Open Access Contents
Int. J. Drug Dev. & Res.
| July - September 2014 | Vol. 6 | Issue 3 | ISSN 0975-9344 |
www.ijddr.in

Evaluation of antimicrobial efficacy of Flavonoids, Alkaloids and Steroids of *Nerium oleander* Linn against some pathogenic bacteria

Meenakshi Fartyal

Padma Kumar

Department of Botany, Laboratory of Plant Tissue culture and Secondary metabolites, University of Rajasthan, India.

Corresponding Authors:
E-mail Id: mksh35@gmail.com, godgift1955@yahoo.co.in

Abstract:
In recent years, infections have increased to a great extent and antibiotic resistance effects have become an ever-increasing therapeutic problem. Natural products of higher plants are effective in the treatment of infectious diseases, while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials. Systematic screening of plant extracts may result in the discovery of novel active compounds. Present work was carried out to assess the antimicrobial activity of *N.oleander* against some multidrug resistant pathogenic bacteria. Different parts (leaf, stem, flower and root) of *N.oleander* were collected and dried. These parts were then soxhlet extracted by using standard methods for flavonoids, alkaloids and steroids. Extracts were then tested for antimicrobial activity using 'Disc Diffusion Assay'. Minimum inhibitory concentration, Minimum bactericidal concentration & Total activity were studied and statistically were analyzed. *E.coli, S.aureus* & *K.pneumonae* were found to be the most susceptible organism followed by *B.subtilis* & *A.tumifaciens*. Free flavonoid extract of stem showed the best activity against *S.aureus* (IZ=18mm, MIC= 0.156mg/ml, MBC= 0.078mg/ml, TA=19.23ml/g). Steroids of flowers were best against *B.subtilis*(IZ=14mm,MIC=0.312mg/ml, MBC= 0.156mg/ml, TA=62.5ml/g), similarly Free flavonoid extract of root were best against *B.subtilis* (IZ=13mm,MIC=0.312mg/ml, MBC= 0.156mg/ml, TA=3.20ml/g) & *E.coli* (IZ=12mm,MIC=0.312mg/ml, MBC= 0.156mg/ml, TA=3.20ml/g). The range of MIC & MBC was found to be 1.25-0.156mg/ml & 0.625-0.078mg/ml respectively. Results of the present study reveal that extracts of *N.oleander* have great antimicrobial potential against tested microorganisms, and may be exploited for future antimicrobial drugs.

Keywords: Flavonoids, Alkaloids, Steroids, Minimum inhibitory concentration, Minimum bactericidal concentration & Total activity.

Introduction:

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural resources. Traditional medicines are important source of potentially useful compounds for the development of chemotherapeutic agents [1]. Although pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs in microorganisms has increased. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents [2]. In the present scenario, there is an urgent and continuous need of exploration and development of cheaper and effective new plant based drugs with better bioactive potentials and least side effects. Hence, recent attentions have been paid to biologically active extracts and compounds from plant species used in herbal medicines [3]. In the present study *Nerium oleander* has been selected for the study.

Nerium oleander (comman name Kaner) is an evergreen shrub belongs to family Apocynaceae. It is native to southern Europe, and is widely cultivated and naturalized in Asia, Europe and

North America. It is four metres in height, occurs along watercourse, gravely place and damp ravines, widely cultivated particularly in warm temperate subtropical regions where it grows outdoors in parks, gardens and along road sides. Various medicinal properties viz. Cardiotonic, Analgesic, Antidiabetic, Anti-inflammatory, Antibacterial, Anticancer/Antineoplastic, Antifungal, Depressant, Antimitotic, Insecticidal, Larvicidal are attributed to this plant. Other properties attribute are inhibition of Nuclear factor-kappa B (NF-/cB) activation, Muscle stimulation, effective against Asthma, Seizures, Cancer, Menstrual pain, Skin problems, Warts, epilepsy, leprosy, malaria, ringworm, indigestion, and venereal disease and also cause abortions. The present investigation was undertaken to find out the antibacterial potential of flavonoids, alkaloids and steroids of different parts of *N.oleander* against some Gram positive and Gram negative bacteria.

Alkaloids are known to have pharmacological effects and are used in medications, as recreational drugs or in entheogenic rituals. Literature indicates that plant alkaloids have considerable biological activity [4], [5]. Alkaloid, flavonoid, saponin, tannin and phenol were identified in the aqueous extracts of *N.oleander* [6]-[8]. Alkaloids of *N.oleander* dissolved in ethanol at 1% were used in integrated pest management [9].

Flavonoids are potent water-soluble antioxidants and free radical scavengers, which prevent oxidative cell damage and have strong anti-cancer activity [10]. It was reported that flavonoids can improve the blood circulation and lower the blood pressure [11]. Flavonoid, alkaloid, phenols and tannin have been reported in Nerium [12]. Kaempferol is a flovonol type flavonoid with a wide spectrum of bioactivity. It has been isolated from N.oleander L. as well as a wide range of medicinal herbs [13].

Steroids are frequently used as signalling molecules, represents highly concentrated energy stores, along with phospholipids function as components of cell membranes Steroid, terpenoid, quinine, saponin, and Phenolic compounds were identified in methanolic and aqueous extract of *Nerium oleander* [14]. The roots of *Nerium oleander* yielded a new cardenolide, 12β-hydroxy-5β-carda-8, 14, 16, 20(22)-tetraenolide. Biological screening of the compound revealed antibacterial and digoxin-like cardiac activities [15].

Considering the rich diversity of plants, it is expected that screening and scientific evaluation of plant extracts for their antimicrobial activity may provide new antimicrobial substances. Review of the current literature reveals that no work has been carried out for extraction and screening of specific compound from selected plant. Hence, in the present work an extraction and screening for antibacterial activity of the flavonoids, alkaloids and steroids of *N.oleander* has been undertaken.

Material and methods:

Different parts of *N.oleander* (leaf, stem, flower and root) were collected in the month of May to July from the western parts of India (Jaipur, Rajasthan). Plants were identified by senior taxonomist at department of Botany, university of Rajasthan and voucher specimen no: RUBL 21176 was submitted to the Herbarium, Botany department, university of Rajasthan.

Preparation of Extracts:

Flavonoid extraction:

Selected plant parts were separately washed with sterilized water; shade dried, and finely powdered using a blender. Each sample was subjected to extraction, following the method of Subramanian and Nagarjan [16]. One hundred grams of each finely powdered sample was soxhlet extracted with 80% hot methanol (500ml) on a water bath for 24 h and filtered. Filtrate was re- extracted successively with petroleum ether (fraction I), diethyl ether (fraction II), and ethyl acetate (fraction III) using separating funnel. Petroleum ether fractions were discarded as being rich in fatty substances, where as diethyl ether and ethyl acetate fractions were analyzed for free and bound flavonoids respectively. The ethyl acetate fraction of each of the samples was hydrolyzed by refluxing with 7% H_2SO_4 for 2 h (for removal of bound sugars) and the filtrate was extracted with ethyl acetate in separating funnel. Ethyl acetate extract obtained was washed with distilled water to neutrality. Diethyl ether (free flavonoids) and ethyl acetate fractions (bound flavonoids) were dried in vaccuo and weighed. The extracts were stored at 4°c and were re-suspended in their respective solvents to get 10mg/ ml concentration for antimicrobial assay.

Alkaloids Extraction:

Alkaloids were extracted from different parts of the selected plant by well established method [17].Finely powered sample (100g) of plant parts were extracted in 20ml methanol after shaking of 15 min. After filtration, filtrate kept for drying then residual mass was treated with 1% H_2SO_4 (5ml. 2 times).Extraction was then done in 10ml. Chloroform ($CHCl_3$) by using separating funnel. Organic layer of chloroform was rejected and aqueous layer was basified with 30% NH_4OH (P^H=9-10). Now again, extraction was done in 10ml. chloroform & organic layer of chloroform (lower layer) was collected in a flask and repetition of step was done with fresh chloroform. Extracts was then dried in vaccuo for further use.

Steroid Extraction:

Steroids were extracted from different parts of the selected plant by well established method [18] after preliminary detection of steroids. Finely powdered sample (100g) of plant parts were extracted in petroleum ether for 2-4hr.After filtration, residual mass was treated with 15% ethanolic HCl for 4hr. Extraction was then done in ethyl acetate followed by washing in dis. water to neutralize the extract. Neutral extract was then passed over Sodium sulphate to remove moisture contents and was dried in vaccuo. Chloroform was used for reconstitution of extract, filtered and dried for further use.

Selected Test Microorganisms:

Three pathogenic bacteria were screened, viz., *Escherichia coli* (MTCC no.46), *Bacillus subtilis* (MTCC no. 121), *Staphylococcus aureus* (MTCC no. 3160), *Klebsiella pneumonae* (MTCC no. 4030) and *Agrobacterium tumifaciens* (MTCC no. 431). The pathogens were procured from IMTECH (Chandigarh, Punjab, India). Bacterial strains were grown and maintained on Muller-Hinton Agar medium.

E.coli is one of the most frequent causes of many common bacterial infections including bacteremia, urinary tract infection (UTI), traveler's diarrhea, neonatal meningitis [19] and pneumonia. Some virulent strains cause serious illness or death in the elderly, the very young or the immunocompromised [20], [21]. Intestinal mucosa

Covered in Scopus & Embase, Elsevier Int. J. Drug Dev. & Res., July - September 2014, 6 (3): 119-127

associated *E. coli* is observed in increased number in the inflammatory bowel diseases, Crohn's diseases and ulcerative colitis [22], [23].***S.aureus*** is the most common hospital acquired pathogen and cause staph infections which is responsible for various diseases including: mild skin infections e.g. folliculitis, invasive diseases e.g. wound infections and bacteremia etc., and toxin mediated diseases e.g. food poisoning, toxic shock syndrome (TSS) and scaled skin syndrome etc. In infants its infection can cause a severe disease Staphylococcal scalded skin syndrome (SSSS) [24]. Recently, the serious emergence of antibiotic resistance staph occurred with a specific strain is Methicillin-Resistant *Staphylococcus aureus* (MRSA) and research being done to investigate hospital acquired MRSA. ***B.subtilis*** bacteria are nonpathogenic. They can contaminate food; however, they seldom result in food poisoning. ***K.pneumonae*** cause destructive changes to human lungs inflammation and hemorrhage with cell death (necrosis) .The range of clinical disease includes pneumonia, thrombophlebitis, UTI, cholecystitis, diarrhea, upper respiratory tract infection, wound infection, osteomyelitis, meningitis and bacteremia and septicemia. ***Agrobacterium tumefaciens*** is a tumor producing pathogenic bacteria and do not benefit the plant. Economically, this pathogen is a serious pathogen of walnuts, grape vines, and stone fruit.

Antimicrobial assay:

'Disc Diffusion Assay' was performed for screening [25]. MH agar base plates were seeded with the bacterial inoculum (inoculum size 1×10^8 CFU/ml). Sterile filter paper discs of Whatmann no.1 (6mm in diameter) were impregnated with 100µl of each of the extract of concentration 10mg/ml to give a final concentration of 1 mg/disc. Discs were left to dry in vaccuo so as to remove residual solvent, which might interfere with the determination. Discs with extract were then placed on the corresponding seeded agar plates. Each extract was tested in triplicate along with streptomycin (1mg/disc) as standard drug for bacteria. The plates were kept at 4°C for diffusion of extract, thereafter were incubated at 37°C for 24h. Activity index for each extract was calculated. (Table 1) by the standard formula viz.

Activity index = IZ produced by the extract/ IZ produced by standard

Where, IZ = inhibition zone.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal (MBC)/ Fungicidal (MFC) Concentration:

Minimum inhibitory concentration (MIC) was determined for each plant extract showing antimicrobial activity against the test pathogens. 'Broth micro dilution' method was followed for determination of MIC values [26]. Plant extracts were resuspended in acetone (which has no activity against test microorganisms) to make 10 mg/ml final concentration. Two fold serially diluted extracts were added to broth media of 96-wells of micro titer plates. Thereafter 100µl inoculum (1×10^8 CFU/ ml) was added to each well. Bacterial suspensions were used as negative control, while broth containing standard drug was used as positive control. Micro titer plates were then incubated at 37°C for 24 h. Each extract was assayed in duplicate and each time two sets of micro plates were prepared, one was kept for incubation while another was kept at 4°C for comparing the turbidity in the wells of micro plate. The MIC values were taken as the lowest

concentration of the extracts in the well of the micro titer plate that showed no turbidity after incubation. The turbidity of the wells in the micro titer plate was interpreted as visible growth of microorganisms. The minimum bactericidal concentration (MBC) was determined by sub culturing 50 µl from each well showing no apparent growth. (Table2). Least concentration of extract showing no visible growth on sub culturing was taken as MBC.

Total activity (TA) determination:

Total activity is the volume up to which test extract can be diluted without losing the ability to kill microorganisms. It is calculated by dividing the amount of extract from 1 g plant material by the MIC of the same extract or compound isolated and is expressed in ml/g. [27] (Table 3)

Results: Alkaloids, Flavonoids (Free and Bound) and Steroids were assessed for their antimicrobial potency by IZ, AI (Table2), MIC & MBC (Table 1). Quantity of extracts per gram of plant material was also calculated (Table3).In the present investigation total 16 extracts were tested, among which `Leaf bound flavonoids (B.F.)'& `flower alkaloid' were found inactive against any of the tested pathogens whereas other 14 extracts were active against atleast one of the tested pathogens. *E.coli, S.aureus* and *K.pneumonae* were observed to be most susceptible organism in the investigation followed by *B.subtilis* and *A.tumifaciens*. Best activity was observed in stem F.F. (IZ=18mm, AI=0.75±0.01, MIC=0.156mg/ml) against *S.aureus*. Flower steroid also showed good activity (IZ=14mm, AI=0.47±0.04, MIC=0.312mg/ml) against *B.subtilis*. Root F.F. also showed good activity against *B.subtilis* (IZ=13mm, AI=0.44±0.04, MIC=0.312mg/ml) and against *E.coli* (IZ=12mm, AI=0.43±0.01, MIC=0.312mg/ml). Steroidal extract of root also showed good activity against *K.pneumonae* (IZ=12mm, AI=0.92±0.02, MIC=0.312mg/ml) and *A.tumifaciens* (IZ=12mm, AI=0.40±0.01, MIC=0.312mg/ml). Leaf alkaloid, stem steroid, stem alkaloid and flower F.F. showed good activity against *A.tumifaciens* (IZ=10mm, AI=0.38±0.01, MIC=0.625mg/ml), *S.aureus* (IZ=10.5mm, AI=0.65±0.03, MIC=0.312mg/ml), *A.tumifaciens* (IZ=10.5mm, AI=0.40±0.02, MIC=0.312mg/ml) and *B.subtilis* (IZ=10mm, AI=0.33±0.01, MIC=0.625mg/ml) & *K.pneumonae* (IZ=10mm, AI=0.62±0.02, MIC=0.625mg/ml) respectively. Among all extracts, steroids found to be the most bioactive metabolite as activity was observed almost all the tested pathogens.MIC and MBC values (Table 2) were evaluated for plant extracts which had shown activity, in diffusion assay. The range of MIC and MBC of extracts recorded was 1.25-0.156mg/ml & 0.625-0.078mg/ml respectively.

In present investigation lowest MIC value 0.156mg/ml was recorded against *S.aureus*, indicating significant antimicrobial potential of test extracts. Quantity of extracts per gram of plant material & TA calculated was recorded (Table 3). TA indicates the volume at which extract can be diluted without losing ability to kill microorganisms. High values of TA were observed against A.tumifaciens (158.4ml/g & 144.8ml/g) followed by *E.coli* (79.2ml/g), *B.subtilis* (62.5ml/g) & *K.pneumonae* (53.6ml/g).

Discussion:

Antibiotic resistance has become a global concern. The development of bacterial super

Covered in Scopus & Embase, Elsevier
Int. J. Drug Dev. & Res., July - September 2014, 6 (3): 119-127

resistance strains is resulting in failure of currently used antibiotic agents for many bacterial infections. Hence, a continuous research for getting new antimicrobial agents is the need of the present scenario, either by designing and synthesizing new agents, chemically or through the search of new natural sources for antimicrobial agents [28].

Present study is an effort towards this direction. In the present investigation *N.oleander* has shown antibacterial potential against all the five tested pathogens which are the major causative agents of various human diseases. Although, the plant (N.oleander) has been studied previously for its antimicrobial activity but so far it has not been worked out for flavonoids, alkaloids and steroids. Mostly the crude extracts have been screened of all four parts (leaf, stem, flower and root), without MIC, MBC and TA determination. Such studies could only indicate their antimicrobial potential but are not helpful in establishing them as an antibiotic, hence can't replace the existing antibiotics. In the present investigation IZ, AI, MIC, MBC and TA have been evaluated for each extract. Extracts recorded for low MIC values, indicate strong bioefficacy of the plant. The findings of the present investigation offer a scientific evidence to support the ethno-medicinal use of the plants as an alternative medicine.

Table 1: Antimicrobial activity of extracts of *Nerium oleander* against some pathogenic bacteria

Plant part	Extract	Microorganisms									
		E. coli		*B. subtilis*		*S. aureus*		*K. pneumonae*		*A. tumifaciens*	
		IZ	AI	IZ	AI	IZ	AI	IZ	AI	IZ	AI
Leaf	E1	9.25	0.46±0.01	-	-	-	-	-	-	9	0.3±0.04
	E2	-	-	-	-	-	-	-	-	-	-
	A1	8.25	0.33±0.01	-	-	-	-	-	-	10	0.38±0.01
	S1	9.25	0.33±0.01	8.5	0.34±0.02	8	0.5±0.02	-	-	-	-
Stem	E1	8.75	0.41±0.04	-	-	18	0.75±0.01	-	-	-	-
	E2	8.75	0.55±0.02	-	-	-	-	7	0.35±0.01	7.5	0.25±0.02
	A2	7.25	0.28±0.01	-	-	-	-	-	-	10.5	0.40±0.02
	S2	-	-	-	-	10.5	0.65±0.03	9	0.69±0.02	-	-
Flower	E1	-	-	10	0.33±0.01	8.5	0.42±0.03	10	0.62±0.02	-	-
	E2	-	-	-	-	8	0.32±0.01	7	0.44±0.04	-	-
	A3	-	-	-	-	-	-	-	-	-	-
	S3	7.25	0.29±0.01	14	0.47±0.04	9	0.56±0.02	7	0.54±0.02	8	0.27±0.01
Root	E1	12	0.43±0.01	13	0.44±0.04	9	0.75±0.02	7	0.47±0.02	10	0.34±0.01
	E2	-	-	7	0.24±0.01	8	0.67±0.02	8.5	0.57±0.04	-	-
	A4	7	0.25±0.01	9	0.30±0.01	-	-	9	0.69±0.02	-	-
	S4	-	-	11	0.37±0.04	10	0.62±0.02	12	0.92±0.02	12	0.40±0.01

IZ=Inhibition zone in mm (value: including 6mm diameter of disc),
AI= Activity index (IZ developed by extract/IZ developed by standard),
E1 = Free flavonoids , E2= Bound flavonoids ,
A1, A2, A3, A4= Alkaloids of respective plant parts,
S1, S2, S3, S4= Steroids of respective plant parts, (-)= no activity, ±=SEM

Table 2: MIC and MBC of active extracts of *Nerium oleander* against different pathogens

Microorganism		E1	E2	A1	S1	E1	E2	A2	S2	E1	E2	S3	B3	E1	E2	A4	S4
E.coli	MIC	0.625	-	1.25	0.625	0.625	0.625	1.25	-	-	-	-	1.25	0.312	-	1.25	-
	MBC	0.312	-	0.625	0.312	0.312	0.312	0.625	-	0.625	-	-	0.625	0.156	-	0.625	0.312
B.subtilis	MIC	-	-	-	0.625	-	-	-	-	0.312	-	-	0.312	0.312	1.25	0.625	0.156
	MBC	-	-	-	0.312	-	-	-	0.312	0.625	0.625	-	0.156	0.156	0.625	0.312	0.625
S.aureus	MIC	-	-	-	-	0.156	-	-	0.156	0.312	0.312	-	0.625	0.625	0.625	-	0.312
	MBC	-	-	-	1.25	0.078	-	-	0.625	0.625	0.312	-	0.312	0.312	0.312	0.625	0.312
K.pneumonae	MIC	-	-	-	0.625	-	1.25	-	0.312	0.625	1.25	-	1.25	1.25	0.625	0.312	0.156
	MBC	0.625	-	0.625	-	-	0.625	0.625	-	0.312	0.625	-	0.625	0.625	0.312	0.312	0.156
A.tumifaciens	MIC	0.312	-	0.312	-	-	1.25	0.312	-	-	-	-	0.312	0.625	-	-	0.312
	MBC				-	-	0.625		-	-	-	-		0.312	-	-	0.156

E1= Free flavonoids, E2= Bound flavonoids ,

A1, A2, A3, A4= Alkaloids of respective plant parts,

S1, S2, S3, S4= Steroids of respective plant parts,

MIC= Minimum inhibitory concentration in mg/ml,

MBC=Minimum bactericidal concentration in mg/ml,

(-)= no activity.

Table 3: Quantity & Total activity of extracts of *Nerium oleander*

Plant part	Extract	Quantity of extract mg/g dwt	Total Activity(ml/g)				
			E.coli	*B.subtilis*	*S.aureus*	*K.pneumonae*	*A.tumifaciens*
Leaf	**E1**	1.5	2.4	-	-	-	2.4
	E2	3.5	-	-	-	-	-
	A1	99	79.2	-	-	-	158.4
	S1	17	27.2	27.2	13.6	-	-
Stem	**E1**	3	4.8	-	19.23	-	-
	E2	1.5	2.4	-	-	1.2	1.2
	A2	90.5	72.4	-	-	-	144.8
	S2	13	-	-	41.67	20.8	-
Flower	**E1**	3.5	-	5.6	5.6	5.6	-
	E2	6	-	-	9.6	4.8	-
	A3	47	-	-	-	-	-
	S3	19.5	15.6	62.5	31.2	15.6	31.2
Root	**E1**	1	3.20	3.20	1.6	0.8	1.6
	E2	1.5	-	1.2	1.s2	2.4	-
	A4	33.5	26.8	53.6	-	53.6	-

Covered in Scopus & Embase, Elsevier Int. J. Drug Dev. & Res., July - September 2014, 6 (3): 119-127

	S4	2	-	6.41	3.2	6.41	6.41

E1 = Free flavonoids, E2= Bound flavonoids,
A1, A2, A3, A4= Alkaloids of respective plant parts,
S1, S2, S3, S4= Steroids of respective plant parts,
TA= total activity (extract per gm dried plant part/MIC of extract).

References:

1) Racio MC,Rios JC,Villar A. A review of some antimicrobial compounds isolated from medicinal plants. Phytotherapy Research. 1989. 3(4):117-125.
2) Cohen ML. Epidemiology of drug resistance: implications for a postantimicrobial era. Science.1992; 257, 1050-1055.
3) Essawi T. Screening of some palesiman medicinal plants for antibacterial activity. Journal of Ethanopharmacology . 2000; 46:343-349.
4) Cowan MM. Clin Microbiol Rev. 1999; 12: 564-582.
5) Okunade AL, Elwin-Lewis MPF and Lewis WH. Phytochemistry. 2004; 65: 1017-1032.
6) Hussain I, Khattak M.U.R., Ullah R., Muhammad Z., Khan N., Khan F.A., Ullah Z. and Haider S., Phytochemicals screening and antimicrobial activities of selected medicinal plants of Khyberpakhtunkhwa Pakistan, African Journal of Pharmacy and Pharmacology. 2011; 5(6):746-750.
7) Khan FA, Khattak M, Shah M, Zahoor M and Shah S. Screening of Crude Phytochemicals and Antimicrobial Activities of Selected Medicinal Plants of Peshawar Region Khyber Pakhtoon Khawa Pakistan. Middle-East Journal of Scientific Research. 2011; 9(2): 200-208.
8) Hussain J, Khan FA, Khattak M, Shah S, Zahoor M and Shah S. Screening of crude Phytochemicals and Antimicrobial activities of Selected Medicinal Plants of Peshawar Region Khyber Pakhtoon Khawa Pakistan. Journal of Pharmacy Research. 2011; 4(10):3712-3716.
9) Tail G, Allal-Benfekih L, Kara F and Aci L. Biocide activity of alkaloids extracted from *Nerium oleander* and *Eucalyptus globulus* on adult of *Schistocerca gregaria*. cab direct. 2011.
10) Del-Rio A, Obdulio BG, Castillo J, Marin RR and Ortuno A. Uses and properties of citrus flavonoids. J Agric Food Chem. 1997; 45: 4505-4515.
11) Blumenthal M. The ABC Clinical Guide to Herbs. Austin: American Botanical Council. 2003; 239.
12) Haniffa MA and Kavitha K. Antibacterial activity of medicinal herbs against the fish pathogen Aeromonas hydrophila. Journal of Agricultural Technology. 2012; 8(1): 205-211.
13) Wagner H and Luck R. Naturwissenschaften. 1995; 42: 607.
14) Gopinath SM, Suneetha TB, Mruganka VD. Chemical prophylaxis and antibacterial activity of methanolic and aqueous extracts of some medicinal plants against bovine mastitis. I.J.A.B.R. 2011; 1(1):93-95.

15) Mostaqul Huq M, Jabbar A, Rashid MA, Hasan CM. A novel antibacterial and cardiac steroid from the roots of *Nerium oleander*. Fitoterapia. 1999; 70(1): 5–9.
16) Subramanian SS and Nagarjan S. Flavonoids of the seeds of Crotolaria retusa and Crotolaria striata. Curr Sci . 1969; 38:65.
17) Ramawat KG and Merillon JM. Biotechnology: Secondary Metabolites. Science Pub Inc. 2000.
18) Tomita Y, Uomori A and Minato H. Steroidal sapogenins and sterols in tissue cultures of Dioscorea tokora. Phytochemistry. 1970; 9:111-114.
19) Venier AG, Talon D, Party I, Mercier-Girard D and Bertrandx. Patient and bacterial determinants involved in symptomatic urinary tract infections caused by *E. coli* with and without bacteraemia. Clin Microbial Infect. 2007; 13(2):205-208.
20) Hudault S, Guignot J and Servin AL. "Escherichia coli strains colonising the gastrointestinal tract protect germfree mice against *Salmonella typhimurium* infection". Gut. 2001; 49(1): 47-55.
21) Nataro JP and Kaper JB. Diarrheagenic *Escherichia coli*. Clin Microbiol Rev. 1998; 11(1): 142-201.
22) Toder K. "Pathogenic *E. coli*". Online Textbook of Bacteriology. University of Wisconsin- Madison Department of Bacteriology. 2007; 11-30.
23) Rolhin N and Darfeuille-Michaud A. Adherent-invasive *Escherichia coli* in inflammatory bowel disease. Inflamm Bowl Dis. 2007; 13(10): 1277-83.
24) Curran JP and Al-Salihi FL. "Neonatal staphylococcal scalded skin syndrome: massive outbreak due to an unusual phage type". Pediatrics. 1980; 66 (2): 285-90.
25) Andrews JM. BSAC standardized disc susceptibility testing method. J Antimicrob Chemother 2001; 4:43-57.
26) Basri DF, Fan SH. The potential of aqueous and acetone extracts of gall of Quercus infectoria as antibacterial agents. Indian J Pharmacol. 2005; 37: 26-29.
27) Eloff JN. Quantifying the bioactivity of the plant extracts during screening and bioassay- guided fractionation. Phytomedicine. 2004; 11 (4):370-371.
28) Bhavnani SM and Ballow CH. New agents for Gram positive bacteria. Current Opin. Microbiol. 2000; 3: 528-534.

Article History: ----------
Date of Submission: 21-08-2014
Date of Acceptance: 10-09-2014
Conflict of Interest: NIL
Source of Support: NONE