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ANTIBACTERIAL ACTIVITY OF CENTELLA ASIATICA LEAVES ACCORDING TO THEIR MATURATION AND THE SYNERGISTIC ACTIVITY OF CENTELLA ASIATICA ANTIBIOTIC DISCS TOWARDS STAPHYLOCOCCUS AUREUS

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ABSTRACT

Centella asiatica (C. asiatica) is a medicinal herb which had been used by many countries around Asia, Africa and Australia. This plant can be used as a green leafy vegetable, medicine or as a cosmetic. This study will focus on the antibacterial activity of C. asiatica leaves according to their maturation stage; as pre mature, post mature and mature. C. asiatica extract was obtained by ethanol extraction method by mixing 15g of C. asiatica leaf powder with 25ml of 95% ethanol. From each extract antibiotic discs were prepared by using No.1 Whatman's filter paper. According to the obtained results, mature C. asiatica leaves had the highest antibacterial activity towards Staphylococcus aureus (S. aureus) with a 12.3mm zone of inhibition in nutrient agar medium. The least antibacterial activity was obtained in the pre mature C. asiatica leaves, which had given 7.2mm zone of inhibition and post mature leaves had shown a moderate antibacterial activity of 10.3mm on Nutrient agar. By using the antibiotic discs, which were created by, using mature C. asiatica extract; synergistic activity with Ciprofloxacin, Gentamycin and Vancomycin was observed. Both Ciprofloxacin and Gentamycin antibiotic discs activity were reduced from 32mm to 26mm and 34mm to 30mm respectively: when they are combined with the C. asiatica extract where Vancomycin had given increased antibacterial activity from 23mm to 24mm when it was synergised with C. asiatica leaf extract. This study will help to understand about the antibacterial activity of C. asiatica leaves' extracts towards S. aureus. Since the antibiotic resistance is a major problem in the presence this study had also focussed on the antibiotic synergism by synergising the created C. asiatica antibiotic discs with well-known antibiotics such as Vancomycin, Gentamycin and Ciprofloxacin.

Key words: Antibiotic Synergism, Centella asiatica, Ethanol extraction

INTRODUCTION

Centella asiatica (C. asiatica) which is also known as pennywort or 'gotu kola (Sri Lanka)' is a commonly eaten raw food (Puttarak et al., 2017). C. asiatica is a perennial creeping weed which grows mostly in moist grounds (Oyedeji and Afolayan, 2004) in Sri Lanka, India, China, Madagascar, Indonesia, Malesia and Africa (James and Dubery, 2009). This herbaceous plant is categorized under Apiaciae family (Figure 1) which consists about 50 species (James and Dubery, 2009). This project is about the antibacterial activity of C. asiatica leaves which are shaped as a spade.

Kingdom: Plantae
Subkingdom: Tracheobionta
Division: Magnoliophyta
Class: Magnoliopsida
Subclass: Rosidae
Order: Apiales
Family: Apiaceae
Genus: Centella
Species: asiatica

Figure 1-Scientific classification of *C. asiatica* (Database of Medicinal and Aromatic Plants in Rajasthan, 2016).

Since the prehistoric time C. asiatica is mostly used as an ayurvedic medicinal plant in China, India, Africa and Sri Lanka (Soyingbe, Mongalo and Makhafola, 2018). They had used this plant as an anti-inflammatory, anti-cancer, anti-oxidant (Dash et al., 2011), anti-microbial, anti-depressive agent in their treatments. Also it has been found that this plant can be used as a brain tonic (Omar et al., 2011), to treat chronic mental disorders (Arumugan et al., 2011) eczema, atherosclerosis, leprosy, rheumatoid arthritis and also to treat kidney disorders (Idriz and Nadzir, 2017). Most of the folk healers had used this plant as a wound healing agent (Gohil, Patel and Gajjar, 2010). Even though this plant consists too many medical values this research will be based on anti-bacterial activity of C. asiatica leaves. At the end of this research it will reveal that which maturation stage has the best antibacterial activity towards Staphylococcus aureus. To prepare the antibiotic discs C. asiatica extracts were obtained using ethanol.

Various antibiotics have been developed over the years to improve human quality of life. However unwise use of antibiotics make the bacteria resistant towards the antibiotics. Therefore, it required antibiotics to counteract with bacteria which may cost more (Dash et al., 2011). Beside the drug resistance, undesirable side effects of certain antibiotics encourage the use of natural sources (mostly plants extracts) as antibacterial agents (Nasution et al., 2018). The growing concern regarding the increase of bacterial resistance to antibiotics and increasing interest towards application of natural medicine have led to the search of new antibacterial agents mainly from plant extract. Medicinal herbs are alternative treatment which is preferable for human and animal health which believe to have least side effects.

According to Nasution et al., 2018 they had discovered that C. asiatica leaves consist antibacterial activity towards Staphylococcus aureus, Staphylococcus albus, Streptococcus pyogenes, Streptococcus pneumonia, Aspergillus niger, Aspergillus flavus, Escherichia coli and Microsporium boulardii. Staphylococcus aureus (S. aureus) is prevalent contagious pathogenic bacteria. So that S. aureus was chosen for this research (Lalitha, Kiran and Raveesha, 2013). It has been found that C. asiatica inhibit the growth of S. aureus and reduce the inflammation. Triterpenes are the most prominent biologically active compound which is present in C. asiatica (Wijeweera et al., 2006). Triterpenes consists asiatic acid, madecassic acid and asiaticoside (Bylka et al., 2013). Among them, asiatic acid is an aglycone of asiaticoside which can be isolated from C. asiatica (Taemchuay et al., 2009), is mostly used for wounds as a healing agent: as an antibacterial and potentially anti-fungal: as an anti-oxidizing agent: as a dermis reconstructing agent which stimulates the collagens synthesis or as an anti-aging agent which reinforce the bio-mechanic properties of mature skins (Taemchuay et al., 2009).

According to Abaza et al., 2017 who were researched on Olive tree plants had discovered that phytochemical content and their characteristics are varying according to their development stage. So that this research was designed to detect changes in the antibacterial activity of C. asiatica leaves according to their maturation.

In Sri Lanka most of the time people tend to buy pre mature C. asiatica leaves as it has more milky taste when compare with mature and post mature leaves, believing that it is much better for health. This study was designed to identify, which maturation stage has the maximum antibacterial activity towards S. aureus which is a gram positive bacteria.

S. aureus was selected as the bacterial strain in this project. According to Soyingbe, Mongalo and Makhafola, 2018 they had discovered that S. aureus gives a considerable zone of inhibition when compared with Escherichia coli when using ethanol as the extraction medium.

Main goal of this project: Identify the antibacterial activity of the ethanol extract of C. asiatica leave towards S. aureus and the synergistic effect of C. asiatica extract with Ciprofloxacin, Gentamycin and Vancomycin.

METHODOLOGY

Centella asiatica powder making.

Centella asiatica leaves were bought from a local market at Ingiriya, Sri Lanka. Leaves were separated from stem and they were collected according to their maturation by palping the texture and by observing the colour and the size of the leaves. Pre mature leaves are light green, in colour and small in size. Mature Centella asiatica leaves are smooth in texture and green in colour where post mature leaves are rough in texture and greenly yellowish in colour.

Figure 2. C. asiatica leaves according to the maturation (a- pre mature, b- mature and c- post mature).

All the leaves were rinsed with water and then soak in the salt water for 15 minutes. After washing all the separated leaves were dried under the direct sunlight and grinded well. Then the C. asiatica extract was obtained by the solvent extraction method.

Before starting the lab work all the glass ware and agar were autoclaved and the working area was cleaned by using 70% ethanol.

First Extraction

15g of mature, pre mature and post mature Centella asiatica powder was measured and added to three 50ml falcon tubes and 25 ml from the 95% ethanol was added to it as follows (Table 1). The samples were placed on the roller mixture let them mix overnight.

Table 1. Samples and the added 95% ethanol volume.

95% ethanol volume Sample	25ml	25ml	25ml
Premature (15g)	✓	✓	✓
Mature (15g)	✓	✓	✓
Postmature (15g)	✓	✓	✓

Then the samples were filtered by using a cotton cloth and then the extract was again filtered with a 'Munktel' filter papers and extracts were separated. By using Whatman's no.1 filter papers empty antibiotic discs (diameter: 7mm) were prepared. Each disc was saturated with C. asiatica extract by pipetting each volume with five minutes breaks (Table 2). Meanwhile the nutrient agar plates were prepared and S. aureus was streaked on the plate by using 'Kerby Beuer' disk diffusion method. The bacterial

concentration (x 108 CFU) was calculated according to the 0.5 McFarland's solution.

Table 2. Volume for each antibiotic disc.

Volume (µl) / Sample	5	5	5	5	4	3	3	2	oven	3	3	3	2	2	oven	3	3
2g:15ml (pre mature)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2g:20ml (mature)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2g:25ml (post mature)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

All the discs were kept on the oven at 400C for 30 min. Then the discs were placed on the petri plates as shown in Figure 3.

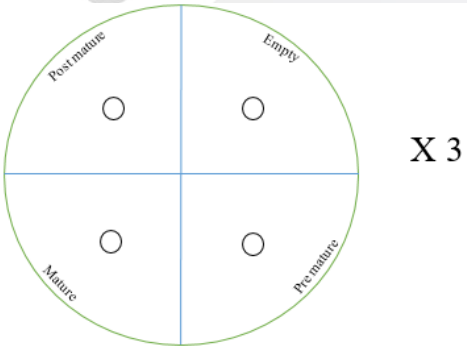

Figure 3. The antibiotic discs organization in First extraction.

The negative and the positive controls were made on nutrient agar as shown in Figure 4. The petri plates were incubated at 370C for 24h and the zone of inhibition was measured.

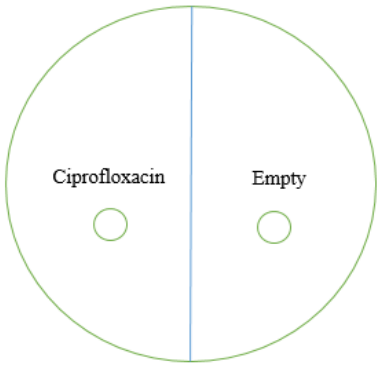

Figure 4. Organization of the control antibiotic discs (Ciprofloxacin- Positive control and Empty disc saturated with ethanol- Negative control).

Second extraction.

15g of mature (2 falcon tubes with same concentrations), pre mature and post mature C. asiatica powder was measured and added to four 50ml falcon tubes and 25 ml (Table 3) from the 95% ethanol was added to it. The samples were placed on the roller mixture let them mix overnight.

Table 3. Samples and the added 95% ethanol volume.

95% ethanol volume / Sample	25ml	25ml	25ml
Pre mature (15g)	✓	✓	✓
Mature (15g)	✓	✓	✓
Mature (15g)	✓	✓	✓
Post mature (15g)	✓	✓	✓

Then the samples were filtered by using a cotton cloth; then the extract was again filtered with a 'Munktel' filter papers and extracts were separated. By using Whatman's no.1 filter papers empty antibiotic discs (diameter: 7mm) were prepared. Each disc was saturated with C. asiatica extract by pipetting each volume with five minutes intervals (Table 4). By adding mature Centella extract: Ciprofloxacin, Gentamycin and Vancomycin antibiotic discs were re-prepared by pipetting each volume with five minutes intervals according to Table 4. Meanwhile the Mueller Hinton agar plates were prepared and S. aureus was streaked on the plate by using 'Kerby Beuer' disk diffusion method. The bacterial concentration (x 108 CFU) was calculated according to the 0.5 McFarland's solution.

Volume (µl) / Sample	5	5	5	4	4	4	4	4 oven	4	3	3	3	3	3 oven	3	2	2 oven
Pre mature	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Mature	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Post mature	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

Table 4. Volume for each antibiotic disc

The antibiotic discs which are made according to the Table 4 were placed on the petri dishes as shown in Figure 5.

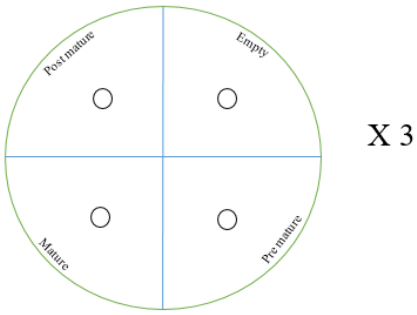

Figure 5. The antibiotic discs organization in forth extraction.

The negative and the positive controls were made on Mueller Hinton agar as shown in Figure 4. The petri plates were incubated at 370C for 24h and the zone of inhibition was measured.

Antibiotic synergism Test.

By using C. asiatica extract which are obtained by the second extraction, following steps were carried out.

Table 5. Volume for each re-preparing antibiotic disk.

Volume (µl) / Sample	5	5	5	5	5	4	4	4	4	oven	5	5	5	5	5	5	5
Ciprofloxacin	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Vancomycin	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Gentamycin	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

Volume (µl) / Sample	oven	5	5	5	5	5	5	oven
Ciprofloxacin	✓	✓	✓	✓	✓	✓	✓	✓
Vancomycin	✓	✓	✓	✓	✓	✓	✓	✓
Gentamycin	✓	✓	✓	✓	✓	✓	✓	✓

All the discs were kept on the oven at 400C for 30 min. The antibiotic discs which are made according to the Table 5 were placed on the petri dishes as shown in Figure 6

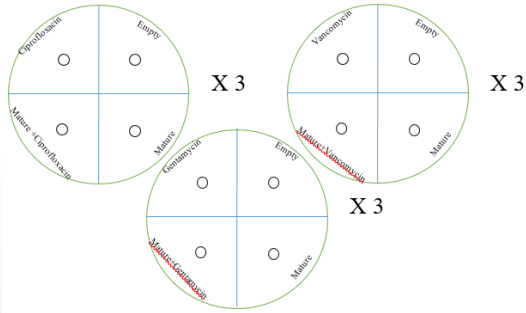

Figure 6. The antibiotic discs organization in the antibiotic synergism test.

When preparing the antibiotic discs as shown in Figure 6 empty discs which is saturated from ethanol was used as the negative control and mature C. asiatica discs was added as the positive control. All the petri dishes were incubated overnight and the zone of inhibition was measured.

RESULTS

First Extraction Results.

Table 6. Results of the first extraction.

Sample	Zone of inhibition			Mean of zone of inhibition
	A	B	c	(a+b+c)/3
Pre mature	7mm	7.2mm	7.4mm	7.2mm
Mature	13mm	12mm	12mm	12.3mm
Post mature	10mm	10mm	11mm	10.3mm

Second Extraction Results.

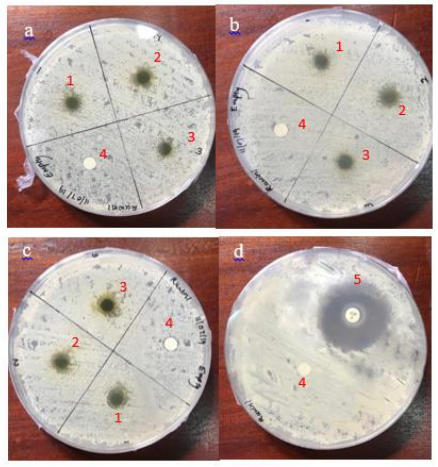

Figure 7. Results of the second extraction Table.4 by using Mueller Hinton agar and the positive control and the negative control. (1: pre mature, 2: mature and 3: post mature, 4-negative control and 5-positive control)

Table 7. Results of the second extraction.

Sample	Zone of inhibition			Mean of zone of inhibition
	A	B	c	(a+b+c)/3
Pre mature	7mm	7mm	7mm	7mm
Mature	9mm	10mm	9mm	9.3mm
Post mature	8mm	8mm	7mm	8.3mm

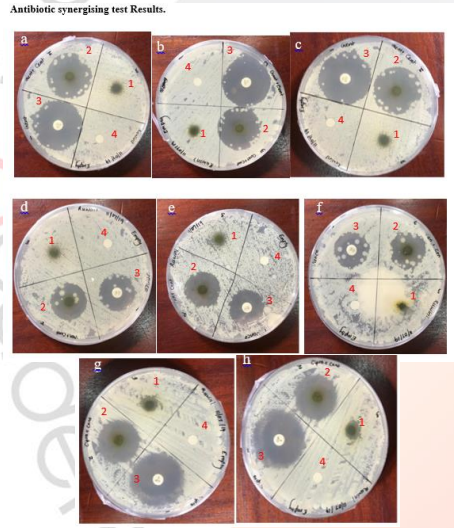

Figure 8. Results of the forth extraction antibiotic synergism. (a, b and c are the results for mature Centella and Gentamycin combination, d, e and f are the results for Vancomycin and mature Centella combination; and g, h and i are the results for mature Centella and Ciprofloxacin combination.1-Mature, 2-Synergised antibiotic, 3-antibiotic alone (positive control) and 4-negative control)

Table 8. Results of the Vancomycin combination.

Sample	Zone of inhibition			Mean of zone of inhibition
	a	b	C	(a+b+c)/3
Vancomycin	23mm	23mm	23mm	23mm
Vancomycin + Mature *C. asiatica*	23mm	25mm	24mm	24mm
Mature	10mm	9mm	9mm	9.3mm

Table 9. Results of the Gentamycin combination.

Sample	Zone of inhibition			Mean of zone of inhibition
	a	b	C	(a+b+c)/3
Gentamycin	34mm	34mm	34mm	34mm
Gentamycin + Mature *C. asiatica*	31mm	30mm	29mm	30mm
Mature	9mm	9mm	9mm	9mm

Table 10. Results of the ciprofloxacin combination.

Sample	Zone of inhibition			Mean of zone of inhibition
	a	b	c	(a+b+c)/3
Ciprofloxacin	24mm	32mm	32mm	32mm
Ciprofloxacin + Mature *C. asiatica*	25mm	27mm	-	26mm
Mature	9mm	10mm	9mm	10.3mm

DISCUSSION

C. asiatica leaves were separated into three age groups by observing and by palping the texture. This method is more reliable rather than cultivating C. asiatica and pluck the leaves according to the date since they were planted. The problem of the cultivation method is the root system of C. asiatica. In this plant the roots are spreading every day and start growing new leaves. So that it is hard to measure the age of each leaf and also it consumes more time. When drying the leaves direct sunlight was used. Other than the direct sunlight, wind or the oven (400C) can be used for drying. Even though the drying method is different it won't be able to affect the phytochemical activity (Nelson and Bugbee, 2015); as the temperature which obtained from the sunlight is also around 400C (Volokin and ReLlez, 2014).

To grind the dried C. asiatica leaves the mixer grinder was used. When the leaves are not dehydrated or dried properly it won't be easy to grind and the grinder will generate heat; which will burn the leaves. Then the grinded leaves were sieved from the siever which is usually used in the kitchens. As the siever had larger pores, the obtained C. asiatica powder was not very fine, so that it might have affect the extraction procedure by increasing the

surface area (Chemistry libretexts, 2019) and by reducing the extract concentration. When using a fine powder for the extraction it will increase the surface area of the solute and give more concentrated C. asiatica extract.

According to Dash et al., 2011 in C. asiatica; ethanol extract gives the highest zone of inhibition (17mm) towards S. aureus in nutrient agar, other than chloroform, methanol and n-hexane. So that 95% ethanol was used to obtain the C. asiatica extract. As the third and fourth extraction mixtures were highly concentrated, a clean, sterile and white colour cotton cloth was used for the filtration. First extraction was done by using 15g from post mature, mature and pre mature C. asiatica powder along with 25ml of 95% ethanol. As it is hard to filter the extract from the filter paper a cotton cloth was used. According to Table 6 it shows that the median zone of inhibition of pre mature C. asiatica leaves was 7.2mm, which was the least value. Post mature C. asiatica leaves have given a moderate value 10.3mm where the mature leaves had given the highest median zone of inhibition of 12.3mm (Table 6). According to these results it can be concluded that mature C. asiatica leaves gives the highest zone of inhibition which describes that it has the greatest antibacterial activity when comparing with post mature and premature leaves. Pre mature leaves had the lowest zone of inhibition which further confirms that it has the lowest antibacterial activity when compared with the post mature C. asiatica leaves.

These changes of the values must have arose due to the maturation of C. asiatica leaves and mature leaves which are at their younger age must be having the highest phytochemical activity where the pre mature leaves which are at their early stage of maturation might not have grown enough phytochemicals (Khoo et al., 2018). So that the pre mature leaves has lowest antibacterial activity. The moderate antibacterial activity was obtained from the post mature C. asiatica leaves. This might happen due to the aging of the leaves which degrade the phytochemicals (Thi and Hwang, 2014).

Nasution et al., 2018 had used rotary evaporator to create the concentrated extract and in this project a rotary evaporator had not been used. So that the concentrations of extracts might be different in these two studies. As there is no use of rotary evaporator step to concentrate the extract, C. asiatica concentration of this study extract should be reduced when compared with Nasution et al., 2018. So that the produced antibiotic discs concentration is also reduced. This might be the reason for the reduction of the zone of inhibition (Table 6) from 17mm to 12.3mm. In all the experiments an empty antibiotic disc which is saturated with ethanol was used as the negative control. Since none of the negative controls had a zone of inhibition it can be concluded that these inhibitions were not due to the solvent (ethanol) but due to the C. asiatica extract. Then the second extraction results which are shown in Figure 7, was cultured on Mueller Hinton agar for further confirmation. It also give similar results as the first extraction which was performed on the Nutrient agar, but the radius of the zone of inhibition (Table 7) was reduced.

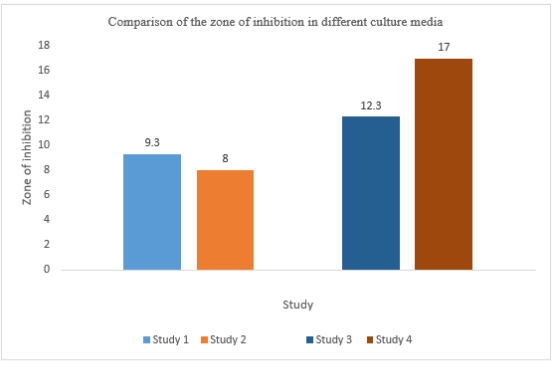

Figure 9. The difference between the zones of inhibition (Study 1-Second extraction, Study 2-Nasution et al., 2018, Study 3- First extraction and Study 4- Dash et al., 2011)|

Figure 9 is a comparison between the studies and this project results. According to Figure 9 it shows that study 2 which was done by Nasution et al., 2018 by using Mueller Hinton agar had given 8mm zone of inhibition where the second extraction give 9.3mm zone of inhibition when using Mueller Hinton agar. Also the study 4 which was done by Dash et al., 2011 by using Nutrient agar had given 17mm zone of inhibition where the first extraction which is similar to study 2 had given only 12.3mm zone of inhibition. According to these results it clearly shows that when using different agar mediums it gives different results. The gold standard culture media for the Kirby Bauer disk diffusion is Mueller Hinton agar (Nassar, Hazzah and Bakr, 2019). Even though Mueller Hinton is the gold standard, the antibiotic discs which are made from the C. asiatica extract had given maximum zone of inhibition in Nutrient agar. So according to these results it can be concluded that for C. asiatica studies using nutrient agar will give visible results when compared to the Mueller Hinton agar. Nassar, Hazzah and Bakr, 2019 and Donkor et al., 2008 had concluded that replacing Mueller Hinton agar with Nutrient agar will give rise to multiple errors on S. aureus etc. in antimicrobial susceptibility tests. Nassar, Hazzah and Bakr, 2019 said that the Mueller Hinton agar is a loose agar which will enhance the diffusion of the antibiotics in the culture medium and the starch which is a component of Mueller Hinton agar also absorb the bacterial toxins; must be the cause for larger zone of inhibition when compared to Nutrient agar (Nizet, 2017).

According to Baym, Stone and Kishony, 2016 synergising antibiotics is a recent development in the pharmaceutical industry. Nowadays most of the scientists are interested in antibiotic synergism as it has given good effect most of the time; on the bacteria and their antibiotic resistant phenotypes (Kuok et al., 2017). Also Dhiman et al., 2016 had performed an in vitro study of herbal extracts including C. asiatica; combinations towards the bacteria's associated with fruit juices. This further confirms that combining herbal antibiotics are effective than the antibiotic alone.

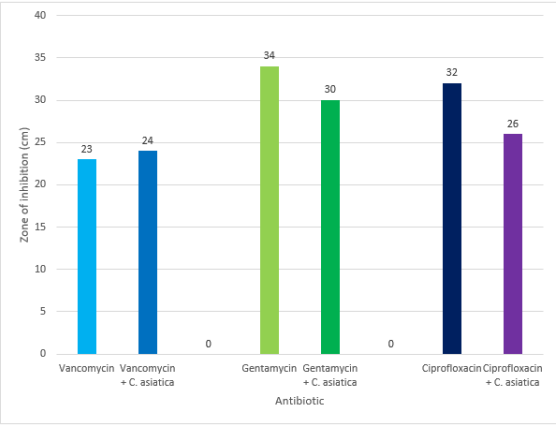

Figure 10. Comparison between different antibiotics and their combination with C. asiatica leave extract.

When combining mature C. asiatica leaves extract with existing antibiotics (Figure 10) such as Vancomycin, Gentamycin and Ciprofloxacin it gives different results. The median zone of inhibition was reduced in combined antibiotic discs such as Gentamycin and Ciprofloxacin. But in Vancomycin it had increase the median zone of inhibition (24mm) from 1mm than the antibiotic alone (23mm).

These reductions of the zone of inhibitions might have arose due to oven incubation at 40¬¬0C. As the antibiotic discs are storing 40C or less than that (Chen et al., 2013) the high heat must have damaged its properties (Cheesman et al., 2017). Also the synergising the antibiotics might have cross reacted with each other. According to Dhama et al., 2014 this reduction might have occurred due to the bacterial resistance towards the antibiotic.

CONCLUSION

According to the obtained results mature C. asiatica antibiotic discs had given the maximum zone of inhibition of 12.3mm. The post mature extract got the second maximum zone of inhibition (10.3mm) and according to this study the least antibacterial activity or the lowest zone of inhibition (7.2mm) was obtained from the pre mature C. asiatica extract. When combining C. asiatica with the other antibiotics such as gentamycin, ciprofloxacin and Vancomycin it shows that Gentamycin and the Ciprofloxacin have a negative impact on their normal antibacterial activity. But when the C. asiatica extract is mixed with Vancomycin it shows that this combination had increased the antibacterial activity of Vancomycin.

According to these results, using mature leaves of C. asiatica will give the maximum antibacterial activity rather than using the other maturation stages.

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