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Short communication

Rare dipeptide and urea derivatives from roots of *Moringa oleifera* as potential anti-inflammatory and antinociceptive agents☆

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Abstract

In the course of our studies on the isolation of bioactive compounds from the roots of *Moringa oleifera*, a traditional herb in southeast Asia, rare aurantiamide acetate **4** and 1,3-dibenzyl urea **5** have been isolated and characterized. And also, this is the first report of isolation from this genus. Isolated compound inhibited the production of TNF-α and IL-2; further compound **5** showed significant analgesic activities in a dose dependant manner. These findings may help in understanding the mechanism of action of this traditional plant leading to control of activated mast cells on inflammatory conditions like arthritis, for which the crude extract has been used.

Keywords: Aurantiamide acetate; 1,3-Dibenzyl urea; Analgesic activity; TNF-α; IL-2; IL-6

1. Introduction

The design and development of new molecules potentially useful in the control of pain is a very important area today. Over the last few years, the amount of information from studies on pain transmission by transient receptor potential channel vanilloid subfamily member 1 (TRPV1) binding ligands has dramatically increased, thus revealing novel targets for the advent of new pain therapies. Furthermore, a gigantic step came with the identification of a protein called TRPV1, cloned in 1997, which is a ligand-gated nonselective cation channel vanilloid receptor with high Ca^{2+} permeability [1]. TRPV1 is activated not only by vanilloid ligands such as capsaicin **1** (Scheme 1), noxious heat (>42 °C) and protons (extracellular pH < 6) but also by endogenous mediators of inflammation such as cannabinoid anandamide and arachidonic metabolites [2,3]. Capsazepine **2**, which has been extensively characterized, was the first reported competitive VR1 antagonist. However, its drawback is its modest potency and poor metabolic and pharmacokinetic properties [4]. Recently the structure–activity relationships of 1,3-diarylalkyl thioureas **3** possessing new vanilloid equivalents have been reported [5]. Accordingly, the idea that TRPV1 functions as an integrator of multiple pain producing stimuli implied that TRPV1 antagonists should have profound antinociceptive effects, especially in inflammatory pain models.

Rheumatoid arthritis (RA) is one of the most typical rheumatic diseases, and is characterized by chronic inflammatory changes that lead to cartilage destruction, joint deformity, and disability [6]. Pain of RA is more unbearable than any other kinds of arthritis. Recently, mast cells are understood as a crucial factor to cause RA because RA subjects have more synovial mast cells, and activated mast cells secrete various inflammatory substances. Although mast cells have been viewed primarily in the central role of immediate-type hypersensitivity reactions, the significant contribution of the mast

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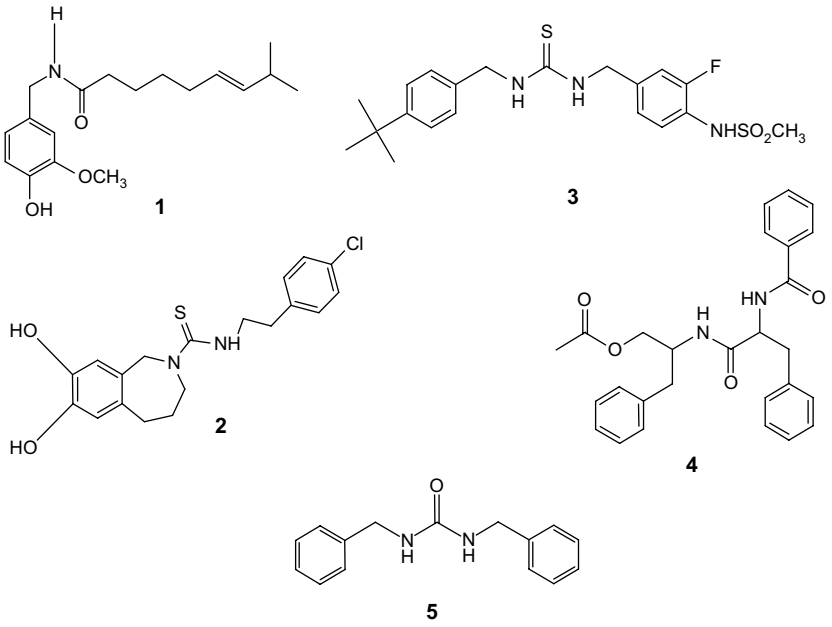

Scheme 1. Capsaicin, **1**; capsazepine, **2**; nonvanilloidal dibenzyl thioureas, **3**; aurantiamide acetate, **4**; 1,3-dibenzyl urea, **5**.

cells in the pathogenesis of rheumatic arthritis recently has become more evident [7]. Activated mast cells synthesize prostaglandins and leukotrienes, and release both preformed and newly synthesized cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-2. Thus infiltrated mast cells and their mediators may contribute to the initiation and progression of the distributive inflammatory process and matrix degradation of RA [8]. TNF-α is an autocrine stimulator as well as a potent inducer of other inflammatory cytokines, including IL-2 and IL-6. TNF-α play crucial roles in the pathogenesis of RA because it is at the apex of inflammatory and destructive processes that operate in the joint. IL-6 is a pleiotropic inflammatory cytokine produced by T cells, macrophages and synovial fibroblasts. IL-2 regulates both T cell's growth and death and is involved in maintaining peripheral tolerance [9].

For decades, natural products have been a wellspring of drugs and drug leads. *Moringa oleifera* Lam. (syn. *Moringa pterygosperma* Gaertn.) (Moringaceae) commonly known as ''sahjna'' is a fast growing ornamental tree, which is widely distributed in tropical areas [10]. Its medicinal value has long been recognized in the indigenous system of medicine [11]. It is a small to medium sized tree with multiple uses, and its different parts are reputed to be used in folk medicine for the treatment of a variety of human ailments such as rheumatism, paralysis, epilepsy, ascites, etc. [12].

In recent decades, the extracts of leaves, seeds and roots of *M. oleifera* have been extensively studied for many potential uses including wound healing [13], anti-tumor [14], antihepatotoxic [15], and analgesic activities [16].

M. oleifera is incorporated in various marketed formulations, such as Rumalaya and Septilin (The Himalaya Drug Company, Bangalore, India), Orthoherb (Walter Bushnell Ltd, Mumbai, India), Kupid Ford (Pharma Products Pvt Ltd, Thayavur, India) and Livospin (Herbals APS Pvt Ltd, Patna, India), which are available for a variety of disorders.

Indian Materia Medica describes the use of roots of *M. oleifera* in the treatment of a number of ailments, including asthma, gout, lumbago, rheumatism, enlarged spleen or liver, internal deep seated inflammations and calculous affections [17,18]. The root extracts of *M. oleifera* have been studied for diuretic and acute anti-inflammatory activities [19].

Several phytochemical investigations of this plant on its leaves and pods have led to isolation of several carbamate, thiocarbamate and isothiocyanate glycosides which were also the hypotensive principles [20,21]. In view of the fact that there has been no systemic phytochemical work on the roots of this plant, in the present work the alcoholic root extract was subjected to a systematic bioassay guided isolation procedure. During the course of this investigation, in addition to other known compounds, we have isolated two major compounds, aurantiamide acetate and 1,3-dibenzyl urea. This is the first report of isolation of these compounds from this genus; aurantiamide acetate is a rare dipeptide that has been reported previously from red alga [22a], also from members of Piperaceae [22b], Leguminosae [22c], Renunculiaceae [22d], *Aspergillus penicilloides* [22e] and several other sources [22f–o]. The occurrence of 1,3-dibenzyl urea is also rare, the only other natural source of this compound being the roots of *Pentadiplandra brazzeana* [23].

As these two compounds could be isolated with a yield of 0.2% and they have never been subjected to systematic pharmacological evaluation, and also these were structurally similar to new TRPV1 antagonists it was deemed as an appropriate candidate for investigation for its analgesic and cytokine inhibitory activities.

In the present study we carried out experiments to investigate the analgesic activity of isolates in hot-plate model [24].

The results presented in Fig. 1 show that compounds **4** and **5** have significant analgesic effect in mice. There was a dose dependant increase in time course of response (latency) to thermal stimulation in the mice. This effect started 15 min after treatment and persisted throughout the 120 min duration of the experiment. However, compound **5** seems to have more potent analgesic activity.

Further both the compounds were subjected to cytokine concentration using whole-blood assay [25]. As depicted in Fig. 2 aurantiamide acetate is showing significant inhibition of TNF-α and IL-2 but not IL-6, whereas 1,3-dibenzyl urea is significantly inhibiting only IL-2, Fig. 3.

The antinociceptive behavior of 1,3-dibenzyl urea can be attributed to being structurally close analog of 1,3-dibenzyl thioureas, which has emerged as one of the promising nonvanilloid TRPV1 antagonists possessing excellent therapeutic potential in pain regulation, and was shown to exhibit Ca^{2+} uptake inhibition in rat DRG neuron with IC_{50} between 10 and 100 nM [26]. Further compound aurantiamide acetate on subcutaneous administration in adjuvant arthritic rat model suppressed hind paw swelling at 10 mg/kg body weight [22e]. Since the isolated compounds significantly inhibited the inflammatory cytokines, it is proposed that they will be promising lead for the treatment of metabolic cartilage disorders.

2. Results and discussion

In conclusion, we have isolated aurantiamide acetate (compound **4**) and 1,3-dibenzyl urea (compound **5**) from roots of *M. oleifera*. Aurantiamide acetate showed significant inhibition of TNF-α and IL-2 but not IL-6, while 1,3-dibenzyl urea showed significant analgesic activity in dose dependant manner and significant inhibition of IL-2. These results indicate that these compounds may be responsible for the anti-inflammatory/antiarthritic and analgesic activities of *M. oleifera* root [22b]. Earlier, compound **4** was reported for its antiarthritic activity and a selective cathepsin inhibitor [22e] and since cathepsins are implicated in matrix turnovers in mammals, conceptually

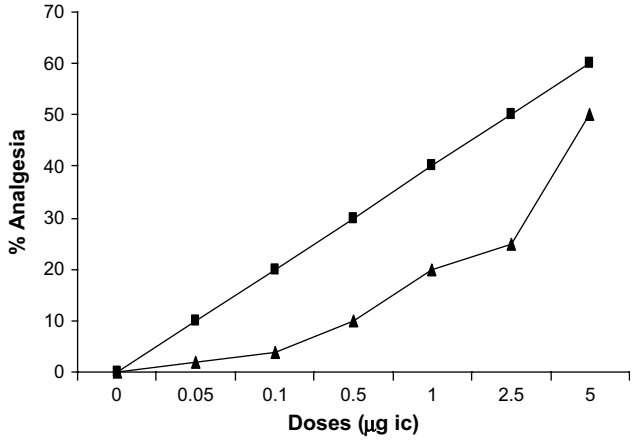

Fig. 1. Dose dependant analgesic effect of aurantiamide acetate (triangles) and dibenzyl urea (squares).

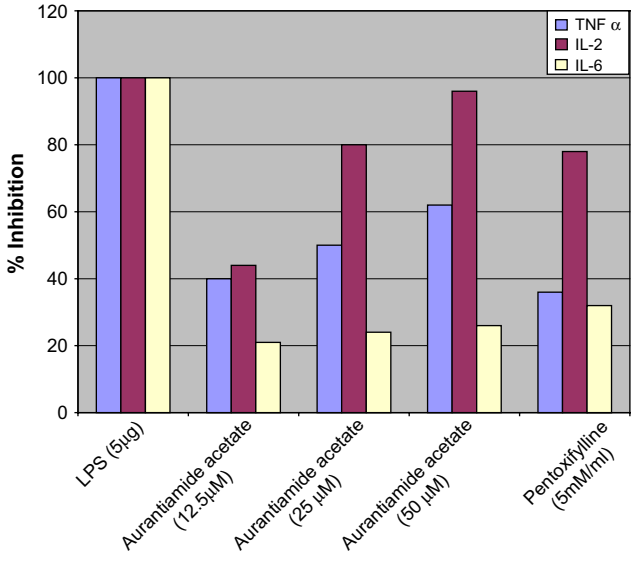

Fig. 2. Effect of aurantiamide acetate on LPS induced cytokines.

hence potential therapeutics for the treatment of metabolic cartilage disorders. The over-expression of pro-inflammatory cytokines has been implicated in a number of autoimmune disorders such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD), psoriasis, systemic lupus erythematosus (SLE), and organ graft rejection.

Since the isolated compounds significantly inhibited the inflammatory cytokines, it is proposed that they will be promising lead for the treatment of metabolic cartilage disorders. Synthesis of appropriate analogs may pave the way to develop a potent drug. Also the above results justify the traditional use of this plant for the various forms of pain including rheumatoid arthritis. These findings may help in understanding the mechanism of action of this traditional plant leading to control activated mast cells on inflammatory conditions like arthritis.

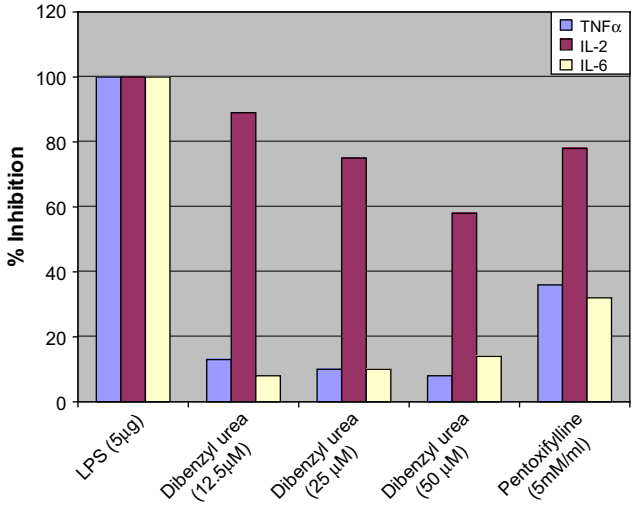

Fig. 3. Effect of 1,3 -dibenzyl urea on LPS induced cytokines.

3. Experimental

3.1. Isolation of compounds

The roots of *M. oleifera* were collected by the Botany division of, CDRI, and voucher specimen has been maintained. The roots (10) were extracted with 8 L of ethyl alcohol four times in a percolator. The resultant alcoholic extract was combined and concentrated under reduced pressure to give 200 g of alcohol extract. This was fractionated with hexane, chloroform and *n*-butanol successively. The resultant chloroform fraction (20 g) was subjected to conventional silica gel column chromatography and eluted with mixtures of increasing polarity of hexane:ethyl acetate (20%) solvent system to give aurantiamide acetate and 1,3-dibenzyl urea which were characterized by using ^{1}H NMR, ^{13}C NMR, 2D NMR, IR and mass spectral data and comparing with the literature data [22a,23].

3.2. Hot-plate test

The analgesic activity was measured by hot-plate test of Eddy and Leimbach [24]. The basal reaction time of each mouse to noxious heat (55 ± 1 °C) was determined twice 10 min apart before the graded doses of test compounds were given intracerebrally in groups of 10 mice each. Reaction time was determined every 10 min after the drug administration till the basal reaction time was restored. The percentage of animals showing analgesia at each dose level was calculated. An analgesic effect was considered to be present when the reaction time was increased by more than 100%. Animals were habituated twice to the hot plate in advance 24 h before the test (1 min) and again 20 min before test (1 min). For testing, mice were placed on hot plate maintained at 55 ± 1 °C. The time that elapsed until occurrence of either a hind paw licking or a jump off the surface was recorded as the hot-plate latency. Mice with baseline latencies of <5 or >30 s were eliminated from the study.

3.3. Whole-blood cytokine assays

3.3.1. Stimulation of cells and collection of supernatants for cytokine analysis

Blood was collected in 15-ml plastic syringes from heart of rat for TNF-α, IL-2 and IL-6 and immediately mixed with sodium heparin at 20 U/ml in 50-ml plastic centrifuge tubes and further diluted to 1/5 with sterile RPMI 1640 tissue culture medium containing 100 U/ml of penicillin/100 μg/ml streptomycin and 2 mM L-glutamine (Sigma). Diluted blood (800 μl/well) was dispensed in 24-well tissue culture plates within 2 h of collection. Cultures were stimulated with 100 μl/well of mitogens (to give final concentration of LPS at 5 μg/ml) and inhibitors are added in each well in concentrations of 25 μM/ml and 50 μM/ml. Pentoxifylline is used as standard synthesis inhibitor for the cytokines in the concentration of 5 mM/ml. Plates were incubated overnight at 37 °C in 5% CO_2. Supernatants were collected from the wells and stored at −70 °C until assay.

3.3.2. Cytokine analysis

Cytokines TNF-α, IL-2 and IL-6 were assayed using ELISA adapting the procedures recommended by the manufacturer (Duo Set, respectively, R&D Systems, UK). Briefly, captured antibodies for all cytokines were coated as recommended by the manufacturer in PBS, pH 7.2–7.4. All standards and samples were run in duplicates. Anti-cytokine-biotinylated antibodies were used at 400 ng/ml for IL-2, 200 ng/ml for IL-6 and 100 ng/ml for TNF-α. Streptavidin–horseradish peroxidase conjugate with H_2O_2 (R&D, UK) substrate was used. Plates were read by BIO-TEK ELISA plate reader and absorbance was transformed to cytokine concentrations (pg/ml) using a standard curve.

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