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Research Article

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Determination of Minimum Inhibitory Concentration of organic extract of *Catharanthus roseus* by a novel modified well diffusion technique

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ABSTRACT

India has a tremendous biodiversity. It has optimum conditions for a number of medicinal plants that thrive naturally here. Catharanthus roseus also known as Sadabahar or Sadhaphuli by vernacular names is one among many medicinal plants commonly occurring here. C. roseus has always remained in limelight for its various anticancerous properties imparted by various alkaloids. In this research paper, a new microbial susceptibility test method is described, which retains the simplicity of Epsilometer test for determination of MIC. In this method, organisms can be tested directly for susceptibility to known concentration of plant extracts and antibiotics in agar well. Ranges of concentrations are used to determine the minimum inhibitory concentration. Using dried leaves of C. roseus, organic extracts were prepared in acetone and ethyl acetate solvent using soxhlet apparatus. MIC values of extracts were determined by E-test method against pathogens like Escherichia coli, Klebsiella pneumoniae and Proteus vulgaris. MIC values evaluated were 25µg/ml and 12.5µg/ml for E.coli, 12.5µg/ml for P. vulgaris and 6.25µg/ml and 50µg/ml for K. pneumoniae in acetone and ethyl acetate extracts respectively. The MIC values obtained by E Test are lower than those reported in literature using broth dilution method. Modified well diffusion method is efficient, less time consuming and simpler. C. roseus has a great potential as an antimicrobial agent, which needs to be explored further intensively.

Keywords: *Catharanthus roseus, Modified well diffusion, MIC, Acetone, Ethyl acetate*

INTRODUCTION

India is endowed with rich wealth of about 47000 species of plants and ranks 8th in the world biodiversity. Out of these, 8000 species are medicinal which have been a valuable sources of natural products for maintaining human health. A large number of these medicinal plants are used in several formulations for the treatment of various diseases caused by microbes. According to World Health Organization, medicinal plants would be the source of obtaining a variety of drugs. Various societies across the world have shown great interest in curing diseases using plants/plant based drugs.

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Microbes are closely associated with the health and welfare of human beings. Some are beneficial and some are detrimental. As preventive and curative measures, plants and their products are used in the treatment of infections for many centuries ago. Herbal medicine – It is also called botanical medicine or phytomedicine - refers to using plants seeds, flowers, roots for medicinal purposes. It is becoming more main stream as improvements in analysis and quality control along with advances in clinical research show the value of herbal medicine in treating and preventing diseases[6]. Resistance to antimicrobial agents such as antibiotics is emerging worldwide for variety of organisms. Multiple drug resistant organisms thus pose serious threats for treating infectious diseases. Hence, plant derived antimicrobial agents have received considerable attention in recent years. There has been an upsurge in demand for pharmaceutical, raw medicinal plant materials, herbs and aromatic plants of the subcontinent origin in western countries[14]. There, the current scenario is to isolate the active constituents present in the plant material to develop medicinal drugs, which are having rare chances of adverse effects[8,11]. The increasing global interest in the medicinal potential of plants during the last few decades is therefore quite logical[10]. The need for the novel pharmaceutical products out from the plant has attained a great interest in the present research world due to the cost and the higher side effects that are associated with the chemically manufactured drugs[2]. Phytochemicals play a critical role in diversity oriented synthesis (DOS) of natural product like pharma compounds[7]. The research for new therapeutic treatments for various disease conditions is expanding. In many poor countries, plants have been looked at as a very promising source of new lead compounds for drug discovery and development.

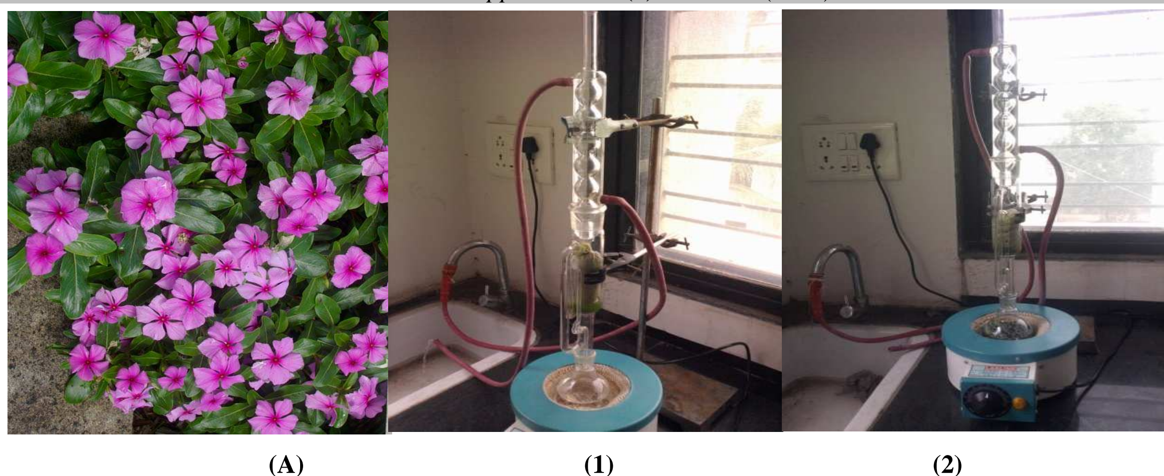
Catharanthus roseus is also known as *Vinca rosea*. It belongs to family Apocynaceae. It is a perennial herbaceous plant growing 1m tall. The flowers are white to dark pink with a darker red centre. It is found in subtropical and tropical areas of the World. The species has long been cultivated as an ornamental plant. It is noted for its long flowering period, throughout the year in tropical conditions, and from spring to late autumn, in warm temperate climates. In traditional Chinese medicine, extracts from it have been used against numerous diseases,including diabetes, malariaand Hodgkin 's lymphoma. It is also used as an astringent, diuretic and expectorant[9]. The alkaloids like actineo plastidemeric, Vinblastin, Vincrestine, Vindesine, Vindeline Tabersonine etc. are mainly present in aerial parts whereas Ajmalicine, Vinceine, Vineamine, Raubasin, Reserpine, Catharanthine etc. are present in roots and basal stem. These are used as indispensable cancer drug and antihypertensives. The tropical plant produces more than 100 monoterpenoids indole alkaloids in different organs[12].

In this present study *C. roseus* dried leaves extract was made in acetone and ethyl acetate solvents. These extracts were used to examine MIC by modified agar well diffusion method against pathogens like *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus vulgaris*.

MATERIALS AND METHODS

Collection of plant leaves and extract preparation

Fresh leaves of *C. roseus* (Fig: 1) from different localities of Nashik district were collected. The leaves were initially washed with tap water to remove any dust particles and later with sterile water. The leaves were then dried in shade for a week. The dried leaves were used for extract preparation using acetone and ethyl acetate extract by Soxhlet apparatus. About 50gm of dried powder of *Catharanthus* leaves was taken into a cotton thimble and extracted with 250ml of solvents like Ethyl acetate and Acetone using Soxhlet apparatus for 6-8 hrs (Fig:1). The extracts were evaporated using rotatory vacuum evaporator. The extract was dissolved in respective quantity of DMSO[12] to make a final concentration of 300ug/ml[3].



(A) **(1)** **(2)**

Fig1: (A) *Catharanthus roseus* plant; (1) Soxhlet extraction of dried leaves of *Catharanthus roseus* in Aetone and (2) Ethyl acetate extract.

MIC

Modified Agar well diffusion technique was used for determination of MIC. The direct colony suspension method recommended by CLSI was used for inoculum preparation. Inoculum of respective pathogen was prepared by making a direct broth suspension of pure culture from an 18hour agar plate. Suspension was adjusted to achieve a turbidity equivalent to a 0.5 McFarland turbidity standard. This resulted in a suspension containing 2 x 10^8 CFU/mL. To perform this step accurately, a photometric device was used to compare the inoculum tube and the 0.5 McFarland standard. Different concentrations of plant extract were prepared using the protocol given below in the table 1. On sterile Mueller Hinton agar plates organisms were spread using sterile cotton swabs and the plates were kept inverted for 5 mins in order for microorganisms toadhere and moisture removal. Then using a sterile borer, 6mm wells were digged and respective concentrations of extracts were added in respective wells using a micropipette. DMSO was used as a negative control. The whole procedure was performed in duplicate and under sterile conditions in Laminar Air Flow. The plates were kept for prediffusion at 4^0C in refrigerator for 20 minutes. The plates were then incubated at 37^0C for 24 hrs in an incubator, the inhibition zones were measured and recorded using digital vernier caliper[15].

Table 1: Protocol for preparation of different concentrations of Plant extracts

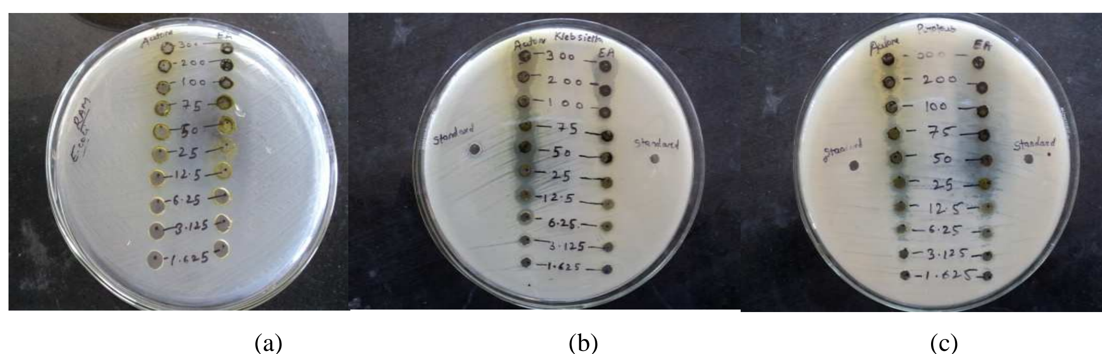
No. of tube	Concentration of plant extract (µg/ml)	Stock of plant extract (ml)	Solvent (DMSO ml)	Total volume (ml)
1.	300	1	0	1
2.	200	0.666	0.334	1
3.	100	0.333	0.667	1
4.	75	0.250	0.750	1
5.	50	0.166	0.834	1
6.	25	0.083	0.917	1
7.	12.5	0.042	0.958	1
8.	6.25	0.021	0.979	1
9.	3.125	0.010	0.990	1
10.	1.625	0.005	0.995	1

RESULTS AND DISCUSSION

The MIC value of acetone and ethyl acetate extracts for *E.coli* were 25µg/ml and 6.25µg/ml using E Test respectively. MIC value of acetone and ethyl acetate extracts for *P. vulgaris* was the same, i.e. 12.5 µg/ml. MIC value of acetone and ethyl acetate extracts for *K. pneumoniae* were 6.25 µg/ml and 50 µg/ml respectively (Table 2 and Fig 2). The MIC values obtained by modified well diffusion method are lower than those reported in literature using broth dilution method. The MIC values reported here are less than the ones foun by Goyal[4] for dry leaves extract of methanol and ethanol extract against *E.coli* and *K. pneumoniae*. These values are significantly less as compared to ones reported by Kamraj[5], for acetone and ethyl acetate leaf extract of *C. roseus* against *E.coli* and *K.pneumoniae*. The MIC value of acetone and ethyl acetate extract against *P. vulgaris* was nearly same as observed in our paper Gaur *et al*, 2016.

Table 2: MIC values of acetone and ethyl acetate leaves extract of *C. roseus* against *E.coli*, *K. pneumoniae* and *p. vulgaris*

Concentration of plant extract (µg/ml)	Inhibition Zone diameter in mm					
	E. coli		*K. pneumoniae*		*Proteus vulgaris*	
	Acetone	Ethyl Acetate	Acetone	Ethyl Acetate	Acetone	Ethyl Acetate
300	15±0.2	27±0.8	19±1.3	15±0.8	31±0.8	30±0.8
200	13±0.6	25±0.6	18±1.3	12±0.8	29±0.5	29±0.5
100	12±0.8	24±0.9	16±0.5	11±0.5	28±0.6	28±0.6
75	11±1.2	22±0.6	16±0.6	10±1.4	23±0.8	27±0.9
50	11±0.5	19±0.6	15±0.8	10±0.2	26±0.4	25±1.4
25	10±0.5	18±0.5	13±0.2	-	25±0.8	14±0.5
12.5	-	15±0.2	11±0.5	-	12±0.8	11±0.5
6.25	-	12±1.2	10±0.5	-	-	-
3.125	-	-	-	-	-	-
1.625	-	-	-	-	-	-
DMSO	7.3±0.2		7.8±0.5		8.8±0.3	



(a) (b) (c)

Fig2: E test for MIC against (a) *E.coli*, (b) *K. pneumoniae* and (c) *P. vulgaris* using acetone and ethyl acetate extract

MIC can be detected using broth dilution method. MIC of antibiotics can also be detected using commercially available E test strip. Using E test strips we get an elliptical inhibition zone on agar plates, whereby easily MIC is read from the concentration impregnated on the strip. However E test strips are not available for plant extracts or any other source whose antimicrobial efficacy is to be detected. Researchers across the World have to still go for the laborious broth dilution technique. In this paper we tried to mimic the E test by modified agar well diffusion method. This technique is based on the same principle on which Epsilometer test (E test) is based for the detection of MIC. Elliptical inhibition zones were observed in the same

manner as a normal E test strip gives. But more clear interpretation and exact elliptical zones can be elucidated if the wells are digged close to each other and suspension is kept optimum. Wells near each other and exactly below each other would produce a clear and elliptical zone if the compound being examined is antimicrobial and MIC can be interpreted easily. The differences and advantages between the actual E test and the modified well diffusion technique described in this paper are as follows: Firstly, in well diffusion technique we have to make wells manually whereas in epsilometer test we do not make wells we use strips. Secondly, in well diffusion technique we have to inoculate different concentration of the antimicrobial compound in the wells in contrast in Epsilometer test we use E test strip having different concentrations already impregnated onto them. Thirdly, there is no E test strip available for different plant extracts. Practically it is impossible to make them commercially available for all the existing flora and who knows whether or not the plant actually has antimicrobial potential or not. Fourthly, it is easy to determine MIC of plant extracts by modified well diffusion technique then by broth dilution because plant extracts are already green to yellow or brown in colour depending upon the plant and its parts used and also turbidity initially in the extract makes it difficult to interpret the results. Lastly it is less time consuming as compared to MIC by broth dilution[13]. So we propose that, researchers should switch over to more easy, convenient, and accurate method of determination of MIC by modified well diffusion technique mentioned here instead of broth dilution, especially for ones who want to determine MIC of their plant extracts due to various drawbacks

CONCLUSIONS

In conclusion this study highlights, extracts of dried leaves of *C. roseus* were prepared successfully in acetone and ethyl acetate solvent using soxhlet apparatus. Using modified agar well diffusion method MIC was determined. The E test was performed successfully and MICs were determined for various extracts against the various pathogens used. MIC obtained was used for the determination and comparison of MIC by broth dilution method in our publication Gaur et al, 2016 and to check how far the E test and MIC by broth dilution correlate (Rolinson *et al,* 1972). The determination of MIC by E test method has been reported for the first time for *C. roseus* acetone and ethyl acetate extracts against *Proteus vulgaris. C. roseus* is exploited for anticancerous properties, but now its antimicrobial properties must also be evaluated against multi drug resistant strains as more and more pathogens are gaining antimicrobial resistance against the conventional antibiotics.

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REFERENCES

1. Bholay, A.D., Ingale, M. and Gaur, A., Therapeutic potential of mangrove and its associate plant extracts from thane creek, against human respiratory tract MDR pathogens, *Journal of Biodiversity and Environmental Sciences*, **7(4):** 118-126 (2015).
2. Gajalakshmi, S., Vijayalakshmi, S. and Devi, R.V., Pharmacological activities of *Catharanthus roseus:* A Perspective review. *Int J Pharm Bio Sci*, 431 – 439 (2013).
3. Gaur, A.P, Bholay, A.D. and Ganeshan, M., Antimicrobial activity of *Catharanthus roseus* against Human Microbial Pathogens. *Journal for Advanced research in applied sciences*, **3:** 120-129 (2016).
4. Goyal, P., Khanna, A., Chauhan, A., Chauhan, G., Kaushik, P., *In vitro* evaluation of crude extracts of *Catharanthus roseus* for potential antibacterial activity. *International Journal of Green Pharmacy*, 176-181 (2008).
5. Kamaraj, C., Rahuman, A.A, Siva, C., Iyappan, M. and Kirthi, A.V., Evaluation of antibacterial activity of selected medicinal plant extracts from south India against human pathogens. *Asian Paicfic Journal of Tropical Disease*, S296-S301 (2012).
6. Kumar, R.A. and Yadav, D., Antibacterial activity of *Nyctanthes arbortristis*, *Nerium oleander* and *Catharathnus roseus*. *IIJRPC,* **3(2):** 509-512 (2013).
7. Kumari, K., Gupta, S., Phytopotential Of *Catharanthus roseus* L. (G.) Don. Var.

"*rosea*" and "*alba*" against various pathogenic microbes In Vitro. *International Journal of Research in Pure and Applied Microbiology,* 77-82 (2013).

8. Mabrouk, M.I., Synergistic and Antibacterial activity of six medicinal plants used In folklore medicine in Egypt against *E.coli* H7. *J. of App. Sci.Rese.,* **157:** 1321-1327 (2012).
9. Patharajan and Abirami S.B., Antioxidant activity and phytochemical analysis of fractionated leaf extracts of *Catharanthus roseus*. *IJP*, 138-143 (2014).
10. Patil, P.J. and Ghosh, S.J., Antimicrobial Activity of Catharanthus roseus – A Detailed Study. *British Journal of Pharmacology and Toxicology*, 40-44 (2010).
11. Rajalakshmi, G., Komathi, S., Poongodi, N., Sasikala, T. and Banuraviganesh, Antimicrobial Activity and Phytochemical Screening of *Catharanthus roseus.IJSR,* 1-2 (2013).
12. Retna, M.A. and Ethalsa, P., A review of the taxonomy, ethnobotany, chemistry and pharmacology of *Catharanthus roseus* (Apocyanaceae). *International Journal of Engineering Research & Technology (IJERT)* 2013, 3899-3912 (2013).
13. Rolinson, G.N. and Russell, E.J., New Method for Antibiotic Susceptibility Testing. *Antimicrobial Agents and Chemotherapy*, 51-56 (1972).
14. Sathiya, S., Karthikeyan, B., Jaleel, A.C., Azooz, M.M. and Iqbal, M., Antibiogram of *Catharanthus roseus* Extracts, *Global Journal of Molecular Sciences*, 01-07 (2008).
15. Bholay, A.D., Rajguru, A., Khandale, P. and Gaur, A., Antimicrobial profiling and synergistic interaction between leaves extracts of *Cryptolepis buchanani* (Roem. and Schult.), *Int. J. Pure App. Biosci.* **4(1):** 240-247 (2016). doi: http://dx.doi.org/10.18782/2320-7051.2219

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