known as Basil Red Rubin or Purple Basil. It is a purple version of Italian large leaf basil and one of the sweet basil cultivars which improved variety from Dark opal basil and possess a traditional sweet basil flavour. This purple basil also belongs to the family Lamiaceae and its chemical composition consists of a significant quantity of phenolics compound especially flavonoids (Pedro et al., 2016). In addition, this variety also a good source of anthocyanins which it is one of the flavonoids that account for a large class of secondary metabolites in plants (Szymanowska et al., 2015). This Purple Basil is among of the most commercial basil species cultivated in producing essentials oils, their dried leaves or as an ornamental foliage and for culinary purposes.

The uses of basil

Basil is widely used for flavouring purposes particularly from the uses of leaves. The leaves of the basil can be used in cooking especially as spices in traditional cuisines while its essential oil used in wide application of perfumery, personal care and cosmetic industries such as shampoos, lotions and soaps (Kaya *et al.*, 2008; Varga *et al.*, 2017). In food products, basil is used in soups, meat pies, fish dishes, certain cheeses, tomato salads, cooked cucumber dishes, cooked peas, squash and string beans as well as vinegars and oils (Pushpangadan and George, 2012). Other than that, basil is widely used as food preservative as they perceived a lower risk to consumers and flavouring agent particularly in non-alcoholic beverage, ice creams and condiments.

Additionally, due to the presence of aromatic compounds in basil leaves, it has traditionally been used as medicinal plant in the treatment of headaches, migraine, stress, fever, coughs, diarrhea, constipation, warts, worms and kidney problems (Joshi, 2014). The plants of this genus are called 'king of herbs' because of its' various of applications particularly in folk medicine, pharmaceutical, perfumery, cosmetics and food preservations (Piras *et al.*, 2018). Besides, the juices form the leaves of basil have therapeutic characteristics which can relive the symptoms of cold and cough and those of croup when mixed with honey. Indeed, all the

parts of basil can be used as treatment to cure every infections and diseases particularly to humans.

Chemical composition in basil

Basil has long been used as traditional folk medicine because of its' healthful properties and the presence of secondary metabolites such as essentials oils, phenols, tannins, anthocyanins, flavonoids and steroids. In the previous study, Naidu et al., (2015) reported that the methanol extract of O. basilicum presence of polyphenols and flavonoids by using high performance liquid chromathography (HPLC) which include quercetin, rutin, kaempferol and caffeic acid. However, in recently research, El-Azim et al., (2017) indicated that methanol extract of basil was found to include twelve active phenolic compounds and they were identified as *p*-hydroxy benzoic acid, ferrulic acid, gallic acid, p-qumaric acid, benzoid acid, kaempferol, catechin, quercetin, chlorogenic acid, cafeeic acid, cinnamic acid and ellagic acid by column fractionation. A study on the ethanol extracts of basil by Guez et al., (2017) was found that to have almost similar constituents with methanol basil extract as stated in previous study which contains polyphenols and flavonoids such as quercetin, rutin, caffeic acid, kempferol in addition to chlorogenic acid, gallic acid and rosmarinic acid.

Other than that, O. basilicum is widely used as essentials oils since it possess source of aroma compound and biological active constituents which effectively as nematicidal, insecticidal and fungistatic properties. In the latest study conducted by Piras et al., (2018), the data presented explained that basil essential oils contain oxygenated monoterpenes, phenylpropanaoids. hydrocarbon sesquiterpenes. linalool, eugenol and 1.8-cineole that have been identified by using gas chromatography combined with mass spectrometry (GC-MS). However, the essential oils may vary with the cultivar type but the main components are phenylpropanoids and monoterpenes.

In the other hand, there is no study reported on methanol extracts of O. basilicum var. purpurascens however there was limited study described on the essential oil in this variety. The main components reported on the O. basilicum var. purprascens was methyl chavicol or methyl cinnamate. In the recent study expressed by Varga et al., (2017), these variety chemical constituents consist of trans-methyl cinnamate, linalool-acetate, methyl chavicol, eugenol, trans-a-bergamotene, 1,8-Cineole, Linalool and others. Varieties of compounds are available in the essential oil of basil however the main compounds present in abundance are eugenol, estragol and linalool. The main active agent is likely to be linalool which acts for its antibacterial activities.

Methods

Bacteria Cultures

The strain of bacteria used were *Eschericia coli, Salmonella typhimurium, Staphylococcus aureus* and *Bacillus cereus*. Each bacteria strain was maintained in glycerol stock at -18°C. Then, all of them were streaked separately on nutrient agar and incubated in the incubator for 24h at 37°C. A colony of each bacterium was grown at 37°C for overnight in the Mueller-Hinton broth (MHB: Oxoid; England). The density of each inoculum bacteria was standardized at optical density (OD) 600 nm in accordance with the McFarland Standard Formula at a concentration of 10⁷ cfu/ml before use for the following procedures. The suspension of all the bacteria was used for further studies in antimicrobial activity.

Samples Preparation

The samples extracts was prepared according to (Al-Talib *et al.*, 2016) with slightly modification. The plants were washed with tap water to remove impurities. Then, the plants were dried by using drying oven for 4 hours at 60° C before further process with extraction method. The dried basil samples were grounded into fine powder using the electric grinder (Waring Commercial, Malaysia).

Extraction of samples was done by using method described by (Sasidharan *et al.*, 2010) with slightly modification. About 30 g of dried powder basil was added with 300 ml of methanol (1: 10; w/v) in conical flask. The mixture then was left to stand for an overnight before filtered using Whatman No. 1 filter paper through Buchner funnel to obtain clear and pure extract. The filtrate then was evaporated using rotary evaporator at 40°C at 65 rpm. About 5 000 mg, 3 000 mg, 1 000 mg and 500 mg of crude extract were weighed and diluted with 1 ml of 99% Dimethyl sulfoxide (DMSO) in order to prepare stock solution. The stock solution were preserved in airtight bottles at 4° C until further use.

Antibacterial activity via Agar Disc Diffusion

Disc diffusion assay method was carried out based on method described by (Balouiri *et al.*, 2016) with slightly modification. The Mueller-Hinton agar (MHA) plates were inoculated with 100uL each test bacteria by using sterile cotton swab. Then, the filter paper was placed onto the MHA plates and allowed to stand for a few minutes for pre-diffusion of samples. About 15uL of each sample at desired concentration was poured onto the disc. As control, 15 μ L of each 50 mg/ml streptomycin was then dispersed into its' respective disc. The plates were incubated for 24 hours at 37°C. The diameter of growth inhibition zone surrounding the wells were measured in milimeters (mm) to determine the sensitivity of the test bacteria. All the tests were performed in triplicate.

Determination of MIC and MBC

Determination for both MIC and MBC were performed according to microdilution method (Al-Mariri, A. and Safi, M., 2013). The concentrations of each plants extract were tested as well as 50 mg/ml of streptomycin as a positive control.

While, the negative control were samples without the addition of bacterial broth culture. All these tests were performed in microtitre plates which contained about 50 µL of test samples (5 000, 2 500, 1 250, 625, 312.5, 156.25, 78.13, 39.07, 19.54, 9.77 mg/mL) and 50 µL bacterial culture. These 96-well microtitre plates was incubated for 24 h at 37°C. Then, each wells were put 20 uL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to observe the changed of color which determine the bacterial growth. Thus, the lowest concentration of the plant extracts was considered when the color remain after been put with MTT while wells that contained bacterial growth were changed to purple color. The first well with unchanged color was considered as MIC. To determine the MBC value, each sample from the MIC results were streaked onto MHA plates using wire loop. All the MHA plates were further incubated at 37°C for overnight. The MBC value was recorded as the lowest concentration of test samples and was determined when no presence of colony growth from directly plates of the wells. All tests were performed in triplicate.

RESULTS AND DISCUSSION

The in vitro antibacterial activities of the two species of *Ocimum (Ocimum basilicum L.* and *Ocimum basilicum var. purpurascens)* methanol extracts against the bacteria employed and their potentials were evaluated qualitatively and quantitatively by the presence or absence of inhibition zone. All the antibacterial effects against the pathogens were summarized in Table 4.2 in mean and \pm standard deviation. According to the results given, *O. basilicum L.* and *O. basilicum var. purpurascens* had a great potential of antibacterial activities which showed inhibitory effects against four strains of bacteria by disc diffusion method as similar in a few previous research.



Table 1: Diameter zone of inhibition (mm) from various concentrations of *Ocimum spp*. (mg/mL)(include disk=6mm)

Ocimum spp.	Tested bacteria	a Diameter of inhibition zone (mm)					
		500 (A)	1 000 (B)	3 000 (C)	5 000 (D)	Streptomycin (E)	
Ocimum	B. cereus	NI	9.00 ± 1.00	13.00 ± 1.73	16.33 ± 1.53	31.00	
basilicum L.	S. aureus	6.67 ± 0.29	7.67 ± 0.58	9.00 ± 1.00	10.67 ± 1.15	30.00	
	E. coli	6.50 ± 0.00	7.00 ± 0.00	7.67 ± 0.58	8.00 ± 1.00	27.00	
	S. typhimurium	6.83 ± 0.29	7.00 ± 0.00	8.67 ± 1.15	9.67 ± 1.15	27.00	
Ocimum	B. cereus	NI	8.67 ± 0.58	10.67 ± 0.58	11.00 ± 1.00	31.00	
basilicum var.	S. aureus	NI	7.33 ± 0.58	10.33 ± 0.58	11.33 ± 1.15	29.00	
purpurascens	E. coli	NI	8.00 ± 1.00	9.33 ± 0.58	10.67 ± 1.15	28.00	
	S. typhimurium	8.00 ± 0.00	8.33 ± 1.53	10.33 ± 0.58	10.67 ± 1.15	27.00	

NI = no inhibition zone

In agar disc diffusion test, *O. basilicum L.* expressed great inhibitory activity against Grampositive bacteria particularly towards *B. cereus* at subsequent concentrations (1 000, 3 000 and 5 000 mg/mL) which were 9.00 ± 1.00 mm, 13.00 ± 1.73 mm and 16.33 ± 1.53 mm respectively. At the same time, *O. basilicum var. purpurascens* showed maximum zone inhibition against *S. aureus* which is 11.33 ± 1.15 mm at 5 000 mg/mL. While, *B. cereus* was also strongly inhibited by *O. basilicum var. purpurascens* at 3 000 mg/mL and 1 000 mg/mL (10.67 ± 0.58 mm and 8.67 ± 0.58 mm). However, both of these extract did not expressed any inhibition zone against *B. cereus* at concentration below 1 000 mg/mL.

The results obtained showed that both of these *Ocimum spp.* least effective against Gram-negative bacteria particularly *E.coli*. The lowest activity of *O. basilicum L. and O. basilicum var. purpurascens* were observed against *E. coli* with the smallest inhibition zone. Nonetheless, *O. basilicum var. purpurascens* extracts expressed higher inhibition zone against *E. coli* than *O. basilicum L.* Other than that, these two *Ocimum*

spp. extracts able to inhibit S. typhimurium at concentration 500 mg/mL. These results indicated that these two Ocimum spp. extracts strongly inhibited Gram-positive bacteria than Gram-negative bacteria. These showed that Gram-positive bacteria is more sensitive towards these two Ocimum spp. extracts compared to Gram-negative bacteria. These findings had been supported by Khattab et al., (2015) which Gram-negative bacteria cell wall has an outer membrane acting as a barrier possess a high level of lipid materials which restricts the diffusion of hydrophobic compounds through its lipopolysaccharides.

Other major screening method used was minimum inhibitory concentration (MIC) which described as the lowest concentration that capable to inhibit bacterial growth and the determination of microbial growth was indicated visually by using MTT staining. This MTT staining convert to formazon and purple colour in the presence of living organisms. The MIC of these two *Ocimum spp.* were determined by microdilution method using 96-well microtiter plates in two-fold serial dilution to obtain final concentrations from 9.77 to 5 000 mg/mL. A lower MIC value indicates an efficient antimicrobial agent due to fewer

extract is required to inhibit the development of the bacteria.

Table 2: N	IIC values (mg/mL)	O. basilicum L.	and O. basilicum var.	purpurascens again	st selected bacteria

	Gram-posit	ive bacteria	Gram-negative bacteria	
	B. cereus	S. aureus	E. coli	S. typhimurium
O. basilicum L.	625	1 250	2 500	1 250
O. basilicum var. purpurascens	625	1 250	1 250	2 500

According to the Table 2, it was observed that the MIC values for both O. basilicum L. and O. basilicum var. purpurascens against the test pathogenic bacteria was ranged from 625 mg/mL to 2 500 mg/mL respectively. The lowest MIC values of O. basilicum L. extracts with the values of 625 mg/mL reflected the highest sensitivity against B. cereus to the test extract however showed lowest inhibition activity against E.coli at concentration 2 500 mg/mL. Whilst, the MIC values of O. basilicum var. purpurascens showed the same influence at 625 mg/mL against B. cereus and the lowest inhibition at 2 500 mg/mL against S. typhimurium. The control using the mixture of streptomycin and the inoculated bacteria did not showed any sign of color changed which indicated that there was no presence growth of bacteria.

The findings in this study support the observations of some researchers regarding the MIC of sweet basil methanol extracts. As reported by Gork *et al.*, (2017), *S. typhimurium* inhibited by methanol extracts of *O. basilicum L.* in 6 500 mg/mL but not inhibited by aqueous extracts in this bacterium as well as *L. monocytogene* that not suppressed by aqueous extracts. Other than that, Baldim *et al.*, (2017) also described in his investigation by using the essential oils

of O. basilicum L. which exhibit antimicrobial activity through MIC against the tested microorganisms. The results showed that the samples were more effective against S.aureus, L., monocytogenes and P. aeruginosa however less effective against B. cereus and Salmonella which indicated weak or moderate activity. Baldim et al., (2017) also suggested that concentration of linalool and concentration of essential components are the most crucial characteristics for effective antimicrobial compounds. In the difference solvent extraction of O. basilicum study conducted by Adiguzel et al., (2005), MIC values of the bacterial strains sensitive to the hexane extracts compared to methanol and ethanol extracts as stated in literature finfings. The basil hexane extracts expressed highest inhibition activity against E. coli, S. auereus, C. albicans, S. epidermis, B.macerans, *B. megaterium* and *P. putida*.

From the reviews and findings in this study, it was showed that the methanol extract of two *Ocimum spp.* was able to inhibit the activity of the tested microorganism. Both these extracts were more sensitive against Gram-positive bacteria particularly *B. cereus* and least inhibition effects against *S. typhimurium* for *O. basilicum var. purpurascens* and *E. coli* for *O. basilicum L.*

Gram-positive bacteriaGram-negative bacteriaB. cereusS. aureusE. coliS. typhimuriumO. basilicum L.ND2 5005 0002 500O. basilicum var. purpurascensND2 5002 5005 000

Table 3: MBC values (mg/mL) O. basilicum L. and O. basilicum var. purpurascens against selected bacteria

ND= not detected

The determination of MBC values were displayed from the MIC results which was spread to MHA plates and incubated for 24 hours. MBC is defined as the lowest concentration of test samples that required to kill a particular bacterium. The MBC values for these two *Ocimum spp*. were expressed in Table 3. In this study, the basil methanol extract successfully kill the tested bacteria at concentrations between 2 500 mg/mL to 5 000 mg/mL. The test sample showed highest bactericidal effect against *S. aureus* and *S. typhimurium*. Whilst, the test sample of O. *basilicum var. purpurascens* appeared to have a weak bactericidal activity against *S. typhimurium* at 5 000 mg/mL however showed strong bactericidal effect against *S. aureus* and *E. coli* at concentration 5 000 mg/mL.

Nonetheless, both of these extracts expressed bacteriostatic activity against B. cereus even at higher concentration which indicated that this Ocimum spp. extracts inhibit bacterial replication without killing the organism. This result obtained means that, the *B. cereus* had highest sensitivity within overnight however the bacterial started to regrowth after 24h which imply that the test samples had less effectiveness against B. cereus. The results showed organism proliferation because the antibacterial agents did not cause death against the B. cereus. These findings correlated to study proclaimed by Moghaddam et al., (2011) that had investigated the essential oil of O. basilicum against B. cereus and had found that the MBC concentration was not detectable. These issues had been explained by Levison and Levison (2013) which the bacterial growth after a brief

exposure of bacteria to an antibacterial agent due to the absence of host defences which commonly referred to as post antibiotic effect (PAE).

Pandey et al., (2014) reported on her study that the action of the essential oil of the sweet basil exhibited minimum bactericidal activity against E.coli strain. However, in Moghaddam et al., (2011) study, the MBC values of O. basilicum L. against E.coli and P. aeruginosa was not countable due to the lower concentrations of the extracts. In the recent study researched by Beatovic et al., (2015), the findings correspond with this study which the essential oils of O. basilicum var. purpurascens were more sensitive against Gram-negative bacteria. E.coli and S. typhimurium and less sensitive against Gram-positive

bacteria such as S. aureus, B. cereus, L. monocytogenes, M. flavus, Ps. aeruginosa and En. faecalis.

According to Krishnan et al., (2010), antimicrobials which in the MBC/MIC > 4 are considered bacteriostatic agents while bactericidal activity has a ratio of MBC to MIC of ≤4. The data presented in the Table 4 had demonstrated that these antibacterial properties of two Ocimum spp. have considerably efficient MBC:MIC ratio that was 2 (i.e <4). Thus, both of these *Ocimum spp*. methanol extract were likely to have a strong bactericidal attributes on these tested pathogenic bacteria (S. aureus, E. coli and S. typhimurium) when exposed to the concentration above the MIC. While, MBC/MIC ratio was not determined against B. cereus since the MBC values were not detectable as the bacteria started to regrowth.

Table 4: MIC, MBC values and MBC/MIC ratio values of O. basilicum L. and O. basilicum var. purpurascens

			extracts				
	Ocimum basilicum L.			Ocimum basilicum var. purpurascens			
	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC	
B. cereus	625	-	-	625	-	-	
S. aureus	1 250	2 500	2	1 250	2 500	2	
E. coli	2 500	5 000	2	1 250	2 500	2	
S. typhimurium	1 250	2 500	2	2 500	5 000	2	
		MOCAUC D	1 ()()				

MBC/MIC= Ratio value of MBC over MIC

Given the results from this experiment indicated that among the crude extract methanol of O. basilicum L. and O. basilicum var. purpurascens, MBC values at 2 500 mg/mL produced the most potent extract for use against Gram-positive and Gramnegative bacteria particularly S. aureus, E. coli and S. typhimurium. A few results from the present study correlated with the findings in this research however apart of the results were generally difference according to the other studies. The observed difference may be probably environmental factors as the basil is strongly dependent on environmental conditions during growth, including temperature, nutrient availability in soil and seasonal geographical and climate version which can influence the bioactive constituents in the basil extract.

CONCLUSION

The present work showed that the methanol extract of O. basilicum L. and O. basilicum var. purpurascens possess potent and antibacterial activity against Gram-positive and Gram-negative bacteria which include B. cereus, S. aureus, E. coli and S. typhimurium. The agar disc diffusion method exhibit good inhibition zone on Gram-positive bacteria and showed less effectiveness against E. coli. Whilst, the MIC determination showed high inhibitory activity towards B. cereus on both of the crude extracts and Gram-negative more resistant against bacteria. However, in the MBC determination the B. cereus did not show any bactericidal effects towards both of these extracts due to regrowth of the bacteria. In the

MBC/MIC ratio, both of Ocimum spp. extracts showed same ratio (<4) which indicate that the three bacteria (S. aureus, E. coli and S. typhimurium) were sensitive towards these methanol extracts.

Further work is required to analyse the chemical components of these extracts particularly for O. basilicum var. purpurascens so in order to isolate the specific antibacterial and determined the action of the mechanism. This is important because these extracts could be potentially used to replace current synthetic antibiotics in treatment of infectious diseases. Besides that, other solvents may be used for the production of the sample extracts since the selection of solvent are crucial to extract variety of bioactive compounds.

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