IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

ANTIYPHOID ACTIVITY OF *ENICOSTEMMA AXILLARE* (POIR EX LAM.) RAYNAL LEAF EXTRACTS BY DISC DIFFUSION METHOD.

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Abstract: The clinically important Salmonella include *Salmonella typhi*, *Salmonella paratyphi*- A, and *Salmonella paratyphi*-B; which cause typhoid fever, paratyphoid A, and B fever, respectively. For this, the leaves of *Enicostemma axillare* were selected. The antityphoidal activity of extracts were studied using standard procedures. The antityphoid activity test performed using the agar disk diffusion method revealed that all four solvent extracts showed activity against *S. typhi* and *S. paratyphi*-A. The highest zones of inhibition were obtained from the ethanol extract of leaves with diameters of 16.00±0.57 mm and 14.16±0.44 mm at 100 mg/ml, for *S. typhi* and *S. paratyphi*-A respectively. The leaf extracts of *Enicostemma axillare* showed significant antityphoid activity which can be used as a remedy against the *Salmonella typhi* and *Salmonella* paratyphi-A and therefore promise to be effective antityphoidal supplement for typhoid patients. The antityphoidal activity of the leaf extracts were compared with azithromycin 17.66±0.88 mm and 19.00±0.57 mm zone of inhibition for *S. typhi* and *S. paratyphi*-A respectively. The present results indicate that the leaf extracts of *Enicostemma axillare* has antityphoid potential against both typhoid pathogenic strains i.e. *Salmonella typhi* and *Salmonella paratyphi*-A. The Minimum inhibitory concentration (MIC) of water, ethanol, chloroform and hexane extracts of leaf extracts were determined by broth dilution method. The water, ethanol and chloroform extracts of *Enicostemma axillare* leaf showed 12.5mg/ml MIC against both strains *Salmonella typhi* and *Salmonella paratyphi*-A.

Key words: Antityphoid, MIC, *Enicostemma axillare* and *Salmonella*

I. INTRODUCTION

The clinically important Salmonella include *Salmonella typhi*, *Salmonella paratyphi* A, and *Salmonella paratyphi* B; which cause typhoid fever, paratyphoid A, and B fever, respectively (Fabrice et al. 2006; Ammah et al. 1999). In experimental animal models, *Salmonella typhimurium* is the species responsible for typhoid fever. Worldwide, an estimated 16 million cases of typhoid fever occur each year, resulting in 600,000 deaths; most infections and deaths occur in developing countries where typhoid fever is endemic (WHO 1996), associated with peritonitis due to perforation of ulcerated Peyer's patches in the small intestine (Everest et al. 2001). These intestinal complications are only caused by *Salmonella enterica serotype typhi*. Traditional antimicrobial drugs are increasingly becoming unavailable to the common man in Africa due to rising prices (Gatsing et al. 2007). In addition, there is widespread resistance to all three first line antimicrobials (i.e. chloramphenicol, ampicillin and co-trimoxazole) (WHO 1992). Furthermore,

chloramphenicol, a long-standing drug of choice for the treatment of typhoid fever, has been withdrawn from the market due to its medullary toxicity (medullary aplasia) (Knoxle and Wilde 2005).

Studies have shown that the pathogenicity and virulence of Salmonella are host specific. *Salmonella typhi* causes systemic infection only in humans, whereas *Salmonella typhimurium* causes systemic infection in rats and mice, similar to *S. typhi* in humans, and only a localized gastroenteritis. Experimental infection of mice with *Salmonella typhimurium* provides a useful model of human typhoid fever caused by *S. typhi.* Salmonellosis in mice shares many similarities with the human disease, with the primary site of colonization in both species being the ileum (Barbel Raupach and Kaufman, 2001). Traditional anti-tuberculosis drugs are becoming increasingly available to the common man in Africa due to their increasing cost. Furthermore, the bacterium that causes typhoid fever, *Salmonella typhi*, has rapidly developed resistance to previously effective drugs such as ciprofloxacin. Therefore, there is a need for new anti-tuberculosis agents. The use of herbal medicines as a complement or alternative to orthodox medicines is increasing due to their cheapness, availability and accessibility. According to WHO (1996), medicinal plants are the best source for obtaining a variety of new herbal medicines. About 80% of individuals in developing countries use traditional medicines, which contain substances derived from medicinal plants (WHO, 1996). Therefore, such plants should be investigated to better understand their properties, safety and efficacy (Nascimento et al., 2000).

Methanol extracts of leaves and stems were tested for anti-typhoid and antioxidant activities. The compounds were isolated and their structures elucidated by analysis of spectroscopic data along with literature data and tested for the same activities. The leaf extract was also tested for its anti-typhoid potential in a *Salmonella typhimurium*-induced typhoid fever model in Wistar rats (**Lunga et al., 2014**).

The literature reviewed suggests that *Enicostema axillare* may have promising antimicrobial properties, including a potential role in combating typhoid bacteria, particularly *Salmonella typhi*. Various studies have shown that compounds isolated from the plant exhibit significant antibacterial activity, but most findings are preliminary and based on in vitro results. Although the potential of *Enicostema axillare* as an adjunct or alternative treatment for typhoid fever is clear, further clinical trials and studies are needed to establish the plant's safety, efficacy, and appropriate dosage for use in humans.

II.MATERIALS AND METHODS

Test isolates

Both *Salmonella typhi* and *Salmonella paratyphi*-A reference strains of bacteria were used in the study. It was obtained from Microbial Type Culture Collection (MTCC), Chandigarh. These bacterial cultures were mixed with a 15 to 20% glycerol solution before freezing and were preserved at − 80 °C temperature. The inoculum of these organisms was prepared by 4 h activation in a suitable broth, followed by its standardization up to 0.5 McFarland standards. The isolates were maintained on nutrient agar slants for further use.

Collection and identification of plant leaves.

The leaves of *Enicostemma axillare* were collected from Nanded, Marathwad. The identification and authentication of the plant material was done at the Herbarium of the Botanical Survey of India, Pune using

the specimen number RVZEN-1 and specimen is kept there for future reference. The leaves were washed thoroughly with distilled water and dried in the shade for 2 weeks. The dried leaves were ground to a powder using a sterile pestle and mortar under laboratory conditions. The powder was kept in an airtight container for future use as described by Ali et al. in 2017.

Preparation of plant extracts

Water, ethanol, chloroform and hexane solvent were used in the extraction process. Fifty grams of plant leaf powder was soaked in 500 ml of distilled water, ethanol, chloroform and hexane respectively. The flask was kept at room temperature for three days with occasional shaking and then filtered using Whatman filter paper no.1. The ethanol, chloroform and hexane extracts were evaporated at 60°C using a rotary evaporator while the dried extract samples were evaporated in a water bath at 40°C to 45°C until they were obtained. All the dried extract samples were separately dissolved in 10% DMSO as stock concentrations. The stock solutions were stored in a refrigerator at 4°C to 5°C for further use.

Disc Diffusion Assay

The antibacterial activity of the extracts was determined using the disc diffusion method as described by Kirby-Bauer, 2009 with slight modifications. The prepared bacterial suspension containing 0.5 McFarland standard (equivalent to 1.5 x 106 CFU) was inoculated into sterile Mueller-Hinton agar medium in sterile Petri-dishes. Then, filter paper discs (about 6 mm in diameter) containing the test compound at the desired concentration (100 mg/ml) are placed on the agar surface. The plates were allowed to stand on the laboratory bench for 1 hr to allow the extract to spread properly in the medium, after which the plates were incubated at 37^0C for 24 h. Typically, the antimicrobial agent diffuses into the agar and inhibits the germination and growth of the test microorganism, and the diameters of the zones of inhibition are then measured. Azithromycin 100 mg/ml was used as a positive control in the experiment. The experiment was performed in triplicate and the average inhibition zone was calculated.

Minimum inhibitory concentration (MIC) studies

The minimum inhibitory concentration (MIC) of the extract was determined by using broth dilution technique. Two-fold serial dilutions of the extracts were prepared by adding 1 ml of 100 mg/ml extract to 1 ml of nutrient broth to form a solution containing 100 mg/ml extract. This process was continued sequentially up to test tube number five, thus producing the following concentrations; 100, 50, 25, 12.5, 6.25 mg/ml. Test tube number six did not contain extract and served as a negative control. 0.5 ml of McFarland equivalent standard was added to the test tube and incubated at 37 °C for 24 hours. After incubation, growth was monitored by checking the turbidity in the test tube

Experimental Results

The antityphoidal activity of water, ethanol, chloroform and hexane extracts of *Enicostemma axillare* leaf against *Salmonella typhi* and *Salmonella paratyphi*-A is presented in Table 1. The result shows that among all the solvent extracts tested, the ethanol extracts of *Enicostemma axillare* leaf showed the highest antityphoidal activity i.e. 16.00±0.57mm zone of inhibition for *Salmonella typhi* and 14.16±0.44mm zone of inhibition for *Salmonella paratyphi*-A at 200 mg/mL. It is followed by solvent chloroform extracts

14.50±0.50mm and 12.33mm zone of inhibition for *Salmonella typhi* and *Salmonella paratyphi*-A respectively. Water extracts shown 11.66±0.66mm and 12.16±0.72mm zone of inhibition against *Salmonella typhi* and *Salmonella paratyphi*-A respectively. The lowest antityphoidal activity shown by hexane extracts 07.50±0.28mm and 09.33±0.88mm zone of inhibition. Zones of inhibition shown by control (100 mg/mL Azithromycin) were 17.66±0.88mm and 19.00±0.57mm for *Salmonella typhi* and *Salmonella paratyphi*-A respectively.

It is evident from the Figure 1 that among the solvent extracts tested for the anti-typhoid activity by Agar Disc-Diffusion assay, ethanol extract was found the best in inhibiting the maximum growth of both pathogens *Salmonella typhi* and *Salmonella paratyphi*-A. It exhibited antityphoid activity at par with Azithromycin used as standard. Graphical representation of antityphoidal potential of *Enicostemma axillare* leaf extracts against *Salmonella typhi* and *Salmonella paratyphi*-A by Agar Disc-Diffusion Assay with control is depicted in Figure 1.

Table 1: The anti-typhoidal potential of *Enicostemma axillare* leaf extracts against *Salmonella typhi* and *Salmonella paratyphi*-A by Agar Disc-Diffusion Assay.

Sr. No.	Solvent extracts	Zone of inhibition in diameter (mm)		
		Salmonella typhi	*Salmonella paratyphi*-A	Mean
1.	Control	00	00	00
2.	Water	11.66±0.66	12.16±0.72	11.91
3.	Ethanol	16.00±0.57	14.16±0.44	15.08
4.	Chloroform	14.50±0.50	12.33±0.88	13.41
5.	Hexane	07.50±0.28	09.33±0.88	08.41
6.	Standard (Azithromycin)	17.66±0.88	19.00±0.57	18.33
7.	Mean	13.46	13.39	13.43

Note: Values are presented as mean ± Standard error (n = 3).

Figure 1: The anti-typhoidal potential of *Enicostemma axillare* leaf extracts against *Salmonella typhi* and *Salmonella paratyphi*-A by Agar Disc-Diffusion Assay.

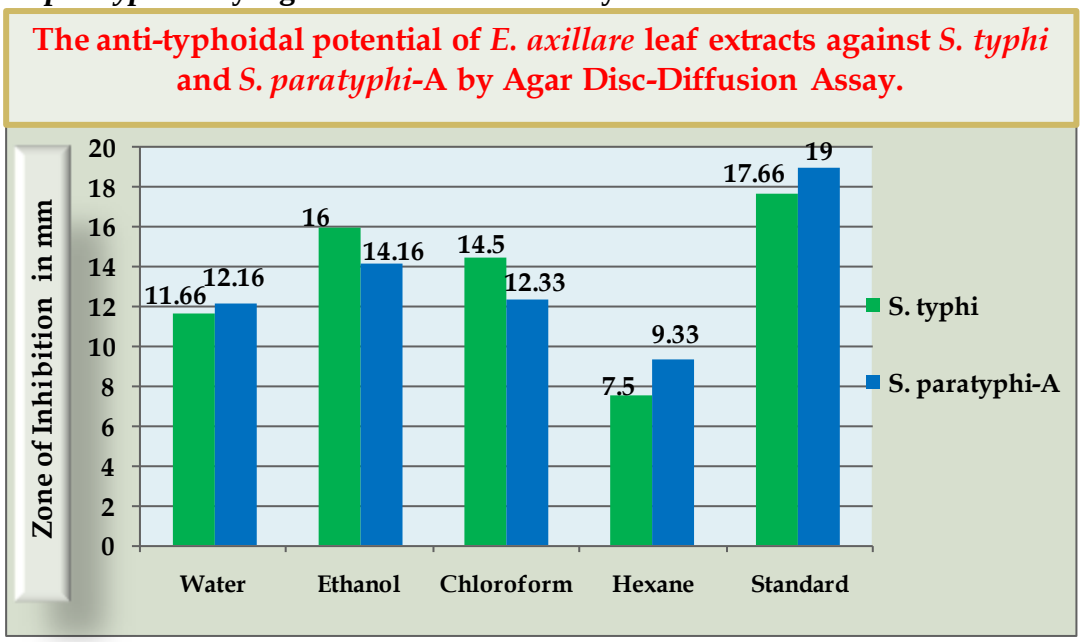

Table 2: The MIC of *Enicostemma axillare* leaf extracts against *Salmonella typhi* and *Salmonella paratyphi*-A.

Sr. No.	Solvents	% of Growth inhibition at concentration (mg/mL)	
		Salmonella typhi	***Salmonella paratyphi*-A**
1.	Water	12.5	12.5
2.	Ethanol	12.5	12.5
3.	Chloroform	12.5	12.5
4.	Hexane	25	25
5.	Standard (Azithromycin)	6.25	6.25

Minimum inhibitory concentration (MIC) of water, ethanol, chloroform and hexane extracts of *Enicostemma axillare* leaf against *Salmonella typhi* and *Salmonella paratyphi*-A is presented in presented in Table 2. The result shows that dilutions of various concentrations of the plant leaf extracts are active against test isolates. The MIC ranges from 6.25 – 25 mg/mL.

Both bacterial strains are tested against five different concentrations of plant extracts (100, 50, 25, 12.5, 6.25 mg/ml) and compared with standard (Azithromycin). The percent inhibition growth is measured and recorded. Each test was performed three times and then corresponding mean value is taken. The water, ethanol and chloroform extracts of *Enicostemma axillare* leaf showed 12.5mg/ml MIC against *Salmonella typhi* and *Salmonella paratyphi*-A. The hexane extract of leaf showed 25 mg/ml MIC against both bacteria tested.

III.DISCUSSION:

The results of the antityphoidal assay obtained by agar disk diffusion method are presented in Table 1 as inhibition zones. The antityphoidal properties are measured in terms of the inhibition zone (mm); it is the area around the disk where the plant extract is able to inhibit bacterial growth. The higher the inhibition zone, the higher the antibacterial properties of the tested plant sample and it was compared with the inhibition zone of the standard antibiotic gentamicin. The extract of both plant samples inhibited Salmonella typhi and Salmonella paratyphi-A. The results of this study showed that the extracts of *Enicostemma axillare* leave have anti-typhoidal potential. Many plants have been scientifically investigated for their immunomodulatory activities and a large number of plant products have been shown to inhibit the growth of pathogenic bacteria (Naima et al., 2012). Some of these metabolites, especially flavonoids, tannins and alkaloids, have been reported to be responsible for the immunomodulatory activities associated with some ethno-medicinal plants (Atto et al., 2017). The antibacterial properties of the extracts against the test isolates showed that the highest zones of inhibition were observed in *S. typhi* and *S. paratyphi*-A, 16.00$\pm$0.57 mm and 14.16$\pm$0.44 mm, respectively. This result supports the findings of Al-Akel et al., 2017. The plant leaf extracts are known to have antibacterial activity against pathogenic bacteria such as *Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhi* and *Bacillus pumilas* (Brindha et al., 2012).

The antimicrobial activity of the leaf extracts was due to the presence of phytochemical constituents present in the leaves of the plant. The ethanol extract was found to be more active than the other extracts and this supported several studies involving aqueous and organic extracts (Nas and Ali, 2017; Ali et al., 2017 and

Sasidharan et al., 2011) as most of the studies reported that organic solvents were good chemical reagents for the consistent extraction of antimicrobial substances from medicinal plants. The MIC of E. axillare against Salmonella typhi and Salmonella paratyphi-A was recorded 12.5 mg/ml for both strains. This shows that E. axillare is effective against Salmonella typhi. The minimum inhibitory concentration (MIC) value ranged from 12.5 to 25 mg/ml among the test organisms. This showed that the leaves of *Enicostemma axillare* have antibacterial properties against *S. typhi* and *S. paratyphi*-A. This is similar to findings of Muhammad et al., 2020, which reported that in test tubes, *A. indica* has been shown to have significant effects on gram-positive and gram-negative bacteria and other bacteria that cause a wide range of human and animal diseases.

IV.CONCLUSION

II. It can be concluded from this study that *Enicostemma axillare* leaf extracts have antityphoidal activity against *S. typhi* and *S. paratyphi*-A. The widespread use of *Enicostemma axillare* is due to the presence of these bioactive compounds, which may explain its many traditional uses against typhoid fever.

V.ACKNOWLEDGMENT

The authors would like to thank the principal, S. C. M. Ardhapur, Nanded. The authors thank the supervisor Dr. Shinde Saheb L., Professor, Department of Botany, Yeshwant Mahavidyalaya, Nanded, for his inspiring and valuable suggestions.

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