Comparison of Methods of Extraction and Antimicrobial Activity of Six *Ocimum* Species against Human Pathogens

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Abstract

Extracts from plants continue to be a vital source of medicines, Ocimum, (family Labiatae) is well known for treatment of a wide range of human illnesses. This study evaluates the extraction methods best suited for the determination of the antimicrobial potential of basil. The methanolic extract of O. gratissimum species had the highest yield (1.48±1.48%), while the essential oil of O. tenuiflorum had the lowest (0.19±0.30%). Comparison of methanolic extracts of different accessions, O.tenuiflorum (MSR1), O.basilicum (PI 172996) showed a growth reduction of 74%, for S. pyogenes, 91% for E. coli respectively; while essential oils of O. basilicum (PI 358472) had a growth reduction of 57% for S. pyogenes, 72% for E. coli. The chloroform extracts exhibited little or no inhibitory effects. In conclusion, the methanolic extracts, and essential oils were equally effective against the tested bacteria. Ocimum species may be used as an alternative source in the search for agents to treat bacteria.

Keywords: *Ocimum*, crude extracts, essential oils, antibacterial activity, bioscreen analyzer, *Streptococcus pyogenes* and *Escherichia coli*

1. Introduction

Since ancient times, plants have played a vital role in the development of human civilizations. Today, plant-derived antimicrobials (PDAs) are being extensively used in alternative therapeutic strategies to combat microbial infections (Upadhyay et al., 2014), and also for preparing drugs, bioactive compounds, pharmacological tools and herbal remedies for various medicinal applications (Fabricant & Farnsworth, 2001). Over time, commercial antimicrobial drugs become ineffective against certain diseases when the causal bacteria become drug resistant (Jiyauddin et al., 2015). In addition to this problem, antibiotics sometimes associated with adverse effects on the host including hypersensitivity, immune-suppression and allergic reactions (Bharathi, Kolanjinathan, & Saranraj, 2014). For these reasons, it is becoming increasingly important to investigate newer drugs. Plants could be cheaper and safer sources of natural antimicrobials (Devi et al., 2017; Jiyauddin et al., 2015). Essential oils and volatile constituents extracted from aromatic plants are frequently used in folk medicine for prevention and treatment of different human diseases (Devi et al., 2017). Some of these extracts have an antioxidant or hormone-like effect which helps in fighting against many diseases including cancer, heart disease, diabetes, high blood pressure, etc., and may also prevent the formation of carcinogens on their target tissues (Prasad et al., 2012).

Some of the microorganisms that need to be controlled include *E.coli* and *S.pyogenes*. Diarrhea caused by *Escherichia coli* is a serious problem, and it is one of the common causes of morbidity and mortality among neonates in animals, and infants in developing countries (Shweash et al., 2014). *Escherichia coli* is a bacterial pathogentransmitted through contaminated food and water (Esena & Owusu, 2013), and also, this bacteria can survive and persist in numerous environments such as soil, as well as in animal reservoirs (Lim, Yoon, & Hovde, 2010).

The bacterial pathogen, *S. pyogenes* is associated with pharyngitis (Limsuwan & Voravuthikunchai, 2013), skin infections such as cellulitis, furuncles, superficial abscesses, and wound infections to life-threatening diseases such as necrotizing fasciitis or gas gangrene(Hindi et al., 2016).

Several species of the Labiatae family serve as sources of spices worldwide. Many of these species have proven medicinal properties considered as a consolidated source of extracts with potent antibacterial and antioxidant properties (Kaya, Yiğit, & Benli, 2008). The genus *Ocimum*, a member of a *Labiatae* family, is considered, an important source of medicinal plants with therapeutic potential (Pandey, Singh, & Tripathi, 2014). Most of the species belonging to genus *Ocimum* are grown and frequently cultivated in several countries of East Asia, Europe, America and Australia (Pandey et al., 2014; Verma et al., 2011). The objective of the study was to evaluate the extraction methods best suited for the determination of the antimicrobial potential of *Ocimum* species against human pathogenic bacteria.

2. Materials and Methods

2.1. Plant Materials

Six-week-old greenhouse grown seedlings of six *Ocimum* species, *O. x africanum, O. americanum*, *O. basilicum*, *O. campechianum*, *O. gratissimum* and *O. tenuiflorum* (Table.1) were transplanted on to raised beds (50 cm wide, 15 cm high, 25 m long, 2 m apart, covered with 4 mm black plastic with drip irrigation tubing underneath the plastic) at the Alabama A&M Winfred Thomas Agricultural Research Station located in Hazel Green, AL (latitude 34°89'N and longitude 86°56'W). Soil at the experimental site is a Decatur silty loam (fine, kaolinitic, thermic Rhodic Paleudult). Fresh leaves from the plants of each species at peak vegetative phase were collected early morning and placed immediately in a cooler with ice packs for transportation to the laboratory for further processing.

2.2. Chloroform extract

About 100g of leaves of plants from were cut into small pieces and soaked in chloroform (1:3 w/v) which incubated at room temperature for seven days. The slurry was filtered allowed to evaporate under room temperature stored under the 4 ^{0}C until analysis. Dry crude leaf extract content expressed in mg/100g dry weight (DW) see Table 1(Mousavi, Salleh, & Murugaiyah, 2014).

2.3. Methanolic extract

About 100g of leaves of plants were cut into small pieces and soaked in methanol(1:3 w/v) which incubated at room temperature for seven days. The slurry filtered allowed to evaporate under room temperature stored under the 4^{0} C until analysis. Dry crude leaf extract content was expressed in mg/100g dry weight (DW) see Table 1 (Mousavi et al., 2014).

2.4. Isolation of Essential Oil

The essential oil (EO) extracted from 100g fresh basil leaf material was hydro-distilled for 3 hours in a Clevenger-type apparatus, per the British Pharmacopoeia specification. Essential oil content expressed in ml/ dry weight (DW) see Table 1. The EO were collected and stored at 4°C until analysis (Ajao et al., 2017; Shadia et al., 2007).

2.5. Bacterial Cultures and Culture Conditions

The chloroform, and methanolic leaf extracts, and EO obtained from the six *Ocimum* species were tested for their relative efficacy on human-animal pathogenic bacteria: Gram-positive bacteria (*S. pyogenes*), and Gram-negative bacteria (*E. coli*).The overnight cultures were grown on BHI broth at 37°C, and diluted to provide a final concentration of approximately 10 ^{5} colony forming units per ml (CFU/ml), and adjusted according to turbidity using 0.5 McFarland tube scale (Ajao et al., 2017; Shadia et al., 2007).

	Plant species	[1]P.I. Number	[*]Country of Origin	[a]CH	[a]ME	[b]EO
1	*O. x africanum*					
		500942	Zambia	0.48	1.44	0.22
		500943	Zambia	0.46	1.11	0.52
		500947	Zambia	0.52	1.38	0.49
	Mean Yield Content			0.49±0.12%	1.31±0.69%	0.41±0.65%
2	*O. americanum*					
		652058	Togo	0.61	1.61	0.25
		500945	Unknown	0.52	1.01	0.19
	Mean Yield Content			0.57±0.18%	1.31±1.17%	0.22±012%
3	*O. basilicum*					
		172996	Turkey	0.3	1.27	0.16
		173746	Turkey	0.16	1.44	0.6
		358472	Macedonia	0.74	0.98	0.21
		652071	California	0.25	1.15	0.13
	Mean Yield Content			0.36±1.0%	1.21±0.76%	0.28±0.86%
4	*O. campechianum*					
		652066	Brazil	1.06	1.77	0.39
5	*O. gratissimum*					
		652067	Brazil	0.42	1.68	0.17
		652069	Brazil	0.21	1.91	0.19
		211715	Taiwan	1.47	1.16	0.43
		500952	Zambia	0.24	1.16	0.1
	Mean Yield Content			0.59 ±2.33%	1.48±1.48%	0.22±0.57%
6	*O. tenuiflorum*					
		414202	Maryland	1.0	1.28	0.16
		652056	Denmark	0.34	1.0	0.17
		652057	Cuba	0.6	1.42	0.3
		MSR1	India	1.27	1.37	0.13
	Mean Yield Content			0.80±1.61%	1.27±0.73%	0.19±0.30%

Table 1: List of plants and their yield of leaf extracts used for crude extracts chloroform, methanol and EO (Plant species,[1]P.I. Number - Plant Introduction Number, [a][amount of crude extract (g)/amount of basil fresh leaf (g)]/100 (w/w), [b][amount of EO (ml)/amount of basil fresh leaf (g)]/100 (w/w))

2.6. Antibacterial Assay- Bioscreen Assay

The bacterial growth rate was determined using the Bioscreen C a wideband filter between 420-580nm (Bioscreen C, Lab system, Helsinki, Finland). Measurements were then processed to generate microbial growth curves, plotting turbidity vs. time according to the manufacturer's instructions(Lambert, Johnston, & Simons, 1998).The streptomycin sulfate served as positive control (Ajao et al., 2017; Mann, 2012). An aliquot of 150 µL of bacterial suspension,20 µL of reconstituted of either chloroform extract, methanolic extract and EOwith 1% DMSO, were placed into different wells of the honeycomb titer plate (Bharathi et al., 2014). Subsequently, the plateincubated at 37°C, the bacterial growth rate determined for a total of 24 h at regular intervals of 30 min each.

2.7. Statistical Analyses

The crude and oil yield mean content, yield percent difference per 100 g leaves of each six *Ocimum* species are observed significantly at ($p < 0.0001$).We compared the yield percentage by

$$\frac{\text{Mean of species A-Mean of species B}}{\text{Mean of species A}} \times 100.$$

The mean OD units ’differences obtained for experimental groups calculated as a mean± standard deviation (SD) of three independent measurements using the Microsoft Excel program, 2004.

Percentage change as compared to control was analyzed. Data subjectedto one-way analysis of Variance (ANOVA), P values ≤ 0.01 regarded has been significant.

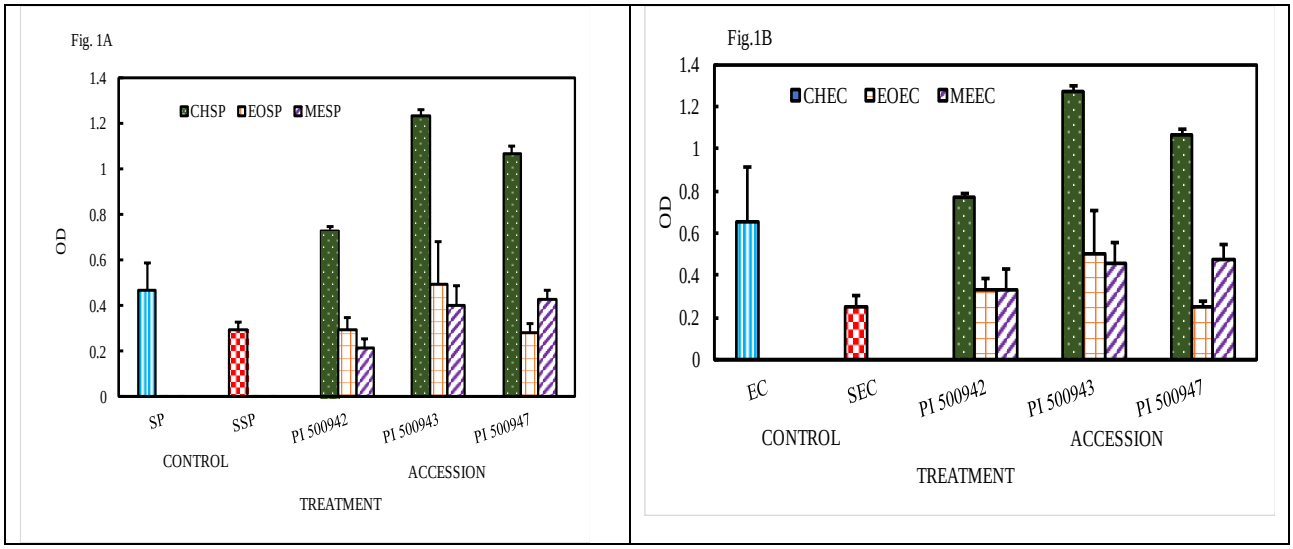
3. Results and Discussion

3.1. Crude yields in g for chloroform, methanolic extracts and *Ocimum* species Essential oil (EO).

Table 1 shows there were significant differences in the relative average crude and EO oil yield of leaf extracts obtained from each *Ocimum* species used in this study. The average methanolic leaf extract yield of O. *x africanum* species was highest at 1.31±0.69%, while the chloroform extract was0.49±0.12%and, EO was 0.41±0.65%. Among the six-species tested, the mean methanolic extracts of O. *x africanum* ranged from 1.11g for (PI 500943) and, 1.44 g (PI 500942). The EO yield of O. *x africanum* species varied from 0.22 g (PI 500942) and, 0.52 g (PI 500943). The EO yields of five different species (*O. tenuiflorum, O. americanum, O. gratissimum, O. basilicum,* and *O. campechianum*) compared to *O. x africanum* were less by 54%, 46%, 46%, 34%, and 5% respectively. The mean methanolic leaf extract yield of *O. americanum* accessions was highest at 1.31±1.17%, while chloroform extract 0.57±0.18% and, EO was 0.22±0.12%. The average methanolic leaf extract yield of *O. basilicum* accessions was highest at 1.21±0.76%, while the yield for the chloroform extract was 0.36±1.0%, and EO was 0.28±0.86%. The mean methanolic yield of *O. basilicum* species ranged from 0.98g (PI 358472) and,1.44g (PI 173746), while EO yield varied from 0.13g (PI 652071) and,0.60 g (PI 173746). The mean methanolic leaf extract from *O. campechianum* was highest (1.77 g) as compared to the chloroform leaf extract (1.06 g) and EO (0.39 g). In comparison, to the yield of *O. campechianum,* the chloroform yields of the five-different species (*O. tenuiflorum, O. americanum, O. gratissimum, O. basilicum,* and *O. campechianum*) were less by 54%, 46%, 66%, 47%, and 25% respectively. The methanolic yields of the five-different species (*O. tenuiflorum, O. americanum, O. gratissimum, O. basilicum,* and *O. campechianum*) compared to *O. campechianum* were less by 61%, 26%, 32%, 16%, and 28% respectively. The mean methanolic yield of *O. gratissimum* was highest at 1.48±1.48%, while the yield for chloroform extract was 0.59 ±2.33% and EO was 0.22±0.57%.The mean methanolic extracts from *O. gratissimum* ranged from 1.16g (PI 211715) and, 1.68g (PI 652069). The mean methanolic yield of *O. tenuiflorum* was highest1.27±0.73%, while the yield for chloroform extracts 0.80±1.61% and EO 0.19±0.30%. The mean methanolic extracts from *O. tenuiflorum* ranged from 1.0g (PI 652056) and, 1.42g (PI 652057).

From the above results, the study shows that the methanolic extracts gave excellent extract yield compared to either chloroform extracts or EO for all the six test-species. Methanol was a better extraction solvent than chloroform, with substantially higher oil yields for all six-*Ocimum* species in this study. This was also observed by (Njume, Jide, & Ndip, 2011), in their study methanol and ethyl acetate. Methanol was quantitatively the best solvent for extraction. The research by (Cock et al., 2016), is also consistent with the results of our study indicating that methanol is a suitable solvent for the extraction of bioactive compounds from plants.

3.2. Antibacterial activity of crude extracts of chloroform, methanol, and EO of *Ocimum* species



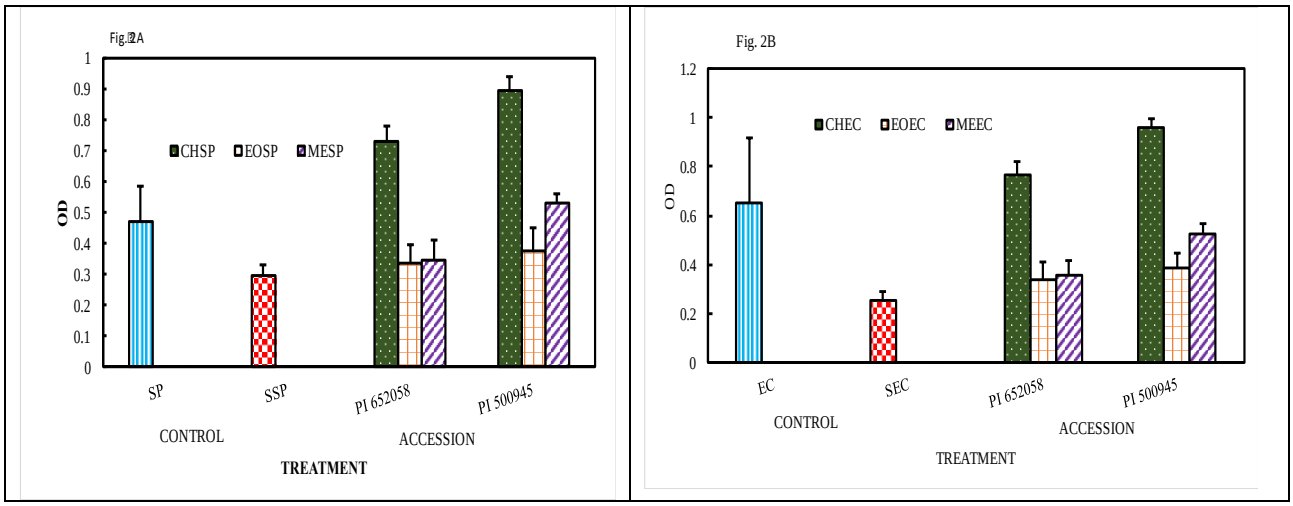
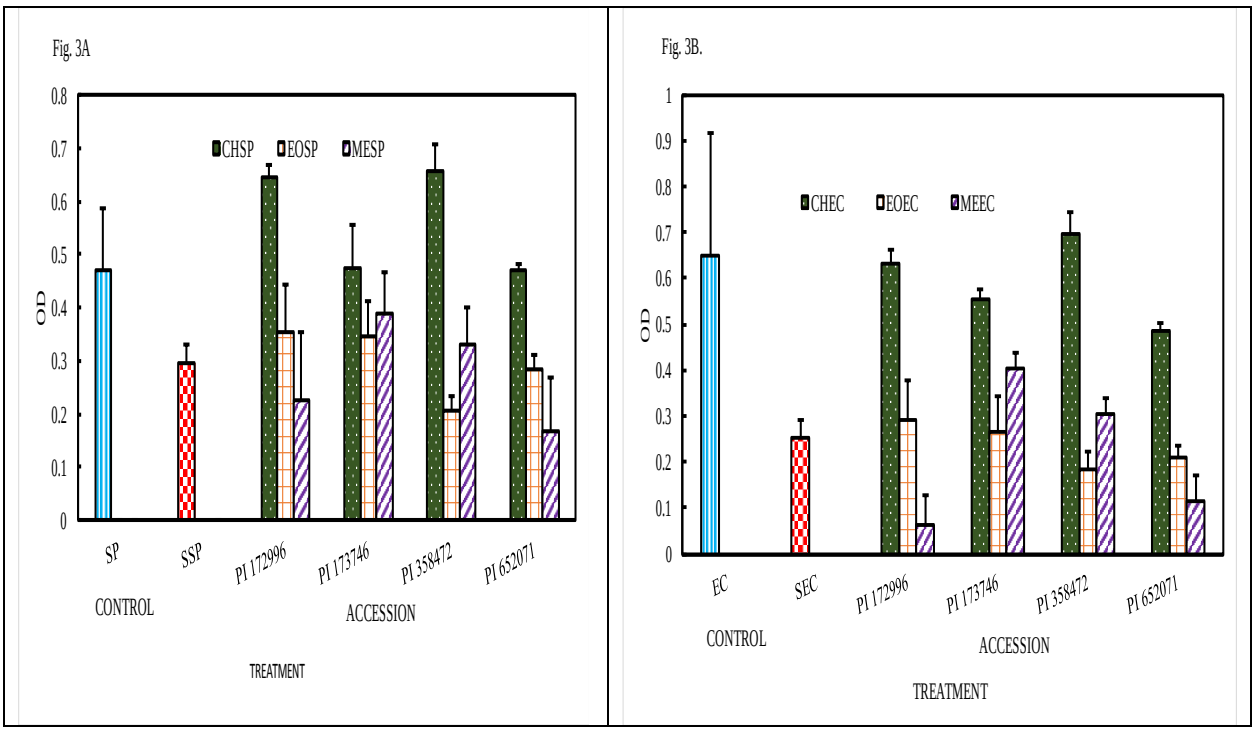


Fig. 1A and 1B: Comparison of growth inhibition over time of *S. pyogenes* and *E. coli* treated with chloroform, methanol and, EO leaf extracts of *O. x africanum.*

Fig. 2A and 2B: Comparison of growth inhibition over time of *S. pyogenes* and *E. coli* treated with chloroform, methanol and, EO leaf extracts of *O. americanum.*

Fig. 1A shows the OD values of the methanolic extract from *O. x africanum* (PI 500942), as 0.22 and growth reduction of 53% for *S. pyogenes*. The OD values of the EO extract from *O. x africanum*, PI 500947 was 0.24 (Fig. 1B), and the growth reduction was 63% for *E. coli*. In this study methanolic and EO extracts from O. *x africanum* inhibited the growth of both *S. pyogenes* and *E. coli*. Kawsud, Puripattanavong, & Teanpaisan, 2014; Teanpaisan, Kawsud, Pahumunto, & Puripattanavong, 2017 in their studies indicated the anticandidal and anti-biofilm effects of ethanol extracts of O. *x africanum.*

The EO from *O. americanum* PI 652058, had OD 0.34, and growth reduction was28% for *S. pyogenes* (Fig. 2A);while the EO from both *O. americanum* PI 652058 and PI 500945 had OD of 0.34 and 0.38respectively. The growth reduction was 48%, and 42% for *E. coli* respectively(Fig. 2B).The EO from *O. americanum* was very effective against both test bacteria in this study. In a previous study by Thaweboon & Thaweboon(2009),using oral bacteria the EO effect was similar to the results obtained in the present study.



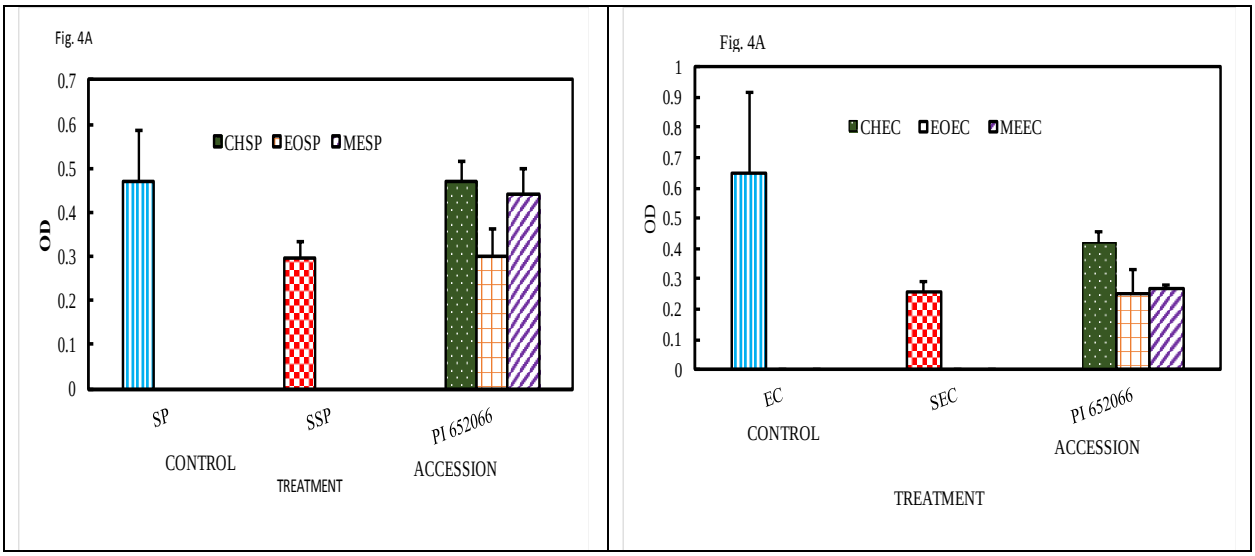


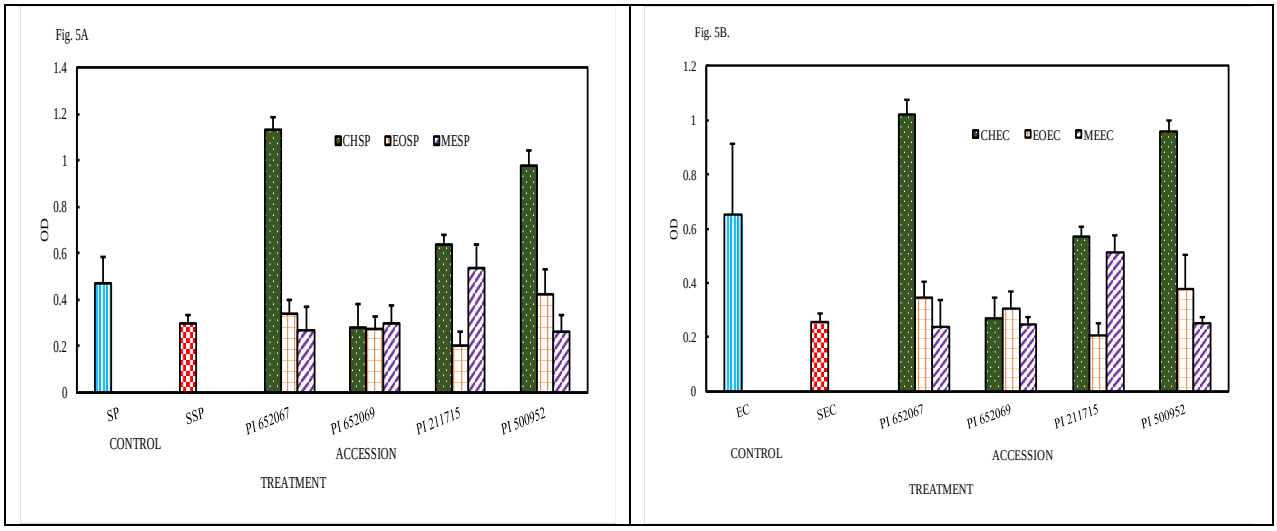
Fig. 3A and 3B: Comparison of growth inhibition over time of *S. pyogenes* and *E. coli* treated with chloroform, methanol and, EO leaf extracts of *O. basilicum*.

Fig. 4A and 4B: Comparison of growth inhibition over time of *S. pyogenes* and *E. coli* treated with chloroform, methanol and, EO leaf extracts of *O. campechianum.*

From the four accessions of *O. basilicum* used in this study, two showed effective antibacterial activity. The EO extract from PI 358472 and methanolic extract of PI 652071 had OD 0.20 and 0.16and growth reductions of 57% and 65% for *S. pyogenes* respectively (Fig. 3A).According to Cock et al., (2016), the aqueous and methanolic leaf extracts of Native Australian basil also inhibited the growth of *S. pyogenes.*

The methanolic PI 172996 and EO extract from PI 358472 had OD 0.06 and 0.18 and, growth reductions of 91%, 72% for *E. coli* respectively (Fig. 3B).These extracts greatly inhibit the growth of *E. coli.* According to (Azam & Saba, 2016), basil EO substantially inhibit the growth of *E.coli.* Figures 4A and 4B show that O. *campechianum* (PI 652066) EO had37% and 61%growth reduction of *S. pyogenes* and *E. coli* as well as OD values of 0.30 and 0.25 respectively. The EO inhibits the growth of *E. coli* more than it inhibits the growth of *S. pyogenes*.

From the four accessions of *O. gratissimum* used in this study, the OD values from the methanolic extract of both PI 652067 and PI 500952 was 0.26, and EO extract from PI 211715 was 0.20 with growth reduction of 45% and 57% for *S. pyogenes* respectively. The methanolic extract from PI 652067 and EO extract ofPI 211715 showed OD as 0.23 and 0.20, and growth reduction was 65% and 69% for *E. coli* (Fig. 5B). The EO inhibits the growth of *E. coli* more than it inhibits the growth of *S. pyogenes*. According to Olamide and Agu, (2013), reported that there was no significant difference in the efficiency of wild basil extractsof *O. gratissimum* and bitter leaves on the growth of *E. coli*andother enteric pathogens used in their study.



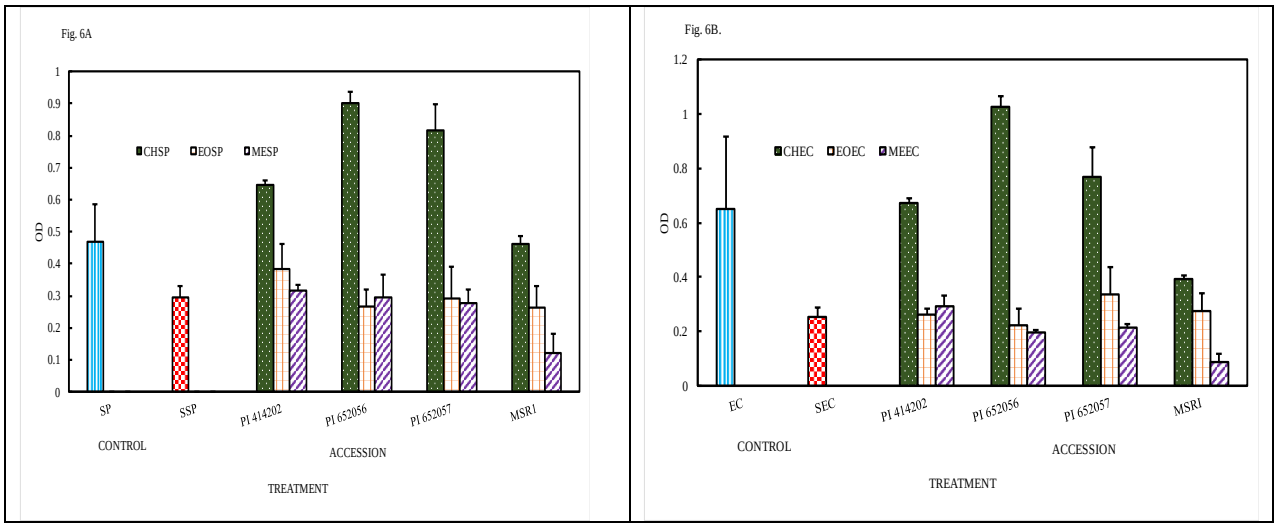
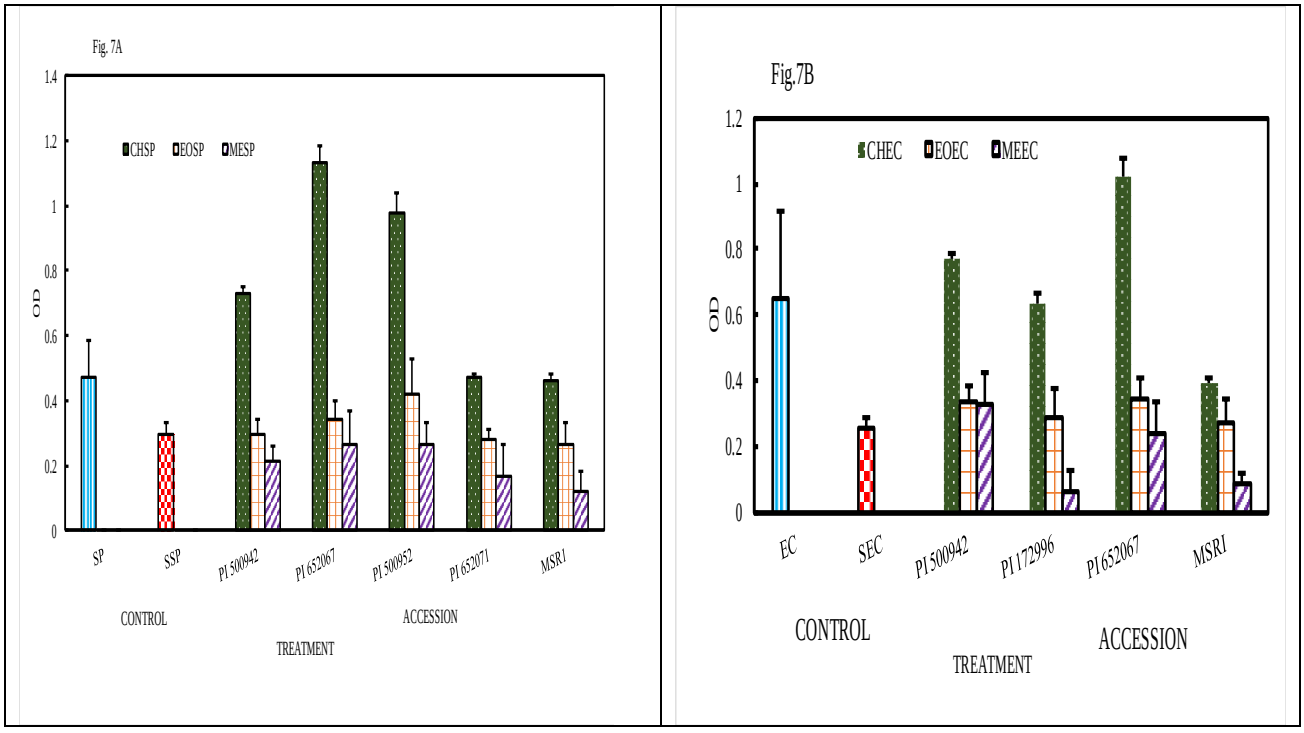


Fig. 5A and 5B: Comparison of growth inhibition over time of *S. pyogenes* and *E. coli* treated with chloroform, methanol and, EO leaf extracts of *O. gratissimum*.

Fig. 6A and 6B: Comparison of growth inhibition over time of *S. pyogenes* and *E. coli* treated with chloroform, methanol and, EO leaf extracts of *O. tenuiflorum*.

Figures 6A and 6B show that four accessions of *O.tenuiflorum*used in this study, two showed effective antibacterial activity. The methanolic extract from MSR1(Indian basil), EO extract of PI 652056 had OD at 0.12, and 0.26 with growth reduction of 74%, and 45% for *S. pyogenes* respectively. The methanolic extract from MSR1 and EO extract of PI 652056; OD as 0.08 and 0.22, with the growth reduction of 88%, and 66% for *E. coli*. The methanolic extract inhibited the growth of *E. coli* more than it inhibits the growth of *S. pyogenes*. Subramanian, 2014 reported that *O. tenuiflorum* extracts inhibited the growth of *Staphylococcus aureus* and *Candida albicans.*



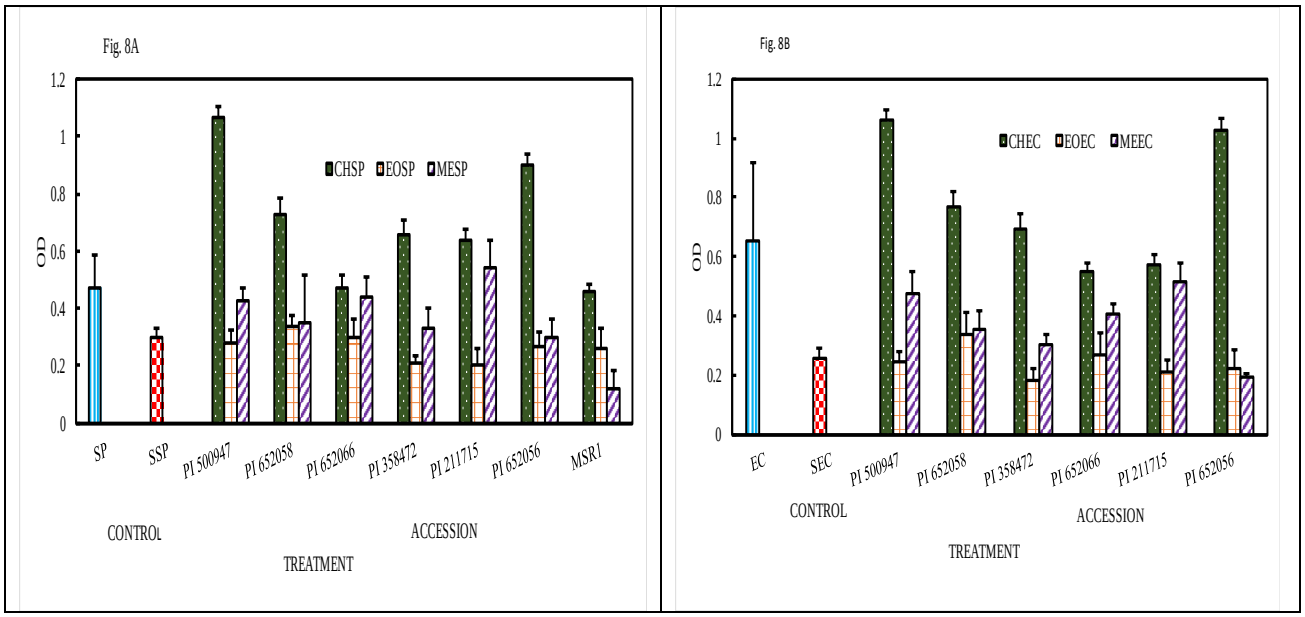


Fig. 7A and 7B: Comparison of methanolic extract inhibitory effect on *S. pyogenes* and *E. coli* treated with chloroform, methanol and, EO leaf extracts of six basil species

Fig. 8A and 8B: Comparison of EO extracts inhibitory effect on *S. pyogenes* and *E. coli* treated with chloroform, methanol and, EO leaf extracts of six basil species

Key: Fig. 1A through 8A: Fig 1B through 8B

CHSP- chloroform extract and *S. pyogenes*;

EOSP- EO and *S. pyogenes*;

MESP-methanolic extract and *S. pyogenes*;

SSP- Streptomycin and S. *pyogenes*

CHEC- chloroform extract and *E. coli*;

EOEC- EO and *E. coli*;

MEEC-methanolic extract and *E. coli*;

SEC-Streptomycin and *E. coli*;

Error bar signifies the ±1 standard deviation from the sample means at OD 420–580.

Figure 7A shows the comparison of methanolic extract treatment of six *Ocimum* species. The OD values for different accessions range from 0.12 to 0.26 for *O. tenuiflorum* MSR1,*O.basilicum* PI 652071, *O.x africanum* PI 500942, *O. gratissimum* both PI 652067, PI 500952 accessions had growth reductions of 74%, 66%, 53%, 45%, and 45% for *S. pyogenes* respectively.The methanolic extract of *O.tenuiflorum* had greatly inhibited the growth of *S. pyogenes* when compared to all other species.Figure7B shows the OD values for methanolic extracts of six-*Ocimum* species, range from 0.06 to 0.33 and growth reduction of 91%, 88%, 72%, 65%, and 49% for accessions of *O. basilicum* PI 172996,*O.tenuiflorum* MSR1, *O. gratissimum* PI 652067 and *O. x africanum* PI 500942 when compared to control wells of *E.coli*. The methanolic extract from *O.basilicum* had highest effect on *E.coli* when compared to all other species.The control wells showed OD units were 0.47 and 0.65 for both *S. pyogenes* and *E. coli*, respectively. From the above results we conclude that methanolic extracts of basil generally are effective in controlling the growth of *S. pyogenes* and *E. coli* because extracts contain flavonoids and therefore are biologically active against human pathogens. This is similar to the conclusion of Rai, Ghosh, & Basheer, (2016). Flavonoid bioactive compounds are soluble in polar solvents such as methanol (Naidu, Naidu, & Sujatha, 2013). In this study, we observed that extracts all six basil species using chloroform solvent exhibited little or no inhibitory effect, probably because chloroform is non-polar solvent and the yield of polyphenols from the plants materials may below. This is similar to the conclusion of Zlotek, Mikulska, Nagajek, & Swieca, 2016. Figure 8A shows the comparison of EO inhibitory effect of six *Ocimum* species. The accessions of *O. basilicum* PI 358472 and *O. gratissimum* PI 211715 showed similar growth reduction of 57%, *O. tenuiflorum*PI 652056 and MSR1 of 45%, *O. x africanum* PI 500947,*O. campechianum* PI 652066 and *O.americanum* PI 652058 of 40%, 36%, and 28%, respectively on *S. pyogenes* with OD values ranged from 0.20, and 0.34.

Figure 8B, the OD values varied from 0.18 to 0.27 were observed for *O. basilicum* PI 358472,*O. gratissimum* PI 211715, *O. tenuiflorum* PI 652056, *O. x africanum* PI 500947, *O. campechianum* PI 652066, and *O. americanum* PI 652058 had growth reductions of 72%, 69%, 66%, 63%, 62%, and 48% for *E. coli*.The EO extract of *O. basilicum* inhibits the growth of *E. coli* more than it inhibits the growth of *S. pyogenes* when compared to all other species. From above results we conclude that susceptibility of E. *coli* for basil EO were higher than Gram-positive bacteria *S. pyogenes.*This may be dueto streptococcal biofilms which have been shown to exhibit high antibiotic resistance(Harper et al., 2014). In our study, all basil EO were effective on *E.coli*, which is similar to the conclusion of (Carović-Stanko et al., 2010).The EO are considered an important source of bioactive substances which are highly potent antimicrobial agents in comparison to conventional antibiotics(Devi et al., 2017). Saharkhiz et al., (2014), using *O. tenuiflorum* extracts indicate that the efficacy of EO mechanisms are enhanced by their unique difference from those of the antibiotics.

4. Conclusion and Future Research

Many human-pathogenic microorganisms, which cause damage to human health, exhibit drug resistance due to the over-use of antibiotics. *S. pyogenes* still show multiple-resistance to drugs such as the macrolide and lincosamide antibiotics. The high antibacterial effects of essential oils from basil species may be useful in developing natural drugs, as well as prevent the spread of resistant bacterial strains. The EO and herbal extracts have attracted a great deal of scientific interest due to their potential as a source of natural antioxidants and biologically active compounds. The present study provides evidence that efficacy of EO is solvent-dependent.

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