Assessment of enzymatic antioxidant and antimicrobial potential of *Reinwardtia indica* from Garhwal region of Uttarakhand

Saklani Pooja*, Rana Pawan Singh, Chandel Chandresh, Mehta Sonam and Thakur Rahul
Hemvati Nandan Bahuguna Garhwal University, Srinagar Garhwal, Uttarakhand, INDIA
*poojasaklani@rediffmail.com

Abstract

The present study estimated the enzymatic antioxidant and antimicrobial potential of medicinal plant, Reinwardtia indica, collected from the Garhwal region of Uttarakhand. The enzymatic antioxidant activities were estimated for six enzymes Catalase, Ascorbate oxidase, Ascorbate peroxidase, Glutathione S transferase, Polyphenol oxidase and Ascorbate oxidase.The antibacterial potential of different extracts of plant leaves was estimated against ten common pathogenic bacteria. Significant antioxidant activity was reported for all the studied enzymes.

Highest antibacterial activity was observed against Cornybacterium diptheriae, Proteus sp.and E. coli in acetone, chloroform and methanol extracts. Reinwardtia indica showed great potential to be used as a source of antioxidants and as a potent antibacterial agent in the present study. The plant can be promising source for developing drugs.

Keywords: *Reinwardtia indica*, Enzymatic antioxidant, Antibacterial activity.

Introduction

In the recent years, there has been a tremendous research and discovery of novel drugs to be used in pharmaceutical industry. Many of them have been developed through recombinant DNA technology. But the use of traditional plants in curing various diseases has also gained pace owing to their safe and natural source[1]. A number of metabolites like alkaloids, flavonoids, tannins, resins, glycosides, volatile oils, saponins, steroids, protein, carbohydrates, phenol etc. have been reported to be present in various herbal medicinal plants[2-8]. *Reinwardtia indica* belongs to the Linaceae family and is a bushy plant mainly found in the foot hills of Himalayas. This plant has been in use by local people to meet various therapeutic uses viz. to increase the lactation period, as a mouth wash, wound healer, treating skin infections etc.[1,9-11]

Almost every part of the plant - root, stem and aerial tissue has been reported to possess medicinal properties. It has also been used for treating head and back ache, indigestion, fever and measles[12]. Stem extracts are used against skin infections of humans as well as cattle[13]. Aerial tissues like branches and leaf extracts have been reported to cure bleeding wounds and paralysis[13,14]. Yellow flowers of the plant have been used for the extraction of colour for dying and painting. The phytochemical contents, radical scavenging activities and TLC profiling of *Reinwardtia indica* have already been reported and showed good promises[15,16].

A plant with such spectacular properties but brief biochemical scientific evidence needs an in depth study so as to explore more of its bioactive compounds. Therefore, in the present study, *Reinwardtia indica* has been chosen to analyze enzymatic antioxidant potential and antimicrobial activity against various human pathogens.

Material and Methods

Sample collection and preparation: The samples of *Reinwardtia indica* were collected from Chandrapuri, district Rudraprayag (30˚ 27’ 0” N, 79˚ 4’ 0” E), Uttarakhand, India. After thoroughly washing the leaves under running tap water, some of the fresh leaves were directly ground in the phosphate buffer for assessment of enzymatic antioxidants while other were air dried at room temperature and then homogenized to fine powder in pestle and mortar. The homogenate was stored in air-tight bottles for further extraction process and chemical testing.

Determination of enzymatic antioxidant activity: For enzymatic antioxidant activity, the fresh leaves were ground using mortar-pestle in 0.15 M potassium phosphate buffer B pH 7.8 and1 mM EDTA. The extract so obtained was centrifuged at 12000rpm for 20 min (4^0C), the supernatant was taken and stored till further use.

Total protein estimation: Total protein was estimated according to the Lowry’s method with slight modification. Bovine serum albumin with known concentrations (1 to 10 mg/ml) was taken as standard and the OD was read at 660 nm using a suitable blank. After that 4.5 ml of reagent I (containing 2% Na_2CO_3 in 0.1 N NaOH, 1% NaK Tartrate in DW and 0.5% $CuSO_4.5H_2O$ in DW) was added in each test tube and incubated for 10 minutes. After incubation 0.5 ml of reagent II **(**5 part Folin-Phenol [2 N]: 6 part water) was added in each test tube and incubated for 30min, absorbance was taken at 660 nm and standard graph was plotted according to absorbance. After that the total amount of protein was estimated which was present in the given sample from the standard curve of BSA.

Catalase: Enzymatic antioxidant activity of catalase was determined by Aebi[17] method. 100 mg of fresh leaf samples were crushed in 2 ml of 5mM potassium phosphate buffer

(pH 7.0). The crushed leaves were then made to centrifuge at 14000rpm for 30 min at 4^0C and supernatant so obtained was mixed with 20mM of H_2O_2 and the rate of disappearance of H_2O_2 was observed as rate of decrease in absorbance at 240 nm at an interval of 3 minute for 9 minutes. The catalase activity was calculated by using the following formula:

Decrease in H_2O_2 concentration (min/mol) = $\Delta A/ \epsilon \times \Delta t \times l \times$ mg of protein

Ascorbate oxidase activity: For the determination of ascorbate oxidase activity, 100 mg of fresh leaves were crushed in 2 to 3ml of solution containing 50mM phosphate buffer (pH 7.0) and 1% polyvinylpyrolidone (PVP). The homogenate was centrifuged at 15000rpm for 30min at 4˚C and supernatant was collected for ascorbate oxidase activity. The activity was determined by using the method of Diallinas et al[18]. 10µl of enzyme extract was mixed with 1 ml of reaction mixture containing 20mM phosphate buffer (pH 7.0) and 2.5mM ascorbic acid. The decrease in absorbance was observed for 9min after 3min interval at 265nm due to ascorbate oxidation and activity was calculated using extinction coefficient, $mM^{-1}\,cm^{-1}$:

Enzyme activity (Units/mg/min) = (ΔAbs × total assay volume) /$\Delta t \times \epsilon \times l \times$ enzyme sample

Ascorbate peroxidase activity: Ascorbate peroxidase activity was measured according to Nakano and Asada[19] with minor modifications. Enzyme extraction buffer was prepared as 50 mM NaH_2PO_4 (pH 7.0), 2% PVP, 0.1 mM EDTA. 0.1 g of fresh leaves was taken and grinded and suspended in 1.5 ml homogenization buffer. After that, the suspension was centrifuged at 14000 rpm for 30 min at 4˚C. Supernatant was taken for enzyme assay and reading was taken at 290 nm. The enzyme activity was calculated by using the following formula:

Enzyme activity (Units/min/mg) = (ΔAbs × total assay volume) /$\Delta t \times$ □ × l× enzyme sample

Glutathione S Transferaseactivity: Glutathione S Transferase activity was assessed by using method of Habig et al[20]. The enzyme extract was prepared by homogenizing 0.5gm of sample in 5ml of phosphate buffer. This homogenate was centrifuged at 5000rpm for 10min and supernatant was taken. The activity of the enzyme was determined by change in absorbance at 340nm.The reaction mixture containing 0.1 ml of GSH, 0.1ml of CDNB and phosphate buffer was made up to 2.9ml. The reaction was initiated by addition of 0.1ml of enzyme extract. The readings were recorded at an interval of 15sec at 340nm for 3min using distilled water blank.

GST activity was calculated using extinction coefficient of the product 9.6 $mM^{-1}cm^{-1}$:

(ΔA_{340})/min×3×DF/9.6× Vol. of enzyme.

Glutathione Reductase: Glutathione reductase was assayed according to the procedure of David and Richard[21]. 0.1ml of sample was mixed with 1ml of potassium buffer (0.12M, pH 7.2), 0.1ml of EDTA, 0.1ml of sodium azide and 0.1ml of oxidized glutathione. The volume was made up to 2ml with water. This mixture was kept at room temperature for 3min and 0.1ml of NADPH is added. The absorbance at 340nm was recorded at intervals of 15 seconds for 2-3 minutes. One unit of Glutathione Reductase (GR) was expressed as micromole of NADPH oxidized min^{-1} gm^{-1}. Enzymatic activity was calculated using following formula:

U /min/mg= ΔA340nm/min×3×Df / (6.22)×(0.1)

Polyphenol Oxidase Activity: Polyphenol oxidase activity was determined by the procedure of Esterbauer et al.[22] 100mg plant leaves were homogenized in about 2ml of extraction buffer solution containing 50mM Tris HCL (pH 7.2), 0.4M sorbitol and 10 mM NaCl. The homogenate was then centrifuged at 14000rpm for 10 min at 7˚C and supernatant was collected the enzyme assay. The assay mixture contained 2.5 ml of 0.1 M phosphate buffer and 0.3 ml of 0.01 M catechol solution. The gradual increase in absorbance was recorded at 495 nm every 30 seconds for the duration of 3 minutes.

One unit of catechol oxidase is defined as the amount of enzyme that transforms 1 µmole of dihydrophenol to 1 µmole of quinine per minute under the assay conditions. Activity of polyphenol oxidase is calculated using the formula:

PPO activity = k ×ΔA_{495} / minute

where k for catechol is 0.272.

Determination of Antibacterial activity of leaf extracts

Preparation of leaf extracts: Extracts of young leaves of *Reinwarditia indica* were obtained by sequential Soxhlet extraction with different solvents of increasing polarity. 100 grams of powdered sample of young leaves were sequentially extracted in a Soxhlet extractor using 800 ml of petroleum ether, chloroform, acetone, methanol and water. The extraction was done until the sample in Soxhlet became colourless. The extracts were then subjected to distillation for preparation of crude extracts in respective solvents.

Bacterial strains: The pathogenic strains *Cornybacterium diphtheriae, Shigella flexneri, Escherichia coli, Salmonella sp., Citrobacter koseri, Klebsiella pneumoniae, Klebsiella oxytoca, Morganella morgani, Enterobacter sp*. and *Proteus* were procured from Department of Microbiology, VCSG Medical Science and Research Institute, Srinagar, Pauri Garhwal. The strains were further revived by inoculating a single colony in 10ml MacConkey broth and incubated. Agar well diffusion method was used to investigate antimicrobial activity of *Reinwardtia indica.*

MacConkey agar media was prepared and then poured in sterile Petri plates within laminar air flow hood and the

plates were allowed to dry. The plates were then inoculated with 24hr old bacterial culture using sterile autoclaved cotton swabs for uniform spreading.

Antibacterial activity: The anti-bacterial activity was performed by the procedure followed by Perez et al.[23] A sterile 1ml micropipette tip was used to cut five wells at an equal distance from one another in each of the plate. 200µl each of all the five solvent extracts in the concentration 10mg/ml was poured in each well. After the extracts diffused properly in the plates, the petri plates were sealed and kept for incubation at 37°C for about 24hr. After 24hr the diameters of the zone of inhibition were measured for each extract using a scale and the results were recorded. Antibacterial activity of plant extracts was also compared with standard antibiotics norfloxacin, levofloxacin and gatifloxacin.

Results and Discussion

Enzymatic Antioxidant Activity: Table 1 shows the enzymatic activities of six different enzymatic antioxidants extracted from *Reinwardtia indica* leaves. Enzymatic system includes Catalase, Ascorbate peroxidase, Ascorbate oxidase, Polyphenol oxidase and Glutathione S Reductase. *Reinwardtia indica* was assayed for six important enzymes having antioxidant activity namely Catalase, Ascorbate Oxidase, Ascorbate Peroxidase, Glutathione Reductase, Polyphenol Oxidase and Glutathione S Transferase. Catalase showed activity of 3.20×10^{-5}U/min/mg of protein, Ascorbate Oxidase showed activity of 2.1×10^{-1}U/mg/min, Ascorbate Peroxidase showed activity of 1.4×10^{-2}U/mg/min, Glutathione Reductase showed activity of 1.35U/mg/min of sample and Polyphenol Oxidase showed activity of 8.7×10^{-3}U/mg/min of protein respectively which shows effective presence in the system.

Plants have always been a common source of food and medicines either in the form of traditional preparations or as pure active principles. Most of the observed therapeutic effects of plants have been linked to their potent antioxidant activity. Khatun et al found positive correlation between enzymatic antioxidant activity and antioxidant potential of plant *Coleus forskohlii*. It has been suggested that free radicals are involved in the pathology of more than 50 human diseases including aging[24].

Table 1
Activity of different enzymatic antioxidants

Enzymes	Activity
Catalse (U/min/mg)	$3.20X10^{-5}$
Ascorbate oxidase (U/min/mg)	$2.1X10^{-1}$
Ascorbate peroxidase (U/min/mg)	$1.4X^{10-1}$
Polyphenol oxidase (U/min/mg)	$8.7X10^{-3}$
Glutathione reductase (U/min/mg)	1.35
Glutathione S peroxidase (U/min/mg)	$8.7X10^{-3}$

The plant extracts in chloroform, acetone, methanol and distilled water were tested against ten pathogenic bacterial strains. Among all the tested strains, significant inhibitory activity was observed against *Cyanobacterium diptheriae*, *Proteus* and *E. coli* (Table 2, Fig. 1).

The extracts which showed inhibitory activity against the three bacterial strains (namely *Cornybacterium diptheriae*, *Escherichia coli* and *Proteus sp.*) were compared with three standard antibiotics viz. norfloxacin, levofloxacin and gatifloxacin (Fig. 2). It was observed that these standard antibiotics were not effective against *Cornybacterium diptheriae* while acetone and methanol extracts of *R. indica* leaves showed considerable activity (Fig. 2).

Table 2
Zone of inhibition of leaf extract against various pathogens

Bacterial strains	Chloroform	Acetone	Methanol	Water
	Zone of Inhibition (mm)			
Cornybacterium diptheriae	0	14	15	0
Shigella flexneri	0	0	0	0
Escherichia coli	13	18	13	0
Salmonella typhimurium	0	0	0	0
Citrobacter koseri	0	0	0	0
Kleb pneumonia	0	0	0	0
Kleb oxytoca	0	0	0	0
Morganella morgana	0	0	0	0
*Anterobac*ter	0	0	0	0
Proteus sp.	0	12	14	0

a) Cornybacterium diptheriae ***b) Escherichia coli*** **c) *Proteus sp.***

(A- Petroleum ether extract, B - Water extract, C - Chloroform extract, D- Acetone extract, E- Methanol extract)
Fig. 1: Antibacterial activity of various extracts against pathogenic bacterial strains in MacConkey agar plates

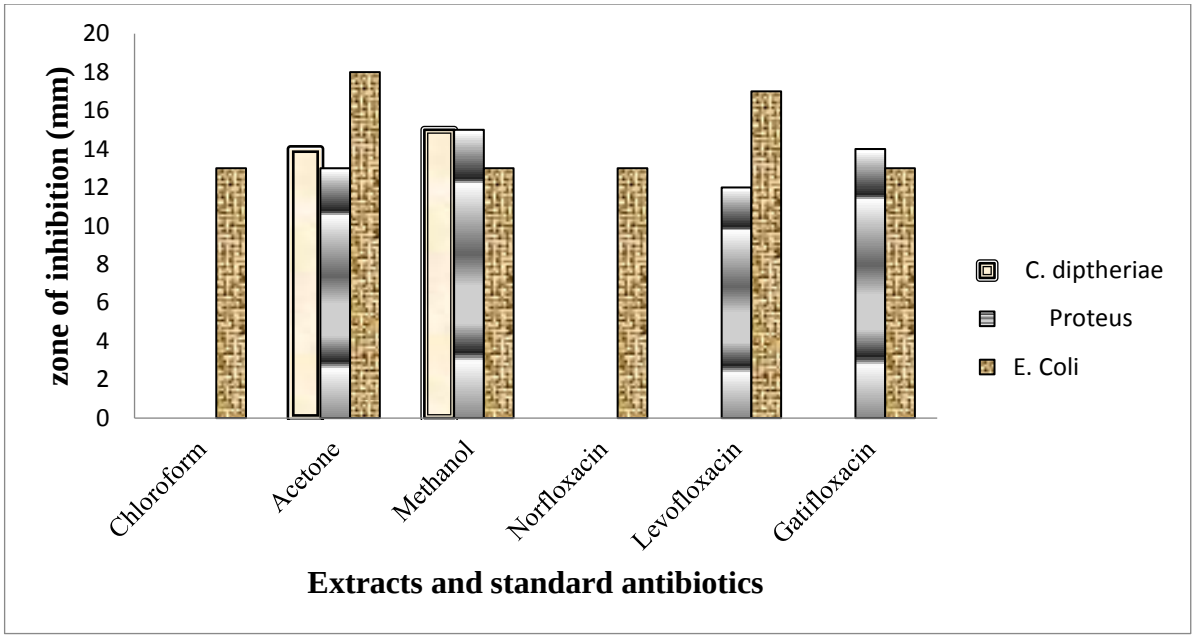

Fig. 2: Comparative potential of *R indica* extracts and standard antibiotics

Among all the extracts, chloroform, acetone and methanol were found effective against *E. coli.* It was observed that acetone extract showed highest inhibition even better than the standard antibiotics (Fig. 2). Acetone and methanol extracts were found significantly effective against *Proteus* sp. in comparison to the standard antibiotics. The comparative studies suggest that *R.indica* has significant antimicrobial potential, so it can be used to prepare effective antibiotics against *Cornybacterium diptheriae*.

Shukla et al[25] have also reported the antibacterial activity of carbinol extracts of *Reinwardtia indica* against *Staphylococcus aureus* and *Pseudomonas aeruginosa.* The silver nanoparticles of *Reinwardtia indica* were found effective against *P. aeruginosa, E. coli* and *S. aureus*[26]. Results of the present study showed good antibacterial activity against the gram-negative pathogens. Pushpa et al[27] have reported differences in antibacterial activityof *Avicenniamarina* against *Staphylococcus* and *Proteus* with change of solvent. Different solvents used have different impact on activation of biomolecules. They have reported highest activity in ethyl acetate followed by ethanol, petroleum ether and chloroform. In the present study highest activity was found in acetone followed by methanol and chloroform.

Sen et al[28] have reported anti-microbial efficiency of *Melia azedarach* leaves extract against a *Bacillus cereus, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*[28]. Most of the medicinal plants studied so far by researchers all over the world have shown antimicrobial activity against a number of pathogens. Antibacterial activity of *Reinwardtia indica* in the current study is quite comparable to the reports of other workers against the same bacterial strains in different plants, higher in some cases too.

Conclusion

Reinwardtia indica has been reported to be effective in body aches, nausea, indigestion, dermatitis and wound healing. These qualities are suggestive of the medicinal properties of the plant. The present study further strengthened this fact as the results have shown good antibacterial activity against

some pathogenic strains and significant enzymatic antioxidant activity. Therefore, it is concluded that the plant *Reinweardtia indica* possesses medicinal properties and can be used for drug formulations.

References

1. Bhattarai S., Chaudhary R.P. and Taylor R.S., Ethno-medicinal plants used by the people of Nawalparasi District, Central Nepal, *Our Nature*, **7(1),** 82-99 **(2009)**

2. Gul R., Jan S.U., Faridullah S., Sherani S. and Jahan N., Preliminary Phytochemical Screening, Quantitative Analysis of Alkaloids and Antioxidant Activity of Crude Plant Extracts from Ephedra intermedia Indigenous to Balochistan, *The Scientific World Journal*, https://doi.org/10.1155/2017/5873648 **(2017)**

3. Madhu M., Sailaja V., Satyadev T.N. and Satyanarayana M.V., Quantitative phytochemical analysis of selected medicinal plant species by using various organic solvents, *Journal of Pharmacognosy and Phytochemistry*, **5(2),** 25 **(2016)**

4. Prabhavathi R.M., Prasad M.P. and Jayaramu M., Studies on Qualitative and Quantitative Phytochemical Analysis of Cissus quadrangularis, *Advances in Applied Science Research*, **7(4),** 11-17 **(2016)**

5. Paritala V., Chiruvella K.K., Ripain I.H.A. and Arifullah M., A recent review on phytochemical constituents and medicinal properties of kesum (*Polygonum minus* Huds.), *Asian Pacific Journal Tropical Biomedicines*, **4(6),** 430–435 **(2014)**

6. Ahmad I. and Beg A.Z., Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens, *Journal of Ethnopharmacology*, **74(2),** 113-23 **(2001)**

7. Kutama R.M., Abdulkadir S., Kwalli S.A. and Chiroma G., Phytochemical Compositions in Some Nigerian Medicinal Plants and Their Pharmacological Properties: A Review, *Journal of Anesthesiology*, **6(1),** 15 **(2018)**

8. Butkutė B., Dagilytė A., Benetis R., Padarauskas A., Cesevičienė J., Olšauskaitė V. and Lemežienė N., Mineral and phytochemical profiles and antioxidant activity of herbal material from two temperate Astragalus species, *BioMed Research International*, https://doi.org/10.1155/2018/6318630 **(2018)**

9. Phondani P.C., Maikhuri R.K. and Bisht N.S., Medicinal plants used in the health care system practiced by traditional Vaidyas in Alaknanda catchment of Uttarakhand, India, *Ethnobotanical Leaflets*, **12**, 4 **(2009)**

10. Shah R., Pandey P.C. and Tiwari L., Traditional veterinary herbal medicines of western part ofAlmora district, Uttarakhand Himalaya, *Indian Journal of Traditional Knowledge*, **7(2),** 355-359 **(2008)**

11. Verma S. and Chauhan N.S., Indigenous medicinal plants knowledge of Kunihar forest division, district Solan, *Indian Journal of Traditional Knowledge*, **6(3),** 494-497 **(2007)**

12. Thakur S. and Sidhu M.C., Phytochemical Screening of Some Traditional Medicinal Plants Research, *Journal of Pharmaceutical, Biological and Chemical Sciences*, **5(4),** 1088-1097 **(2014)**

13. Pande P.C., Tiwari L. and Pande H.C., Ethnoveterinary plants of Uttaranchal — A review, *Indian Journal of Traditional Knowledge*, **6(3),** 444-458 **(2007)**

14. Malla B., Gauchan D.P. and Chhetri R.B., Medico-ethanobotanical investigations in Parbat district of Western Nepal, *Journal of Medicinal Plants Research*, **8(2),** 95-108 **(2014)**

15. Rawat A., Rana P.S., Molpa D. and Saklani P., Phytochemical composition and antioxidant activity of Reinwardtia indica from selected location of Uttrakhand, *Int. J. Prog. Eng. Manag, Sci and Hum*, **1(3),** 22-29 **(2015)**

16. Sonam M., Singh R.P. and Pooja S., Phytochemical screening and TLC profiling of various extracts of Reinwardtia indica, *International Journal of Pharmacognosy and Phytochemical Research*, **9(4),** 523-527 **(2017)**

17. Aebi H., Catalase in vitro, In Methods in, Enzymology, Academic Press, New York, 121–126 **(1984)**

18. Diallinas G., Pateraki I., Sanmartin M., Scossa A., Stilianou E., Panopoulos N.J. and Kanellis A.K., Melon ascorbate oxidase: cloning of a multigene family, induction during fruit development and repression by wounding, *Plant Molecular Biology*, **34(5),** 759-770 **(1997)**

19. Nakano Y. and Asada K., Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts, *Plant and Cell Physiology*, **22(5),** 867-880 **(1981)**

20. Habig W.H., Pabst M.J., Fleischner G., Gatmaitan Z., Arias I.M. and Jakoby W.B., The identity of glutathione S-transferase B with ligandin, a major binding protein of liver, Proceedings of the National Academy of Sciences, 3879-3882 **(1974)**

21. David M. and Richard J.S., In Methods of enzymatic analysis, Bergmeyer J. and Grab M., Eds., Verlag Chemie Wenhein Deer Field, Beach Floride, 358 **(1983)**

22. Esterbauer H., Schwarzl E. and Hayn M., A rapid assay for catechol oxidase and laccase using 2-nitro-5-thiobenzoic acid, *Analytical Biochemistry*, **77(2),** 486-494 **(1977)**

23. Perez C., Paul M. and Bazerque P., Antibiotic assay by agar-well diffusion method, *Acta Biol Med Exp*, **15**, 113-115 **(1990)**

24. Halliwell B., Drug antioxidant effects, *Drugs*, **42(4),** 569-605 **(1999)**

25. Shukla A., Vats S. and Shukla R., Preliminary phytochemical screening, antibacterial and nitric oxide radical scavenging activities of *Reinwardtia indica* leave extract, *International Journal of Pharm Tech Research*, **5(4),** 1670-1678 **(2013)**

26. Upadhyay P., Mishra S.K., Purohit S., Dubey G.P., Singh Chauhan B. and Srikrishna S., Antioxidant, antimicrobial and cytotoxic potential of silver nanoparticles synthesized using flavonoid rich alcoholic leaves extract of *Reinwardtia indica*, *Drug and Chemical Toxicology*, 10.1080/01480545.2018.1488859, **19 (2019)**

27. Pushpa D.A. and Chaminda D.S., Screening of petroleum ether, chloroform, ethyl acetate, ethanol and water extracts of medicinal

plant, *Avicenniamarina* for antibacterial activity against antibiotic resistant bacteria species, *Staphylococcus* and *Proteus, Journal of Pharmaceutical and Medical Sciences,* **11(18),** 1-5 **(2011)**

28. Sen A. and Batra A., Evaluation of antimicrobial activity of different solvent extracts of medicinal plant: *Melia ezedarach L, International Journal of Current Pharmaceutical Research*, **4(2),** 67-73 **(2012)**.

(Received 23rd January 2020, accepted 27th March 2020)