Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
https://www.phytojournal.com
JPP 2023; 12(1): 35-40
Received: 13-10-2022
Accepted: 11-12-2022

Md. Mozibullah
Department of Biochemistry and Molecular Biology, Mawlana Bhashani Science and Technology University, Santosh, Tangail-1902, Bangladesh

Hasib Ahammad
Department of Biochemistry and Molecular Biology, Mawlana Bhashani Science and Technology University, Santosh, Tangail-1902, Bangladesh

Tasmina Tarin
Department of Biochemistry and Molecular Biology, Mawlana Bhashani Science and Technology University, Santosh, Tangail-1902, Bangladesh

Md. Jahinur Islam
Department of Biochemistry and Molecular Biology, Mawlana Bhashani Science and Technology University, Santosh, Tangail-1902, Bangladesh

Marina Khatun
Assistant Professor, Department of Biochemistry and Molecular Biology, Mawlana Bhashani Science and Technology University, Santosh, Tangail-1902, Bangladesh

Mohammod Johirul Islam
Department of Biochemistry and Molecular Biology, Mawlana Bhashani Science and Technology University, Santosh, Tangail-1902, Bangladesh

Md. Asaduzzaman Sikder
Department of Biochemistry and Molecular Biology, Mawlana Bhashani Science and Technology University, Santosh, Tangail-1902, Bangladesh

Corresponding Author:
Marina Khatun
Assistant Professor, Department of Biochemistry and Molecular Biology, Mawlana Bhashani Science and Technology University, Santosh, Tangail-1902, Bangladesh

Evaluation of anti-oxidant and antibacterial activities of *Ixora chinensis* and *Cascabela thevetia* leaf extracts: An *in vitro* study

Md. Mozibullah, Hasib Ahammad, Tasmina Tarin, Md. Jahinur Islam, Marina Khatun, Mohammod Johirul Islam and Md. Asaduzzaman Sikder

DOI: https://doi.org/10.22271/phyto.2023.v12.i1a.14545

Abstract
The purpose of this study was to assess the antioxidant and antibacterial activities of *Ixora chinensis* and *Cascabela thevetia* leaf. Leaf parts of *Ixora chinensis* and *Cascabela thevetia* plants were subjected to prepare the extracts with pure methanol and ethanol as solvent. These leaf extracts were evaluated for their antioxidant and antibacterial potentialities by utilizing the DPPH assay and disc diffusion methods, respectively. All the leaf extracts exhibited DPPH radical scavenging ability, among them ethanolic leaf extracts of *Ixora chinensis* and combined methanolic extracts of both leaf demonstrated the substantial antioxidant potentiality with the 18.52 and 29.64 µg/ml as IC_{50} value, respectively. In the context of antibacterial activity, both leaf extracts (Methanol and ethanol) of *Cascabela thevetia* showed low to moderate antibacterial activity, generating almost similar zone of inhibition against three (*Staphylococcus aureus, Klebsiella pneumonia,* and *Vibrio cholerae* C6706) of eleven investigated bacterial strains with the 7 to 10 millimeters (Diameter). The current outcomes conclude that leaf extracts of the *Ixora chinensis* and *Cascabela thevetia* could be utilized to develop the biopharmaceutical agents.

Keywords: *Ixora chinensis, Cascabela thevetia,* leaf esxtracts, antioxidant activity, antibacterial activity

1. Introduction
Reactive Oxygen Species (ROS) implies reactive molecules and free radicals such as superoxide, hydroxyl radicals, hydrogen peroxide, singlet oxygen, peroxynitrite, and nitric oxide. Xenobiotics such as radiation, drugs, habits like smoking, as well as environmental agents, interact with cellular sources of ROS, inducing its generation [1]. Excessive ROS that cannot be neutralized by our body due to the depletion of endogenous antioxidants causes various pathologies such as cancer, neurodegenerative diseases, diabetes, and cardiovascular diseases (CVD), due to its role promoting inflammation, damaging DNA and proteins, as well as lipid peroxidation [2, 3]. As antioxidants can neutralize ROS thus prevent ROS-mediated various diseases, there is growing interest to investigate substances exhibiting antioxidant activity. Synthetic and natural are two basic categories of antioxidants. The use of synthetic antioxidants is restricted due to the carcinogenic effect [4].
Infectious disease is the leading cause of death in many countries, specifically in developing countries. The most remarkable thing is that mortality rates are surprisingly augmenting in developed countries as well. Antibiotics or antimicrobial drugs are the treatment option for most of the infectious diseases [5]. Antibiotic-resistant bacteria have been reported to be evolved due to the indiscriminate utilization of existing antibiotics. Bacteria are becoming resistant to almost all of the existing antibacterial agents [6]. Public health treatment is becoming an alarming condition due to the increasing antibiotic resistance phenomena. Regarding the frightening incidence of antibiotic resistance, it is of utmost need to develop new and promising therapeutic agents [7]. In addition, the development of totally novel antibiotics has reduced over the previous fifteen years despite these circumstances [6].
Traditional medicinal plants have long been widely recognized for centuries in numerous portions of the globe for the therapeutic purpose of various diseases [8]. A large population of the world estimating four billion relies on plants based medicinal products as a chief source of treatment and traditional therapy practice [9]. Modern clinical medicines, more than fifty percent of all, are demonstrated to be developed from natural products [10]. The comparatively lower rate of adverse effects and reduced cost encourage the healthcare institution and public consuming plant-based medicines as a promising substitute to synthetic drugs [11].

Plants are the sources of the plethora of active compounds, a large proportion of which has been shown to have antimicrobial, antioxidant features [12, 13]. *Cascabela thevetia*, belonging to the Apocynaceae family, is an evergreen shrub. Different parts of the plant have long been widely used as a medicine for various ailments including leaf decoction for loosening the bowels and effective for intermittent fever, seeds for skin infections, rheumatism, jaundice, and also as insecticidal [14]. *Ixora chinensis* belongs to the Rubiaceae family, which is extensively found in Bangladesh as a gardening plant. It is traditionally utilized as a treatment option for a wide variety of diseases like abnormal menstruation, hypertension, rheumatism, skin and external diseases, and gastralgia [15].

In this study, experiments were carried out to evaluate the antioxidant and antibacterial activities of *Ixora chinensis*, *Cascabela thevetia* leaves for their methanolic and ethanolic extracts using 2, 2 diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and disc diffusion assays, respectively. In addition, their combined methanolic extracts were also subjected to asses these medicinal properties.

2. Materials and Methods

2.1. Plant material

The leaves of *Cascabela thevetia* and *Ixora chinensis* (Family: Apocynaceae) were collected from Mawlana Bhashani Science and Technology University, Santosh, Tangail-1902 campus area and authenticated by Horticulture Research Center of Bangladesh Agricultural Research Institute (BARI) in Gazipur, Bangladesh.

2.2. Preparation of extracts

The leaf powder of *Cascabela thevetia* and *Ixora chinensis*, which was dried in absence of direct sunlight, was extracted in methanol and ethanol solvent. The leaf powder (25 gram) of both plants was then immersed in 250 ml of pure methanol and ethanol, maintaining a 1:10 (w/v) ratio. For combined methanol extract, 12.5 gm of each plant's leaf powder (total 25 gram) was also immersed similarly. The conical flask containing the powder immersed in solvent was placed in an orbital shaker at 140 rpm for three hours and then subjected to soaking for four days at room condition. The removal process of unwanted dry parts of powder was mediated through the filtration using a cotton cloth and then Whatman No.1 filter paper three times. The solvent of the filtrate was evaporated to get the desired extract [12, 16]. The obtained extract was then re-suspended in the corresponding solvent that was utilized in preparing the extract.

2.3. Antioxidant activity (DPPH assay)

The ability to scavenge the 2, 2 diphenyl-1-picrylhydrazyl (DPPH) free radicals of prepared leaf extracts was assessed utilizing the method of Chew, Jessica, and Sasidharan 2012 with slight modification [12]. Different concentrations (1 ml) of the re-suspended methanolic and ethanolic leaf extracts were added with newly made 0.1mM DPPH (3 ml) in methanol and ethanol solution, respectively. Each mixture was then vortexed and placed in the dark for 30 minutes and the absorbance of each dark incubated solution was measured at 517 nm. Gallic acid was used as a standard to compare the scavenging activity of leaf extracts. The experiment was performed three times and the percentage scavenging activity was calculated by utilizing the equation:

Percentage scavenging activity $=\left(1-\frac{As}{Ac}\right)\times 100$, Where As is the absorbance of sample/standard and Ac is the absorbance of the control.

2.4. Antibacterial activity of the leaf extracts

2.4.1. Microorganisms

Gram positive *Streptococcus mutans*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and Gram negative bacteria *Salmonella typhi*, *Salmonella paratyphi*, *Vibrio cholerae C6706*, *Vibrio cholerae* N-16961, *Enterotoxigenic Escherichia coli* (ETEC), *Enteropathogenic Escherichia coli* (EPEC), *Klebsiella pneumoniae and Pseudomonas aeruginosa*. All bacterial strains from -80 °C were cultured and managed on Muller Hinton Broth at 37 °C.

2.4.2. Assessment of antibacterial activity

Pure Dimethyl sulfoxide (DMSO) as solvent was used to prepare the working extracts for assessing the antibacterial activity to the concentration of 50 mg/ml of 0.5 ml. Antibacterial activity of prepared leaf extracts was assessed by disc diffusion method [17]. The cotton swab was used to uniformly spread the overnight bacterial culture into the Muller Hinton Agar (MHA) plate at the surface and permitted to dry. Sterilized paper discs that were prepared by using What man No.1 filter paper (5 mm in diameter) were impregnated with 20 µl of 50 mg/ml leaf extracts and DMSO as a negative control. Kanamycin (30 µg/disc) and Erythromycin (15 µg/disc) were applied as the positive control. Antibiotics (Kanamycin and Erythromycin) and impregnated discs with leaf extracts and DMSO were distributed and pressed gently on MHA plates by using sterile forceps and needles. The study was performed three times. The MHA plate was then incubated at 37 °C for 18 hours and after that presence and/or absence of antibacterial activity were observed and measured, if the zone of inhibition was present, by using the transparent scale in diameter (Millimeter).

2.5. Statistical analysis

The results were expressed by calculating mean with standard deviation (SD). Mean with ± standard deviation (SD) calculation and linear regression analysis (to reveal the dose-response relation of leaf extracts and calculate the IC_{50} value) were performed by using Excel 2013.

3. Results

3.1. Antioxidant activity

Antioxidant activity, in terms of capacity to scavenge free radicals, of leaf extracts was evaluated by employing the DPPH assay. DPPH assays revealed that leaf extracts specifically *Ixora chinensis* exhibited remarkable scavenging activity (Figure 1). IC_{50} value refers to the concentration of extracts at which 50% free radicals are neutralized, which can be calculated from the linear regression of concentration versus percentage scavenging activity. Table 1 indicates the IC_{50} value of leaf extracts in comparison to gallic acid as positive control or standard. The calculated IC_{50} value of methanolic and ethanolic extracts of *Ixora chinensis* was notably higher 39 and 18.52 µg/ml, respectively than *Cascabela thevetia* leaf extracts (315.6 µg/ml for methanolic and 313 µg/ml for ethanolic extracts). Combined methanolic leaf extracts showed substantial synergistic effects on DPPH radicals and the computed IC_{50} value was 29.64 µg/ml.

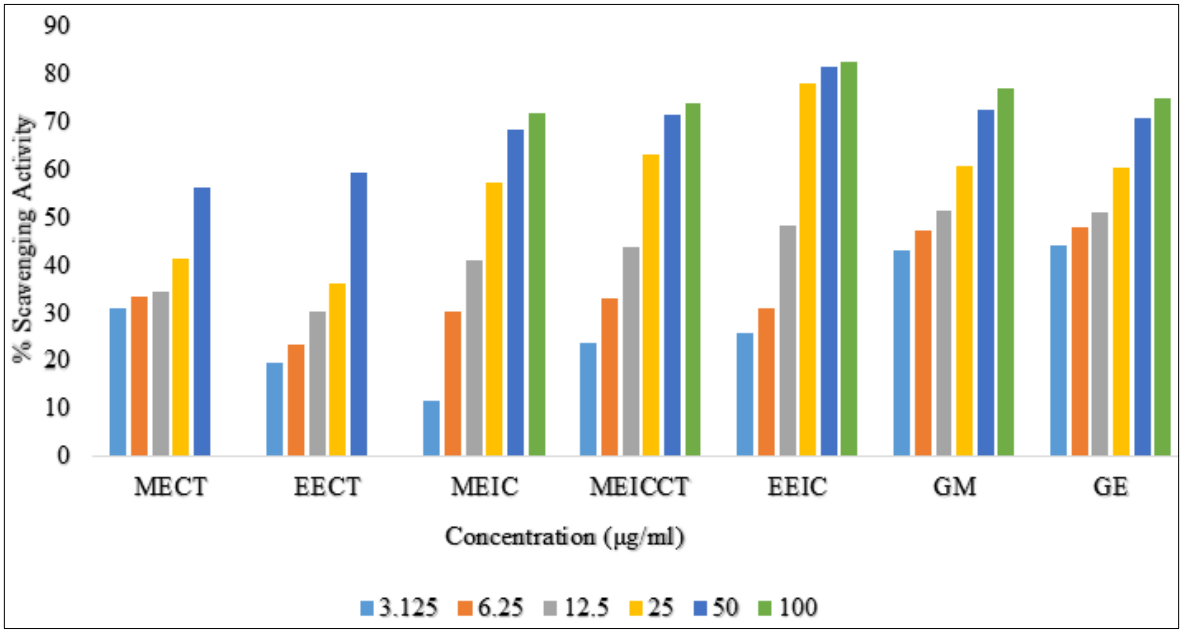

Fig 1: DPPH percentage scavenging activity of investigated extracts and Gallic acid. Data are presented as mean ± standard deviation and are triplicate of the experiments. MECT- Methanolic Extract of *Cascabela thevetia* leaf, EECT- Ethanolic extract of *Cascabela thevetia* leaf, MEIC- Methanolic extract of *Ixora chinensis* leaf, MEICCT- Combined methanol extract of *Ixora chinensis* and *Cascabela thevetia* leaf, EEIC- Ethanolic extract of *Ixora chinensis* leaves, GM- Gallic acid in methanol, GE- Gallic acid in ethanol

Table 1: IC_{50} value of our investigated leaf extracts and Gallic acid

Extract/Standard	Type of solvent used	IC_{50} (µg/ml)
Ixora chinensis	Pure methanol	39
	Pure ethanol	18.52
Cascabela thevetia	Pure methanol	315.6
	Pure ethanol	313
Combined leaf extract (Synergistic effect)	Pure methanol	29.64
Gallic acid	Pure methanol	7.66
	Pure ethanol	6.6

3.2. Antibacterial activity

The Table 2 and Figure 2 demonstrate the detailed comprehensive result of antibacterial activity of our investigated extracts, which exhibits that all extracts did not show antibacterial property except for the extract of *Cascabela thevetia* leaf. Methanol and ethanol extracts of *Cascabela thevetia* leaf were active against the three tested bacteria (*Klebsiella pneumonia, Staphylococcus aureus, Vibrio cholerae C6706),* while these extracts produced the zone of inhibition on *Klebsiella pneumonia* (10 mm for methanol extracts and 9.5 mm for ethanol extracts), *Staphylococcus aureus* (8.5 mm for methanol extracts and 9 mm for ethanol extracts), and *Vibrio cholerae C6706* (7 mm for both extracts). In addition, antibiotic kanamycin achieved the zone of inhibition on tested bacterial strains (6 strains) between 16 to 21 mm, while erythromycin was found to be resistant against all applied (5 strains) microorganisms.

Table 2: Antibacterial activity of our investigated leaf extracts and control (antibiotics and DMSO)

Name of bacteria	Zone of inhibition (diameter in millimeter)							
	C. thevetia		*I. chinensis*		MEICCT	Erythromycin	Kanamycin	DMSO
	ME	EE	ME	EE	ME			
Staphylococcus aureus	8.5±0.5	9±0	0	0	0	R	NU	0
Staphylococcus epidermidis	0	0	0	0	0	R	NU	0
Streptococcus mutans	0	0	0	0	0	R	NU	0
Klebsiella pneumoniae	10±0	9.5±0.5	0	0	0	R	NU	0
Pseudomonas aeruginosa	0	0	0	0	0	R	NU	0
Salmonella typhi	0	0	0	0	0	NU	21±0	0
Salmonella paratyphi	0	0	0	0	0	NU	18±0	0
Vibrio cholerae C6706	7±0	7±0	0	0	0	NU	16±0	0
Vibrio cholerae N-16961	0	0	0	0	0	NU	20±0	0
Enterotoxigenic Escherichia coli (ETEC)	0				0	0	0	0
Enteropathogenic Escherichia coli (EPEC)	0	0	0	0	0	NU	18±0	0

Data are expressed as Mean ± Standard deviation. ME- Methanol Extract, EE- Ethanol extracts, MEICCT-Combined methanolic extract of *I. chinensis* and *C. thevetia* leaf, R- Resistant, NU- Not Used.

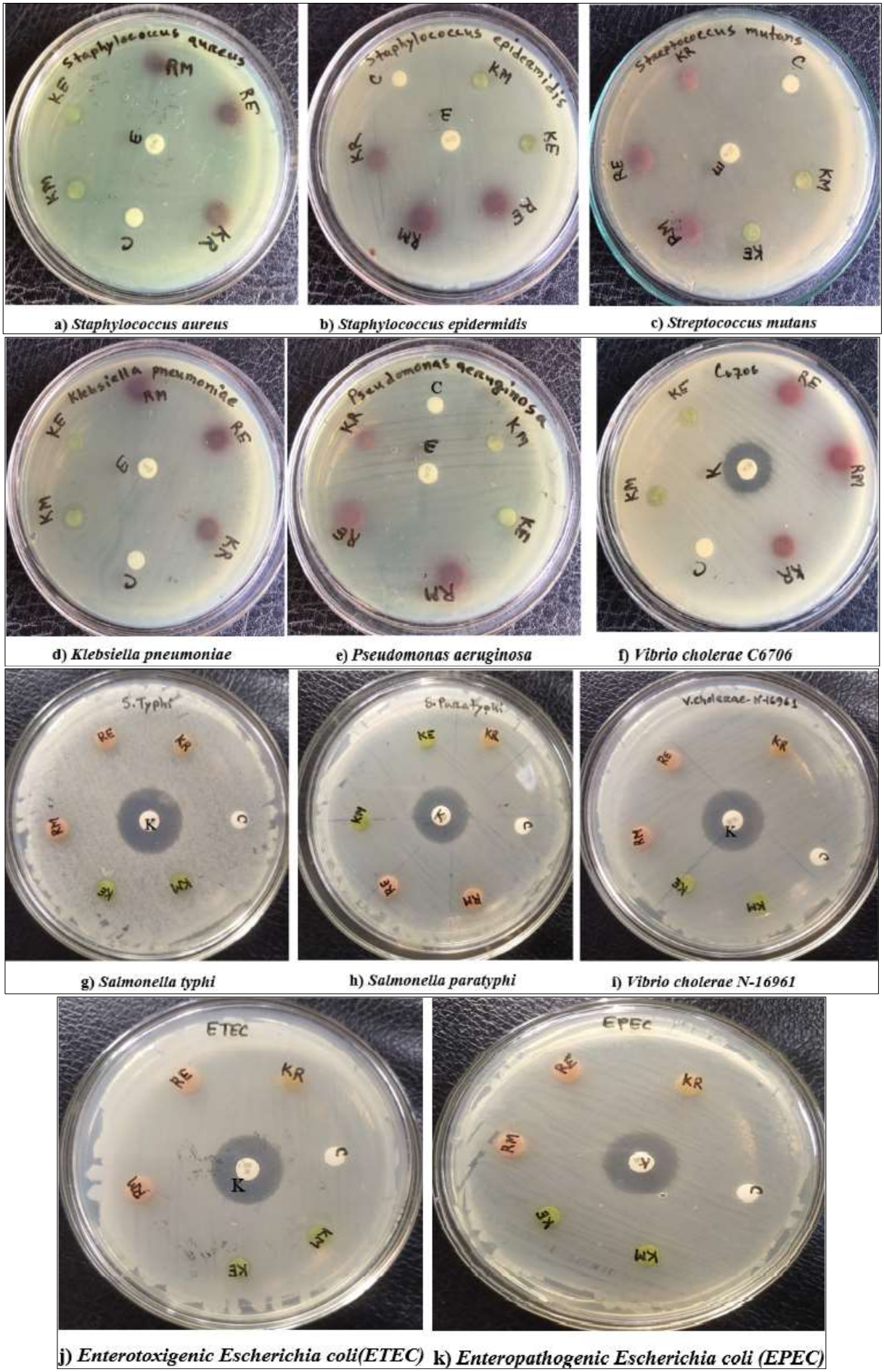

Fig 2: Antibacterial activity of plant extracts. RM and RE stand for methanolic and ethanolic extracts of *Ixora chinensis* leaf, respectively. KM and KE indicate methanolic and ethanolic extracts of *Cascabela thevetia* leaf, respectively. KR refers to the combined methanolic extracts of *Ixora chinensis* and *Cascabela thevetia* leaf. C, K, and E stand for DMSO, Kanamycin and Erythromycin, respectively

4. Discussion

Antioxidants derived from natural origin can furnish a defensive role against ROS and hence ensure chemoprevention against ROS mediated diseases [18]. Hence there is an increasing interest to assess the antioxidant activity of natural origin. DPPH assay, a commonly employed method to determine the antioxidant activity, is appraised to be the most commodious, quick, and unsophisticated assay [19, 20].

In the present study, all extracts exhibited the scavenging activity in a dose-dependent fashion, where extracts of *Ixora chinensis* leaf (IC_{50} value of methanolic; 39 µg/ml and ethanolic extracts; 18.52 µg/ml) showed substantial higher antioxidant activity than extracts *Cascabela thevetia* leaf *(*IC_{50} value *of methanolic;* 315.6 µg/ml *and ethanolic extracts;* 313 µg/ml*).* Combined methanolic leaf extracts also displayed notable *antioxidant activity, implying its remarkable* synergistic effects on DPPH radicals. In particular, the ethanolic extract was manifested to be a superior system for radical scavenging activity than methanolic extract owing to its higher proton-providing capacities, which was consistent with the previously reported study [12]. As reported by Seetharaman *et al*. 2017, methanolic extracts of the whole *Cascabela thevetia* plant showed substantial antioxidant *activity with the 60.20* µg/ml as calculated IC_{50} value [21]. This discrepancy in the outcomes is likely attributable to the whole plant extracts utilized and different extraction procedures for assessing the antioxidant potentiality. In addition, the growth area of plants also influences the active compounds of corresponding plants, causing different medicinal property [12].

Plant extracts have long been widely utilized as a prominent origin of drugs, and plenty of plants have been assessed for the compound with therapeutic value like antibacterial property [12, 22, 23]. In the current study, the antibacterial property of leaf extracts (separately and combinedly) of *Ixora chinensis* and *Cascabela thevetia* was investigated by disk diffusion technique, where leaf extracts of *Cascabela thevetia* produced a zone of inhibition against three of the 11 investigated bacteria *Staphylococcus aureus*, *Klebsiella pneumonia*, and *Vibrio cholerae* C6706 and rest of the extracts did not exhibit any antibacterial activity. Both methanolic and ethanolic leaf extracts of *Cascabela thevetia* generated almost the same mean zone of inhibitory area ranging from 7 to 10 diameter in millimeters (mm). This moderate to low activity could be due to the fact that crude form of plant extracts contain low amounts of bioactive compounds [24]. Seetharaman *et al*. 2017 [12], demonstrated that the methanolic extracts of the whole *Cascabela thevetia* plant produced the zone of inhibition against *Staphylococcus aureus* (9.4 mm), *Klebsiella pneumonia* (7 mm), and *Pseudomonas aeroginosa* (10.4 mm), while our leaf extracts exhibited dissimilar results. This inconsistency is due to the difference in the plant part that was utilized for preparing extracts (only leaf extracts were utilized in our study, while whole plant extracts were utilized in that study) and dissimilar extraction methods. *Moreover,* the growth area of plants also influences the active compounds of corresponding plants, causing different medicinal property [12].

5. Conclusion

From the current investigation, it can be concluded that both methanolic and ethanolic *Ixora chinensis* leaf extracts, specifically ethanolic extracts showed potent DPPH radical scavenging activity, indicating substantial antioxidant activity. Combined methanolic leaf extracts were also revealed to have outstanding antioxidant activity. Further investigation by utilizing other methods of assessing antioxidant activity need to be carried out to confirm the present outcomes. In case of antibacterial activity, *Cascabela thevetia* leaf extracts were exhibited to have low to moderate antibacterial activity against three of eleven tested bacterial strains. Further phytochemical analysis should be performed to identify the compounds that behave to have aforementioned medicinal properties. This study indicates that *Ixora chinensis* and *Cascabela thevetia* could be a potent source of biopharmaceutical agents.

6. Data Availability

Any data or information used in this current study is available from the corresponding author on reasonable request.

7. Conflict of interest statement

The authors declare that there are no conflicts of interest.

8. Acknowledgements

The authors would like to express their gratitude to the Department of Biochemistry and Molecular Biology, Mawlana Bhashani Science and Technology University, for providing laboratory facilities. In addition, the authors thank the Research cell, MBSTU, for providing the funding necessary to complete this research endeavor.

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