International Journal of Advanced Biotechnology and Research
ISSN 0976-2612, Online ISSN 2278–599X,
Vol 4, Issue 4, 2013, pp 511-517
http://www.bipublication.com

EVALUATION OF ANTIBACTERIAL ACTIVITY ON SELECTED BACTERIA AND SCREENING OF SECONDARY METABOLITES OF *Avicennia Alba* STEM

P. Satya Veni *, S. Sunita and A. Srinivasulu

Department of Biochemistry, V S Lakshmi Women's Degree and PG College, Kakinada,
East Godavari District, Andhra Pradesh, India
* Corresponding Author, E-Mail: styveni@yahoo.co.in

[Received-11/07/2013, Accepted-15/11/2013]

ABSTRACT

Most of the human diseases are mediated by microorganisms. The discovery of antimicrobials provides an increasingly important tool to combat microbial diseases. Improper usage of antimicrobials results in the development of microbial resistance. Therefore, identification of new antimicrobial compounds is essential. Traditional medicine still plays an important role in the health care in India and other countries. The mangrove ecosystem is a largely unexplored source for the potential biologically active secondary metabolites. Consequently, we set out to screen the stem of *Avicennia alba* collected from the Coringa Mangrove Reserve Forest, Kakinada, East Godavari, Andhra Pradesh, India for antibacterial activity. In the present study, the stem extract of *Avicennia alba* in Hexane, Benzene, Chloroform, Ethylacetate, Methanol, Acetone, Absolute Alcohol & Water were screened for the antibacterial activity by Agar well diffusion method against selected microorganisms, viz, *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Klebsiella pneumonia* (drug resistant strains) *Pseudomonas aeruginosa, Enterobacter aerogenes, Clostridium perfringens & Bacillus subtilis* (drug sensitive strains). The extracts were also screened for secondary metabolites like flavanoids, alkaloids, saponins, terpenoids, steroids and tannins. None of the extracts were found effective against the tested drug resistant strains irrespective of Gram nature. While, Methanol extract is active against *E.aerogenes & Bacillus subtilis* and Chloroform, Acetone, Absolute Alcohol & aqueous extracts exhibited only anti *E. aerogenes* activity. However, Hexane, Benzene & Ethyl acetate solubles does not show any activity on the tested cultures. Phytochemical studies support the antibacterial activity of methanol, acetone, alcohol & water solubles of *A.alba*. This clearly indicates that the active principles of *A.alba* stem are positive against drug sensitive strains but not to the drug resistant strains.

Key Words: Mangrove, *Avicennia alba*, Antimicrobials, Minimum Inhibitory Concentration, Phytochemicals.

[I] INTRODUCTION

Currently, the haphazard use of commercial antimicrobial drugs has caused multiple drug resistance in human pathogenic microorganisms [3]. In addition to this problem, hypersensitivity, immune-suppression and allergic reactions are sometimes present from the adverse effects of antibiotics on the host [20].This situation forced scientists to search for new and effective antimicrobial agents to replace the current practice [12]. Plants remain the most common source of antimicrobial agents. Their usage as traditional health remedies is the most popular for 80% of world population in Asia, Latin America and Africa and is reported to have minimal side effects [24]. In recent years, pharmaceutical companies have spent a lot of time and money in developing natural products

extracted from plants, to produce more cost effective remedies that are affordable to the population. The rising incidence in multidrug resistance amongst pathogenic microbes has further necessitated the need to search for newer antibiotic sources. It is well known that some plants containing active compounds are able to inhibit the microbial growth. Studying plant-based antimicrobial properties provides additional information in developing natural antibiotics and discovering the alternative of antimicrobial drugs for the treatment of infectious diseases. Mangroves are diversified group of plants that grow in estuarine environment, have been a source of interest for their novel natural products like alkaloids, flavanoids, glycosides, saponins, tannins, etc., are known to exhibit antiviral, antibacterial and antifungal activities [6]. *Avicennia alba* (A. alba) belongs to Avicenniaceae family. The common name for this plant is "Vilava mada". In India it occurs along the east and west coasts from Sunderbans up to Maharashtra. *A. alba* is a rich source of naphtha-quinones [7]. The bark and seeds of *A.alba* are used as a fish poison and the resin used in birth control, ulcers treatment, skin diseases and also used to cure tumors[27]. It provides a rich source of steroids, triterpenes, saponins, flavonoids, alkaloids and tannins. Therefore, the present study has designed to evaluate the antimicrobial activity of *A. alba* stem extracts against eight human pathogenic microbes.

[II] MATERIALS AND METHODS

2.1. Plant Material

The healthy plant of *A. alba* was collected from Coringa Reserve Forest, Kakinada, Andhra Pradesh, India. The stems were surface sterilized with 1% mercuric chloride and thoroughly washed with plenty of distilled water. Later, the stems were shade dried after chopped into small pieces.

2.2. Extraction

The chopped stem material of *A. alba* (100g) was extracted in to different solvents in the increasing order of polarity viz, Hexane, Benzene, Chloroform, Ethylacetate, Methanol, Acetone, Absolute Alcohol & Distilled water. The chopped material was extracted sequentially into 500 ml of the respective solvent by initial soaking for 12 hours followed by refluxing for about 10 hours below the boiling point of the respective solvent. Resulting extracts in different solvents were evaporated and concentrated using the rotary evaporator. Concentrated extracts were dissolved in 1 – 2 ml of DMSO and the concentration was adjusted to 100mg/ml with water and stored at 4°C. Plant extracts were tested for antibacterial activity by agar well diffusion technique.

2.3. Bacterial Strains

Pure cultures of *Staphylococcus aureus* (MTCC 87), *Bacillus subtilis* (MTCC 441), *Escherichia coli* (MTCC 40), *Klebsiella pneumonia* (MTCC 39) (drug resistant strains), *Pseudomonas aeruginosa* (MTCC 424), *Enterobacter aerogenes* (MTCC 111), *Clostridium perfringens* (MTCC 450) *& Bacillus subtilis* (MTCC 121) (drug sensitive strains) were procured from Microbial Type Culture Collection (MTCC) Chandigarh to determine the antibacterial activity.

2.4. Determination of Antibacterial activity

The anti-bacterial activity of *A. alba* extracts were performed by Agar well diffusion method [19]. Bacterial suspensions of the test cultures were prepared by using 24 hour old bacterial culture. The amount of bacteria needed to undertake the study was determined using UV/Vis spectrophotometer (ELICO, India) at 625 nm so that the absorbance of the suspension was held at 0.1 which was assumed to contain $1\text{-}2\times10^{8}$CFU/ml. About 20 ml of melted (at about 50°) Mueller Hinton agar was mixed with 1 ml of bacterial suspension homogeneously and allowed to solidify in petri dishes (143 mm diameter). Wells (8mm dia.) were made using a sterile cork borer on the solidified medium. Wells were filled with 100 µl of the original crude extraction of each extract obtained by the above extraction method. All the tests were performed in duplicates. The diameters of the inhibition zones were measured and their means were calculated. DMSO in water was taken as control.

2.5. Determination of Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) was determined by broth dilution method [26,25]. Each

inoculum was prepared in Mueller Hinton broth and density of bacteria was adjusted as described above. To determine the MIC, the identified extract in DMSO was serially diluted in Muller Hinton broth medium to get the concentration of 5, 10, 15, 20 & 25 µg/100µl. Later, each tube was inoculated with 0.2 ml suspension of reference strain. After incubation, the test tubes were observed for turbidity and the least concentration where no turbidity was observed and the MIC values were noted.

2.6. Phytochemical Analysis

The crude extracts were subjected to preliminary phytochemical analysis by following standard protocols from Pharmacopia for identification of different phytochemical constituents.

Test for flavanoids

a) Ferric chloride test - About 2ml of the test solution was boiled with distilled water and then filtered. Then, few drops of 10% ferric chloride solution was added to the filtrate. A green-blue or violet coloration indicates the presence of a phenolic hydroxyl group.

b) Shinoda's test - About 0.5gm of each extract was dissolved in ethanol, warmed and then filtered. Small pieces of magnesium chips were then added to the filtrate followed by few drops of conc.HCl. The pink, orange, or red to purple coloration indicates the presence of flavanoids.

c) Sodium hydroxide test - About 0.2gm of the each extract was dissolved in water and filtered; to this 2ml of the 10% Na OH was added to produce a yellow coloration. A change in colour from yellow to colourless on addition of dilute hydrochloric acid was the indication for the presence of flavonoids.

d) Lead acetate test - About 0.5gm of the each extract was dissolved in water and filtered.To the 5ml of each filtrate, 3ml of lead acetate solution was then added.Appearance of a buff-coloured precipitate indicates the presence of flavanoids.

Test for alkaloids

0.5 g of crude extracts were stirred with 1% aqueous HCl on water bath and then filtered. To 1ml filtrate few drops of dragendroff's reagent was added. Formation of orange –red precipitate was considered as positive to alkaloids. To another 1ml filtrate few drops of Mayer's reagent was added and J. Ethnopharmacolappearance of buff-coloured precipitate will be taken as presence of alkaloids.

Test for Saponins

About 0.5gm of crude extract was shaken with water in a test tube and it was warmed in a water bath. The persistent froth indicates the presence of saponins.

Test for Terpenoids

Small quantity of crude extract was dissolved in ethanol. To this, 1ml of acetic acid was followed by the addition of few drops of conc.H_2SO_4. A change in colour from pink to violet confirms the presence of terpenoids.

Test for steroids

a) Salkowskii test - About 0.2gm of each extract was dissolved in 2ml of chloroform, followed by the addition of conc. H_2SO_4 to form reddish brown colour at interphase indicates the presence of steroids.

b) Keller-Killiani test - To 0.5ml of test solution, 2ml of 3.5% $FeCl_3$, small amount of glacial acetic acid and 2ml of conc. H_2SO_4 were added carefully. Appearance of reddish brown ring at interphase is a positive indication for the presence of steroids.

c) Liebermann-Burchard test - To 0.2gm of each extract, 2ml of acetic acid was added and the solution was cooled well in ice followed by the addition of conc. H_2SO_4 carefully. Colour development from violet to blue or bluish-green indicates the presence of a steroidal ring

Test for tannins

About 0.5gm of each extract was stirred with about 10ml of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2ml of the filtrate. Occurence of a blue-black, green or blue-green precipitate indicates the presence of tannins.

a) Borntrager's test - About 0.2gm of each extract to be tested was shaken with 10ml of benzene and then filtered. The filtrate was shook well after the addition of 5ml of the 10% ammonia solution. Appearance of pink, red or violet colour in the ammonical (lower) phase was taken as the presence of free anthraquinones.

b) Phlonatanins test - About 0.2gm of each extract was added with 1% HCl solution. Formation of red precipitate indicates the presence of tannins.

[III] RESULTS & DISCUSSION

Extracts from different mangrove plants are reported to possess diverse medicinal properties [27,1]. For example, *A. illicifolius, A. marina* and *E. agallocha* showed significant analgesic activity [13]. Mangroves and mangrove associates possess novel agrochemical products, compounds of medicinal value, and biologically active compounds [5]. Extracts from different mangrove plants are also active against human and plant pathogens [8]. In this study, *A.alba* was selected for evaluating the antibacterial activity on selected eight bacteria. The active constituents of *A.alba* were extracted in to different solvents in the order of increasing polarity. The physical characteristics and percentage yield of the extracts are summarized in Table-1. As indicated in the table, there were differences in the yield of extraction products which might be due to polarity difference of solvents used for extraction, solubility of various ingredients, etc. [16].

A.alba stem extracts in different solvents in the order of increasing polarity were used to determine the antibacterial activity against drug resistant and sensitive strains of bacteria by agar well diffusion method as it is a better method than the disc diffusion method. Discs of crude extracts are not suitable for the complete diffusion of material into the agar. In the present study, all the 8 extracts of *A. alba* were tested against the selected bacteria and the data was presented in table-2. These results indicate that, the stem extracts of *A. alba* did not show any anti bacterial activity against Gram +ve or Gram –ve strains drug resistant microorganisms. However, Chloroform, Methanol, Acetone, Absolute Alcohol & aqueous extracts exerted different degree of zone of inhibition with Gram –ve drug sensitive *E. aerogenes* while the methanol extracts exerted little anti *B. subtilis* activity. While the A.alba constituents in hexane, benzene and ethyl acetate found to be ineffective against any of the tested microorganisms. Hence it is confirmed that, *A.alba* stem to contain strong anti Gram –ve principles. These observations are in concurrence with the previous studies on *A.alba* [22].

Several reports are documented in literature on the determination of MIC values of many plant extracts [21]. In our study, the positive extracts for antibacterial activity were further tested to determine the MIC and the data was represented in figure-1. The MIC values of stem extracts were found to be in the range of 5 to 20 mg/100 μl against *E. aerogenes.*

Secondary metabolite such as flavanoids, alkaloids, saponins, terpenoids, steroids, tannins were reported to exhibit different anti biological activities [14]. For example, Saponins are glycosides occurring widely in plants. These are abundant in many foods consumed by animals and human beings. These are widely used as mild detergents. These are known to associate with hypercholesterolemia, hyperglycemia [23] , anti-cancer, anti-inflammatory [17] activities. Plant steroids are known for their anti-inflammatory [2], analgesic [18], anti-microbial and on central nervous system [4]. Tannins have been widely recognized for their pharmacological properties. These are well studied for anti-diabetic [9], anti-inflammatory [15] and anti-bacterial activities. Secondary metabolites of *A.alba* stem revealed that acetone, methanols were found to contain most of the tested secondary metabolites as shown in Table-3. Alkaloids and flavonoids are known to associate with strong anti-bacterial activities [11,10]. Phytochemical data also indicates that the common secondary metabolites in all the positive extracts are alkaloids and flavonoids. Hence, the anti-bacterial activity of A.alba might be because of the alkaloids and flavonoids. The results of the present study support the traditional use of *A.alba* as an ethnomedicine. The profound chemical diversity with in the mangroves provides an opportunity for the discovery of new drugs. The present work on *A.alba* stem needs further extension to isolate and characterize the active anti-bacterial principles.

[IV] CONCLUSION

A.alba stem collected from mangrove forest of Coringa Reserve Forest, Kakinada, East Godavari, Andhra Pradesh, India was tested for anti-bacterial activity and phytochemical screening. This study has revealed the preliminary evidence of antibacterial ability of *A.alba* stem extracts in various solvent. Polar solvents like methanol, acetone, ethanol and

water and non-polar solvent chloroform showed maximum antibacterial activity. Flavonoids and alkaloids are known for the antibacterial activity and the phytochemical analysis of different solvent extracts indicates that flavonoids and alkaloids are common in all the extracts with antibacterial properties. These studies also conform that it can be considered as a potential ethno pharmacological plant for the treatment bacterial disorder of Gram –ve bacteria. Therefore the *A.alba* stems extracts can be used to discover new bioactive natural products that may serve as natural antibiotics to replace the synthetic pharmaceutical to control microbial disorders. The plant studied here can be used as a potential source of useful drugs. There is need for further studies on the plants parts in order to isolate, identify, characterize and elucidate the structure of antimicrobial bioactive compounds.

ACKNOWLEDGEMENT

The authors of this paper are very much thankful to the Department of Science and Technology, New Delhi, India for financial support through WOS-A scheme.

REFERENCES

1. Agoramoorthy G, Chandrasekaran M, Venkatesalu V, Hsu MJ. (2007). Antibacterial and antifungal activities of fatty acid methyl esters of the blind-your eye mangrove from India. Braz J Microbiol. 38:739–42.
2. Akindele AJ and Adeyemi OO. (2007). Anti-inflammatory activity of the aqueous leaf extracts of *Byrsocarpus coccineus.* Fitoterapia. 78, 25–28.
3. Aliero A, Aliero BL, Buhari U. (2008). Preliminary phytochemical and antibacterial screening of *Scadoxus multiflorus.* Int J Pure Appl Sci. **2**: 13-17.
4. Argal A and Pathak AK. (2006). CNS activity of *Calotropis gigantea* roots. J.Ethnopharmacol. 106, 142-145.
5. Bandaranayake WM. (2002). Bioactivities, bioactive compounds and chemical constituents of mangrove plants. Wetlands Ecology and Management. 10:421–52.
6. Bhimba BV, Meenupriya J, Joel EL, Naveena DE, Kumar S, Thangaraj M. (2010). Antibacterial activity and characterization of secondary metabolites isolated from mangrove plant *Avicennia officinalis*. Asian Pac J Trop Med. 3: 544-546.
7. C, Ito, S, Katsuno, Y, Kondo,.H.T. Tan, and H. Furukawa. (2000). Chemical constituents of *Avicennia alba*. Isolation and structural elucidation of new naphthoquinones and their analogues. Chem. Pharm. Bull. 48: 339-343.
8. Chandrasekaran M, Kannathasan K, Venkatesalu V, Prabhakar K. (2009). Antibacterial activity of some salt marsh halophytes and mangrove plants against methicillin resistant Staphylococcus aureus. World J Microbiol Biotechnol. 2:155–60.
9. Cherian S and Augusti KT. (1995). Insulin sparing action of leucopelargonidin derivative isolated from *Ficus benghalesis* Linn. Indian J. Exp. Biol. 33, 608-611.
10. Ernest K. Ruttoha, Paul K. Tarusb, Christine C. Biic, Alex K. Machochod, Lucas K. Karimie and Paul O. Okemo. (2009). Antibacterial activity of *Tabernaemontana stapfiana* britten (apocynaceae) extract. Afr. J. Trad. CAM. 6 (2): 186 – 194.
11. Gurinder J Kaur and Daljit S Arora. (2009). Antibacterial and phytochemical screening of *Anethum graveolens*, *Foeniculum vulgare* and *Trachyspermum ammi*. BMC Complementary and Alternative Medicine. 9:30 : 1 – 10
12. Jacquelyn GB. (2002). Microbiology principles and exploration. 5th ed. USA: John Wiley and Sons Inc.
13. Kokpol U, Chittawong V, Mills HD. (1984). Chemical constituents of the roots of *Acanthus illicifolius.* J Nat Prod. 49:355–6.
14. Kubmarawa D, Khan ME, Punah AM and Hassan. (2008). Phytochemical screening and antibacterial activity of extracts from *Parkia clappertoniana* keay against human pathogenic bacteria. J. Med. Pl. Res. 2(12), 352-355.
15. Latha, R.M., T. Geetha and P. Varalakshmi. (1998). Effect of *Vernonia cinerea* less flower extract in adjuvant induced arthritis. General Pharmacol. 31: 601-606.
16. Mahour K, Mishra A, Kumar A, Vihan VS. (2008). Preliminary Pharmacognostical and phytochemical investigation on *Feronia elephantum corr*. Fruit. J Pharm Res. 1:45–8.
17. Manach C, Regerat F and Texier O. (1996). Bioavailability, metabolism and physiological impact of 4-oxo-flavonoids. Nutr. Res. 16, 517-544
18. Malairajan P, Geetha G, Narasimhan S and Jessi Kala Veni K. (2006). Analgesic activity of some Indian medicinal plants. J. Ethnopharmacol. 19, 425-428.

19. N.Sharief Md and V.Uma Maheswara Rao. (2011). Antibacterial activity of stem and root extracts of *Avicennia officinalis* L. International Journal of Pharmaceutical Applications. 2 (4) 231-236.
20. Nebedum J, Ajeigbe K, Nwobodo E,Uba C, Adesanya O, Fadare O, et al. (2009). Comparative study of the ethanolic extracts of four Nigerian plants against some pathogenic microorganisms. Res J Med Plant. **3**: 23-28.
21. Nostro A, Germano MP, Angelo VD, Marino A. (2000). Cannatelli MA. Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. Lett Appl Microbiol. 30:379–84.
22. P.Nagababu and V.UmaMaheswara Rao. (2012). Antibacterial ativity and phytochemical screening of leaves and stem extracts of *Avicennia alba* Blume. International Journal Of Applied BiologyAnd Pharmaceutical Technology. Vol-3 (4) 399-405.
23. Rupasinghe HP, Jackson CJ, Poysa V, di Berado C,Bewley JD and Jenkinson J. (2003). Soyasapogenol A and B distribution in Soybean (*Glycine max (*L.) Merr.) in relation to seed physiology, genetic variability and growing location. J. Agric. Food Chem. 51, 5888-5894.
24. Safary A, Motamedi H, Maleki S and Seyyendnejad SM. (2009). A preliminary study on the antibacterial activity of Quercus brantii against bacterial pathogens, particularly enteric pathogens. Intl. J. Botany. 5(2),176–180.
25. Usman H, Abdulrahman FI, Ladan AH. (2007). Phytochemical and Antimicrobial Evaluation of *Tribulus terrestris* L.(Zygophylaceae) growing in Nigeria. Res J Bio Sci.2:244–7.
26. Vollekova AD, Kostalova, Sochorova R. Isoquinoline. (2001). Alkaloids from *Mahonia aquifolium* stem bark is active against Malassezia Sp. Folia Microbiol. 46:107–11.
27. W.M. Bandaranayake, Traditional and medicinal uses of mangroves. (1998). Mangroves and Salt Marshes. 2: 133-148.

Table-1: Yield of stem extracts of *Avicennia alba* in different solvents

TEST ORGANISM	Diameter of Zone of inhibition (mm)							
	H	B	C	EA	M	A	E	W
Drug resistant bacteria								
Escherichia coli	-	-	-	-	-	-	-	-
Klebsiella pneumonia	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-	-
Bacillus subtilis	-	-	-	-	-	-	-	-
Drug sensitive bacteria								
Pseudomonas aeruginosa	-	-	-	-	-	-	-	-
Enterobacter aerogenes	-	-	4.5	-	4	4	5	5
Clostridium perfringens	-	-	-	-	-	-	-	-
Bacillus subtilis	-	-	-	-	1.5	-	-	-

Table-2: Antibacterial activity of *Avicennia alba* stem extracts in different solvents

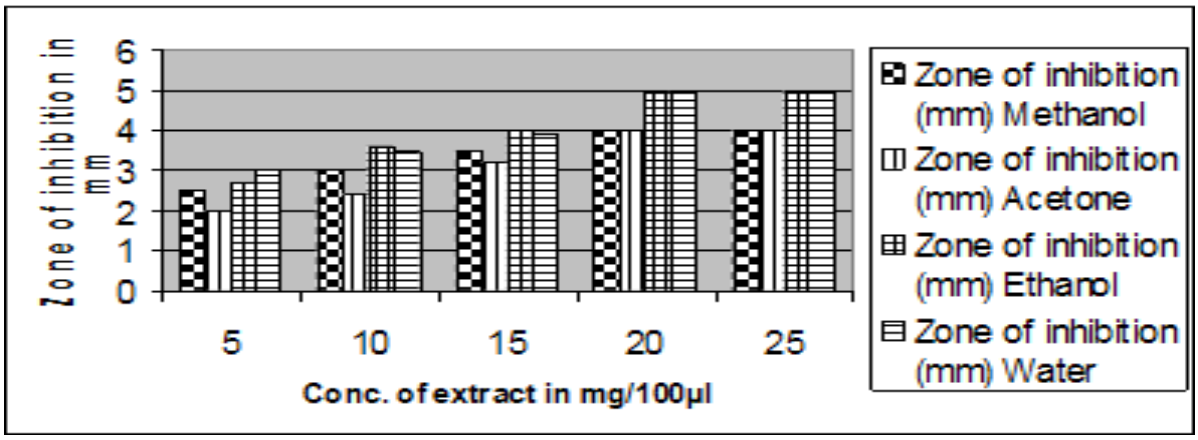
S.No	Name of the solvent	Physical Characteristics	Extract weight in gms
1	Hexane	Powder	0.329
2	Benzene	Powder	0.356
3	Chloroform	Powder	4.805
4	Ethylacetate	Powder	0.647
5	Methanol	Viscous	1.082
6	Acetone	Viscous	0.372
7	Ethanol	Powder	0.585
8	Sterile Distilled water	Powder	3.673

Table-3: Phytochemical analysis of *Avicennia alba* stem extracts in different solvents.

S. No	**Phytochemical**	**H**	**B**	**C**	**EA**	**M**	**A**	**E**	**W**
1	Flavanoids	+	-	+	+	+ +	+ +	+	+
2	Alkaloids	+	+	+	-	+	+	+	+
3	Saponins	-	-	-	-	+ +	+ +	+	+
4	Terpenoids	+	-	-	-	+ +	+ +	-	-
5	Steroids	+	-	-	-	+ +	+ +	+	+ +

H- Hexane; B- Benzene; C- Chloroform; EA- Ethyl Acetate; M- Methanol; A- Acetone; E- Ethanol

Figure – 1 : MIC of *Avicennia alba* stem extracts against *E. aerogenes*



H- Hexane; B- Benzene; C- Chloroform; EA- Ethyl Acetate; M- Methanol; A- Acetone; E- Ethanol (Absolute alcohol); W- Water.