International Annals of Science
ISSN: 2456-7132
Volume 8, Issue 1, pp. 47-53, 2020
DOI: https://doi.org/10.21467/ias.8.1.47-53

RESEARCH ARTICLE

Antibacterial Activity of *Melia azedarach* Leaves against *Salmonella typhi* and *Streptococcus pneumoniae*

Charlene Mwale, Kuda Nelia Makunike, Rumbidzai Mangoyi*

Department of Biochemistry, University of Zimbabwe

* Corresponding author email: rrumbie.2000@gmail.com

Received: 17 May 2019 / Revised: 07 August 2019 / Accepted: 19 August 2019 / Published: 30 August 2019

ABSTRACT

Antimicrobial drug resistance is increasingly becoming an important global problem. Among the major causes for concern is drug resistant *Streptococcus pneumoniae* and *Salmonella typhi*, which have become resistant to at least one antibiotic. This challenge has lead scientists to investigate plants as potential sources of antimicrobial agents since they have been used to treat diseases long before the discovery of antibiotics. In Zimbabwe, typhoid is a leading cause of mortality and morbidity due to poor sanitation and poor treatment regimes. Traditionalists are using *Melia azedarach* leaves for the treatment of diarrhea, a typhoid symptom. Thus, this study focused on validating the use of *M. azedarach* leaves for medicinal purposes by determining their antibacterial activity against *S. pneumoniae* and *S.typhi*, the causative agent of typhoid fever. *Melia azedarach* leaf constituents were extracted using ethanol, ethylacetate, hexane, dichloromethane and methanol. Their antibacterial activities were assessed using the agar disk diffusion, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays. Haemolysis assay was carried out to determine the toxicity of the potent extracts. The ethanol and hexane extracts showed antibacterial activity against *S. typhi* whilst dichloromethane and hexane extracts showed antibacterial activity against *S. pneumoniae*. Minimum inhibitory concentrations for ethanol and hexane against *S. typhi* were < 1 µg/ml and 15.6 µg/ml respectively, whilst their minimum bactericidal concentrations were 31.25 µg/ml and 250 µg/ml. The MICs for dichloromethane and hexane extracts against *S. pneumoniae* were 31.25 µg/ml and 62.5 µg/ml respectively, whilst their MBCs were 31.25 µg/ml and 125 µg/ml. The extracts ethanol, hexane and dichloromethane had haemolytic activity of 63 %, 62 % and 59 % respectively. Therefore, these results validate the use of *M. azedarach* leaves for medicinal purposes. However, these leaves may be toxic to human consumption, thus there is need for further investigation on their toxicity in *vivo*.

Keywords: antibacterial, Melia azedarach, plant extract, Salmonella typhi, Streptococcus pneumonia, toxicity

1 Introduction

Most cases of death are due to infectious diseases, particularly in low-income countries [1]. A number of antibiotics have been introduced to treat these diseases, accounting to the fall in the number of deaths [2]. However, disease causing agents are still a threat to society due to resistance of these causative agents to the antibiotics [3]. The overuse and misuse of these antibiotics has led to the resistance of these strains to treatment [4]. Typhoid fever, being an important infectious disease, has received considerable control efforts at national, regional and district levels with the aid of World Health Organisation. However, despite all the efforts the disease continues to persist leading to significant morbidity and mortality rates due to antibiotic resistance. Typhoid fever is caused by *salmonella typhi*, which has emerged and become prevalent in typhoid outbreak areas, suggesting that the mortality rate will likely to increase in the future if new treatment options are not developed [5; 6]. Typhoid fever is a life-threatening infectious disease that is common in Zimbabwe especially in the summer seasons according to the City of Harare council report of

2011. The leading cause of the outbreak of the disease is due to the inadequate sanitary provisions for everyone in the country. Water supplies are not sufficient enough and hence, individuals tend to use other water sources like unprotected wells and river water, contaminated boreholes [7]. The sewage reticulation systems installed in the colonial era have not been improved as they were installed to accommodate a low population number. These systems are failing due to the growing populations leading to sewage spillage and raw sewage flowing in suburb streets. Substandard waste removal systems are also a threat to the spread of the disease. According to the UNICEF Zimbabwe Mid-Year Situation Report, from January 2017-June 2017, a total of 2,415 suspected typhoid cases with six typhoid related deaths were reported, out of which 79 were laboratory confirmed.

On October 16, 2017, the Harare City Health Department (HCHD) identified a cluster of 17 suspected typhoid cases in residents of Harare's Mbare suburb. As of 24 February 2018, more clusters were detected in Harare's western suburbs, including Kuwadzana, where high rates of ciprofloxacin-resistant Typhi were identified. A total of 3,187 suspected and 191 confirmed cases were identified with no reported deaths among confirmed cases. Among suspected cases, 1,696 (53%) patients were male, and median age was 17 years (range = 1 month–90 years). [8]

The standardized collection and analysis of clinical and laboratory information during an outbreak in which an unusual regional antibiotic resistance pattern featured prominently prompted public health officials to recommend third-generation cephalosporins as first-line treatment for patients residing in areas with high rates of ciprofloxacin resistance [9]

On the other hand, the incidence of antimicrobial resistance in the leading causative agent of pneumococcal infections, *S. pneumoniae* is a major public health concern worldwide. *Streptococcus pneumoniae* has become resistant to most commonly prescribed antibiotics including β lactams and macrolides by acquiring genes from closely related species residing in the same environment resulting in transformation. By this process, different strains of *S. pneumoniae* have become resistant to most classes of antibiotics [10]. The lethality of pneumococcal infection arises as a complication of immunosuppression due to the presence of a debilitating disease. *Streptococcus pneumoniae* is the leading cause of bacterial meningitis, pneumonia and sepsis in children and the elderly. Haematologic cancers, anatomic asplenia, acquired immune deficiency like HIV infections and congenital immune deficiency like SCID as well as immunosuppressive therapy (including chemotherapy) are risk factors for infections with invasive *S. pneumoniae*.

Lately, there has been an interest in traditional medicines driven by interest in complementary medicine. *Melia azedarach* is one of the plant species that has been used in the history of herbal medicines. The tree is native in south of Asia and has been naturalized in Zimbabwe and most parts of the world including America [11]. In Zimbabwe, it is known as *mukin*a or *musiringa*, but in English it is known as china berry tree or Indian lilac. The tree belongs to the mahogany family, Meliaceae. *Melia azedarach* has been used traditionally to treat symptoms associated with typhoid infection such as headache, abdominal discomfort, vomiting, diarrhea, cough, or constipation, fever and other infections. Scientific and clinical research has proven that extracts of the tree have antimicrobial, anticancer, antioxidant, male contraceptive, antipyretic, antiplasmodial, antiulcer as well as analgesic properties and have been used to make pesticides and insecticides [12; 13].

Therefore, this study seeks to justify the antibacterial activity of *Melia azedarach* leaf extract being used traditionally to treat stomach disorders and symptoms of bacterial meningitis.

2 Materials and Methods

2.1 Collection of plant leaves

Identification and authentication of the plant was done at Harare Botanical Gardens with the help of Mr Chris Chapano (Botanist). Mature fresh green leaves of *M. azedarach* were collected at the University of Zimbabwe campus opposite the Science lecture theater in August 2017. Collected leaves were dried in open air, ground to a fine

powder and stored in airtight containers at room temperature prior to antimicrobial assays.

2.2 Preparation of plant extracts

The ground powder of the leaves was divided into 5 equal portions and to each portion, solvent was added and mixed thoroughly. The solvents used were dichloromethane, ethyl acetate, ethanol, hexane and methanol. Extraction was carried out for three days with agar disk diffusion assay was used to determine the susceptibility of the plant regular mixing of contents. The mixture was filtered using Whatman No 1 filter paper and cotton wool and the filtrate was collected. The solvents were then allowed to evaporate under room temperature and pressure. Dried extracts were then stored at 4 ºC until use.

2.3 Test organisms

Salmonella typhi and *Streptococcus pneumoniae* strains were provided by Prof. Mukanganyama from the Department of Biochemistry at the University of Zimbabwe, Zimbabwe. Cells were resuscitated separately by culturing them in nutrient broth at 37 °C and 150rpm. The bacteria were then streaked on a nutrient agar plates to determine if the cultures were not contaminated. Presence of different colonies after incubation overnight at 37°C would mean that another bacterium could have contaminated the strains. The nutrient agar plates and the broth cultures were stored in the fridge at 4°C.

2.4 Disc diffusion assay

The extracts against *S. typhi* and *S. pneumoniae*. Sterile filter paper discs (6 mm) were impregnated with 500µg of crude extract of each solvent. The *S. typhi* and *S. pneumoniae* inoculums were prepared to a final concentration of 10^6 CFU/ml by using 0.5M McFarland's standard and poured in agar plates. The impregnated discs were then loaded onto the agar plates and incubated in the fridge at 4°C for 2 hours (pre-diffusion time). After incubation, the bacteria were allowed to grow at 37°C overnight. Pure solvents used for extraction were used as controls. The results were recorded by measuring the zones of growth inhibition. Clear inhibition zones around discs indicated the presence of antimicrobial activity.

2.5 MTT assay

The MTT assay was carried out to determine the minimum concentration of the potent plant extracts that had visible growth inhibitory activity against *S. typhi* and *S. pneumoniae*. Various concentrations of the plant extracts were prepared in a 96 well ELISA plate, from 500 µg/ml to 0.1µg/ml by two fold dilution. A volume 100 µl of the standardized inoculums 10^6 CFU/ml were added to each dilution well containing a different plant concentration. The plate was then incubated overnight at 37°C. Growth of the bacterial cells was observed by turbidity, however because it was not conclusive, 25 µl of 2 mg/ml of MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) was added. MTT assesses the viability of live bacterial cells through a reduction reaction of the yellow tetrazole to give purple formazan with the intensity depending on the concentration of the bacterial cells present. This reaction is catalaysed by oxidoreductase enzymes in viable cells. Thus, the intensity was measured using a spectrophotometer at 540 nm. The lowest concentration of the extract that inhibits growth was recorded as the MIC

2.6 Minimum Bactericidal Concentration determinations

Minimum bactericidal concentration was determined by re-culturing cells from wells that had no viable cells present from the MTT assay. Cells were streaked onto nutrient agar and incubated at 37°C overnight. Growth of the bacteria on agar shows presence of viable cells in the well. The lowest extract concentration which showed no growth of bacteria on the media is the MBC value.

2.7 Haemolytic assay

A volume of 5 ml of sheep blood in EDTA was centrifuged at 1000 rpm and 4 °C for 10 minutes [14]. The supernatant and buffy coat layer were

carefully discarded. The pellet collected was washed 3 times with phosphate buffered saline (PBS). The erythrocyte pellet (100 µl) was diluted using 900µl of PBS and 50 µl of the diluted sample was mixed with 100µl of plant extract. This was done for plants that showed antibacterial activity against both *S. typhi* and *S. pneumoniae*, at their MIC values. For the positive control, 50µl of diluted erythrocytes were mixed with 10 % Sodium Dodecyl Sulphate (SDS). The negative control had 100µl of PBS. The contents were then incubated at 37°C for 60 minutes. After incubation 850µl of PBS were added to each reaction vessel and thoroughly mixed. The tubes were centrifuged at 300 rpm for 3 minutes and the supernatant was put into respective wells of a 96 well ELISA plate. The absorbance was read at 545nm. Percentage lysis was determined using the positive control as 100 % lysis. Percentage haemolysis of the extracts was calculated by the formula:

(Absorbance of red blood cells with plant extract ÷ Absorbance of red blood cells with SDS) ×100%

2.8 Statistical analysis

Data analysis was done by Graph Pad Prism 5 to obtain the standard deviation of the inhibition zones of the extracts and streptomycin obtained from the agar disk diffusion test. Graph Pad Prism 5 was used in calculating the standard deviation for the haemolytic assay absorbances.

3 Results

3.1 Inhibition of growth of *S. typhi* and *S. pheumoniae* growth by *M. azedarach* extracts

The effects *M. azedarach* extracts on the growth of *S. typhi and S. pheumoniae* is shown by zones of inhibition in Table 3.1. The hexane extract inhibited both the bacteria species with *S. phemoniae* being inhibited the most. The ethanol extract inhibited *S. typhi* only and dichloromethane inhibited *S. pheumoniae* only. The methanol and ethyl acetate extracts did not show any inhibitory activity against these two bacterial species. Although both plant extracts inhibited the bacterial species, the effects were less than that of streptomycin, a known antibiotic, except for hexane extract which showed an inhibition zone of 17 ± 4 mm against *S. typhi* whilst streptomycin had 12 ± 2 mm.

Table 3.1: *Susceptibility of S. typhi and S. pheumoniae to 500µg of plant extract.*

Plant extract	Inhibition zone (mm)	
	S. pheumoniae	*S. typhi*
Dichlomethane	9.6±0.78	6
Ethyl Acetate	6	6
Ethanol	6	10 ± 3
Hexane	11.7 ± 2	17 ± 4
Methanol	6	6
Streptomycin	22.3 ± 4.4	12 ± 2

Antibacterial activity of *M. azedarach* extracts. The commercial drug streptomycin was used as the control for the experiment. Methanol, ethanol and ethyl acetate extracts did not inhibit the growth of bacteria on the agar plates therefore there was no zone of inhibition. The value 6 is the diameter of the disc. Values are means ± SD for N=2 incubations.

3.2 MIC and MBC determination

The ethanol and hexane extracts of *M. azedarach* were found to have MICs of <1µg/ml and 15.6 µg/ml against *S. typhi* respectively and the MBC values were found to be 31.25 and 250 µg/ml (Table 3.2). Dichloromethane and hexane extracts were also found to have the MIC values of 31.25 and 62.5 µg/ml respectively against *S. pheumoniae* and the MBCs values were 31.25 and 125 µg/ml. The positive control, streptomycin had an MIC of 7.8 against both species but the MBC value was 15.6 against *S. pheumonia*e and 500 against *S. typhi.*

Table 3.2: *MIC and MBC determination*

Plant extract	MIC (µg/ml)		MBC (µg/ml)	
	S. pheumoniae	*S. typhi*	*S. pheumoniae*	*S. typhi*
Dichloromethane	31.25	-	31.25	-
Ethanol	-	<1	-	31.25
Hexane	62.5	15.6	125	250
Streptomycin	7.8	7.8	15.6	500

MIC was determined by MTT assay where concentrations of the plant extracts were diluted from 500 µg/ml to 1 µg /ml. MBC was

determined by reculturing cells from wells that did not show viability by MTT assay. The lowest concentration that showed growth was regarded as the MBC. Values are means ± SD for N=2 incubations.

3.3 Toxicity effects

Haemolysis assay was carried out to assess the effect of the active extracts on mammalian (sheep) red blood cells. The haemoglobin released by red blood cells when they lyse was measured at 545nm against a positive control of 10 % SDS. The MIC of plant extracts obtained against both bacterial species was used to determine haemolytic activity. **Table 3.3** shows that all tested extracts had haemolytic activity. Ethanol had 62.9 ± 0.4 %, hexane had 61.8 ± 1.8 % and dichloromethane had 58.7%.

Table 3.3: *Haemolytic activity of plant extracts that showed antibacterial activity*

Plant extract	% haemolysis
Dichlomethane	58.7
Ethanol	62.9 ± 0.4
Hexane	61.8 ± 1.8
SDS	100

The MICs of plant extracts obtained against both extracts were used to determine haemolysis activity. Values are means ± SD for N=2 incubations

4 Discussion

The combination of poor water quality and sanitation and urban overcrowding continues to be a persistent driver of seasonal outbreaks of waterborne diseases in Harare. Therefore, comprehensive measures are needed to improve the water treatment and delivery system in Harare as well as treatment of typhoid. One such measure includes development of new antibacterial agents for the treatment of typhoid. Thus, in this study, the antibacterial activity of *M. azedarch* leaf crude extracts was assessed by measuring inhibition zones (Table 3.1). Inhibition growth of the highest zone was that of the hexane extract with 17 mm growth inhibition zone, followed by that of ethanol with 10 mm, against *S. typhi*. The extracts of dichloromethane, ethyl acetate and methanol showed no antibacterial activity at 500 µg against *S. typhi* by the disk difussion assay. The probability that these solvents failed to extract compounds with antibacterial activity is high. Dichlorometahne and hexane extracts showed antibacterial activity against *S. pheumoniae* with average zone of 10 mm and 12mm respectively. Phytochemicals that can be extracted with ethanol, hexane and dichloromethane are mostly terpenes and flavonoids, glycosides, alkaloids, saponnins, tannins, tritepenoids and steroids [15]. Flavonoids have a wide range of pharmacological and biological functions including anticancer, anti-inflammatory, antioxidant, anti-allergic and antibacterial properties. Different phytochemicals in the class of flavonoids affect bacterial growth in different ways. Studies done using *Proteus vulgaris* and *S aureus, E.coli* and *S. typhimurium* show that flavonoids strongly inhibit DNA and RNA synthesis [16]. Terpenes have antimicrobial activity against a wide variety of microorganisms including fungi, gram-positive and gram-negative bacteria. The most active terpenoid class of phytochemicals found in *M. azedarach* leaf include azedirachitn, nimbin and nimbidine. Research has reported that nimbin has antibacterial, antifungal, anti-inflamatory, antipyretic, antiseptic and antihistamine properties [17]. Terpenoids are known to inhibit respiration as well as alter ion transport processes [18]. Alkaloids also have been shown to have growth inhibition mechanisms. These phytochemicals may be responsible for inhibiting growth in the two bacterial species investigated in this study.

The minimum inhibitory concentration (MIC) was used to determine the potency of extracts that were found to have antibacterial activity using the disk diffusion assay. The assay was done using the 3-(4, 5-dimethylthiozol-2-yl)-2-5-diphenyltetrazolium bromide assay (MTT assay). The MTT assay is used to assess the presence of metabolic activity in cells. The assay quantifies cellular oxidoreductases that depend on NAD(P)H to reflect the amount of viable cells present in the assay. MTT is yellow in colour and is reduced by mitochondrial succinate dehydrogenase to an insoluble formazan product

which is purple in colour. This reaction occurs only in cells that are metabolically active. The intensity of the purple colour is a measure of the level of activity and amount of viable cells in the assay. The concentration of the extracts decreased across the plate inorder to determine the lowest concentration of the extract at which bacteria growth is inhibited [19]. The wells whose colour changed to purple had live cells whilst those that remained yellow had metabolically inactive cells. When cells cease to be metabolically active they die. Therefore, using the MTT assay, the ethanolic extract had an MIC of <1 μg/ml, while the hexane extract was 15.6 μg/ml against *S. typhi*. Inoculumns were recultured to obtain the MBC values which were found to be 31.5 μg/ml and 250 μg/ml respectively. These two extracts were bacteriostatic at low concentrations and bactericidal at high concentrations. The MIC values of dichloromethane and hexane against *S. pheumonia*e were 31.25 μg/ml and 62.5 μg/ml respectively.

Haemolytic assay was performed for the extracts that exhibited antibacterial activity at their MIC values. SDS (10 %) was used as a positive control because it causes 100 % haemolysis of red blood cells. Haemolytic activity of the all the plant extracts was concentration-dependant, at higher concentrations, the extracts showed high haemolytic activity. Haemolytic activity of 62.93 ± 0.4 % for ethanol and 61.86 ± 1.8 % for hexane extract, 58.7% for dichloromethane extract was observed. The presence of saponnins in the extracts could be the cause for the haemolytic activity. Saponnins tend to react with the lipids in the phospholipid bilayer causing increased permeability of the erythrocytes thereby allowing influx of other molecules eventually leading to the cell bursting, releasing haemoglobin. Also, the plant fruits according to recent studies, proved that they are poisonous for human consumption because they contain meliatoxins [11], which might as well support the evidence that the leaves have haemolytic activity. However further studies have to be done to investigate the actual component in the leaf extracts that exhibits anti-bacterial activity and check for its toxicity rather than testing the crude extracts which might have constituents that maybe have no anti-bacterial activity and yet toxic.

5 Conclusion

Salmonella typhi and *Streptococcus pneumoniae* have increasingly become multi drug resistant by developing various mechanisms to defend against the effects of the drugs. The ineffectiveness of some antibiotics such as ciprofloxacin in the treatment of typhoid, and penicillin, macrolides and cephalosporins in the treatment of pneumococci infections has led to the investigations of antibacterial properties of phytocompounds found in plants. The results of this study validates the use of *M. azedarach* as a medicinal plant, as the ethanolic and hexane extracts of *M. azedarach* leaves were found to have the antibacterial activity against *Salmonella typhi* whilst dichloromethane and hexane were found to have antibacterial activity against *S. pheumoniae.* However, the potent plant extracts were found to be toxic *in vitro*. Since crude extracts were used in this study, further experiments need to be done in order to isolate the active compound or compounds and determine their toxicity in their pure form. Non-toxic compounds may serve as templates in production of new antibiotics. These compounds may also be used to make teas and lotions.

6 Declaration

6.1 Funding Source

This study was sponsored by the Department of Biochemistry at the University of Zimbabwe, and the University of Zimbabwe Research Board (Harare, Zimbabwe) is also acknowledged.

6.2 Competing Interests

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

How to Cite this Article:

C. Mwale, K. Makunike, and R. Mangoyi, "Antibacterial Activity of Melia azedarach Leaves against Salmonella typhi and Streptococcus pneumoniae", *Int. Ann. Sci.*, vol. 8, no. 1, pp. 47-53, Aug. 2019. doi:10.21467/ias.8.1.47-53

References

[1] C. Dye, "After 2015: infectious diseases in a new era of health and development" *Philos Trans R Soc Lond B Biol Sci*, 12, 369, 1-9, June, 2015.

[2] A. Penesyan, M. Gillings, and I. T. Paulsen. Antibiotic Discovery: Combatting Bacterial Resistance in Cells and in Biofilm Communities. *Molecules*. 20, 5286-5298, March, 2015.

[3] M. Bassetti, M. Merelli, C. Temperoni, and A. Astilean, "New antibiotics for bad bugs: where are we?, *Ann of Clinic Microb and Antimicrob*, 12, 22, August, 2013.

[4] I. K. Wang, S.Y. Lin-Shiau, and J. K. Lin. "Induction of apoptosis by apigenin and related flavonoids through cytochrome c release and activation of caspase-9 and caspase-3 in leukaemia HL-60 cells". *EJC*, 10, 35, 1517-1525, September 1999.

[5] N. A. Feasey, C. Masesa, C. Jassi, E. B. Faragher, J. Mallewa, M. Mallewa, C.A. MacLennan, "Three Epidemics of Invasive Multidrug-Resistant *Salmonella* Bloodstream Infection in Blantyre, Malawi, 1998–2014", *Clin Infect Dis*. 61,4, 363-371, October 2015.

[6] A. Chong, S. Lee, Y.Yang, and J. Song. "The Role of Typhoid Toxin in *Salmonella typhi* Virulence", *Yale Jour Biol Med*, 90, 2, 283-290, June 2017.

[7] W. W. Davis, P. Chonzi, and K. P. E. Masunda, "Notes from the field: typhoid fever outbreak—Harare, Zimbabwe, October 2016–March 2017". *MMWR* 67:342–3. March, 2018.

[8] H. S. N'cho, K. P.E. Masunda, I. Mukeredzi, P. Manangazira, E. Govore, C. Duri, R. D. Aubert, H. Martin, E. Gonese, M. Vere, B. A. T. Barr, S. Balachandra, J. Strysko, W. W. Davis, G. D. Appiah, and E. Mintz, "Typhoid Fever Outbreak — Harare, Zimbabwe, October 2017–February 2018" *MMWR*, 68, 2, January, 2019.

[9] World Health Organization. Guidelines for the management of typhoid fever. Geneva, Switzerland: World Health Organization; 2011.

[10] E. L. Nuermberger, and W.R. Bishai, "Antibiotic resistance in Streptococcus pneumoniae: what does the future hold?" *Clin lnfect Dis*, 38, 4, 363-371, May, 2004.

[11] D. Sharma, and Y. Paul, "Preliminary and Pharmacological Profile of *Melia azedarach L*". *Jour Appl Pharm Sci*. 3, 133-138, December 2013.

[12] M. M. Azam, R. A. Mamunor, N. M. Towfique, M. K. L. Sen, and S. Nasrin, "Pharmacological potentials of *Melia azedarach L*", *AJBIO*, 1, 44-49, July, 2013.

[13] K. K. Shekhawat, D.V. Rao, and A. Batra, "Phyto-Morphological Overview of Medicinal Plant: *Melia azedarach Linn*". *JFEB*. 6, 2, 318-327, February 2017.

[14] S. Henkelman, G. Rakhorst, J. Blanton, and W. Oeveren, "Standardization of incubation conditions for hemolysis testing of biomaterials". *Mat Sci Eng C – Biomimetic and Supramolecular Systems*. 29, 1650-1654, June, 2009.

[15] N. Packialakshmi, and S. Naziya, "Green synthesis of silver nanoparticles from stem extracts of *Caralluma fimbriyata* and its antibacterial activity". *Int J Appl Sci Biotechnol*. 3, 305-310, September, 2014.

[16] T. T. Cushnie, and A. J. Lamb, "Antimicrobial activity of flavonoids", *Inter jour antimicrob agents*, 5, 343-356, 2005.

[17] S. Gunasekaran and B. Anita, "Analysis of phytochemical variability in Neem formulations" *IJNPR*. 3, 1, 291-295, September, 2010.

[18] D. Trombetta, F. Castelli, M.G. Sarpietro, V. Venuti, M. Cristani, C. Daniele, A. Saija, "Mechanisms of antibacterial action of three monoterpenes". *Antimicrob Agents Chemother*. 49, 6, 2474-8, June, 2005.

[19] M. V. Berridge, P. M. Herst, and A. S. Tan, "Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction", *Biotech ann rev*. 11, 127 -147, 2005.