TOTAL FLAVONOID, PHENOLIC CONTENT AND CYTOTOXIC ACTIVITIES TO STUDY THE COMBINATION EFFECT OF TWO MALAYSIAN MEDICINAL PLANTS

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ABSTRACT: *In this regard the herbs involved more appropriate Ficus deltoidea female, male leaves, Gynocthodes sublanceolata and combination for two active extracts (50%) from each one. The present study was conducted to evaluate the antioxidant activity and cytotoxic activities of two medicinal plants individually and in combination. Method Were determined Total phenolic and Flavonoid Content (TPC, TFC) and cytotoxic activities (MTT) assay of two medicinal plants individually and in combination. F. deltoidea female leaves extract were indicated a higher antioxidant activities than G. sublanceolata leaves and male leaves. The highest result with, TPC and TFC for combination extract. Combination which treats cancer cells be more effective than individually. For (MTT) assay in this test was used two cancer lines Breast cancer cell line MCF-7, Human Prostatic cancer cell line PC3 and Human Umbilical vein Endothelial cell line (HUVEC) as normal cell line. The combination revealed the best inhibition on cancer cell lines and lowest effect on normal cell line compared to the one individually. On the other hand the combination showed more morphological effects on cancer cells than two extracts individually and lowest effects on normal cell line. The combination showing increase in the effectiveness between phytochemicals and polyphenols which present in plants when worked together on cancer cells.*

Keywords: *cytotoxic*ity, Flavonoid, Phenol anti-cancer, MTT

1. INTRODUCTION

Female leaves of *F. deltoidea* and *G.sublanceolata* can be used as traditional medicine. Similar to other species of Ficus, most of these plants' parts are used for different medical purposes .*F.deltoidea*, known as "Mas Cotek" of Moraceae family, is one of the famous herbal plants in Malaysia [1]. This plant has two types of leaves: female and male leaves of F.deltoidea. Female leaves were selected due to their higher active ingredients. G. Sublanceolata (Pitang) leaves are known as hail of Rubiaceae family. These leaves turn brown when dried. Pitang leaves are a rare plant; the largest number of these plants can be found in Thailand. The Pigments of this plant are diverse including xanthophyll, chlorophyll, flavonol, flavones, carotene and anthocyanin [2].

Cancer is a serious disease caused by losing the control of normal cell growth due to mutation, as well as a change in a genomic material of cells; for example: DNA and RNA lead to the unbalance of cell proliferation .Hence, cancer treatment has become a major issue as there is no specific remedy that acts on all kinds of cancer. Chemotherapy involves various combinations of toxic drugs applied on living cells is associated with grave side effects and chemo resistance Patients try to avoid such treatments because it is painful and harmful to their bodies and make them weaker .

In recent years, there has been a growing interest in determining medicinal plants and plants used as food that contain abundant antioxidants .The plants studied in this research could clarify antioxidant activities and prevent oxidative stress as well as any illnesses.

The compounds have radical scavenging potential or antioxidant elements called chemo preventive. A large numbers of studies have interpreted the anti-oxidative properties of plant products [3]. These studies indicated that these plants have therapeutic anti-cancer properties. However, in traditional medication, most of these herbal products are usually given as a combination of herbs and not individually. Therefore, the present study aimed to compare the total phenols, flavonoids, antioxidants and cytotoxicity on cancer and normal cell lines for leaves of these plants collaboratively and individually in order to determine their pharmacological properties in these conditions.

2. EXPERIMENTAL DETILES

a. Sample Collection and Extraction

F. deltoidea was collected from a covered greenhouse garden of School of Bioprocess Engineering and G. sublanceolata was collected from Tumpat, Kelantan cleaned through the running tap water to remove debris and contamination. The leaves of F. deltoidea were classified into two groups: male and female plants. Each group was dried at room temperature for two weeks. The dried leaves were grounded. Each 100 gm was then mixed with 200 ml of methanol and distilled water (60:40% v/v) was put in a beaker and covered with aluminum foil at ambient temperature for 24 h and shaken during the extraction time.

The extracts were filtered through Whatman filter paper No.1. Then, the solvent was removed from samples using a rotary evaporator (Switzerland). The weight obtained of the crude leaves extract was 4.2 gm, 3.7gm, 4.5gm of female, male leaves of F.deltoidea and G. sublanceolata respectively. Finally, the extract was placed in airtight amber bottles and stored in a freezer to prevent the oxidation damage until further use.

3. Antioxidant Test

a. Determination of total phenolic content

The total phenolic content (TPC) was specified by spectrophotometry, gallic acid as a standard, followed the method of Singleton and Rossi with a simple deviation [4]. 0.5 mL of the diluted sample extract was added into the tubes containing 1.0 mL of dilution of Folin-Ciocalteu's reagent in water. Ten minutes later, 0.8 mL of a sodium carbonate solution (7.5% w/v) was added to the sample. The tubes were then kept at room temperature for 30 min before absorbance at 743 nm was measured using TECAN Multi-mode micro plate reader Model Infinite® 200 (Mannedorf, Switzerland). The TPC was indicated as gallic acid equivalents at (GAE) in

mg/100 mL; the calibration curve of gallic acid was illustrated in Figure 1. The concentration of polyphenols in samples was taken from a standard curve of gallic acid.

b. Determination of the total Flavonoid content

This method was used for the assessment of the total flavonoids [5]. mixed. 0.5 ml solution of plant extract was mixed with 1 .5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. The mixture was kept at room temperature for30 min. Then, the absorbance of the reaction mixture was measured at 415 nm using spectrophotometer (Perkin Lambda 45). Standard calibration curve was produced using quercetin as a reference standard for this study; the calibration curve of quercetin was shown in Figure 2. Stock solution of quercetin was made by dissolving 10 mg in methanol and transferred to volumetric flask and completed the volume to 10 ml, After that, serial dilution was prepared to make concentration at (10-100 µg/ml) in methanol.

4. Cytotoxicity assay

The MTT cytotoxicity test was done as explained previously [6]. Cells were seeded at 1.5×10^4 cells in each well of 96-well plate in 100 µl of fresh culture medium and left it to attach for overnight. For screening, the cells (70 - 80% confluence) were treated with the extracts individually at 100µg and in combination (1:1) 50 µg from each extract, and then the concentration of (3.1-200) µg /ml was used to gain a dose–response curve, and with IC_{50}, the extracts were tested for cytotoxicity. After 48h of the treatment, the medium was removed, and the cells were exposed to MTT solution prepared at 5 mg/ml in sterile PBS, which was added to each well at 10% v/v in the specific medium and incubated at 37°C in 5% CO_2 for 3 h. The water insoluble formazan salt was solubilized with 200 µl DMSO/well. Absorbance was measured by infinite® Pro200 TECAN Group Ltd., (Switzerland) at a wave length of 570 nm and reference wavelength of 620 nm. Each plate contained the samples, negative control and blank. DMSO (1% v/v) was used as a negative control and control. The assay was performed in quadricate.

Cell line HUVEC (Passage No. 3), catalogue number (C2517A) and Breast cancer cell line MCF-7 (catalogue.

5. RESULTS AND DISCUSSION

The results of antioxidant tests were indicated for the one individually and in combination for *F.deltoidea* (male, female and *G. sublanceolata*. Statistical significant between inhibitions and extract concentrations (20-100) was calculated by using ANOVA showed significant effect represents ($p < 0.05$) two assays.

The total phenolic content of the female and *G. sublanceolata* extracts, the subsequent quantities are explained in Table I, depending on Gallic acid (Fig.1). The female leaves extract contained the highest phenolic content from male of *F.deltoidea and G. sublanceolata* leaves.

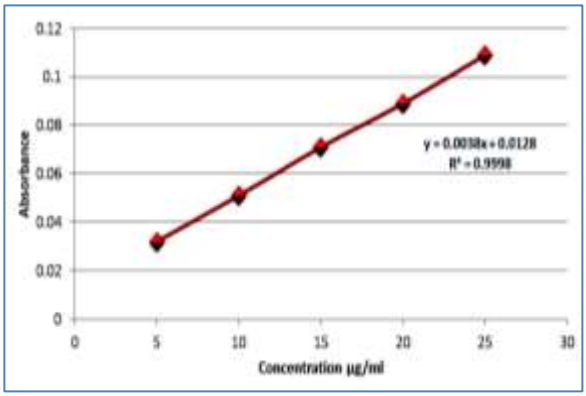

Fig (1) Standard Curve of Gallic Acid

The total flavonoid content of female and *G. sublanceolata* extract was determined and subsequent quantities are illustrated in Table I, depending on Quercetin (Fig.2). The combination obtained the highest TPC and TFC at 170.12µg/mg and 51.19µg/mg.

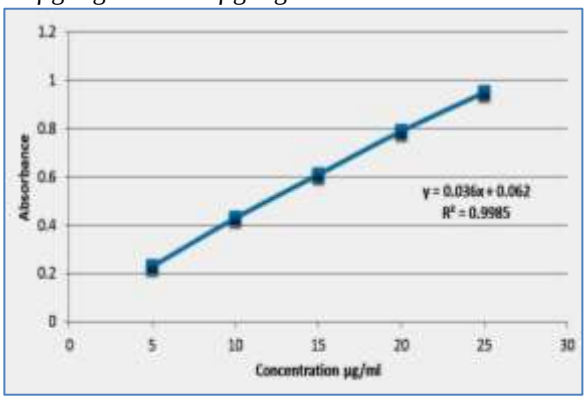

Fig (2) Standard Curve of Quercetin

TPC and TFC for each extract results are shown in Table II. The value of the total phenolic compounds in each extract are calculated by the linear equation of calibration curve: y = 0.0038x + 0.0128, R^2=0.999, and indicated as Gallic acid equivalent in µg /mg of extracts; and the value of the total flavonoid content is calculated by equation: y = 0.036×+ 0.062 R^2 =0.9985.

Table I: Total content of flavonoid and phenolic in female leaves of *F. deltoidea* and *G. sublanceolata* individually and in combination.

Plant extract (µg\ml)	TFC (µg \mg)	TPC (µg \mg)
*F.*female	44.33 ± 0.06	155.33 ± 0.1 5
G.sublanceolata	29.13 ± 0.05	107.12 ± 0.1
Combination	51.19 ± 0.03	170. 12± 0.10

The results were presented as mean ± SD, n =3

The results indicated that combination plants were tested and exhibited a remarkable cytotoxicity activity more each one individually all inhibition on cancer cell lines was enhanced, whereas inhibition on normal cell lines was still less than on cancer cell lines as illustrated in (Fig.3) (Fig.5) and (Fig7). All inhibition at 100µg /ml values showed in (Table II).In addition, the plants showed a significant anti-proliferative effect on human breast (MCF-7) and prostate (PC3) cancer cells individually, but indicated mild-cytotoxic to the tested normal cells. However, for combination, there was no influence on normal cell line, but the best inhibition on cancer cell lines was found. The IC_{50} values were determined for combination. Meanwhile, the IC_{50} values and estimated against PC3, MCF-7 and HUVEC cell lines (Table II).The morphological change on cancer cell lines was clearer when cancer cells were treated in combination (Figure 8d and 9d) compared with those treated individually (Fig.6b,c.7b,c.) Meanwhile, there was no change on normal cells when treated in combination (Fig.8d) but less cytotoxicity when treated individually (Figure 8c and d) compared to negative control DMSO (Fig.8a), which is offensively proliferative.

Table II. . IC_{50} for two plants individually,and in combination (*F.deltoidea* + *G.sublanceolata*) individually and in combination

Cell Lines	IC_{50} µg/ml	IC_{50}µg/ml *combination*
PC3	*138*	*83*
MCF-7	*159*	*95*
HUVEC	*168*	*196*

The results were presented as mean ± SD, n =4

Reactive Nitrogen Species (RNS) and Reactive Oxygen Species (ROS) have a tendency to accumulate originating from the mitochondria within the living systems [7]. Charged molecules are unstable primarily because of the electrical charge they carry as one of the definitions of free radicals. Free radicals have an impact on cell molecules such as DNA, poly saturated fatty acids and proteins damage and cause changes in genetic expression, which also influences cellular metabolism. Noticeably, the poly saturated fatty acids and proteins in cell membranes tend to undergo oxidative damage resulting in the loss of membrane integrity and enzymes function [8]. It should be interpreted that the presence of reactive oxygen species and endogenous antioxidants can be still accumulated within cells. There is an oxidative damage, which causes genetic disturbances to the materials such as DNA, cellular proteins and lipids leading to the development of cancer [9]. Therefore, this research aimed to estimate the cytotoxic activities of these extracts individually and in combination of two medicinal plant leaves extracts. To get photochemical and antioxidant capability, assay was used to evaluate anti-carcinogenic properties of plant extracts named the MTT assay, which provides an uncomplicated method for the determination of cell viability through mitochondrial effectiveness in living cells, and two antioxidant tests were used to determine this ability such as TPC and TFC. The activity of extracts is towards two human cancer cells lines (PC3 and MCF-7) and normal cell line (HUVEC). For the examination, cells were treated with the extracts individually and in combination.

The results of this study indicated that the extracts of *F.deltoidea and G. sublanceolata increased* when used as combination. Which demonstrates selective cytotoxicity towards human breast MCF-7 and prostate cancer PC3 cell lines, whereas less cytotoxic against the normal cells HUVEC was found. However, there was less inhibition on cancer cell lines when used individually. Such a cytotoxic activity occurs because active molecules interact with cancer cell molecules. This technique could kill cancer cells .In monitoring under the digital microscope, properties was spotted including nuclear condensation, loss of pseudopodia-like cellular projections, separated apoptotic bodies and membrane blebbing (Fig.4). In addition, the treated cells showed the vital signs of apoptosis such as chromatin condensation, shrinkage of cells and spaces between cells (Fig.6).

Flavonoids enhance their anticancer effect due to the technique related to cancer development. Flavonoids are considered as effective antioxidant molecules [10]. In the initial phase of disease, Flavonoids prevent metabolic energizing for carcinogenic materials. In the advanced stages, they stimulate apoptosis, prevent cancer cell generation, angiogenesis and development of cancer [11]. Besides that, the antioxidant supplementation can block NF-KB (nuclear factor kappa-light-chain enhancer of activated B cells) activation as well as inhibit NF-KB activity. Since NF-KB is responsible for cancer and inflammatory, thus it indirectly plays an important role in inhibiting cancer and inflammation through mechanisms distinguished from redox regulation. Combinations can help treat cancer or prevent it by the interaction between Phytochemicals in plants. Both of them have an interaction to create a higher anti-cancer activity more than the one works individually .The combination of two or more Phenolics compounds has shown the signs of anticancer activities. *Roxburghii Tratt* and *Fagopyrum cymosum* are considered as Chinese medicines used for improving persons' immune system and their ability to digest. These medicines act as anti-aging agents in some cases [12].

Therefore, research intensive on studying phenolics and flavonoids which are naturally accumulated in plants to explain the ability of these compounds to minimize the generation of reactive oxygen species (ROS) in the biological system. ROS enhance growth of cancer through the uncontrolled proliferation of cancerous cells, DNA mutations and change in gene expression. Moreover, the elevation in ROS levels is spotted in the number of cancer cells. Mentioned that ROS mission is to open the signaling molecules to promote various abnormal growths such as angiogenesis and tumorigenesis [13]. Studies showed an important role of phenolics and flavonoid inhibition and cytotoxic effect on prostate cancer when they influence the vascular endothelial growth factor (VEGF) which is responsible for new angiogenesis. Flavonoid has inhibition on cancer cell lines influencing the replication of DNA stops it and Bcl-2 gene inhibition. Plants have various phytochemical properties depending on an exporter of normal antioxidants such as diterpenes, flavonoids, polyphenolic acids and tannins [14]. In this view, this study was undertaken to show the cytotoxic effect and antioxidant activities of female leaves of *F.deltoidea* and *G. sublanceolata* leaves extracts prepared

to use it in combination to evaluate and investigate its cytotoxic effects on breast and prostate human adenocarcinoma. The combination of two or more active compounds for curing may be useful for decreasing the toxic and adverting the influence of cancer. The cure effect has sufficient results such as enhancing the effectiveness and the need for minimizing concentrations at the same or increasing the level of efficacy, as well as reducing unfavorable actions during the treatment.

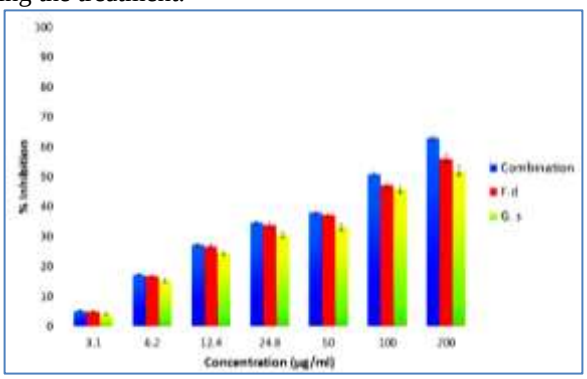

Fig (3) Comparative inhibitions for different extracts on MCF-7

The statistical analysis of variances by ANOVA proved there are difference between concentrations of the extracts and the inhibition of the cells, where the significant variance (P) value was less than the standard value 0.05 ($P \leq 0.05$). The results for all extracts at all concentrations on MCF-7 cancer cell line individually and in combination which have higher inhibition. Combination showed significant differences between inhibitions with each extract individually in some concentrations and illustrated significant differences between inhibitions with each extract individually of *F. deltoidea* and *G. sublanceolata*.

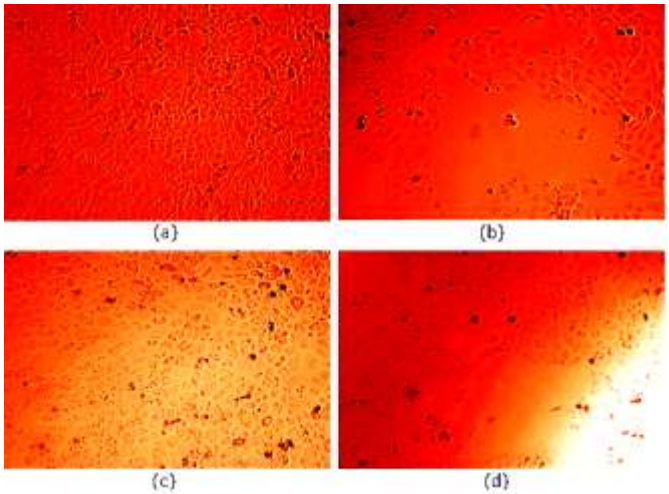

Fig (4) MCF-7 Cancer Cell line. (a) Negative Control, (b) *F. deltoidea*, (c) *G. sublanceolata*, (d) combination of b+c. The results were presented as mean ± SD, n =4

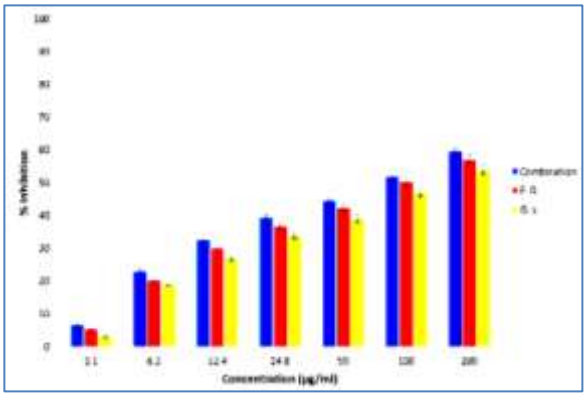

Fig (5) Comparative inhibitions for different extracts on PC3

The results for all extracts at all concentrations on PC3 cancer cell line individually and in combination which have higher inhibition and significant differences between inhibitions with each extract individually.

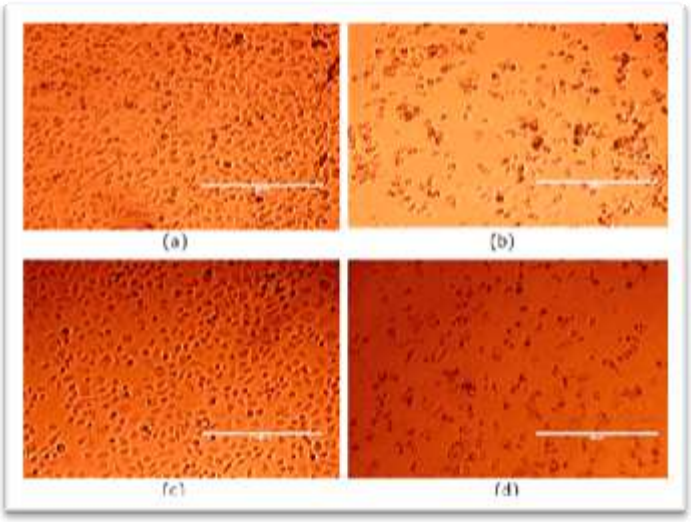

Fig (6) PC3 Cancer Cell line, (a) Negative Control, (b*) F. deltoidea*, (c) *G. sublanceolata*, (d) combination of b+c. The results were presented as mean ± SD, n =4

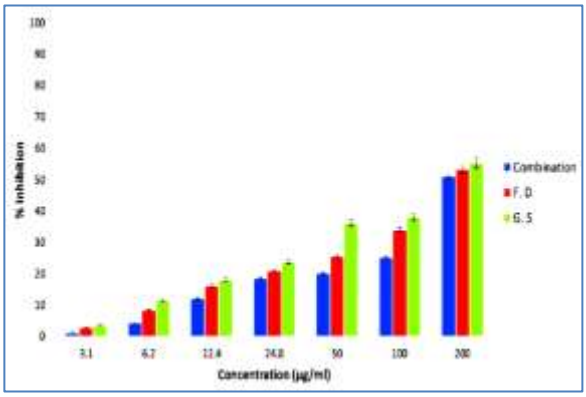

Fig (7) Comparative inhibitions for different extracts on HUVEC

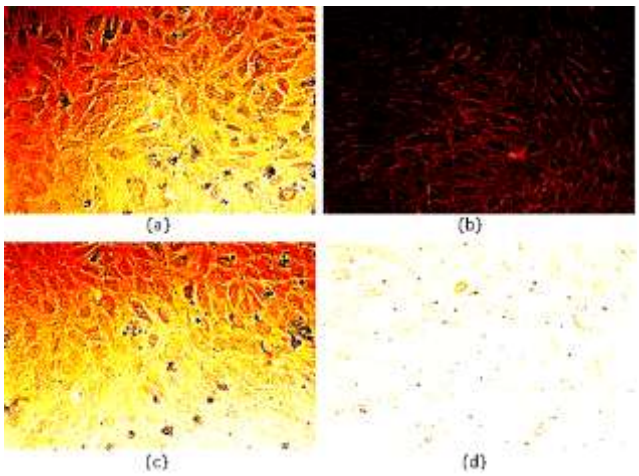

Fig (8) HUVEC Normal Cell line .(a) Negative Control, (b) *F. deltoidea,* (c) *G.sublanceolata,* (d) Combination of b+c

In control wells, the inhabitance of the cells was significantly more than that of the treated wells ($p < 0.05$, $p < 0.01$) The graphical performance clarify the percentage of plating efficiencies after the treatment of the cells with extract in comparison with negative control. The results were presented as mean ± SD, n =4.

The pictures of cancer cells (PC3 and MCF-7) and normal (HUVEC) cell line under an inverted phase-contrast microscope at × 100 enlargement with a digital camera for 48 h after the treatment with the extracts in individual and combination at 100 µg\ml on cells. The treatment with the extracts individually showed a moderate reduction in the density of cell-inhabitants. Besides that, treating with the extracts does not make an important cytotoxicity towards normal cells (HUVEC) compared to negative control DMSO.

The pictures of cells treated with extract as combination showed spaces between the cells and suffered from morphological changes in their cellular properties. Treatment caused cells to lose the pseudopodial like membrane projections. Clearly, the features of apoptosis such as the membrane blebbing, nuclear thickness and apoptotic bodies called apo bodies in the treated cancer cells are clearer than those in individual.

6. CONCLUSIONS

The results of this study showed combination have higher Antioxidant, TPC, TFC and Cytotoxic activities against PC3 and MCF-7 than individual extract. These results consistency with the botanical uses of these plants to protect the body from oxidative stress. Curing these types of cancer and the protectiveness for normal cells which is influenced by all the traditional treatments for cancer. On the other hand, further research needs to separate the effective ingredients and searches for the mechanisms of this combination compound in vivo model against pathological malignant neoplasm.

7. REFERANCE

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