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Original Research Article

In Vitro Antibacterial Activity of Different Stem-Bark Extracts and Fractions of *Lophira Lanceolata*

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Abstract

Background: Infectious diseases are the leading cause of death world-wide despite the vigorous campaigns that have been made to combat them. This has been occasioned by drastic growth of drug resistant pathogens. Plant based antimicrobials represent a vast untapped source of medicines and a further exploration of plant antimicrobials is called for. Plant extracts have led to the discovery of many clinically useful drugs such as emetine, berberines and quinine. There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases. Therefore, researchers are increasingly turning their attention to exploring untapped opportunities of natural products, looking for new leads to develop better drugs against microbial infections and other diseases. The objective of the study is to determine *In-vitro* antibacterial activity of different extracts and fractions of *Lophira lanceolata* plant. In this study, the plant extracts were screened for their antibacterial activity against selected strains of bacteria, including Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram-positive *(Bacillus subtilis and Staphycoccus aureus)*. The diameter zone of inhibition was determined using agar well diffusion method. Ethanol extract was found to have the highest activity while aqueous extract has the least against the tested strains. The n-hexane, ethylacetate and residual aqueous fraction have no activity whereas the n-butanol fraction has activity. The MIC of ethanol extract was found to be inhibitorier than methanol and aqueous extracts. The MBC of the extracts revealed that methanol, ethanol and aqueous extracts are bactericidal on one or more organisms while n-butanol fraction is bacteriostatic to all test strains at the concentration used. ***Conclusion:*** Conclusively, the stem-bark of *Lophira lanceolata* possessed in-vitro antibacterial activity with highest active observed in ethanol extract.

Keywords: antibacterial activity, Minimum inhibitory concentration, Minimum Bactericidal concentration, Zone of inhibition.

INTRODUCTION

The use of plants in indigenous cultures are multiple and very diverse. For many people they still form an important economic basis and are used as food, medicine, construction material, firewood, dyes, as ritual paraphernalia and ornaments [1]. For thousands of years' plants have been the foundation of traditional medicine systems where the knowledge on the plants has been passed on from generation to generation [1]. The abundance of plants on the earth surface has led to an increasing interest in the investigations of different extracts obtained from traditional medicinal plants as potential sources of new antimicrobials agents [2]. People have used plants for millennia and vast information of the medicinal uses of plants has therefore accumulated especially in the tropical parts of the world. In African, Indian and Chinese communities, plants have formed the main ingredient of traditional medicines [3, 4]. Plants produce compounds of varying diversity as a means of defense against bacteria, fungi, pests and predators, hence the plants are efficient natural chemical factories, producing compounds of various structures that result in different physiological effects in the body once ingested [5]. In 1971, substances isolated from plants were used as important drugs in one or more countries and that 60% of these compounds were discovered as a result of phytochemical studies on plants used for medicinal purposes [6]. The relationship between man and plants has been very close throughout the development of

almost all civilizations. The plant kingdom is abundant and natural products of higher plants may give a new source of antimicrobial agents with possibly novel mechanisms of action [7, 8]. Antibacterial agent is an agent that interferes with the growth and reproduction of bacteria. These agents kill or prevent growth of bacteria by inhibiting some cellular functions of the bacterial cell [9]. Antibacterial agents are either bacteriostatic (inhibiting growth of bacterial cells) or bactericidal (causing death of bacteria). Bactericidal drugs are usually independent in their actions while bacteriostatic ones are dependent on the host's defense mechanisms for the eventual elimination of pathogenic microorganisms [10].

Sulfamethoxazole (1) is an important drug of sulphonamides group, and has been extensively employed in medicine in the treatment of pneumonia, staphylococci, gonococci, streptococcal infections, meningococcal meningitis, and in the treatment of open wounds to prevent gangrene [11]. The most selective antibiotics are those that interfere with bacterial cell wall synthesis. Drugs such as pencillin, cephalosphorins, vancomycin, and bacitracin have high therapeutic index because they target structures not found in eukaryotic cells. Most penicillin (e.g. penicillin G or benzyl penicillin) are derivatives of 6-aminopenicillanic acid and differ from one another with respect to the side chain attached to the amino group. The most crucial feature of the molecule is the B-lactam ring, which is responsible for bioactivity. Many penicillin-resistant bacteria produce penicillinase (also called B-lactamase), an enzyme that inactivate the antibiotic b penicillin-resistant bacteria produce penillinase (also called B-lactamase), and an enzyme that inactivate the antibiotic by hydrolyzing bond in the B-lactam ring [12]. Penicillin G has the greatest activity against streptococci, meningococci, enterococci, non-β-lactamase producing staphylococci, *Treponema pallidum* and many other spirochetes, clostridium species, actinomyces and other Gram-positive rods and non-β-lactamase producing Gram-negative anaerobic organisms. Unfortunately, it has little activity against Gram-negative rods and it is susceptible to hydrolysis by β- lactamases [13]. The increase in infectious diseases and resistance to antimicrobial drugs has called for development of newer, safe and effective medicines. This has necessitated studies on other potential sources of effective, safe and cheap antimicrobial drugs, and plants have been considered to be an alternative source. There is need for a reliable, bioassay guided fractionation of active extracts in order to detect a broad spectrum antimicrobial agent.

MATERIALS AND METHODS

Materials/equipment

Autoclave, Hot air sterilizing oven GRX-9053(B. Bran scientific and instrument company, England), Micropipette (Axiom, Germany), Multiple micropipette, Incubator (Precision Scientific, England. Model 6, model number MCP 7743), Inoculating loop

Plant collection and identification

The plant was collected in Bukuyun local government of Zamfara state Nigeria in May 2018 by a staff of Pharmacognosy and ethnomedicine department, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto. The plant was identified at the herbarium unit of the department, and was authenticated by a Pharmacognost in the same department and the specimen was deposited at the herbarium and a voucher number was obtained (PCG/UDUS/ochna 001) for the deposited sample.

Preparation of plant extract

The stem bark of the plant was washed, air dried and powdered using pestle and mortar. It was labeled and kept in air-tight container prior to commencement of the extraction process.

The extraction was carried out by maceration technique 100g each of the dried powder was accurately weighed and soaked in 500ml of 70% ethanol, 80% methanol and in distilled water using 5 liters volumetric flask respectively. It was shaken for 10 minutes and then allowed to stay at room temperature overnight. The filtrate was decanted in a separate container. This process was repeated 3 times to ensure complete extraction. The mixtures were first filtered with cheese cloth, then with Whatman No 1 filter paper (24cm). The filtrate was separately concentrated in vacuum using rotary evaporator (Model EYELA SB 1100, China) to 10% of its original volume at 40^0C. These were concentrated to complete dryness in water bath in order to obtain the crude extract [14].

Antibacterial test

Preparation of sample

The extract 1000mg was dissolved in 2mL of 10% dimethyl sulphoxide (DMSO). This stock solution 1000mg/2ml was again diluted thus 6 concentrations of the extract were prepared that is 1000, 500, 250, 100, 50, 25mg/ml. The solutions of the extracts were used for test control. Standard antibiotics Tinidazole/ciprofloxacin and 10% DMSO were used as positive and negative control respectively.

Preparation of media for bacteria

Four strains of bacteria were used in the study which was collected from Department of microbiology Faculty of pharmaceutical sciences, Usmanu Danfodio University Sokoto. Two were gram positive *Staphylococcus aureus, Bacillus specie, Escherichia coli* and *Pseudomonas aeruginosa*. The organisms were maintained on nutrient agar medium at 4^0c.

Standardization of inoculum

The organisms were harvested from overnight culture using normal saline solution. The organisms

were standardized using spectrophotometer to 0.5 Mcfarland turbidity standards at wavelength of 625nm and absorbance of 0.08-0.1.

Determination of antimicrobial activity of the extracts

Agar well diffusion technique as described by Cheese [15] was used to determine the antibacterial activity of the extracts. 300mL of nutrient agar was prepared and 15mL of the nutrient agar was poured in each sterile petri dish (20 petri dishes) and allowed to solidify. The plates were seeded with 2mL of overnight standardized culture of each of bacterial isolate. The seeded plates were allowed to set after uniform distribution of bacterial isolate following slow rotation of the petri dish. A standard sterile cork-borer of 8mm diameter was used to cut uniform wells on the surface of the agar. The wells filled with the extracts with the aid of a sterile syringe. One of the well in each nutrient agar plate was filled with 10% DMSO as negative control. Standard antibiotic disc (Tinidazole/ciprofloxacin) was placed on the nutrient agar as positive control. The plates were then allowed to stand for 1 hour at room temperature to allow proper diffusion of the extract to occur. All the plates were incubated at 37^0C for 24 hours and observed for zones of inhibition. A zone of clearance round each well signifies inhibition and the diameter of such zones were measured in millimeter (mm) with ruler.

Determination of minimum inhibitory concentration (MIC)

The MIC of the extracts for *S. aureus, B. subtilis, P. aeruginosa, and E. coli* was determined using the broth microdilution method. Culture of each tested bacterium were diluted in normal saline solution and the turbidity of the inoculums was adjusted to 0.5 Mcfarland standard spectrophotometrically at (0.08 – 0.13) OD 625nm [16].

100µl aliquots were then aseptically in wells of a 96 well plate. The extracts were diluted with 10% DMSO. 50µL of the organisms were added and were serially diluted to concentrations ranging from 1000mg/2ml to 0.5mg/2ml.50µL of the organism were added to each well. The plates were incubated at 37˚C for 24hours.Negative control and blank control was maintained for each test. After 24hours incubation, the absorbance of the wells was taken using microplate spectrophotometer. The MIC was taken as the minimum concentration in which there is no microbial growth.

Determination of minimum bactericidal concentration (MBC)

20µL of sample were collected from wells which did not show any growth and inoculated on sterile prepared nutrient agar. The plates were then incubated overnight at 37˚C. The MBC was read as the lowest concentration of *L. lanceolata* which did not show any visible growth on agar plate.

Fractionation of crude extract

The crude extract with highest antibacterial activity was fractionated (ethanolic extract). The crude ethanolic extract was fractionated using different solvents based on their polarity. The solvents used are n-hexane, ethylacetate, n-butanol and water. The fractionation was done using separating funnel, 10g of the crude ethanolic extract was suspended in 50ml of distilled water and was successfully extracted with organic solvents of increasing polarity (50mL six times each) from the least polar n-hexane, ethylacetate, n-butanol and residual aqueous fraction respectively. The fractions obtained were dried.

Determination of antibacterial activity of fractions of ethanolic extract of *L. lanceolate*

Agar well diffusion techniques as described by Cheese [15] was used to determine the antibacterial activity of the different fractions.

Determination of Minimum inhibitory concentration (MIC)

The MIC of the fraction that showed inhibition in the antibacterial screening was determined. The MIC was determined using the broth micro-dilution method. Cultures of each tested bacterium were diluted in normal saline solution and the turbidity of the inoculum was adjusted to 0.5 McFarland standard spectrophotometrically at (0.08-0.13) OD 625nm [16].

100µl aliquots were then aseptically in wells of a 96 well plate. The fraction was diluted with n-butanol. 50µL of the organisms were added and were serially diluted to concentrations ranging from 1000mg/ml to 0.5mg/ml.50µL of the organism were added to each well. The plates were incubated at 37˚C for 24hours.Negative control and blank control was maintained for each test. After 24hours incubation, the absorbance of the wells was taken using microplate spectrophotometer. The MIC was taken as the minimum concentration in which there is no microbial growth.

Determination of minimum bactericidal concentration

20µL of sample were collected from wells which did not show any growth and inoculated on sterile prepared nutrient agar. The plates were then incubated overnight at 37˚C. The MBC was read as the lowest concentration of *L. lanceolata* which did not show any visible growth on agar

RESULTS

Extraction yield

The result of percentage yield of extract obtained from 100g of dried powder of stem bark of *L. lanceolata* is given in table1. The result indicated that,

Ethanol extract had the highest percentage of yield of 15.23% per 100g dried extract when compared to methanol and aqueous extracts.

Table-1: Percentage yield of stem bark extracts of *L. lanceolata*

Extract	Yield (%)
Ethanol	15.23
Methanol	12.98
Aqueous	6.25

Percentage yield of fractions from Liquid -liquid fractionation

The result of percentage yield of fractions of *L. lanceolata* obtained from 10g of ethanolic crude extract is shown in table1. The result indicated that, N-hexane fraction had the highest percentage of yield of 22.1% when compared to other fractions.

Table-1.1: Percentage yield of fractions of ethanolic extract of stem bark of *L. lanceolata*

Fractions	Yield (%)
N-hexane	22.1
Ethylacetate	31.8
N-butanol	12
Residual aqueous fraction	2.1

Antimicrobial susceptibility testing

The result of antibacterial activity (diameter zones of inhibition) of stem bark extracts of *L. lanceolata* was tested using agar well diffusion method. The results of the assay showed that, aqueous fraction has antibacterial activity against *S. aureus, P. aeruginosa, E. coli and B. subtilis*. The activity of aqueous fraction was found to increase with increasing dosage and this is expressed by measured zone of inhibition of bacteria. The values for diameters of zone of inhibitions were from…..mm for aqueous fraction, while antibiotics (positive controls) the values were from ….mm. The result is represented in table ……

Table-1.2: Antibacterial activity of ethanol extract of *L. lanceolata*

Sample	Concentration (mg/mL)	Organisms [(zones of inhibitions (mm)]			
		S. aureus	***P. Aeruginosa***	***E. coli***	***B. subtilis***
ethanol extract of *L. lanceolata*	31.3	14 ± 0.6	12 ±0.8	11 ± 0.4	14 ± 1.0
	62.5	16 ± 0.8	14 ± 0.5	12 ± 0.3	14 ± 1.5
	125	17 ± 0.6	16 ± 1.0	19 ± 1.0	15 ± 0.9
	250	18 ± 0.8	18 ± 1.3	20 ± 1.5	18 ± 0.8
	500	21 ± 0.4	20 ± 1.8	20 ± 1.8	20 ± 0.7
	1000	23 ± 0.2	30 ± 1.2	30 ± 0.8	25 ± 0.7
TCP(µg/mL)	50	40 ± 0.2	32 ± 3.0	32 ± 0.8	31 ± 2.0
DMSO	0	0	**0**	0	0

Key; TCP Tinidazole/ciprofloxacin (+ve control) DMSO=dimethyl sulphoxide (-ve control) 0=no activity

Antibacterial activity values measured by the diameter of zones of inhibition. Data were mean ± SD of three independent experiments.

Table-1.3: Antibacterial activity of aqueous extracts of *L. lanceolata*

Sample	Concentration (mg/mL)	Organisms [(zones of inhibitions (mm)]			
		S. aureus	***P. Aeruginosa***	***E. coli***	***B. subtilis***
aqueous extract of *L. lanceolata*	31.3	12 ± 1.0	0 ±0.0	10 ± 0.4	10 ± 0.8
	62.5	13 ± 1.0	10 ± 0.5	10 ± 0.3	11 ± 0.2
	125	15 ± 0.8	10 ± 1.0	11± 1.0	11 ± 0.3
	250	16 ± 1.1	10 ± 1.3	11± 1.5	12 ± 0.7
	500	20 ± 0.5	17 ± 1.8	12 ± 1.8	13 ± 0.6
	1000	28 ± 0.7	20 ± 1.2	15 ± 0.8	16 ± 0.8
TCP(µg)	50	30 ± 1.5	32 ± 1.0	40 ± 2.0	31 ± 0.5
DMSO (-ve control)	0	0	**0**	0	0

Key; TCP Tinidazole/ciprofloxacin (+ve control) DMSO=dimethyl sulphoxide (-ve control) 0=no activity

Antibacterial activity values measured by the diameter of zones of inhibition. Data were mean ± SD of three independent experiments.

Table-1.4: Antibacterial activity of methanol extract of *L. lanceolata*

Sample	Concentration (mg/mL)	Organisms [(zones of inhibitions (mm)]			
		S. aureus	***P. Aeruginosa***	***E. coli***	***B. subtilis***
methanol extract of *L. lanceolata*	31.3	11 ± 0.8	11 ± 0.6	0 ± 0.0	0 ± 0.0
	62.5	15 ± 1.0	15 ± 0.5	11 ± 0.3	0 ± 0.6
	125	17 ± 1.0	18 ± 1.0	13 ± 1.0	17 ± 0.6
	250	20 ± 1.2	23 ± 1.3	15 ± 0.5	19 ± 0.7
	500	22 ± 0.8	24 ± 1.8	20 ± 0.8	21 ± 0.6
	1000	24 ± 0.5	26 ± 1.2	25 ± 0.8	23 ± 0.8
TCP(µg)	50	32 ± 0.4	31 ± 0.7	30 ± 0.9	40 ± 1.8
DMSO (-ve control)	0	0	**0**	0	0

Key; TCP Tinidazole/ciprofloxacin (+ve control) DMSO=dimethyl sulphoxide (-ve control) 0= no activity

Antibacterial activity values measured by the diameter of zones of inhibition. Data were mean ± SD of three independent experiments.

Table-1.5: Antibacterial activity of fractions of *L. lanceolata* ethanolic extract

Organisms	Diameter of zone of inhibition (mm)/ Different fractions of ethanol extract					
	N-hexane	Ethyl acetate	aqueous	N-butanol	TCP(µg)	DMSO
P. aeruginosa	0	0	0	14 ± 0.58	34 ± 0.00	0
E. coli	0	0	0	15 ± 1.00	42 ± 0.00	0
S. aureus	0	0	0	17 ± 0.58	31 ± 0.00	0
B. subtilis	0	0	0	16 ± 0.00	30 ± 0.00	0

Key; TCP=Tinidazole/ciprofloxacin (+ve control), DMSO=Dimethyl sulphoxide (-ve control)
0=no activity

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of Extracts and Fractions of *L. lanceolata*

The minimum inhibitory concentration (MIC) is considered as the lowest concentration of a compound that inhibited visible growth of bacteria. The results of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of stem bark extracts and fractions of stem bark of *L. lanceolata* showed that, aqueous fraction, the MIC values for *S. aureus* and *Ps. aeruginosa* were found to be 3.125 µg/mL, while that of for *E. coli* was 6.25 µg/mL. This showed that, the MIC for aqueous fraction was good when compared to that of standard tetracycline antibiotic that had MIC value of 3.125 µg/mL for *E. coli* and *S. aureus* and 6.25 µg/mL for *Ps. aeruginosa*. This is illustrated in table 1.4 and 1.5

Table-1.6: Determination of MIC and MBC of ethanol and methanol extracts of *L. lanceolata*

Bacterial isolates	Ethanolic extract (mg/mL)		Methanolic extract (mg/mL)	
	MIC	MBC	MIC	MBC
S. aureus	15.6	+	62.5	+
B. subtilis	31.3	62.5	62.5	62.5
P. aeruginosa	31.3	+	62.5	62.5
E. coli	62.5	+	62.5	+

Key: + =Growth

Table-1.7: MIC and MBC of aqueous extract and N-butanol fraction *L. lanceolate*

Organisms (mg/mL)	Aqueous extract (mg/mL)		N-butanol fraction	
	MIC	MBC	MIC	MBC
S. aureus	62.5	+	125	+
B. subtilis	62.5	+	125	+
P. aeruginosa	31.3	62.5	62.5	+
E. coli	62.5	+	125	+

DISCUSSION

This study revealed the antibacterial activity of different extracts and fractions of stem bark of *L. lanceolata* in methanol, ethanol and aqueous extract of stem bark of *L. lanceolata* against the selected bacterial strains (*S. aureus, B. subtilis, P. aroginosa*, and *E. coli*). However, ethanol extract and n-butanol fraction were found to be most active. Ethanol extract have the highest antibacterial activity with zone of inhibition of (32mm) as compared to methanol and aqueous extracts. Ethanol extract is bacteriostatic to *S. aureus*, *P. aeruginosa*, and *E. coli* where as to B. subtilis it is bactericidal. This finding was similar to that of López *et al*. [17]. Who found that 8-10% (v/v) concentrations of *Origanum vulgare* phytochemicals can completely inhibit the growth of E. coli and other Gram-negative bacteria? Methanol extract is bacteriostatic to *S. aureus* and *E. coli* where as it is bactericidal to *B. subtilis* and *P. aeruginosa*. A similar finding was reported by Reuben *et al*. [18]. Aqueous extract has the least activity. It's antibacterial activity against the tested organisms is little. According to Koduru *et al.* [19] water extract usually has little or no activity against bacteria. This may be due to the fact that, the phytochemicals responsible for the antimicrobial activity are non-polar compounds and hence cannot be solubilized by polar solvent. The fractionation of ethanol crude extract of *L. lanceolata* yielded four fractions with varying or no degree of antibacterial activity. The n- hexane, ethylacetate and residual aqueous fraction have no antibacterial activity. This finding was contrary to the report of Aiman and Mazen [20], who reported sensitivity by some gram-negative organisms in similar solvents, employed using different plants. n-butanol fraction were active against the test organisms with MIC value of 62.5mg/mL in all the organisms and no MBC was detected indicating that n butanol fraction is bacteriostatic to both gram positive (*S.aureus and B.subtilis)* and gram negative *(E.coli and P.aeruginosa).*This finding is in line with the work carried out by Reuben *et al*.

CONCLUSION

There was notable activity of the extract with MIC's against the tested strains of bacteria and the ethanol extract is recorded with greatest activity against the tested bacterial strains, also the n-butanol fraction is bacteriostatic to all the bacterial strains while others are bactericidal to one or more bacterial strain at the different concentrations. It is recommended for future work to isolate, characterize and elucidate the chemical structure of the compound(s) responsible for the observed activity.

REFERENCES

1. Koehn, F. E., & Carter, G. T. (2005). The evolving role of natural products in drug discovery. *Nature reviews Drug discovery*, *4*(3), 206.
2. Bonjar, G. S., & Farrokhi, P. R. (2004). Anti-Bacillus activity of some plants used in traditional medicine of Iran. *Nigerian Journal of Natural Products and Medicine*, *8*(1), 34-39.
3. Gurib-Fakim, A. (2006). Medicinal plants: traditions of yesterday and drugs of tomorrow. *Molecular aspects of Medicine*, *27*(1), 1-93.
4. Magassouba, F. B., Diallo, A., Kouyaté, M., Mara, F., Mara, O., Bangoura, O., & Lamah, K. (2007). Ethnobotanical survey and antibacterial activity of some plants used in Guinean traditional medicine. *Journal of ethnopharmacology*, *114*(1), 44-53.
5. Edeoga, H. O., Okwu, D. E., & Mbaebie, B. O. (2005). Phytochemical constituents of some Nigerian medicinal plants. *African journal of biotechnology*, *4*(7), 685-688.
6. Farnsworth, N. R., Akerele, R. O., Bingel, A. S., Soejarto, D. D., & Guo, Z. (1985). Sources, action and uses of plant-derived drugs and their corrections. *Bull. WHO*, *63*, 965-981.
7. Shahidi, B.H. (2004) Evaluation of antimicrobial properties of Irananian Medicinal Plants against *micrococcus luteus, serratia marcescens, klebsiella pneumonia and Bordetella brochoseptica. Asian Journal of Plant Science, 3*: 82-86.
8. Runyoro, D., Matee, M., Olipa, N., Joseph, C., and Mbwambo, H. (2006). Screening of Tanzanian Medicinal Plants for *anti-candida* activity. *BMC Complement Alternative Medicine*, *6*(11): 1-10.
9. Medical Dictionary. (May, 2008). Webster's New World™ Medical Dictionary, 3rd Edition. Wiley Publishing, Inc. *www.medterms.com/script/main/art.asp.*
10. Grahame-Smith, D. G., & Aronson, J. K. (1992). *Oxford textbook of clinical pharmacology and drug therapy*. Oxford University Press.
11. Garg, S.K., Ghosh, S.S., Mathur, V.S. (1986). "Comparative pharmacokinetic study of four different sulfonamides in combination with trimethoprim in human volunteers". *International Journal of Clinical Pharmacology, Therapy, and Toxicology*, *24* (1): 23–25.
12. Prescott, J.M. (2011). Microbiology 5th edition. The McGraw Hill Companies Incorpation, 806-821.
13. Jacoby, G.A., & Munoz-Price, L.S. (2005). The New Beta-Lactamases. *New England Journal of Medicine*, 352: 380.
14. Won, J.O., Cheong, Moon, H.P., Gyoung, W.K., Joung, H.O.K.O. and You, J.S., (2005). Bull Korean chem. SOC.26;5.
15. Cheesbrough, M. (2006). *District laboratory practice in tropical countries*. Cambridge university press.
16. Wiegand, I., Hilpert, K., & Hancock, R. E. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature protocols*, *3*(2), 163.

17. López, P., Sánchez, C., Batlle, R., & Nerín, C. (2007). Development of flexible antimicrobial films using essential oils as active agents. *Journal of Agricultural and Food Chemistry*, *55*(21), 8814-8824.
18. Reuben, K. D., Abdulrahman, F. I., Akan, J. C., Usman, H., Sodipo, O. A., & Egwu, G. O. (2008). Phytochemical screening and in vitro antimicrobial investigation of the methanolic extract of Croton zambesicus Muell ARG stem bark. *Eur J Sci Res*, *23*(1), 134-140.
19. Koduru, S., Grierson, D. S., & Afolayan, A. J. (2006). Antimicrobial Activity of Solanum aculeastrum. *Pharmaceutical biology*, *44*(4), 283-286.
20. Al-Mariri, A., & Safi, M. (2014). In vitro antibacterial activity of several plant extracts and oils against some gram-negative bacteria. *Iranian journal of medical sciences*, *39*(1), 36.