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Metabolic Amalgam of *Calotropis gigantean* and Its Anti-Inflammatory Activity

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Abstract: The present investigation exposed that the extracts of *Calotropis gigantean* leaf have potent phytochemical and antimicrobial activity which explains its use in traditional medicines. he extracts of *Calotropis gigantean* were originate to be more or less active against almost all tested pathogenic strains. Hence, *Calotropis gigantean* can source of natural antimicrobials that can serve as a substitute to conventional medicine The qualitative analysis of the extracts from the leaf sample of *Calotropis gigantean* showed the presence of phytochemical constituents such as tannins, saponin, flavonoids, steroid, lipids, amino acids and terpenoids. The active compounds such as amino acids, flavonoids, alkaloids, lipids have *in-vitro* anti inflammatory activity in comparison to Standard drug.

Key words: *Calotropis gigantean* · Saponin · Flavonoids and Steroid

INTRODUCTION

Many of the pharmaceuticals currently available to physicians have a long history of use as herbal remedies, including opium, aspirin, digitalis and quinine. Among the 120 active compounds currently isolated from the higher plants and widely used in modern medicine today, 80 percent show a positive correlation between their modern therapeutic use and the traditional use of the plants from which they are derived. More than two thirds of the world's plant species - at least 35,000 of which are estimated to have medicinal value, come from the developing countries. At least 7,000 medical compounds in the modern pharmacopoeia are derived from plants. Many medicinal and aromatic plants (MAPs) significant variations of plants characteristics have been ascertained with varying soil traits and the selective recovery and subsequent release in food of certain elements have been demonstrated. Great attention must be paid to choose soil and cropping strategies, to obtain satisfactory yields of high quality and best-priced products, respecting their safety and nutritional value.

Inflammation is the body's attempt at self-protection; the aim being to remove harmful stimuli, including damaged cells, irritants, or pathogens and begin the healing process. Inflammation does not mean infection, even when an infection causes inflammation. Infection is caused by a bacterium, virus or fungus, while inflammation is the body's response to it. However, sometimes inflammation can cause further inflammation; it can become self-perpetuating. More inflammation is created in response to the existing inflammation. Plaque in coronary artery disease linked to inflammation, scientists from Stanford University, California, linked 25 new genetic regions to coronary artery disease. They found that people with coronary artery disease, the leading cause of death globally, are most likely predisposed to the disease because they have gene variants linked to inflammation.

Inflammation is a part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells or irritants. It is characterized by redness, swollen joints, joint pain, its stiffness and loss of joint function. Inflammation is currently treated by NSAIDs. Unfortunately these drugs cause increased risk of blood clot resulting in heart attacks and strokes.

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Therefore, the developments of potent anti- inflammatory drugs from the natural products are now under considerations. Natural products are rich source for discovery of new drugs because of their chemical diversity. A natural product from medicinal plants plays a major role to cure many diseases associated with inflammation. The conventional drug available in the market to treat inflammation produces various side-effects. Due to these side-effects, there is need for the search of newer drugs with less or no side-effects. There are hundreds of phyto constituents reported to have many pharmacological activities although most of these reports are of academic interest and very few find entry in clinical trials Kumar *et al.* [1].

MATERIALS AND METHODS

Collection of Samples and Extractions: Leaves of *Calotropis gigantean* were collected from Herbal garden, Nehru Arts and science Coimbatore. The Leaves were washed under running tap water to eliminate dust and other foreign particles and to clean these thoroughly. The fresh Leaves were trodden into small pieces, powdered and mixed in 1:10 ratio with distilled water and methanol separately. The extractions were obtained through continuous grind using mortar and pestle followed by filtration using Whattman No.1 filter paper. The residues were re-dissolved with the appropriate solvents for the further analysis.

Phytochemical Components: Phytochemical analyses were carried out according to the methods described by Trease and Evans[2] of the extracts of leaves for the identification of phytochemicals like, alkaloids, saponin, steroids, flavonoids, Cynogenic glycosides, Cardiac glycosides, Phlobatannins and phenols.

Protein Determination: Protein content in the plant extracts were estimated by the method described by Lowry *et al.* [3]. 0.2ml and 0.4ml of extract was used to determine the protein content.

Estimation of total Antioxidant [Phosphomolybdenum method-Prieto *et al.* [4]: Aliquot of 0.1 ml sample was obtained with 1 ml of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95ºC for 90 mints. After the samples were cooled to room temperature, the absorbance of the aqueous solution of each tube was measured at 695 nm against blank, a typical blank solution contained 1ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same as rest of the sample. For sample of unknown composition, water soluble antioxidant capacity was expressed as equilates of ascorbic acid (1 ml of extracts).

Phenolic Content as Tannic Acids Jayaprakasha *et al.* [5]: The extracts were dissolved in a mixture of methanol and water (6:4 v/v).sample (0.2ml) were mixed with 1 ml of tenfold diluted folin-ciocalteus reagents and 0.8 ml of 7.5% sodium carbonate solution, after standing for 30 mints at room temperature, the absorbance was measured at 765 nm. The estimation of phenolic compound in the fractions was carried out in triplicate and the results were averaged.

Antibacterial Activity: *Klebsiella, Proteus, Shigella, Escherichia coli* and *Salmonella* were used for the study. Three or four isolated colonies were inoculated in the 2 ml nutrient broth and incubated till the growth in the broth was equivalent with Mac-Farland standard [0.5%] as recommended by WHO(2009). Antibacterial activity of the different extracts was determined by cup diffusion method on Muller Hinton agar medium by Anon [6]. Wells are made in Muller Hinton agar plate using cork borer [5 mm diameter] and inoculums containing bacteria were spread on the solid plates with a sterile swab moistened with the bacterial suspension. 20-80 µl of the working suspension/solution of plant extracts and same volume of distilled water and methanol for control were filled in the wells with the help of micropipette. Plates were kept for some time till the extract diffuse in the medium and incubated at 37°C for 24 h. After incubation, the plates were observed for the zone of inhibition [ZI], the diameter of the inhibition zone were measured and recorded.

Minimum Inhibitory Concentration (MIC)–Dilution Method: To determine Minimum Inhibitory Concentration (MIC) 1 ml of nutrient broth was taken in 10 test tubes. Different sets were prepared for each bacterium. Different concentrations of plant extracts ranging from 1mg to 10mg/ml were added into test tubes. To this 50µl of an overnight broth culture of each bacterium were inoculated in the respective tubes and the tubes were incubated for 24 hours at 37°C. One tube was inoculated with 1ml sterile nutrient broth with the 50µl of an overnight broth culture of each bacterium and placed at +4°C in a refrigerator overnight to be used as standard for the determination of complete inhibition. MIC is expressed as the lowest

dilution, which inhibited growth judged by lack of turbidity in the tube. Because very faint turbidity may be given by the inoculum itself, the inoculated tube kept in the refrigerator overnight may be used as the standard for the determination of complete inhibition.

Separation of Active Compound from Extracts from *Calotropis gigantean* by Thin Layer Chromatography (TLC): The glass slides were cleaned and dried in hot air oven. Slurry was prepared by mixing silica gel with double the volume of distilled water in a clean beaker. One drop of slurry was placed on the slide by using another slide edge, the drop of slurry was scattered all over to make thin film. The slides were kept as such for few minutes. Then the chromo plates were activated by heating in hot air oven at 120°C for 30 min. The slides were allowed to cool at room temperature and marked about 2 cm from the bottom as the origin. The working suspensions were loaded at the center of the each slide above from the edge.

The development tank was saturated with suitable solvent according to Eskil Hultin [7].

Alkaloids :	Benzene/ Methanol-80:20
Flavanoids:	Chloroform/Methanol-70:30
Lipid :	Chloroform/Methanol/water-10:10:3
Terpeniods :	Acetic acid/water-1:3

The slides were kept in the tank without touching baseline by solvent. The final solvent front was marked and the slides were dried.

Spot Visualization: For visualization of Flavonoids 1% ethanolic solution of Aluminium chloride was used and viewed under 560nm UV light. Alkaloids and Terpenoids were visualized under UV light and they were visible as yellow and orange fluorescent spots. Few pieces of iodine crystals were kept in the tank and covered with glass plate to saturate the tank with iodine vapor for detecting lipids. The plate was then kept in iodine vapor saturated tank and left for few hours and brown colored spots were visualized.

Retrieval of the Active Compound: Spots on the preparative silica gel slides were scratched with the help of clean and dry spatula and collected in beaker containing appropriate solvents [Bishnu Joshi,[8]] and left overnight. The content in the beaker was stirred and filtrated through Whattsman no.1 filter paper. The filtrate was collected in clean and dry beaker. The filtrate containing active compound was used for the determination of antimicrobial effect against *Salmonella* and *Klebsiella* by cup diffusion method.

***In vitro* Anti-inflammatory Activity**

Inhibition of Albumin Denaturation: Methods of Sakat *et al.* [9] followed with minor modifications. The reaction mixture was consisting of test extract and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted. The sample extracts were incubated at 37°C for 20 min and then heated to 51°C for 20 min. after cooling the samples the turbidity was measured by spectrophotometrically at 660nm. The experiment was performed in triplicate. Percent inhibition of protein denaturation was calculated as follows,

% inhibition= Abs control- Abs sample
Abs control X 100

Where Abs control is the absorbance without sample, Abs sample is the absorbance of sample extract/standard.

Membrane Stabilization Test: Preparation of red blood cells (RBCs) suspension

Fresh whole human blood (10 ml) was collected and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10min and were washed three times with equal volume of normal saline. The volume of blood was measured and re constituted as 10% v/v suspension with normal saline Sadique *et al.*[10]; Saket *et al.* [9].

Heat Induced Hemolytic: The reaction mixture (2 ml) consisted of 1 ml of test sample solution and 1 ml of 10% RBCs suspension, instead of test sample only saline was added to the control test tube. Ibuprofen was used as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in water bath at 56°C for 30min. At the end of the incubation the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. The experiment was performed in triplicates for all the test samples. Percent membrane stabilization activity was calculated by the formula mentioned above Shinde *et al.* [11], Saket *et al.* [9].

Protein Inhibitory Action: The test was performed according to the modified method of Oyedepo *et al.* [12] and Sakat *et al.* [9]. The reaction mixture (2 ml) was containing 0.06mg trypsin, 1ml of 20mM Tris HCl buffer

(pH7.4) and 1ml test sample of different concentrations of different solvents. The reaction mixture was incubated at 37°C for 5min and then 1ml of 0.8% (W/V) casein was added. The mixture was inhibited for an additional 20 min, 2ml of 70% perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210nm against buffer as blank. The experiment was performed in triplicate. The percentage of inhibition of proteinase inhibitory activity was calculated.

RESULTS

Phytochemical Analysis: Qualitative phytochemical analyses were performed according to the methods described for the detection of alkaloids, saponin, steroids, flavonoids, cardiac glycosides, cynogenic glycosides and phlobatannins. The observed results were tabulated [Table 1].

Protein Determination: Standard graph was plotted for bovine serum albumin using spectrophotometric reading at 660 nm and protein content in aqueous extract about 2.8mg/g and methanolic extract was about 8.4 mg/g.

Total Anti Oxidant and Phenolic Content: The absorbance of the aqueous solution of each tube was measured for total anti oxidant content at 695 nm against blank it was observed that leaves contain 13.71 mg/g and 10.70 mg/g of anti oxidant in methanol and aqueous extract. The absorbance was measured at 765 nm for Phenolic content. The estimation of phenolic compound in the fractions was carried out in triplicate and the results were averaged. Leaves contain 11.56 mg/g and 12.55 mg/g of phenolic content in methanol and aqueous extract. The results were tabulated in Table 3.

Antibacterial Property: Minimal inhibitory concentration assay was carried out for methanolic extraction *Calotropis gigantean* against *Shigella, Proteus, Klebsiella, Escherichia coli* and *Salmonella*. Table 4 illustrated the minimal concentration level which required to kill the pathogen. Antibacterial properties of the *Calotropis gigantean* extracts were carried out by well diffusion method. The pathogens *Shigella, Proteus, Klebsiella, Escherichia coli* and *Salmonella* were tested for antibacterial assay. *Calotropis gigantean* leaves showed 1.7 cm the maximum zone of inhibition in Shigella followed by Proteus and minimum zone of inhibition found in methanol extract in *Escherichia coli* and *Klebsiella* which is tabulated in Table 5.

Table 1: Phytochemical analysis of *Calotropis gigantean*

Phytochemical Constituents	leaves ---------- Aqueous	Methanol
Alkaloids	+	+
Steriods	+	+
Saponin	+	+
Cynogenic glycosides	+	+
Cardiac glycosides	-	-
Flavonoids	+	+
Phlobatannins	-	-
+Phenols	+	+

'+' – Present '-'–Absent

Table 2: Total protein content of *Calotropis gigantean*

Samples	Concentration of proteins (mg/g)
Aqueous	2.8
Methanol	8.4

Table 3: Total anti oxidant and phenol content of *Calotropis gigantean*

Sample	Anti oxidant content OD at 695 nm (mg/g)	Phenolic content OD at 765 nm (mg/g)
Methanol	13.71	11.56
Water	10.70	12.55

Table 4: MIC assay (methanol extraction) of *Calotropis gigantean*

Test organisms	Leaf OD at 540 nm ---------- 60µl	80µl	100µl
Salmonella typhi	0.57	0.35	0.13
*pseudomonas*sp.	0.42	0.32	0.10
Escherichia coli	0.16	0.50	0.42
Klebsiella	0.79	0.21	0.19
Shigella	0.53	0.21	0.09

Separation of Active Compound from Thin Layer Chromatography (TLC): Table 6 represented the methanol and aqueous extract contain alkaloids, flavonoids, lipids and terpenoids in the Rf value of 0.45, 0.5, 1.2 and 0.66. The separated active compounds alkaloid, flavonoids, lipids and terpenoids were found that effective against *Salmonella* and *Klebsiella*, the zone of inhibition tabulated in table 7 which represented the maximum zone of inhibition of leaves extract found in *Klebsiella* in the active compound of flavanoids and minimum zone of inhibition found in active compound of lipid. Figure 1 represented the zone of inhibition of active compounds of alkaloids and lipids.

Table 5: Antimicrobial activity of methanolic *Calotropis gigantean*

	Zone of inhibition(in diameter)				
	Leaf				Standard drug (amp)
Test organisms	20µl	40µl	60µl	80µl	20µl
Salmonella typhi	0.8cm	1.3cm	1.4cm	1.7cm	1.2
Shigella	-	1.2cm	1.3cm	1.5cm	1
Escherichia coli	-	1.2cm	1.4cm	1.6cm	1.1
Proteus	-	1.2cm	1.6cm	2.0cm	0.9
Klebsiella	-	0.8cm	1cm	1.5cm	-

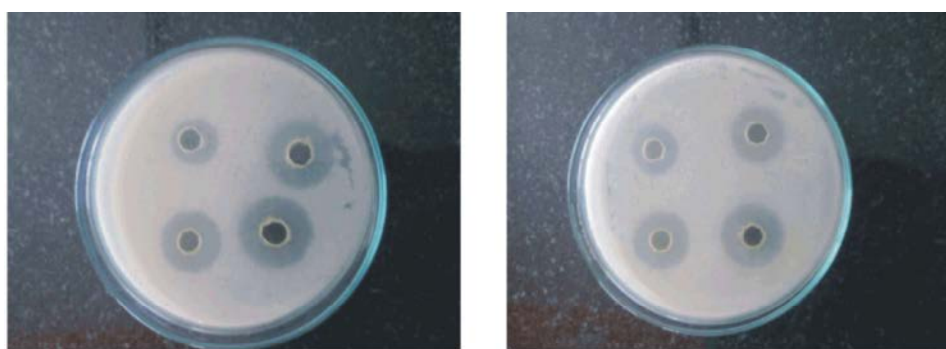

Fig. 1: Antimicrobial activity of active compounds from *Calotropis gigantean*

Table 6: Rf values of phytochemicals of *Calotropis gigantean*

Sample	Flavonoids	Alkaloids	Lipids	Terpenoids
Methanol	0.5	0.45	1.2	0.66
Aqueous	0.45	0.45	1.1	0.62

Table 7: Antimicrobial activity of active compounds of methanolic extract of *Calotropis gigantean*

	Zone of inhibition (in diameter)	
	Test organism	
Active compounds	*Salmonella*	*Klebsiella*
Lipid	1.9	2
Flavonoids	2	2.5
Alkaloids	2.3	2.1
Trepanoids	2.2	2.4

In-vitro Anti Inflammatory Activity

Inhibition of Albumin Denaturation: Denaturation of proteins is a well documented cause of inflammation. As part of the investigation on the mechanism of the anti inflammation activity, ability of extract protein denaturation was studied. It was effective in inhibiting heat induced albumin denaturation [Table 8]. Leaf extract had inhibition about 40%. Ibuprofen, a standard anti-inflammation drug showed the maximum inhibition 56.4% at the concentration of 200 µg/ml.

Membrane Stabilization Test: Stabilization of RBCs membrane was studied for further establishes the mechanism of anti-inflammatory action of aqueous extract of *Calotropis gigantean*. The extracts were effectively inhibiting the heat induced hemolysis.

Table 8: Effect of water extract of *Calotropis gigantean* on albumin denaturation, membrane stabilization and proteinase inhibitory activity percentage inhibition

Samples	Albumin denaturation	Membrane stabilization	Proteinase inhibition
Aqueous extract of *Calotropis gigantean*	40%	45%	48%
Lipid	12%	13%	15
Flavonoids	10%	11%	14.6%
Alkaloids	5%	6.8%	9%
Trepanoids	8%	10.1%	13%
Ibuprofen(200 µg/ml)	54.6	57.6%	58.2%

These results provide evidence for membrane stabilization as an additional mechanism of their anti inflammatory effect. This effect may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation. The extracts inhibited the heat induced hemolysis of RBCs to varying degree [Table 8]. The inhibitions of leaf was about 45 %. The Ibuprofen standard drug standard drug showed the maximum inhibition 57.6%.

Proteinase Inhibitory Activity: The *Calotropis gigantean* aqueous extract exhibited significant anti proteinase activity from different parts. The maximum inhibition was observed from leaves [48%]. The standard Ibuprofen [58.2%] drug showed the maximum proteinase inhibitory action [Table 8].

DISCUSSION

Medicinal herbs as potential source of therapeutics aids has attained a significant role in health system all over the world for both humans and animals not only in the diseased condition but also as potential material for maintaining proper health. Determining the biological (activity) properties of plants used in traditional medicine is helpful to the rural communities and informal settlements. Several scientific investigations are currently being undertaken to isolate the active compounds by bioassay-guided fractionation from the species that showed high biological activity during screening.

The presence of alkaloids, saponin, steroids, flavonoids, cardiac glycosides and cynogenic glycosides. The aqueous and methanolic extract of both leaves and stem of *Calotropis gigantean* contain alkaloids, saponin, steroids, flavonoids, cardiac glycosides and cynogenic glycoside. Phlobatannins was absent in both methanol and aqueous extract of *Calotropis gigantean*. The medicinal values of the secondary metabolites are due to the presence of chemical substances that produce a definite physiological action on the human body. The most important of these substances include, alkaloids, glucosides, steroids, flavonoids, fatty oils, resins, mucilages, tannins, gums, phosphorus and calcium for cell growth, replacement and body building according to Kubmarawa *et al.* [13].

The pathogens *Shigella, Proteus, Klebsiella, Escherichia coli* and *Salmonella* were tested for antibacterial assay. The methanol extract of *Calotropis gigantean* leaves showed 1.7 cm the maximum zone of inhibition in *Shigella* followed by Proteus and minimum zone of inhibition found in methanol extract in *Escherichia coli* and *Kelbsiella*.

The antimicrobial activity of many plant extracts had been previously reviewed and classified as strong, medium or weak according Zaika [14]. The *in vitro* antimicrobial activity of methanolic extracts of some medicinal plants against *Escherichia coli, Salmonella Typhimurium, Staphylococcus aureus* and *Enterococcus sp*. The methanolic extract of *Caryophyllus aromaticus* presented the highest anti-*S. aureus* activity and was effective against all bacterial strains tested by Priscila Ikeda Ushimaru *et al.,* [15].

The inhibition produced by the plant extracts against particular organism depends upon various extrinsic and intrinsic parameters. Due to variable diffusability in agar medium, Therefore Zone of inhibition value has also been computed in this study. *Calotropis gigantean* popularly known as Madagascar periwinkle is a potential source for anti-leukemic alkaloids. To determine antibacterial activity, crude extracts from different parts of *C. roseus* were tested against bacterial strains of clinical significance. Extracts prepared using organic solvents exhibited higher antibacterial activity when compared to their corresponding aqueous extracts. However, no activity was observed in the aqueous extracts. Among the extracts that were significantly active, extract obtained using ethanol exhibited maximum activity against bacterial strains tested. Gram negative stains were more sensitive when compared to Gram positive bacteria. The study implicates that bio-active compound(s) of *C. Roseus* could potentially be exploited as antibacterial agents Ramya *et al.* [16].

Thin layer chromatography techniques were carried out using respective solvents as mentioned in the materials and methods in order to detect the presence of alkaloid, flavonoids, lipids and terpenoids. Reproducible TLC preparations can be guaranteed only if standardized adsorption layers are used. Silica gel is an efficient adsorbent for the separation of most plant extracts [Wagner and Bladt [17]. The system selection entails choosing the mobile phase, the stationary phase and the detection method. While recovering the separated components from silica plates, it should be considered that polar eluants, e.g., alcohol, can dissolve not inappreciable amounts of colloidal silica in it Stahl [18]. Ethanol, methanol and water can dissolve appreciable amounts of silica gel which appear as a residue when the eluant is concentrated Houghton and Raman [19].

Thin layer chromatography has been used for the analysis of natural and synthetic steroids in various environmental materials. The review focused mainly on steroid analysis in environmental materials such as pharmaceuticals, plant products and other biological specimens. The most widely investigated biological specimens are urine and blood plasma or serum. Aqueous extracts of *Azadirachta indica* (Neem) was subjected to thin layer chromatography (TLC) by using different solvent system for the analysis of lipid, alkaloids, flavonoids present in plant extract. The active components separated through TLC were subjected to antimicrobial activity against the pathogens. The present study will be successful in identifying candidate plant with different antimicrobial activity which could be further exploited for isolation and characterization of the novel phytochemicals in the treatment of infectious diseases especially in light of the emergence to produce more effective antimicrobial agents Susmitha *et al.* [20].

Denaturation of proteins is a well documented cause of inflammation. As part of the investigation on the mechanism of the anti inflammatory activity, ability of extract protein denaturation was studied. It was effective in inhibiting heat induced albumin denaturation. Stabilization of RBCs membrane was studied for further establishes the mechanism of anti-inflammatory action of aqueous extract of *Calotropis gigantean*. The extracts were effectively inhibiting the heat induced hemolysis. These results provide evidence for membrane stabilization as an additional mechanism of their anti inflammatory effect. This effect may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation. The extracts inhibited the heat induced hemolysis of RBCs to varying degree Bamidele V *et al.* [21].

CONCLUSION

The extracts of *Calotropis gigantean* were originate to be more or less active against almost all tested pathogenic strains. Hence, *Calotropis gigantean* can source of natural antimicrobials that can serve as a substitute to conventional medicines. From the above investigation it can be conclude that the extract of above *Calotropis gigantean* plants can be considered as a resource for potential anti inflammation and antimicrobial agents.

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REFERENCES

1. Kumar, P. and S. Kalavathy, 2013. Review on a potential herb Calotropis gigantea. Scholars Academic. Journal of Pharmacy. Sch. Acad. J. Pharm., 2: 135-143.
2. Trease, G.E. and W.C. Evans, 1989. Pharmacognosy. London: 13th edn. Bailliere, pp: 176-180.
3. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem., 193: 265.
4. Prieto, P., M. Prenda and M. Aguilar, 1999. Spectrophotometric quantification of antioxidant capacity though the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. Anal. Biochem., 269: 337-341.
5. Jayaprakasha, G.K., R.P. Singh and K.K. Sakariah, 2001. J.Agric Food Chem., 55: 1018-1022.
6. Anonymous, 1996. The Indian pharmacopoeia. Government of India New Delhi. Ministry of Health and family welfare.
7. Eskil Hultin, 1966. Thin layer chromatography of plant extracts. Act a chemica scandinavica, 20: 1588-1592.
8. Bishnu Joshi, 2011. Phytochemical extraction and antimicrobial properties of different medicinal plants. Journal of Microbiology and Antimicrobials, 3: 1-7.
9. Sakat, S., A.R. Juvekar and M.N. Gambhire, 2010. *In vitro* antioxidant and anti-inflammatory activity of methanol extract of Oxalis corniculata Linn. I. J. Pharm. Pharm. Sci., 2: 146-155.
10. Sadique, J.A., W.A. Rqobahs, E.I. Bughaith and A.R. Gindi, 1989. The bioactivity of certain medicinal plants on the stabilization of RBS membrane system. Fitoterapia, 60: 525-532.

11. Shinde, U.A., A.S. Phadke, A.M. Nari, A.A. Mungantiwar, V.J. Dikshit and M.N. Saraf, 1999. Membrane stabilization activity-a possible mechanism of action for the anti-inflammatory activity of Cedrus deodara wood oil. Fitoterapia, 70: 251-257.
12. Oyedapo, O.O. and A.J. Famurewa, 1995. Antiprotease and membrane stabilizing activities of extracts of Fagara Zanthoxyloides, Olax subscorpioides and Tetrapleura tetraptera. Int. J. Pharmacogn, 33: 65- 69.
13. Kubmarawa, D., M.E. Khan, A.M. Punah and Hassan, 2008. Phytochemical Screening and antibacterial activity of extracts from Pakia Clapperotoniana keay against human pathogenic bacteria. Journal of Medicinal Plants Research, 2: 352-355.
14. Zaika, L.L., 1988. Spices and herbs: their antibacterial activity and its determination.. Food Saf., 23: 97-118.
15. Priscila Ikeda Ushimaru, Mariama Tomaz Nogueira da Silva, Luiz Claudio Di Stasi, Luciano Barbosa and Ary Fernandes Junior, 2007. Antibacterial activity of medicinal plant extracts. Brazilian Journal of Microbiology, 38: 717-719.
16. Ramya, S., V. Govindaraji, K. Navaneetha, R. Kannan and Jayakumararaj, 2008. *In vitro* Evaluation of Antibacterial Activity Using Crude Extracts of Catharanthus roseusL. (G.) Don. Ethnobotanical Leaflets, 12: 1067-72
17. Wagner, H. and S. Bladt, 1996. Plant drug analysis-A thin layer chromatography. atlas. Springer, 2: 1-2.
18. Stahl, E., 1969. Thin Layer Chromatography. 2nd edition. Springer-Verlag; Berin, Heinderberg, New York.
19. Houghton, P.J. and A. Raman, 1998. Chromatographical procedures using columns a liquid system in Laboratory handbook for fractionation of natural extraction, 8: 82-93.
20. Susmitha, S., K.K. Vidyamol, P. Ranganayaki and R. Vijayaraghavan, 2013. Phytochemical Extraction and Antimicrobial Properties of Azadirachta indica (Neem). Global Journal of Pharmacology, 7(3): 316-320.
21. Bamidele, V., Owoyele, M. Olubori, Adebukola, A. Adeoye, Funmilayo, E.O. Ayodel and Soladoye, 2008. Anti-inflammatory activities of ethanolic extract of Carica papaya leaves. Journal of Inflammo pharmacology, 16: 168-173.