ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

A novel treatment approach towards Emerging Multidrug Resistant Enteroaggregative *Escherichia coli* (EAEC) causing acute/persistent diarrhea using medicinal plant extracts.

[1]Bangar Raju, [2]Mamatha Ballal*, [3]Indira Bairy

[1]Assistant Professor, Dept of Clinical Microbiology & Immunology, KMC-International Center, Udupi Dst, Manipal-576104, Karnataka, India.
[2] Prof & Head, Dept of Clinical Microbiology & Immunology, KMC-International Center, UDUPI DST, Manipal-576104, Karnataka, India.
[3]Prof & Head, Dept of Microbiology, Kasturba Medical College, Udupi Dst, Manipal- 576104, Karnataka, India.

ABSTRACT

Diarrheal diseases are a leading cause of mortality and morbidity especially among children in developing countries resulting in a major health care problem. Medicinal plants constitute the major component of traditional medicine practiced worldwide due to the economical viability, accessibility, and ancestral experience. Two medicinal plants (*Punica granatum, Syzygium cumini*) used in diarrheal treatment in Karnataka state, India were investigated. This study evaluates the potential antidiarrheal activity of aqueous and methanolic extracts of dry rind of pomegranate fruit and bark of Jamun fruit. This study was carried out on isolated and confirmed strains of biofilm producing multidrug resistant strains of Enteroaggregative Escherichia coli (EAEC) causing acute and persistent diarrhea in children and adults in Manipal. The antidiarrheal activity of the two plant extracts against multidrug resistant EAEC strains was performed by agar well diffusion method using standard guidelines. The results obtained revealed that the aqueous and methanolic extracts of Punica granatum and alcoholic extract of Syzygium cumini plants investigated have good pharmacological activity against biofilm producing MDR EAEC strains. However aqueous extract of Syzygium cumini had no action on these strains. On the basis of these findings it can be assumed that Punica granatum and to a lesser extent Syzygium cumini could be a potential source for novel lead discovery for antidiarrheal drug development towards MDR EAEC strains causing persistent diarrhea in general population.

Keywords: herbal extracts, multidrug resistance, Enteroaggregative Escherichia coli, *Punica granatum, Syzygium cumini,* biofilm, persistent diarrhea.

Corresponding author
Email: mamatha_98@yahoo.com

ISSN: 0975-8585

INTRODUCTION

Diarrheal diseases are a leading cause of mortality and morbidity, especially in children in developing countries resulting in a major health care problem [1, 2]. There are large numbers of epidemiological and experimental evidence pertaining to world-wide acute /persistent diarrheal disease, which is one of the principal causes of death in the infants, particularly in malnourished and which is of critical importance in developing countries. Considering this fact the World Health Organization has constituted a diarrheal disease control programme, which includes studies of traditional medicinal practices, together with the elevation of health education and prevention approaches.

Antibiotics provide the main basis for the therapy of microbial (bacterial and fungal) infections. Since the discovery of these antibiotics and their uses as chemotherapeutic agents there was a belief in the medical fraternity that this would lead to the eventual eradication of infectious diseases [3-5]. However, overuse of antibiotics has become the major factor for the emergence and dissemination of multi-drug resistant strains of several groups of microorganisms. Thus, in light of the evidence of rapid global spread of resistant clinical isolates, the need to find new antimicrobial agents is of paramount importance. However, the past record of rapid, widespread emergence of resistance to newly introduced antimicrobial agents indicates that even new families of antimicrobial agents will have a short life expectancy. For this reason, researchers are increasingly turning their attention to herbal products, looking for new leads to develop better drugs against MDR microbe strains. Use of herbal drugs has been an inseparable part of human civilization as many food materials like ginger, garlic, etc. have long been used as medicines [6-8]. A vast majority of the people of developing countries relies on herbal drugs for the management of diarrhea. Medicinal herbs constitute the major component of the traditional medicine practiced worldwide due to the economical viability, accessibility and ancestral experience. It thus becomes important to identify and evaluate commonly available natural drugs as alternative to currently used anti-diarrhoeal drugs which are not completely free from adverse effects (Goodman and Gilman, 1992). In recent years, there has been a surge of interest in herbal remedies for a number of ailments [9,10].

Punica granatum L. (Punicaceae) is a shrub, usually with multiple stems, that commonly grows 1.8–4.6m tall. The deciduous leaves are shiny and about 7.6 cm long. Punica granatum has orange-red, trumpet-shaped flowers with ruffled petals. The flowers are about 5 cm long, often double, and are produced over a long period in summer. The fruit is globose, 5–7.6 cm in diameter, and shiny reddish or yellowish green when mature. The fruit is technically a berry. It is filled with crunchy seeds, each of which is encased in a juicy, somewhat acidic pulp that is itself enclosed in a membranous skin (Polunin & Huxley, 1987). Punica granatum grows well in warm areas and is cultivated in south western & northern states of India. Almost all parts of this plant are used in traditional medicine for the treatment of various ailments. Bark and rind of the fruit are used in dysentery, diarrhea, piles, bronchitis, to reduce the risk of cardiovascular disease, and as an antihelmintic (Polunin & Stainton, 1985; Morton, 1987). Methanol extract of Punica granatum seed and dried peels has antidiarrheal activity and wound healing activity,

respectively (Das et al., 1999; Murthy et al., 2004). In a recent study, Punica granatum juice was found to reduce cholesterol oxidation by almost half and reduce the retention of disproportionate low-density lipoprotein (LDL) cholesterol (Singh et al., 2002). Punica granatum rind extract has been shown to have gastro protective activity through its antioxidant mechanism [11-13]. A breast cancer chemopreventive property of Punica granatum fruit extracts has been found in a mouse mammary organ culture (Mehta & Lansky, 2004). Other studies have also evaluated the Punica granatum extract and established it to be effective as an antibacterial and anti-candidal agent (Navarro et al., 1996; Rani & Khullar, 2004). A symbol of fecundity and divine femininity emerges, whose fruit rinds, bark and roots are used worldwide as taenicides, owing to alkaloids, and treatment of diarrhea and oral and genital lesions, owing to tannins and astringency [14-17]. Both the juice and the oil contain numerous and diverse bioflavonoids, which have been shown to be both potently antioxidant and inhibitory of one or both of the enzymes cyclooxygenase (catalyzing arachidonic acid to prostaglandins) and lipoxygenase (catalyzing arachidonic acid to leukotrienes). Extracts of the rinds have been shown to be bactericidal, antiviral and antitumor. A 1998 medical monograph recommends the use of pomegranates in the treatment of Acquired Immune Deficiency Syndrome (AIDS) owing to their antioxidant properties and botanical uniqueness. The potential toxicity of pomegranate is also considered.

Despite the relatively wide use of this plant in popular medicine in India and other Middle East countries for its antidiarrheal properties, surprisingly no research has been carried out to examine the antidiarrheal activity of its peels extract. The current study thus was carried out to evaluate the antidiarrheal effect of aqueous and alcoholic extracts of Punica granatum peels using multidrug resistant strains of EAEC and to determine if the folk medicinal use has a scientifically justified basis.

The Jambul fruit is a well-known common fruit in India. It has two varieties. The big one is oval in shape and is commonly called as *Suva-jumun.* The small one is round in shape and is commonly called as *Kutta-jamun.* The bigger variety is sweeter than the smaller one. The fruit is a juicy berry with a single stone. It is black outside and violet inside; has a sourish-sweet pulp and greenish yellow seed. The Jambul fruit has been cultivated in the south western region of India for a long time. It is considered to be native of India or further east, but is now found in all tropical regions and grows abundantly during the rainy season. It is a common tree, found wild or cultivated in most parts of India. The Jambul fruit regarded in traditional medicine as a specific against diabetes because of its effect on the pancreas. The fruit as such, the seeds and fruit juice are all useful in the treatment of this disease. The seeds contain glucose 'Jamboline' which is believed to have the power to check the pathological conversion of starch into sugar in cases of increased production of glucose. They are dried and powdered [18-21]. In *Ayurveda,* the inner bark of the Jambul tree is also used in the treatment of diabetes. The powder of the seeds is valuable in Polyuria or production of excess urine. Powder of the seed is an effective remedy for diarrhea and dysentery. An infusion of the tender leaves, which contain a high concentration of gallic and tannic acid is also given as a medicine in diarrhea and dysentery. A decoction of the bark taken with honey is also a useful medicine for chronic diarrhea and dysentery.

ISSN: 0975-8585

The current study thus was carried out to evaluate the antidiarrheal effect of aqueous as well as alcoholic extracts of Punica granatum peels and bark of Jambul fruit against various multidrug resistant strains of EAEC (isolated during the study period), control strain of EAEC 042, and E coli ATTCC strain. The present study was aimed to evaluate the potentiality of aqueous and alcoholic extracts of Indian medicinal plants Pomegranate and Jambul fruit against Enteroaggregative Escherichia coli. Similar screening studies were done against selected pathogens of Enterobacteriaceae. As pomegranate and jumbal fruit is widely available, cheap and commonly used in this part of India, we thought it would be worthwhile to study its potential anti-diarrheal activity, which if proved would be an easily accessible home remedy.

METHODOLOGY

Ethno medical information and plant collection

Fresh plants or plant parts were collected randomly from different regions of Udupi Dt , Karnataka state, India. Fresh plant material were washed under running tap water, air dried, homogenized to fine powder, and stored in airtight bottles.

Plant extraction for aqueous extract

10 g of air-dried powder was taken in distilled water and boiled on slow heat for 2 h. It was then filtered through filter paper and centrifuged at 5000 g for 10 min. The supernatant was collected. This procedure was repeated twice. After 6 h, the supernatant was collected at an interval of 2 h, pooled together, and concentrated to make the final volume one fourth of the original volume. It was then autoclaved at 121 °C under 15 lbs pressure and stored at 4 °C. An equivalent of 2 g of powder was obtained from 150 g of dried peels and bark of Jambul fruit. Solutions were prepared by dissolving the resultant powder in physiologic salt solution (PSS).

For solvent extraction

10 g of air-dried powder was taken in 100 ml of organic solvent (methanol) in a conical flask, plugged with cotton, and then kept on a rotary shaker at 190-200 rpm for 24 h. After 24 h, it was filtered through filter paper and centrifuged at 5000 g for 10 min. The supernatant was collected and the solvent was evaporated to make the final volume one-fourth of the original volume and stored at 4 °C in airtight bottles. An equivalent of 2 g of powder was obtained from 150 g of dried peels of pomegranate and bark of Jambul fruit. Solutions were prepared by dissolving the resultant powder in physiologic salt solution (PSS).

Microorganisms

The microbial strains investigated are identified and confirmed strains isolated during the study period and control strains were obtained from National Institute of Cholerae and Enteric Diseases (NICED), Kolkata, India. The E. coli strains were identified as EAEC using multiplex PCR targeting two important specific genes AggR, and EAST. The property of Biofilm

ISSN: 0975-8585

production by these EAEC stains was identified using quantitative microtitre plate assay following standard guidelines. The studied bacterial strains include Escherichia coli ATCC, Enteroaggregative Escherichia coli 042, Multidrug resistant strains of EAEC (n=25) which were maintained at 4 °C on nutrient agar slants.

Antibacterial activity

Agar well diffusion method for aqueous extract and for solvent extract

The Mueller Hinton Agar medium was prepared and sterilized at 15 pounds for 15 min in autoclave. The sterilized agar medium was poured into sterile Petri dishes under aseptic conditions and allowed to solidify. The 24-h-old cultures of control strains and test strains of EAEC were inoculated and evenly spread on the surface of the agar by sterile swab to get uniform lawn culture of the organism. The standard antibiotic discs each were placed over the agar plate gently to give better contact with agar. For agar well diffusion method, a well was prepared in the plates with the help of a cup-borer (0.85cm). Into the well, 100 µl of the test compound was introduced. The Petri dishes were incubated at 37C for 16- 18 h in inverted position, the zone of inhibition was observed and the results were recorded.

Microbial growth was determined by measuring the diameter of the zone of inhibition. For each bacterial strain, negative controls were maintained where pure solvents were used instead of the extract. The control zones were subtracted from the test zones and the resulting zone diameter is shown in the Table. For positive control, 2 antibiotics, namely, Gentamicin (10 mcg/disc), and Meropenem (10 mcg/disc) were used.

RESULTS

TABLE 2: Screening of 2 Indian Medicinal plants species for potential Antimicrobial activity against Multi Drug Resistant EAEC Strains.

S.NO	Botanical Name	Extract	EAEC 042	MDR EAEC (n=25)	E. coli ATCC
1	*Punica granatum*	Aqueous	18 mm	17.5 mm	20 mm
		Methanol	18 mm	17 mm	20 mm
2	*Syzygium cumini*	Aqueous	3 mm	-----	-----
		Methanol	13 mm	13 mm	15 mm

ISSN: 0975-8585

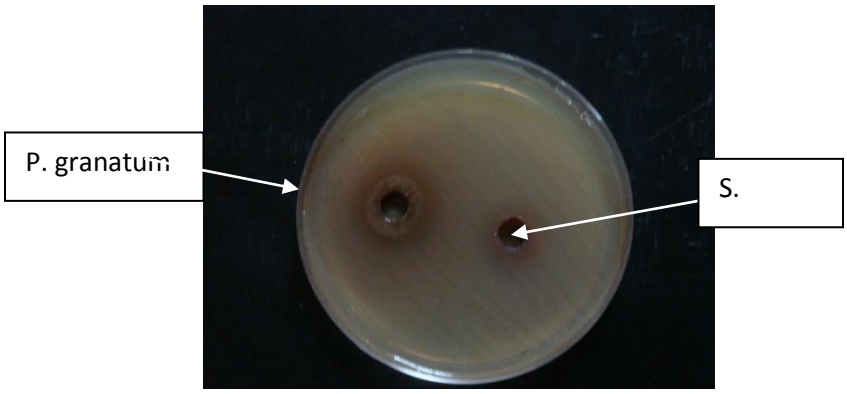


Fig 1: Zone of inhibition as seen due to action of aqueous extract of P granatum and S. cumini against EAEC 042

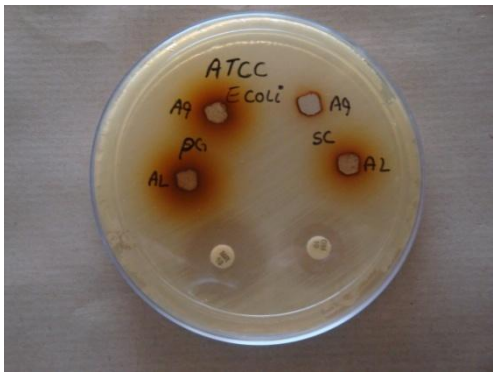
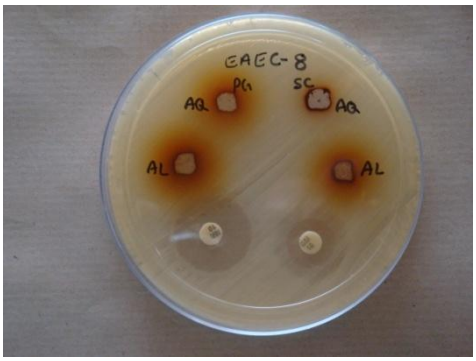


Fig 2: Zone of inhibition as seen due to action of aqueous and alcoholic extracts of P granatum and S. cumini against ATCC E coli strain.

(Note: The zones of inhibition of two Standard antibiotics seen below at the base of the plate are in comparison with zone of inhibition produced by the extracts)



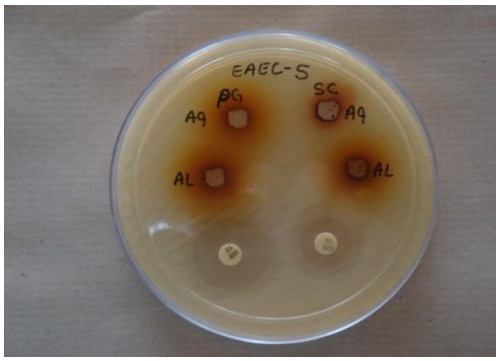


Fig 3: Zone of inhibition as seen due to action of aqueous and alcoholic extracts of P granatum and S. cumini against MDR EAEC strains.

(EAEC= Enteroaggregative Escherichia coli; PG= P. granatum; SC= S. cumini, AQ= aqueous extract; AL= alcoholic extract; MR=Meropenem; GM= Gentamicin)

In this study, we have tested the aqueous and methanolic extracts of two plants for their antimicrobial (antidiarrheal) activity against 25 known, identified and characterized multi-drug resistant strains of EAEC. E coli ATCC strain, control strains of EAEC 042 were also used as control sensitive strains. The aqueous and alcoholic extracts of P. granatum showed potent antimicrobial (antidiarrheal) activity against all the multidrug resistant strains of EAEC by an agar well diffusion assay (Table 2). Alcoholic extracts of S. cumini showed no activity on any of the test as well as control strains tested. The antimicrobial activity of the aqueous and alcoholic extracts is summarized in table 2. The zone of inhibition as seen with MDR EAEC strains is in comparison with standard antibiotics. In comparison zone of inhibition due to alcoholic and aqueous extracts of P. granatum was much higher than with alcoholic extract of S. cumini.

CONCLUSION

The traditional medicinal methods, especially the use of medicinal plants, still play a vital role to cover the basic health needs in the developing countries, and moreover, the use of herbal remedies has risen in the developed countries in the last decade [1, 6, 18, 21]. In this manner, plants continue to be a rich source of therapeutic agents. It is anticipated that phytochemicals with adequate antibacterial efficacy will be used for the treatment of bacterial infections. The need of the hour is to screen a number of plants that are traditionally used and also to evaluate their probable phytoconstituents.

Table 1: Ethano-botanical information of 2 Indian medicinal plants species screened for antidiarrheal activity.

S.NO	Botanical Name (Family, Genus, Species)	Vernacular Name	Habit	Part(s) extracted	Known Action/Therapeutic use
1	*Punica granatum*	Ana	shrub	Rind of fruit	Digestive disorders, diarrhea, dysentery, intestinal worms, anal itching, kidney and bladder stones, teeth and gum disorders
2	*Syzygium cumini*	Jamun, Rose apple, Indian black berry, java plum	tree	Bark	Diabetes, Polyuria, diarrhea, dysentery, piles, liver disorders, female sterility

Table 1 summarizes the ethobotanical information of the 2 plant species belonging to 2 different families. The antibacterial activity of the bacteria (control + MDR EAEC strains) against the plants screened is shown in Table 2. S. cumini did not show any activity in aqueous extract. However, negative results do not indicate the absence of bioactive constituents, nor that the plant is inactive. Active compound(s) may be present in insufficient quantities in the crude extracts to show the activity alternatively, if the active principle is present in high enough quantities, there could be other constituents exerting antagonistic effects or negating the positive effects of the bioactive agents. With no antibacterial activity, extracts may be active against other bacterial species which were not tested [9, 10, 25, 26, 27, and 29].

ISSN: 0975-8585

Out of 2 plants screened our study revealed that S. cumini did not show any activity in aqueous extract while the aqueous and alcoholic extract of P. granatum plant showed maximum antibacterial activity. However, methanol and aqueous extract of P. granatum showed activity towards the control strains (EAEC042, ATCC E coli) too.

TABLE 3: Antibacterial susceptibility of standard antibiotics against EAEC 042, MDR EAEC, E. coli ATCC Strain.

S.NO	Antibiotics	Zone of inhibition (mm)		
		EAEC 042	MDR EAEC (n=25)	E. coli ATCC
1	Gentamicin	18 mm	15 mm	18 mm
2	Meropenem	23 mm	20 mm	23 mm

Table 4: Resistance Profile of Multi-Drug Resistant Isolates of EAEC, versus control strains (EAEC 042, and ATCC E coli).

Microorganism	Source	Resistance pattern	Isolates
ATCC E coli	NICED, KOLKATA	----------	1
EAEC 042	NICED KOLKATA	Na, Ak.	1
MDR EAEC	MANIPAL	Ca, Ch, Cpm, Ci, G, Na, Cf, T,C, Nf, Tb	n=14
MDR EAEC	MANIPAL	Ca, Ak, Ci, G, Na, Cf, Pc T,C, Nf	n = 6
MDR EAEC	MANIPAL	Ca, Ci, G, Na, Cf, T,C	n = 5

Agent: Cephalosporins: Ch=Cephalothin (30 µg), Ca=Ceftazideme (30 µg), Ci=Ceftriaxone (30 µg), Cpm=Cefepime (30 µg). Ao=Aztreonam (30 µg), Pc=Piperacillin (100 µg).
Aminoglycosides: Ak=Amikacin (30 µg), G= gentamycin (10 µg), Tb=Tobramycin (10 µg); Fluoroquinones: Na=Nalidixic acid (30 µg), Cf=Ciprofloxacin (5 µg).
Others: Nf=Nitrofurantoin (300 µg), T=Tetracycline (30 µg), C=Chloramphenicol (30 µg).

Table 3 reports the antibacterial susceptibility of various standard antibiotics against all the tested strains. The antibacterial activity of the two plant species can be compared with the standard antibiotics. This work may provide essential information in the selection of plant extract for further isolation of constituents responsible for the activity against the studied species, thereby aiding to explore an antibacterial lead that is helpful in combating the acute/persistent diarrhea caused by biofilm producing EAEC.

REFERENCES

[1] Coates A Hu, Y Bax, and R Page C. Nat Rev Drug Discov 2002; 1: 895-910.
[2] Braga LC, Leite AAM, Xavier KGS, Takahashi JA, Bemquerer MP, Chartone-Souza E, Nascimento and AMA. Can J Microbiol 2005; 51: 541-547.
[3] Betoni JEC, Mantovani RP, Barbosa LN, Di-Stasi LC and Fernandes A. Mem Inst Oswaldo Cruz 2006; 101: 387-390.
[4] Perez C, Paul M, and Bazerque P. Acta Biol Med Exp 1990; 15: 113-115.
[5] Nair R, and Chanda S. Indian J Pharm Sci 2005; 67: 239-243.
[6] Balandrin MF, Kjocke AJ, and Wurtele et al. Science 1985; 228: 1154-1160.

[7] Parekh J, and Chanda S. Afr J Biomed Res 2007C; 10: 175-184.
[8] Taylor JLS, Rabe T, and McGraw LJ et al. Plant Growth Regul 2001; 34: 23-37.
[9] Lewis K and Ausubel FM. Nat Biotechnol 2006. 24, 1504 - 1507.
[10] Lee SB, Cha KH, Kim SN, Altantsetseg S, Shatar S, Sarangerel O and Nho CW. J Microbiol 2007; 45: 53-57.
[11] Bonjar S. J Ethnopharmacol 2004; 94: 301-305.
[12] Clardy J, and Walsh C. Nature 2004; 32: 829-837.
[13] Verpoorte R, Choi YH, and Kim HK. J Ethnopharmacol 2005 ;100: 53-56.
[14] Mitscher LA, Drake S, and Gollapudi SR et al. J Nat Prod 1987; 50: 1025-1040.
[15] Palombo EA, and Semple SJ. Antibacterial J Ethnopharmacol 2001; 77: 151-157.
[16] Zgoda-Pols JR, Freyer AJ, and Killner LB et al. J Nat Prod 2002; 65: 1554-1559.
[17] Parekh J, Jadeja D, and Chands S. Turk J Biol 2005; 29: 203-210.
[18] Nair R, and Chanda S. Indian J Pharmacol 2006; 38: 142-144.
[19] Parekh J, and Chands S. Turk J Biol 2007; 31: 53-58.
[20] Parekh J, and Chands S. Afr J Microbiol 2007; 1: 92-99.
[21] Bauer AW, Kirby WMM, and Sherris JC et al. Am J Clin Pathol 1966; 45: 493-496.
[22] Brantner A, Grein E. J Ethnopharmacol 1994; 44: 35-40.
[23] Somchit MN, Reezal I, Nur IE and Mutalib AR. J Ethnopharmacol 2003; 84: 1-4.
[24] Nair R and Chanda S. Strains. Indian J Pharmacol 2006; 38: 142-144.
[25] Walsh SE, Maillard JY, Russel AD, Catrenich CE, Charbonneau AL and Bartolo RG. J Appl Microbiol 2003; 94: 240-247.
[26] Suffredini IA, Paciencia ML, Nepomuceno DC, Younes RN and Varella AD. Mem Inst Oswaldo Cruz 2006; 101: 287-290
[27] Kang CI, Kim SH, Bang JW, Kim HB, Kim NJ, Kim EC, Oh MD and Choe KW. J Kor Med Sci 2006; 21: 816-822.
[28] Prabuseenivasan S, Jayakumar M and Ignacimuthu S. BMC Comp Alternat Med 2006; 6: 39.
[29] Chamin N, Chami F, Bennis S, Trouillas J and Remmal A. Braz J Infect Dis 2004; 8: 217-226.
[30] Rani P and Khullar N. Phytother Res 2004; 18: 670-673.
[31] Aboaba O and Efuwape BM. Bio Res Comm 2001; 13: 183-188.
[32] Chakrabarti A, Ghosh A, Kanta A and Kumar P. Indian J Med Res 1995;102:13-19.
[33] National Committee for Clinical Laboratory Standards. Methods for Disk Susceptibility Tests for Bacteria that Grow Aerobically, NCCLS Document M2-A7. National Committee for Clinical Laboratory Standards: Wayne, USA, 2000; Volume 7.