RESEARCH REVIEW JOURNALS
Volume-04
Issue-04
April -2019

ISSN: 2455-3085 (Online)
RESEARCH REVIEW International Journal of Multidisciplinary
www.rrjournals.com [UGC Listed Journal]

Antiinflammatory activity of Equal Combination of *Calotropis gigantia* and *Calotropis procera* dried flower extract in experimental Rats

[1]Dileep Bharati & [2]Dr. Swapnil Goyal

[1]Associate professor, Department of Pharmacolog, Technocrats institute of technology- pharmacy, Bhopal, Madhya Pradesh (India)
[2]Associate professor, Department of Pharmacology, Mandsaur Univesity, Mandsaur, Madhya Pradesh (India)

ARTICLE DETAILS

Article History
Published Online: 15 April 2019

Keywords
Calotropis gigantea, antiinflammatory

ABSTRACT

In this study we found the combination of dried flower extract of equal combination of Calotropis gigantea and Calotropis procera, In this experiment we got antiinflammatory activity which is higher then individual performance of each plant, in this experiment we got three different plant doses 200mg/kg of Calotropis gigantea, 200mg/kg Calotropis procera and 200mg/kg of equal combination of both. carageenan induced paw edema and cotton pellet induced granuloma method were applied in this experiment and found positive response.

1. Introduction

Inflammation is protective and defensive mechanism of the body. During inflammatory conditions various pathological changes are take place. The production of active inflammatory mediators is triggered by microbial products or by host proteins such as proteins of the complement, kinins and coagulation systems that are themselves are activated by microbes and damaged tissues.

The inflammatory process is closely intertwined with the process of repair. Both of these processes proceed simultaneously; however the repair activities of either tissue regeneration or scarring predominate after the injurious agent has been eliminated. The inflammatory process may be destructive to tissues when activated repeatedly or in appropriately such as in autoimmune diseases in which native antigens are recognized as foreign by the individual's immune system.

Normally, the inflammatory-anti-inflammatory cycle is somewhat like an "on-off" switch: inflammation is turned on when needed for healing and repair (by inflammatory chemicals), then turned off when not needed (by anti-inflammatory chemicals).

There are two types of inflammation: acute and chronic. Acute inflammation is characterized by a rapid onset and short duration. It manifests with exudation of fluid and plasma proteins, and emigration of leukocytes, most notably neutrophills. Chronic inflammation is of prolonged duration and manifests histologically by the presence of lymphocytes and macrophages and results in fibrosis and tissue necrosis. When inflammation continues for prolonged periods of time, it can be thought of as the healing process in overdrive, and deleterious changes can occur to localized tissues as well as the entire body.

These inflammatory mediators come from plasma proteins or cells including mast cells, platelets, neutrophils and monocytes/macro-phages. They are triggered by bacterial products or host proteins. Chemical mediators bind to specific receptors on target cells and can increase vascular permeability and neutrophil chemotaxis, stimulate smooth muscle contraction, have direct enzymatic activity, induce pain or mediate oxidative damage. Most mediators are short-lived but cause harmful effects.[1] Examples of chemical mediators include vasoactive amines (histamine, serotonin), arachadonic acids (prostaglandins, leukotrienes) and cytokines (tumor necrosis factor and interleukin–1).

Acute inflammation is the body's initial response to a physical or chemical stress that requires healing and repair. Chronic inflammation is an abnormal condition that can cause or is associated with ill health and disease. When acute inflammation does not, or cannot, complete its task, chronic inflammation results. This transition may take place due to continued physical or chemical stress, such as smoking, or infection. More often it's due to biochemical influences such as the imbalance of dietary fats, absence of specific substances that adversely affect our anti-inflammatory production, and specific nutrient problems.

1.1 SIGNS OF INFLAMMATION:-There are 4 cardinal signs of inflammation which was named by Roman Writer Celsus as:

(1) Rubor (redness)
(2) Tumor (swelling)
(3) Calor (heat)
(4) Dolor (pain)

2. PLANT PROFILE

2.1 Taxonomical classification

- Kingdom: Plantae
- Phylum: Angiosperm
- Class: Eudicots
- Sub-class Asterids
- Order : Gentinales
- Family : Apocynacae
- Subfamily: Asclepiadaceae
- Genus : Calotropis
- Species: gigantea

2.2 Botanical description

The plant is a large erect shrub or small tree up to 3m high. Two varieties of the plant are described by Sanskrit writer, viz; the white flowered or "alarka" (*Calotropis procera*) and the purple or red flowered or "arka" (*Calotropis gigantea*).The leaves are opposite, broad and sub sessile glaucous green and 5-20cm by 3.8-10cm. Flowers are 3.8-5cm diameter, inodorous, purplish (*Calotropis gigantea*) or white (*Calotropis procera*).Seeds are numerous, 6-5mm, broadly ovate, flattened, narrowly margined, minutely tanentose, brown, coma 2.5-3.2 cm long. It is found chiefly in waste land in Lower Bengal, Himalayas, Punjab, Assam, Madras, South India, Ceylon, Singapore, Malay Island and South China [7-11].

2.3 Synonyms

The plant is known by various names in different languages:

- ***Sanskrit:*** Arka, Alarka
- ***English:*** Gigantic swallow wort, Mudar
- Hindi: Madar
- *Kannada*: Ekkemale
- ***Telugu:*** Mandaramu, Ekke, Jilledu, Arka
- Malayalam: Errikka

2.4 Traditional uses

It has been reported as traditional medicinal plant in Ayurveda, Unani and Homeopathic system of medication for the treatment of different ailments [12, 13]. According to Ayurveda the milky juice of the plant is bitter, heating, oleaginous, purgative, cures leucoderma, tumors, ascites, and disease of the abdomen. According to Unani the milk is caustic, acrid, expectorant, depilatory, anthelmintic, useful in leprosy, scabies, ring worms of the scalp, piles, eruptions on the body, asthma, enlargement of spleen and liver, drop-sy, and also applied to painful joints and swelling. The root bark is diaphoretic, cures asthma, elephantitis, cough and syphilis (Ayurveda). The dried bark of the root is an excel-lent substitute for Ipecacuanha for the treatment of dysentery in small doses, but in large doses it is an emetic. Root bark is tonic, antispasmodic, expectorant, anthelmintic and laxative [7-9]. In syphilitic infection it is regarded as a great remedy so much so that it is called as vegetable mercury. The mixture of the powder of root bark with black pepper twice a day is also used to cure jaundice [8]. As per Ayurveda the flowers are bitter, digestive, astringent, stomachic, anthelmintic, tonic and analgesic, normally used to cure inflammation, tumors, kapha, asthma, loss of appetite and ascites [7-9, 14-16]. According to Unani the flowers are stomachic and good for the liver, dried flowers in 1 to 2 grains doses with sugar is given in leprosy, secondary syphilis and gonorrhoea with milk diet [7, 8]. According to Unani the leaves are useful in the treatment of paralyzed parts, the oil in which leaves have been boiled, is also applied to paralyzed parts. The leaves are also used in the treatment of arthralgia, swelling and intermittent fever [7]. The powder of the dried leaves is dusted upon wounds and ulcer to prevent excessive granulation and promote healthy action [8]. Its expectorant, depurative, anthelmintic, fungicidal and insecticidal properties are also reported in Ayurveda which makes the plant medicinally most useful.

2.5 PHYTOCHEMISTRY

The latex of *C. gigantea* always was an area of interest for researchers. The latex contain calotropin, calotoxin, uscharin, voruscharin, uschridin, uzarigenin, syriogenin, calotonic acid, proceroside, calotropin DI and DII [17,18] a cysteine proteinases calotropain FI and FII and a proteinas-es [19]. Apart from these latex also contains constituents like α-amyrin, β-amyrin, taraxasterol, ψ tarexosterol and β-sitosterol [12]. Isorhamnetin-3-O- rutinoside, isorhamnetin-3-O-glucopyranoside, teraxosteryl acetate and a new flava-nol glycosideisorhamnetin- 3-O- [2-O- beta- D-galactopyranosyl -6-O-alpha-L-rhamnopyranosyl]-beta-D-glycopyranoside were isolated from aerial parts [21,22]. Cyanidine-3-rhamnoglucoside isolated from flowers of *C. gigantea* [21]. Two new cardinolides (19-Nor- and 18, 20-epoxy-cardinolides) have been isolated from the leaves of *C. gigantea* and the structural elucidation was accomplished by spectroscopic methods [23]. From the methanol extract of *C. gigantea* root bark a new novel non-protein amino acid giganticine has been isolated [24]. Three cardenolide glycosides, calotropin, frugoside, and 4'-O-beta-D-glucopyranosylfrugoside were also reported as the cytotoxic principles of root bark [25]. Along with calotroposides A and B, two new oxypregnaneoligo glycosides from the roots of *C. gigantean*

Taxonomical classification

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- Genus : Calotropis
- Species: procera

3. Animals

Female albino rats (100-150 g) were used to study the anti-inflammatory activity. The animals (six per cage) were maintained under standard laboratory conditions (light period of 12 h/day and temperature 27° C ± 2° C), with access to food and water *ad libitum.*

The experiment was carried out according to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines and the Institutional Animal Ethical Committee (IAEC) approved all the procedures.(Provide CPCSEA Number of Institution where the work was carried out) Experimental studies were undertaken according to their rules and regulations.

Acute Toxicity

All the extracts were tested for acute toxicity test according to OECD guideline 423. No toxicity was observed at the doses of 500, 1000, 2000 mg/kg of body weight but it was observed that more than 50% of animals were died at the dose of 2000 mg/kg of body weight. Thus, for the screening of anti inflammatory activity, the dose selected was 200 mg/kg of body weight (i.e., 1/10 of the 2000 mg/kg of body weight) as per the OECD guidelines.

Chemicals

Ibuprofen 400mg tablet (Brufen, Abbott) was purchased from Anant Shri medical store, Bhopal. All other chemicals used for this study were of analytical grade.

4. Anti-Inflammatory Activity:-

Purpose and Rationale

Anti inflammatory activity of drugs can be observed by oedema produced in the hind paw of the rat after injection of a phlogistic agent such as brewer's yeast, formaldehyde, dextran, egg albumin, kaolin, Aerosil®, sulphated polysaccharides like carrageenin ornaphthoyl heparamine. The effect can be measured in several ways. The hind limb can be dissected at the talocrural joint and weighed. Usually, the volume of the injected paw is measured before and after application of the irritant and the paw volume of the treated animals is compared to the controls. Many methods have been described how to measure the paw volume by simple and less accurate and by more sophisticated electronically devised methods. The value of the assessment is less dependent on the apparatus but much more on the irritant being chosen. Some irritants induce only a short lasting inflammation whereas other irritants cause the paw oedema to continue over more than 24 h.

Carrageenan-induced rat paw oedema

Acute inflammation was caused by injecting 0.1 ml of 1 % (w/v) carrageenan in saline into thesub-plantar region of the right hind paw of each rat. The paw volume was measured plethysmometrically, after the carrageenan injection. Oedema was expressed as mean increase in paw volume relative to control animals. The percentage inhibition of oedema was calculated by the following equation: % inhibition of oedema= 100 (1-Vt/Vc), where Vc is the oedema volume in the control group and Vt is the oedema volume in tested groups. A single daily dose of test drug pretreatment was given 1 h before the injection of carrageenan.[Winter CA, *et al*, 1962]

Experimental Design

In the experiment rats were divided into 5 groups comprising of 4 animals in each group.

Group **I:** Control**,** were inject carrageenan (0.1 ml.)

Group **II:** Were treated with Ibuprofen I.P.400mg (100mg/kg, orally),

Group **III:** Were treated with *Calotropis procera* (200mg/kg, p.o.) for 7 days

Group **IV:** Were treated with *Calotropis gigantia* (200mg/kg, p.o.) for 7 days

Group **V:** Were treated with combination of *Calotropis gigantia* & *Calotropis procera* (200mg/kg, p.o.) for 7 days

Cotton pellet induced granuloma

The five groups of rats, six in each group was included in this study. After shaving off the fur the animals were anaesthetized. Sterile preweighed cotton pellets (10 ± 1mg) were implanted in the axilla region of each rat through a single needle incision [15]. Ethanol extract of *Calotropis procera, Calotropis gigantia, combination of Calotropis procera and Calotropis gigantia* at 200 mg/kg, Ibuprofen at 100mg/kg (standard) or 1% w/v carboxymethyl cellulose (control) were administered orally to the respective group of animals for seven consecutive days, from the day of cotton-pellet implantation. On the eighth day, the animals were anaesthetized again; the cotton pellets were removed surgically and made free from extraneous tissues. The pellets were incubated at 37 °C for 24 h and dried at 60°C to constant weight. The increase in the dry weight of the pellets was taken as a measure of granuloma formation.

5. Experimental Design

In the experiment, a total of 30 rats were used. The rats were divided into 5 groups comprising of 6 animals in each group.

Group **I:** (negative control**)** were inject CMC 1%(1 ml.)

Group **II:** was treated with Ibuprofen I.P.400mg (100mg/kg, orally),

Group **III:** were treated with *Calotropis procera* (200mg/kg, orally)

Group **IV:** were treated with *Calotropis gigantia* (200mg/kg, orally)

Group **V:** were treated with combination of *Calotropis gigantia* & *Calotropis procera* (200mg/kg, orally)

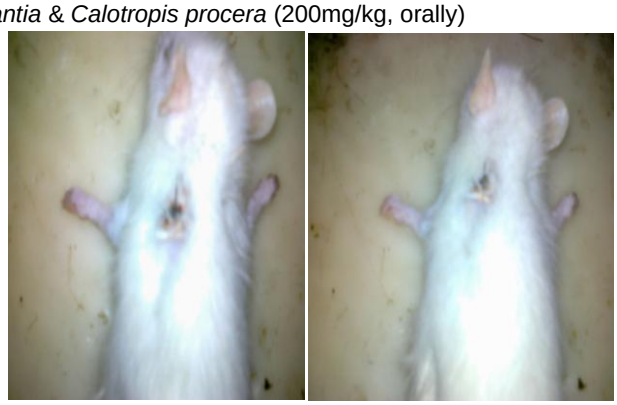


FIG.7 FEMALE ALBINO RAT TO BE OPERATED FOR INSERTION OF COTTON PELLET

5.1 EXTRACTION

Extraction of Calotropis gigantia was performed by Soxhlet apparatus

5.2 Extraction Procedure

5.2.a Solvent extraction:

- Flowers were collected from the vicinity of NRI College Bhopal (MP).the collected flowers was shed dried for 10-12 days.
- After drying the flowers were pulverized into the coarse powder.
- The weighed amount of the powdered drug was the first defatted with petroleum ether and then filtered.
- The filtrate and powder was separated and it was kept for drying for complete removal of petroleum ether.
- The dried powder drug was then extracted with 90% ethanol (90:10, ethanol and water) for about 32 hours.
- After the complete solvent extraction extract was separated and solvent was removed by solvent evaporator in reduced pressure by rotary evaporator until the solvent was completely removed and get dried extract with minimal solvent.
- After that weigh the accurate amount of dried extract and calculate the percentage yield of the extract.
- Determination of % yield:
- The percentage yield of each extract wsa calculated by using following formula:-

$$\% \text{ yield} = \frac{\text{Weight of extract}}{\text{Weight of powder drug taken}} \times 100$$

Solvent extraction steps

Powdered flower preliminary defatting petroleum ether Residue extracted with Ethanol: water (90:10) Hydro alcohol Filtrate(90%) Filtered, Concentrated Crude hydroalcoholic extract

(2) Chemical Evaluation

The extract obtained from the above mentioned procedure was subjected to qualitative test for the identification of various constituents.

6. RESULTS & DISCUSSION

6.1 PHYTOCHEMICAL INVESTIGATION

(1) Percentage yield of extract (w/w):

Percentage yield (%w/w) of hydroalcholic extract (CGE) obtained by solvent extraction was determined by following formula:

$$\% \text{ yield} = \frac{}{} \times 100$$

- Weight of Extract: 3.5 g
- Weight of powdered drug taken: 50 g
- Percentage yield was found to be : 7.0%w/w (table no 1)

Table 1 Percentage yield of extract (5w/w)

S.No.	Solvent	Yield(% w/w)	Characteristics	Consistency
1.	Hydro alcoholic (90%)	7.0	Reddish brown	Semisolid

6.2 Qualitative Chemical Evaluation

S.N.	Chemical Constituents	Hydroalcoholic Extract
1	Alkaloid	+ve
2	Steroid	+ve
3	Tannin	+ve
4	Saponin	+ve
5	Flavonoid	+ve
6	Anthraquinone	-ve
7	Triterpenoids	+ve
8	Carbohydrate	-ve

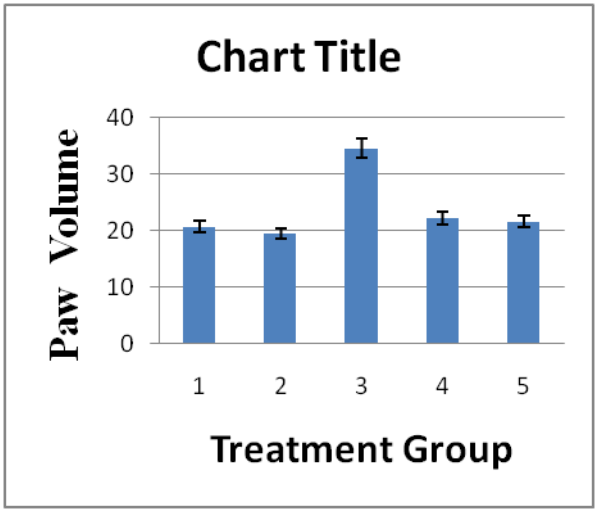
6.3 Table -3 Effect of Calotropis procera & Calotropis gigantia on carrageenan induced paw oedema in rats:

Serial no	Group	Paw volume (ml) (Mean±SEM)
II	Control	0.62±0.06
III	Standard Ibuprofen	0.18±0.02**
IV	Test group 1	0.47±0.02*
V	Test group 2	0.55±0.03*
VI	Test group 3	0.36±0.04**

All values are mean ± SEM, n = 4. $^{*}p<0.05$, $^{*}p<0.01$as compared to negative control group.

Statistical Analysis

The results were presented as mean ± SEM. One way analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons were used for statistical evaluation. p values less than 0.05 were considered as significance

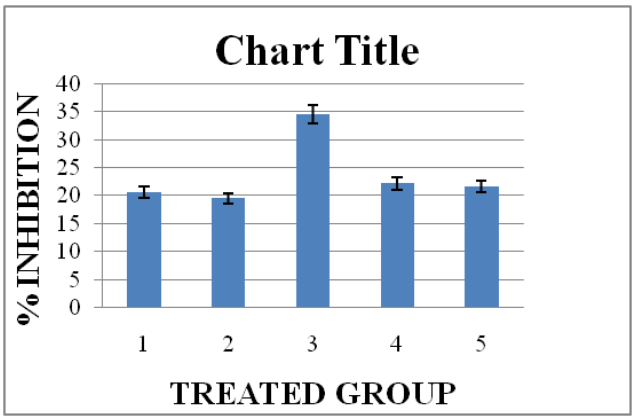


Graph 1

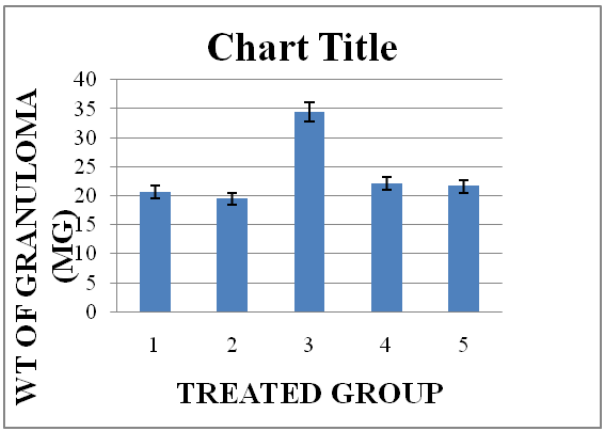
6.4 Table 4 : Effect of Calotropis procera & Calotropis gigantia on granuloma formation in rats :

Serial no	Group	Weight of Granulation(mg) (Mean±SEM)	% Inhibition
I	Negative control	20.66±0.55	27
II	Standard Ibuprofen	19.5±0.42	32
III	Test group 1	34.5±0.61**	23
IV	Test group 2	22.16±0.79	36
V	Test group 3	21.66±0.95	29

Each value represents the mean ± S.E.M.,n=6.*p<0.05 compared with control, Dunnett's t-test after analysis of variance.



Graph -2



Graph 3

Statistical Analysis

The results were presented as mean ± SEM. One way analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons were used for statistical evaluation. p values less than 0.05 were considered as significance.

The ethanolic flower extract of *Calotropis gigantia* & *Calotropis procera* were evaluated for anti inflammatory activity in acute and chronic experimental animal models and results are summarized in table 4& 5.

6.5 DISCUSSION

Carrageenan-induced paw oedema model is used as experimental model for acute inflammation. Were Carrageenan used as phlogistic agent of choice for testing anti inflammatory drugs as it is not known to be antigenic and is devoid of apparent systemic effects.

Carrageenan induced rat paw oedema is commonly used as an experimental animal model for evaluation of the anti inflammatory potential of natural products [10] and is believed to be biphasic. The initial phase is due to the release of histamine, serotonin and kinin in the first hour after the administration of carrageenan, a more pronounced second phase is attributed to release of bradykinin, prostaglandin and lysosome.

The cotton pellet granuloma bioassay is considered a model for studies on chronic inflammation and considered as a typical feature of established chronic inflammatory reaction [19]. Ethanolic extract of *Calotropis gigantia* exhibited significant reduction in the granuloma formation in the cotton pellet-induced granuloma in rats. This reflected that EECG may be effective in chronic inflammatory conditions. Anti-inflammatory activities of many plants have been attributed to their high sterol/triterpenoid saponins [20]. Though at this stage it is not possible to identify the exact phytochemical constituent(s) responsible for anti-inflammatory activities of *Calotropis gigantia*, it may be assumed that the effects could be due chemicals present in the methanolic extract examined by qualitative test and these constituents were confirmed using thin-layer chromatography (TLC). The result of present study indicates that ethanol extract of *Calotropis gigantia* flower possess significant anti-inflammatory activity on both acute and chronic inflammation. Further detailed investigation is underway to determine the exact phytoconstituents, which are responsible for the anti-inflammatory activity.

CONCLUSION

We may conclude that these results support the traditional use of *Calotropis giagntia flower* in some inflammatory conditions.

As for as the comparison between anti inflammatory activity in the flower extract of *Calotropis gigantia* & *Calotropis procera* is concerned, *Calotropis gigantia* flower extract at the dose of 200mg/kg is found to be more effective .

The ethanolic extract *Calotropis gigantea* showed significant anti – inflammatory activity, suggesting that it predominantly inhibits the release of inflammatory mediators. However, animal study and other studies are necessary to identify and isolate the active constituents responsible for its anti inflammatory activity and also there is a need to elucidate its mechanism/s of anti inflammatory action.

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