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Total steroid and terpenoid enriched fraction from *Euphorbia neriifolia* Linn offers protection against nociceptive-pain, inflammation, and *in vitro* arthritis model: An insight of mechanistic study

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ABSTRACT

The plant *Euphorbia neriifolia* Linn has been successfully used for the management of acute inflammatory, arthritic, nociceptive pain and relieves the asthmatic symptom as a tribal folk medicine in India. The present study was conducted to evaluate the anti-inflammatory, analgesic, anti-arthritic activity from total steroid and terpenoid rich fractions derived from hydro-alcoholic extract of *Euphorbia neriifolia* stem (STF-HAENS). STF-HAENS fraction demonstrated 68.58 ± 2.5% and 75.25 ± 5.1% protection against acetic acid-induced pain and central neuropathic pain at 80 mg/kg. It also showed 98.47% protection against acute inflammation at 100 mg/kg with 1.7 fold higher protective activity than the standard drug. The fraction exhibited this efficacy *via* inhibition of proinflammatory cytokines TNF-α, IFN-γ, IL-12 and IL-6 by 74%, 81.26%, 92.10% and 93.4% respectively at 100 μg/ml. It also showed dual inhibition of cyclooxygenase (COX) and lipooxygenase (LOX) activity in a dose-dependent manner that elicited the desired pharmacological action. The fraction downregulated nitric-oxide production from lipopolysaccharide (LPS) stimulated PBMC derived macrophages. The spectrophotometric analysis reveals the STF-HAENS induced ameliorative effect against heat-induced denaturation of BSA protein and exhibited significant antiproteinase activity. Our findings suggest that STF-HAENS could be used as an effective safe therapeutic agent for treatment of nociceptive pain, acute inflammation and arthritis.

1. Introduction

Euphorbia neriifolia Linn, a xerophytic succulent plant belong to Euphorbiaceae family, is a tribal folk medicine according to Indian traditional health care system (Ayurveda) and has been identified as a potential healer of wound, inflammation, pain, asthma, arthritis, ulcer and cancer according to indigenous resources of India, Burma, Thailand, Malaysia, Philippines, China, and Japan [1–4]. Presently, there is no specific preclinical study on anti-nociceptive pain, anti-inflammatory and *in vitro* anti-arthritic effect from the stem of this plant. The pharmacological advantages of this plant in pathology are related to its method of extraction, ratio of bioactive compounds and their synergistic actions. This plant has anti-oxidant, anti-inflammatory and wound healing agent but its mode of action is unclear [5,6].

Pain is an unlikable sensory and emotional experience related to potential tissue damage due to key activation of visceral or somatic nociceptors that can be induced by disease, trauma, peripheral or central nervous system damaging stimuli [7]. Therefore, management of such nociceptive pain through central or peripheral route *via* safe natural drug therapy is necessary.

WHO (World Health Organization) reported that acute inflammatory pain-related diseases, autoimmune syndromes such as inflammation and arthritis affect 500 million people globally resulting in morbidity and severe economic impact [8,9]. Arthritis, a chronic inflammatory auto-immune disease, is characterized by inflammation of connective tissue with swelling, severe pain in joints, progressive destruction of cartilage and subchondral bone coupled with enhanced production of pro-inflammatory cytokines [10].

Most bodily pains, wounds and pathological infections are due to inflammation. Generally, leukocyte extravasations and cell-derived mediators like IFN-γ, histamine, IL-8, IL-6, leukotriene B4, nitric oxide (NO), and prostaglandins are involved in inflammation. On the other hand, it is well known that nociceptive pain mediators like prostaglandins and leukotrienes utilize COX and LOX pathways to aggravate pain,

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inflammatory and arthritic syndromes. Therefore, suppression of such inflammatory mediators like IL-1 and TNF-α [11] or development of enzyme inhibitors of COX and LOX [12] would be an effective therapeutic intervention.

The treatment of pain, inflammation and arthritis with classical non-steroidal anti-inflammatory allopathic drugs or opiate analgesics have undesirable side effects thereby limiting its clinical effectiveness [13, 14]. Drugs like Indomethacin, diclofenac and aceclofenac sodium, rofecoxib, celecoxib and tramadol induces serious side effects like tachycardia, muscle tremors, restlessness, nervousness, throat irritation, ankle edema, respiratory depression, sedation, dizziness, dry mouth, nausea, weight gain gastrointestinal bleeding, gastrointestinal, renal toxicity and bone loss [15]. No permanent cure or satisfactory relief of pain, inflammation and arthritis has been found so far through the conventional mode of treatment.

Globally, patients suffering from arthritis, cancer, microbial disease, dental caries and accident victims suffer traumatic pain and inflammation, requiring powerful and safe anti-inflammatory drugs.

Earlier reports showed that steroidal, terpene like compounds and also their mixtures have potential analgesic, anti-inflammatory and anti-arthritic activity [16–19]. The active fractions of STF-HAENS (total steroid and terpenoid rich fractions derived from hydro-alcoholic extract of *Euphorbia neriifolia* stem) was behaving like the Chinese phytomedicine [20] and are devoid of untoward effect at pharmacological relevant therapeutic doses compared to pure drugs. This was supported by our toxicity studies as described follows.

In the present work, we have evaluated the anti-nociceptive pain and anti-inflammatory activity along with *in vitro* anti-arthritic effect of STF-HAENS to justify its traditional folk use. We have also elucidated the mechanism of action of STF-HAENS as strong pain reliever and anti-inflammatory agent that could emerge as a promising drug candidate.

2. Materials and methods

2.1. Experimental animals

Albino mice of both sex (20–25 g) and healthy male albino adult rats (150–230 g) were approved by Institute animal ethical committee (Ref. No. BCRCP/IAEC/5/2013) and housed under standard laboratory conditions for pharmacological screenings. Albino rats and mice were used in the proposed studies to evaluate the anti-inflammatory and analgesic activity of STF-HAENS. Carrageenan induces acute-inflammation in albino rats. Hot plate model and acetic acid mediated peritoneal constrictions develop central and peripheral nociceptive pain in albino mice as per established model. Wister rats were used to isolate the lungs derived 12 LOX enzymes as well.

2.2. Chemicals

All chemicals and reagents used in this study are the analytical grades, procured from SRL and SIGMA, India.

2.3. Extraction and preparation of steroid-terpenoid rich fraction from hydro-ethanolic extract of Euphorbia neriifolia stem

The test plant was identified by the in-house Botanist through taxonomical identification procedure and preserved as voucher specimen reference as BCRCP/PBI/1/2013. Air-dried triturated stem powder of *Euphorbia neriifolia*, 1000 g (W_i) was taken for hydro-alcoholic extraction through cold maceration process. The whole powder was taken in 5-liter conical flask and hydro-ethanolic solutions in the ratio of 30:70 were poured into the flask. After complete soaking of dry powder into the extracting solvent, the menstruum was shaken vigorously for 1 h and then kept it at 4 °C room for 15 days. The solution was shaking with a stirrer to diffuse out the compounds into solvent efficiently at every 3 days interval. After 15 days, the hydroethanolic extract was decanted into another container. Remaining phytocompounds were pulled out from marc (residual cellular debris available after extraction from organized dried powdered stem) through re-maceration with fresh solvents. The final solution was filtered. The filtrates were evaporated to dryness through rotary vacuum evaporator to get dry crude drug residue (W_f) containing phyto-extract. Finally, the % of yield of extractive value (We) was calculated as per indicated formula. $We = 100 \times W_f / W_i$. This extract was partitioned against n-hexane and ethyl-acetate solvents to afford two fractions of weight 70 g and 56 g respectively. N-hexane fraction was subjected to silica gel column chromatography, eluted with hexane–acetone (90:10–10:90) to obtain 12 fractions (A–L). These fractions were evaluated and the presence of steroid and terpenoid supported by thin layer chromatography (TLC) and phyto-chemical test. Finally, the remaining 7 fractions were mixed and evaporated to dryness in rotary vacuum to obtain dry residue of 12.7 g that coded as STF-HAENS and validated by TLC study and chemical test.

2.4. High performance liquid chromatographic study (HPLC) study

HPLC chromatogram was developed by injecting STF-HAENS in the reverse phase C-18 column with the mobile phase methanol: water (40:60) in 0.1% formic acid at 1 ml/min flow rate for 15 min runtime. Euphol and β-amyrin (Gift sample) were evaluated in similar HPLC system as mentioned above in order to characterize them with the chromatogram of the STF-HAENS as phytomarkers. The characterization of these marker compounds within the STF-HAENS chromatogram was interpreted based on their individual identified retention-time.

2.5. Inhibition of protein denaturation, proteinase inhibitory action and membrane stabilization studies

The final reaction mixture (5 ml) consisted of 5% aqueous 2.40 ml bovine serum albumin and 0.10 ml solution containing STF-HAENS (10, 25, 50 and 100 μg/ml of final volume) dissolved in 0.2% DMSO (respect to final reaction volume). pH was adjusted at 6.3 using a small amount of 1 N HCl. All the samples were incubated at 37 °C for 20 min and then heated at 57 °C for 30 min. After cooling the samples, 2.5 ml phosphate buffer saline (pH 6.3) was added to each tube to make up the final reaction volume. Turbidity was measured including positive controls containing aspirin (100 μg/ml) spectrophotometrically [23] at 660 nm compared to untreated controls. Test 0.10 ml DMSO was used instead of fraction while product control tests lacked bovine serum albumin as per method adopted. The percentage inhibition of protein denaturation was calculated as follows.

$$\text{Percent inhibition} = 100 - \frac{(\text{O.D. of test} - \text{O.D. of product control}) \times 100}{\text{O.D. of Control}}$$

The reaction mixtures (2.0 ml) contained 0.06 mg trypsin, 1.0 ml. 25 mM Tris-HCl buffers (pH 7.4) and 1.0 ml solution containing 10, 25, 50 and 100 μg/ml of STF-HAENS dissolved in 0.2% DMSO. The mixtures were incubated at 37 °C for 5 min, and then 1.0 ml of 0.8% (w/v) casein was added. The mixtures were further incubated for 20 min. 2.0 ml of 70% (v/v) perchloric acid was added to the mixtures to terminate the reaction. The cloudy suspension was centrifuged. The absorbance of the supernatant of each group of the solution was measured at 280 nm against buffer as blank [24]. The percentage of inhibition was calculated as per the following the formula and compared with positive controls of aspirin (100 g/ml).

$$\%\text{of Inhibition} = 100 - [(\text{O.D of test solution} - \text{O.D.of only STF} - \text{HAENS fraction without casein} - \text{trypsin enzyme reaction}) \div (\text{O.D of control}) \times 100].$$