Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
https://www.phytojournal.com
JPP 2024; 13(1): 399-403
Received: 12-11-2023
Accepted: 19-01-2024

Khushboo Vaghela
Associate Professor, Sharda School of Pharmacy, Pethapur, Gandhinagar, Gujarat, India

Sonal Patel
Sharda School of Pharmacy, Pethapur, Gandhinagar, Gujarat, India

Divyakant Patel
Principal, Sharda School of Pharmacy, Pethapur, Gandhinagar, Gujarat, India

Maitreyi Zaveri
Associate Professor, K. B. Institute of Pharmaceutical Education and Research, Gandhinagar, Gujarat, India

Corresponding Author:
Khushboo Vaghela
Associate Professor, Sharda School of Pharmacy, Pethapur, Gandhinagar, Gujarat, India

Collagenase type II inhibitory activity of stem, root and leaf of *Cocculus hirsutus*

Khushboo Vaghela, Sonal Patel, Divyakant Patel and Maitreyi Zaveri

DOI: https://doi.org/10.22271/phyto.2024.v13.i1e.14858

Abstract
Herbal products prepared either from single or multiple botanical ingredients are usually complex and variable in nature. Undoubtedly, the plant kingdom still holds many species of plants containing substances of medicinal value that have yet to be discovered. For this reason, *Cocculus hirsutus* (L.) a medicinal plant belonging to family *Menispermaceae*, the perennial climber was selected for the present study. The study was formulated with the objective to assess the Collagenase inhibitory activity and standardization of *Cocculus hirsutus*. Pharmacognostical study, physicochemical study and Phytochemical study were performed of leaf, stem and the root of *Cocculus hirsutus.* The leaf, stem and the root of *Cocculus hirsutus* were extracted with solvents namely ethanol and 70% hydro-alcohol for the determination of Collagenase inhibitory activity. *Cocculus hirsutus* contained steroids, saponin, flavonoid and phenolics. Stem ethanolic extracts showed highest collagenase inhibitory activity than all other extracts. Again fractionation of stem ethanolic extracts of *Cocculus hirsutus* was done with petroleum ether, ethyl acetate, n-Butanol and residue. From that petroleum ether and ethyl acetate showed significant collagenase inhibitory activity. So it may conclude that collagenase inhibitory activity may be due to steroids and flavonoids.

Keywords: Collagenase type II, stem, root, leaf, *Cocculus hirsutus*

Introduction
Nature always stands as a golden mark to exemplify the outstanding phenomena of symbiosis. Natural products from plant, animal and minerals have been the basis of the treatment of human disease. Today estimate that about 80% of people in developing countries still relays on traditional medicine based largely on species of plants and animals for their primary health care.
Cocculus hirsutus belonging to the family *Menispermaceae* consists of 20 different species distributed in tropics and subtropics regions. *Cocculus hirsutus* is a scandent shrub, known as Chilahinta in Ayurveda and Kattu kodi in Siddha system of medicine. The plant grows all over India, especially in dry regions. Leaves are simple, alternate, ovate, sub deltoid or three lobed, obtuse and mucronate. The base of leaf is subcordate or truncate. Petioles are very short, dark green, usually subauriculate at the base. The plant is a climber with green flowers bloom in February-March and fruits in May-June [1, 2].
The roots and leaves are used to treat polyuria, eczema, dysuria, abdominal disorders, rheumatoid arthritis, fevers, piles, syphilis, disorders of blood and as an aphrodisiac. Roots and leaves possess antimicrobial, cardiotonic, hyperglycaemic, diuretic, laxative and epileptic activity. In Sind, leaves are used in headache and neuralgic pains (Murray). The juice of the leaves, mixed with water, has the property of coagulation into a green jelly-like substance, which is taken internally, sweetened with sugar, as cure for gonorrhea [3, 4].
Arthritis is a form of joint disorder that involves inflammation of one or more joints [5]. There are over 100 different forms of arthritis. The most common form, osteoarthritis (degenerative joint disease), is a result of trauma to the joint, infection of the joint, or age. Other arthritis forms are rheumatoid arthritis, psoriatic arthritis, and related autoimmune diseases. Septic arthritis is caused by joint infection. The major complaint by individuals who have arthritis is joint pain. Pain is often a constant and may be localized to the joint affected. The pain from arthritis is due to inflammation that occurs around the joint, damage to the joint from disease, daily wear and tear of joint, muscle strains caused by forceful movements against stiff painful joints and fatigue [6].
Collagen Type II is composed of a triple helix of three identical a chain. These molecules associate to form a fibril that is stabilized by intermolecular crosslinks.

The fibrils provide the tensile strength and maintain the integrity of mammalian articular cartilage by forming a network that resists the swelling pressure resulting from the hydration of the polyanionic proteoglycan aggregates in the extracellular matrix. Damage to this fibrillar meshwork, made up of primarily type II collagen (90–95%), may be a critical event in the pathology of many arthritis, due in part to the very slow rate of collagen turnover within the cartilage. In early degeneration in articular cartilage, which may lead to osteoarthritis (OA), there is a loss of the tensile properties, indicative of damage to the fibrillar network [7].

Materials and Methods

Collection of raw material of *Cocculus hirsutus* [7]

Fresh plant material was collected from widely grown plant *Cocculus hirsutus* from Dhandhiya village of Rajkot district, Gujarat, India. Raw material was subjected to washing with distilled water and then allowed for drying for 5 days under shade and powdered to 60# separately and stored in well close container.

Authentication of raw material of *Cocculus hirsutus*

The procured material of *Cocculus hirsutus* was authenticated by taxonomist and further authenticated by comparing the microscopy with reported literature. Herbarium specimen was deposited (PH/013/001) at Pharmacognosy department, K.B.I.P.E.R., Gandhinagar.

Preparation of different extracts and fractions of leaf, stem and root of *Cocculus hirsutus* [8]

Ethanolic and 70% hydro-alcoholic extracts were prepared by maceration of powder of leaf, stem and root of *Cocculus hirsutus* 48 hours. Solvents were removed by rota evaporator. Dried extract was taken and assay was performed with this extract. For the assay different concentration was prepared like 10 µg/ml, 100 µg/ml, and 500 µg/ml.

Preparation of sub fraction

Successive fractionation was carried out by using Soxhlet assembly according to polarity index in following order:

1. Petroleum ether
2. Ethyl acetate
3. n-Butanol
4. residue(ethanol)

After getting different fractions of different solvents, evaporate the solvents to dryness to obtained semisolid residue. All fractions

Weigh and % yield of these extracts was calculated.

Collagenase inhibitory activity of leaf, stem and root of *Cocculus hirsutus*

Methodology for collagenase inhibitory assay [9]

Collagenase cause hydrolysis of protein to form amino acids which reacts with ninhydrin reagent, give purple color. Collagenase inhibitory activity reaction was carried out in appendorff's tubes. 500 µl of leaf, stem and root of *Cocculus hirsutus* and 10 µl collagenase enzyme were added to all tubes and incubated it for 3.5 hours.

After completion of 3.5 hours all tubes were taken back and 2.5 mg collagen was added and diluted with 500 µl of Tris-buffer (pH 6.8, 50 mM) to all tubes. All tubes were mixed well and incubated at 37 °C for 1.5 hrs. After 1.5 hrs, tubes were centrifuged at 5000 rpm and supernatant was taken for color development.

a) Control sample was run without extracts and a blank sample was run without extracts and enzyme.
b) O-phenanthroline was taken as an inhibitor of metalloproteinase.

Method for color development

20 µl of supernatant was taken for color development in a 96 well plate and 200 µl of ninhydrin reagent was added in that. The plate was placed on water bath for 20 minutes for the reaction to take place. After completion of the reaction plate was cooled to room temperature. 50 µl of above reaction mixture was again taken to another 96 well plate and 200 µl of n-butanol was added for the dilution. Mixed well above solution and the absorbance was read at 570 nm. The % inhibition was calculated by using mean control absorbance valve. % inhibition was calculated by following formula:

$$\% \text{ Inhibition} = \frac{\text{Abs. of con.} - \text{Abs. of test}}{\text{Abs. of con.}} \times 100$$

Method optimization

1) Activation of collagenase enzyme
2) Complexation (reaction) time between collagen and collagenase enzyme.
3) Inhibition time of collagenase enzyme by O-phenanthroline (standard).

Method optimization for the activation of collagenase enzyme [10]

1.25 mg of collagenase enzyme was taken in appendorff tube and 1 ml of Tris-HCl buffer was added in that tube. Enzyme was incubated at 37 °C for 5 hr. Another 5 appendorff's tubes were taken and in every appendorff tube 2.5 mg of collagen diluted with 0.5 ml of Tris-buffer was added.

At the end of every one hour one appendorff tube was taken and 10 µl of collagenase enzyme was added in that and again incubate that tube for 1.5 hr. Same procedure was done for all remaining four appendorff's tube containing collagen after 2, 3, 4 and 5 hours of enzyme incubation. Incubate all tubes for one hour after adding of enzyme.

After 1.5 hr that tube was taken back. 20 µl of supernatant was taken for color development in a 96 well plate and 200 µl of ninhydrin reagent was added in tubes. The plate was placed on water bath for 20 minutes for the reaction to take place.

After completion of the reaction plate was cooled to room temperature. 50 µl of above reaction mixture was again taken to another 96 well plate and 200 µl of n-butanol was added for the dilution. Mixed well above solution and the absorbance was read at 570 nm. The % inhibition was calculated by using mean control absorbance valve.

Method optimization for the complexation (reaction) time between collagen and collagenase enzyme [11-12]

Six appendorff's tubes were taken and in all tubes 2.5 mg of collagen diluted with 0.5 ml of Tris-buffer was added. After that 10 µl of collagenase enzyme was added in all tubes.

All tubes were incubated at 37 °C for different time interval like 15 min, 30 min, 1 hour, 2hours and 3 hours. At the end of every time interval one tube was taken.

From that tube 20 µl of supernatant was taken for color development in a 96 well plate and 200 µl of ninhydrin reagent was added in that. The plate was placed on water bath for 20 min for the reaction to take place.

After completion of the reaction plate was cooled to room temperature. 50 µl of above reaction mixture was again taken

to another 96 well plate and 200 µl of n-butanol was added for the dilution.

Same procedure was done for all remaining tubes. Mixed well above solution and the absorbance was read at 570 nm. The % inhibition was calculated by using mean control absorbance valve. Above assay was performed in duplicate.

Method for optimization of the inhibition time of collagenase enzyme by O-phenanthroline (standard) [13-15]

Six appendorff's tubes were taken and in all tubes 500 µl of standard solution was added of concentration (1-10 µg/ml). 10 µl of collagenase enzyme was added in all tubes.

All tubes were incubated at 37 °C for different time interval like 30 minutes, 1 hr, 2 hr, 3 hr and 4 hours. After completion of every interval of time an appendorff tube was taken and 2.5 mg of collagen diluted with 0.5 ml of Tris-buffer was added. Again, incubate all tubes for 1 hour. After 1 hr tubes were taken back.

From that tube 20 µl of supernatant was taken for color development in a 96 well plate and 200 µl of ninhydrin reagent was added in that. The plate was placed on water bath for 20 minutes for the reaction to take place.

After completion of the reaction plate was cooled to room temperature. 50 µl of above reaction mixture was again taken to another 96 well plate and 200 µl of n-butanol was added for the dilution.

Same procedure was done for all remaining tubes. Mixed well above solution and the absorbance was read at 570 nm. The % inhibition was calculated by using mean control absorbance valve.

Results

Collection and Authentication of raw material of *Cocculus hirsutus*

Plant *Cocculus hirsutus* was collected in the month of June, when it fully grown and flowering. Total 1500 gm of herb was collected to get 1000gm of dry powder. Authentication was done by taxonomist and Herbarium sheet (PH/013/001) was deposited at the Pharmacognosy and Phytochemistry department of K. B. Institute of Pharmaceutical Education and research.

Preparation of different extracts of leaf, stem and root of *Cocculus hirsutus*

Fig 1: Preparation of extracts

Using two solvents ethanolic & hydro-alcoholic (70%) different extracts were prepared. Leaf hydro-alcoholic extracts showed highest % yield as showed in Table 1.

Table 1: % Yield of various extracts of leaf, stem and root of *Cocculus hirsutus*

Sr. No.	Extract	Colour and consistency	% Yield (w/w)
1	Leaf ethanolic	Dark green-sticky solid	14.60
2	Stem ethanolic	Dark green-sticky solid	10.15
3	Root ethanolic	Light brown-dry	00.34
4	Leaf hydro-alcoholic	Green-sticky solid	21.90
5	Stem hydro-alcoholic	Light green-sticky solid	15.60
6	Root hydro-alcoholic	Light brown-dry	03.90

Method optimization

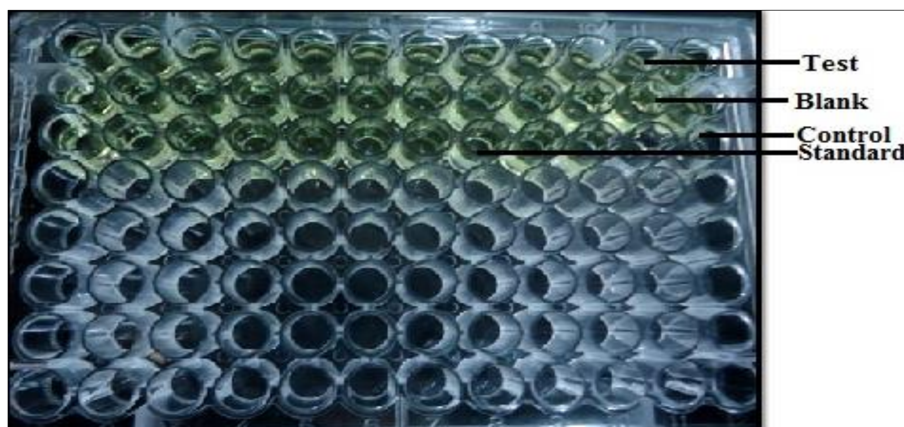

Fig 2: 96 well plate of extracts of *Cocculus hirsutus*

Activation time of collagenase enzyme

Time of activation of collagenase enzyme was optimizing with absorbance taken at different time interval. At the end of 3 hr, maximum blue color showed with ninhydrin reagent and after 5 hr, it became stable. Maximum activation was observed after 3hr as showed in table 2.

Table 2: Method for optimization for time of activation of collagenase

Time(hours)	Absorbance
1	0.147
2	0.237
3	0.297
4	0.314
5	0.315
6	0.316

Complexation (reaction) time between collagen and collagenase enzyme

Complexation time of collagen and collagenase was observed with change in absorbance. At the end of 3hr maximum blue color observed with ninhydrin reagent. At the end of 4 and 5 hr absorbance became stable. Digestion of collagen by collagenase was maximum at 3 hr because collagenase enzyme requires 3hr for activation. Reaction time required for collagen and collagenase was 1-1.5 hr after activation of collagenase enzyme as showed in table 3.

Table 3: Complexation (reaction) time between collagen and collagenase enzyme

Time (hours)	Absorbance
0 minutes	0.078
15 minutes	0.089
30 minutes	0.094
1	0.108
2	0.223
3	0.452
4	0.454
5	0.455

Inhibition time of collagenase enzyme by O-phenanthroline (standard)

O-phenanthroline was used as a standard drug which inhibits collagenase enzyme. Absorbance was decreases as time of reaction of collagenase and o-phenanthroline increases. Inhibition of collagenase enzyme was started after 1 hr and became stable after 5 hr as showed in table 4.

Table 4: Optimization for Inhibition time of collagenase enzyme by O-phenanthroline (standard)

Time(hours)	Absorbance
30 minutes	0.090
1	0.085
2	0.076
3	0.064
4	0.053
5	0.052
6	0.050

Collagenase inhibitory activity assay of extracts of leaf, stem and root of *Cocculus hirsutus*

All the ethanolic and hydro-alcoholic extracts of leaf, stem and root of *Cocculus hirsutus* showed more than 70% of collagenase inhibitory activity. From that stem ethanolic extract showed highest collagenase inhibitory activity i.e. 98.21 as showed in table 5.

Table 5: % inhibition of collagenase inhibitory assay by different extracts of leaf, stem and root of *Cocculus hirsutus*

Test extract (500 µg/ml)	% inhibition	Con of standard drug (µg/ml)	% inhibition
LHA	89.78	0.1	55.79
SHA	83.79	0.5	73.3
RHA	94.81	1.0	67.58
LE	80.70	1.5	55.10
SE	98.21	2.0	69.85
RE	75.36		

Discussion

There were many reported articles which showed collagenase inhibition helps in arthritis. Collagen digests by collagenase enzyme and converted into amino acids. Due to increase the concentration of collagenase in cartilage and bone there was a progression of arthritis and many other diseases.

Collagenase inhibitory activity was done for all extracts of leaf, stem and root of *Cocculus hirsutus*. All the extracts showed the presence of collagenase inhibitory activity. The highest collagenase inhibitory activity was present in stem ethanolic extract.

There are many reported articles showed the anti arthritis activity due to presence of B-sitosterol.

Various reports suggested that several plants containing anti arthritis activity *Tinospora cordifolia* [16], *Alangium salviifolium* [17], *Linum usitatissimum* [18], *Glycyrrhiza glabra* [19], *Boswellia serrata* [20], Rutin, Quercetin and Hesperidin [21].

In present study *Cocculus hirsutus* contain triterpenoid, phenols, flavanoids, steroids and saponins like B-sitosterol hence collagenase inhibitory activity may be due to all these constituent. Stem ethanolic extracts shows highest collagenase inhibitory activity.

The results of the present study substantiate to *Cocculus hirsutus* use to find out active compound responsible for this collagenase inhibitory activity.

References

1. Deepak Kamboj has reported Kamboj and medical plant published in kamboj society.com; c2009
2. Kirtikar KR, Basu BD. Indian Medicinal Plants, 2nd ed., Lalit Mohan Basu Publication, Allahabad. 1933;1:86-88.
3. Bhavna HM, Bothara SB. Ethnopharmacological properties of *Cocculus hirsutus* (L.) Diels- a review, International Journal of Pharmaceutical Sciences Review and Research. 2011;7(1):022.
4. Sharma PV, Dravyaguna V. Chaukambha Bharati academy, Varanasi. 2005;2:777.
5. Arthritis. CollinsDictionary.com. Collins English Dictionary - Complete & Unabridged 11th Edition; c2012.
6. Wollenhaupt J, Zeidler H. Undifferentiated arthritis and reactive arthritis, Current opinion in rheumatology. 1998;10(4):306-313
7. Jethva KD, Bhatt DR, Zaveri MN. Antimycobacterial Screening of Selected Medicinal Plants against Mycobacterium tuberculosis H37 Rv Using Agar Dilution Method and the Microplate Resazurin Assay. The International Journal of Mycobacteriology. 2020 Apr 1;9(2):150-155.
8. Henriksen K, Karsdal MA. Biochemistry of Collagens, Laminins and Elastin: Structure, Function and

Biomarkers. Elsevier Inc.; Amsterdam, The Netherlands. Type I Collagen; c2016. p. 1-11.
9. Dr. Pulok Mukherjee. quality control of herbal drug, 1st edition; c2002. p. 183-215
10. Berry L, Shuttleworth C. Bacterial Collagenase and Collagen Identification, Conn Tissue Res. 1988;17:153.
11. Cawston T. Measurement of activity of collagenolytic MMP and inhibitors of MMPs using radiolabeled collagen substrate, Nat Protoc. 2009;4:286.
12. Eisen A, Jeffrey J, Gross J. Human Skin Collagenase, Isolation and Mechanism of Attack on the Collagen Molecule, Biochim Biophys Acta. 1968;151:637.
13. Evanson J, Jeffrey J, Krane S. Collagenase and Rheumatoid Arthritis. New England Journal of Medicine. 1968;279:942.
14. Gallop P, Seifter S, Meilman E. Studies on Collagen. I. The Partial Purification, Assay, and Mode of Activation of Bacterial Collagenase. Journal of Biological Chemistry. 1957;227:891.
15. Harper E. Collagenases. Annual Review of Biochemistry. 1980;49:1063.
16. Woolley D, Lindberg K, Glanville R, Evanson J. Action of Rheumatoid Synovial Collagenase on Cartilage Collagen. Different Susceptibilities of Cartilage and Tendon Collagen to Collagenase Attack. European Journal of Biochemistry. 1975;50:437.
17. Tirkey R, Tiwari P. Effect of Cocculus hirsutus leaves extract on Freund's complete adjuvant and formaldehyde-induced arthritis. International Research Journal of Pharmacy. ISSN 2230-8407.
18. Paval J, Kaitheri SK, Kumar A, Govindan S, Mohammed CA, Kumar RS, *et al*. Anti-arthritic activity of the plant Tinospora cordifolia Willd. Journal of Herbal Medicine and Toxicology. 2011;5(1):11-16.
19. Paval J, Kaitheri SK, Kumar A, Govindan S, Mohammed CA, Kumar RS, *et al*. Anti-arthritic activity of bark extracts of Alangium salviifolium Wang. Rasayan Journal of Chemistry. 2008;1(3):433-436.
20. Kaithwas G, M., Dwivedi D. K. Therapeutic effect of Linum usitatissimum (flaxseed/linseed) fixed oil on acute and chronic arthritic models in albino rats. Inflammopharmacology. 2010;18(3):127-136.
21. Mishra NK, Bstia S, Mishra G, Chowdary KA, Patra S. Anti-arthritic activity of Glycyrrhiza glabra, Boswellia serrata and their synergistic activity in combined formulation studied in Freund’s adjuvant-induced arthritic rats. Journal of Pharmacy Education and Research. 2011;2(2).
22. Gujral ML, Sareen KN, Tangri KK, Roy AK, Gupta GP, Amma MK. Anti-arthritic activity of Glycyrrhiza glabra Linn. Indian Journal of Psychology. 1959;3(1):39-47.