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EXTRACTION OF ESSENTIAL OILS FROM CLOVE BUD (SYZYGIUM AROMATICUM), AND CEYLON CINNAMON (CINNAMOMUM ZEYLANICUM), AND THE DETERMINATION OF THEIR ANTIBACTERIAL ACTIVITY ON S. AUREUS AND E. COLI

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ABSTRACT

In recent times, bacteria have been shown to display acquired resistance against antibiotics that were previously effective in treating pathogenic infections caused by such bacteria, by altering their metabolic processes, cellular structure and genetic material. In a bid to combat this drug resistance, the use of natural sources of antibacterial agents have been explored, and essential oils of herbs and spices offer a potential outlook. Clove bud (Syzygium aromaticum), and Ceylon Cinnamon (Cinnamomum zeylanicum) are spices that are commonly used in Sri Lankan households, especially in the preparation of food. The objectives of this study was to extract the essential oils from these spices via hydrodistillation, and to determine their antibacterial activity individually, and in combination with antibiotics. The spices were dried and processed to obtain a fine powder, following which, were subjected to hydrodistillation and then centrifugation, to obtain the essential oil. The antibacterial activity of the essential oil alone, and in combination with ciprofloxacin and chloramphenicol were determined using the disc diffusion method against Staphylococcus aureus (S. aureus) and Escherichia coli (E. coli). The effect of storage on the antibacterial activity of the essential oils were also determined by storing extracted oils for 7 days. Results showed clove bud and cinnamon essential oils to possess antibacterial activities alone, as well as in combination with the tested antibiotics. Results also showed an overall reduction in the antibacterial activity of the 7 day-stored essential oil than the fresh essential oil sample.

Keywords: antibacterial activity, Cinnamomum zeylanicum, Escherichia coli, Staphylococcus aureus, Syzygium aromaticum

INTRODUCTION

Essential oils, also known as volatile oils are liquid extracts obtained from plants that have been known to contain numerous health benefits. The use of these medicinal plants as cosmetics, food preservatives, and for the treatment of various ailments, date back to centuries ago. With the advancements made in science, a deeper approach into the various uses of essential oils and their constituents have received great interest (Dilworth, Riley and Stennett, 2017). Such uses of essential oils can be seen in the food industry as flavourings, aromatics and preservatives, in the cosmetics industry as fragrances, and in the pharmaceutical industry as natural alternatives for various medications (Man et al., 2019). Bioactive compounds found to be present in essential oils have been found to result in the various properties of essential oils such as their anti-fungal, anti-oxidative and anti-bacterial effects. Essential oils are

made up of a complex of bioactive compounds such as aromatic compounds, terpenes, and terpenoids (such as aldehydes, ketones, phenols and alcohols) which give rise to these properties (Elshafie and Camele, 2017; Rezzoug et al., 2019).

Oils can be extracted from various plant parts such as the roots, barks, berries, seeds, rhizomes, leaves and flowers, using various techniques. Some of the more frequently used extraction methods are hydrodistillation, steam distillation, solvent extraction, and maceration. Hydrodistillation is one of the oldest and commonly used method of extraction. It does not require the use of various organic solvents for the extraction, and does not require the sample to be dehydrated prior to extraction (Oreopoulou, Tsimogiannis and Oreopoulou, 2019). Hydrodistillation requires the sample to be immersed in water and boiled in direct heat, from which the water vapour travels along with the essential oil and is condensed in a separate container. Unlike other methods of extraction, hydrodistillation can be used to extract oils from samples which are powdered or finely cut. Additionally, hydrodistillation is a relatively simple setup with a low expense, and can be carried out easily, to obtain a highest possible yield Of the numerous medicinal plants available, in Sri Lanka, several herbs and spices stand out as those possessing very high antibacterial properties, and are used in day-to-day food preparations. These include; Clove bud (Syzygium aromaticum), and Ceylon Cinnamon (Cinnamomum zeylanicum) (Silva and Domingues, 2015). Clove is a spice plant that is commonly found to grow in Spice Islands, and is used in African, Asian and Middle Eastern cuisines. Mith et al., 2014 found that Clove bud oil possessed strong antibacterial activity against Gram-positive and Gram-negative bacteria. Cinnamon is referred to as the dried bark of the Cinnamomum zeylanicum tree, and is used in various industries across the world. Its main uses are the food industry, cosmetic industry and as an alternative medicine. Cinnamon essential oil possesses an anti-bacterial effect on numerous bacteria, and is sometimes used as an alternative to synthetic pharmaceuticals (Nabavi et al., 2015; Kaya, Yigit and Benli, 2008).

The scope for the use of essential oils as natural alternatives to various synthetic pharmaceuticals have increased in the recent past. Currently, with the misuse of antibiotics, an increase in various antibiotic-resistant strains of bacteria have been seen to be arising. This has led to an increased interest the use of essential oils against various antibiotic-resistant bacteria (Chouhan, Sharma and Guleria, 2017). In Sri Lanka, a trend in the increase of antibiotic-resistant cases of S. Aureus and E. Coli infections has been observed. A prospective remedy for this is the use of essential oils from herbs and spices commonly found in Sri Lanka, and the determination of the anti-bacterial activity (Tissera et al., 2017; Jayatilleke and Bandara, 2019). For this study, the essential oils of clove bud, and Ceylon cinnamon are tested for their anti-bacterial properties against S. Aureus and E. Coli.

Agar well diffusion and Kirby-Bauer disk diffusion are two of the most commonly Anti-bacterial susceptibility tests (ABST) in determining the antibacterial activity of compounds. The Kirby-Bauer disk diffusion is the more frequently used method, due to its convenience, efficiency and cost. In Kirby-Bauer disk diffusion, bacterial suspensions of 0.5 macfarland standard turbidity are inoculated onto the surface of a Mueller-Hinton agar plate and discs infused with the sample of choice are placed along with negative and positive controls to visualize inhibitory zones and determine the antibacterial activity (Sandle, 2016). For this study, ABST was

carried out for each essential oil against S. Aureus and E. Coli using two different known positive controls of ciprofloxacin and chloramphenicol. The change in antibacterial activity of the essential oils were monitored, by carrying out ABST on fresh oil samples and one week stored oil samples, as studies have shown to observe a difference in antibacterial activity of essential oils when stored (Turek and Stintzing, 2012). Antibiotics are created to be effective against bacteria that cause diseased conditions, however due to misuse or overuse of antibiotics, in recent times, an increase in antibiotic resistant bacteria has been observed. Research has been carried out to combine antibiotics with supplementary components such as essential oils as a mode of treating these antibiotic resistant variants (Yap, Lim, Hu and Yiap, 2013). As such, the combinatory effect of the essential oil with the individual positive controls were also tested to determine a synergistic or antagonistic effect.

METHODOLOGY

Sample preparation

Samples were collected, cleaned of any visible impurities, washed with distilled water, and allowed to air-dry. 50g of each sample was measured, and processed until a uniform powder was obtained. Each sample was prepared within 24 hours of extraction of essential oils.

Extraction of essential oils from samples

Extraction of essential oil carried out adapting the procedure discussed by (Mahadagde, 2018). The Soxhlet's apparatus was assembled and connected to a water inlet and a water outlet. 50g of prepared sample was weighed out using an analytical balance. 50 mL of distilled water was measured using a measuring cylinder. Next, the sample and the distilled water was added to the boiling flask of the setup. The boiling flask was swirled to ensure mixing of the sample and water. This setup was then placed on the heating mantle and heated at 100°C until no liquid was observed in the boiling flask. The heating was then discontinued and the setup was allowed to come to room temperature. Next, the hydrosol present in the extraction column was dispensed into 50mL falcon tubes, placed in a centrifuge, and centrifuged at 3500 rpm for 20 minutes. Once centrifuged, the falcon tubes were carefully placed in a falcon tube rack, and the oil present found either above or below the aqueous layer was dispensed into a 1.5 mL microcentrifuge tube using a micropipette. The mass of the obtained oil was measured using an analytical balance to determine the yield.

Antibacterial Susceptibility test

Disc diffusion method for fresh extracted essential oil

The ABST for each sample was carried out against S. Aureus and E. Coli using ciprofloxacin and chloramphenicol as positive controls for each bacteria. Two Mueller-Hinton agar prepared petri dishes were obtained, and using a marker, the dishes were divided into 4 quadrants and labelled as P (positive control), N (negative control), Eo (Essential oil), Syn (positive control+ essential oil). Next, 120µl of E coli bacterial dilution was added on to each agar dish and spread across the plate using sterile cotton swabs. Once spread, the negative controls previously prepared were placed on the agar. The essential oil infused discs were then placed on the agar. To one plate, a ciprofloxacin antibiotic disc was placed as the positive control, and to the other dish, a chloramphenicol disc was placed. The final quadrants, the relevant positive control infused with the essential oil was placed. The Petri dishes were closed and sealed with parafilm. Each test was carried out in triplicates. The above procedure was

repeated for the same sample against S. Aureus bacterial dilution. The inoculated petri dishes were placed in an incubator at 37°C for 24hours, and the resulting zones were observed and recorded.

Disc diffusion method for 1-week old extracted essential oil

The remaining extracted essential oil was stored in the refrigerator for 1 week. Once stored for 1 week, the above procedures for the preparation of the discs and the disc diffusion method were repeated using the 1-week old essential oil sample. The prepared dishes were incubated at 37°C for 24 hours and the observations were recorded.

RESULTS

Hydro-distillation of samples

The volume and mass of essential oils obtained from Clove bud (Syzygium aromaticum), and Cinnamon (Cinnamomum zeylanicum) obtained from hydro distillation was measured, and the yield was recorded.

Table 1: *Volume and yields (w/w %) of essential oils obtained by hydro distillation*

Sample	Volume of oil (mL)	Mass of oil (g)	Mass of sample used (g)	Yield (w/w %)
Syzygium aromaticum	5.20	5.408	50.00	10.82
Cinnamomum zeylanicum	0.63	0.649	50.00	1.30

Antibacterial Susceptibility Test

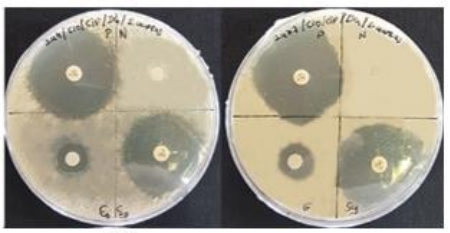
The antibacterial activity of the freshly extracted and 1-week stored essential oils were tested against S aureus and E coli, independently and in combination with the positive controls on Mueller Hinton Agar plates. Positive controls used were; Ciprofloxacin and Chloramphenicol antibiotic discs.

Antibacterial activity of Syzygium aromaticum oil

Zones of inhibition were obtained for fresh and 1-week old clove bud oil against S aureus and E coli independently and in combination with the positive controls, with Ciprofloxacin and Chloramphenicol being used as positive controls (Table 2) (Figures 1,2,3, and 4).

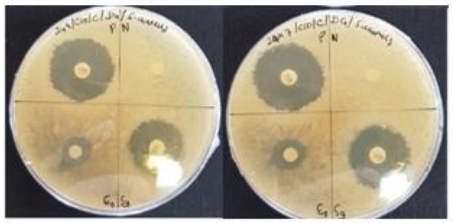
Table 2: *Mean zones obtained for the antibacterial activity of fresh and 1-week old clove bud oil against S aureus and E coli.*

	S aureus		***E coli***	
	Fresh sample (mm)	1 week old (mm)	Fresh sample (mm)	1 week old (mm)
Negative control	00.00	00.00	00.00	00.00
Ciprofloxacin	37.00	39.34	28.00	29.34
Chloramphenicol	26.67	24.67	26.00	25.00
Clove oil	15.34	13.67	15.67	13.34
Clove oil + Ciprofloxacin	39.34	37.00	29.34	28.34
Clove oil + Chloramphenicol	27.34	23.34	26.67	24.67



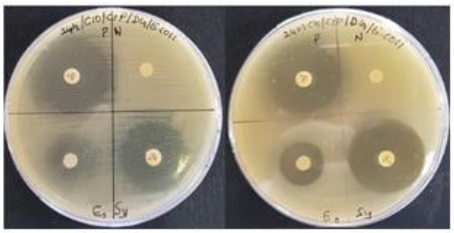
(A) (B)

Figure 1: Zones of inhibition for fresh (A) and 1-week (B) old clove bud oil against S aureus, with ciprofloxacin as the positive control, individually (Eo), and in combination with the positive control (Sy), along with a negative control (N).



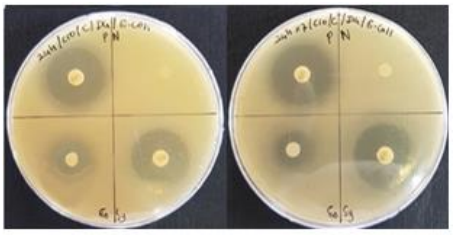
(A) (B)

Figure 2: Zones of inhibition for fresh clove bud oil (A) and 1-week old clove bud oil (B) against S aureus, with chloramphenicol as the positive control, individually (Eo), and in combination with the positive control (Sy), along with a negative control (N).



(A) (B)

Figure 3: Zones of inhibition for fresh clove bud oil (A) and 1-week old clove bud oil (B) against E coli, with ciprofloxacin as the positive control, individually (Eo), and in combination with the positive control (Sy), along with a negative control (N).



(A) (B)

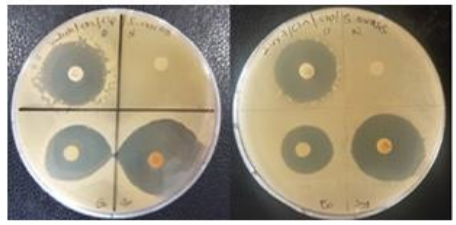
Figure 4: Zones of inhibition for fresh clove bud oil (A) and 1-week old clove bud oil (B) against E coli, with chloramphenicol as the positive control, individually (Eo), and in combination with the positive control (Sy), along with a negative control (N).

Antibacterial activity of Cinnamomum zeylanicum oil

Zones of inhibition were obtained for fresh and 1-week old cinnamon oil against S aureus and E coli independently and in combination with the positive controls, with Ciprofloxacin and Chloramphenicol being used as positive controls (Table 3) (Figures 9, 10, 11, and 12).

Table 3: *Mean zones obtained for the antibacterial activity of fresh and 1-week old cinnamon oil against S aureus and E coli.*

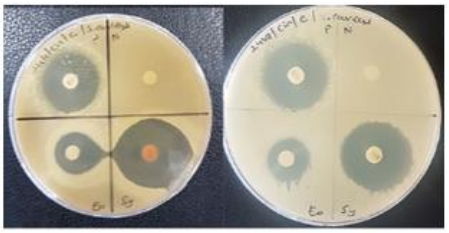
	S aureus		***E coli***	
	Fresh sample (mm)	1 week old (mm)	Fresh sample (mm)	1 week old (mm)
Negative control	00.00	00.00	00.00	00.00
Ciprofloxacin	27.76	27.67	31.00	31.00
Chloramphenicol	27.67	27.67	27.67	27.67
Cinnamon oil	28.00	23.67	19.67	15.33
Cinnamon oil + Ciprofloxacin	38.00	31.67	37.00	33.67
Cinnamon oil + Chloramphenicol	40.00	32.00	34.00	28.67



(A) (B)

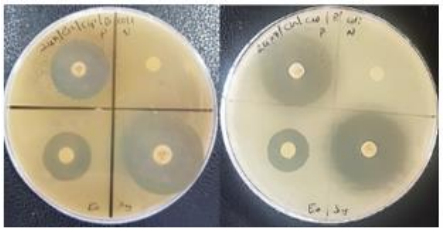
Figure 9: Zones of inhibition for fresh cinnamon oil (A) and 1-week old cinnamon oil (B) against S aureus, with ciprofloxacin as the positive control, individually (Eo), and in combination with

the positive control (Sy), along with a negative control (N).



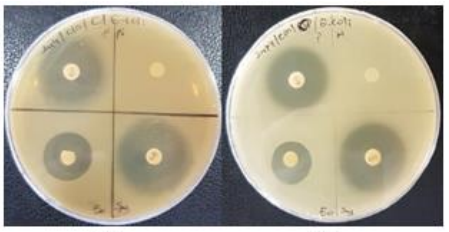
(A) (B)

Figure 10: Zones of inhibition for fresh cinnamon oil (A) and 1-week old cinnamon oil (B) against S aureus, with chloramphenicol as the positive control, individually (Eo), and in combination with the positive control (Sy), along with a negative control (N).



(A) (B)

Figure 11: Zones of inhibition for fresh cinnamon oil (A) and 1-week old cinnamon oil (B) against E coli, with ciprofloxacin as the positive control, individually (Eo), and in combination with the positive control (Sy), along with a negative control (N).



(A) (B)

Figure 12: Zones of inhibition for fresh cinnamon oil (A) and 1-week old cinnamon oil (B) against E coli, with chloramphenicol as the positive control, individually (Eo), and in combination with the positive control (Sy), along with a negative control (N).

DISCUSSION

For the extraction of essential oils from the clove bud, and cinnamon via hydrodistillation using the soxhlet apparatus, 50g of crushed samples was extracted in 300mL of water to ensure consistency in obtained yields. In this work, the hydrodistillation of crushed clove bud resulted in a final volume of 5.20 mL, with a yield of 10.82%, which was slightly lesser than a yield of 11.50% that was obtained by Guan et al., 2007. For the extraction of cinnamon oil, a yellow oil of a volume of 0.63mL was obtained, resulting in a yield of 1.30%, which was similar to yields obtained by similar previous research (Kasim et al., 2014; Pistelli et al., 2016). The variation in the yields obtained for the samples can be affected by several factors; quantitative factors such as the ratio between the mass of sample and the volume of distilled water used, along with the extracting time, and qualitative factors such as the harvest period of the samples and the harvest location which would result in variations in amount of essential oil present (Moghaddam and Mehdizadeh, 2017).

Up to present times, numerous studies have been carried out in determining the antibacterial activity of various essential oils against a multitude of bacteria. Essential oils have been used for many years to treat human health from infectious diseases and against notorious pathogens. More recently, there are increasing evidence indicating the use of essential oils in the field of medicine, notably that of Yap et al., 2013 who looked in to the use of essential oils in combination with antibiotics to reduce antibiotic resistance against multidrug resistant bacteria. For this study, the antibacterial activity of the extracted essential oils of Clove bud

(Syzygium aromaticum), and Cinnamon (Cinnamomum zeylanicum) obtained from hydro distillation was determined against Staphylococcus aureus (S aureus) and Escherichia coli (E coli), two pathogens which are commonly found to cause infective diseases such as urinary tract and enteric infections (E coli), and skin infections (S aureus) in Sri Lanka (Arulnesan et al., 2015; Fernando et al., 2017; Samaranayake, Karunanayake and Patabendige, 2019). Antibacterial susceptibility test (ABST) was carried out on the extracted essential oils against S. Aureus and E. Coli. Via the kirby-Bauer disc diffusion method, with the use of Ciprofloxacin and Chloramphenicol – two broad spectrum antibiotics, as positive controls. The combined effects of the essential oils with Ciprofloxacin and Chloramphenicol was tested to determine a synergistic or antagonistic effect. Turek and Stintzing, 2012 determined the effect of storage on the composition of essential oils, which was also tested in this study to determine if there was a difference in antibacterial activity of freshly extracted essential oil and the same oil stored for one week.

ABST results observed for clove oil showed the essential oil to possess antibacterial activity against both S. Aureus and E. Coli. (Table 2), with zone of inhibition radius of 15.34mm and 15.67mm respectively, which showed similarities to previous studies (Prabuseenivasan, Jayakumar and Ignacimuthu, 2006; Mith et al., 2014). Eugenol, the major constituent of clove oil is most likely the main contributor of its antibacterial effect (Guan et al., 2007). Fresh clove oil was found to show synergistic inhibition of S. Aureus and E. Coli. When combined with ciprofloxacin (39.34mm, 29.34mm) and chloramphenicol (27.34mm, 26.67mm), when compared with clove oil alone. 7-day stored clove oil was found to show antagonistic inhibition of S. Aureus and E. Coli. When combined with ciprofloxacin (37.00mm, 28.34mm) and chloramphenicol (23.34mm, 24.67mm), when compared with clove oil alone. The overall antibacterial results of clove oil (Table 2) showed a reduction in activity of 7 day stored essential than fresh extracted clove oil, which can be due to the reduction in its major antibacterial component; eugenol. Clove essential oil was found to be effective against both the gram positive and gram negative bacteria, which corresponded to the work of Marchese et al., 2017, who discussed the antibacterial modes of action of eugenol to be the increase of cytoplasmic membrane permeability, thus leading to cell death.

ABST results observed for cinnamon oil showed the essential oil to possess antibacterial activity against both S. Aureus and E. Coli. (Table 3), with zone of inhibition radius of 28.00mm and 19.67mm respectively, which was relatively in agreement with previous studies carried out (Prabuseenivasan, Jayakumar and Ignacimuthu, 2006). The high antibacterial activity observed in cinnamon essential oil may be due to the action of transcinnamaldehyde, considered as its single major compound (Singh, Maurya, delampasona and Catalan, 2007). Fresh and 7 day stored cinnamon oil were both found to show synergistic inhibition of S. Aureus when combined with ciprofloxacin (38.00mm, 31.67mm) and chloramphenicol (40.00mm, 32.00mm). Fresh and 7 day stored cinnamon oil were both found to show synergistic inhibition of E. Coli when combined with ciprofloxacin (37.00mm, 33.67mm) and chloramphenicol (34.00mm, 28.67mm). These results showed similarities to tests carried out by El Atki et al., 2019.When observing the overall results of cinnamon oil (Table 3), a reduction in antibacterial activity of 7 day stored essential oil was observed, indicating a change in its

composition, most likely due to a reduction in transcinnamaldehyde.

CONCLUSION

The aim of this study was to determine the antibacterial activity of essential oils of clove bud and cinnamon against two pathogens which commonly cause disease, the effect of storage on their antibacterial activity, and the combinatory effect of the essential oils with widely used antibiotics. The essential oils of clove bud, and cinnamon bark were obtained by hydrodistillation, and yields of obtained essential oils were in relative agreement to studies carried out previously. The antibacterial effects of the fresh and 7 day stored extracted essential oils were determined, and a reduction in the activity by storage was observed in clove bud, and cinnamon oil. Fresh and 7 day stored essential oils of clove bud, and cinnamon showed varying synergistic and antagonistic effects against both bacterial species, when combined with either ciprofloxacin or chloramphenicol.

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