

Evaluation of *In-vitro* Anti-inflammatory Activity of Aqueous Extract of *Andrographis paniculata*

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Abstract: The studies on metabolic compound of *Andrographis paniculata* shows that it possess potential anti-inflammatory and antimicrobial activity. The aqueous extract of leaf samples was qualitatively analyzed for the presence of phytochemicals and it was conformed that the plant extract contain metabolites such as alkaloids, flavonoids, terpenoids, steroids, aminoacids. The plant extract showed potential antibacterial activity. The chromatographical nalysis revealed the presence of possible metabolites. The purified active compounds through TLC and crude plant extract were subjected to analyze the anti-inflammatory activity and shows potent inhibitory activity against the adverse effects of the inflammation such as tissue damage and protein denaturation.

Key words: Flavonoids • Terpenoids • Antibacterial • Active Components and TLC

INTRODUCTION

Inflammation is the word derived from '*inflammare*' which means burning, defence mechanism of the human body against pathogenic microorganisms, physical or chemical agents and any injuries. The process can be classified as acute and chronic inflammation. Acute inflammation is the initial response to the harmful stimuli resulting in redness, pain in the area of infection or injury. Chronic inflammation which is also known as prolonged inflammation involves the lymphocytes and macrophages resulting in fibrosis and tissue necrosis. This increases the development of diseases such as arthritis, heart disease, asthma etc.

The inflammatory process is a combination of many pathways like synthesis of prostaglandin, interleukin, platelet-activating factors. It initiates with any stress on the membrane or by other trigger which activates hydrolysis of membrane phospholipid by phospholipase-A into arachidonic acid. Several cytokines also play essential roles, especially interleukin-1 (IL-1) and tumor necrosis factor-a (TNF-a). They are secreted by monocytes and macrophages, adipocytes and other cells.

Working in concert with each other and various cytokines and growth factors (including IL-8 and granulocyte-macrophage colony-stimulating they induce gene expression and protein synthesis in a variety of cells to mediate and promote inflammation. Steroids e.g. betamethasone and the non-steroidal anti-inflammatory drugs (NSAIDs) e.g. acetyl salicylic acid are used for the treatment of inflammatory diseases. However, these agents cause severe side effects such as adrenal suppression for steroids and gastric ulceration. It is now accepted that cyclooxygenase (COX) enzyme exists in two isoforms – COX I (constitutive) and COX II (inducible). The therapeutic activities of NSAIDs are attributed to the inhibition of COX II. Therefore, an ideal anti-inflammatory drug is expected to inhibit prostaglandin synthesis mediated by COX II while sparing COX I, inhibition of which is believed to mediate the side effects. And so, though arthritis is one of the oldest known diseases, there is yet no drug leading to a permanent cure and which is devoid of adverse effects.

Andrographis paniculata belongs to the family Acanthaceae. The herb is exceedingly bitter and has been in use for many centuries in Asia, where it is regarded as

the 'King of Bitters'. It grows erect to a height of over one meter in moist shady habitats. It has ovate, pinnate or lanceolate leaves of various sizes.

Scientific Classification

Kingdom:	Plantae
Order:	Lamiales
Family:	Acanthaceae
Genus:	<i>Andrographis</i>
Species:	<i>A. paniculata</i>
Binomial name:	<i>Andrographis paniculata</i>

The flowers have minute white petals bearing purplish spots. The stem is deep green, with diameter ranging from 2 mm to 6 mm or more. The flowers give rise to oblong capsules bearing numerous, minute brown seeds. The plant reproduces by seeds and is widely distributed in tropical Asia. Its centre of origin and diversity is thought to be Sri Lanka and south India, but the herb is found in north India, China and the entire Southeast Asia. Unlike other species of *Andrographis*, the herb called. Occurs quite commonly in the entire India subcontinent, which accounts for its widespread use since ancient times against a variety of disorders, in both Ayurvedic and Chinese Medicine.

MATERIAL AND METHODS

Collection of Samples and Extraction: *Andrographis paniculata* plant was collected from Herbal garden, Agricultural university, Thrissur, Kerala. The Leaves were washed under running tap water to eliminate dust and then with distilled water thoroughly. The shade dried leaves were trodden into small pieces, powdered and mixed in 1:10 ratio with distilled water. The extractions were obtained through continuous shaking for about 48hrs followed by filtration using Whatman No.1 filter paper.

Phytochemical Components and Protein Determination: Phytochemical analyses were carried out according to the methods described by Trease and Evans [1]. Protein content in the plant extracts were estimated by the method described by Lowry *et al.* [2].

Estimation of total Antioxidant: (Phosphomolybdenum method- Prieto *et al.* [3].

Aliquot of 0.1 ml samples were 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 95°C

for 90 mins. 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 samples and it was incubated under same as rest of the sample. For sample of unknown composition, water soluble antioxidant capacity was expressed as equivalents of ascorbic acid (1 ml of extracts).

Phenolic Content as Tannic Acids [4]: 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 mins 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.

Minimum Inhibitory Concentration (MIC)-Dilution Method: To determine Minimum Inhibitory Concentration (MIC) 1ml 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.

Antibacterial Activity: *Staphylococcus*, *Proteus*, *Klebsiella*, *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 MacFarland 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 [4]. Wells are made in Muller Hinton agar plate using cork borer [6 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 for control were 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.

Separation of Active Compound from Extracts from *Andrographis paniculata* Suspension by Thin Layer Chromatography (TLC)

Preparation of Chromaplate: 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.

Development of Chromatogram: The development tank was saturated with suitable solvent according to Eskil Hultin [6].

Alkaloids : Benzene/ Methanol-80:20

Flavanoids : Chloroform/Methanol-70:30

Lipid : Chloroform/Methanol/water-10:10:3

Terpenoids : 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 Flavanoids 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 according to Bishnu Joshi [7] and left overnight. The content in the beaker was stirred and filtrated through Whatman 0.1 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 *Staphylococcus* and *Salmonella* by cup diffusion method.

***In-vitro* anti-inflammatory Activity**

Inhibition of Albumin Denaturation: Methods of Mizushima and Kobayashi [8] and Saket *et al.* [9] followed with minor modifications. The reaction mixture was consisting of test extracts and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted with 1N HCl. 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,

$$\% \text{ inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control} \times 100}$$

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 (10ml) was collected from healthy volunteers 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 according to Sadique, [10] and Saket *et al.* [8].

Heat Induced Hemolytic: The reaction mixture (2 ml) consisted of 1 ml of test samples solution and 1 ml of 10% RBCs suspension, instead of test sample only saline was added to the control test tube. Diclofenac sodium 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 [8, 11].

Proteinase Inhibitory Action: The test was performed according to the modified method of Oyedepo *et al.* [12]. The reaction mixture (2 ml) was containing 0.06 mg trypsin, 1ml of 20mM Tris HCl buffer (pH7.4) and 1ml test sample of different concentrations. The reaction mixture was incubated at 37°C for 5min and then 1ml of 0.8% (W/V) casein was added. The mixture was incubated 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 and Protein Content: Qualitative phytochemical analyses were performed for the detection of alkaloids, saponin, steroids, flavonoids, cardiac glycosides, terpenoids and phenols. The observed results were tabulated (Table 1). 0.02 ml and 0.04 ml of aqueous extracts were used to determine the protein content. Standard graph was plotted for bovine serum albumin using spectrophotometric reading at 660 nm and it contained 3.4 mg/g of protein in aqueous extract of leaves.

Total Antioxidant 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 11.64 mg/g of antioxidant in 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 4.38 mg/g of phenolic content in aqueous extract.

MIC and Antibacterial Property: Minimal inhibitory concentration assay was carried out for methanolic extraction *Andrographis paniculata* leaves against *Shigella*, *Proteus*, *Klebsiella*, *Escherichia coli* and *Salmonella*. Table 2 illustrated the minimal concentration level which required to kill the pathogen. Antibacterial properties of the *Andrographis paniculata* extracts were

Table 1: Phytochemical analysis of *Andrographis paniculata*

Phytochemical constituents	Aqueous extract
Alkaloids	+
Flavonoids	+
Terpenoids	+
Steroids	+
Cardiac glycosides	-
Saponins	-
Phenols	+

‘+’ – Present ‘-’ – Absent

Table 2: MIC assay of *Andrographis paniculata*

Test organism	Optical Density at 540nm		
	60µl	80µl	100µl
<i>Salmonella typhi</i>	0.56	0.34	0.23
<i>Staphylococcus sp.</i>	0.37	0.18	0.07
<i>Klebsiella sp.</i>	0.69	0.45	0.28
<i>Escherichia coli</i>	0.43	0.26	0.17
<i>Shigella sp.</i>	0.72	0.37	0.25

Table 3: Antimicrobial activity of *Andrographis paniculata*

Test organism	Zone of inhibition(in diameter in cm)				Standard drug (amp) 20µl
	Zone of Inhibition (diameter in mm)				
	20µl	40µl	60µl	80µl	
<i>Staphylococcus</i>	0.5	0.8	1.2	1.6	-
<i>Escherichia coli</i>	-	0.6	1	1.3	2
<i>Proteus</i>	-	1.1	1.5	1.8	1
<i>Shigella</i>	-	1.2	1.4	1.7	2
<i>Salmonella typhi</i>	0.8	1.4	1.8	2.0	1.2

carried out by well diffusion method. The aqueous extract of *Andrographis paniculata* leaves showed 2 cm the maximum zone of inhibition in *Salmonella typhi* and minimum zone of inhibition found in *Escherichia coli* which is tabulated in Table 3.

Separation of Active Compound from Thin Layer Chromatography (TLC): 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. Table 4 represented the extract of leaves contain alkaloids, flavonoids, lipids and terpenoids in the Rf value.

The separated active compounds alkaloid, flavonoids, lipids and terpenoids were found that effective against *Salmonella* and *Klebsiella* the zone of inhibition tabulated in Table 5 which represented the maximum zone of inhibition of leaves extract found in *Klebsiella* in the active compound of flavonoids and minimum zone of inhibition found in active compound of lipid.

Table 4: Rf values of phytochemicals of *Andrographis paniculata*

Phytochemical constituents	Alkaloids	Flavonoids	Lipids	Terpenoids
Rf value	0.56	0.4	1.2	0.7

Table 5: Antimicrobial activity of active compounds of *Andrographis paniculata*

Active compounds	Zone of inhibition (in diameter mm)	
	<i>Salmonella</i>	<i>Klebsiella</i>
Lipids	1.6	1.5
Flavonoids	2.2	1.8
Alkaloids	1.7	1.6
Terpenoids	2.4	2.2

Table 6: Effect of *Andrographis paniculata* on Anti-inflammatory activity

Active compounds	Albumin denaturation	Membrane stabilization	Proteinase inhibition
Alkaloids	8.6%	8.7%	8.5%
Lipids	6%	5.4%	6.5%
Flavonoids	7.8%	8.2%	7.6%
Terpenoids	10%	9.2%	10.1%
Aqueous extract	72%	75%	75%
Diclofenac sodium (200µg/ml)	65.4%	66%	67.8%

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 to inhibit protein denaturation was studied. It was effective in inhibiting heat induced albumin denaturation (Table 6). Maximum inhibition was observed from leaf extract. Diclofenac sodium, a standard anti-inflammation drug showed the maximum inhibition 65.4% 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 *Andrographis paniculata*. 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 (Table 6). The Diclofenac sodium, standard drug showed the maximum inhibition 66%.

Proteinase Inhibitory Activity: The *Andrographis paniculata* aqueous extract exhibited significant anti-proteinase activity from different parts. The extract effectively inhibited the proteinase activity. The standard Diclofenac sodium drug showed the maximum proteinase inhibitory action (Table 6).

DISCUSSION

The qualitative phytochemical screening was done for the analysis of secondary metabolites and reducing sugars (viz alkaloids, terpenoids, steroids, flavonoids, tannins, cardiac glycosides) and reducing sugars which was reported by Susmitha *et al.* [13]. The aqueous extract of leaves contains alkaloids, terpenoids, flavonoids, steroids and aminoacids. The extract of *Andrographis paniculata* shows 2cm maximum zone of inhibition in *Salmonella typhi* and 1.6cm in *Staphylococcus sp.* The minimum zone of inhibition was found in *Escherichia coli*.

The antibacterial activity of aqueous, ethanol and acetone extracts of *Corriander sativum*, *Abutilon indicum*, *Boerhavia diffusa* and *Rographis paniculata*, *Plantagoovata*, *Euphorbia ligularia*, *Zinziber officinale*, *Terminalia chebula*, *Azadirachta indica*, *Ocimum sanctum* and *Cinnamomum cassia* was determined against 33 UTI isolates i.e. *Proteus mirabilis*, *Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Providencia pseudomallei*, *Pseudomonas aeruginosa* and *Klebsiella oxytoca* by disc diffusion method. The studies concluded that crude extracts of the selected plants especially the acetone and ethanol extracts exhibited significant activity against UTI pathogens reported by Anjana Sharma [14].

Sanjeet Kumar [15] reported that the thin layer chromatography is a simple, cost-effective and easy-to-operate planar chromatographic technique which has been used in general chemistry laboratories for several decades to routinely separate chemical and biochemical compounds. Traditionally, chemical and optical methods are employed to visualize the analyte spots on the TLC plate. Also it has a wide application in identifying impurities in a compound. Study highlights the review on TLC and its application of qualitative and quantitative estimation of bio-active compounds from medicinal plants.

The aqueous extract of leaves contain alkaloids, flavonoids, lipids and terpenoids in the Rf value of 0.56, 0.4, 1.2 and 0.7. The separated active compounds alkaloid, flavonoids, lipids and terpenoids were found that effective against *Salmonella* and *Klebsiella* the zone of

inhibition. Chromatographical analysis of *Andrographis paniculata* revealed that the presence of terpenoids, lipids, alkaloids and flavanoids. Chromatographic profiles of crude extracts obtained through different solvents were similar. The visualization of chromatographic profiles for each extraction technique and solvent used permit to evaluate the qualitative and quantitative variations in secondary metabolites content by Cristiane [16]. In addition, these data present compound profiles related to the biological effects and medicinal use.

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 to inhibit protein denaturation was studied. It was effective in inhibiting heat induced albumin denaturation. Maximum inhibition was observed from leaf extract. The active compounds obtained from the plant part also had more or less anti-inflammatory activity. Diclofenac sodium, a standard anti-inflammation drug showed the maximum inhibition 0.65% at the concentration of 200 µg/ml.

Stabilization of RBCs membrane was studied for further establishes the mechanism of anti-inflammatory action of aqueous extract of *Andrographis paniculata*. 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. Eugenol (1-hydroxy-2-methoxy-4-allylbenzene) a naturally occurring phenolic compound is a major component of basil oil and exists to a lesser extent in oil of several other plants according to Nagababu *et al.* [17].

Nitin gupta *et al.* [18] reported that the anti-inflammatory activity of spilanthos, for centuries, *Spilanthos acmella* (L.) Murr. (Fam. Compositae) has been recommended in traditional medicine for treatment of toothache, rheumatism and fever. Plant phenolic compounds have been found to possess potent anti-inflammatory activity which was reported by Roy *et al.* [19]; Garg *et al.* [20]. According to Lin *et al.* [21] flavonoids have been found to possess anti-inflammatory properties in various studies.

CONCLUSION

Medicinal plants have been used for thousands of years, in pharmaceuticals, as alternative medicines etc.

The extracts of these plants contains significant active components especially against bacterial pathogens. In this study it shows that the *Andrographis paniculata* plant extracts possess metabolic compound which inhibits bacterial growth and have anti-inflammatory activity.

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