

## Cytotoxicity Evaluations on *Vitex negundo* Anti-inflammatory Extracts

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**ABSTRACT** *Vitex negundo* that is locally known as legundi has been used traditionally as tonic, febrifuge and anti-inflammatory medicaments. FRIM already made an attempt to proof its anti-inflammatory activities. *V. negundo* dichloromethane and ethanolic extracts were scientifically proven for its anti-inflammatory activities through TPA-induced mouse ear oedema assay with percentage of inhibition 72.2% and 54.1% respectively. Cytotoxicity effects on these anti-inflammatory extracts were evaluated using sulphorhadamine B (SRB) assay on mammary (MCF-7 cell line) and genito-urinary (VERO cell line) systems. Based on WHO 1972, extracts with  $IC_{50}$  values less and equal to 20 $\mu$ g/ml can be preliminary considered as cytotoxic. The  $IC_{50}$  values of the extracts on both cell lines were more than 50  $\mu$ g/ml. Hence, these anti-inflammatory extracts were not cytotoxic on both mammary and genito-urinary systems teste.

**Key words:** *Vitex negundo*, anti-inflammatory extracts, TPA-induced mouse oedema assay, sulphorhodamine B assay, not cytotoxic.

### INTRODUCTION

*Vitex negundo*, a deciduous shrub plants can be found from Africa to the Pacific [1]. The leaves have been used in the Philippines in reducing inflammation, rheumatic swellings of the joints and testes due to gonorrhoeal epididymitis [2]. Various anti-inflammatory assays had been used to scientifically proof the anti inflammatory effects of its leaves. The leaves were processed and extracted following bioassay guided fractionation approach to search for its most active ingredient, which later will be used for standardisation of the effective extract. The anti-inflammatory effects of the extracts were analysed using TPA-induced mouse ear oedema assay. The most active anti-inflammatory extract was then evaluated for its cytotoxic effects on cell proliferation. Human cell lines or cell cultures have long being used to assess the acute toxicity effects for natural products and chemicals for preliminary safety evaluation. A great variety of *in vitro* cellular models are used depending on the aspect dealt with [3]. In this study, we had cultured cellular model from mammary (MCF7) and genito-urinary (VERO

kidney) systems *in vitro* to assess the cytotoxic effect of two *V. negundo* anti-inflammatory extracts.

### MATERIALS AND METHODS

#### Cell and Media

The breast carcinoma (MCF-7) and African green monkey normal kidney (VERO) were purchased from American Type Culture Collection (ATCC), Maryland, USA. Dubelcco's Modified Essential Medium (DMEM, PAA Lab., UK) was supplemented with 5% Fetal Calf Serum (PAA Lab., UK), 1% fungizone, 1% penicillin streptomycin and 0.125% gentamycin sulphate (Flowlab, Australia).

#### Plant Sample

*V. negundo*'s leaves was collected from Perlis in January 2002. A voucher specimen has been deposited at Institute's herbarium for reference.

#### Extraction

Powdered dried leaves of *Vitex negundo* (1kg) were extracted with ethanol at a temperature of 40°C via soxhlet preparation for a period of 12 hours. After filtration, the filtrate was evaporated to a small volume in vacuo and lyophilized. The dried ethanol crude extract was then partitioned

between dichloromethane and water to yield dichloromethane fraction and aqueous layer. The aqueous layer was repartitioned with ethyl-acetate. All fractions were evaporated and lyophilized to dryness. All fractions were stored at -20°C prior to testing.

#### Treatment of Cultured Cells with *V. negundo* Extracts

Anti-inflammatory TPA-induced ear oedema assay on dichloromethane, ethyl acetate, ethanol and aqueous *V. negundo* extracts were initially conducted. The results indicated that dichloromethane and ethanol extracts were active and its cytotoxic effect was currently studied. Cells were cultured in DMEM until it reached at its sub-confluent state. 0.1 ml DMEM containing cells ( $1 \times 10^5$ ) were plated into each well of 96 well plates and incubated overnight at 37°C, pH 7.4 and 5% carbon dioxide in air. The media were removed and replaced with new media (0.2ml/well). *V. negundo* extracts and paclitaxel (positive control) were dissolved in ethanol at 10 mg/ml and further diluted by 10 fold dilutions which finally produced 5 different concentrations ranging from 10 to 0.01 mg/ml. Extract at each concentration was then added by 1% amount of the media in different well. Controlled wells were treated with ethanol only. The treated cells in 96 well plates were incubated at same conditions mentioned above for 72 hours. The treatment of cells per well was duplicated and the experiment was repeated three times.

#### Sulphorhodamine B (SRB) Assay

The extracts reactions were stopped and the living cells were fixed at the bottom of the well using 50% trichloroacetic acid (TCA). It was then incubated at 4°C for one hour. The media in the 96 well plate was then removed and the wells were washed with deionised water. The cells were stained using 0.4% sulphorhodamine B (100 µl/well) [4]. The 96 well plate was then rinsed with acetic acid to remove the unbound dye. 100 µl TRIS buffer was added in each wells and the optical density (OD) was read using ELISA reader at 492 nm.

#### TPA-induced mouse ear oedema

Anti-inflammatory activity of *V. negundo* extracts were evaluated using a modification of the methods of [5] and [6]. A 25 µg/ml stock solution of 12-O-tetradecanoylphorbol-13-acetate or TPA was prepared in acetone. Each mouse

was then 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 minutes before TPA application). The left ear (control) received the same volume of acetone. After 6 hrs, the mice were killed by cervical dislocation and a 7-mm diameter section of both ears obtained and weighed.

The swelling induced by TPA was 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 then calculated as the ratio of the weight increase of the ear sections, according to the following formula:

$$\begin{aligned} \text{Inhibitory effect (IE\%)} &= \frac{L - R}{L - C} \times 100 \\ &= \frac{L - R}{L - (L/2.41^*)} \times 100 \end{aligned}$$

Where L – weight of left ear which is treated with TPA only  
 R – weight of right ear which is treated TPA plus tested extract  
 C – calculated weight of untreated ear (\*-C is calculated weight. It had been found that treating a normal ear with 0.5µg TPA resulted in a 2.41 times increase in weight of the ear)

## RESULTS

### Anti-inflammatory Activities

TPA induced-mouse ear oedema assay was used to determine the percentage of inhibition for anti-inflammatory activities. The initial crude ethanolic extract of leaf showed an inhibitory activity of 54.1%. Further fractionation of the leaf extract revealed that the inhibitory activity was distributed over the dichloromethane (72.2%), ethyl-acetate (23.5%) and aqueous fraction (3.3%) respectively (Table 1).

**Cytotoxicity Evaluations**

SRB assay was used to determine the percentage of cell viability for cytotoxicity evaluations. Percentage of cell viability were analysed as:

$$\frac{OD_{492nm} \text{ reading of treated cells}}{OD_{492nm} \text{ reading of controlled cells}} \times 100$$

The graph of percentage of cell viability versus final concentrations of extracts were plotted to obtain the concentration at 50% cell populations death (IC<sub>50</sub> values). The IC<sub>50</sub> values were used as bench mark to evaluate the cytotoxic effects of the extracts. [7] and [8] had denoted that extracts with IC<sub>50</sub> values equal or less than 20 µg/ml has a high cytotoxic potential, whereas, values more than that can be categorised as non-cytotoxic.

From Figure 1, the IC<sub>50</sub> values of *V. negundo* ethanolic and dichloromethane when treated on MCF7 mammary cell line were 82 and 65 µg/ml, respectively and considered as non-cytotoxic to mammary cell line tested. Whereas, *V. negundo* ethyl acetate and aqueous extracts did not inhibit the proliferations of at least 50% cell population, hence, the IC<sub>50</sub> would be more than the highest concentration tested (>100 µg/ml). In comparison, paclitaxel showed very high cytotoxic activity with IC<sub>50</sub> value of 0.05 µg/ml. The toxic effect of paclitaxel on proliferation of MCF-7 (mammary cell lines) had been supported

by other studies [9]. Hence, it had been used for treatment of breast cancer for its toxic effect. An established anti-inflammatory drugs like NSAIDs (non-steroidal anti-inflammatory drugs) will be included in the future studies as comparison to investigate whether the established anti-inflammatory drugs showed significant inhibition during cell proliferations.

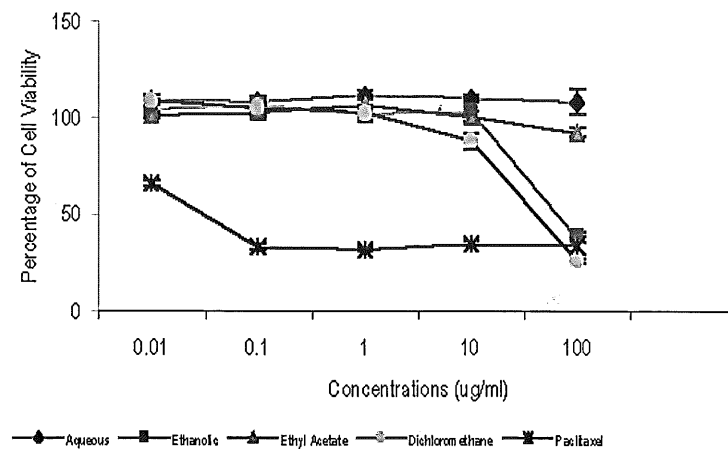
From Figure 2, all *V. negundo* extracts and paclitaxel tested on VERO kidney cell line did not inhibit the proliferations of at least 50% cells population. Hence, the IC<sub>50</sub> values would be more than the highest concentration tested (>100 µg/ml). VERO cell line had been used not only to evaluate the hepato-cytotoxicity of natural products and chemicals [10] but also used for cytophotogenicity evaluations [11].

**CONCLUSION**

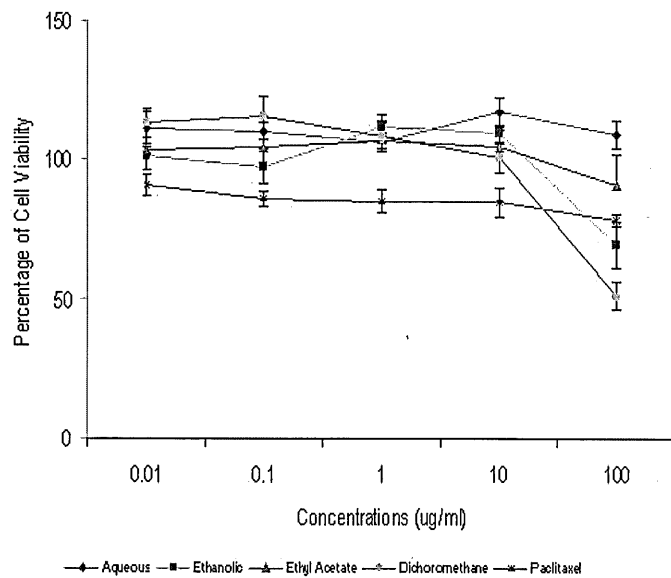
From all the four *V. negundo* extracts tested, only dichloromethane and ethanolic extracts had been scientifically proven to have high anti-inflammatory activities through the TPA-induced-mouse ear oedema assay. These anti-inflammatory extracts did not exert significant cytotoxic effects on both mammary and genitor-urinary cell lines.

**Table 1. Percentage of inhibitions for anti inflammatory activities of *V. negundo* extracts.**

| Sample                    | % inhibition ± SEM |
|---------------------------|--------------------|
| Leaf extract (ethanol)    | 54 ± 3             |
| Dichloromethane fraction  | 72±3               |
| Ethyl-acetate fraction    | 24 ± 2             |
| Aqueous fraction          | 3 ±1               |
| Indomethacin (0.5 mg/ear) | 76 ±7              |



**Figure 1** The effect of *V. negundo* extracts and paclitaxel (positive control) on the proliferation of mammary cell line (MCF-7). Each point represents the mean value (n=3) and the bar represents  $\pm$  S.D. < 5%.



**Figure 2.** The effect of *V. negundo* extracts and paclitaxel (positive control) on the proliferation of genitor urinary cell line (VERO). Each point represents the mean value (n=3) and the bar represents  $\pm$  S.D. < 5%.

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