CURATIVE EFFECT OF FORMONOETIN-7-*O*-B-D-GLUCOPYRANOSIDE FROM METHANOLIC EXTRACT OF *OPERCULINA TURPETHUM IN* FREUND'S COMPLETE ADJUVANT INDUCED ARTHRITIS

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Abstract:

Objective: To investigate the anti arthritic potential of isolated compound Formonoetin-7-O-β-D-glucopyranoside from Methanolic Extract of Operculina turpethum. ***Methods:*** *F-7-β-D-g was subjected to assess the anti arthritic activity by foot pat thickness, body weight measurement, rheumatoid factor, spleen index score assessment and hematological estimation using a model Complete Freund's Adjuvant (CFA) induced arthritis in wistar rat.* ***Result:*** *Administration of F-7-β-D-g at the doses of 10, 20, 40, 50 mg/kg b. w. p. o exhibited statistically significant (p < 0.001) inhibition of the edema, rheumatoid and spleen index factors significantly increased (P < 0.001) the Heamoglobin (Hb) level, Red Blood Cells (RBCs), White Blood Cells (WBCs) count, Erythrocyte sedimentation Rate (ESR) compared to arthritic treated groups.* ***Conclusion:*** *F-7-β-D-g from the methanol extract of Operculina turpethum holds anti arthritic property by modulating bone erosion.*

Keywords: *Operculina turpethum, Formonoetin-7-O-β-D-glucopyranoside, Rheumatoid arthritis, Freund's complete adjuvant-induced arthritis.*

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INTRODUCTION:

Rheumatoid arthritis (RA) is a systemic auto immune disease that causes chronic inflammation of connective tissue primarily in the joints that involves synovial proliferation, and cartilage destruction [1]. In RA, bone deformations and disability of joint function occurred due to progressive erosion of articular cartilage in synovial joint and infiltration of auto antibodies in it, leading to severe pain, swelling and redness within the joints [2]. Amongst the various experimental animal models of arthritis, induction of arthritis by Freund's complete adjuvant (FCA) is one of the standardized methods which mimic the human pathophysiological state including chronic swelling in multiple joints due to accumulation of inflammatory cells, bone and joint destruction.

Rheumatoid arthritis (RA) is a major auto immune disorder and up to 6 % of the world population develops immunity deficiency in their lifetime [3]. RA affects an estimated 7 million people in India, and 60 million worldwide. Approximately 30 % of them are middle age and women. In 2013 it resulted in 35,000 deaths up from 23,000 deaths in 1980. The prevalence of RA is 0.7 % in India, which is comparable to the U.S. and other developing nations [4]. The World Health Organization (WHO) estimated that approximately 50 % people with RA live in developing countries and most of them do not get adequate medical treatment. The drugs commonly in use for the treatment of arthritis include glucocorticoids namely cortisone and prednisone, NSAIDS like ibuprofen and naproxen, disease modifying anti inflammatory and anti rheumatic drugs like Methotrexate and leflunomide and newer therapies such as biological response modifiers like tumor necrosis factor, alpha blocking agents, which are often required to inhibit the underlying immune processes [5].

However, besides high costs, all of these drugs are associated with numerous side effects, severe adverse reactions and toxicity [6]. In recent days, researchers are directed towards traditional system of medicine for the discovery of drugs that are long acting with minimum side effects [7]. However, herbal medicines are being accepted and used increasingly by general populations because of the ethnic acceptability and compatibility having fewer side effects [8].

It is imperative to examine the therapeutic activity of herbal products against autoimmune disorders as well as other conditions involving inflammation [9]. In Ayurveda, root of operculina turpethum is used internally to treat fevers, anorexia, edema, anaemia, ascites, constipation, hepto-splenomegaly, hepatitis, intoxication, abdominal tumors, ulcers; wounds worm infestation, pruitus and other skin disorders.

Root is also administered to treat obesity, haemorrhoids, cough, asthma, dyspepsia, flatulence, paralysis, gout, rheumatism, melancholia, scorpion sting, and snake bites [10]. The paste of root powder of Trivitis used topically to treat vitiligo and other skin disorders, alopecia, cervical lymhadeitis, haemorrhoids, fistulas, ulcers and chancres [11]. Oil extracted from the root bark of Trivitis used in skin diseases of a scaly nature [12].

A processed ghee with Trivritor fresh juice of Trivit leaves is dropped into the eyes to treat diseases like corneal opacity used to treat hematemesis, tuberculosis and herpes [13]. In this scenario, the current study focuses the curative effects of isolated compound Formonoetin-7-O-β-D-glucopyranoside from Methanolic Extract of **Operculina** *turpethum* on Rheumatoid arthritis. Further to provide scientific justification to evaluate its folk claims and also propose its probable mechanism of action.

MATERIALS AND METHOD:

Plant material

Operculina turpethum was collected from the herbal garden of Tirunelveli district of Tamilnadu during the month of December 2015. The plant material was identified and authenticated by Dr. J. Jayaraman, Director, Plant Anatomy Research Centre, West Tambaram, and Chennai. Voucher specimen (No. PARC/2015/986) was preserved for future references.

Animals

The experiment was performed on healthy male and female Sprague Dawley (SD) rats of eight weeks old and body weight of 140-160 g. The female rats were nulliparous and non-pregnant. The rats were fed with standard laboratory diets, given water *ad libitum* and maintained under laboratory conditions of temperature 22°C (± 3°C), with 12 h light and 12 h dark cycle. All experimental procedures were carried out in strict accordance with the guidelines prescribed by the Committee for the Purpose of Control and Supervision on Experimentation on Animals (Regd. No. 1696/PO/a/13/CPCCSEA).

Drugs and chemicals

All the reagents used were of analytical grade. Methotrexate from Akums Drugs & Pharmaceuticals, India. Complete Freund's adjuvant (CFA) was a 5mg/ml suspension of heat-killed *Mycobacterium tuberculosis* (H37Rv strain) obtained from the (Tuberculosis Research Centre, ICMR, Chennai) triturated in paraffin oil (Ernest Chemist, Accra, Ghana) was used for the induction of arthritis.

Extraction and isolation of compound
Collection of plant material and preparation of extract
Fresh plant materials of Operculina turpethum were collected from the herbal garden of Tirunelveli, Tamilnadu. The dried plant material was extracted for 72 h with 7 L of 70 % methanol (v/v) at 50 °C. The extract was evaporated under reduced pressure to furnish a dark brown thick semi solid residue. The preliminary phytochemical investigations were carried out with methanolic extract of *Operculina turpethum* (MEOT) for qualitative identification of phytochemical constituents.

Isolation of the compounds
MEOT was subjected for fractionation to isolate a compounds using flash column, HPLC, sephadex LH-20. The isolated compounds were identified as Quercetin (**1**), Formonoetin (**2**), and Formonoetin-7-*O*-β-D-glucopyranoside (**3**) by spectral techniques. In this study, F-7-β-D-g (compound **3**) was used in the treatment protocol because of minimal yields of compounds **1** (0.75 mg) and **2** (0.82 mg).

Acute Toxicity Studies
Acute toxicity study for isolated compound - Formonoetin-7-*O*-β-D-glucopyranoside (F-7-β-D-g) was performed as per OECD guideline 425 [14]. Animals were divided into 3 groups of 3 animals in a group.
Female, nulliparous and non-pregnant mice weighing between 18-22 g was selected for this study. The animals were kept fasting overnight provided only with water. The dose progression study was carried out at three different dose levels of 100 mg/kg, 250 mg/kg and 500 mg/kg and observed for mortality during 48 h study period. The dose was administered only once for each group. The dose at which mortality was observed in two out of three mice was considered as toxic dose. All the animals were observed twice daily for health condition, morbidity and mortality for 14 days. Based on the result obtained from this study, the dose for this study was fixed.

Complete Freund´s Adjuvant Induced Arthritis
Wistar albino rats of either sex weighing between 200 to 300 g were taken and divided into seven groups each containing six animals [15]. On day zero, all rats were administered with 0.1 ml of complete Freund's adjuvant (CFA) into the sub plantar region of the left hind paw. The adjuvant contained heat killed *Mycobacterium tuberculosis* in sterile paraffin oil (10 mg/ml). Dosing with the test and standard compounds was started on the first day and continued for 12 days according to the following

schedule: Group I: Normal control (0.5 ml normal saline), Group II: Disease control (arthritic treated), Group III – VI: F-7-β-D-g (10, 20, 40, 50 mg/kg b.w.p.o.), Group VII: Methotrexate (5mg/kg b.w.p.o.). From day 13th to 21st, the animals were not dosed with the test compound or the standard. The following parameters were measured.

Evaluation of the development of Arthritis
The animals were weighed using digital weighing balance and foot pad volume of left hind limbs were recorded on the day of CFA injection, and again measured on 3rd, 7th, 14th and 21st day using mercury column Plethysmometer (Ugo Basile, Italy) [16,17].

Arthritic Index
Inflammation in each paw was graded according to the extent of erythematic and edema of the periarticular tissues using a scale of 0 – 4. Animal were scored 0 for no inflammation, 1 for unequivocal inflammation of one joint of the paw, 2 for unequivocal inflammation of at least two joints of the paw or moderate inflammation of one joint, 3 for severe inflammation of one or more joints, 4 for maximum inflammation of one or more joints in the paw. The arthritis score for each rat on day 0 was determined to be 0. The scores for each paw were then added to get the total arthritis score on day 22, designated as the arthritic index [18].

Rheumatoid Factor
On the day 22nd blood was withdrawn from the each animal through retro-orbital plexus under light ether anesthesia. The blood was collected into vials containing EDTA and the RF of synovial joints was analyzed. The latex turbidimetry method was used in the present study using RF turbilatex kit of SPINREACT. Latex particles coated with gamma globulin are agglutinated when mixed with samples containing RF. The agglutination causes an absorbance change, dependent upon the RF content of sample that can be quantified by comparison from a calibrator of known RF concentration. Calibration was carried out for linear range up to 100 IU/ml. The reading of RF factor of all the groups obtained was compared with the control animals and was expressed as IU/ml RF [19].

Spleen-Index
At the end of the experiment, after sampled for serum separation, all rats were sacrificed by ether anesthesia [20]. The spleen of all the rats were removed and weighed immediately. The spleen indexes were calculated by using the following formula:

Spleen Index = Spleen weight of CFA rat/ Body weight of CFA rat
                     Spleen weight of normal rat/Body weight of normal rat

Hematological estimation

The collected blood samples were subjected to hematological parameter. In which, Hemoglobin content was estimated by the method of Austin and Drabkin. Red Blood Cell (RBC) and White Blood Cell (WBC) counts were estimated according to the method of Chesbrough and MC Arthur in an improved Neubauer chamber. Estimation of Erythrocyte Sedimentation Rate (ESR) was carried out by the method of Westergren [21].

Statistical analysis

The experiment results are expressed as means ± SE. Statistical analysis of the data obtained from the experiment was carried out using the one way analysis of variance (ANOVA) followed by Dunnett's multiple range test. P-values < 0.05 were considered as statistically significant using SAS software -Version 6 (SAS Institute, Cary, North Carolina).

RESULT:

Acute oral toxicity test (AOT)

In AOT test, F-7-β-D-g administration does not produce mortality and any clinical signs of toxicity within 48 h and over a period of 14 days. No Statistically significant difference was found at 500 mg/kg b. w. p. o., 1/10th of this dose (50 mg/kg b.w.p.o.) Was taken as the test dose for further study and so it was found to be safe.

CFA induced rat paw edema

The hind paw injected with complete Freund's adjuvant became gradually swollen and reached its peak on 21st day. Table-1 showed the results obtained for the test and standard drug treatment in the complete Freund's adjuvant-induced (CFA) paw edema test at specific time intervals. It was obvious that during 21st day paw edema in disease control inflamed paw is increased in time dependent manner and all administration test groups (F-7-β-D-g at the dose of 10, 20, 40, 50 mg/kg , and Methotrexate 5 mg/kg) significantly inhibited the development of joint swelling induced by complete Freund's adjuvant.

Table 1: Effect of Formonoetin-7-*O*-β-D-glucopyranoside on paw edema

Treatments	Mean paw thickness (mm)				
	0 day	**3th day**	**7th day**	**14th day**	**21st day**
Group I	3.91±0.003	3.91±0.003	3.94±0.003	3.85±0.02	3.86±0.002
Group II	3.73±0.05***	10.01±0.03***	10.13±0.01***	10.15±0.05***	10.8±0.02***
Group III	3.9±0.04***	7.21±0.04***	6.73±0.03***	5.03±0.02***	4.26±0.02***
Group IV	3.66±0.034***	7.72±0.054***	6.28± 0.02***	5.43±0.04***	4.06±0.09***
Group V	3.65±0.03***	7.86±0.02***	7.13±0.02***	6.3±0.02***	6.12±0.03***
Group VI	3.68±0.02***	7.66±0.02***	6.75± 0.03***	5.31±0.03***	4.71±0.04***
Group VII	4.21±0.010***	7.23±0.010***	6.5±0.02***	5.23±0.02***	4.36±0.02***

N = 6, values were expressed as Mean ± SEM, ***p value <0.001 statistically significant, Group- I; Normal control. Group- II; Disease control. Group III-VI; treatment group. Group- VII; standard group.

Body weight changes

Body weight in CFA treated rats was significantly reduced compared to control (P<0.001). The increase in body weight was observed in (F-7-β-D-g at the dose of 10, 20, 40, 50 mg/kg, and Methotrexate 5 mg/kg) when compared to arthritic treated rats. (Table- 2).

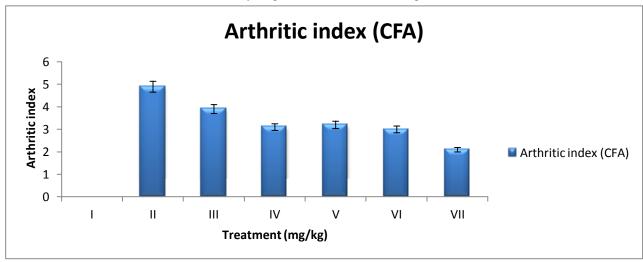**Table 2: Effect of Formonoetin-7-*O*-β-D-glucopyranoside on average body weight.**

Treatment	Average Body weight				
	0 day	3rd day	7th day	14th day	21st day
Group I	276.55± 0.32	275.32±0.43	274.50±0.98	273.60±0.12	271.93±0.72
Group II	264.19±0.34**	222.16±0.56**	216.40±0.34**	190.31±0.72**	180.41±0.45**
Group III	263.18±0.84**	211.16±0.96**	205.40±0.24**	194.31±0.92**	190.41±0.32**
Group IV	260.16±0.74**	240.16±0.46**	230.40±0.54**	229.31±0.82**	220.41±0.52**
Group V	259.13±0.34**	250.16±0.87**	248.40±0.97**	243.31±0.32**	243.41±0.92**
Group VI	260.14±0.24**	261.16±0.57**	247.40±0.77**	252.31±0.82**	249.01±0.32**
Group VII	277.83±0.37**	270.33±0.38**	262.16±0.57**	257.66±0.29**	252.32±0.32**

N = 6, values were expressed as Mean ± SEM, **p value <0.001 statistically significant, Group I; Normal control. Group II; Disease control. Group III VI; treatment group. Group VII; standard group.

Arthritic Index

Sub plantar administration of CFA results in significant increase (P < 0.001) in arthritic score in all arthritic treated rats as compared to control rats. Albino rats treated with various doses of (F-7-β-D-g 10, 20, 40, 50 mg/kg and Methotrexate 5 mg/kg) showed significant and dose dependant decrease in arthritic score (P < 0.001) as compared to arthritic treated rats (Figure 1).
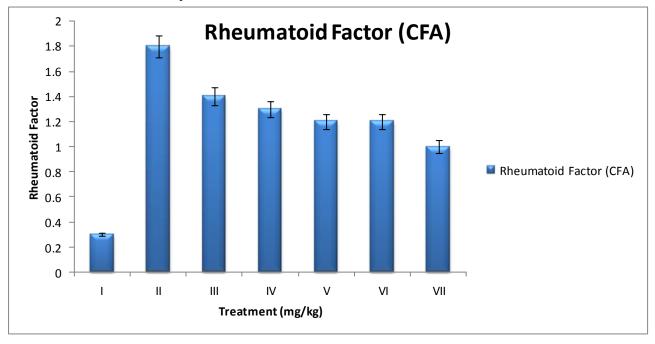

N = 6, values were expressed as Mean ± SEM, ***p value <0.001 statistically significant, Group I; Normal control. Group II; Disease control. Group III – VI; treatment group. Group VII; standard group

Fig 1: Effect of Formonoetin-7-*O*-β-D-glucopyranoside on arthritic index.
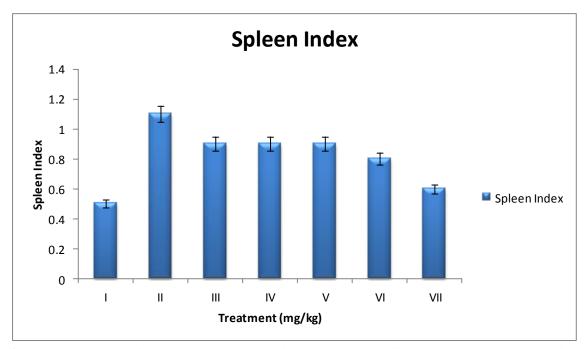
Rheumatoid factor & Spleen Index
As shown in Figure- 2, 3 rheumatoid factor and spleen index were significantly increased (P < 0.001) in all arthritic treated rats as compared to control rats.

The rheumatoid factor and Spleen Index reduction in of (10, 20, 40, 50 mg/kg and Methotrexate 5 mg/kg) treated rats were significantly (P < 0.001) lesser than that of arthritic treated rats.

N = 6, values were expressed as Mean ± SEM, ***p value <0.001 statistically significant, Group I; Normal control. Group II; Disease control. Group III – VI; treatment group. Group VII; standard group
Fig 2: Effect of Formonoetin-7-*O*-β-D-glucopyranoside on Rheumatoid factor.

N = 6, values were expressed as Mean ± SEM, ***p value <0.001 statistically significant, Group I; Normal control. Group II; Disease control. Group III – VI; treatment group. Group VII; standard group.
Fig 3: Effect of Formonoetin-7-*O*-β-D-glucopyranoside on spleen index.

Table 3: Effect of Formonoetin-7-*O*-β-D-glucopyranoside on Hematological Parameter of CFA induced arthritis in albino rats

Treatment	Hb (g/dl)	RBC (x10^6/mm^3)	WBC (x10^3/mm^3)	ESR (mm)
Group I	12.98 ± 0.040	4.51 ± 0.030	6.35 ± 0.002	3.65 ± 0.034
Group II	11.05 ± 0.034***	2.7 ± 0.025***	10.48 ± 0.030***	10.9 ± 0.025***
Group III	12.5 ± 0.025***	5.2 ± 0.025***	6.8 ± 0.025***	4.51±0.030***
Group IV	10.8 ± 0.025***	4.5 ± 0.025 ***	8.3 ± 0.03***	7.46±0.021***
Group V	10.25 ± 0.004***	4.76 ± 0.021***	7.5 ± 0.02***	5.11 ±0.03***
Group VI	11.73 ± 0.004***	4.9 ± 0.057***	7.21 ± 0.030***	6.6 ± 0.025***
Group VII	12.73 ± 0.004***	7.9 ± 0.057***	7.78 ± 0.030***	6.2 ±0.025***

N = 6, values were expressed as Mean ± SEM, ***p value <0.001 statistically significant, Group I; Normal control. Group II; Disease control. Group III – VI; treatment group. Group VII; standard group.

Hematological parameters

The changes in hematological parameters in adjuvant induced arthritic rats are shown in Table- 3. There was a significant (p < 0.001) decrease in RBC count and hemoglobin, and increase in WBC count and ESR of arthritic rats as compared to control rats. The drug treatment has significantly brought back the altered hematological changes in both developing and developed phases of adjuvant induced arthritis.

DISCUSSION:

RA is a chronic inflammatory disease characterized by fibroblastic proliferation, infiltration of the synovial lining by inflammatory cells which leads to expression of pro inflammatory cytokines [23]. FCA is used to initiate induction of arthritis. This model has been extensively used in preclinical screening of new anti arthritis compounds and has successfully predicted activity in multiple new therapeutics. After a single injection of the adjuvant, a rapid, reliable, robust, and easily measurable poly arthritis developed [24]. In the present study, rats were selected to induce arthritis because they develop a chronic swelling in multiple joints due to accumulation of inflammatory cells, and erosion of joint cartilage. The determination of paw thickness is apparently simple, sensitive and quick procedure for evaluating the degree of inflammation and the therapeutic effects of drugs. The Freund's adjuvant model is chosen as it develops chronic swelling in multiple joints with influence of inflammatory cells, erosion of joint cartilage and paw edema. Chronic inflammation involves the release of number of mediators like cytokines (IL-1B and TNF-alpha), Interferon's and Platelet derived growth factor (PDGF) [25].

These mediators are responsible for the pain, along with swelling of the limbs and joints that can lead to severe disability. On the 21st day, a significant decrease in paw thickness was observed in F-7-β-D-g (10, 20, 40 and 50 mg/kg b.w.p.o.) and the Methotrexate (5mg/kg b.w.p.o.) Treated group as compared to the arthritic treated rats.

The change in body weight of rats was measured as one of the parameters to assess the course of the disease and the response to therapy of arthritic drugs. Finding from the studies implicated that with increase the incidence and severity of arthritis, a decrease in body weight of the rats occurred during the course of the experimental period due to alterations in the metabolic activities of diseased rats [26]. Earlier findings suggest that absorption of 14C- glucose and 14C-leucine in rat's intestine was reduced in the case of inflamed rats but on the treatment with anti-inflammatory drugs, the decrease in absorption was nullified and it shows that the anti-inflammatory drugs correct the decreased absorption capacity of intestine during inflammation [27]. The increased body weight during treatment of standard drug, and test compound may be due to the restoration of absorption capacity of intestine.

Arthritic index includes the combined index of inflammation, formation of nodules, and extent of spread of the disease to other organs. Administration of Freund's adjuvant induced severe chronic disease of arthritic index in disease control group which was significantly different from normal control group. Test drug treated group produced statistically significant reduction in arthritic score compared to arthritic treated rats [28].

Rheumatoid factor is the true marker of clinical presentation of RA. Rheumatoid factor generation in arthritis involves B-cell activation and several genetic predispositions to arthritic diseases. Higher the levels of serum rheumatoid factor, higher the development

of inflammation. The Arthritic treated rats showed significantly elevated levels of serum rheumatoid factor compared to the normal control group.

The treatment with Methotrexate (5mg/kg b.w.p.o.) and F-7-β-D-g (10, 20, 40 and 50 mg/kg b.w.p.o.) significantly reduced the levels of inflammation and autoimmune stimulation in the treated rats. The results of the present study indicate that the anti-inflammatory effects of F-7-β-D-g may be due to the inhibition of activation of B cell [29].

Spleen is a vital organ involved in immune responses. In adjuvant arthritis, spleen serves as the reservoir for the cells and antibody formation which involved in the immune response. Increase in the weight of spleen is associated with the splenomegaly, generalized lymphadenopathy and altered hepatic function [30]. In the present study treatment with F-7-β-D-g (10, 20, 40 and 50 mg/kg) significantly decreases spleen weight which might be due to anti-inflammatory and immune stimulant effect of test drug. Erythrocyte sedimentation rate (ESR) is an estimate of the suspension stability of RBCs In plasma. ESR in the arthritic treated group is found to be several folds high when compared to drug treated groups. ESR is strongly related with the ability of red cells to aggregate into orderly stacks or rouleaux. Proteins are thought to affect the repellant surface charges on red cells and cause them to aggregate into rouleaux and hence the sedimentation rate increases.

In the present investigation the rate of sedimentation was increased in arthritic treated control where as F-7-β-D-g (10, 20, 40 and 50 mg/kg) treated groups significantly restores the decreased level of RBC along with Hb and reduce the elevated level of ESR attributing its anti inflammatory potential.

White blood cells (WBCs) are a major component of the body's immune system. Increased white blood cell counts are a common feature of inflammatory reactions, especially those induced by microbial infection. IL-IB increases the production of both granulocyte and macrophages colony stimulating factor. In arthritis condition there is a mild to moderate rise in WBC count due to release of IL-IB. WBC count was increased in arthritic group.

In the current study, the migration of leukocytes is significantly suppressed in test drug treated groups as seen from the significant decrease in the WBC count. All the rectification of hematological parameters supports the anti arthritic effect of F-7-β-D-g [31].

CONCLUSION:

Anti- arthritic activity of Formonoetin-7-*O*-β-D-glucopyranoside from Methanolic Extract of *Operculina turpethum* appears to be possessing anti–inflammatory activity showed in arthritic parameters like foot pat thickness, arthritic index, rheumatoid

factor, improving bone erosion. All these results thus predict that the drug provide pharmacological rationale for the traditional use of the drug against inflammatory disorders such as rheumatoid arthritis.

Conflict of interest

The authors declare that they have no conflict of interest.

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