ANTI-INFLAMMATORY ACTIVITY OF EXTRACTS AND COMPOUNDS FROM *VITEX NEGUNDO*

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RASADAH, M. A., FAREDIAH, A., WONG, C. L. & ONG, B. K. 2005. **Anti-inflammatory activity of extracts and compounds from *Vitex negundo***. The anti-inflammatory activity of the leaf extract of *Vitex negundo* was investigated on topical administration using the TPA (tetradecanoylphorbol acetate)-induced mouse ear inflammation model. The crude methanolic extract of *V. negundo* showed moderate inhibition (74%) at a dose of 2 mg per ear. The methanol extract was then subjected to liquid–liquid fractionation with petroleum ether, chloroform, ethyl-acetate and ethanol. Bioassay-guided chromatographic fractionation of these fractions led to the isolation of 3,4-dihydroxybenzoic acid as the bioactive principle responsible for the anti-inflammatory activity. The structures of the pure compounds were elucidated using various spectroscopic techniques.

Key words: Verbenaceae – 4-hydroxybenzoic acid – negundoside – 3,4-dihydrobenzoic acid – luteolin

RASADAH, M. A., FAREDIAH, A., WONG, C. L. & ONG, B. K. 2005. **Aktiviti antiradang bagi ekstrak dan sebatian tulen *Vitex negundo*.** Aktiviti antiradang daripada daun *Vitex negundo* dikaji menggunakan ujian edema telinga aruhan tetradekanoilforbol asetat (TPA). Menerusi model TPA ini, ekstrak metanol *V. negundo* menunjukkan perencatan yang sederhana (74%) pada dos 2 mg setiap telinga. Fraksi kromatografi berasaskan bioesei berjaya memisahkan komponen aktif, asid 3,4-dihidroksibenzoik yang bertanggungjawab terhadap aktiviti antiradang. Struktur sebatian tulennya telah dicirikan melalui kaedah spektroskopi.

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Introduction

Inflammation is the body's immediate defensive response to injury, which may be caused by infections, chemicals or physical agents. Its treatment requires the application or ingestion of an anti-inflammatory drug, the earliest of which was the natural products salicylic acid prepared from *Spiraea* species (Isel 1991). Numerous synthetic anti-inflammatory drugs are now available but most of them can cause side effects such as gastric irritation as well as peptic and duodenal ulcers. Thus, plants, especially medicinal plants, are still a valuable resource for new alternative and safer anti-inflammatory drugs.

Inflammation is known to occur via a series of complex pathophysiological pathways, influenced by various mediators such as prostaglandins and leukotrienes. These mediators can cause oedema such as heat, pain, disturbed tissue function, reddening and swelling. Assay models which focus on blocking the production or the action of these mediators have been used in evaluating extracts and purified compounds for anti-inflammatory properties. A general topical method to assay for anti-inflammatory activity of plant samples is the 12-*O*- tetradecanoylphorbol-13 acetate (TPA) induced mouse ear oedema model (Carlson *et al.* 1985, Bird *et al.* 1986). The model proved useful in determining the *in vivo* activities of non-steroidal anti-inflammatory drugs (NSAIDs), mixed lypoxygenase (LO)/cyclooxygenase (COX) inhibitors or 5-LO inhibitors (Carlson *et al.* 1985).

In the course of a random screening of plants for potential anti-inflammatory properties using the above model, the methanolic leaf extract of *Vitex negundo* (Verbenaceae) demonstrated significant activity. *Vitex negundo* or locally known as lenggundi is a medium-sized tree. It is found throughout India, Sri Lanka, Myamar, China, Pakistan, Afghanistan, Malaysia, Africa and the Philippines. It can be propagated readily by vegetative cutting. Published reports indicated that it is used in a variety of traditional preparation. The roots and leaves are used for pain, tonic expectorant and diuretic. Sap from crush leaves is used for coughs and sore throat (Burkill 1966). The leaf of this plant was traditionally used to treat asthma, fever, headaches as well as arthritis. The plant is also used as a commercial drug in the indigenous system of medicine. In the Philippines, the extract of this plant has been formulated into cough syrup and tablet/capsules to treat cough and asthma respectively.

Previous chemical studies on *V. negundo* included triterpenoids as well as flavonoids from the seeds (Bhargava 1989, Chawla *et al.* 1992). Many biological activities on *V. negundo* have been reported. These activities were central nervous system (Gupta *et al.* 1999), insecticidal (Hebbalkar *et al.* 1992) as well as analgesic activities (Shrivastava & Sisodia 1970). This paper reports the *in vivo* anti-inflammatory activity of *V. negundo* and the bioassay-guided isolation of its bioactive constituents.

Materials and methods

Collection

Vitex negundo was collected from Taman Sri Pulai, Skudai, Johor. A voucher specimen (AZ7078) was deposited at the Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia.

Extraction and fractionation

Powdered, dried *V. negundo* leaves (747 g) were extracted with methanol for 12 hours. The methanol extract was then concentrated to half its volume and subjected to liquid–liquid fractionation with petroleum ether, chloroform, ethyl-acetate and ethanol. All fractions were concentrated in vacuo and stored at -20 °C prior to testing. All fractions were evaluated for anti-inflammatory activity on the TPA-induced mouse ear oedema assay. The active chloroform and ethyl-acetate fractions (40.5 g) were combined and subjected to fractionation using silica gel vacuum column chromatography with hexane, chloroform, ethyl-acetate, acetone followed by a gradient of methanol up to 100%. The fractionation process gave 39 fractions. In each case, fractions with the same thin layer chromatography (TLC) profiles were combined and subjected to column chromatography and preparative TLC to yield 4-hydroxybenzoic acid (288.6 mg), 3,4-dihydrobenzoic acid (44.5 mg), negundoside (49.6 mg) and luteolin (23.1 mg). Each of these compounds was tested for their anti-inflammatory activities.

Structure determination

The identities of the isolated compounds were carried out by spectroscopic techniques (^{1}H NMR, ^{13}C NMR , UV-VIS and MS) and compared with published data.

Animals

Groups of six female BALB/c mice weighing 17–22 g were purchased from the Institute for Medical Research, Kuala Lumpur. The animals were housed at 23 ± 4 °C with 12 hours lighting schedule. The animals were fed on pellet chow and water.

Chemicals

12-*O*-tetradecanoylphorbol-13-acetate (TPA) was purchased from the Wako Pure Chemical Industries Ltd. Japan and indomethacin from Sigma.

TPA-induced mouse ear oedema

The anti-inflammatory activity of *V. negundo* extracts and the pure compounds were evaluated using modified methods by Gschwendt *et al.* (1984) and Hirota *et al.* (1990). A 25 µg ml^{-1} stock solution of 12-*O*-tetradecanoylphorbol-13-acetate or TPA was prepared in acetone. Each mouse was treated with 20 µl (0.5 µg/ear) of TPA on the inner surface of both ears. Test extracts, pure compounds and the standard drug were each prepared in acetone and applied topically to the right ear (2 mg/ear) 40 min before TPA application. The left ear (control) received the same volume of acetone. After six hours, the mice were killed by cervical dislocation and 7 mm diameter sections of both ears were obtained and weighed.

Swellings induced by TPA were assessed in terms of the increase in the weight of the right ear punch biopsy over that of the left ear. The inhibitory effects (IE%) of each extract were calculated as the ratio of the weight increase of the ear sections, according to the following formula:

$$\text{Inhibitory effect (IE\%)} = \frac{L-R}{L-C}\times 100$$

$$= \frac{L-R}{L-(L/2.41^{*})}\times 100$$

where

L = weight of left ear which was treated with TPA only

R = weight of right ear which was treated with TPA plus the tested extract

C = calculated weight of untreated ear

(* treating with 0.5 mg TPA resulted in 2.41 times increase in weight of the ear)

Results and discussion

The anti-inflammatory activity of each extract was evaluated based on the inhibitory effect value (IE) from the TPA-induced oedema test. At a dose of 2 mg per ear, extracts showing IE greater than 50% were considered to have significant activity.

The initial crude methanolic extract induced 74% reduction in the TPA-induced oedema and further fractionation revealed that the activity was distributed over the petroleum ether (59%), chloroform (68%), ethyl-acetate (65%) and ethanol (50%) fractions (Table 1). The notably high topical activity of the chloroform and ethyl-acetate justified their further subjection to a bioassay-guided fractionation to yield pure compounds.

Table 1 Anti-inflammatory effect of the crude extract (5 µg/ear), fractions (5 µg/ear) and purified components (0.5 µg/ear) from *Vitex negundo* (leaf) on mouse ear oedema

Sample	Oedema inhibition (%)
Crude methanolic extract	74 ± 7
Petroleum fraction	59 ± 3
Chloroform fraction	68 ± 1
Ethyl-acetate fraction	65 ± 1
Ethanol fraction	50 ± 1
4-Hydroxybenzoic acid	24 ± 2
3,4-Dihydroxybenzoic acid	41 ± 4
Negundoside	27 ± 3
Luteolin	25 ± 1
Indomethacin (standard compound)	97 ± 3

4-Hydroxybenzoic acid, 3,4-dihydrobenzoic acid, negundoside and luteolin (Figure 1) were compounds isolated from the active fractions of *V. negundo*. 3,4-Dihydrobenzoic acid showed moderate activity in TPA-induced oedema with percentage inhibition 41% as compared with the reference standard, indomethacin, which gave 97% inhibition. In contrast, the other three components (4-hydroxybenzoic acid, negundoside and luteolin) showed low activity in the range of 24 to 27% . The study shown here is an example of the activity of a plant extract being due to more than one type of constituent. Synergic effects are often claimed for such situation but the work reported here cannot show if this occurs.

The leaves of *V. negundo* have been used in the Philippines in reducing inflammation, rheumatic swellings of the joints and testes due to gonorrheal epididymitis (Quasumbing 1978). In Malaysia, the leaves of *V. negundo* are traditionally used to treat asthma, fever, headaches as well as arthritis (Burkill 1966). This study has confirmed the traditional use of this plant in reducing inflammation. In this case, negundoside can also been used as a marker component for *V. negundo* in the quality control activity for the herbal industries.

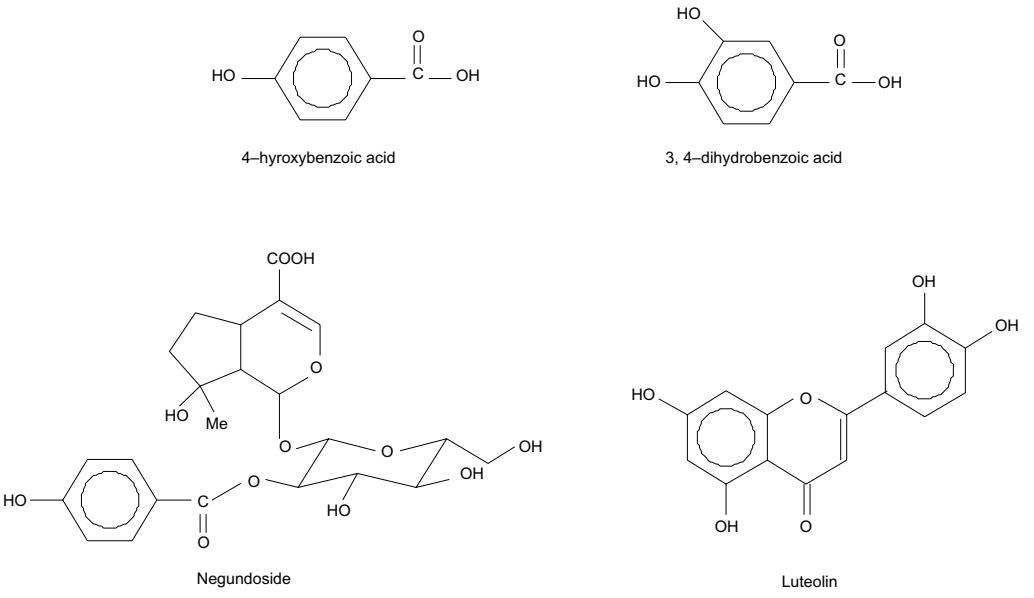

Figure 1 Chemical structure of compounds isolated from *Vitex negundo*

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