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ANTIBACTERIAL ACTIVITIES OF *PARKIA JAVANICA* EXTRACT AGAINST MULTIDRUG RESISTANT GRAM-NEGATIVE BACTERIA PREDOMINANTLY FOUND IN SKIN WOUND

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ABSTRACT

Aim: *To evaluate the antibacterial activity of Parkia javanica against gram negative MDR bacterial strains which are predominantly found in skin wound.* ***Methods:*** *The crude methanol extract of Parkia javanica (Crude MEPJ) was screened for antibacterial activity against gram negative multi drug resistant bacterial strains namely Enterobacter aerugenes, Pseudomonas aeruginosa and Klebsiella pneumonia by serial dilution technique. Growth kinetics study was performed, and percentage of ROS production was measured by NBT reduction assay. Reporter gene assay was performed to understand the mode of action and finally phytochemical analysis was done according to standard protocol.* ***Results:*** *The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were obtained with a range of IC_{100} 5-20 mg/ml in case of MDR bacterial strains. The lag phase of all extract treated bacteria is extended compared to untreated cells. The normalized % of ROS is increased in presence of crude extract. Crude MEPJ is responsible for breakdown of the plasmid DNA.* ***Conclusions:*** *This study suggests that, the crude methanol extract of Parkia javanica possesses promising antimicrobial substances which are having activity against MDR bacterial strains and direct or ROS induced indirect DNA damage could be the possible mediator of its antimicrobial activity.*

KEY WORDS

Parkia javanica, anti-bacterial activity, MDR bacterial strains, growth curve, ROS.

INTRODUCTION

Bacterial infections are generally caused by pathogenic bacteria which affect millions of people worldwide [1]. The acquired antibiotic resistance to the recent drugs, may be due to the over expression of MDR efflux pump [2]. In multidrug resistant gram-negative bacteria, the effect of efflux pumps in combination with reduced drug uptake, due to presence of double membrane barrier, is responsible for the antibiotic resistance [3]. Thus, in light of the evidence of rapid global spread of resistant bacteria, there is need to identify new substances with effective antimicrobial activity. However, the past record of rapid, widespread emergence of resistance to newly introduced antimicrobials indicates that even new families of antimicrobial agents will have a short life expectancy [4]. In case of diabetic patient, the skin infection, especially skin of the foot, is frequently infected by different pathogenic microbes, causing chronic skin to wound [5]. For this reason, researchers are increasingly turning their attention to herbal products, looking for new leads to develop better drugs, against these pathogenic microbes [6]. Plants are

valuable source of antimicrobial agents and the plant based antimicrobial compounds have enormous therapeutic potential as they can serve the purpose without any side effects that are often associated with synthetic antimicrobials [7]. Thus, discovery of new antimicrobial agent or formulation could be a great advantage to this emergence of MDR strains causing treatment failure. *Parkia javanica*, of leguminece family, has large ethnomedicinal history. This plant is widely used by tribal population of north-east region of India [8,9,10]. Therefore, this work has been undertaken to explore the antibacterial properties of this plant against gram negative multi drug resistant bacterial strains with possible mode of action.

MATERIALS AND METHODS

Plant collection & Authentication

Fresh stem barks of *P. javanica* were collected from Suryamaninagar, Tripura, India. The plant was initially identified by Dr. B. K. Dutta, Taxonomist, Department of Botany, Tripura University and finally authenticated by Dr. H. J. Chowdhery, Joint Director, Central National Herbarium, Botanical Survey of India, Shibpur, Howrah, West Bengal and respective voucher specimen No. ≠BD-01/06 has been deposited in the Herbarium.

Preparation Plant Extract

Fresh stem barks of *Parkia javanica* was collected from Suryamaninagar, Tripura, India. After washing with water these barks were allowed to dry in shade. Then barks were cut into small pieces. Then 500 gm of powdered bark was soaked in 2000 ml of methanol to prepare the crude extract and then kept in a shaker for 48 hours. After that the solutions were filtered through Whatman filter paper no. 1 for 3 times. Then these solutions were dried in rotary evaporator at 70°C. Finally, these solutions were freeze- dried and stored at - 20°C [11].

Bacterial Culture and Growth Conditions

All the multidrug resistant (MDR) strains *Enterobactor aerugenes* (ATCC 13048), *Pseudomonas aeruginosa* (ATCC 10145) and *Klebsiella aerugenes* (ATCC BAA-1705) were grown, cultured and maintained on Muller Hinton Broth. For long time storage 15% glycerol solution was used and vial was stored at -80° C [12].

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC was determined by serial dilution technique, with an inoculum of 10^6 CFU/ml of gram negative MDR bacterial strains in separate 96 well plate, in presence of increasing concentrations of crude MEPJ. The bacterial cultures were incubated at 37° C and shaken at 200 rpm for 24 hours. Then the bacterial cell viability was determined by measuring the OD value at 600 nm. Here, extract with media, used as blank; extract, media and bacterial culture, used as experiment; media with bacterial culture and 25% DMSO, used as positive control; and media with only 25% DMSO, used as negative control. Then, % of Inhibition was calculated by following formula,

% of Inhibition = [1- {(Exp. - Blank) / (Positive Control – Negative Control)} * 100]

Then MBC for each bacterial species were determined by treating the bacterial strains with 3 different doses, IC_{50}, IC_{100} and $>IC_{100}$ dose. After incubation with these 3 doses, one loop full bacterial culture from each tube was streaked on Muller Hinton agar plate in respective zone and again these plates were incubated at 37° C for overnight. IC_{100} value indicates the concentration which inhibits 100% of bacterial growth, whereas, MBC value indicates the concentration at which a drug can kill the bacterial species [13].

Measurement of Bacterial Growth Kinetics

To determine the bacterial growth kinetics, in presence of crude MEPJ, each bacterial species were grown in Muller Hinton Broth in presence and absence of extracts separately, at 37° C at 200 rpm for 12 hours. Here, bacterial cells were treated with respective IC_{50} dose. Then, the bacterial concentration in presence and absence of extract were determined by measuring the OD at 600 nm in every 1-hour interval. Bacterial growth kinetics was plotted graphically with time versus OD_{600} [12].

Estimation of Reactive Oxygen Species (ROS)

0.1ml of each bacterial suspension (where OD_{600} = 1.0) in Hank's balanced salt solution (HBSS) was incubated with respective IC_{50} dose of crude MEPJ for 2 hours with 15 min interval at 37° C. Then 500 µl of 1 mg/ml NBT was added and again incubated for 30 min at 37° C. After incubation, 0.1 (M) HCl was added and tubes were centrifuged at 3000 rpm for 10 min. The pellets were

treated with 0.6 µl of DMSO to extract the reduced NBT. Then, 0.5 µl of HBSS was added and OD was measured at 575 nm (intracellular ROS) [14]

DNA damage Assay

To examine the effect of crude MEPJ on DNA inside bacterial cell, reporter (*β*-galactoside) gene expression assay was performed. In this assay, pUC19 transformed DH5α cells were incubated for 3 hours at 37° C in presence or absence of IC_{50} dose of crude MEPJ. Then these bacterial cells were inoculated on Muller Hinton agar plate (*amp⁺)* containing X-gal and IPTG in medium and incubated for 12 hours at 37° C to observe the blue colour forming colonies [14].

Phytochemical Analysis

The phytochemical screening of crude MEPJ was assessed by standard method [15,16,17]. Phytochemical screening was performed to identify the important natural chemical groups such as alkaloids, flavonoids, phenolic compounds, steroids, terpinoids etc. Biochemical reactions in this screening revealed the presence and absence of these compounds in the study plant [18].

Statistical Analysis

We repeated these experiments for 3 times and data were expressed by calculating the standard deviation of all 3 experiments. ANOVA single factor (using Microsoft Office Excel) was used to determine statistical significance for multiple comparisons. $P<0.05$ was accepted as statistically significant.

RESULTS

Determination of MIC:

Antibacterial activity of crude MEPJ on multidrug resistant (MDR) bacterial strain, was obtained by determining the minimum inhibitory concentrations by serial dilution technique. As shown in Table 1 and Fig. 1, the growth of *E. aerugenes* is completely inhibited at lower concentration of crude MEPJ (5 mg/ml) followed by *P. aeruginosa* (10 mg/ml) *and K. pneumonia* (20 mg/ml). The order of observed sensitivity to crude MEPJ, of 3 MDR strains were, *E.aerugenes>P. aeruginosa > K. pneumoniae.*

Minimum bactericidal concentration of crude MEPJ on each bacterial strain was also determined. According to Table 1, the ratio between MBC and MIC for each bacterium is same (~1, for all bacteria). This result indicated that, crude MEPJ and different fractions of *P. javanica* are a bactericidal agent rather than bacteriostatic agent.

Measurement of Bacterial Growth Kinetics:

As shown in Table 1, crude MEPJ can kill the MDR bacterial species, so, we next measured the growth curve of three MDR strains to examine the pattern of the growth with time in presence and absence of crude MEPJ. All the bacterial strains were exposed to crude MEPJ separately, at a concentration of IC_{50} dose for each bacterium. As shown in Fig 2, the lag phase of all crude MEPJ treated bacteria was extended compared to control and the pattern of extension is slightly higher in case of *E. aerugenes*. Among three MDR strains, growth of *E. aerugenes* is mostly affected by the *P. javanica* extract

Estimation of ROS:

Finally, to understand the mechanism of antibacterial activity of *P. javanica*, intracellular reactive oxygen species (ROS) were estimated after treatment with crude MEPJ at IC_{50} dose. As shown in Fig 3, after treatment of crude MEPJ, the production of ROS was increased drastically with time. It was highest in *E. aerugenes*, in which ROS production increased about 65% in 3 hours compared to control. The order of observed ROS production on 3 MDR bacterial strains were, *E. aerugenes > P. aeruginosa > K. pneumonia.*

DNA Damage assay:

As shown in Fig 3, ROS production was increased drastically after treatment with crude MEPJ compared to control. ROS usually targets the cellular DNA, so, to observe the effect of ROS inside bacterial cells, we used plasmid-based reporter gene assay. In Fig 4, reporter gene *β*-galactosidase was assayed by transforming the bacteria with the *pUC*19 plasmid and then the bacterial cells were treated with crude MEPJ. The blue colonies, formed due to hydrolysis of X-gal by *β*-galactosidase enzyme, were completely absent in case of bacterial cells treated with crude MEPJ.

Phytochemical Screening

The phytochemical screening was performed by testing the extract in presence of certain chemicals according to the protocols [15,16,17]. The extract showed the presence of major classes of secondary metabolites such as alkaloids, flavonoids, phenolic compounds, steroids and terpinoids etc (Table 2).

Table 1: MIC and MBC values of Crude MEPJ on MDR Strains

	IC_{100}*	MBC*	MBC/MIC
K. pneumoniae	20 ± 0.03	20 ± 0.01	1
P. aeruginosa	10 ± 0.04	10 ± 0.02	1
E. aerugens	5 ± 0.07	5 ± 0.06	1

***Concentration of extracts in mg/ml. MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; Experiments were performed in triplicate and all the MIC and MBC values are significant at the level of p< 0.05.**

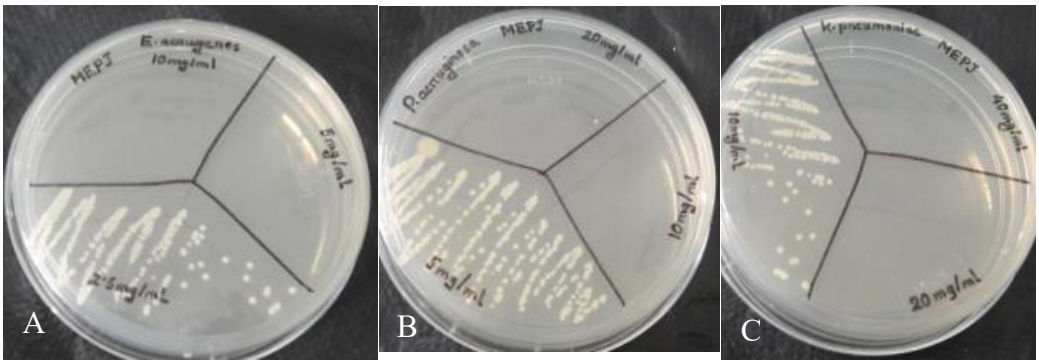

Fig. 1: Muller Hinton agar plate showing the minimum bactericidal concentration (MBC) of MEPJ on MDR strains of gram negative bacterial species; (A) *E. aerugens*; (B) *P. aeruginosa*; (C) *K. pneumoniae*.

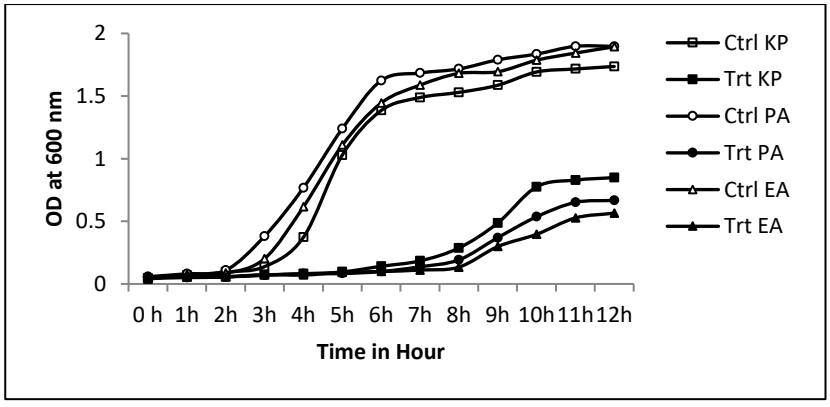

Fig. 2: Effect of crude MEPJ at respective IC_{50} dose on pattern of growth curve of MDR gram negative bacterial strains; Ctrl: Control; Trt: treated with respective IC_{50} dose of MEPJ of each bacterium; KP: *Klebsiella pneumoniae*; PA: *Pseudomonas aeruginosa*; EA: *Enterobacter aerugenes*.

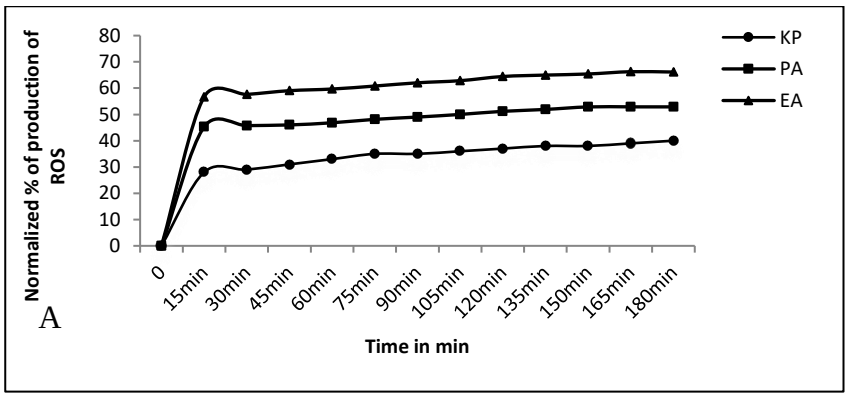

Fig. 3: Effect of crude MEPJ at respective IC_{50} dose of each bacterium, on normalized % of ROS production in standard gram negative MDR bacterial strains; KP: *Klebsiella pneumoniae*; PA: *Pseudomonas aeruginosa*; EA: *Enterobacter aerugenes*.

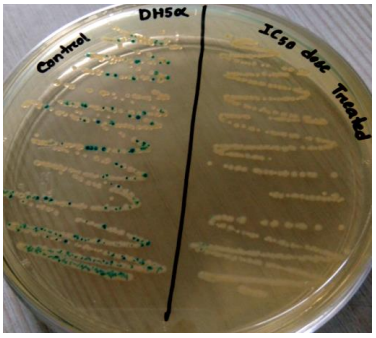

Fig .4: Reporter gene (*β*-galactosidase) assay on crude MEPJ of *P. javanica* treated pUC19 Transformed *E. coli DH5α*.

Table 2: Chemical composition of crude MEPJ

Chemical Constituent	Crude MEPJ
Alkaloids	+
Flavonoids	+++
Phenolic compounds	+++
Steroids	++
Terpinoids	++

+++: abundant; ++: average; +: trace

DISCUSSION

The emergence and widespread occurrence of drug resistant bacteria presents a serious global medical crisis, requiring constant surveillance, which continuously challenges the scientific community [19]. The diminishing efficacy and increasing toxicity of synthetic drugs further aggravate this problem. Thus, researchers are directed to seek more natural or organic materials to solve this health problem [13]. Traditional medicine has been used worldwide for centuries, especially the herbal plants for therapeutic purposes against bacterial strains [20]. In this study, *Parkia javanica*, a large plant used as traditional folk medicine in north-east region of India, has been screened in vitro for antibacterial activity against three multidrug resistant bacterial species known to aggravate the skin wound of diabetic patient.

The crude methanol extract showed antimicrobial activity against all the tested gram negative MDR strains (*E. aerugenes, P. aeruginosa, K. pneumonia*) with a range of MIC (IC_{100}) values. The two-fold serial dilution technique was used to determine the MIC values and it was observed that, the ratio between IC_{100} dose and MBC of *P. javanica*, for each MDR strains is 1. A sample or any agent is bactericidal when the ratio MBC/MIC ≤ 4 and bacteriostatic when this ratio is > 4 [21]. Therefore, this study plant has bactericidal effect on gram negative MDR strains. From growth kinetics study, it is found that, the lag phase of all extract treated bacteria is extended compared to untreated cells.

The same condition also observed in ROS production. The normalized % of ROS is increased about 70% (in MDR strain), in presence of crude MEPJ. The normalized % of ROS is increased in presence of crude extract. Reactive by products of oxygen, such as superoxide anion radical (O2), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radicals (·OH), are generated continuously in cells grown aerobically because these aerobic bacteria use molecular oxygen of nutrients to obtain energy [22]. These species cause damage to proteins, lipids, and nucleotides, negatively impacting the organism [23]. Living organism's own mechanisms to protect themselves against oxidative stress, with enzymes such as catalase and superoxide dismutase, small proteins like thioredoxin and Glutaredoxin, and molecules such as glutathione [24]. However, the damage ensues when the concentration of active oxygen increases to a level that exceeds the cell's defence capacity [25]. Preliminary screening tests of this plant for phytochemicals revealed that, alkaloids, triterpinoids, flavonoids, steroids, phenolic compounds are present in it. It is reported that, all these compounds possess anti-infective or bactericidal activity [26, 27]. Among them phenolic compounds, more specifically flavonoids, exerts their antibacterial activity through inhibition of DNA [28]. The trace amount of alkaloid can increase the ROS production, which is too much harmful

for organisms [29]. Therefore, direct or ROS induced indirect DNA damage, due to the presence of different secondary metabolites, could be the reason for the death of the microorganism found in this study in response to the *Parkia javanica* extract. Other macromolecular damages due to increased ROS generation may also contribute to bactericidal activity which needs further studies.

CONCLUSION

In this study, we reported the antibacterial activity of crude methanol extract of *P. javanica* with their mode of action. The study showed that, crude MEPJ was effective at lower concentrations on MDR strains. The increased ROS production may be the possible mechanism of antibacterial activity of of *Parkia javanica*.

COFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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REFERENCES

1. Solanki R., Some Medicinal plants with antibacterial activity, Pharmacie Globale (IJCP), 4(10); 1-4, (2010).
2. Hancock E. W., Mechanisms of action of newer antibiotics for gram positive pathogens. Lancet Infect Dis, 5; 209-218, (2005).
3. Lomovskaya O., Bostian K. A., Practical applications and feasibility of efflux pump inhibitors in the clinic-a vision for applied use. Biochem pharmacol, 71; 910-918, (2006).
4. Coates A., Hu Y., Bax R., Page C., The future challenges facing the development of new antimicrobial drugs. Nat. Rev. Drug Discov, 1; 895-910, (2010).
5. Bader M. S, Diabetic foot infection. Am Fam Physician, 78(1); 71-79, (2008).
6. Bagra L. C., Leite A. A. M., Xevier K. G. S., Takahashi J. A., Bemquerer M. P., Chartone-Souza E., Nascimento A. M. A., Synergic interaction between pomegranate extracts and antibiotics against Staphylococcus aureus. Can. J. Microbiol., 51; 541-547, (2005).
7. Sukanya S. L., Sudisha J., Hariprasad P., Niranjana S. R., Prakash S. H., Fathima S. K., Antimicrobial activity of leaf extracts of Indian medicinal plants against clinical and phytopathogenic bacteria, 8(23); 6677-6682 (2009).
8. Sinha J., Medicinal plants of Manipur**,** Mass and Sinha Manipur Cultural Integration Conference, Imphal, (1996).
9. Majumder K., Dutta B. K., Roy D., Inventory and status of medicinal trees of Tripura, Indian Medicinal Plants. Editor P. C. Trivedi. Avishkar publishers, Distributors, Jaipur, (2009), pp 93- 123.
10. Bhardwaj S., Gakhar SK, Ethnomedicinal plants used by the tribals of Mizoram to cure cuts & wounds. Indian J. Traditional knowledge, 4(1); 75- 80, (2005).
11. Nikolic M., Vasic S., Durđevic J., Stefanovic O., Comic L., Antibacterial and anti-biofilm activity of ginger (*Zingiber officinale* (roscoe)) ethanolic extract. Kragujevac J. Sci, 36 (2014); 129-136 (2014).
12. Bhattacharya D., Samanta S., Mukherjee A., Santra C. R., Ghosh A. N., Niyogi S. K., Karmakar P., Antibacterial Activities of Polyethylene Glycol, Tween 80 and Sodium Dodecyl Sulphate Coated Silver Nanoparticles in Normal and Multi-Drug Resistant Bacteria. J Nanosci and Nanotech, 12; 1–9 (2012).
13. Demetrio L. V. J., Jeannie I. A., Juliana J. M. P., Esperanza C. C., Antibacterial activities of ethanol extracts of Philippine medicinal plants against multidrug-resistant bacteria. Asian Pac J Trop Biomed, 5(7); 532-540 (2015).
14. Pramanik A., Laha D., Bhattacharya D., Pramanik P., Karmakar P., A novel study of antibacterial activity of copper iodide nanoparticle mediated by DNA and membrane damage. Colloids and Surfaces B: Biointerfaces, 96; 50– 55 (2012).
15. Brinda P., Sasikala P., Purushothaman K. K., Pharmacognostic studies of Merugan Kizhangu. Bull Med Eth Bot Res, 3; 84-96 (1981).
16. Siddiqui A. A., Ali M. Practical Pharmaceutical chemistry. 1st ed. New Delhi: CBS Publisher and Distributors; p. 126-131 (1997).
17. Savithramma N., Linga R. M., Bhumi G., Phytochemical screening of *Thespesia populnea* (L.) *Soland* and *Tridax procumbens* L. J chem. Pharm Res, 3(5); 28-34 (2011).
18. Anbukkarasi M., Dhamotharan R., Janarthanam B., Studies on phytochemical screening, tannins content and antibacterial activity from leaf and callus extracts of *Memecylon umbellatum*. Asian J Pharm Clin Res, 10(5); 265-269 (2017).
19. Lai C. C., Lee K., Xiao Y. H., Ahmad N., Veeraraghavan B., Thamlikitkul V., High burden of antimicrobial drug resistance. Asia J Glob Antimicrob Resist, 2(3); 141-147 (2014).
20. Quisumbing E., Medicinal plants of Philippines. Quezon: Katha Publishing Co., Inc (1978).
21. Cabiscol E., Tamarit J., Ros J., Oxidative stress in bacteria and protein damage by reactive oxygen species. Int. Microbiol, 3; 3-8 (2000).

22. De Orue Lucana D. O., Wedderhoff I., Groves M. R., ROS mediated signalling in bacteria: zinc-containing cys-x-x-cys redox centres and iron based oxidative stress. J Sig Trans, (2012). doi: 10.1155/2012/605905.
23. Storz G., imlay J. A., Oxidative stress. Curr opin Microbial, 2; 188-194, (1999).
24. James A., Imlay J. A., Cellular defences against superoxide and hydrogen peroxide. Annu Rev Biochem, 77; 755-776, (2008).
25. Kashmiri Z. N., Mankar S. A., Free radical and oxidative stress in bacteria. Int J Curr Microbiol App Sci., 3(9); 34-40, (2014).
26. Stafy F., The Natural Guide to Medicinal herbs, plants. London: Tiger Bookds International; P. 12-16, (1998).
27. Britto J. D., Sebastian S. R., Biosynthesis of silver nano particles and it's antibacterial activity against human pathogens. Int J Pharm Sci., 5; 257-259, (2011).
28. Cushnie T. P., Lamb A. J., Antimicribial activity of flavonoids. Int J Antimicrob Agents. 26(50); 343-356, (2005).
29. Marshall K. M., Andjelic C. D., Tasdemir D., Concepcion G. P., Ireland C. M., Barrows L. R., Deoxyamphimidone, a pyridoacridine alkaloid, damages DNA via the production of reactive oxygen species. Mar. Drugs, 7; 196-209, (2009).

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