ISSN: 0067-2904

Antibacterial activity of different part of Neem (*Azadirchta indica*) growing in Sharjah, United Arab Emirates

Kadhim Hashim Yaseen
Department of Biology, College of Science, Al Mustansiriyah University , Baghdad , Iraq

Abstract

Aqueous and ethanolic extracts of different parts (seeds, leaves, bark) of neem plant (*Azadirachta Indica)* were screened for antibacterial activaties against five species of bacteria (*Staphylococcus aurous*, *Staphylococcus epidermises*, *Acinetobacter baumanni*, *Psedommonas aeruginosa*, *and Escherishia coli*). Different extracts 40-80 mg/L were tested using Ager-well diffusion method. Neem parts potent demonstrated for anti-bacterial activaties against all microorganisms tested. The results showed that neem *seeds* aqueous and ethanolic, extract have significant effects for all tested bacteria, the maximum inhibition zone by seeds cold aqueous and cold ethanolic extracts were 22 & 13 mm for *E. coli* and *S. epidermidis* respectively; while leaves extracts were given 15 & 13 mm inhabitions zones against *S. aureus* and *E. coli* respectively. But extracts were showed inhibition zone 22 & 13 mm for *S. aureus* and *S. epidermidis* respectively. The fatty acids were determine in seeds neem, the compositions resulted in detection of eight fatty acids, the maximum dominant compound is Linoleic Acid and the minimum construction is Palmiticoleic Acid, the ethanolic extracts contained, *Phenol*, *Alkaloids*, *Tannins*, *Glycosides*, while the Steroids were absent, the results gathered from this study indicated that neem plant has anti-Gram negative and Gram positive bacteria.

KeyWords: Neem, *Azadirchta indica*, Fatty acids, Chemical composition, Antibacterial activity.

دراسة الفعالية المضادة للبكتريا لأجزاء من نبات النيـم النامية في الشارقة – الامارات العربية المتحدة

كاظم هاشم ياسين
قسم علوم الحياة ، كلية العلوم ، الجامعة المستنصرية، بغداد، العراق

الخلاصة

اجري هذا البحث لمعرفة الفعالية التثبيطية للبكتريا لكل من المستخلص المائي والايثانولي للأجزاء (البذور، الاوراق، القلف) لنبات النيم Azadirchta indica تجاه خمسة من الانواع البكتيريـة الممرضـه Staphylococcus aurous, Staphylococcus epidermidis , Acinetobacter baumanni, Escherichia coli, Pseudomonas aeruginosa وبالتراكيز 40mg/L و 80mg/L اظهر التركيز 80mg/L اعلى تأثير معنوي للأجزاء النباتية المدروسة .كان المستخلص المائي والكحولي للبذور ذا تأثير تثبيطي معنوي لكافة الاحياء المجهرية المدروسة لذا بلغت اقطار التثبيط للمستخلص المائي البارد والمستخلص الكحولي البارد 22mm و 13 mm لبكتريا E. coli و S. epidermidis على التوالي،

*Email: Khzwaini@yahoo.com

واظهر المستخلص للأوراق اعلى تأثير اذ بلغ 15 mm و 13mm لبكتريا S. aureus و E. coli على التوالي، في حين سجل اعلى تأثير لمستخلص القلف 22mm و 13mm على بكتريا S. aureus و S. epidermidis على التوالي ايضا ، قدرت الاحماض الدهنيه لبذور النيم فوجد ان اعلى تركيز للحامض Linolic acid واقل تركيز للحامض Palmitic acid ، أجريت بعض الاختبارات الكيميائيه الكميه على اجزاء من نبات النيم فوجد انه يحتوي على المركبات الفينوليه ، القلويدات ، التانينات الكلوكوسيدات ، الصابونيات وقد خلت من الستروييدات، أشارت النتائج ان نبات النيم يمتلك فعاليه مضاده للبكتيريا السالبه والموجبه لصبغة كرام.

Introduction

Neem tree (*Azadirchta indica* A. Juss) is related to the family Meliaceae. It is tropical evergreen tree at altitudes between sea level and 700 m, the best growth for neem is in 9.5-37°c ,it could tolerate 50°c but it could not grow in less than 4°c, and the annual rainfall (450 – 1200mm) . It has been used in ayurvedic medicine for more than 4000 years due to its medicinal properties, Most of the plant such as fruits, seed, leaves, bark and root contain compounds wich provided to be antiseptic, antiviral , antipyretic , anti-Inflammatory , antiulcer and antifungal. Neem tree is one of best tree in the world, it is the most useful plant used in traditional medicine as a source of many therapeutic in the Indian culture, the plant grows well in the tropical and semi-tropical countries [1-3]. The drugs were derived from natural sources which play a important role in the prevention and treatment of human diseases. Which shown by (WHO) and their reports in 2008 [4].

We can use neem as alternative therapy to treat antibiotic – resistant microorganisms is a (Combination therapy) which uses a combination of plant extract and antibiotic against resistant pathogens. [5].

Earlier studies on neem have shown that it contains active substances in its parts including seed, leaves, roots, bark, trunk and branches with multiple medicinal properties, neem seeds are used in traditional medicine to treat infection conditions especially those affecting the eye and ear, in addition the neem leaf extract has a good therapeutic potential as anti-hyperglycemic agent, the plant has antibacterial properties and could be used for controlling airborne bacterial contamination in the residential premise[6].

In this study the antimicrobial activity of aqueous and ethanolic extracts of neem seeds , leaves , bark against some bacterial species was studied .

Materials and Methods

Plant materials: Neem seeds, leaves and bark were collected from Sharjah-UAE in August 2014, the plant was authenticated by a Botainist in the Department of Biology, college of science at University of Al-Mustansiryha. The plant was cleaned by distilled water, dried in the shade at room temperature, they were chopped into small pieces then powdered with mechanical grinder, the powder was dried at room temperature for 24 hrs. and were used for extraction.

Preparation of plant extract

The cold water extract was prepared by steeping 15g of the plant powder in 100 ml of cold distilled water and the extract was lefted in a cooled orbital incubator for 24hrs, then filtered and put it in oven at 40°c to prepare the stock solution and achieve the desirable concentration [7].

The hot water extract was prepared by boiling 15 g of plant powder in 100 ml of distilled water for 30 min, then desirable concentrations were obtained [8].

The cold alcoholic extract was prepared by taken 100g of the prepared plant parts powder and dissolved in 400 ml (70%) ethanol and the extract was left in a cooled orbital Incubator for 24 hrs. The method was continued to achieve the desirable concentrations as indicated above [9].

The hot alcoholic extract applied by using approximately 50g of dried plant materials which extracted by 200 ml of ethanol (70 %) (Hexane for seeds) and extracted using Soxholet apparatus of 500 ml for 7hrs with continuous slow mixing, the extract solution was filtered and the solvent was removed using rotary evaporator at 45c to obtain the crude extract , which kept in sterile bottles at 4°c until use. The resulted deposit was dissolved in sterile distilled water to prepare the concentrations [10].

Antibacterial sensitivity test

The effect of the extract against bacterial growth was tested separately using agar well diffusion method [11].Tested organisms included both Gram-negative and Gram-positive bacteria which included to evaluate the antibacterial activity of the three neem parts extracts, the inoculum was performed by making bacterial suspension from 4-5 colonies of an overnight culture grown on nutrient agar, the resulted turbidity was compared with turbidity standard 0.5 McFarland solution which corresponds to a density of 1.5×10^8 cells /ml The suspension was inoculated on Mueller-Hinton agar plates, in each plate a single well of 5mm diameter was made using a sterile cork borer, the extracts were tested at two concentrations 40mg/L, 80mg/L, one well contained normal saline and served as control.

Antibacterial assay plates were incubated at 37±2°c for 24hrs to allow maximum growth of bacteria, antimicrobial activity was evaluated by measuring the diameter of the inhibition zone and measured in mm, the assay was repeated three times and the mean diameter was recorded.

Statistical analysis

The results of antibacterial activity were analyzed according to SAS 2012 significant differences between mean values which determined at a level of $p\leq0.05$.

Fatty acid composition analysis

The analysis was performed by Gas Chromatography Shimadzo 2014 modified procedure , (200°c capillary colum polar 220°c FID detector, N2 carrier gas) . The preparation of test samples were done after taken 0.1g of oil then add 1ml of normal heptane was added followed by the addition of methanolic 0.1 ml /KOH solution (5 g KOH dissolved in 100 ml methanol), mixed and left for half hours, a two layers formed. 1 µl from upper layer was injected in the apparatus during 15 mints [12].

chemical analysis of plant extracts

The chemical analysis was performed to identify the component of each extract. Dragendorffs reagent (solution) was used to detect the alkaloids in the plant extracts, Ferric Chloride solution (1% aqueous solution of $FeCl_3$) was used to detect the phenolic compound in the extracts, One percent Ferric Chloride solution was used to detect tannins, the Benedict's and Molisch's reagents were used for the detection of glycosides. Five ml from extract in a test tube was shaken strongly during over half min, appear fuming during 3-5 min indicated the presents of sponging in the extracts, steroids in ethanolic extract was done by adding to 1gm of extract a little pit of chloroform followed by the addition of one drop of concentration sulfuric acid, a blue color formation indicates the presence of steroids.[13]

Results and Discussions

The aqueous and ethanolic extracts of neem seeds were screened against five tested bacteria, all microorganisms were found to be sensitive to the extracts as shown in Table 1, Both 40 and 80mg/L concentration of seeds extracts gave excellent effect as antibacterial especially at concentration of 80mg/L. The bioassay showed the maximum inhibition which was 13 & 22mm towards *S. epidermidis* and *E. coli* respectively at the concentration 80mg/L cold ethanolic and cold aqueous extracts after 48h of exposure time compared to distilled water. Seeds fatty acids and composition may be attributed to the origin' plant species and their growth conditions [14].The results were also influenced by many factors such as climate and topography. [15]. Many factors such as type of bacteria, type of solvent, extraction methods, temperature and pH of extracts were found to be influential [16]. Neem seeds oil used as therapeutic agent for fighting pathogenic bacteria, and another oil was used as well such as: Anised Oil, Calamus Oil, Camphor Oil, Cedar wood, Clove Oil, Lime Oil and Rosemary Oil [17,18,19], Combination of neem seeds oil with another plants oil gave synergistic effect as antibacterial agent [5,2o]. The aqueous extract was more effective than the ethanolic extract of neem leaves. Table-2. shows the maximum antimicrobial activity that was observed on *S. aureus*, at 80mg/L of cold aqueous, with an inhibition zone of 15mm, while it gave a 13mm on *E. coli* at 80mg/L of cold ethanolic extract with an inhibition zone. This result is in agreement with the findings of Dhanya Kumar *et al.* ,2011. [21]. The results in Table 3 Shows the effect of bark extract on different types of bacteria which agree with Md Mohashine[22].In general gram negative have more resistance because of their cell wall and have susceptible bacterium to aqueous and ethanol extract of neem. [23,24].

The results were agreed with the study that done by Mamman *et al*. (2001) that the bark has more effect than leaf extract [25].

The antibacterial activities of the plant parts reported in this study might be attributed to the types of compounds recorded from this plant agreed with many studies that its include the major eight fatty acids , the Linoleic acids has the highest percentage that is 35.36%. The chromatogram in Figure-1 explain the retention time of different types of fatty acids that found in the neem seed.when the peaks of Linoleic acid , Oleic acid , Stearic acid . Table- 4, Figure-1 [26,17,18,19,12] . Alkaloid, phenol compounds, tannins, glycosides as well as saponines found in the ethanolic and aqueous extract of the neem plant parts and this agreed with other studies shown inTable- 5 [27,13].

In this respect, we noticed that approximately 135 chemical compounds identified from different parts of neem. These compounds are divided into two major groups, the first isoprenoids are includes diterpenoids and triterpenoids containing protomeliacins, limonoids, azadirone and its derivatives, gedunin and its derivatives, vilasinin type of compounds and c-secomeliacins such as nimbin, salanin and azadirachtin. The second which non-isoprenoids included carbohydrates (polysaccharides), proteins (amino acids), sulphur compounds, polyphenols such as flavonoids and their glycosides, dihydrochalcone, coumarine and tannins, aliphatic compounds, phenolic acids, etc.. Bioactivities of only few compounds have been studied, this compounds could be antibacterial as reported by [28,29,30,31], the theme emerging from this study is to further characterize the active antibacterial substances that affected both Gram negative as well as Gram positive microorganisms, seen in this report which requires further studies.

Table 1- Antibacterial activity of aqueous and ethanolic (cold and hot) extracts from the seeds of neem on test bacteria

Test bacteria	Mean inhibition zone (mm)								Lsd
	Cold aqu. Ext.		Hot aqu. Ext.		Cold ethan. Ext.		Hot. Ethan. Ext.		
	40 mg/L	80 mg/L	40 mg/L	80 mg/L	40 mg/L	80 mg/L	40 mg/L	80 mg/L	
Staphylococcus aureus	15.0	17.0	15.0	17.0	10.0	12.0	6.0	6.5	* 2.92
Staphylococcus epidermidis	13.0	15.0	12.0	14.0	10.0	13.0	5.5	6.0	* 3.07
Acinetobacter Baumanni	5.5	6.0	8.0	10.0	9.0	11.0	5.5	5.5	* 2.61
Pseudomonas aeruginosa	6.0	6.0	5.5	5.5	8.0	10.0	5.5	6.0	* 2.05
Escherichia coli	21.0	22.0	7.0	8.0	8.0	11.0	6.5	7.0	* 5.38
Lsd	4.52 *	3.95 *	3.47 *	3.69 *	2.75 NS	3.15 NS	2.37 NS	2.29 NS	…… …….

Distilled water was served as negative control and gave zero inhibition zone.
* Significant at P < 0.05, NS is not significant at P < 0.05

Table 2- Antibacterial activity of aqueous and ethanolic (cold and hot) extracts from the leaves of neem on test bacteria

Test bacteria	Mean inhibition zone (mm)								Lsd
	Cold aqu. Ext.		Hot aqu. Ext.		Cold ethan. Ext.		Hot. Ethan. Ext.		
	40 mg/L	80 mg/L	40 mg/L	80 mg/L	40 mg/L	80 mg/L	40 mg/L	80 mg/L	
Staphylococcus aureus	13.0	15.0	8.0	8.5	9.0	11.0	6.5	7.0	* 3.26
Staphylococcus epidermidis	10.0	13.0	8.0	9.0	7.0	9.0	5.5	6.0	* 2.55
Acinetobacter Baumanni	5.5	7.0	6.0	6.5	5.5	6.0	6.0	6.5	* 2.06
Pseudomonas aeruginosa	5.5	6.0	6.5	7.0	6.0	7.0	6.0	6.5	* 2.15
Escherichia coli	8.0	10.0	6.0	7.0	10.0	13.0	7.0	7.5	* 2,63
Lsd	2.57 *	2.33 *	2.24 NS	2.61 NS	2.59 *	2.42 *	1.97 NS	2.08 NS	…… …….

Distilled water was served as negative control and gave zero inhibition zone.
Statistical symbols as indicated in Table- 1.

Table 3- Antibacterial activity of aqueous and ethanolic (cold and hot) extracts from the barks of neem on test bacteria

Test bacteria	Mean inhibition zone (mm)								Lsd
	Cold aqu. Ext.		Hot aqu. Ext.		Cold ethan. Ext.		Hot. Ethan. Ext.		
	40 mg/L	80 mg/L	40 mg/L	80 mg/L	40 mg/L	80 mg/L	40 mg/L	80 mg/L	
Staphylococcus aureus	17.0	18.0	20.0	22.0	8.0	9.0	9.0	10.0	* 4.26
Staphylococcus epidermidis	7.0	7.5	8.0	8.0	10.0	13.0	9.0	9.5	* 2.79
Acinetobacter baumanni	5.5	6.0	6.0	6.0	7.0	7.5	7.0	8.0	* 2.34
Pseudomonas aeruginosa	7.0	9.0	9.0	12.0	6.0	6.5	7.0	7.0	* 2.61
Escherichia coli	6.0	6.5	7.0	8.0	8.0	8.5	9.0	9.0	* 2.52
Lsd	3.91 *	3.68 *	3.71 *	3.58 *	2.48 *	2.72 *	2.48 NS	2.63 *	…… …….

Distilled water was served as negative control and gave zero inhibition zone. Statistical symbols as indicated in Table-1.

Table 4- Fatty acids composition of neem seeds

Fatty acid	Systematic name	Formula	Structure	Ret. time	Area %
Linoleic acid	9,12-octadecadienoic acid	C18H32O2	C18.2	3.118	35.36
Oleic acid	9-octadecenoic acid	C18H34O2	C18.1	3.508	27.74
Stearic acid	Octadecanoic acid	C18H36O2	C18.0	1.925	18.23
Palmitic acid	Hexadecanoic acid	C16H32O2	C16.0	2.947	9.26
Arachidic acid	Eicosanoic acid	C20H40O2	C20.0	4.831	1.28
Behenic acid	Docosanoic acid	C22H44O2	C22.0	5.501	0.25
Lignoceric acid	Tetraacosanoic acid	C22H48O2	C24.0	---	0.18
Palmiticoleic acid	9-hexadecenoic acid	C16H30O2	C16.1	---	0.10

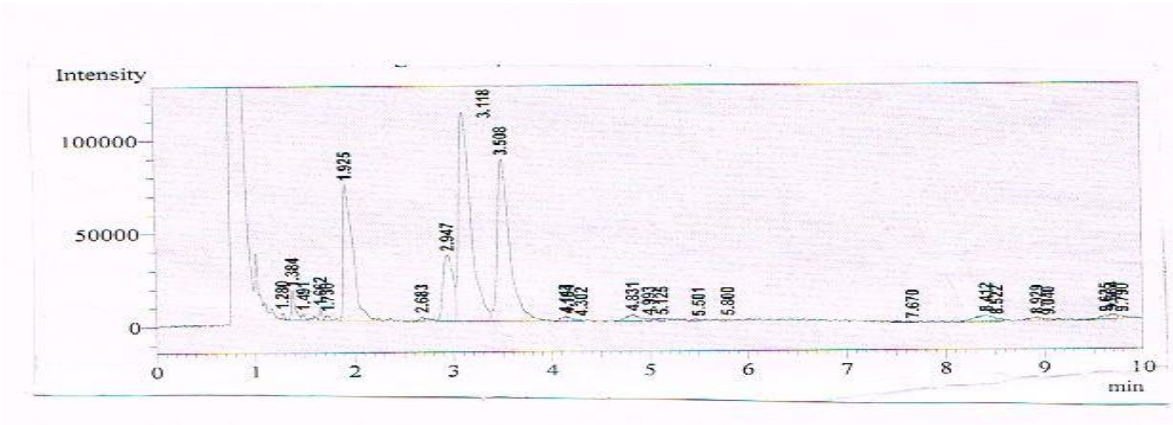

Figure 1- G.C. chromatogram for separation of fatty acids composition in seeds of neem .

Table 5- detection of some chemical constituents of neem plant parts.

Chemical constituents	Seeds		leaves		Barks	
	Cold ethanolic extract	Hot water extract	Cold ethanolic extract	Hot water extract	Cold ethanolic extract	Hot water extract
Alkaloids	+	+	+	+	+	+
Phenol compounds	+	+	+	+	+	+
Tanins	+	+	+	+	+	+
Glycosides	+	+	+	+	+	+
Saponines	+	+	+	+	+	+
steroides	____	____	____	____	____	____

References

1. Sharma, P., Tomar, L., Bachwani, M.I. and Bansal, V. **2011**. Review on Neem *Azadirachta indica*: thousand problems one solution. *International research J. of , pharmacy, 2(12),97-102.*
2. Puri, H.S. **1999**. Neem divine tree. *Harwood, Academic publishers, Amsterdam.*
3. Singh, R.P., Chari, M.S., Raheja, A.K. and Kraus, W. **1996**. Neem and Environment. *Oxford & IBH publishing, New Delhi, Vol.1and11, pp.1-1198.*
4. Kumral, A. and Sahin, T. **2003.** Effects of some spice extracts on *Escherichia coli*, *Salmonella typhimurium, Lisetiar monocytogenes, Yersinia enterocolitica* and *Enterobacter aero gene. Ann. Microbiol*.53, 427-435.
5. Sevil, T. **2011**. In-vitro antimicrobial activity and synergistic antagonistic effect of interaction between antibiotics and some spice essential oils. *J. of Environmental Biology, Jan.32 (1)23-29.*
6. Biswas, K., Chattopadhyay , I. and Banerjee , R.K. **2002**. Biological activities and medicinal properties of neem (Azadirachta indica) . *Curr. Sci. , 82: 1336 – 1345.*
7. Abhishek, M., Mamta; Niketa; Poonam, Pranjul and Priyanka **2013**. Antibacterial effects of crude extract of *Azadirachta indica* against *Escherichia coli* and *Staphylococcus aureus. Inter. J. of Sci. Env. and Tech. Vol. 2, No.5, 989*-993.
8. Trease, G. E. and Evans, W. C. **2002**. Pharmacology. 15thg. End, Saunders Publishers, LONDON, PP:42-393.
9. Andesine, C. and Perez, C**. 1993**. Screening of plants used in Argentine folk medicine for antimicrobial activity *J. of Ethno pharmacology, 39(2):119-128.*

10. Annegowda, H. V., Mordi, M. N., Ramanathan, S. and Mansor, S.M. **2010,** Analgesic and antioxidant properties of ethanolic extract of Terminalia catappa L. leaves. *Int. J. Pharmacology. 6(6):910-915.*

11. Mahmood, M. j., Jawad, A. J., Hussein, A. M., Al-Omari, M. and Al-Naib, A. **1989**. In vitro antimicrobial activity of *Salsola rosmarinus and Adiantum capillus veneris. Int. J. Crude Drug Res., 27:14-16.*

12. Jacinta, S., Azhari, H. N., Saiful Nizam, B. T. and Abdurrahman, H. N. **2013**. Fatty acid composition and antibacterial activity of Neem (*Azadirachta indicia*) seed oil. *The Open Conference Proceedings Journal, 4, (Suppl-2, M11) 43-48.*

13. Ayeni, K. E. and Yahaya, S. A. **2010**. Phytochemical screening of three medicinal plants neem leaf *(Azadirachta indica*), Hibiscus leaf(*Hibiscus rosasinsis*) and spear grass leaf*(Imperata cylindrical*). *Continental J. pharma-ceutical Sciences 4:47-50.*

14. Atabani, A. E.; Silitonga, A. S.; Ong, H. C.; Mahlia, T. M. I.; Masjuki, H. H.; Irfan, A. B.; Fayaz, H**. 2013**. Non-edible vegetable oils: Acritical evaluation of oil exeraction,fatty acid compositions, biodiesel production. *Renew. Sustain. Energy Rev., 18, 211-214.*

15. Stoney, C. **1997.** Fact sheet on *Azadirachta indica* (neem)-a versatile tree for the tropics and subtropics, publication of Forest,Farm, and Community Tree Network (FACT Net), Arkansas, United States, www. winrock. Org/forestry/factnet.htm

16. Goutam Brahmachari. **2004**. Neem-An Omni-potent plant: A Retrospection. *Chem. Bio. Chem. (Wiley-VCH)- 5:408-421*.

17. Tuhin Jahan, Zinnat Ara Begum and Sayeeda Sultana. **2007.** Effect of neem oil on some pathogenic bacteria. *Bangladesh, J. Pharmacol. 2, 71-72.*

18. El-Mahmood, A. M., Ogbonna, O. B. and Raji, M. **2010**. The antibacterial activity of *Azadirachta indica* (neem) seeds extracts against bacterial pathogens associated with eye and ear infections. *J. Medi. Plants Res.,4,1414-1421.*

19. Chindo, I. Y., Osiude, J. O. and Yongabi, K.A. **2011**. *Azadirachta indica* (neem) seed oil as adjuvant for antimicrobial activity. *Int. Res. J. Appl. Basic Sci.,2,299-302.*

20. Chanda, S. and Rakholiya, K. **2011.** Combination therapy: Synergism between natural plant extracts and antibiotics against infectious diseases. Science against microbial pathogens*: Communicating current research and technological advances.520-529.*

21. Dhanya Kumar, N. M. and Sidhu, P. **2011**. The antimicrobial activity of *Azadirachta indica*, *Glycyrrhiza glabrat*, *Cinnamum zeylanicum*, *Syzygium aromaticum*, *Acacia nilotica*, on *Streptococcus mutans* and *Enerococcus faecalis.* An in vitro study. Endodontology , 23: 18 – 25.

22. Md Mohashine, B., Michiko, N., Seishi, M. and Tsutomu, S. **1997**. Antibacterial effects of the crude *Azadirachta indica* Neem bark extract on *Streptococcus sobrinus*. *Pediatric Dental. J. 7(1):61-64.*

23. Gajendrasinh , P.R., Bhavik , M.K., Rohit , S., Hetal , A. and Prajapati , P.K. **2009**. In vitro antibacterial study of two commonly used medicinal plants in ayurved : Neem (*Azadirachta indica* L.) and Tulsi (*Ocimum sanctum* L.). *IJPBA* ; 3 (3) : 582-586.

24. Uwimbabazi, F., Uwimana, J. and Rutanga , J.P. **2015**. Assessment of antibacterial activity of neem plant (*Azadirachta indica*) on *Staphylococcus aureus* and *Escherichia coli* . *Journal of medicinal plant studies*; 3(4) 85-91.

25. Mamman , P.H., Mshelia , W.P., Susbatrus , S.C. and Sambo , K.W**. 2013**. Antibacterial effects of crude extract of *Azadirachta indica* against *Escherichia coli* , *Salmonella* spp. And *Staphylococcus aureus.Int. J. Med. Sci.; 5 (1) : 14-18.*

26. SaifRam, M. LLavazhagan, G., Sharma, S. K., Dhanraj, S. A., Suresh, B., parida, M.M., Jana, A.M.; Devendra, K. and Selvamurthy, W. **2000.** Anti-microbial activity of a new vaginal contraceptive NIM-76 from neem oil *(Azadirachta indica). J. Ethnopharmacol. 71(3):377-382.*

27. Singh, U. P., Maurya, S. and Singh, D. P. **2005**. Phenolic acids in neem (Azadirachta indica):a major pre existing secondary metabolites. *J. Herb.Pharmacother,5:35-43.*

28. Chawla, A. S., Kumar, M., M. and Bansal, I. **1994.** Chemical constituents and biological activity of neem areview. *Indian Drugs, 32:57-62.*

29. Luo, X., Ma. Y. and Wu, S. **1999**. Two novel azadirachtin triterpenoids from the leaves of *Azadirachta indica. J. Nat. Prod. 62:1022-1024.*

30. Siddiqui, B. S., Afshan, F. and Gulzar, T. **2004.** Tetracyclic triterpenoids from the leaves of *Azadirachta indica. Phytochemistry*. *65:2353-2367*.

31. Susmitha, S., Vidyamol, K. K., Ranganayaki, P. and Vijayaragavan, R. **2013.** Phytochemical extraction and antimicrobial properties of *Azadirachta indica* (Neem). *Global J. of Pharamacology. 7(3):316-320.*