Abstract

Argyreia nervosa (AN) is traditionally used in rheumatic disorders, cerebral disorders, syphilis, leucocohhrea and as wound healing agent. We aimed to validate the anti-arthritic effect of AN in Freund Complete Adjuvant (FCA) induced arthritis rats. The effects of hydroalcoholic extract of Argyreia nervosa leaves on the changes in rheumatoid factor (RF), Haemoglobin (Hb) content, erythrocyte sedimentation rate (ESR), prostaglandin E₂ (PGE₂), superoxide dismutase (SOD), catalase, reduced glutathione (GSH), lipid peroxidation levels and histopathology were investigated. The results showed that prophylactic treatment with ANLE suppressed the histopathological changes in joints and significantly reduced the FCA induced paw edema, RF, ESR, Lipid peroxidation and significantly elevated the FCA decreased SOD, Catalase and GSH levels as compared to arthritic rats. These results suggest that ANLE possess anti-arthritic activity at least in part by preventing oxidative stress.

Keywords: Antioxidant, antiarthritic activity, rheumatoid factor, Catalase, Lipid peroxidation, Superoxide dismutase, Argyreia nervosa.

Introduction

Rheumatoid arthritis affects about 1–1.5% of the population throughout the world, is a polyarticular inflammatory disorder that leads to joint swelling, stiffness, deformity and loss of joint function with systemic manifestations that include vasculitis, rheumatoid nodules and anaemia (1). Mediators of inflammation generate reactive oxygen species (ROS). Tumor necrosis factor – α (TNF - α), Interleukin-1β (IL-1β), interleukin-6 (IL-6), prostanoids, leukotrienes, proteases and rheumatoid factor (RF) in which cause tissue destruction of synovial membrane (2, 3). Secondarily formed radicals like hydroxylradicals destroy membrane lipids, proteins, deoxyribonucleic acid (DNA), hyaluronic acid and cartilage (4). Adjuvant arthritis is best experimental model not only for studying biochemistry, pharmacology of human arthritis and but also for the development of potential drugs for arthritis (5).

Argyreia nervosa (Syn: Argyreia speciosa) belongs to family Convolvulaceae and commonly known as Elephant creeper in English, Chandrapada, Samudrpalapal in Telugu, Samundar-ka pata in Hindi and Vriddhadaruka in Ayurveda (6). In Ayurvedic preparations, AN is used as Rasayana drug. Argyreia nervosa is traditionally used in Rheumatic disorders, cerebral disorders, inflammation (7), to treat wounds (8), syphilis, diarrhea (9). Leaves of AN are used by tribals in chittoor district of Andhra Pradesh to treat rheumatoid arthritis (10). The present study was designed to authenticate the traditional claim of ANLE in rheumatoid arthritis.

Evaluation of Antiarthritic Activity of Argyreia nervosa Leaf Extract

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Antiarthritic activity of Argyreia nervosa leaf
Materials and Methods

Plant material and Hydroalcholic extraction: The leaves of *Argyreia nervosa* (AN) 1 kg were collected from Tirumala Hills in Tirupathi, Chittoor District, Andhra Pradesh, India. The plant was authenticated by Dr. Madhava Chetty, Professor, Department of Botany, Sri Venkateshwara University, Tirupati. A voucher specimen of same was already present (Voucher specimen No. 786) in the Department of Botany, S.V. University, Tirupati. The collected leaves were shade dried at room temperature and coarsely powdered, and macerated in 70% alcohol for 7 days with an intermittent vigorous shaking. Then resulted extract was dried by flash evaporation to obtain dark green residue and the percentage yield was 22.8% W/W.

Preliminary Phytochemical analysis: The total phenolic content of the extract was determined using the Folin–Ciocalteus’s reagent (11). Total flavonoid content was determined using aluminium chloride (AlCl₃) according to the method described previously (12). IsoQuercetin was used as a standard. Total tannin content was determined by the method described earlier (13).

Animals: Pathogen free adult albino rats weighing 120-150 gm were used and housed 4 per cage in polypropylene cages under 12 hrs light/dark cycle in rooms maintained at 25±3°C fed with standard laboratory chow (Hindusthan Lever Limited, Mumbai, India) and water ad libitum. Animals were acclimated to their surroundings for 10 days to eliminate the effects of stress prior to initiation of experiments. The experimental was conducted after prior approval from Institutional Ethical Committee No.1677/PO/a/12/CPCSEA.

**Complete Freund’s Adjuvant (FCA) Induced arthritis in rats:** Adjuvant arthritis was induced by the subplantar injection of 0.1 ml Complete Freund’s Adjuvant (each ml contains 1 mg of heat killed and dried *Mycobacterium tuberculosis* (strain H37Ra, ATCC 25177), 0.85 ml of paraffin oil and 0.15 ml of mannide monooleate) procured from Sigma-Aldrich, St. Louis, Mo, USA (14). The rats were randomly divided into five groups: Vehicle control (2 % gum acacia), Disease control (2 % gum acacia), ANLE (200 mg/kg/p.o), ANLE (400 mg/kg/p.o), standard Diclofenac (5 mg/kg/p.o) animals received respective treatment for 21 days and were sacrificed on day 22. The dose of ANLE was selected from the previous reports (15). The lethal dose (LD₅₀) of ANLE was more than 2000 mg/kg as none of the rats shown toxic symptoms after the dose of the extract for 14 days (15).

**Paw volume assessment:** Paw volumes (16) were measured with Digital Plethysmograph (Model No. 7140, UGO Basile, Comerio, Italy) on 1, 3, 6, 9, 12 and 21st day.

**Biochemical measurements:** On the day 22, over night fasted animals were sacrificed by diethyl ether asphyxiation and blood samples were collected by cardiac puncture. ESR was estimated by Westergren's method (17) and Haemoglobin content was estimated by the method of Sahili (18).

**Prostaglandin estimation (PGE):** Both the left control and right inflammatory hind paw were removed from above ankle and stored in Phosphate buffer containing normal saline (pH – 7) for 60 min and homogenised and centrifuged at 3000 rpm for 10 min and to 0.1 ml of
supernatant, 2ml of 0.5 mol potassium hydroxide (KOH)—methanol was added, the mixture was incubated at 50°C for 20 min, and then diluted to 20 ml with methanol. The PGE\textsubscript{2} level in the mixture was determined (19) with a Spectrophotometer (Model No. 177, ELICO Ltd, Hyderabad) at 278 nm and the values were expressed as µg/mg.

**Rheumatoid factor (RF):** Rheumatoid factor was estimated with a Commercial kit (ORGENTEC Diagnostics, Germany). To the serum sample, RF reaction buffer and RF antiserum was added according to the manufacturer's instructions. The values were expressed as IU/ml.

**Superoxide dismutase:** The enzyme superoxide dismutase (SOD) was determined in erythrocytes using photo-oxidation method. 3ml of packed blood cells were lysed by the addition of equal volume of cold deionised water. Hemoglobin was then precipitated by the addition of chloroform and ethanol (1:5:1). Then 500µl of water was added and centrifuged for 15 minutes at 3000rpm. The supernatant containing SOD was used for the measurement of activity. To 0.88ml of riboflavin solution (1.3x10^{-5}M in 0.01M potassium phosphate buffer, PH7.5), 60µl of O-dianisidine solution (10^{-2}M in ethanol) and 1000µl of clear supernatant was added and optical density was measured at 460nm. Then cuvette along with reaction mixture was transferred to the illuminating box, illuminated for 4min and optical density was measured against blank containing ethanol in place of enzyme. The change in the optical density was determined (20).

**Catalase:** 2.5 ml of Phosphate buffer and 0.1 ml of serum was incubated at 25°C for 30 minutes. The absorbance was measured at 240 nm then 650µl of hydrogen peroxide solution was added to initiate the reaction. The change in absorbance was measured for 3 minutes and activity was expressed as the µmoles of H\textsubscript{2}O\textsubscript{2} degraded /ml/ min (21).

**Reduced glutathione:** To citrated blood, 5% trichloro acetic acid (TCA) solution was added and centrifuged at 3000rpm for 20 minutes. To 0.1ml of supernatant, 1ml of sodium phosphate buffer and 0.5ml of DTNB reagent were added (22). The absorbance of yellow color developed was measured at 412nm. The values were expressed in mM/ml.

**Lipid Peroxidation:** 0.1ml of Plasma was treated with 2 ml of 37% Thiobarbituric acid (TBA), 25N Hydrochloric acid (HCL) and 15% trichloro acetic acid (TCA) in 1:1:1 ratio and placed in water bath for 15min, cooled and centrifuged and clear supernatant was measured at 535nm against reference blank and the values were expressed in µM/ml/min (23).

**Histopathological Evaluation:** The hind paws were dissected out, ankles were removed and fixed in a mixture of 10% formaldehyde for 48 hrs for fixation, specimens were then placed in 5% formic acid for decalcification for 7 days. Ankles were cut into 5 µm thick sections using microtome and stained with hematoxyline and eosin (H&E) for histological evaluation using light microscopy.

**Statistical analysis:** All the values were expressed as Mean±SEM. The data was analyzed using Analysis of variance followed by Dunnett’s multiple comparison t-test. In all the tests, the criterion for statistical significance was P<0.05.

**Results and Discussion**

By performing preliminary phytochemical analysis to ANLE, the total phenolic content was found to be 1.082 mg/g of GAE (gallic acid equivalent), flavonoid content was 0.784 mg/g of QE (isoquercetin equivalent) and the tannin content was 1.301 mg/g TAE (tannic acid equivalent).

Rheumatoid arthritis is a chronic inflammatory disease affecting about 1% of the population in developed countries. Adjuvant

Antiarthritic activity of *Argyreia nervosa* leaf
induced arthritis is one of the most widely used model for studying the anti-inflammatory/anti-rheumatic properties of compounds in rats. Intrarticular injection of FCA is known to induce inflammation as well as immune response and features produced resemble rheumatoid arthritis in humans (24, 25). FCA induced arthritis is a model for chronic polyarthritis, which induces arthritis through cell-mediated autoimmunity by structural mimicry between mycobacteria and cartilage proteoglycans, which activate macrophages, lymphocytes and monokines (26). In the present study, FCA immunization resulted in swelling and erosion of joints, further bone was also destroyed. Determination of paw swelling is apparently simple, sensitive and quick procedure for the evaluation of degree of inflammation and the therapeutic efficacy of drugs. In the present study paw volume of both hind limbs were recorded on the day of FCA injection, and again measured on day 1, 3, 6, 9, 13, 21. The day 6 and 12 measurements are indicative of primary lesions and secondary lesions respectively, 1% increase in the paw volume was observed in the control paw as well indicating systemic effect of FCA immunization. On the day 21, the secondary phase of rheumatoid arthritis becomes more evident and inflammatory changes spreads systemically and becomes observable in the limb not injected with Freund’s adjuvant (27, 28). Fig.1 showed the inhibitory effect of ANLE on FCA induced paw edema in rats. Rats treated with vehicle (arthritic rats) displayed visible arthritic signs on day 1 after immunization and showed maximum paw swelling on day 3, while treatment with ANLE 200 mg/kg, 400 mg/kg and diclofenac 5 mg/kg (std) significantly lowered the incidence of arthritis and markedly reduced paw swelling throughout the disease progression.

Erythrocyte sedimentation rate (ESR) is an estimation of the suspension stability of RBC’s in plasma, is a nonspecific marker of disease. It is related to the number and size of the red blood cells to the concentration of plasma proteins, especially fibrinogen, α and β globulins. Acute phase proteins in ESR and C-reactive protein concentrations are elevated in response to stress and inflammation. In the present study, arthritic group of animals showed a significant increase in ESR and reduced Hb levels indicating the presence of organic disease and anaemia respectively (41). Rats treated with FCA (arthritic) showed anaemia characterized by significant decrease in the Hb levels and significant elevation in ESR (Table 1) indicating the development of arthritis, whereas treatment with ANLE 200 & 400 mg/kg and diclofenac (5 mg/kg) showed increased Hb levels and decreased ESR significantly.

Values are expressed as Mean ±SEM [n=5]; Data were analyzed by One-way analysis of variance (ANOVA) followed by Dunnet’s t-test; *(p<0.05), **(P<0.01), *** (p<0.001) Vs Disease control; +(p<0.05), ++(P<0.01), +++(p<0.001) Vs Normal control.

FCA treatment increased PGE₂ levels in the arthritic rats indicating development of inflammation. PGE₂ is generated from arachidonic acid by cyclooxygenase-2 and mediates many features of inflammation (29). Proinflammatory cytokines in synovial fluid stimulate collagenase and PGE₂ production by synovial cells which turn inhibit bone formation, stimulate resorption of proteoglycans and inhibit its biosynthesis in explants of cartilage (30). Treatment with FCA (arthritic group) showed increased PGE₂ levels significantly indicating the development of inflammation (Table 1). While the
The intensity of rise in PGE2 was dose dependently reduced in ANLE 200 mg/kg & 400 mg/kg treated rats and the results were comparable with standard diclofenac (5 mg/kg) treatment group. Decrease in PGE2 levels may be either due to inhibition of cyclooxygenase or phospholipase A2.

RF was markedly elevated in arthritic group indicating activation of immune system and recognition of immunoglobulin G (IgG) as nonself. T-cells, IL-6, act as potent stimulus for the maturation of B cells to plasma cells to produce antibodies, serum RF recognizes immunoglobulin molecule as “non-self” results in the generation of immune complexes, which inturn bind complement and eventually lead to destruction of synovium, cartilage, and bone. Higher the levels of serum RF, higher the development of inflammation. Serum RF measures the amount of antibody IgG titre present in the serum (31, 32). ANLE dose dependently decreased PGE2 and RF which inturn ameliorated PGE2 and RF mediated inflammatory changes in the synovial membrane of joints. The results of histopathology confirmed it. Arthritic rats showed significant increase in the levels of Rheumatoid factor indicating FCA immunization produced arthritis. ANLE (200 and 400 mg/kg) and Diclofenac (5 mg/kg) markedly reduced the RF and values reached normal with ANLE (400 mg/kg) treatment.

The sections of the left tibiotarsal joints of normal rats (A) revealed that there was no bone matrix destruction and loss of cell integrity. Adjuvant induced arthritis group of animal (B) showed bone matrix destruction and loss of cell integrity. Diclofenac (5 mg/kg), ANLE (200 and 400 mg/kg) C, D and E respectively showed reduced bone matrix destruction and loss of cell integrity.

Values are expressed as Mean ±SEM [n=5]; Data were analyzed by One-way analysis of variance (ANOVA) followed by Dunnett's t-test; *(p<0.05), ***(p<0.01), ****(p<0.001) Vs Disease control; +(p<0.05), ++(P<0.01), +++(p<0.001) Vs Normal control.

**Table 1.** Effect of ANLE on Haemoglobin, Erythrocyte sedimentation rate (ESR), Prostagnandin-E2 (PG-E2), Rheumatoid factor (RF)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Haemoglobin(gm/dl)</th>
<th>ESR(mM/hr)</th>
<th>PG-E2(μg/mg)</th>
<th>Rheumatoid Factor(RF) (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>13.75±0.25</td>
<td>4.25±0.43</td>
<td>0.05±0.06</td>
<td>14.48±1.59</td>
</tr>
<tr>
<td>Disease control</td>
<td>9.25±0.25***</td>
<td>9.6±0.23***</td>
<td>0.174±0.06***</td>
<td>43.18±2.07***</td>
</tr>
<tr>
<td>Diclofenac(5mg/kg)</td>
<td>11.50±0.28*, +</td>
<td></td>
<td>0.123±0.50*, +++</td>
<td>33.73±2.37***, +++</td>
</tr>
<tr>
<td>ANLE(200mg/kg)</td>
<td>12.00±0.40**,+++</td>
<td>6.5±0.28***, +++</td>
<td>0.104±0.04**, +++</td>
<td>26.45±2.30**, ++</td>
</tr>
<tr>
<td>ANLE(400mg/kg)</td>
<td>12.75±0.25+++</td>
<td>5.37±0.23+++</td>
<td>0.082±0.03*, +++</td>
<td>20.33±2.15, +++</td>
</tr>
</tbody>
</table>

**Fig. 2.** Histological changes in hematoxylin and eosin stained tarsal joint sections in arthritis induced rats. (A) Normal (B) Disease control, (C) Diclofenac (5 mg/kg), (D) ANLE (200 mg/kg), (E) ANLE (400 mg/kg).
peroxidation in arthritic rats indicating development of free radicals induced oxidative stress. ANLE (200 mg/kg and 400 mg/kg) markedly elevated SOD, Catalase, GSH levels with a consistent decrease in the levels of lipid peroxidation (Table 2).

In the present study, arthritic rats showed decreased levels of SOD, GSH and Catalase indicating generation of free radicals during the development of arthritis, which was substantiated by the marked increased levels of lipid peroxides, a marker of cell damage which is online with previous results (33, 34). ROS are produced during many metabolic processes including mitochondrial respiration and enzyme activities. The superoxide anion plays a pivotal role in inflammatory joint disease. Super oxide anion is produced by one electron reduction of oxygen by several different oxidases including Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NADPH) oxidase, xanthine oxidase, cyclooxygenenase as well as by several mitochondrial electron transport chain during the course of normal oxidative phosphorylation which is essential for generating adenosine triphosphate (ATP) (35). Superoxide free radical causes fibroblast proliferation and damages endothelial cells, increases the permeability of the microvasculature and promotes the migration of neutrophils to foci of inflammation and produces more aggressive free radicals (36), which is dismutated to hydrogen peroxide (H$_2$O$_2$) by manganese superoxide dismutase in the mitochondria and by copper superoxide dismutase in the cytosol (35). Superoxide also reduces iron complexes to ferrous state which reacts with hydrogen peroxide to generate hydroxyl radical by Fenton reaction. This reaction is accelerated in the synovial membrane due to high content of Iron complexes. Hydroxyl radical is extremely reactive and probably is the final mediator of most free radical induced tissue damage (37), including membrane lipids, proteins, deoxy ribo nucleic acid (DNA), cartilage, hyaluronic acid, and further damages IgG, initiates autoimmunity in rheumatoid arthritis.

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synovial liquid contains α-antiproteases which suppress catalase activity derived from neutrophils, the activity of these proteins can be decreased by the action of oxygen free radicals and myeloperoxidase, which was prominent in the arthritic group as observed by the decreased GSH>SOD, Catalase and increased lipid peroxides. Online with this our finding of increased lipid peroxidation and decreased antioxidants in arthritic group supports the involvement of oxidative stress in arthritis. The decreased activities of catalase, SOD, GSH may be a response to increased production of hydrogen peroxide and superoxide anion. This effect was blunted by the ANLE and diclofenac providing evidence that ANLE impart exerts its antiarthritic effect by free radical scavenging.

Phytochemical studies ANLE showed the presence of flavanoids, tannins, alkaloids, polyphenols have potent antioxidant activity (except alkaloids), which was confirmed by increased SOD, GSH, Catalase and decreased lipid peroxidation levels. As ROS is closely associated with the increase in PGE$_2$, RF and inflammation, free radical scavenging activity of ANLE provides close relationship between antioxidant and anti-arthritic activity. Further work is required for the clear understanding of the mechanism of action and phytochemical responsible for the activity.

Values are expressed as Mean ±SEM [n=5]; Data were analyzed by One-way analysis of variance (ANOVA) followed by Dunnett’s t-test; *(p<0.05), **(P<0.01), *** (p<0.001) Vs Disease control; +(p<0.05), ++(P<0.01), +++(p<0.001) Vs Normal control.

**Conclusion**

The results of present study indicate that ANLE possess anti-arthritic activity in FCA model. This was demonstrated by marked decrease in paw volume, ESR, RF, PGE$_2$ and improvement in Haemoglobin levels. Histopathological changes also supported its antiarthritic activity. Phytoconstituents like flavanoids, phenols and tannins present in hydroalcoholic extract of ANLE may be contributing for the antiarthritic activity as they have potent antioxidant activity. Further studies can be designed on the identification of phytochemical responsible for the anti-arthritic activity.

**Acknowledgements**

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**References**


**Table 2.** Effect of ANLE on Superoxide Dismutase (SOD), Catalase, Reduced glutathione (GSH), Lipid peroxidation.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Superoxide dismutase (IU/ml)</th>
<th>Catalase (μmoles/ml/ min)</th>
<th>Reduced glutathione (mM/ml)</th>
<th>Lipid peroxidation (μM/ml/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>201.1±10.59</td>
<td>166.4 ±4.96</td>
<td>3172 ±77.38</td>
<td>159.3 ±23.30</td>
</tr>
<tr>
<td>Disease control</td>
<td>121±7.651***</td>
<td>60.53±3.41***</td>
<td>1033±102.9***</td>
<td>2505±100.1***</td>
</tr>
<tr>
<td>Diclofenac(5mg/kg)</td>
<td>163.2±3.286**, ++</td>
<td>79.50±2.59*, +</td>
<td>2447±50.5*, ++</td>
<td>1021±82.36*, +***</td>
</tr>
<tr>
<td>ANLE(200mg/kg)</td>
<td>172.9±5.81*, +++</td>
<td>102.1±3.49**, +++</td>
<td>2744±78.65**, +++</td>
<td>768±50.72*, +++</td>
</tr>
<tr>
<td>ANLE(400mg/kg)</td>
<td>180.6±5.08****</td>
<td>139.9±5.65*, +++</td>
<td>2916±78.68+++</td>
<td>444.3±68.12*, +++</td>
</tr>
</tbody>
</table>

Antiarthritic activity of Argyreia nervosa leaf


