Research Article

In Vitro Anti-Inflammatory, Antioxidant and *Ex Vivo* Anti-Arthritic Activity of *Operculina turpethum*

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ABSTRACT

Operculina turpethum (OT) has long since been used in Ayurveda to treat inflammatory conditions. In this investigation, we aimed to assess the antioxidant, anti-inflammatory and anti-arthritic properties of the plant. Phytochemical analysis of whole plant methanolic and aqueous extracts showed the presence of carbohydrates, amino acids, proteins, tannins, alkaloids, cardiac glycosides, terpenoids, flavonoids, and steroids; with higher amounts in methanolic extracts. We also quantified phenols and flavonoids in ethanolic, methanolic, and butanolic extracts. Anti-inflammatory activity was measured in vitro by the protein denaturation inhibition assay and the hRBC membrane stabilization method. The IC_{50} values were found to be 380.55 µg/mL and 878.608 µg/mL respectively, with aspirin as the standard drug. Anti-arthritic activity was measured ex vivo using collagen denaturation inhibition assay. Whole plant methanolic extracts were found to inhibit heat-induced collagen denaturation maximally at a concentration of 500 µg/mL. 82.176 % protection was achieved, and the IC_{50} value was found to be 341.094 µg/mL. These results suggest that OT possesses significant anti-inflammatory and anti-arthritic effects. However, further studies are warranted to evaluate the potential of OT as an anti-arthritic drug.

Keywords: Anti-arthritic, anti-inflammatory, *O. turpethum*, protein denaturation inhibition, membrane stabilization.

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INTRODUCTION

Arthritis is a painful inflammatory disorder of the joints and peri-articular tissues that afflicts more than 350 million people worldwide[1-3]. It is characterized by auto-antibody (rheumatoid factor and anti–citrullinated protein antibody [ACPA]) production, synovial inflammation and hyperplasia (swelling), cartilage as well as bone destruction[4], leading to restricted angular movement[2]. Western medicine approaches treatment symptomatically by the administration of non-steroidal anti-inflammatory (NSAID) drugs like indomethacin[5], aspirin, and diclofenac[6] which block the responsible inflammatory pathways. However, their long-term usage leads to gastrointestinal, cardiovascular, and renal complications[7].

Herbal medicines have a wider range of therapeutic effects, are easier to metabolize, have minimal to no toxicity and are administered in the form of nutraceuticals. They offer holistic as well as safe and effective healing at the root of the disease[8]. *Operculina turpethum*, the plant in this study, was used in traditional Indian medicine to treat skin and gastrointestinal issues, tumors[9], paralysis[10], myalgia, cardiovascular diseases, ocular problems, and other inflammatory conditions[11].

Previous studies focused on the assessment of anti-inflammatory and anti-arthritic effects of *O. turpethum* extracts on rat paw edema[12,13] and other parameters such body weight, spleen index score, rheumatoid factor and hematological estimation[14]. However, no studies have been conducted on the plant's protective effect on collagen and therefore, cartilage. Hence, the intention of our study was to appraise the anti-arthritic potential through an *ex vivo* collagen denaturation inhibition assay, in addition to *in vitro* anti-inflammatory assays.

MATERIALS AND METHODS

70% methanol, distilled water, hydrochloric acid, ethanol, n-butanol, benzene, sodium hydroxide, Mayer's reagent, Benedict's reagent, Wagner's reagent, α-naphthol, FC Reagent, Fehling's reagent, ferric chloride ($FeCl_3$), chloroform, lead acetate, Ninhydrin reagent, Million's reagent, acetic anhydride, glacial acetic acid, bovine serum albumin (BSA), sulphuric acid, trisodium citrate, sodium chloride, quercetin, aluminium chloride, potassium acetate, catechol, sodium carbonate, nitric acid, Dragendroff's reagent, aspirin.

Sample collection and preparation: The whole plant samples of *Operculina turpethum* were collected from Foundation for Revitalization of Local Health Traditions (FRLHT), Bengaluru, Karnataka in the month of February 2022. The whole plants were washed thoroughly in tap water, sun dried for about 6 h, and then ground into a fine powder by using a mixer grinder. It was weighed, stored in

airtight containers, and used for different analyses in the laboratory.

Preliminary phytochemical screening: The aqueous and methanolic extracts were used to test for the presence of phytochemicals[15,16] such as tannins, alkaloids, cardiac glycosides, phytosterols, terpenoids, flavonoids, saponins, and nutrients such as carbohydrates and proteins.

Detection of Alkaloids: 10% aqueous and methanolic extracts were treated with in dilute HCl and filtered. The extract was tested for the presence of alkaloids as follows:

Mayer's Test: The formation of a cream or whitish coloured precipitate with Mayer's reagent is an indication that alkaloids are present in the sample being tested.

Dragendroff's Test: The formation of a red precipitate with Dragendroff's reagent is indicative of the presence of alkaloids.

Wagner's Test: The presence of a brown or crimson precipitate when using Wagner's reagent is conclusive evidence that alkaloids are present.

Test for Tannins

Ferric chloride test: The appearance of bluish black colour with 1 ml of 5% $FeCl_3$ solution shows the presence of phenols/tannins.

Test for cardiac glycosides

Keller-Killiani Test: The appearance of blue colour in the acetic acid layer on treatment with 0.4 ml of glacial acetic acid containing trace amounts of $FeCl_3$ and 0.5 ml of concentrated H_2SO_4 indicates the presence of cardiac glycosides.

Test for flavonoids

Alkaline reagent test: The change in colour of the extract from intense yellow coloration to colourless on the addition of a few drops of dilute sodium hydroxide solution followed by dilute HCl indicates the presence of flavonoids.

Test for steroids

Liebermann Buchard test: The appearance of a brown ring at the junction of the two layers and a green layer in the upper layer when 1 ml of extracts, boiled and cooled, is treated with concentrated H_2SO_4 confirms the presence of steroids.

Test for terpenoids

Salkowski Test: Appearance of reddish-brown colour with 1 ml of the extracts, 2 ml chloroform and few drops of H_2SO_4, at interface indicates the presence of terpenoids.

Test for saponins

Froth test: Persistence of froth for at least 2 min with 1 ml of extracts and 2 ml water, shaken well, indicates the presence of saponins.

Test for proteins and amino acids

Ninhydrin test: Appearance of violet colour with extracts and 0.2% w/v ninhydrin solution boiled for a few minutes indicated the presence of amino acids.

Million's test: Appearance of reddish-brown colour or precipitate with extracts and Million's reagent heated gently indicates the presence of tyrosine residues.

Xanthoproteic Test: Appearance of yellow colour of extracts when treated with a few drops of concentrated nitric acid indicates the presence of proteins.

Test for carbohydrates: The extracts were dissolved in 5 ml of distilled water and filtered. Filtrates were used to test for presence of carbohydrates.

Fehling test: Appearance of a brick red coloration or precipitate with 1 ml of filtrates and 0.5 ml of Fehling A and Fehling B, boiled together, suggests the presence of carbohydrates.

Molisch's test: The formation of a reddish violet ring at the junction with 1 ml of the filtrates, two drops of Molisch's reagent and concentrated sulphuric acid indicates the presence of carbohydrates.

Benedict's test: The appearance of an orange red precipitate with Benedict's reagent suggests the presence of reducing sugars.

Estimation of total phenols: The total phenolic content in different plant extracts was determined using the Folin-Ciocalteau colorimetric method[17] based on oxidation-reduction reaction. Catechol was used as standard.

A stock solution of 1 mg/mL catechol was made in distilled water. It was diluted a hundred times with distilled water and used as a working standard solution. From the working solution, 0.2-1 mL aliquots were taken into separate test tubes. The volumes were made up to 3 mL with distilled water, after which 0.5 mL of FC reagent followed by 2 mL of 20% Sodium Carbonate solution were added. Tubes were incubated at room temperature for 15-20 min. The same procedure was followed for 0.1mL of the 3 different plant extracts. Absorbance was read at 560 nm against a suitable blank using the colorimeter. The phenol content in the extracts was measured in terms of catechol equivalent (mg of CE/g of tissue).

Estimation of total flavonoids: The total flavonoid content was estimated using aluminium chloride colorimetric assay[18] with little modifications. Quercetin was used as a standard.

The stock solution contained a concentration of 1 mg/mL and was prepared in methanol. A working standard solution was prepared by diluting it ten times with distilled water. From the working solution, 0.2-1 mL aliquots were taken into separate test tubes. The volume in each test tube was made up to 2 mL using methanol, after which 0.1 mL of 10% $AlCl_3$ followed by 0.1 mL of 1 M potassium acetate solution were added. Volumes in each tube were

made up to 5 mL using distilled water. The tubes were incubated at room temperature for 30 min. The same procedure was followed for 0.1 mL of the three different plant extracts. The absorbance was read at 415 nm against a suitable blank. The flavonoid content in the extracts was measured in terms of quercetin equivalent (mg of QE/g of tissue).

Evaluation of Anti-Inflammatory Activity

Inhibition of protein denaturation assay: The anti-inflammatory activity was studied using inhibition of albumin denaturation technique[19,20] with slight modifications. The reaction mixture contained methanolic extract of the plant sample with different concentrations (100, 200, 300, 400, 500 µg/mL) made up to 2 mL using 0.1M, pH 7.8 Tris-HCl buffer, 1 mL of 1% BSA/Bovine Serum Albumin. Aspirin (1 mg/mL) was used as a standard drug. The tubes were incubated at room temperature for 10 min and then placed in a water bath at 51 ^{o}C for 20 min. After cooling the test tubes, turbidity was measured at 660 nm using a suitable blank. The experiment was conducted in triplicates. The percentage of protein denaturation inhibition was calculated from the below equation. IC50 was calculated from y = 0.1908x - 12.417 for the extract.

$$\%\ Inhibition = \frac{OD\ of\ control - OD\ of\ sample}{OD\ of\ control}\ x\ 100$$

Human RBCs membrane stabilization assay: This assay involved two parts, discussed below.

(a) Preparation of RBC Suspension: The anti-inflammatory activity was studied using the hRBC membrane stabilization method according to Azeem et al., [21] with modifications. About 10 mL of fresh human blood was collected and transferred into a centrifuge tube containing equal volumes of 3.2% trisodium citrate (w/v). This was centrifuged at 3000 rpm for 10 min. The supernatant was removed, and the pellet was washed thrice with isosaline. The volume of blood was measured and made into a 10% (v/v) suspension isosaline.

(b) Hypotonicity induced hemolysis: The reaction mixture consisted of methanolic extract of the plant sample of varying concentrations (100, 200, 300, 400, 500 µg/mL) made up to 0.5 mL with 0.1M phosphate buffer (pH 7.4), 0.5mL of hRBC suspension and 2 mL of hyposaline[22]. Aspirin (1 mg/mL) was used as standard drug. The test tubes were incubated at 37 ^{o}C for 30 min and then centrifuged at 10,000 rpm for 20 min. The absorbance of the supernatants was read at 560 nm against a suitable blank. The experiment was carried out in triplicate. The percentage inhibition of either membrane stabilization or hemolysis was determined using the below equation. IC50 was calculated from y = 0.0388x + 15.91.

$$Inhibition = \frac{OD\ of\ control - OD\ of\ sample}{OD\ of\ control}\ x\ 100$$

Evaluation of Anti-arthritic Activity

Inhibition of collagen denaturation assay:

(a) Isolation of collagen from chicken cartilage: Collagen was isolated from the fore limbs of the chicken that contains good amount of cartilage. Approximately 0.2 g of isolated collagen was weighed. It was homogenized in 10ml of 0.15 M, pH 7.4 ice cold phosphate buffer using a mortar and pestle. The solution was maintained in ice cold conditions and stirred for 10 min using a magnetic stirrer. The homogenate obtained was incubated in the phosphate buffer for 30 min in ice cold conditions. It was centrifuged at 10,000 rpm for 10 min. The supernatant was collected and used to perform the collagen denaturation inhibition assay to assess the anti-arthritic activity of the samples[23].

(b) Inhibition of collagen denaturation assay: The methanolic extract of the plant sample of various concentrations (100, 200, 300, 400, 500 µg/mL) were added to 500 µl of isolated collagen in the ice-cold buffer taken into different test tubes. The standard drug utilized for this experiment was Aspirin. The reaction mixtures were incubated for 20 min at 51 ºC[24]. Absorbance was read at 660 nm. The percentage inhibition of collagen denaturation was determined using the below equation. IC50 was computed from the equation y = 0.1982x - 17.605.

$$Inhibition = \frac{OD\ of\ control - OD\ of\ sample}{OD\ of\ control}\ x\ 100$$

RESULTS AND DISCUSSION

Secondary metabolites, also known as phytochemicals, are bioactive compounds manufactured by plants via accessory pathways of metabolism. They fulfil roles such as flowering, growth maintenance, defense, and abscission[25]. In the present study, the phytochemical screening was carried out using methanolic and aqueous extracts. Aqueous extract showed good results for alkaloids, tannins, flavonoids, steroids, proteins, and carbohydrates and negative results for saponins. Methanolic extract shows better results for all tested phytochemicals compared to the aqueous extract.

Phenols are a broad class of secondary plant products commonly found in various vegetables. It is well-established that a diet containing higher amounts of polyphenols will increase the plasma antioxidant capacity, thereby reducing the threat of degenerative, infectious, and chronic human diseases. They do so by lowering the levels of free radicals in the body[26]. In the present study, the total phenol content in the various extracts of OT was spectrophotometrically determined and it was found that the methanolic extract showed the highest phenolic content followed by the ethanolic extract, i.e., 11.68 ± 1.2 and 5.97 ± 0.6 mg catechol Eq/g tissue (Figure 1).

Table 1: Screening for phytochemical constituents: Both aqueous and methanolic extracts were screened qualitatively for the presence of bioactive primary and secondary metabolites.

Constituents	Test	Aqueous Extract	Methanolic Extract
Alkaloids	Mayer's test	++	+++
	Dragendroff's test	++	+++
	Wagner's test	+	+++
Tannins	Ferric Chloride test	++	++
Cardiac-glycosides	Keller-Killiani test	+	++
Flavonoids	Alkaline reagent test	++	+++
Terpenoids	Salkowski test	+	++
Saponins	Froth test	-	++
Steroids	Liebermann Buchard test	+++	+++
Protein and amino acids	Ninhydrin test	++	+++
	Millon's test	++	+++
	Xanthoproteic test	++	+++
Carbohydrates	Fehling's test	+++	+++
	Molisch's test	++	+++
	Benedict's test	+++	+++

-Indicates absence, + denotes low quantities, ++ indicates average quantities, +++ indicates high quantities.

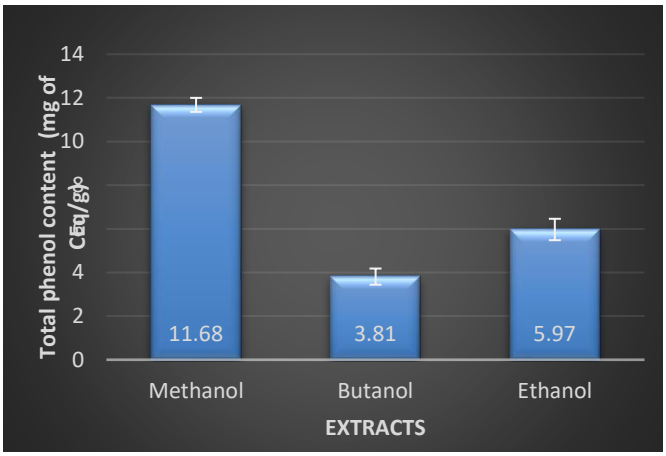

Figure 1: Phenol content in methanolic, butanolic and ethanolic extracts of OT

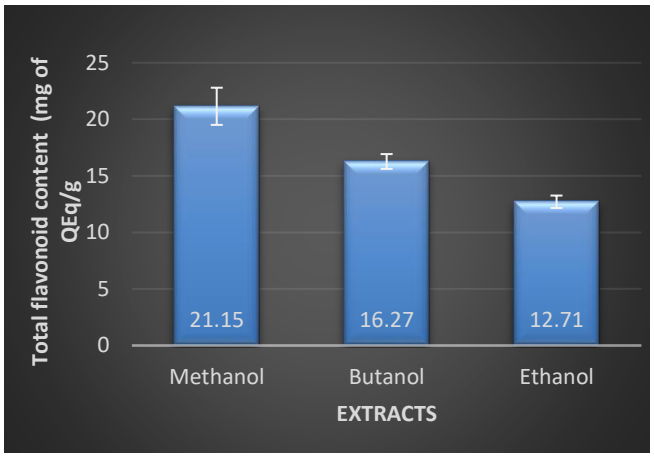

Figure 2: Flavonoid content in methanolic, butanolic, and ethanolic extracts of OT

Flavonoids, a class of polyphenol secondary metabolites, play a crucial role in a variety of biological activities in plants, animals, and bacteria. They exhibit anti-cancerous, anti-inflammatory, anti-mutagenic, as well as anti-oxidative properties[27]. The total flavonoids in three extracts of OT were spectrophotometrically determined in this investigation, with the methanolic extract exhibiting the highest flavonoid content followed by butanolic extract, i.e., 21.15 ± 3.2 and 16.27 ± 2.9 mg of QEq/g tissue, respectively (Figure 2).

Denaturation of proteins is one among many other causes of inflammation in the system. This results in many inflammatory disorders, such as arthritis. Inflammation is characterized by edema, accumulation of leucocytes, and necrosis. Inflammation is a process that occurs in response to physical injury, infections by pathogens, heat, toxic chemical irritants, and many other factors. Inflammation can trigger many diseases, including stroke, arthritis, and cancer[28]. The inflamed tissue is distinguished by redness, heat, protuberance, and pain with impaired physiological functions. The study indicates that the anti-inflammatory activity rises with an increase in the concentration of the plant extract. It can also be inferred that the methanolic extract of OT exceeds the activity of aspirin (Figure 3).

Similar results were reported in *Andrographis paniculata*[29], *Terminalia arjuna*[30], and seeds of *Artocarpus heterophyllus*[31].

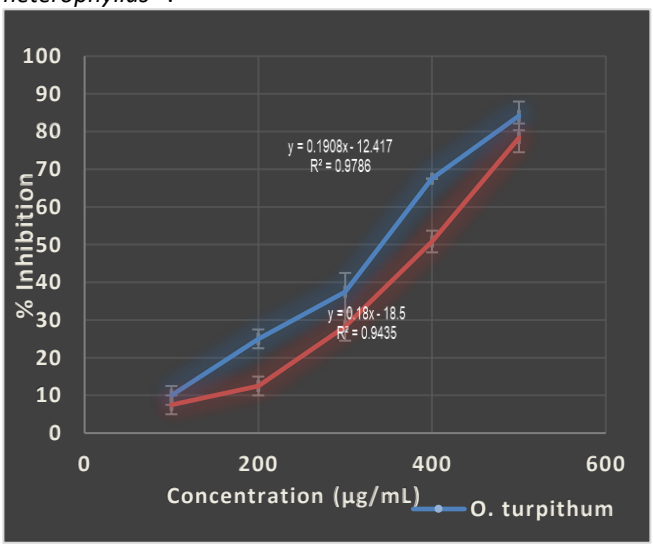

Figure 3: Inhibition of protein denaturation by methanolic extract of OT

Commonly used method to evaluate the *in vitro* anti-inflammatory activity is the RBC membrane stabilization assay. This is because the cell membranes of erythrocytes resemble those of lysosomal membrane[4]. Therefore, stabilisation of the RBC membrane indicates that the extract can also stabilise lysosomal membranes. This is crucial because stabilisation of lysosomes can help to reduce inflammatory responses by inhibiting the release of the constituents of the lysosome in the activated neutrophil. The present study indicates that anti-inflammatory activity is enhanced with an increase in the concentration of the plant extract. Aspirin has better membrane stabilisation activity compared to the plant extract (Figure 4).

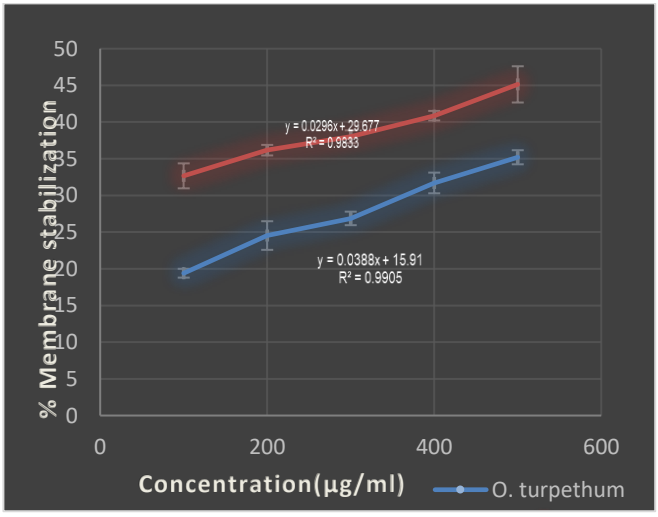

Figure 4: RBC membrane stabilisation by methanolic extract of OT

Collagen is abundantly present in humans. Denaturation of collagen is a marker of multiple diseases and injuries such as cancer, osteoporosis, and osteoarthritis. Rheumatoid arthritis is characterized by auto-antibody (rheumatoid factor and anti–citrullinated protein antibody [ACPA]) production, synovial inflammation and hyperplasia, cartilage as well as bone destruction[33]. Cartilage is mainly made up of collagen and thus the anti-arthritic activity was screened by evaluating the percentage of collagen denaturation inhibition assay (Figure 5). The study reveals that the anti-arthritic activity is enhanced with an increase in the concentration of the plant extract.

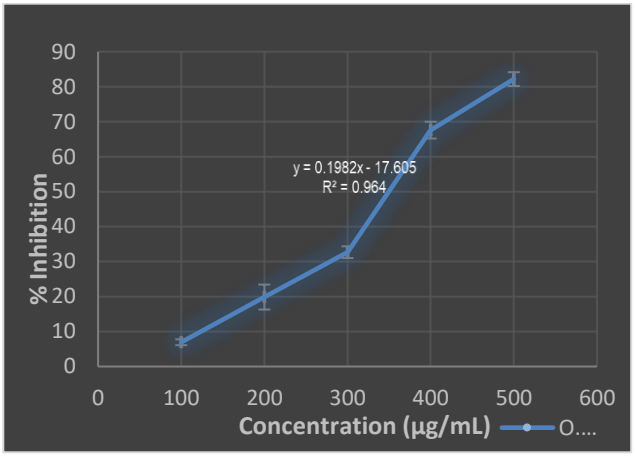

Figure 5: Anti-arthritic activity of methanolic extract of OT evaluated by collagen denaturation inhibition assay

CONCLUSION

This study elucidates the anti-inflammatory and anti-arthritic potential of *Operculina trupethum*. Methanolic extracts showed the highest concentrations of beneficial phytochemicals. This is the first study that evaluates the plant's protective effect on collagen in conditions of heat denaturation via a simple, efficient, and reproducible *ex vivo* protocol. Since its extracts safeguard collagen, a major component of cartilage, OT shows promise as an anti-arthritic drug.

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