IN VITRO ANTIBACTERIAL EFFECTS OF *PIPER LONGUM* FRUIT EXTRACTS ON HUMAN PATHOGENS AND PHYTOCHEMICAL ANALYSIS

Neha Chauhan[1], Pooja Uniyal[2], Renu Chauhan[3], Chhaya Singh[4] & Deepak Kumar[5]
[1, 2, 3, 4, 5]SGRR College of Basic and Applied Sciences, Shri Guru Ram Rai University, Dehradun, Uttarakhand, India

Received: Deecember 13, 2018 Accepted: January 23, 2019

ABSTRACT: *The study focused on determining the antibacterial properties of P. longum against pathogenic bacteria and analyzing the phytochemical constituents responsible for these medicinal activities.The plant fruits were collected, shade dried, powdered and subjected to extraction by Cold Maceration in polar and non-polar solvents. The extracts were used to determine the antibacterial activity by Agar-well Diffusion method and MIC assay.The significant botanicals were also analyzed.The result indicates that the ethyl acetate extract of the fruits of P. longum was found to be most effective against the selected pathogenic bacteria. Phenolic content was found as a major phytochemical constituent in various extracts of P. longumfruit which is responsible for inhibiting the pathogens in reference.The plant based formulations are an effective means of treatment for combating bacterial diseases as they exhibit negligible side effects and also loaded with beneficial antioxidants.*

Key Words: *P. longum, Cold Maceration, Antibacterial activity, Agar-well diffusion, Phytochemicals and MIC.*

INTRODUCTION

Medicinal plants have been used in traditionallyprepared medicine since long time. Assurance of safety, quality and efficacy of medicinal plants and herbal products has now become a key issue in industrialized and in developing countries. The widespread use of herbal remedies and healthcare preparations is described in the Vedas and the Bible. Medicinal plants have been used for thousands of years to flavor and conserve food, to treat health disorders and to prevent diseases including epidemics. The knowledge of their healing properties has been transmitted over the centuries within and among human communities. Active compounds produced during secondary metabolism are usually responsible for the biological properties of plant species used throughout the globe for various purpose, including treatment of infectious diseases **(Singh R., 2015)**

The term medicinal plants include some various types of plants used in herbalism and some of these plants have potent medicinal properties. Medicinal plants are the "backbone" of traditional medicine, which means more than 3.3 billion people in the less developed countries utilize medicinal plants on a regular basis **(Davidson-Hunt I.2000)**. These medicinal plants are considered to be rich resource of bioactive compounds which can be used in drug development and synthesis. Besides that, these plants play a critical role in the development of human cultures around the whole world **(Singh R.2015).**

The Indian sub-continent has a very rich diversity of plant species in a wide range of ecosystems. There are about 17,000 species of higher plants, of which approximately 8,000 species are considered medicinal and used by village communities, particularly tribal communities, or in traditional medicinal systems, such as the Ayurveda. The use of traditional medicine and medicinal plants in most developing countries, as a basis for the maintenance of good health, has been widely observed by UNESCO, 1996**(UNESCO, 1996).** Furthermore, an increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants as well as from traditionally used rural herbal remedies**(UNESCO, 1998)**.

Plants have been a proved source of medicines as they are a reservoir of chemical agents with therapeutic properties since ancient times. Nowadays People are relying on herbal medicines as dietary supplements to relieve and treat many different human afflictions.

Herbs and spices are an important part of our nutrition. They have been used for thousands of years to enhance the flavor, color and aroma of food. In addition to boosting flavor, herbs and spices are also known for their preservative and medicinal value, which forms one of the oldest sciences. So it has been found in recent years that modern researchers has started paying attention to the protective properties of spices **(F. A. Draughon, Food Technology, 2004)**.

With the increasing incidents of remerging infectious diseases, there is urgent requirement of new microbial compoundswith diverse chemical structures and novel mechanism. Development of antibiotic resistance is one of the main concerns. The major health problems in developing countries are the infections caused by resistant microbes **(Adwan G, Abu-Shanab B and Adwan K 2010)** as the major part of the population cannot afford the high cost antibiotics. Therefore, we are moving towards naturopathy to discover new antimicrobial agents. The widespread use of herbal remedies and health care preparations, such as those described in ancient texts like the Vedas and Bible, has been followed to the occurrence of natural products with medicinal properties. In fact, plant produces a wide range of bioactive molecules, making them effective source valuable medicines. Plants with possible antimicrobial activity should be tested against an appropriate microbial model to confirm the activity and to ascertain the parameters associated with it.

*Piper longum*belongs to Piperaceae family. The word *pepper* is derived from the Tamil word used for long pepper, *pippali.* It is sometimes called Indian long pepper (Pipli). The plant itself is a native of India. Dried fruits are usedas spice and for seasoning. *Piper longum* is an herb which is found in the hotter regions of India like Tamil Nadu, Andhra Pradesh, from Central Himalayas to Assam, Khasi and Mikir hills and also in forest of Western Ghats from Kolkata to Kerala. Malaysia, Singapore, South East Asian region, Sri Lanka and Indonesia are some countries other than India where this plant found to grow. Beneficial uses of long pepper for dietary purpose as well as for various health purposes have been documented in Ayurveda **(Sesha Iyengar, 1989, Rawlinson, 2001).** It is originally used for the maintenance of healthy respiratory system and is also beneficial foractive metabolism and good digestion as well. The fruits contain volatile oil, starch, protein and alkaloids, saponins, carbohydrates as potent phytoconstituents and amygdalin and tannins are absent **(Dasgupta, A. and Dutta, P.C, 1980).***P. longum* seeds contain Sylvatine and dieudesmin**(Dutta, C.P, et al., 1975).**Crushed seeds containvariety of significant Fatty acids such as palmitic, hexadecenoic, stearic, linoleic, oleic, higher saturated acids, arachidic, and behenic acids **(Beel. K.L, et al., 1971).**

Plant is small creeping shrub having height up to 3mm with numerous creeping, jointed stems, thickened at the nodes. The leaves are alternately arranged and are ovate or heart shaped. They have dark color above and pale green below. They are spreading and are without stipules. The blade of leaves varies greatly in size. The leaves are about 2 to 3 inches in length. The uppermost leaves are 2-3 cm long, whereas, the lowest leaves are 5-7 cm long. The plant bears flowers during rainy season. Flowers are monoecious. Both male and female flowers are borne on different plants. The flowers are borne in solitary spike. Spike turns red in color when get ripened. Once the spike gets matured, it is collected, dried and powdered to form the commercial form of pippali.Fruits are oval shaped having orange and yellowish color. They grow in early winters. Drupes are about 1 inch in diameter. The fruits are fleshy and embedded in spikes. They are 2.5-3.5 cm long and 5mm thick, oblong, blunt and blackish green in color. Roots are grayish brown in color and longitudinally wrinkled. They are perennial woody roots.

MATERIALS AND METHODS

Plant material and extraction

The dried mature fruits of *Piper longum* were purchased from local market of Dehradun, India. 25g of fruits powder was taken and soaked in 250ml of organic solvent (Ethyl acetate (E4), Methanol (E5)) and Aqueous (E6) for extraction using Cold Maceration method **(Akthar et al., 2014).** All the extracts were made solvent free and concentrated using rotatory evaporator and preserved at 4^{o}C in airtight bottle until further use.

Chemicals and Reagents

The chemicals and reagents used for the study are of pure grade. Ethyl acetate, Methanol(20%),Sodiumhydroxide(4%),Sulphuricacid(72%), DMSO(Dimethylsulphoxide), Muller Hinton Agar and Broth, Molisch's reagent, Ferric Chloride, Dragondroff's reagent.

Microorganisms

Five different microorganism representing Gram- positive and Gram- negative bacteria were used in this study as *Escherichia* coli (B1), *Bacillus subtili*s (B2), *Pseudomonas aeruginosa* (B3), *Salmonella typhi* (B4) and *Staphylococcus aureus* (B5).

Antimicrobial activity

Antimicrobial activities of all the extracts (ethyl acetate, methanol, aqueous) were determined by agar well diffusion method. The extracts were dissolved in DMSO (dimethylsulphoxide). The microbial cultures were incubated at 37^{o}C for 18h in a broth medium. Then the inoculum was prepared in accordance with McFarland turbidity standards **(Sawhney, S.S et al., 2011).** The 20µl of the culture was spread on Muller Hinton agar plates and wells of 9mm diameter were punched into the agar plated. 100 µl of the extracts

concentration (0.5mg/100ml and 1mg/100ml) were used for determination of ZOI (Zone of Inhibition). The plates were incubated at 37°C for 18h-24h. Commercial antibiotic (Gentamicin) and DMSO was used as positive and negative control respectively. The test was performed in triplicates and the final results were presented as the men zone of inhibition.

Broth Dilution MIC tests (NCCLS, 1993)

This test was done to check the minimum zone of inhibition. This process is named as macro broth dilution assay. Muller- Hinton broth diluents was taken and 2-fold series dilutions of all extracts were prepared in the well on the basis of result obtained from agar well diffusion method. Gentamicin and DMSO are positive and negative control respectively 20µl of test culture of concentration (5×10^5 Cfu/ml) was inoculated and plates were incubated for 24h at 37°C. Plate with minimum growth was taken and concentration is noted as minimum inhibitory concentration. Another value minimum bacterial count was calculated by spreading 20µl of MIC test broth on a new plate incubating for 18-24h at 37°C. Dilution of plates showing no single bacterial growth was taken as MBC concentration. Triplicates were used to perform. Test and mean MIC and MBC value were calculated and noted **(Chauhan, Neha et al., 2012).**

Phytochemical Analysis

Qualitative Analysis

Qualitative analysis was done in accordance to **(Chauhan, Renu et al., 2017).**

Test for Glycosides (Sulphuric Acid Test)

To 1ml of plant extract, few drops of sulphuric acids were added and the mixture was allowed to stand for few minutes. Presence of glycosides was confirmed by the formation of Reddish precipitate.

Test for Carbohydrate (Molisch's Test)

To 1ml of extract, 2ml of Molisch's Reagent was added. Now to this mixture, 2ml conc. Sulphuric acid was added along the sides of the test tube. Presence of carbohydrates was confirmed by the formation of reddish violet ring at the junction of two liquids.

Test for Flavonoids (Aqueous Test)

To 1ml of plant extract, add 1ml of aqueous NaOH. Yellow color formation showed the presence of flavonoid.

Test for Saponin (Aqueous Test)

To 1ml of extract of plant add 5ml water and shake well in a test tube shaker. Later formation shows the presence of saponin.

Test for Tannin (Ferric Chloride Test)

To 1ml of plant extract add 1ml of ferric chloride. Formation of greenish black color confirmed the presence of tannins.

Test for Alkaloids (Dragondroff's Reagent)

TO 1ml of plant extract add 5-6 drops of Dragondroff's reagent. Formation of creamish/ brownish-red/ orange precipitate confirmed the presence of alkaloid.

RESULT

Antibacterial activity

The antibacterial activity of the plants was evaluated using agar well diffusion method. All the fruit extracts of *P. longum* showed the antibacterial activity against the selected microbes. Gentamicin used as a positive control showed the maximum ZOI against B1 for both the concentrations of 0.5mg/100µl and 1mg/100µl *i.e.* 31mm and 35mm respectively followed by B3, B2, B4 and B5. For 0.5mg/100µl concentration of E1 extract, B1 and B4 showed the maximum ZOI *i.e.* 21mm followed by B2 (ZOI-17mm), B5 (ZOI-16mm) and the minimum zone of inhibition was B3 *i.e.* 14mm.For 1mg/100µl concentration of E4 extract the most susceptible bacteria was B3 with ZOI of 16mm. It was followed by B4 (ZOI-15mm), B5 (ZOI-12mm) and the B1 which was the least susceptible bacteria having zone of inhibition 10mm followed by B2 (ZOI-11mm). For 0.5mg/100µl concentration of E4 extract, B3 and B4 showed the maximum ZOI *i.e.*mm and B1 and B5 showed same ZOI *i.e.* 09mm and B2 showed minimum ZOI *i.e.* 8mm which makes it least susceptible. For 1mg/100µl concentration of E5 extract, B2 and B3 showed least susceptibility having no zone of inhibition. The highest susceptibility was observed in B4 having zone of inhibition of 12mm followed by B1 and B5 having zone of inhibition of 09mm and 10mm respectively. This shows that the B4 is the most susceptible followed by B1 and B5 while the B2 and B3 are the least susceptible for E5 extract. For 0.5mg/100µl concentration ofE6 extract, B2 have maximum zone of inhibition *i.e.* 12mm and B5 have least zone of inhibition *i.e.* 06mm followed by B1(ZOI-09mm) while B3 and B4 has same ZOI of 08mm. For 1mg/100µl concentration, the maximum zone of inhibition was observed in B2 *i.e.* 15mm. The zone of inhibition for B1 and B4 is equal *i.e.* 13mm while the B5 is least susceptible for E6 extract having ZOI of 9mm.So it shows that

B2 is the most susceptible bacteria for E6 extract of fruits of *P. nigrum.*The results obtained were tabulated in table 2.The extracts showing high efficacy against selected pathogens were subjected to minimum inhibitory concentration (MIC) assay by two-fold serial dilution method (2:2).

Qualitative analysis

Phytochemical assessment of various extracts of *P. longum* was done using qualitative method. Qualitative assessment of E4 extracts showed the presence of glycosides, carbohydrates, flavonoids, tannins, saponins and alkaloids. In E5 extracts, glycosides, carbohydrates, flavonoids, tannins, saponins and alkaloids were present. In E6 extracts also all the phytochemicals were present. The result has been tabulated in table 6.

MIC

MIC is the lowest concentration of the test sample or drug at which it shows the highest inhibitory activity against microorganisms. The extracts showing high efficacy against microorganisms were subjected to minimum inhibitory concentration (MIC) assay by twofolds serial dilution method (2:2) **(Florey *et al.,* 1989 and Drummond *et al.,* 2000).** From the data it was observed that the MIC value ranged from0.156-0.0625mg/ml. To determine whether an extract is bactericidal (MIC/MBC <4) or bacteriostatic (MIC/MBC >4) in nature MIC Index (MIC/MBC) was performed. The E4 extract showed the lowest concentration of MIC for all the microbes *i.e.* 0.25mg/ml. MIC concentration for E5 extract was found to be lowest for all the bacterial culture *i.e.* 0.25mg/ml and E6 extract showed lowest concentration of MIC for B4 *i.e.* 0.125mg/ml whereas it showed same MIC value for the other microbes *i.e.* 0.25mg/ml. The result have been tabulated in tables 3, 4 & 5.

DISCUSSION

Antibacterial activity

It is evident from our result that E4, E5 an E6 fruit extracts of *P. longum* possess effective antimicrobial activity against the selected bacterial strains. Among all the tested solvent fruit extracts, E4 fruit extract had the maximum bacterial activity. It strongly inhibited the growth B3 and B4 followed by B5, B1 and B2. For E5 extract highly susceptible bacteria was B4 having maximum zone of inhibition followed by B5 and B1. B2 and B3 were found to be resistant against the E5 extract. E6 extract showed the maximum inhibition zone for B2, B1 and B4 showed almost equal susceptibility for E6 followed by B3 and B5.The antibacterial activity of E4 showed maximum ZOI value against B3 (16mm), E5 extract showed maximum value against B4 (12mm) and E6 extract showed maximum value against B2 (15mm) at 1mg/100µl. In the study done by **Khan, M. and Siddiqui, M, 2007,** the ethyl acetate and aqueous extract of *P. longum* showed moderate antibacterial activity with zones of inhibition ranging between 5-9mm for all the same bacteria. But results of our study showed that E4 extract strongly inhibit the growth of all the bacteria with inhibition zones ranging between 10-16mm and E6 extract also showed effective antibacterial activity with inhibition zones ranging between 9-15mm. Also, in this study *B. megaterium* and *P. aeruginosa* found to be resistant for ethyl acetate and aqueous extract respectively but in our results they both were found to be highly susceptible having effective inhibition zones. **Lokhande, P.D, et al., 2007** also reported the same in their study where aqueous extract was found to be less effective as compared to our study which showed effective antibacterial property of E6 extract. The MIC value observed in our work were found to be 0.125mg/ml in case of E6 extract against B4 whereas the study performed by **Sawhney et al.,** in **2011** exhibited the MIC value as 0.0625mg/ml for *S. aureus* for all the three extracts which is bactericidal in nature.

MIC

MIC values of all the extracts were same in case of *P. longum.* E4 extract and E5 extract both showed equal MIC values for all the selected pathogens (0.25mg/ml). E6 extract showed the lowest MIC value for B4 (0.125mg/ml) and for rest of the bacteria the values were same as E4 and E5 (0.25mg/ml). Effective results were obtained by our study as the MIC value of 0.25mg/ml was efficiently inhibited the pathogens in reference in comparison to the study done by **Priyadarshani et al.,** in **2015,** which stated the MIC values of ethyl acetate and methanol extract of *P. longum* were 4mg/ml.

Phytochemical analysis

On the basis of results obtained it is clear that all the fruit extracts of *P.longum* contain significant botanicals as saponins, Flavonoids, alkaloids, carbohydrates essential for strong medicinal value. Phytoconstituents have four types of bactericidal effects: 1. they inhibit cell wall synthesis2. They stop microbial protein and nucleic acid synthesis 3. They disrupt microbial membrane structure and function 4. They block metabolic pathways through inhibition of key enzymes **(Latha*et al.,* 2006 and Zulfiler*et al.,* 2011)**. The various extracts of *P. longum* have exhibited strong inhibitory activity against selected microbes.

Table 1: Antibacterial activity of Gentamicin

S. No.	Bacterial Cultures	Concentration of Gentamicin	
		0.5mg/100µl (ZOI in mm)	1mg/100µl (ZOI in mm)
1	B1	31mm	35mm
2	B2	28mm	32mm
3	B3	27mm	31mm
4	B4	25mm	29mm
5	B5	22mm	27mm

Table 2: Antibacterial activity of *P. longum* E4 extract

S. No.	Bacterial cultures	Concentration of E4 extract	
		0.5mg/100µl (ZOI in mm)	1mg/100µl (ZOI in mm)
1	B1	09mm	10mm
2	B2	08mm	11mm
3	B3	13mm	16mm
4	B4	13mm	15mm
5	B5	09mm	12mm

Table 3: Antibacterial activity of *P. longum* E5 extract

S. No.	Bacterial cultures	Concentration of E5 extract	
		0.5mg/100µl (ZOI in mm)	1mg/100µl (ZOI in mm)
1	B1	05mm	09mm
2	B2	No Zone	No Zone
3	B3	No Zone	No Zone
4	B4	09mm	12mm
5	B5	07mm	10mm

Table 4: Antibacterial activity of *P. longum* E6 extract

S. No.	Bacterial cultures	Concentration of E6 extract	
		0.5mg/100µl (ZOI in mm)	1mg/100µl (ZOI in mm)
1	B1	09mm	13mm
2	B2	12mm	15mm
3	B3	08mm	11mm
4	B4	08mm	13mm
5	B5	06mm	09mm

Table 5: The MIC, MBC and MIC Index values of E4 extract against different pathogens

Organisms	MIC (control) (mg/ml)	MBC (control) (mg/ml)	MIC (extract) (mg/ml)	MBC (extract) (mg/ml)	MIC Index (control)	MIC Index (extract)
B1	0.0156	0.0312	0.25	0.5	2	2
B2	0.0156	0.0312	0.25	0.5	2	2
B3	0.0156	0.0312	0.25	0.5	2	2
B4	0.0156	0.0312	0.25	0.5	2	2
B5	0.0156	0.0312	0.25	0.5	2	2

Table 6: The MIC, MBC and MIC Index values of E5 extract against different pathogens

Organisms	MIC (control) (mg/ml)	MBC (control) (mg/ml)	MIC (extract) (mg/ml)	MBC (extract) (mg/ml)	MIC Index (control)	MIC Index (extract)
B1	0.0156	0.0312	0.25	0.5	2	2
B2	0.0156	0.0312	0.25	0.5	2	2
B3	0.0156	0.0312	0.25	0.5	2	2
B4	0.0156	0.0312	0.25	0.5	2	2
B5	0.0156	0.0312	0.25	0.5	2	2

Table 7: The MIC, MBC and MIC Index values of E6 extract against different pathogens

Organisms	MIC (control) (mg/ml)	MBC (control) (mg/ml)	MIC (extract) (mg/ml)	MBC (extract) (mg/ml)	MIC Index (control)	MIC Index (extract)
B1	0.0156	0.0312	0.25	0.5	2	2
B2	0.0156	0.0312	0.25	0.5	2	2
B3	0.0156	0.0312	0.25	0.5	2	2
B4	0.0156	0.0312	0.125	0.5	2	2
B5	0.0156	0.0312	0.25	0.5	2	2

Table 8: Represents the presence of different phytochemicals in various extracts of *P. longum*

S. No.	Phytochemicals	*Piper longum*		
		E4 extract	E5 extract	E6 extract
1	Glycosides	Present	Present	Present
2	Carbohydrates	Present	Present	Present
3	Flavonoids	Present	Present	Present
4	Saponins	Present	Present	Present
5	Tannins	Present	Present	Present
6	Alkaloids	Present	Present	Present

CONCLUSION

Medicinal Plants serves as reservoir of numerous bioactive compounds which have strong have inhibitory effects on pathogenic microbes and can be used as an alternative means of therapy for the treatment of bacterial diseases. Plant based drugs exhibits wide safety profile with negligible side effects when compared with antibiotics. Variety of food borne diseases associated with Gram positive and Gram negative pathogens can be easily treated with the medicinal plants as they have high antibacterial and antioxidant value because of significant bioactive compounds.

ACKNOWLEDGEMENT

Authors of the research paper are highly thankful to Authorities and R&D division of SGRRU for allowing us to carry experimentation work.

REFERENCES

1. Singh R., Medicinal Plants: A review. Journal of Plant Sciences. Special Issue: Medicinal Plants. Vol. 3, No.1-1, 2015.
2. Davidson-Hunt I, Ecological ethno botany: stumbling toward new practices and paradigms. MASA J 2000.
3. UNESCO. Culture and Health, Orientation Texts- World Decade for Cultural Development 1988-1997, Document CLT/DEC/PRO-1996, Paris, France, 1996.
4. UNESCO.FIT/504-RAF-48 Terminal Report: Promotion of Ethno botany and the Sustainable Use of Plant Resources in Africa, Paris, 1998.
5. Draughon, F.A., Food Technology, 2004, 58, 20-28.
6. Adwan G, Abu-Shanab B and Adwan K 2010.Antibacterial activities of some plant extracts alone and in combination with different antimicrobials against multidrug-resistant Pseudomonas aeruginosastrains. Asian Pac J Trop Med 3(4):266-269

7. Saranraj, P., S Sivasakthi, 2014.Global Journal of Pharmacology, 5, 81-86.
8. Costa, E., Ines, A., Mendes-Fala A, Saavedra MJ, Mendes- Ferreira A., 2010.Potential virulence factors of Candida spp. Isolated from clinical and food sources. J Hosp Infect; 75(3): 240-241.
9. Lund, B.M., O'Brien. S.J., 2011. The occurrence and prevention of foodborne diseases in vulnerable people. Foodborne Pathog Dis; 8(9): 961-973.
10. SeshaIyengar, T.R (1989). "Dravidian India"
11. Rawlinson, H.G (2001). "Intercourse between India and the Western World: From the Earliest Times of the fall of Rome".
12. Barnett, Lionel D (1999). "Antiquities of India: An Account of the History and Culture of Ancient Hindustan".
13. Dasgupta, A. and Dutta, P.C. (1980). Medicinal species of piper. Pharmacognostic delimitation. Q J Crude DrugRes. **18**(1): 17-20.
14. Dutta, C.P., Benerjee, N. and Roy, D.N. (1975). Studies of genus Piper longum in the seeds of Piper longum, Phytoche.**14**: 2090-92.
15. Beel, K.L., Atal, C.K., Acharya, K.T. (1971). Study of Indian seed oils-VIII. Component fatty acids of some seed fats of piperaceae. Lloydia. **34**: 256-260.
16. Akthar, Mohd. Sayeed, etal.AsianPac J Trop Dis 2014; Antimicrobial activity of Piper nigrumL. and Cassia didymobotyraL. leaf extract on selected food borne pathogens. 4 (Suppl 2):S911-S919.
17. S.S Sawhneyet. et.al, 2011. Evaluation of bactericidal and anticancer properties of fruits of P. longum International Journal of Pharmacy and Pharmaceutical Sciences.
18. Chauhan, Neha, Singh, Dolly, Paniuli, R.M, 2012, Screening of bioprotective properties and phytochemical analysis of various extracts of Eclipta alba whole plant, International Journal of Pharmacy and Pharmaceutical science, Vol 4, Issue 2, 553-560
19. Florey, HW. Chain E and Florey ME, 1989. The Antibiotic. Vol 1. New York: Oxford University Press.
20. Chauhan, Renu, Chaudhary, Ekta, Chauhan, Neha, 2017, Investigation of inhibitory activity and bioactive compounds of Piper nigrum seeds on selected human pathogens, International Journal of Current Microbiology, Vol 6 Number 7 (2670-2679).
21. Drummond, AJ, and RD Waigh. 2000 (The development of microbial methods for phytochemical screening. Recent Res.Devel. Phytochem; 4:143-152)
22. Latha, M, KM, Ramkumar, Pari L, PN, Damodaran, V, Rajeshkannan and T, Suresh, 2006 Fall; 9(3): 391-4, Phytochemical and antimicrobial study of an antidiabetic plant: Scoparia dulcis L.