38th Meeting of National Working Group on Indonesian Medicinal Plant

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The Future of Medicinal Plants:
From Plant to Medicine

Surabaya, 21-22 July 2010

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PROCEEDING OF INTERNATIONAL CONFERENCE ON MEDICINAL PLANTS

in occasion of

the 38th Meeting of National Working Group on Indonesian Medicinal Plant

21-21 July 2010
Surabaya, Indonesia

Editors:

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Kuncoro Foe
Sendy Junedi
Bambang Soekardjo
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Angelica Kresnamurti

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FACULTY OF PHARMACY
WIDYA MANDALA CATHOLIC UNIVERSITY

in collaboration with
National Working Group on Indonesian Medicinal Plants
and German Academic exchange Service

PREFACE

Earth is perfectly made by God for His people to live. It consists of different bodies of land and water where thousands of species of plants and animals can be found. The human race is called to explore this order, to examine it with due care and to make use of it for the benefits of human being. Since very early in human history, people have relied on medicinal plants to cure them of their various ills. This can be partly attributed to the simple yet highly effective forms of traditional medicine. Knowledge of medicinal plants is a part of the Indonesian national heritage known as ***jamu***. To facilitate networking, collaboration, exchange of information, experiences and and knowledge in the key issues of medicinal plants development, the Faculty of Pharmacy of Widya Mandala Catholic University Surabaya in collaboration with National Working Group on Indonesian Medicinal Plants (POKJANAS TOI) and German Academic Exchange Service (DAAD) held the International Conference on Medicinal Plants on 21-22 July 2010 in Surabaya. The conference provided a evaluation in pharmacology, pharmacognosy, ethnobotany, standardization, cultivation, cell culture and chemistry for medicinal and aromatic plant species. There were over 250 participants, 8 plenary speakers, 101 contributed speakers in oral presentation, and 101 posters presented.

The papers contained in the first volume of the proceeding report the submitted papers on 'The Future of Medicinal Plants: From Plant to Medicine'. Keynote speakers and authors of selected contributed oral and poster presentations were given the opportunity to submit a manuscript for publication.

The conference organizers gratefully acknowledge the financial and other support from the following:

National Working Group on Indonesian Medicinal Plants (POKJANAS TOI)
German Academic Exchange Service (DAAD)
PT. Landson
PT. Gujati 59
PT. Pasifik Sarana Cantik
Herbal Plus

I hope that this publication will raise international awareness of the value of medicinal plants in Indonesia and hence makes a contribution towards promoting the proper use of medicinal plants.

Dr.phil.nat. Elisabeth Catherina Widjajakusuma
Conference Chairman

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TOXIC COMPOUNDS EXTRACTED FROM *EUGENIA UNIFLORA* L AGAINST T47D CELL LINE

Nita Supriyati[1], Esti Wahyu Widowati[2]

[1] Medicinal Plant and Traditional Medicine Research and Development Office, Tawangmangu, NIRDH
[2] Faculty of Science and Technology, UIN Sunan Kalijaga, Yogyakrata

Abstract : Breast cancer is a type of cancer most commonly had by women and is the second most frequent cause of death after lung and bronchial cancer. Today, cancer treatment using natural products has attracted more attention as the existing treatments do not provide satisfactory results. Dewandaru (*Eugenia uniflora*.L), also known as the Surinam Cherry, has been reported to have antioxidant activity and can suppress DNA polymerase of EBV (Eipstein-Bar Virus); however, research on its anticancer activity has not been reported. Therefore, the objective of this research is to study the potential application of *E. uniflora* as anticancer and isolate the toxic compound on T47D cell line.

Petroleum ether, dichlormethane and methanol are used to extract *E.uniflora* leaves. The extract was tested for its cytotoxic activity on T47D cell line using MTT method. The toxic compounds were separated using vacuum column chromatography and Preparative Thin Layer Chromatography methods. Cytotoxicity test on T47D cells was performed for extracts of each separation stage. Chemical type identification of the toxic compounds was performed using TLC, UV and IR spectrophotometry analysis.

The results showed that dichlormethane extracts of *E. uniflora* has cytotoxic potential, having IC_{50} value of 96 µg/ml and The IC_{50} values of the compounds was 8 µg/ml. Finally, the isolated compounds are believed to be of triterpenoid group.

Keywords: *Eugenia uniflora* L, toxic compound, T47D

INTRODUCTION

Cancer is a group of diseases of higher multicellular organisms. It is characterized by alterations in the expression of multiple genes, leading to dysregulation of the normal cellular program for cell division and cell differentiation. This results in an imbalance of cell replication and cell death that favors growth of a tumor cell population (Ruddon, 2007). Cancer is a major public health problem in the United States and many other parts of the world. Currently, one in four deaths in the United States is due to cancer.. The three most commonly diagnosed types of cancer among women in 2009 will be cancers of the breast, lung and bronchus, and colon and rectum, accounting for 51% of estimated cancer cases in women. Breast cancer alone is expected to account for 27% of all new cancer cases among women (Jemal et al, 2009)

The ultimate goal of any cancer drug discovery process is to discover and develop effective and non-toxic therapies (Collota, 2008). Cancer treatment using natural products has attracted more attention as the existing treatments do not provide satisfactory results. One of the potential plants is Dewandaru (*Eugenia uniflora* L), also known as the Surinam Cherry

Extracts from *E. uniflora* leaves have been found to show pronounced anti-inflammatory action (Schapoval, *et al.*, 1994), considerable contractile activity, with a resulting effect on intestinal transit (Gbolade, *et al.*, 1996), endothelium-dependent vasorelaxant effects (Wazlawik et al., 1997) and hypotensive effects (Consolini, *et al.*, 1999; Consolini & Sarubbio, 2002), and to inhibit the increase of plasma glucose and triglyceride levels (Arai *et al.*, 1999; Matsumura *et al.*, 2000). Some compounds present in *E. uniflora* leaf extracts have also been shown to inhibit the Epstein– Barr virus, known to be closely associated with nasopharyngeal carcinoma (Lee, *et al.*, 2000), and to have antimicrobial activity (Adebajo, *et al.*, 1989; Holetz et al., 2002) and antifungal activity (Lima, *et al.*, 1993; Souza *et al.*, 2002). However, research on its anticancer activity has not

been reported. Therefore, the objective of this research is to study the potential application of *E. uniflora* as anticancer and isolate the toxic compound on T47D cells.

MATERIALS AND METHODS

Plant and extracts

Fresh leaves of *E. uniflora* were collected from Tawangmangu, Indonesia in August 2008a and was identified at Medicinal Plant and Traditional Medicine Research and Development Office, Tawangmangu. Their powder was macerated with petroleum ether for 24 hours, three times. After filtration the resulting extracts were combined and evaporated to dryness. The residu from petroleum ether was macerated with diclormethane for 24 hours, three times. After filtration the resulting extracts were combined and evaporated to dryness. The residu from diclormethane was macerated with methanol for 24 hours. Supernatant was evaporated to dryness. Each extracts was tested for cytotoxicity test on T47D cell line.

Fractionation

The toxic extract was partitioned with petroleum ether, petroleum ether/chloroform {(9 :1, 8 : 2, 7 : 3, 6 : 4, 5 : 5, 4 : 6, 3 : 7, 2 : 8, 1 : 9) v/v}, chloroform, chloroform/methanol {(9 :1, 8 : 2, 7 : 3, 6 : 4, 5 : 5, 4 : 6, 3 : 7, 2 : 8, 1 : 9) v/v}, and methanol. The chemical composition of each fraction was monitored on thin layer chromatography.The subfractions, were similar on TLC analysis, were combined. Each fraction was tested for cytotoxicity test on T47D cell line.

The toxic fraction were partitioned with petroleum ether, petroleum ether/chloroform {(9 :1, 8 : 2, 7 : 3, 6 : 4, 5 : 5, 4 : 6, 3 : 7, 2 : 8, 1 : 9) v/v}, chloroform, and metanol. The fractions that showed similarity on TLC were combined. Each fraction was tested for cytotoxicity test on T47D cell line.

Preparative thin layer chromatography

The toxic fraction were separated with preparative thin layer chromatography to give two fractions. Each fractions were tested for cytotoxicity test on T47D cell line. Purification of the toxic compound was carried out by preparative thin layer chromatography.

Cytotoxic assay by MTT method

Cell Culture

Human breast cancer cell lines T47D and MCF-7 were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) in an incubator with humidified air with 5% CO2 at 37°C.

Cell viability assay

The viability of the cells was assessed by MTT (3, 4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) (Sigma) assay which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product (Mosmann, 1983). Cells were plated onto 96-well plates (2x103 cells/well) (Iwaki). After 24 h incubation, cells were treated with each extract/fraction with various concentrations for 48 h. Then, MTT solution was added in each well and cells were incubated for 4 h at 37°C and then incubated with 100 µl of solubilization solution at 37°C overnight. The quantity of formazan product was measured by using a spectrophotometric microtiter plate reader (Bio-Rad) at 595 nm wavelength.

Identification of toxic compound

The toxic compound were identified with TLC analysis, UV and IR spectrometry.

RESULTS AND DISCUSSION

Extraction and Cytotoxicity Assay

Maceration of powdered leaf of Dewandaru was conducted using petroleum ether, dichloromethane and methanol. The four extracts was then tested on T47D breast cancer cells

using concentration series as follows: 500, 250, 125, 62,5 dan 31,25 µg/mL. Each treatment was observed after 48 hours incubation. Figure 1 showed a curved of cell viability versus various concentration extract which is used on the treatment. The results indicate that dichloromethane extracts has the smallest IC_{50} value of 96 µg/ml, whereas the IC_{50} for petroleum ether extract and methanol extract was 144 µg /ml and 148 µg/mL respectively.

There were no significant differences among IC_{50} values of petroleum ether extract, dichloromethane extract and methanol extract. This is due to distribution of toxic compounds in each extract. Extraction process by maceration technique enables the toxic compounds can't extracted perfectly. Dichloromethane extract was then chosen for fractionation because of its smallest IC_{50} value.

The portion of dichlormethane extract that showed the strongest cytotoxic activity was partitioned by vacuum liquid chromatography (VLC). The fraction that showed similarity on TLC were combined to give four fractions. Each fractions were tested for cytotoxicity test on T47D cell line. Two fraction had cytotoxic activity (fraction II and III) and the other fraction not toxic. It was found that fraction II had the highest cytotoxic activity with the IC_{50} value of 19 µg/mL. The viability of T47D cells after 48 h treatment showed at figure 1.

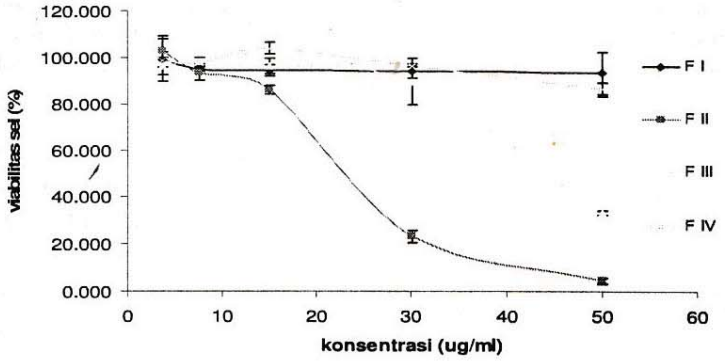

Figure 1. The viability of T47D cells affected fraction of *E. uniflora* dichloromethene extract. FI = combined fraction 1-3; F2 = combined fraction 4-6; FIII = combined fraction 7-9 and FIV = combined fraction 10-21

The most toxic fraction (fraction II) were further were partitioned by VLC using gradient elution. The chemical composition of each fraction was monitored on thin layer chromatography. The fractions that showed similarity on TLC were combined and evaporated given two fraction. At the concentration of 30 µg/ml, citotoxicity activity of F2V2 fraction against T47D cells stronger than F2V1 fraction.

F2V2 fraction were further separated by preparatif thin layer chromatography. The TLC spot were scraped into two portion, upper and lower part, and diluted in mixture chloroform/methanol 4:1. After filtration each fraction were evaporated and tested for cytotoxic activity. It was found that the lower part of PTLC had cytotoxic activity. The toxic compounds were purified by preparatif TLC given two portion upper and lower part. After 48 treatment of 30 µg/ml each portion, it was found that the upper part of preparative TLC had cytotoxic activity with the IC_{50} value of 8 µg/mL.

Furthermore, the purity ot the toxic compound were tested by TLC. It was found that toxic compound had single spot in various eluent (figure 2).

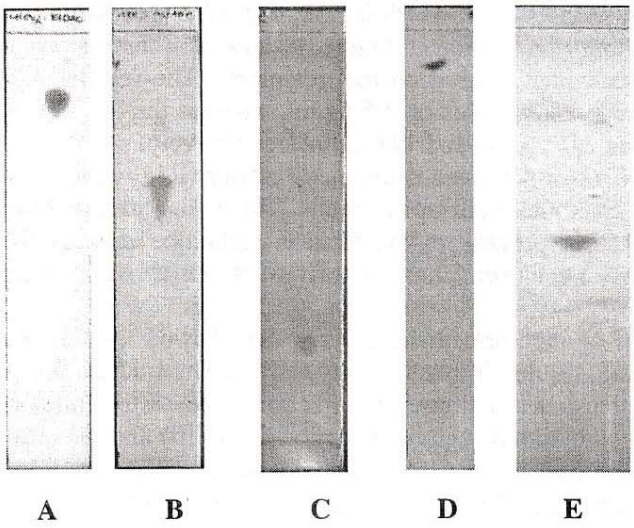

Figure 2. Thin layer Chromatogram of toxic compound from *E. Uniflora* detection : serium (IV) sulphate; adsorbent:silica gel GF_{254} (E-Merck) ; eluen : (A) chloroform : ethyl acetate (2 : 1), (B) n-heksana : acetone (2:1), (C) petroleum ether : diethyl ether (2 : 1), (D) chloroform : acetone (2 : 1), dan (E) n-heksana : ethyl acetate (2 : 1).

Toxic compounds analysis was done using various spray reagents to determine a groups of its compounds (Cannel, 1998). TLC profile showed blue spot after spraying with ammonium molybdate (IV), purple when sprayed with vanillin-sulfuric acid and pink after sprayed with cerium sulfate. All of the result supported a possibility of terpene compound therein. When it was sprayed using dragendorff's reagent, there is no color reaction neither nor fluorescein can be observed. It indicated the absence of alkaloids and lipid (figure 3).

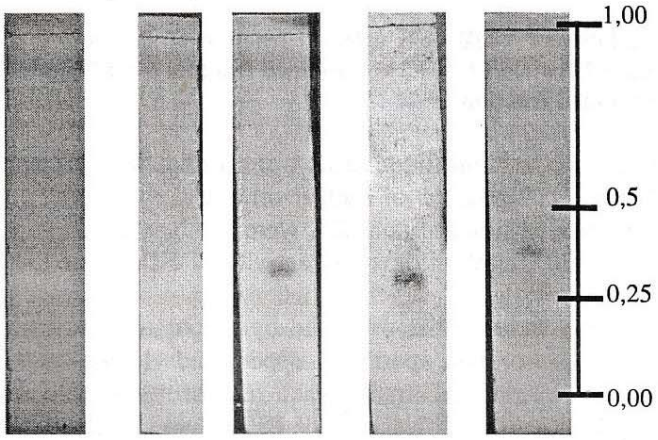

Figure 3. Thin layer cgromatogram of toxic compound from *E. Uniflora*. *Detection :* (1) dragendorff's reagent, (2) sitroborat,(3) vanilin-sulphate (4) antimon chlorida (IV), (5) serium (IV) sulphate; adsorbent : silica gel GF_{254} (E-Merck).

Some literature reported that terpenoid groups had anticancer activity. Salminen *et al* (2008) reported that terpenoid can suppresed *nuclear factor-KB(NF-KB) signaling*; regulator in pathogenesis of inflamation and cancer. Taxol, a diterpen ester is being in the treatment of ovarian and breast cancers, non-small-cell lung cancers, and cancers of the head and neck (Dewick, 2002). Furthermore, identification of toxic compound were determined with UV and IR spectrometry.

Based on UV spectra it can be inferred that toxic compounds against T47D breast

cancer cells have λ max at 215 nm. This result indicates that there is a chromophore group, that is covalently unsaturated group that can absorbs a radiation in ultraviolet and visible regions

Table 1. Detection of toxic compound from *E. uniflora* using various detection reagent spray for TLC (Harborne, 1996; Cannel, 1998)

No	Reagent spray	Chemical group	Interpretation	Result
1	Dragendorff	Alkaloid	Orange spot	-
2	Sitroborat	Flavonoid	Yellow spot	-
3	Vanilin-sulphate	Terpenoid	Brown, violet spot	+
4	Antimon(III) chlorida	Di dan triterpenoid	Red to blue spot	+
5	Serium (IV) sulphate	Terpenoid	Brown, violet spot	+

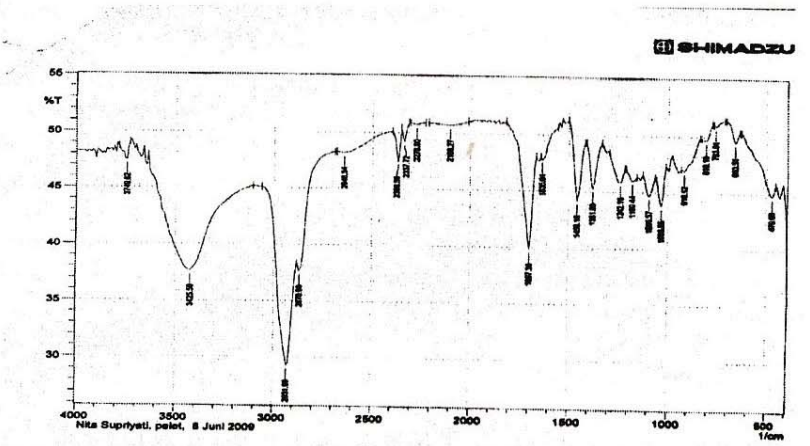

Figure 4. IR spectra (KBr pellet) of toxic compound from *E. uniflora*

Figure 4 shows the spectrum of infrared (IR) of toxic compounds against T47D cells. Absorption at 3425 cm $^{-1}$ originating from the vibrations of the hydroxyl group, OH. Two absorption at 2931 cm $^{-1}$ and 2870 cm $^{-1}$ is typical for symmetric CH stretch vibration and the asymmetric cluster of -CH_3 and-CH_2 -. This absorption was supported by the absorption at 1458 cm $^{-1,}$ and 1381 cm $^{-1}$ which is a CH bending vibration of methylene and methyl group. Absorption at 1695 cm $^{-1}$ is the stretch vibration of carbonyl group, C = O. The absorption at 1072.3 cm $^{-1}$ related to CO stretch vibration. It can be concluded that the toxic compounds have some functional groups OH,-CH_3,-CH_2-, C=O and CO. From the result of TLC and spectroscopy analysis, the toxic compounds was proposed as terpene compound. Terpene compounds were derived from C5 (isoprene units) and combined together through the condensing head to tail. Terpenoids are classified according to the number of C5 units, namely: hemiterpen (the C5), monoterpenes (c10), sesquiterpen (C15), diterpene (C20), sesterterpen (C25), triterpen (C30), tetraterpen (C40) and if more than four units terpene was called as politerpen (Dewick, 2002).

CONCLUSIONS

The dichlormethane extracts of *E. uniflora* has cytotoxic potential, having IC_{50} value of 96 μg/ml. The IC_{50} values of the compounds tested on T47D was 8 μg/ml. The isolated compounds are believed to be of triterpenoid group.

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WIDYA MANDALA CATHOLIC UNIVERSITY SURABAYA
FACULTY OF PHARMACY
Jl. Dinoyo 42-44 Telp. (031) 5678478 Pes. 114 Fax. 5630169 Surabaya – 60265

SURAT KETERANGAN

No : 281/ICoMP/III/10

Panitia International Conference on Medicinal Plants dengan ini menerangkan bahwa makalah dengan:

Judul : Toxic Compunds Extracted from *Eugenia uniflora* L. against T47D Cell Line

Penulis :
1. Nita Supriyati
   Peneliti pada Balai Besar Litbang Tanaman Obat dan Obat Tradisional
   Badan Litbang Kesehatan, Kementerian Kesehatan
2. Esti Wahyu Widowati
   Staf Pengajar Program Studi Kimia Fakultas Sains dan Teknologi
   UIN Sunan Kalijaga Yogyakarta

telah dipresentasikan pada International Conference on Medicinal Plants dengan tema "The Future of Medicinal Plants: From Plant to Medicine" yang diselenggarakan di Universitas Katolik Widya Mandala Surabaya (UKWMS) pada:

Hari, tanggal : Rabu 21 Juli 2010

Waktu : 16.30 – 17.30

Tempat : Plaza Universitas Katolik Widya Mandala lantai 1

Surabaya, 22 Juli 2010

Ketua Panitia

Elisabeth Catherine Widjakusuma