COLLAGENASE INHIBITORY ACTIVITY AND PHYTOCHEMICAL PROFILE OF LEAF OF JUSTICIA GENDARUSSA

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ABSTRACT
Ethnomedicinally Justicia gendarussa is used for treatment of chronic rheumatism. The alcoholic extract of leaf was reported for its anti-inflammatory and antiarthritic activity. This research is aim to find collagenase inhibitory fraction. The leaf was fractionated based on polarity. Methanolic extract and its fractions of leaf of J. gendarussa were screened phytochemically. Based on phytochemical screening the resultant fractions were evaluated for their collagenase (Matrix metalloproteinases) inhibitory activity at 100, 250, 500 and 1000 µg/ml concentration. Among the tested extracts, toluene fraction of leaf of J. gendarussa showed maximum Matrix metalloproteinases inhibition with IC$_{50}$ value 44.75µg /ml. Further, the phytochemical analysis of isolated compounds indicated the alkaloids and saponins in leaf of J. gendarussa respectively. The Matrix metalloproteinases inhibitory activity may be because of high content of alkaloids and saponins. Based on Matrix metalloproteinases inhibitory activity, it could be suggested that the toluene fraction of J. gendarussa might have an influence on arthritis.

KEYWORDS: Matrix metalloproteinases, Collagenase, Toluene fraction, Leaf.

INTRODUCTION
The collagen, the major component of the cartilage, has been thought to undergo only minimal turnover under normal circumstances but is known to be actively destroyed in rheumatoid and septic arthritis. Joint cartilage is composed of a type II collagen-based fibrillar network complexed to proteoglycans. Type II collagen consists of 3 identical α chains arranged in a triple helix that form fibrils. Within this fibrillar meshwork resides the large aggregating proteoglycan aggrecan. In arthritis type II collagen is extensively cleaved and destroyed by the activity of collagenases, which results in loss of type II collagen. [1-3]
Different collagenases, namely matrix metalloproteinases (MMP)-1, MMP-8 and MMP-13, can cleave type II collagen. In synovial fluid, MMP-8 is stored as latent proenzyme in polymorphonuclear neutrophils.[2] The loss of the collagen components may be a function of articular cartilage collagenase that leads to the arthritic condition.[4] The discovery and development of compounds with collagenase inhibitory activity is therefore an effective method for preventing arthritis.

Ethnomedicinally the fresh leaves of *J. gendarussa (Acanthaceae)* are pounded into a paste, warmed and rubbed or applied onto the affected area which is then bandaged to ease muscle pains, broken/fractured bone, muscle sprains and cuts or added to coconut oil to treat boils.[5] The leaves are used in Vietnamese folk medicine as a poultice to treat rheumatism and arthritis. In Sri Lanka traditional medicine, the leaf of *J. gendarussa* are used as an analgesic to treat hemiplegia, rheumatism, arthritis, headache and earache.[6] It is used in India for the treatment of chronic rheumatism, cephalalgia, cough, and bronchitis. In Brazil, it is used to treat pains, fever and for the treatment of diseases of magical-religious origin.[6] The methanolic extract of leaf is reported to have effect in inflammation[7]—while methanolic extract of leaf have proven antiarthritic activity.[8] Therefore, we hypothesized that *J. gendarussa* may inhibit collagenase (matrix metalloproteinases) enzymes, which destroy type –II collagen.

**MATERIALS AND METHODS**

**Chemicals**

Collagen (Sigma–Aldrich Chemical Laboratories), Collagenase Type-II (Dutt enterprise, Nadiad), O- Phenanthrolin (Yucca, Mumbai), UV spectrophotometer (Shimadzu-1800), Micropipette (DK Scientific Technologies). All the other chemicals and reagents used in this study were of analytical grade.

**Collection of plant material**

*J. gendarussa* was authenticated by a taxonomist Dr. S.K. Patel, Head of the Department of Botany, School of Science, Gandhinagar, Gujarat, India. A voucher specimen (PH/08/001 and PH/509/008) was deposited at the Department of Pharmacognosy, K.B. Institute of Pharmaceutical Education and Research, Gandhinagar, Gujarat, India. It was also authenticated by comparing their morphological and microscopical characters with reported literature.[10-13]
Preparation of extracts

The leaves were coarsely powdered to 60 # and stored in air tight container which was used for the present work. The powder (500 gm) of leaf of *J.gendarussa* was subsequently extracted in a Soxhlet extraction unit (Borosil, Mumbai) using petroleum ether, methanol and water to get non-polar, semipolar and polar constituents. The resulting extracts were stored in vials and used for *In-vitro* assay.

These three extracts were standardized by studying their phytochemicals by chemical test[12-13] and also by Thin Layer Chromatography (TLC) before and after hydrolysis with 2 M Hydrochloric acid (HCl). According to the chemical constituents present in all extracts, finally methanolic and water extract of leaf was selected for *In-vitro* screening using collagenase inhibitory assay. As a result of this study methanolic extract was selected for further fractionation to Toluene, chloroform, Ethyl acetate, N- butanol and residue. These all fractions of methanolic extract of leaf were evaluated for its collagenase inhibitory action.

**TLC study of Fractions**

TLC was developed for fingerprinting of above prepared extracts of leaf of *J. gendarussa* with Camag HPTLC System. The standard solutions of 10 µl volume were applied on TLC plate. The samples of all extract of leaf of *J. gendarussa* were spotted. All plates were run in the mobile phase as mentioned in Table 1. The resolved bands on TLC plates were observed under UV light at 254 nm and 366 nm and after derivatization with different reagent.

**Collagenase (Matrix metalloproteinases) inhibitory assay**

Collagenase causes hydrolysis of protein to form amino acids, which reacts with ninhydrin reagent and it, gives purplish blue color. Collagenase inhibitory activity was carried out in eppendorfs tubes, 500 µl of different fractions of leaf of *J. gendarussa* (100, 250, 500 and 1000 µg/ml) solutions were serially diluted with 500 µl of tris buffer and 10 µl collagenase enzyme (0.125 %). All the tubes were mixed well and incubated at 37°C for 3.5 hours. Then 2.5 mg collagen was added to all the tubes, the tubes were incubated for equilibration at 37°C for 1.5 min. After 1.5 min, tubes were centrifuged at 5,000 rpm and the supernatant was taken for colour development. 20 µl of the supernatant was taken for colour development in a 96 well plate and to this 200 µl of ninhydrin colour reagent was added and the plate was placed over boiling water bath for 20 min for the reaction to take place, after completion of the reaction the plate was cooled to room temperature. 50 µl of the reaction mixture was again taken in to another 96-well plate and to this 200 µl of n-butanol was added. The solution was
mixed well and the absorbance was read at 570 nm. The percentage inhibition was calculated by using mean control absorbance value. Control samples were run without drug and blank samples were run without drug and enzyme. O-phenanthroline (a known zinc chelator), is an inhibitor of metalloproteinases. It is used for validation of assay in concentration range from 200 -2000 ng.\textsuperscript{[14-15]}

**Statistical analysis**

The results were presented as mean ± SEM of three determinations. The IC\textsubscript{50} concentration was calculated by Finney software.

**RESULTS AND DISCUSSION**

**Phytochemical study of extracts**

Preliminary phytochemical tests and TLC study indicated the presence of phytoconstituents such as phenolics, carbohydrates, flavonoids (flavanone), steroids, carotenoids, alkaloids and triterpenoids in the leaf of *J. gendarussa* (Figure 1). These are useful for quality evaluation and standardization of *J. gendarussa*. The leaf of *J. gendarussa* was found to be rich in carotenoids (7.88 % w/w), phenolics (2.21 % w/w), alkaloids (1.62 % w/w), flavonoids (2.03 % w/w) and saponins (foaming index- 125. Wide ranges of phytoconstituents were responsible for anti-arthritic activity which includes alkaloids, glycosides, tannins, phenolics, anthocyanins, sterols and triterpenoids. These phytoconstituents present in *J. gendarussa* exert desired pharmacological effect on body and thus act as natural anti-arhritic agents.\textsuperscript{[16]}

Based on phytochemical screening, extraction of the leaf of *J. gendarussa* was performed as per the polarity of the different solvents. They were extracted in petroleum ether (steroids), semipolar solvent like methanol (other alkaloids, flavanoids, saponins and phenolics) while in polar solvent such as water (carbohydrates). Based on above profile probably active extract may be methanolic extract of leaf of *J. gendarussa*. For further confirmation of chemical profile of petroleum ether, methanolic and aqueous extract of leaf of *J. gendarussa*, they were screened by TLC study. TLC study of petroleum ether extract of leaf of *J.gendarussa* shows presence of lupeol and β-sitosterol and other steroidal and triterpenoid constituents (Figure 1). Lupeol and β-sitosterol showed anti-inflammatory action in acute and chronic inflammation in rats and mice. Methanolic extract of leaf of *J.gendarussa* shows the phenolic and alkaloidal constituents and also shows same pattern of constituents at 254 nm and 366 nm before and after hydrolysis of material.
From the result of collagenase inhibitory action methanolic extract of leaf shows better protection against collagen type –II at IC\textsubscript{50} value 52.63 µg/ml. Therefore, methanolic extract was selected for further fractionation. Methanolic extract of leaf having phenolics glycosides and alkaloids that’s why it was first fractionated with toluene, chloroform, ethyl acetate and n-butanol.

**Figure 1. HPTLC analysis of leaf of Justicia gendarussa**

**Collagenase inhibitory assay**

The result shows that methanolic extract and toluene fraction of leaf showed maximum enzyme inhibition at IC\textsubscript{50} value 52.63 µg/ml and 44.75 µg/ml (Table 1). The collagenase inhibitory activity was gradually increased from methanolic extract to toluene soluble fraction which showed purity and potency leaf of *J. gendarussa*. Number of alkaloids were isolated from Justicia genus which have anti-inflammatory action\textsuperscript{[17]} The Wide ranges of phytoconstituents were responsible for anti-arthritis activity includes alkaloids, glycosides, tannins, phenolics, anthocyanins, sterols, triterpenoids etc.\textsuperscript{[16]} like, the drugs nimesulide\textsuperscript{[18]} and doxycycline\textsuperscript{[19]} inhibited collagenase in cartilage derived from osteoarthritis patients *in-vitro*\textsuperscript{[20]}; quinic acid esters from *Pluchea indica* with collagenase, MMP-2 and MMP-9.
inhibitory activities \cite{21}; green tea polyphenols show gelatinase inhibitory activity. \cite{22, 23} Because of the alkaloidal nature, it showed cytotoxic action and collagenase inhibitory action, it can shows inhibitory effect on arthritis.

Table 1 Collagenase inhibitory activity of extracts and fractions of leaf of J. gendarussa

<table>
<thead>
<tr>
<th>TEST EXTRACTS</th>
<th>IC$_{50}$ µg/ml</th>
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<tbody>
<tr>
<td>Methanolic extract</td>
<td>0052.63</td>
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<tr>
<td>Toluene fraction</td>
<td>0044.75</td>
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<tr>
<td>Chloroform fraction</td>
<td>0272.76</td>
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<tr>
<td>Ethyl acetate fraction</td>
<td>1564.41</td>
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<tr>
<td>n-Butanol fraction</td>
<td>0349.87</td>
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<tr>
<td>Residue</td>
<td>0311.19</td>
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<tr>
<td>Standard – O- Phenanthroline</td>
<td>0007.24</td>
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CONCLUSION
This finding may be physiologically significant, since this enzyme can be activated in arthritis. The toluene fraction of leaf of J. gendarussa is a highly potent inhibitor of collagenase. Such a level of potency of toluene fraction from J. gendarussa (IC$_{50}$ value 44.75 µg /ml) has pharmaceutical significance for drug development programs. In summary, inhibition of collagenase activity by the isolated compound may be an important mechanism underlying its chondroprotective effects.

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REFERENCES