



# Assessment of anti-inflammatory and anti-arthritic properties of *Acmella uliginosa* (Sw.) Cass. based on experiments in arthritic rat models and qualitative gas chromatography-mass spectrometry analyses

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**Received:** March 18, 2016

**Accepted:** May 03, 2016

**Published:** May 30, 2016

## ABSTRACT

**Aim:** The principle objective of the study was to explore the anti-arthritic properties of *Acmella uliginosa* (AU) (Sw.) Cass. flower in a rat model and to identify potential anti-inflammatory compounds derived from flower extracts. The synergistic role played by a combination of AU flower and *Aloe vera* (AV) gel crude extracts was also investigated. **Materials and Methods:** Male Wistar rats induced with Freund's complete adjuvant (FCA) were used as a disease model of arthritic paw swelling. There were three experimental and two control groups, each consisting of five rats. Paw circumference and serum biochemical parameters were evaluated to investigate the role of the flower extracts in disease amelioration through a feeding schedule spanning 21 days. Gas chromatography/mass spectrometry (GC/MS) analyses were performed to search for the presence of anti-inflammatory compounds in the ethanolic and n-hexane solvent extracts of the flower. **Results:** As a visual cue to the experimental outcomes, FCA-induced paw swelling decreased to the normal level; and hemoglobin, serum protein, and albumin levels were significantly increased in the treated animals. The creatinine level was estimated to be normal in the experimental rats after the treatment. The combination of AU and AV showed the best recovery potential in all the studied parameters, confirming the synergistic efficacy of the herbal formulation. GC/MS analyses revealed the presence of at least 5 anti-inflammatory compounds including 9-octadecenoic acid (Z)-, phenylmethyl ester, astaxanthin, ð-N-Normethadol, fenretinide that have reported anti-inflammatory/anti-arthritic properties. **Conclusion:** Our findings indicated that the crude flower homogenate of AU contains potential anti-inflammatory compounds which could be used as an anti-inflammatory/anti-arthritic medication.

**KEY WORDS:** *Aloe vera*, Freund's complete adjuvant, rheumatoid arthritis

## INTRODUCTION

Ethnic people have used herbal resources from ancient times to fulfill their nutritional and medicinal needs. A vast majority of the nutritional supplements and edible medicinal materials are either consumed as such from herbs or synthesized from herbal resources [1]. The interest of producing herbal formulations for medicinal purposes has developed due to their low side effects, low costing, and ready availability in countries such as India and China. In ethnobotanical knowledge, daily consumption of different plant parts and products are said to have disease-modifying and disease-improving activities. However, scientific explanations and working principles of such crude plant parts

or herbal formulations are not well-established experimentally. *Acmella uliginosa* (AU) (Sw.) Cass. (Family Asteraceae) is a plant found in the Northern part of West Bengal (known as North Bengal) and has a worldwide distribution [Figure 1]. These herbs grow up to 1 m, generally creep or sometimes stand erect, rooting at nodes, and their stems are sub-glabrous to scabrid-pilose. It has been used as food by many human populations all through the world. The Malay people, as well as the Rajbanshi people from Northern part of West Bengal, consume the plant and its flowers for symptoms such as tooth ache, mouth ulcer, and mouth ache [2]. When consumed, the flower has a characteristic pungent taste which is soon followed by a characteristic tingling and numbness of the tongue. The antinociceptive activity, anti-



**Figure 1:** *Acmella uliginosa* flower and plant body in Medicinal Plant Garden of the University of North Bengal (Photograph courtesy: APD)

inflammatory, and immunomodulatory effects of this plant have been reported by few researchers [3-5]. Antimicrobial activity and antioxidant effect of the plant were also evaluated, and AU has been reported as an effective phytochemical-containing plant [6].

Rheumatoid arthritis (RA), which is a form of an autoimmune bone destructive disease, affects at least 1% of the population in the industrialized world with higher frequency in women. In severe cases of RA, the synovial inflammation leads to particular cartilage damage, bone erosion, and subsequent change in joint integrity. Usually, peripheral joints are involved [7,8]. Based on the results and observations made on the biological and analgesic activities of the plant, we assumed that the plant flower may have an anti-arthritis activity as well. To the best of our knowledge, such activities of AU have not been explored in appropriate model systems. Therefore, we have designed and performed animal model-based experiments to evaluate the anti-arthritis potential of the plant flower at the primary level and correlated the result with the gas chromatography/mass spectrometry (GC/MS) qualitative analyses.

## MATERIALS AND METHODS

### Preparation of Plant Extract

AU (Sw.) Cass. (Family Asteraceae) flowers were collected from the University Medicinal Garden during October-November, 2015. Because the flowers are chewed by the ethnic populations, the extract was prepared in a crude form, and no alterations were made in the unprocessed whole plant flower before monitoring its activity. Flowers were collected and washed thoroughly with water before the extract preparation. Flowers were then crushed and mixed with 3 ml of distilled water per gram of plant material thoroughly in a homogenizer. The mixture was kept for an hour at room temperature, and the process was again repeated. After homogenization, the extract was centrifuged at 3000 rpm and the supernatant was taken for experimentation. Every time the

extracts were made freshly before the feeding of the animals. For GC/MS analysis, the flower was dried at room temperature for 6-7 days, crushed in a grinder, and the fine dust was collected in sterile capped Tarson tubes. It was followed by overnight stirring the powdered *Acmella* flower sample in appropriate solvents, i.e. ethanol and n-hexane, respectively. The supernatants were collected after 24 h and filtration was done using Whatman No 1 filter paper. The extracts were concentrated using nitrogen flow which facilitated enhanced diffusion of the solvent from the sample. This concentrated solution was used as the starting sample for the GC/MS analyses.

### Animal Maintenance

Male Wistar rats, weighing 100-120 g, were used as experimental animals. Rats used for all the experiments were procured from an authorized animal dealer (Ghosh Enterprise, Kolkata, India). Animals were kept in the Departmental Animal House of the Department of Zoology, University of North Bengal with water *ad libitum* and standard pellet food was given. The room temperature was kept between 25°C and 30°C. All the experiments were approved by the Departmental Animal Ethical Committee.

### Chemicals

Freund's complete adjuvant (FCA) was purchased from Sigma-Aldrich, USA; biochemical assay kit for total protein, albumin, and creatinine was procured from Coral Clinical Systems, India; HPLC grade chemicals (ethanol and n-hexane) for GC/MS were purchased from SD Fine Chem Ltd., Mumbai. All the other chemicals required were purchased from Himedia, Mumbai, India.

### Anti-inflammatory Effects of AU

#### *Lethal dose 50% (LD50) test for LD determination*

LD50 test for AU flower extract was done as per the OECD guidelines. The doses considered for the tests were 140 and 280  $\mu$ l of homogenate taken from a stock of 1 g/3 ml solution. These doses corresponded to 50 g flower/60 kg body weight and 100 g flower/60 kg body weight. Three rats were taken in each group to observe the lethality induced by the sample.

#### *Curing potential of AU in FCA-induced arthritic condition*

The animals were divided into five groups, each containing five rats. Adjuvant-induced arthritis model was produced as per the method described by Bendele *et al.* [9], and all animals were administered subcutaneously a dose of 0.1 ml of FCA in the left hind paw interplanetary region with the help of a sterile single-use insulin syringe, excluding the positive control group animals. The animals were administered a dose of 0.1 ml FCA again on the 14<sup>th</sup> day in the same region to boost up the immune response. The first two groups were considered as the control groups. Among them, one group was considered

as a non-treated or positive control that received neither FCA nor the plant extracts. The other group was considered as a negative control (NC), in which only FCA injection was administered, but no treatment regime was initiated. The third and fourth groups were considered as experimental groups. These groups were fed daily doses of aqueous extract of AU flower to the tune of 70  $\mu$ l (AU1) and 140  $\mu$ l (AU2), respectively, corresponding to 25 and 50 g flower/60 kg body weight, from a stock solution of 1 g/3 ml extract. The fifth group (AVAU) was fed 1:1 (w:w) doses of *Aloe vera* (AV) flower and AU gel together to investigate the combined effect of the extracts, as AV has proven role as an anti-inflammatory product [10]. The doses were calculated on the basis of the daily possible consumable amount of the flower by an average healthy person. The animals were sacrificed on the 21<sup>st</sup> day to study the biochemical parameters such as total protein, total albumin, and serum creatinine [11]. The paw circumferences were measured with the help of a vernier caliper at regular interval of 3/4 days from the 1<sup>st</sup> day of the experiment, using the formula  $2\pi\sqrt{(A^2+B^2)}/2$ , where A and B are the measures of the paw diameters at two different planes [12]. Total protein and total albumin were measured using kits from Coral Clinical Systems, following manufacturer's instructions. Hemoglobin from the rat blood was estimated using Sahli's hemoglobinometer. Serum creatinine was measured biochemically by studying reactions between creatinine and alkaline picrate.

### Instrumentation and Chromatographic Conditions

The GC/MS analyses of the ethanolic and n-hexane extracts of *Acmella uliginosa* flower were performed in Thermo Scientific Trace 1300 GC equipped with ISQ MS and an AI/AS 1310 auto-sampler. The instrument had a TG 5 ms fused silica capillary column of 30 m length, 0.25 mm diameter, and 0.25  $\mu$ m film thickness. The column oven temperature was kept at 80°C, with a gradual increase in steps of 5°C/min to 305°C; injection temperature was set at 250°C at a pressure of 5 kPa, with total flow and column flow of 10 ml/min and 1 ml/min, respectively. The rate of purge flow was 3.0 ml/min. The GC program ion source and interface temperatures were 220°C and 305°C, respectively, with a solvent cut time of 5 min. The MS program starting time was 5 min which ended at 51.00 min with event time of 0.50 s and mass range 50-650. Injection volumes of each ethanolic and n-hexane soluble *Acmella* fractions were 1  $\mu$ l (split ratio 10:1). The samples were repeatedly used to find the best result. The interpretation of GC/MS mass spectrum was done using the Xcalibur software version 2.0.1.3 with the help of the NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library Version 2.0g, built May 19, 2011.

### Statistical Analysis

All the statistical analyses were done using MS-Excel 2007 and Kyplot version 2.0 beta. In the Kyplot analysis, the data represented mean  $\pm$  standard deviation which was analyzed by one-way ANOVA. The results were considered significant when  $P > 0.05$ .

## RESULTS

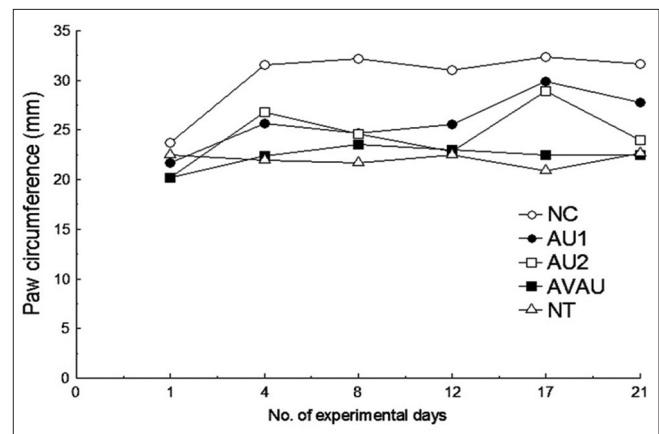
### Anti-inflammatory Effects of AU

#### *LD<sub>50</sub> test for LD determination*

Experimental groups fed with AU flower homogenates showed no toxicity, no mortality, or behavioral changes during the period of 7-day after the commencement of feeding (data not shown). It can be concluded that doses up to 100 g flower/60 kg body weight were non-toxic to the body of a model animal.

#### *Curing potential of AU in FCA-induced arthritis*

Rat paw circumference was reduced significantly in the experimental groups that were fed with AU flower aqueous extract. Interestingly, the AVAU group, which was fed with a combination of *Aloe* and *Acmella* aqueous extracts, showed the better result [Figure 2 and Table 1]. All the treated groups showed an increased level of hemoglobin which is reported to decrease in arthritic conditions. The combined doses (*Aloe* + *Acmella*) of the extract were followed by a high dose of *Acmella* that showed the best result in increasing the blood hemoglobin levels [Figure 3a]. Estimation of total protein showed that there was a significant role of the plant extract to bring the altered

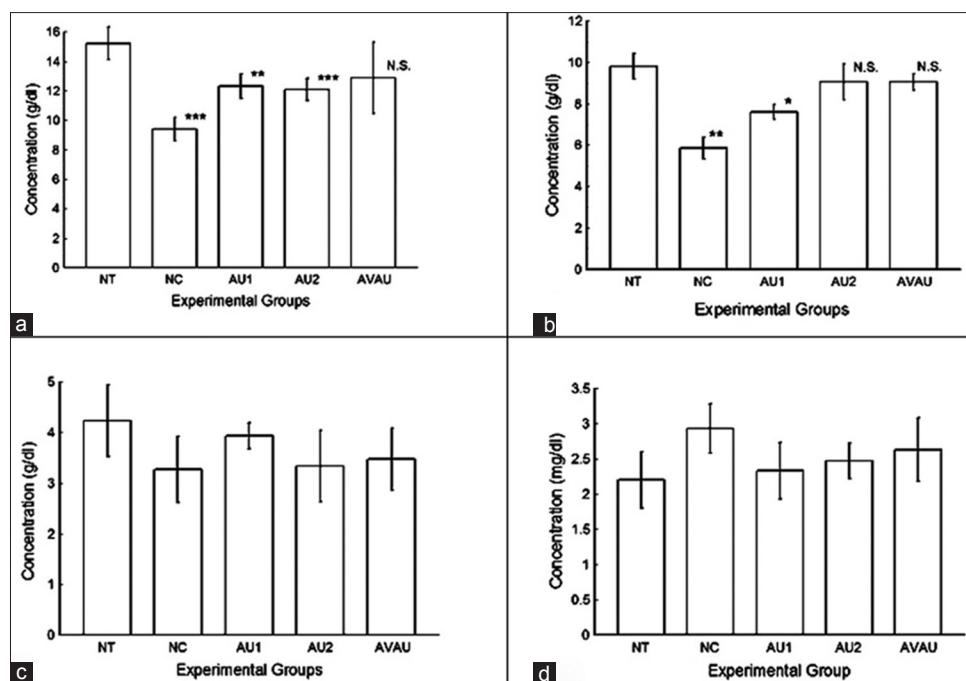


**Figure 2:** Change of paw circumference (mm) in different rat groups on different days after the initiation of treatment in arthritic rat models. Swelling is maximum in the diseased control (negative control [NC]) group, whereas combined dose of *Aloe vera* and *Acmella uliginosa* (AVAU) showed the best inhibition against arthritic paw swelling

**Table 1:** Mean paw circumference (mm) of rats of different experimental groups in different day intervals from the induction of arthritis

Groups	Day 1	Day 4	Day 8	Day 12	Day 17	Day 21
NC	23.74	31.56	32.17	31.01	32.31	31.62
AU1	21.64	25.65	24.64	25.51	29.85	27.75
AU2	20.16	26.82	24.62	22.80	28.87	23.97
AVAU	20.19	22.34	23.50	22.99	22.85	22.43
NT	22.57	22.46	22.24	22.56	22.14	21.99

NC: Negative control, AU1/AU2: *Acmella uliginosa* dose groups, AVAU: *Aloe vera*-*Acmella uliginosa* combination (1:1=w/w), NT: Non-treated



**Figure 3:** Measurement of different serum physiological parameters: (a) Hemoglobin in different rat groups: *Aloe vera-Acmella uliginosa* (AVAU) group showed the best efficacy in normalizing the altered (decreased) hemoglobin level of diseased animals, (b) estimation of total protein in different rat groups: AU2 and AVAU showed the best effect in the normalization of altered (lowered) total protein level, (c) estimation of total albumin level, negative control (NC) showing the most decreased value of albumin, AU1 shows the best restoration of altered albumin level, (d) serum creatinine values: AU1 followed by AU2 showed the best restoration property of serum creatinine compared to diseased condition

total protein levels toward normalcy. There has been a very significant elevation in the serum protein levels of experimental groups up to the normal levels. Both AU2 and AVAU groups showed normal protein levels after treatment, and there was no significant deviation from the normal value [Figure 3b]. Since total albumin level is reported to decrease with the arthritic severity, we measured total albumin level in all the experimental groups and have recorded an elevation of total albumin in all the experimental groups. The experimental groups showed 5.91%, 21.74%, and 17.07% decrease in the albumin levels in AU1, AU2, and AVAU groups, respectively, compared to 21.74% decrease in the disease control (NC) rats [Figure 3c]. Creatinine level generally increases in arthritic condition. In our experiment, it is clearly visible that AU flower extract can normalize the increased serum creatinine level. The NC group showed 31% increase in the creatinine level, whereas the experimental groups AU1, AU2, and AVAU showed only 9.09%, 13.63%, and 18.18% increase, respectively, in serum creatinine levels [Figure 3d]. This clearly indicates that the plant has a protective role against altered creatinine levels.

### GC/MS Analyses

The GC/MS analyses of both ethanolic and n-hexane fractions of AU flower homogenate supports the presence of 96 compounds from the ethanolic fraction and 80 compounds from the n-hexane fraction, of which some anti-inflammatory compounds were identified through the literature search that related to the anti-arthritic properties of the plant. We have identified some key compounds which are already documented

as potent inflammation inhibitors by other researchers. The principle anti-inflammatory and/or anti-arthritic compounds from the plant are listed in Table 2, and the structures of these compounds are depicted in Figure 4.

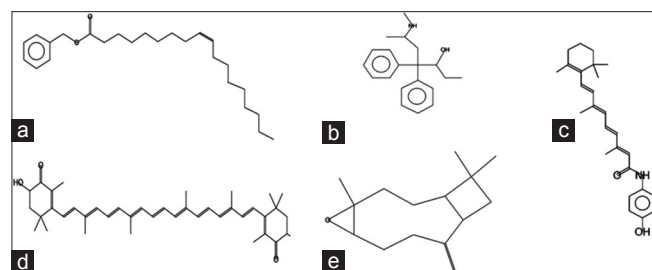
### DISCUSSION

The FCA-induced arthritis model in rats is the most commonly used method of simulating a human disease condition. FCA-induced arthritis has been used as a model of chronic inflammation in rats and of considerable relevance for the study of pathophysiology and pharmacological control of inflammatory processes [17]. In this study, a dramatic cessation of rat paw edema was indicated in the treated experimental groups of animals from the 1<sup>st</sup> week of study, similar to others' findings [17-19]. Paw swelling is a visual cue to the inflammation, and hence, it can be concluded that AU flower aqueous crude extract can significantly reduce paw swelling in FCA-induced arthritic rats when fed orally. As seen in other studies, total protein and hemoglobin generally decrease in arthritic conditions, which is similar to our results. In our study, we also found that experimental group treated with aqueous extracts of AV and AU, combined in equal proportion showed the best result in increasing serum protein and blood hemoglobin levels, which is followed by a similar effective activity of the higher doses of *Acmella* [Figure 3a and b] [17]. The total albumin level also decreased along with the arthritic severity [18]. We estimated total albumin levels in all experimental groups and recorded elevations of total albumin in all experimental groups except that in the NC group. Creatinine level generally increases

**Table 2: List of phytochemicals with anti-inflammatory activities identified from *A. uliginosa* with details; references confirming their anti-inflammatory potentials as pure or conjugated compounds**

Peak RT	Peak area	Peak area (%)	Compound name	SI	RSI	Molecular formula	Molecular weight	P	CAS	References
n-Hexane fraction										
5.46	88,207.11	0.09	9-Octadecenoic acid (Z)-, phenylmethyl ester	605	735	C <sub>25</sub> H <sub>40</sub> O <sub>2</sub>	372	32.51	55130-16-0	[13]
34.20	153,335.35	0.16	à-N-Normethadol	352	456	C <sub>20</sub> H <sub>27</sub> NO	297	4.15	38455-85-5	[14]
40.09	267,475.99	0.28	Astaxanthin	460	520	C <sub>40</sub> H <sub>52</sub> O <sub>4</sub>	596	51.05	472-61-7	[15]
Ethanol fraction										
19.14	1,222,149.73	1.38	Caryophyllene oxide	669	793	C <sub>15</sub> H <sub>24</sub> O	220	15.04	1139-30-6	[16]
19.46	85,133.22	0.10	Fenretinide	355	434	C <sub>26</sub> H <sub>33</sub> NO <sub>2</sub>	391	7.15	65646-68-6	[17]
20.74	161,146.11	0.18	Astaxanthin	460	520	C <sub>40</sub> H <sub>52</sub> O <sub>4</sub>	596	51.05	472-61-7	[15]

RT: Retention time, SI: Strength index, RSI: Relative strength index, CAS: Chemical abstracts service number, P: Probability, *A. uliginosa*: *Acmella uliginosa*



**Figure 4:** Structures of identified anti-inflammatory compounds of *Acmella uliginosa*, (a) 9-Octadecenoic acid (Z)-, phenylmethyl ester, (b) à-N-Normethadol, (c) astaxanthin, (d) caryophyllene oxide, (e) fenretinide

in arthritic condition as mentioned in previous studies [18]. In our experiment, we also found similar results.

AU is reported to have local anesthetizing properties often used by ethnic people as a pain-relieving agent. Considering the studies of different other workers on different other potent anti-arthritic plants such as *Nyctanthes arbor-tristis* [12] and *Aristolochia bracteata* [19], it can be said that AU flower possesses promising arthritis inhibitory active principles which can significantly bring altered parameters toward normalcy in disease models. Moreover, the combined effect of AU flower extract and AV gel in disease amelioration suggested a synergistic role of the plant extracts. Low hemoglobin level clearly states that during arthritis, the bone marrow loses its normal functioning property and low red blood cell (RBC) count results in low hemoglobin level. The rise in hemoglobin levels in experimental groups indicates the increased RBC concentration and bone marrow health. A rise in total protein and albumin in experimental groups clearly demonstrates the restoration of metabolic imbalances during arthritic condition. Creatinine formation in serum was decreased in the experimental groups that confirmed the stability in the physiology of metabolism [Figure 3d]. A similar study on *Strychnos potatorum* Linn. seeds in FCA-induced arthritic rat models by Ekambaram *et al.* [18] also showed similar changes in the above said parameters.

The animal experimental data were supported by the GC/MS analyses of the plant flower extracts. A good number of fatty acids, steroidal, and other products were profiled including different anti-inflammatory compounds. Ayurveda is a system

which establishes the synergistic role in an herbal formulation of more than one plant for a better result against complex diseases. The synergistic role of these compounds may offer an amelioration of the disease condition. The group AVAU, which contains AV and AU in equal proportion showed the best inhibitory role against arthritis generation and progression in experimental rat models that suggested a possible synergistic role of these plant-derived drugs. This work thus also established the efficacy of synergistic activity of the herbal formulations.

## CONCLUSION

In this study, the role of AU flower homogenate in amelioration of arthritic paw swelling in rat models, as well as assessment of some biochemical parameters was established. All the experimental outcomes confirmed that this plant possessed significant curative properties against arthritis. Taking into consideration that different parameters are affected by a network of different complex biochemical pathways and need a different period to settle down, the preliminary 21 day long study promises that effective dose determination and further study would establish AU as a potent arthritis inhibitor. This study further confirmed the effect of crude AV gel in inflammatory conditions. The potential therapeutic values of the unprocessed crude flower homogenate of AU, as a remedy for inflammation as well as arthritic condition, were well documented in this work. The efficacy of the combined crude flower extracts of AU and AV gel further suggested of a possible synergistic activity leading to better remediation of disease conditions in the animal model.

## ACKNOWLEDGMENTS

Authors acknowledge the expertise of Professor A. P. Das (APD), Department of Botany, University of North Bengal in identification and maintenance of the plant. The authors also thank the Department of Zoology for providing the animal house and DST-FIST instrumental facilities for the work. The research was partially funded by the UGC-BSR fellowship scheme (File no. 7-134 [2007]).

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**Source of Support: Nil, Conflict of Interest: None declared.**