CODEN [USA]: IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

http://doi.org/10.5281/zenodo.1098076

Available online at: http://www.iajps.com Research Article

AN *IN-VITRO* STUDY ON ANTI-INFLAMMATORY AND ANTI-BACTERIAL ACTIVITIES OF ETHYL ACETATE EXTRACT FROM THE LEAVES OF *CYCLEA PELTATA*

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Abstract:

The present study was to evaluate the anti-inflammatory activity and their inhibitions way were investigated by Ethyl acetate extract of Cyclea peltata. *Initially the phytochemical analysis was performed to identify the bioactive compounds responsible for the inflammatory activity. These plant possessed alkaloid, flavonoid, terpenoid and polyphenols, glycosides. Among the phytochemicals identified flavonoid extract found to have higher anti-inflammatory activity against inflammatory enzymes like cycloxygenase and lipoxygenase. The strategies of in-vitro cycloxygenase and lipoxygenase inhibitory activity were used to evaluate the efficacy of these compounds as anti-inflammatory properties. The Ethyl acetate extracts of* Cyclea peltata *was studied, effective antibacterial activity by using the disc diffusion method. The Bacteria used for the determination of antibacterial activities were Staphylococcus aureus,* Enterococcus faecalis, *Escherichia coli and Pseudomonas aeruginosa.*

Key words: Cyclea peltata, *flavonoid, Staphylococcus, Ethyl acetate, cycloxygenase, anti-inflammatory*

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Please cite this article in press as S. Sridhar et al., ***An In-Vitro Study on Anti-Inflammatory and Anti-Bacterial Activities of Ethyl Acetate Extract From the Leaves of Cyclea Peltata****, Indo Am. J. P. Sci, 2017; 4(12).*

INTRODUCTION:

Plants synthesize a vast range of organic compounds that are traditionally classified as primary and secondary metabolites although the precise boundaries between the two groups can in some instances be somewhat blurred. Primary metabolites are compounds that have essential roles associated with photosynthesis, respiration, and growth and development. These include phytosterols, acyl lipids, nucleotides, amino acids and organic acids. Other phytochemicals, many of which accumulate in surprisingly high concentrations in some species, are referred to as secondary metabolites. These are structurally diverse and many are distributed among a very limited number of species within the plant kingdom and so can be diagnostic in chemotaxonomic studies. Although ignored for long, their function in plants is now attracting attention as some appear to have a key role in protecting plants from herbivores and microbial infection, as attractants for pollinators and seed-dispersing animals, as allelopathic agents, UV protectants and signal molecules in the formation of nitrogen-fixing root nodules in legumes. Secondary metabolites are also of interest because of their use as dyes, fibers, glues, oils, waxes, flavorings agents, drugs and perfumes, and they are viewed as potential sources of new natural drugs, antibiotics, insecticides and herbicides [1].

Enzymes also play an essential role in inflammation and other functions of the immune system. Inflammation is one of the body's most important mechanisms for protecting itself against danger. If you've ever had an insect bite, a sprained ankle, a sore throat, or bad sunburn, the process associated with the inflammatory response are complex but important aspects which have been exploited for screening of anti-inflammatory compound are the various functions of neutrophils, the metabolic products of arachidonic acid and the role played by reactive oxygen species (ROS) [2].

Cyclooxygenase an enzyme that acts to speed up the production of certain chemical messengers, called prostaglandins, in a variety of areas of the body such as the stomach, kidneys, and sites of inflammation. In the stomach, prostaglandins promote the production of a protective natural mucus lining. They also interact within certain cells that are responsible for inflammation and other functions. Cyclooxygenase (COX) are lipid metabolising enzymes that catalyse the oxygenation of polyunsaturated fatty acids (PUFA), preferably arachidonic acid (AA), to form the prostanoids, which are potent cell-signalling molecules associated with the initiation, maintenance and resolution of inflammatory processes [3].

Lipoxygenases convert fatty acids into proinflammatory leukotrienes, important local mediators of inflammation. Several potent inflammatory leukotrienes are produced by 5-LOX in mammals. Lipoxygenase enzymes, and the pro-inflammatory factors they produce, have a fundamental role in the inflammatory process by aiding in the recruitment of white blood cells to the site of inflammation. They also stimulate local cells to produce cytokines, which amplifies the inflammatory response [4].

Cyclea peltata (Lam) Hook. F. & Thomas also belongs to Menispermaceae family, which is known as Rajpatha in various parts of India. A much-branched, climbing shrub found throughout South and East India and in the Andaman and Nicobar Islands. Roots tuberous; Leaves deltoid or ovate, acute, truncate or slightly sinuate at the base with rounded angles, mucronate, more or less hairy on the nerves and veins, margin often ciliate; flowers in axillary panicles. Male flowers subsessile, interruptedly spicate or collected into heads. Female flowers racemose, sepals' oblong, glabrous.

MATERIALS AND METHODS:

Plant samples / sources

The Leaves of *Cyclea peltata* were collected from Medicinal Plant Garden at Government Siddha Medical College, Arumbakkam, Chennai-600 106, a recognized institution of Government of Tamil nadu and the Department of AYUSH, Government of India. This plant identified and authenticated by Dr. S. Sankaranarayanan, Head of the Department, Department of Medicinal Botany, Government Siddha Medical College, Arumbakkam, Chennai.

Phytochemical analysis of *Cyclea peltata*

The aqueous extract of *Cyclea peltata* was freshly prepared and various chemical constituents were analysed according to methods described by Harbone (1976) [5]. The different chemical constituents tested for included tannins, saponin, glycosides, alkaloids, terpenoids, anthocynin, polyphenol and flavonoids.

The partial characterization of ethyl acetate extract of *Cyclea peltata* by thin layer chromatography

The ethyl acetate extract of *Cyclea peltata* leaves were loaded on to pre coated TLC (60 F_2 54) and it was developed using solvent system in the ratio of 9.5:2.5:0.4 (Toluene, Dioxan and Acetic acid) visible

and the non visible spot given and it is fluorescent with UV light at 360nm.

The cyclooxygenase inhibition assay was performed according to a modified method of Larsen *et al*., (1996) [6]. A spectrophotometric assay for determination of Lipoxygenase inhibition activity was used as reported [7] (Kemal *et al*., 1987) with slight modification. *In vitro* anti-arthritic activity of ethyl acetate extract from the leaves of *Cyclea peltata*, the method used was "inhibition of protein denaturation" Mishra *et al.*, 2011[8] using diclofenac sodium a standard. The principle involved membrane stabilization is stabilization of human red blood cell membrane by hypo tonicity induced membrane lysis was also conducted.

Antibacterial activity of ethyl acetate extract from the leaves of *Cyclea peltata*

The antibacterial activities of the crude alkaloid extracts were assayed using the disc diffusion method. Bacteria were grown overnight on Mueller Hinton agar plates, five colonies were suspended in 5 ml of sterile saline (0.9%) and the bacterial population in the suspension was adjusted to ~$3x10^8$ CFU/ml. The minimum inhibitory concentrations of the isolated compounds were determined by dilution method [9].

RESULTS AND DISCUSSION:

Phytochemical screening of aqueous of *Cyclea peltata*

The phytochemical screening of the *Cyclea peltata* leaves studied presently showed the presence of alkaloids, flavonoids, phenol, Terpenoids, glycosides and saponin, and absence of glycosides and tannin (Table -1).

Table 1: Phytochemical screenings of extract of *Cyclea peltata*

Sl. No.	Phytochemical Constituents	Observation	Extract of *Cyclea peltata*
1	**Alkaloids** -Dragendorff's test -Mayers test	Orange / red precipitate Creampieppt	+ +
2.	**Flavonoids** -Alkalai Reagent -Lead aceate test	Intense yellow colour Precipitate formed	+ +
3.	**Glycosides** -Keller-Killiani test	Pink colour (Ammonia layers)	-
4.	**Tannin** -$FeCl_3$ test	Blue-blackcolour	+
5.	**Saponins** -Frothing test	Foam	-
6.	**Terpenoids** -Salkowski test	Reddish brown colour ring formed in interface	+
7.	**Polyphenols** -Ferrozine test	Raddish blue	+
8.	**Anthocyanin** **-Ammonia test**	Pink color in ammonia layer	-

The Partial characterization of ethyl acetate extract from the leaves of *Cyclea peltata* by TLC

The ethyl acetate extract from the leaves of *Cyclea peltata* was loaded on Pre-coated TLC plates (60 F_2 54 Merck) and developed with a solvent system of toluene, dioxan and acetic acid in the ratio of 9.5:2.5:0.4 was efficient to extract the anti-inflammatory compound it is used for further studies. The developed plate was viewed under UV 240nm and 360nm (Table-2 and Fig-1).

Table 2: The Partial characterization of ethyl acetate extract from the leaves of *Cyclea peltata* by TLC

Component No.	Ethyl acetate extract from the leaves of *Cyclea peltata*		
	240nm UV light	360nm UV light	Normal visible light
1	-	0.42	--
2	-	0.57	--
3	0.63	0.63	0.63
4	0.68	0.68	0.68
5	-	0.77	-
6	-	0.87	-
7	0.95	0.95	-

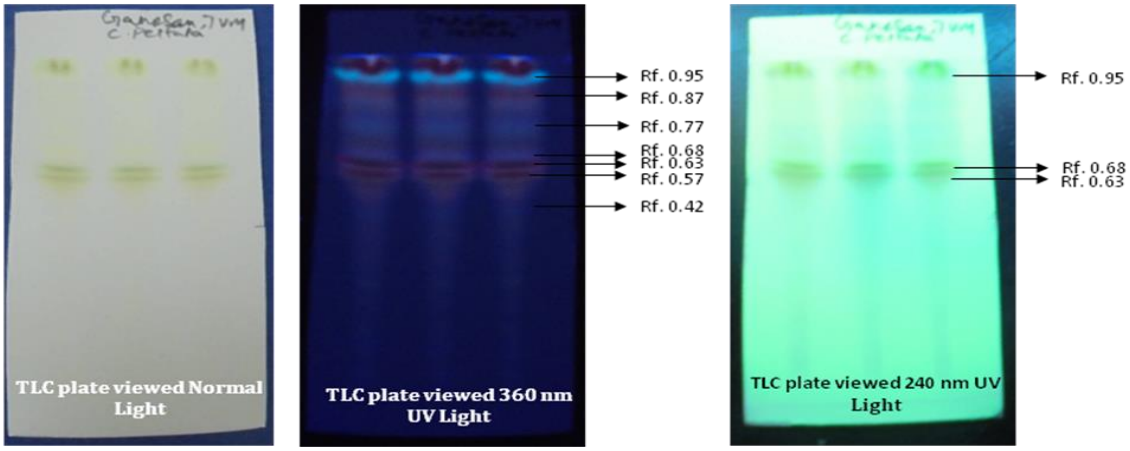


Fig. 1: The Partial characterization of ethyl acetate extract from the leaves of *Cyclea peltata* by TLC

Cycloxygenase 2 inhibition activity of ethyl acetate extract from the leaves of *Cyclea peltata*

The inhibitory effects of COX-mediated N,N,N′,N′-tetramethyl phenylenediamine oxidation activity were examined using purified COX as enzyme sources COX-2 activity was stronglyinhibited by used in this study ethyl acetate extract from the leaves of *Cyclea peltata*, inhibition potency quite different. Based on theresults obtained in Table-3. The following experiments focused on theinhibition potential of ethyl acetate extract from the leaves of *Cyclea peltata* for COX-2 activity. Whereas, the standard drug Celecoxib inhibited the COX_2 enzyme with an72.63µl/ml. Some studies have demonstrated that flavonoid possess anti-inflammatory activities by inhibition of cyclooxygenase-2 (COX-2) expression in lipopolysaccharide (LPS)-activated RAW 264 cells or inhibiting inducible nitric oxide (iNOS) protein and mRNA expression in LPS-activated murine J774 macrophages and such activities appear to be structure dependent. COX-2 seems to be involved in many inflammatory processes. Some antioxidants inhibit the expression of COX-2 by interfering with the signalling mechanisms that regulate the COX-2 gene [10].

Table 3: Inhibition activity of Cycloxygenase 2of ethyl acetate extract from the leaves of *Cyclea peltata*

Ethyl acetate extract Concentration	Inhibition percentage of COX 2
5 µl	19.85±1.26
10 µl	33.65±0.84
15 µl	55.48±0.69
20 µl	75.69±2.54
Diclofenac Sodium (+ve control)	72.63±0.58

Results are expressed as percentage inhibited diphenalase formation with respect to control. Each value represents the mean + SD of five experiments

Lipoxygenase inhibition activity of ethyl acetate extract from the leaves of *Cyclea peltata*

The inhibition of LOX using linoleic acid as substrate was determined for the anti-inflammatory activity in the ethyl acetate extract from the leaves of *Cyclea peltata.* The ethyl acetate extract at 20µl/ml concentration exhibited more inhibition than the other concentration. The inhibition percentage was above 82.47% at20µl/ml (Table-4). The standard diclofenac sodium was showed 76.25% inhibition at 20µg/mL. The ethyl acetate extract from the leaves of *Cyclea peltata* was showed higher inhibition activity than positive control. Lipoxygenase catalyzes the addition of molecular oxygen to fatty acids containing a *cis, cis*-1, 4-pentadiene system. This reaction originates unsaturated fatty acid hydroperoxides. These products are further converted into others that play a key role in inflammatory processes. Hence, *Cyclea peltata* compounds which are able to inhibit that enzyme can be considered as antioxidants and possessing anti-inflammatory properties. LOXs are sensitive to antioxidants as antioxidants are involved in inhibition of lipid hydroperoxide formation due to scavenging of lipidoxy or lipidperoxy-radicals. This could lead to less availability of lipid hydroperoxide substrate required for LOX catalysis [11]. Another hypothesis proposed indicated that inhibition by antioxidant could be attained via chelation of its non-heme bound iron or by reduction of its ferric form Lin *et al*., 2001 [12], suggesting a competitive kind of inhibition as reported for *Mahonia aquifolium* (Rackova *et al*., 2007). The present work would like to speculate that LOX inhibition could be due to antioxidant properties of the anthocynin extract with the mechanism of action to be elucidated.

Table 4: Inhibition activity of Lipoxygenase of ethyl acetate extract from the leaves of *Cyclea peltata*

Ethyl acetate extract Concentration	Inhibition percentage of LOX
5 µl/ml	16.58±2.56
10 µl/ml	33.98±1.49
15 µl/ml	55.63±2.41
20 µl/ml	82.47±1.69
Diclofenac Sodium (+ ve control)	76.25±1.53

Results are expressed as percentage inhibited Lipoxygenase with respect to control. Each value represents the mean + SD of five experiments

Protein denaturation inhibition of ethyl acetate extract from the leaves of *Cyclea peltata*

Examination of ethyl acetate extract from the leaves of *Cyclea peltata* of momentous activity on inhibition of protein denaturation and its effect was compared with the standard drug Diclofenac sodium. The production of auto antigen in certain arthritic disease may be due to denaturation of protein. From the results of present study it can be stated that alkaloid extract is proficient of controlling the production of auto antigen and inhibits denaturation of protein in rheumatic disease. The maximum percentage inhibition of protein denaturation was observed as 69.87% at 20µg/ml which was close to the percentage of inhibition of diclofenac sodium (65.24%) (Table-5).

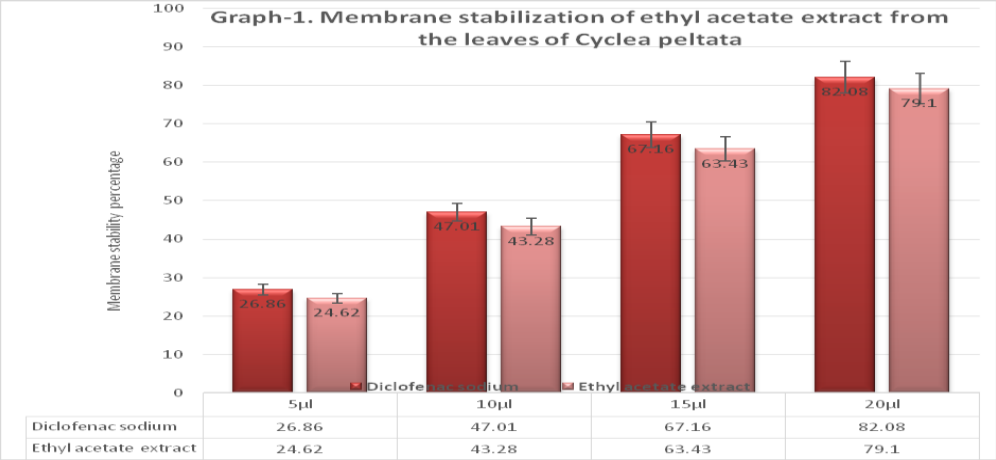
Table 5: Inhibition activity of protein denaturation of ethyl acetate extract from the leaves of *Cyclea peltata*

Flavonoid Concentration	Inhibition percentage of LOX
5 µl/ml	17.63±2.56
10 µl/ml	30.49±1.49
15 µl/ml	52.59±1.63
20 µl/ml	69.87±1.47
Diclofenac sodium (+ve control)	65.24±0.7

Results are expressed as percentage inhibited inhibition of protein denaturation with respect to control. Each value represents the mean + SD of five experiments

Membrane stabilization of ethyl acetate extract from the leaves of *Cyclea peltata*

The ethyl acetate extract from the leaves of *Cyclea peltata* exhibited membrane stabilization effect by inhibiting hypotonic induced lysis of erythrocyte membrane. The erythrocyte membrane is related to the lysosomal membrane and its stabilization implies that the fraction may also well stabilize lysosomal membrane. From the obtained results (Graph-1) it was concluded that the ethyl acetate extract from the leaves of *Cyclea peltata* has significant membrane stabilizing activity which was comparable to the standard diclofenac sodium. The ethyl acetate extract from the leaves of *Cyclea peltata* was effective in inhibiting the heat induced hemolysis of erythrocyte membrane and its effectiveness was dose dependent. This fact provides an evidence for membrane stabilization as an additional mechanism of its anti-inflammatory effect. These fractions may possibly inhibit the release of lysosomal content of neutronphils at the site of inflammation.



Effect of the Ethyl acetate extract from the leaves of *Cyclea peltata* on the growth of Pathogenic bacteria by disc diffusion method

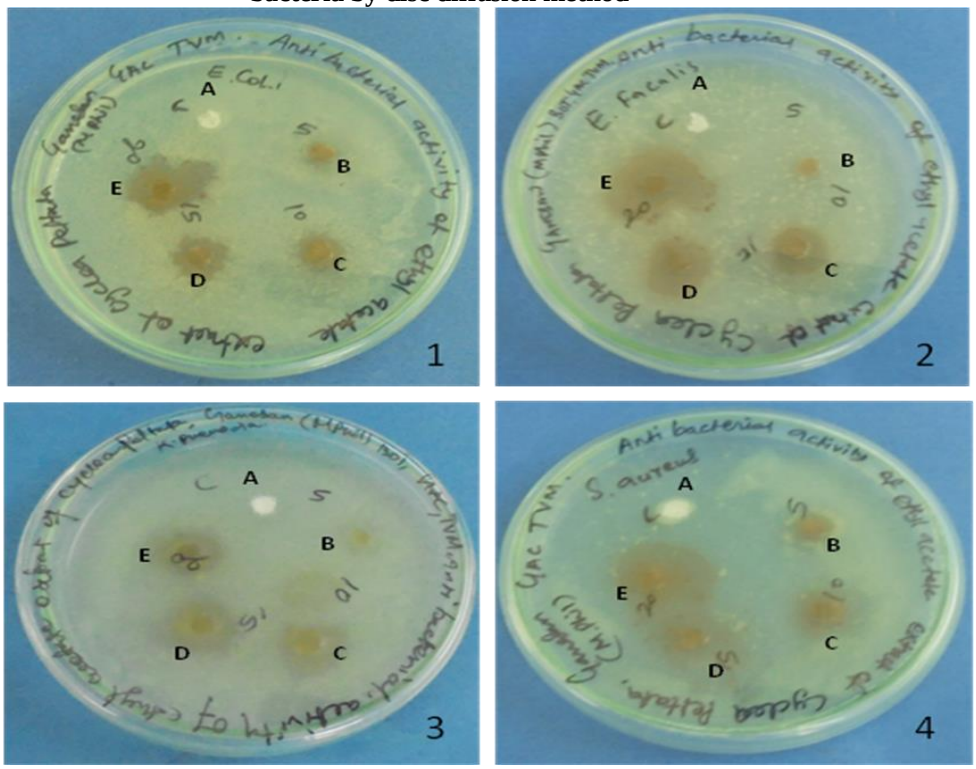
The Ethyl acetate extract from the leaves of *Cyclea peltata* at different concentration (5, 10, 15 and 20 µl/ml) was tested against *Staphylococcus aureus* and *Escherichia coli*, *Enterococcus faecalis* and *Klebsiella pneumoniae*. The Ethyl acetate extract from the leaves of *Cyclea peltata* exhibited more bactericidal action in against *Staphylococcus aureus*, *Klebsiella pneumonia* than *Escherichia coli* with higher inhibition zone was found at 20µl/ml concentration (Table-6 and Fig-2). Similar results were obtained by different workers in various systems[13]. The inhibitory effect of the extract on the growth of microorganisms could be attributed to the presence of some phytochemicals that were found present in the plant extract. The demonstration of antibacterial activity against both gram positive and gram negative bacteria by this plant may be indicative of the presence of broad spectrum antibiotic compounds [14]. The present study justifies the claimed uses of *C. peltata* in the traditional system of medicine to treat various infectious diseases caused by the microbes.

Table 6: The antibacterial activity of the Ethyl acetate extract from the leaves of *Cyclea peltata* by disc diffusion method.

Pathogenic bacteria	The Ethyl acetate extract from the leaves of *Cyclea peltata* exhibited the Zone of inhibition (mm)[a]				
	Positive control 10 µl Ampicillin	Different concentrations Crude extract (µl/ml)			
		5 µl	**10** µl	**15** µl	**20** µl
Staphylococcus aureus	13mm	9mm	11mm	13mm	15mm
Escherichia coli	15mm	7mm	9 mm	11mm	13mm
Enterococcus faecalis	14mm	8 mm	10 mm	12 mm	13 mm
Klebsiella pneumoniae	12mm	10mm	12mm	15mm	17mm

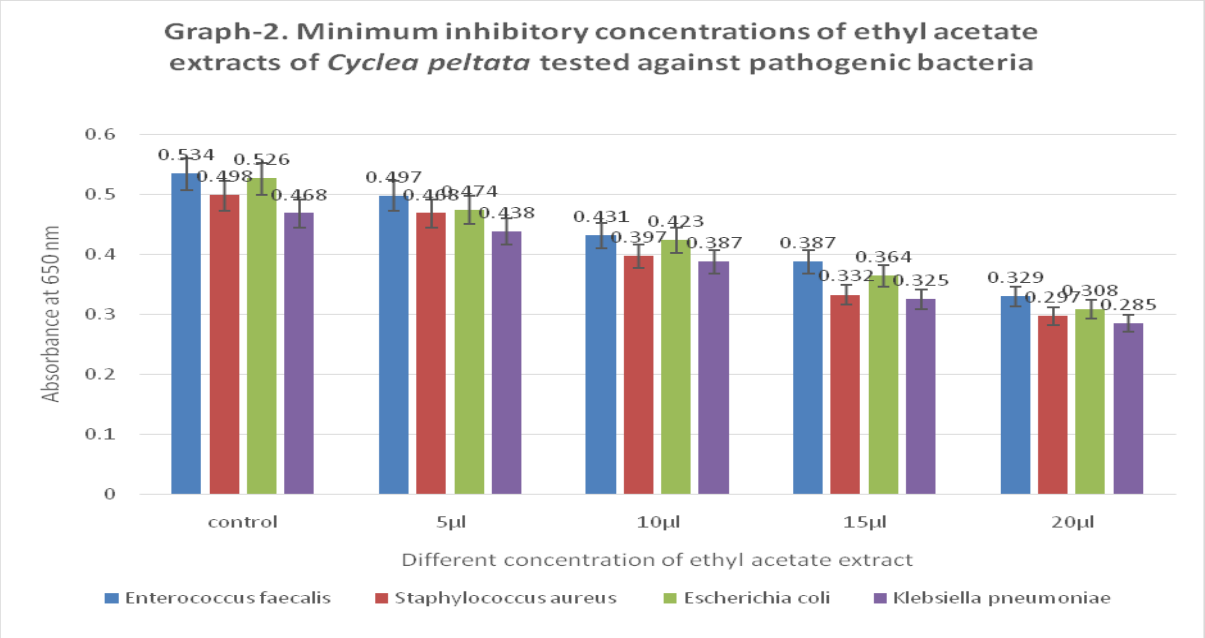
[a]The inhibitory diameter was measured by means of calipers. All the assays were duplicated, and the mean values were recorded.

Fig. 2: Effect of the Ethyl acetate extract from the leaves of *Cyclea peltata* on the growth of pathogenic bacteria by disc diffusion method



Minimum inhibitory concentrations of ethyl acetate extracts of *Cyclea peltata* tested against pathogenic bacteria

Ethyl acetate extracts of *Cyclea peltata* was investigated for their antibacterial activities. Ethyl acetate extracts showed inhibitory activities against *Escherichia coli, Klebsiella pneumoniae, Enterococcus faecalis, and Staphylococcus aureus* strains. The MIC concentrations were mostly very high and ranged from 5 to 20 µg/mL.The Ethyl acetate extracts of *Cyclea peltata* was most active against *E.coli, K. pneumoniae, E. faecalis, S. aureus* were resistant to all tested extracts. This will be of substantial advantage in fighting the menace of antibiotic refractive pathogens that are so prevalent in recent times (Graph-2).



CONCLUSION:

Ethyl acetate extracts of *Cyclea peltata* hold great secure for natural treatments of inflammation that are safe and effective and can be provided as dietary supplements, added to multiple vitamins, and incorporated into food products to create functional foods. In addition, the novel bioactives identified in the Ethyl acetate extracts of *Cyclea peltata* when fully characterized, could prove to be promising new drug leads for Cycloxygenase, and Lipoxygenases as well as triple inflammatory enzyme inhibitors for treatment of a range of inflammatory and infective bacterial diseases that are safe and efficacious.

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