การตรวจสอบฤทธิ์ทางชีววิทยาของส่วนสกัดจาก *Colocasia gigantea*

นายอภิชัย พรประเสริฐผล

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BIOLOGICAL ACTIVITY INVESTIGATION OF *COLOCASIA GIGANTEA* EXTRACTED FRACTIONS

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ในการตรวจสอบฤทธิ์ทางชีววิทยาของสารสกัดจากต้นบอนคูน (*Colocasia gigantea*) วงศ์ Araceae แบ่งเป็น 3 ส่วนคือ การออกแบบระบบตัวทำละลายที่เหมาะสมในการสกัดสารประกอบในกลุ่มฟีโนลิกจากต้นบอนคูน ,ฤทธิ์การต้านเชื้อจุลชีพ และฤทธิ์ความเป็นพิษต่อเซลล์ของสารสกัดจากต้นบอนคูน ในส่วนของการศึกษาการออกแบบตัวทำละลายในการสกัดสารประกอบกลุ่มฟีโนลิกของต้นบอนคูนนั้นพบว่า ตัวทำละลายผสมระหว่างเมธานอลกับน้ำนั้น ค่าการละลายที่เหมาะสมในการสกัดสารประกอบกลุ่มฟีโนลิกจากต้นบอนคูนในส่วนของลำต้นใต้ดินและใบคือ 33.6 และ 42.5 $MPa^{0.5}$ ตามลำดับ และในส่วนของการศึกษาฤทธิ์ในการต้านเชื้อจุลชีพศึกษาโดยวิธี disc diffusion method พบว่าสารสกัดที่ได้จากส่วนของใบนั้น มีความสามารถในการยับยั้งเชื้อ *Escherichia coli*, *Bacillus subtilis* และ *Staphylococcus aureus* ได้ดีกว่าสารสกัดที่ได้จากส่วนของลำต้นใต้ดิน และในส่วนของฤทธิ์ความเป็นพิษต่อเซลล์นั้นได้ทำการศึกษากับเซลล์มะเร็งปากมดลูก (Hela), มะเร็งผิวหนัง (A375) และเม็ดเลือดขาวปกติของมนุษย์ จากผลการทดลอง สารสกัดที่ได้ในส่วนของใบโดยเฉพาะสารสกัดที่ได้โดยใช้ไดคลอโรมีเทนเป็นตัวทำละลายในการสกัดนั้น เร่งการเจริญเติบโตของเซลล์มะเร็งทั้งสองชนิด แต่ไม่เร่งการเจริญเติบโตเซลล์เม็ดเลือดขาวปกติ ในทางตรงข้ามสารสกัดที่ได้จากส่วนลำต้นใต้ดินโดยใช้เฮกเซนเป็นตัวทำละลายในการสกัดนั้น พบว่ามีความเป็นพิษต่อเซลล์มะเร็งทั้งสองชนิด และเร่งการเจริญเติบโตเซลล์เม็ดเลือดขาวปกติ

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APICHAI PORNPRASERTPOL: BIOLOGICAL ACTIVITY INVESTIGATION OF *COLOCASIA GIGANTEA* EXTRACTED FRACTIONS. ADVISOR: CHUTIMON SATIRAPIPATHKUL, Ph.D., CO-ADVISOR: AMORNPUN SEREEMASPUN, M.D., Ph.D., SUCHADA SUKRONG, Ph.D., 89 pp.

In order to supply new information on the biological activity of *Colocasia gigantea* (Araceae) extract, This study investigates the optimal solvent system for phenolic compounds extraction, antimicrobial activity and cytotoxicity of *C. gigantea*'s extract. The methanol-water binary solvents of varying methanol fractions were used for phenolic compounds extraction. The optimal extraction of the solvent can be described by the solvent polarity represented by the solubility parameter. The optimal condition for the solvent to extract total phenolic compounds from *C. gigantea* was predicted at solubility parameter 33.6 and 42.5 $MPa^{0.5}$ in the tuber part and leaf respectively. The antimicrobial activity of *C. gigantea* was determined by disc diffusion method. All extracts from leaf of *C. gigantea* were found to be more effective than extracts from tuber against *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 25923. In the study of cytotoxicity, we investigated cytotoxicity of *C. gigantea* extract on cervical cancer (Hela), melanoma (A375) and human white blood cell (WBC) *in vitro*. The experimental results showed that not at all part of *C. gigantea* had cytotoxicity on Hela and A375 cells whereas some of *C. gigantea* extract from leaf (especially used dichloromethane as a solvent) could promote Hela and A375 cells proliferation significantly. Additionally, only the extract (used n-hexane as a solvent) from the tuber had cytotoxicity on Hela and A375 cells and also enhanced WBC cells proliferation.

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List of Abbreviations

α	alpha
ASTM	American Society of Testing and Materials
ATCC	American Type Culture Collection
BAY32-5915	8-hydroxyquinoline-2-carboxylic acid
β	beta
CC	column chromatography
^{o}C	degree Celsius
cm	centimeter
DMEM	modified Eagle's medium
DNA	deoxyribonucleic acid
DOX	doxorubicin, adriamycin
ED_{90}	dose of a therapeutic agent that eradicates 90% of the target pathogen.
FCC	flat column chromatography
GC-MS	gas chromatography–mass spectrometry
g	gram
His+	polyhistidine-tag
IC_{50}	half maximal inhibitory concentration
IKKα	inhibitor of nuclear factor kappa-B kinase subunit alpha
IKKβ	inhibitor of nuclear factor kappa-B kinase subunit beta
kDa	kilodalton
KINK-1	inhibitors of IKKβ
LPS	lipopolysaccharide
mRNA	messenger ribonucleic acid
mg	milligram
MTT	colorimetric assay
µg	microgram
µl	microliter

μM	micromolar
mm	millimeter
MTCC	microbial type culture collection
min	minute
ml	milliliter
MPa	megapascal
MIC	minimum inhibitory concentration
NF-κB	nuclear Factor-Kappa B
NCIM	national collection of industrial microorganism
No.	number
nm	nanometer
PBS	phosphate buffered saline
PGE_2	prostaglandin E_2
ROS	radical by reactive oxygen species
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
R^2 (predicted)	correlation coefficient between the observed and modeled data values
TLC	thin layer chromatography
Trp-P-1	3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole
UV	ultraviolet

Chapter I

Introduction

Human know the benefits from plants and use them in many applications of food, seasoning, beverage, medicine, cosmetic, dye, household and energy for a long time (Prajapati et al., 2011). Traditional medicine from nature has been important in the global healthcare as well. They have studied in many functions such as promote health, prophylaxis, antioxidant, anticancer, antitumor, antipathogenic bacteria, antipathogenic parasite and other pharmacologically active compounds (Jang et al., 1997). Several articles all over the world are the best evidences to show that the traditional medicine from plants have used for development the quality of life and treatment any diseases with their natural bioactive compounds (Prajapati et al., 2011). Natural bioactive compound is refer to secondary metabolite organic substance. It is not produced for normal growth, metabolic activity and survival like primary metabolite but for defense itself in their living environment (Jason, 2005).

Plant is a huge source of natural bioactive compounds. Various natural bioactive compounds have cytotoxic activity effect to organisms and have anticancer activity potential (Nassr-Allah et al., 2009; Bonham et al., 2002; Hu et al., 2002; Lee et al. 2002; El-Shemy et al. 2007). Natural products can treat several diseases with their natural bioactive compounds such as penicillin is used for antibiotic medicine(Jang et al., 1997; Nassr-Allah et al., 2009). Many of natural sources are selected for investigation anticancer activity. For the clinical therapeutics, cytotoxic activity must not harmful to the patient (Jang et al., 1997). The bioactive compound toxicity must specify to the target such as cancer cells and safety for the patient (Jang et al., 1997).

Many of plant bioactive compounds, phenolic compounds are generally found in plants (Fu et al., 2012). Phenolic compounds such as phenolic acids and flavonoids are the common phytochemical compounds in plants that have strong antioxidant and free radical scavenging capacity (Fu et al., 2011; Javanmardi et al., 2003). Free radical by reactive oxygen species (ROS) and oxidative stress are the cause of ageing, cancer and many diseases (Azizova, 2002; Young et al., 2001). Phenolic compounds are considered to be a high potential substances for treatment and defense ROS and oxidative stress (Kahkonen et al., 2001; Robards et al., 1999). Nowadays, many articles report about total phenolic contents in natural products (Fu et al., 2011; Javanmardi et al., 2003; Cai et al. 2004; Gan et al. 2010). They are the best evidences to show the important of phenolic compounds and phenolic compounds's potency.

At the present time, phenolic compounds are interested in functional foods and used in medicine, cosmetic and supplementary food for promote health by many companies. The effective extraction process are required to

extract bioactive compounds from plants (Kim et al., 2007). Generally, bioactive compounds in plants are extracted by various solvents depend on their solubility in solvents (Kim et al., 2007). For example, n-hexane is used for extraction low polarity bioactive compounds while methanol is regularly used for extraction various bioactive compounds. The important role in extraction process is a solvent polarity. The suitable solvent polarity for specific bioactive compounds can improve extraction process to get high efficiency. Although, many articles have already studies about suitable solvent for extraction specific bioactive compound groups (Kim et al., 2007). For example, methanol was the best solvent among water, ethanol, chloroform and n-hexane for extraction the bioactive ingredents from *Quercus infectoria* galls for purpose of antimicrobial activity against a wide variety of pathogenic bacteria (Satirapipathkul et al., 2011). Although, above previous example showed the suitable solvent for extraction the bioactive ingredents from *Quercus infectoria* galls but above suitable solvent was found by trials and no scientific method for investigation the optimal extraction solvent system for specific bioactive compounds. The previous example also implied that the extraction process efficiency based on suitable solvent system polarity.

Many of pathogenic bacteria species such as *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus epidermidis*, *Pseudomonas testosterone* and *Klebsiella pneumonia* are cause of many infectious diseases. Potential of plants antimicrobial activity should be tested against an appropriate microbial model to investigate their activity (Singh, 2011).

Cancer is a main health crisis problem for people in many parts of the world (Jang et al., 1997; Rebecca et al., 2011). Rebecca and co-workers reported that cancer is the major 1 in 4 deaths of people in the United States (Rebecca et al., 2011). In 2011, the data from the National Center for Health Statistics was reported that 1,596,670 new cancer case patients and 571,950 died from cancer in the United States (Rebecca et al., 2011). National Cancer institute, Department of medical services, Ministry of public health, Thailand reported that there were 3,314 all new cancer patients in Thailand in 2009 (Attasara et al., 2010). Cancer is a disease of uncontrollable cells fission, forming to malignant tumors and invade the normal human body system (Jang et al., 1997; Anand et al., 2008). Human can have a cancer in many causes. Many factors can increase chance to have a cancer for example; environmental pollutant, radiation, stress, obesity and lack of diet (Anand et al., 2008). Cancer patients are obtained painfulness, suffering and gloominess by cancer attack. Although, cancer can treat with anticancer medicines called as a chemotherapy but anticancer medicines are not suitable for a long term therapy. Side effect from anticancer medicines is injurious to the patient. Cancers may have more refractory to present anticancer medicines (Jang et al., 1997).

Selection of plant depended on application of traditional medicines and easily provided in Thailand. *Colocasia gigantea Hook. f.* was selected. *C. gigantea* is belonging to family Araceae (Figure 1) as same as *Colocasia*

Table 1: *Colocasia gigantea* scientific classification (Brown, 2000)

	Scientific classification
Kingdom	Plantae (plants)
Phylum	Magnoliophyta (flowering plants)
Class	Liliopsida
Order	Arales
Family	Araceae (arum family)
Genus	*Colocasia*
Specie	*Colocasia gigantea*

esculenta (*C. esculenta*, Taro). It is about 110 genera and 2500 species. Araceae's family plants spread generally in subtropics and tropical region of the world (Ara et al., 2006). *C. gigantea* is found commonly in humid moist climate region, upland, swamp and bilateral stream banks (Harley, 2011). It was classified in table 1 (Brown, 2000). It has many common name such as; giant taro, Egyptian lily, elephant's ear and giant alocasia (English) (Harley, 2011); biga, aba, aba-aba, badiang (Philippines) (Harley, 2011); salad kachu (Bangladesh) (Ara et al., 2006) and koon (Thailand) (Laohabutr, 2000). It spreads commonly in Thailand and other Southeast Asia countries (Harley, 2011). Although, there are many reports highlighting biological activities of the phytochemicals in *C. esculenta*, there are few reports on *C. gigantea*'s biological activities and its bioactive ingredients.

Figure 1: *Colocasia gigantea* (เพ็ญพิชญา เตียว, 2012)

Research Questions

- What is the suitable solvent system for extraction phenolic compounds from *Colocasia gigantea* ?
- Do the extracts from various solvents differ in their bioactivities of *C. gigantea* ?
- If the *C. gigantea*'s extracts from various solvent polarities had cytotoxic activity on primary cell line and continuous cell line *in vitro*, which fractions or bioactive compounds would have cytotoxic effect to both cell lines *in vitro* ?

The aim of the study

- Evaluate total phenolic content and design the optimal solvent system for extraction phenolic compounds from *C. gigantea*.
- Screening of *C. gigantea*'s extracts from various solvent polarities for potential antimicrobial activity against select test microorganisms.
- Screening of *C. gigantea*'s extracts from various solvent polarities for potential cytotoxicity against select primary cell line and continuous cell line *in vitro*.
- Bioassay guided partial purification or isolation of *C. gigantea*'s extracts which exhibit cytotoxic to select primary cell line and continuous cell line *in vitro*.

Expected Benefits

— - Supply new information about total phenolics contain in *C. gigantea* and optimal solvent system for total phenolics extraction.

— - New knowledge of *C. gigantea* active fraction for antimicrobial and cytotoxic properties .

— - Provide an alternative for development of cancer treatment by chemotherapy.

Chapter II

Review of Literature

2.1 Background of *Colocasia gigantea*

Colocasia gigantea morphology was reported by Ara and Hassan, 2006 (Figure 2) and that could be described as following:

"Perennial, evergreen herbs with stout short above-ground stem, creeping to decumbent, clothed with marcescent leaf bases, 20-50 cm long, 4-6 cm diam., stolons 2-4, trailing horizontally, branching, thin, pale green, 30-40 cm long, 0.4-0.5 cm diam. Leaves several together; petiole light green, pruinose, 80-120 cm long, lower half of the length sheathing; blade ovate-cordate, apex short acuminate, undulate along the margin, base deeply cordate, sinus open, peltate, 50-58 cm long, 30-52 cm wide, green or pale green above, glaucous below; primary lateral veins 6-7 pairs. Inflorescences 5-8 in each axil of leaves. Peduncle cylindric, shorter than petiole, 30-54 cm long, 1-2 cm diam, each one with a membranous cataphyll, nearly equalling the length of peduncle. Spathe white, oblong, distinctly constricted, 12-24 cm long, tube light green, ellipsoid, inrolled, 3.5-5 cm long; limb white, erect, 8.5-19 cm long, boat-shaped, 3-5.5 cm diam., deciduous. Spadix sessile, shorter than spathe, 9-20 cm long, female portion yellow, conic, 1.5-2 cm long, 1.5-2 diam.; ovaries numerous, narrow, ovules scattered, parietal placentation; style distinct but very short, less than 0.5 mm long; stigma light yellow, 2 mm diam.; sterile portion slendar, 3-4.5 cm long; male portion 5-14 cm long, 1.1 cm diam.; appendix very short, acute, 1-5 mm long, surface slightly and irrregularly rugose. Flowers unisexual, naked; berry oblong, 5 mm; seeds many, spindle-shaped, with many distinct longitudinal striae. Flowering and fruiting time: April to September. Flowering of the plant is not an annual event rather it takes an interval of several (6 to 7) years."

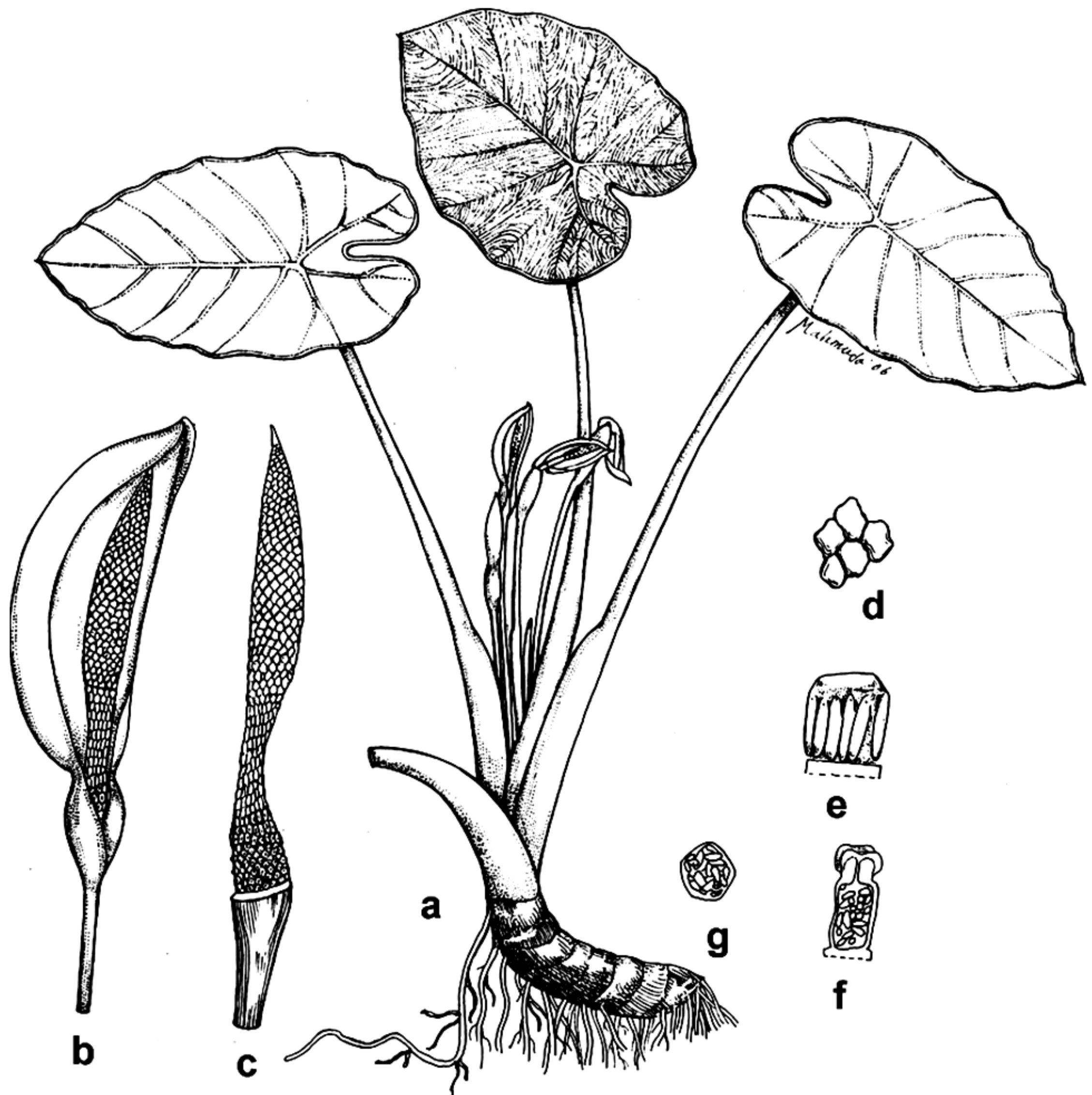


Figure 2: Sketch figure of *Colocasia gigantea*, (a) Habit sketch (×0.9); (b) Inflorescence (×0.5); (c) Spadix (×0.5); (d) Synandria (×3); (e) Synandrium side view (×8); (f) Longitudinal section of gynoecium (×4); (g) Transverse section of gynoecium (×4) (Ara and Hassan, 2006).

2.1.1 Advantages and application of *Colocasia gigantea*

Colocasia gigantea has been considered a minor food crop in Thailand mainly utilized as a stem vegetable. *C. gigantea*'s stem is usually used for making homemade Thai food called "Bon curry" (Figure 3) (รัตนา, 2546).

In Thai traditional medicine, Tuber is heated over a fire. It is use to reduce "internal heat", fever and also for "drowsy" symptom treatment. Fresh tuber can cure stomach problems, pus and wounds. In the northern part of Thailand, fresh or dried tuber is used for phlegm treatment by mixing it with honey (ดวงจันทร์, 2549).

Figure 3: Bon curry (รัตนา, 2546)

2.2 Bioactivities of the *Colocasia* genus

2.2.1 Antimicrobial activity

Both of chloroform and methanolic extracts from leaf of *Colocasia esulenta* showed inhibitory activity against *Staphylococcus aureus* NCIM 2079, *Bacillus subtilis* NCIM 2063, *Escherichia coli* NCIM 2065, *Pseudomonas aerugenosa* NCIM 2036 (Kubde et al., 2010). *Klebsiella pneumoniae* NCIM 2719 was inhibited by methanolic extract (Nair et al., 2005).

Aqueous extract from leaf of *C. esulenta* showed inhibitory activity against *Citrobacter freundii*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio harveyi*, *Vibrio vulnificus*, *Vibrio cholera* (Wei et al., 2008), *Streptococcus mutans* (MTCC-890) and *Pseudomonas fragi* (MTCC-2458) (Singh et al., 2011).

2.2.2 Antimutagenic activity

Nakahara et al., 2002 investigated *in vitro* antimutagenic activity in stem of *Colocasia gigantea* and *Colocasia esculenta*. Crude extract using 80% methanol in aqueous as a solvent was tested antimutagenic effect against Trp-P-1 in *Salmonella typhimurium* TA98 (Nakahara et al., 2002). The activity was tested using the formula defined by Kanazawa et al., 1995 and calculated by decrease number of His+ revertant by extract (Nakahara et al., 2002). The result showed that ED_{90} values (amount of plant extract which suppressed 90% of the mutagenesis) were higher than 50 µl/plate (approximate more than 0.10 mg of dry plant material equivalent) both *C. gigantea* and *C. esculenta* (Nakahara et al., 2002).

In 2011, Tangkanakul et al., 2011 evaluated *in vitro* antimutagenic activity of giant taro stem of *C. gigantea* and use method to determine as same as Nakahara et al., 2002 but converted solvent of extraction process to 100% methanol. The result showed 40% inhibition of antimutagenic activity on 100% methanol extract of giant taro stem of *C. gigantea*. Tangkanakul et al., 2011 explained on their experiment result that antimutagenicity depended on type of plant. Authors claimed report of Bu-Abbas et al., 1994; Luthria and Mukhopadhyay, 2006 that chlorogenic acid (dominant phenolic component, accounting for more than 75% of pea eggplant total phenolic content) was a major component of antimutagenic agent. Chlorogenic acid was implied the major bioactive antimutagenic component of plant.

2.2.3 Antimetastatic activity

Namita et al., 2012 reported bioactive compounds derived from *C. esculenta* (taro) that mentioned potently and specifically inhibition tumor metastasis. Water-soluble extract from root of *C. esculenta* restrained *in vivo* murine lung-colonizing ability and spontaneous metastasis from mammary gland-implanted tumors. Bioactive components were isolated from root of *C. esculenta*. Isolation compound's structures were similar to three taro proteins: 12-kDa storage protein, tarin and taro lectin. The extract cyctotoxicity effected moderately on breast and prostate cancer cell lines. Cell morphology was changed after treat with extract. Moreover, authors found out that extract could blocked cancer cell relocation and also inhibited prostaglandin E_2 (PGE_2) synthesis and downregulated cyclooxygenase 1 and 2 mRNA expression.

2.2.4 Anticancer activity

Brown et al., 2005 published *in vitro* anticancer activity of *Colocasia esculenta* on colonic adenocarcinoma cells. In the experiment, rat YYT colon cancer cell lines were incubated with soluble extract of *C. esculenta* (used phosphate buffered saline (PBS) at 0.1 g/ml as a solvent) at concentration 100 mg/ml for 2 days. The result showed that extract from *C. esculenta* had ability to antiproliferative activity against the rat YYT colon cancer cell line. The study of ^{3}H-thymidine incorporation clearly showed that extract inhibited the proliferation of YYT colon cancer cells in a concentration-dependent. The experimenter observed that the optimal suppression of these cancer cells growth occurred when 25% concentration was used. In addition, extract from *C. esculenta* induced apoptosis on YYT colon cancer cells and not simply toxic to all cells but even had a positive immunostimulatory role. Researcher suggested two mechanisms for anti YYT colon cancer cell activity of *C. esculenta*. First, by induced apoptosis within colon cancer cells and second, by non-specifically activated lymphocytes, which in turn could lyse cancerous cells. Authors referred Cambie and Ferguson, 2003 report to demonstrated bioactive compounds in *C. esculenta* for chemoprotection and anticancer activity. The tuber of *C. esculenta* contains anthocyanins, cyanidin 3-glucoside, pelargonidin 3-glucoside and cyanidin 3-rhamnoside. These compounds have antioxidant and anti-inflammatory ability which can protect the intestine from carcinogens Cambie and Ferguson, 2003. *Colocasia esculenta* contains a specific antioxidative cancer preventative potential; nicotinamide, adenine and dinucleotideoxidase (Marteau et al., 2001). Cichewicz et al., 2004 described which these knew antioxidant substances could be working in tandem with yet unidentified anti tumor substances in *C. esulenta* to yield a greater effect to tumor cells than otherwise possible with an anti tumor agent alone.

Nassr-Allah et al., 2009 noticed *in vitro* anticancer effect against acute myeloid leukemia (AML) and acute lymphocyte leukemia (ALL) and *in vivo* against acute Ehrlich ascites carcinoma cells (EACC) by extract from

Colocasia antiguorum (taro). The result of *in vitro* cytotoxicity analysis 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay of mitochondrial dehydrogenases indicated cytotoxicity effect on AML and ALL cells by *C. antiguorum*'s extract. The extract (using cold water as a solvent) induced ALL cell death by 85% at concentration 200 µg and AML cell death by 67% at concentration 200 µg/ml for 24 hr incubation respectively. When changed extraction condition (low temperature to high temperature), the experimenter observed that the extract (using hot water as a solvent) induced ALL cells death by 66% at concentration 200 µg and AML cells death by 81% at concentration 200 µg/ml for 24 hr incubation respectively. Writers mentioned that polar organic solvents such as phenolic compounds, most glycosides and many types of tannins were the major bioactive substances for cause of leukemia and carcinoma cells death (El-Shemy et al, 2007; Bravo, 1998). Extract from *C. antiguorum* also less cytotoxic on non-Hodgkin's lymphomas (normal bone marrow cells) and induced cells death only 8% (cold water) and 10% (hot water) at 20 µg concentration respectively (Nassr-Allah et al., 2009). In this article reported suggested that hot water extract of *C. antiguorum* possibly had an immuno-modulatory potential via stimulating antiproliferation of tumor cells and significantly obstructed growth both *in vitro* and *in vivo* of AML, ALL and EACC cells (Nassr-Allah et al., 2009).

Sooklert, 2010 evaluated *in vitro* cytotoxic effect of tuber of *Colocasia gigantea* extract on melanoma A375 cell line by using 95% ethanol as a solvent and MTT assay for cell viability measurement. The result showed the IC_{50} value (half maximal inhibitory concentration) of the *C. gigantea* ethanolic extract on melanoma A375 cell lines was 210.25 µg/ml. In apoptosis analysis's result (Annexin V and propidium iodide staining method and measured by flow cytometer) clearly confirmed that extract from tuber of *C. gigantea* induced apoptosis of melanoma A375 cell lines.

Wei et al., 2011 proclaimed *in vitro* anticancer property of corm, stem and leaf of *Colocasia esculenta* extract on human breast adenocarcinoma (MCF-7) cell line by using 70% methanol as a solvent and colorimetric MTT (tetrazolium) assay for cell viability measurement. They demonstrated that only corm and stem of *C. esculenta* inhibited growth of human breast adenocarcinoma (MCF-7) cell line (approximate 70% cell viability at 30 µg/ml extracts concentration both corm and stem part).

2.2.4.1 Role of opposition of doxorubicin in term of general anticancer medicine in chemotherapy

Nowadays, there are many anticancer medicines. Anticancer medicines can be classified to 6 groups (alkylating agent, antimetabolite agent, plant alkaloid, antibiotics, hormone and miscellaneous) depended on sources of each medicine. One of well known anticancer medicine is doxorubicin.

Doxorubicin (trade name Adriamycin, Figure 4) is a medicine that propose is used for cancer chemotherapy (Kataokaa et al., 2000). It is a group of antibiotic and specifically uses for treatment some leukemias, Hodgkin's lymphoma, cancer of the bladder, breast, stomach, lung, ovaries, thyroid, soft tissue sarcoma, multiple myeloma, and others (Saeed et al., 2008). Although, it can be used for treatment various cancer types but it also has side effect to the patients such as cardiomyopathy, arrhythmia, congestive heart failure and subduction the bone marrow. There are some report of mechanism of action that doxorubicin act on cancer cells as follow;

Muller et al., 1997 reported the cytotoxic mechanisms of various doxorubicin concentrations in MOLT-4 ALL-cells. Doxorubicin could induce apoptosis in MOLT-4 ALL-cells at maximum effect concentration at 1 µM, which depended on RNA synthesis and involved oxidative stress. Doxorubicin at concentration 1 to 5 µM destroyed DNA strand in MOLT-4 cells. The authors found that doxorubicin concentration higher than 3 µM did not induce apoptosis but significantly inhibited RNA synthesis. At higher concentration (more than 5 µM) found no oxidation of DNA bases in apoptosis cells. The author suggested that pharmacology concentration ranges in apoptosis and not oxidative DNA damage were the main killing mechanism of doxorubicin against ALL-cells.

Pletz et al., 2012 proclaimed that doxorubicin induced profound of NF-κB activation in A375 melanoma cells. NF-κB activation is the one of the cause of drug resistance in cancer chemotherapy (Camp et al., 2004). In the experiment's results, doxorubicin concentration 1 µM gave the highest NF-κB activation in A375 melanoma cells. BAY32-5915 (8-hydroxyquinoline-2-carboxylic acid, as a IKKα inhibitor, Figure 5) did not affect doxorubicin induced NF-κB activation. On the other hand, completely abrogated of NF-κB activation occurred when KINK-1 (as a IKKβ inhibitor) was used. The result was increase in apoptosis response to doxorubicin (Pletz et al., 2012).

Figure 4: Structural formula of doxorubicin (DOX) (Kataokaa et al., 2000)

Figure 5: BAY32-5915 (8-hydroxyquinoline-2-carboxylic acid)
(Pletz et al., 2012)

2.3 Phytochemistry of *Colocasia* genus

2.3.1 Tuber

Mainly tuber of *Colocasia esculenta* contains starch in the range of 73-76%. The nitrogen content varies from 0.33-1.35%. The starch contains lipid and phosphorus in from of phosphate monoester derivatives varies from 0.23-0.52% and 0.017-0.025% respectively (Anonymous, 2005). The tuber also contains natural polysaccharide with 40% anionic components and 56% neutral sugars. Steamed corms contain 3% sugar and 30% starch (Khare, 2007).

Eighty percent of globulins of the total proteins in tuber (Anonymous, 2005), total amino acids in the range of 1,380-2,397 mg/100g, Amount of lysine was relatively low when compare with another amino acid (Khare, 2007).

From the tuber of *C. esculenta*; mucilage, dihydroxysterols, calcium oxalate, vitamin B, iron (Sheth, 2005). In the tuber part; 2 dihydroxysterols, 14α-methyl-5α-cholesta-9, 24-diene-3α, 7α-diol and 14α-methyl-24-methylene-5α-cholesta-9, β-sitosterol, stigmasterol, nonacosane, cyanidin 3-glucoside, 5-novel aliphatic compounds, tetracos-20-en-1, 18-diol, 25-methyl triacont-10-1, octacos-10-en-1, 12-diol, pentatriacont-1, 7-dien-12-ol, 25-methyl-tritriacont-2-en-1,9,11-triol (Prajapati et al., 2011); pelargonidin 3-glucoside, cyanidin 3-rhamnoside, apigenin, 3’,4’-dimethoxyluteonin, hydroxyl-cinnamoyl amides, benzaldehyde-3, 4-di-O-β glucoside, vitamin C, carotenes, oxalic acid and colocasia sterols (World Health Organization, 1998). Anonymous, 2005 reported in The Wealth of India: A Dictionary of Indian Raw Materials and Industrial Products that 9,12,13-trihydroxy-(E)-10-octadecenoic acid, 2 enzymes, lipoxygenase and lipid hydroperoxide converting enzyme were found in *C. esculenta*’s tuber and have antifungal properties of *Ceratocystis fimbriata*.

Wei et al., 2011 reported bioactive compounds in *C. esculenta*’s tuber 70% methanol extract [47]. Bioactive compounds were identified by comparing the spectra with known compounds stored in internal library. From the tuber of *C. esculenta*; 8, 11-octadecadienoic acid, methyl ester, hexadecanoic acid, methyl ester, 9, 12, 15-octadecatrienoic acid, methyl ester (Z,Z,Z)-, 9-octadecenoic acid, methyl ester, (E)-, 3, 5 - di - tert - butyl - 4 - trimethylsiloxytoluene and cyclohexanol and 2-methyl-5-(1-methylethenyl)-,(1.α., 2.β., 5.α.)-.

Chemical structures of some substances reported for *C.esculenta*’s tuber are shown in Figure 6.

β-sitosterol (Oja et al., 2009)

stigmasterol (Oja et al., 2009)

nonacosane (Channon and Chibnall, 1929)

cyanidin 3-glucoside (Tsuda et al., 1994)

pelargonidin 3-glucoside (Kader et al., 1999)

cyanidin 3-rhamnoside (Jing et al., 2008)

Figure 6: Some of chemical structure compounds reported for tuber of *Colocasia esculenta*.

α-Carotene

β-Carotene

γ-Carotene

δ-Carotene

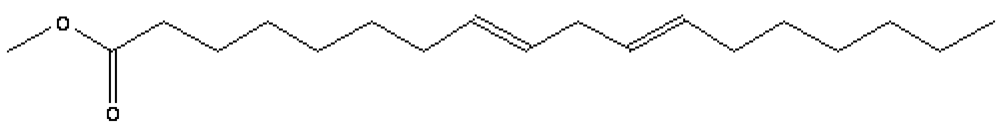
ε-Carotene

chemical structure of carotene series (Barbosa-Filho et al., 2008)

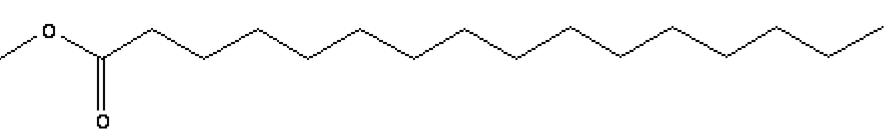
apigenin (Leopoldini et al., 2004) vitamin C (Rice-Evans et al., 1996)

oxalic acid (Ahmed et al., 1953) calcium oxalate (Grases et al., 1988)

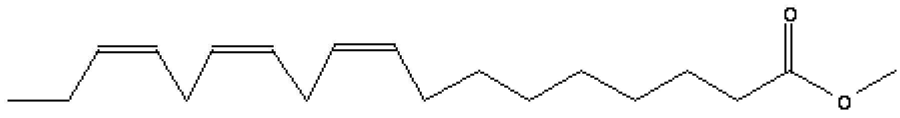
Figure 6 (cont.): Some of chemical structure compounds reported for tuber of *Colocasia esculenta*.



8, 11-octadecadienoic acid, methyl ester (Xuan et al., 2008)



hexadecanoic acid, methyl ester (Guckert et al., 1985)



9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z)- (Görena et al., 2003)

9-octadecenoic acid, methyl ester, (E)- (Rogers et al., 1975)

Figure 6 (cont.): Some of chemical structure compounds reported for tuber of *Colocasia esculenta*

2.3.2 Leaf

Colocasia esculenta's leaf are rich in calcium, phosphorus, iron and approximate 2.26 ppm of iodine (Rummel and Dietmar, 2005). Calcium oxalate, fibers, minerials, vitamin A, B, C and starch are the main constituents in *C. esculenta*'s leaf (Prajapati et al., 2011). In the leaf; flavones, apigenin, luteolin, anthocyanins (Prajapati et al., 2011). Alkaloids, flavonoids, saponins, tannins, sterols, glycosides are the major phytochemical groups contain in leaf of *C. esculenta* (Kumawat et al., 2010). A bitter substance acorine appears to be a nitrogenated glucoside and an aromatic essential oil (Guerrero et al., 1931).

Iwashina et al. (1999) isolated and identified of the flavonoids in *C. esculenta*'s leaf extract using 50% methanol as a solvent for extraction. The flavonoids were orientin, isoorientin, isovitexin, vicenin-2, orientin 7-O-glucoside, isovitexin 3'-O-glucoside, vitexin x"-O-glucoside and luteolin 7-O-glucoside.

Wei et al. (2011) reported bioactive compounds in *C. esculenta*'s leaf 70% methanol extract [47]. Bioactive compounds were identified by comparing the spectra with known compounds stored in internal library. From the leaf of *C. esculenta*; cyclohexanol, 5-methyl-2-(1-methylethyl)-, [1S-(1.α.,2.β.,5.β.,)]-, 4H-pyran-4-one, 2,3-dihydro-3, 5-dihydroxy-6-methyl-, 3,5-di-tert-butyl-4-trimethylsiloxytoluene, 2-propanone, 1-hydroxy-, 2-propanone, 1-hydroxy-, 9,11-octadecadienoic acid, methyl ester, (E,E)-, hexadecanoic acid, methyl ester, formic acid, 2-propenyl ester and megastigmatrienone.

Chemical structures of some substances reported for *C. esculenta*'s leaf are shown in Figure 7.

luteolin (Leopoldini et al., 2004) megastigmatrienone (Montenegro et al., 2009)

2-propanone, 1-hydroxy- (Weast and Grasselli, 1989)

formic acid, 2-propenyl ester (Patnaik, 2007)

cyclohexanol, 5-methyl-2-(1-methylethyl)-, [1S-(1.α.,2.β.,5.β.,)]- (De-Oliveira et al., 1999)

4H-pyran-4-one, 2,3-dihydro-3, 5-dihydroxy-6-methyl- (Lucie et al., 2011)

9,11-octadecadienoic acid, methyl ester, (E,E)- (Rogers and Siddiqui, 1975)

Figure 7: Some of chemical structure compounds reported for leaf of *Colocasia esculenta*.

Substance	R_1	R_2	R_3	R_4	R_5
vitexin	H	Glucose	H	H	H
isovitexin	Glucose	H	H	H	H
isoorientin	Glucose	H	H	OH	H
luteolin 7-O-glucoside	Glucose	H	Glucose	OH	H

Flavonoid's structures isolated from *Colocasia esculenta*'s leaf were reported by Iwashina et al., 1999.

Figure 7 (cont.): Some of chemical structure compounds reported for leaf of *C. esculenta*

2.3.3 Stem

Colocasia esculenta's stem contains myricetin flavonoid approximately 133.5 ± 0.05 mg/kg of dry weight (Miean et al.)

Wei et al., 2011 reported bioactive compounds in *C. esculenta*'s stem 70% methanol extract. Bioactive compounds were identified by comparing the spectra with known compounds stored in internal library. From the stem of *C. esculenta*; acetic acid, 4H-pyran-4-one, 2,3-dihydro-3, 5-dihydroxy-6-methyl-, propanoic acid, 2-furancarboxaldehyde, 5-(hydroxymethyl)-, 9,12-octadecadienoic acid, methyl ester, (E,E)-, 9, 12, 15-octadecatrienoic acid, methyl ester (Z,Z,Z)-, 2-furanmethanol, butanoic acid, 2-methyl-3-oxo-, ethyl ester, cyclopentanol, 2,2'-bioxirane, propanoic acid, 2-oxo-, methyl ester and butanoic acid, 4-hydroxy-.

Chemical structures of some substances reported for *C. esculenta*'s stem are shown in Figure 8.

myricetin (Leopoldini et al., 2004) acetic acid (Jones and Templeton, 1958)

propanoic acid (Nagahara et al., 1994) 2,2'-bioxirane (Smith and Kohl, 1972)

2-furancarboxaldehyde, 5-(hydroxymethyl)- (Mercadier et al., 1981)

2-furanmethanol (Marstokk and Mollendal, 1994)

butanoic acid, 2-methyl-3-oxo-, ethyl ester (Dawson, 1959)

cyclopentanol (Wells, 1984)

Figure 8: Some of chemical structure compounds reported for stem of *Colocasia esculenta*

propanoic acid, 2-oxo-, methyl ester (Zawalich et al., 1997)

butanoic acid, 4-hydroxy- (Wiberg and Waldron, 1991)

Figure 8 (cont.): Some of chemical structure compounds reported for stem of *Colocasia esculenta*.

Chapter III

Total phenolic content and optimal solvent design for extraction of phenolic compounds

Phenolic compounds are general natural bioactive compounds which are found in various plants. They are powerful natural antioxidant compounds in other words, the plant contains high level of total phenolic compounds whose possesses the high level of antioxidant activity (Fu et al., 2011). Therefore, the level of total phenolic compounds can imply the antioxidant activity of the plant.

Until now, many articles have suggested the suitable solvent system for extraction the phenolic compounds from plants that they have interested. Nevertheless, they have found the suitable solvent system for extraction the phenolic compounds by trial and error or reviewed the similar works in the article. There are no scientific method for investigation the optimal extraction solvent system for specific bioactive compounds (Kim et al., 2007).

The objective of this study was to investigate the quantity of total phenolic compounds and also to design the optimal solvent system for extraction the phenolic compounds from *Colocasia gigantea* both tuber and leaf.

3.1 Materials and Methods

The tuber and leaf of *C. gigantea* were collected from Ban Phaeo district, Samut Sakhon province (consist with sample number 013425 (BCU). All samples were washed with water, cut into small pieces and dried by dehydrator at 40 ^{o}C 5 days. They were crushed into small particles by grinder machine.

3.1.1 Extraction for total phenolic contents investigation

Extracts from dried plant materials were prepared by using methanol-water binary solvents of varying polarity. The dried plant materials of 5 g each were extracted by maceration in different polarity solvents (150 ml) for 3 days at room temperature in a dark place. The methanol fractions were 0-100% in water. Following the solvent maceration, the extract was filtered with vacuum filter and was evaporated by rotary evaporator under reduced pressure to

obtained crude extract. These extracts were subjected for further investigation of total phenolic contents

3.1.2 Total phenolic assay

Total phenolic contents were modified and determined with the Folin-Ciocalteu reagent using the method of Fu et al., 2011. Briefly, 0.5 ml of each extract was diluted with 4.5 ml distillated water. Took 40 µl of dilute sample, 200 µl of Folin-Ciocalteu reagent and 3.16 ml of distillated water were added. After 8 min, 600 µl of saturated sodium carbonate solution (75 g/l) were added. The absorbance of the solution was measured at 765 nm using a UV-visible spectrophotometer (UV-2450 Shimadzu Japan) after 1 hour incubation at room temperature. Gallic acid was used as a reference standard. The result was expressed as milligram gallic acid equivalents (mg gallic acid/g dryweight).

3.1.3 Optimal solvent design method

The optimal solvent design method in this study was applied from the method of Kim et al., 2007. Briefly, the common knowledge, the specific bioactive compounds were extracted by the solvent which had suitable solvent polarity. The extraction efficiency was better than using solvent which had inconsonant solvent polarity. Thereby, solvent polarity could be presented in the simple way in the form of the solubility parameter (δ) whereas the solubility parameter has been used for prediction the miscibility and solubility of materials with a particular solvent (Hertz, 1989).

Microsoft Excel Software (Microsoft Software Inc.) and GraphPad Prism 5 (GraphPad Software Inc.) were used to analyze data and find a suitable equation. The detail of calculation has presented in the appendix A.

3.2 Results and Discussions

3.2.1 Total phenolic content

In the Figure 9, the content of total phenolic varied in range from 42.5 to 196 mg gallic acid/g of dry weight in tuber and from 98.4 to 153 mg gallic acid/g of dry weight in leaf based on Folin-Ciocalteu method. The highest total phenolic level was obtained in crude extract of *Colocasia gigantea* by

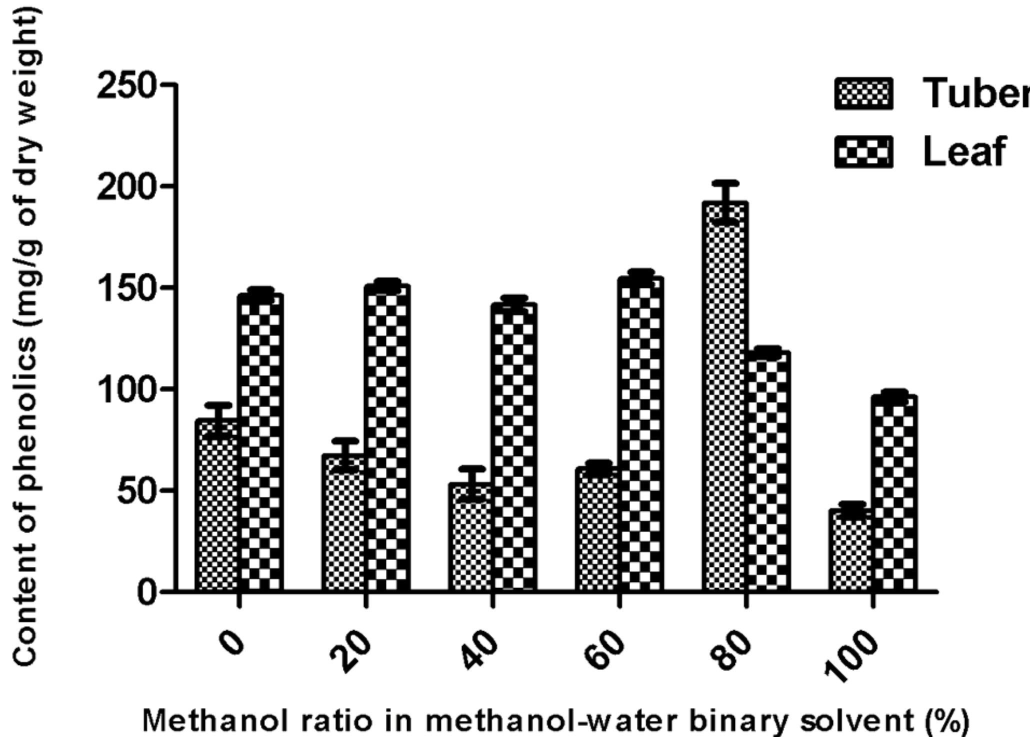


Figure 9: Total phenolic contents of *Colocasia gigantea* using methanol-water binary solvents extraction

using 80% of methanol in tuber (196 mg gallic acid/g) and 60% of methanol in leaf (153 mg gallic acid/g) respectively. The lowest yield was obtained when using 100% of methanol in tuber (42.5 mg gallic acid/g) and 100% of methanol in leaf (98.4 mg gallic acid/g) respectively.

Kahkonen et al., 1999 explained that typical phenolics which have had an antioxidant activity that can be classified to phenolic acids and flavonoids. Phenolic acids such as cafeic acid, ferulic acid, vanillic acid and rosmarinic acid are a nature antioxidant compounds (Larson, 1988 and Mazumder et al., 1997). Moreover, Satirapathkul and Leela, 2011 suggested that phenolic compounds in *Quercus infectoria* galls were the main compounds which inhibited the growth of wide variety of pathogenic bacteria. They implied that phenolic compounds not only has an antioxidant activity but also an antimicrobial activity.

3.2.2 Design of the optimal solvent system for extraction of phenolic compounds from *Colocasia gigantea*

As show in Figure 9, the content of total phenolics from tuber and leaf of *C. gigantea* depended on methanol ratio in the binary solvent. The extraction of phytochemicals is dependent on the compatibility of the phytochemical components to the solvent. Therefore, when the phytochemical components are compatible in polarity with the solvent, they can be easily extracted into the solvent (Kim et al., 2007). From method of Kim et al., 2007; the solubility parameter (δ_i, [$MPa^{0.5}$]) is representative of the relationship between extraction efficiency of the phenolic contents and polarity of the used solvent. The solubility parameter of methanol and water at 25°C are 29.7 and 48 respectively (Barton, 1983). The solubility parameter for binary mixtures can be estimated based on a simple mixing rule in equation 1.

$$\delta_m = \sum_i x_i \delta_i \quad (1)$$

whereas, δ_m is the binary mixture solubility parameter and x_i is the volume fraction of species i.

The equation above give the solubility parameter of the binary mixture. Figure 10 shows the relation of total phenolic content and polarity of the binary mixture solvent. The optimal solvent polarity was 33.6 $MPa^{0.5}$, equivalent to a 78.6% of methanol from tuber and 42.5 $MPa^{0.5}$, equivalent to a 30% of methanol from leaf respectively.

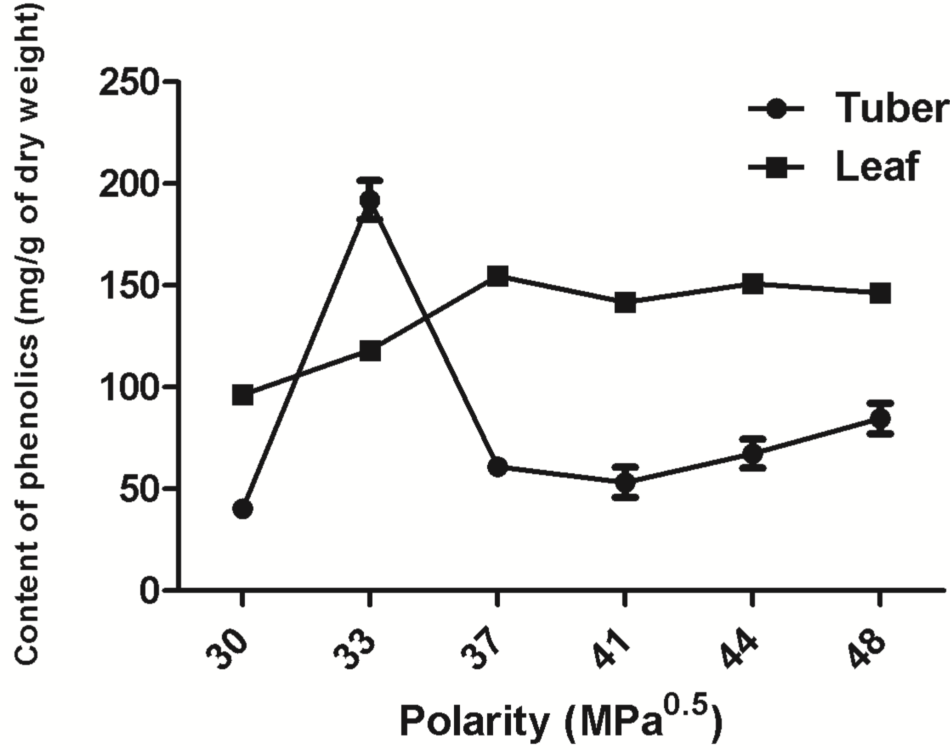


Figure 10: Correlations of content of phenolics from *Colocasia gigantea* with polarity of solvent

This experimental result was consistent with total phenolic contents from *Colocasia esculenta* tuber (Prasad et al.). The highest total phenolic content (equivalent to gallic acid) from *C. esculenta* was obtained by using 80% methanol as a solvent and hardly extracted by using 100% methanol (Prasad et al.). This study provided the information of the optimal solvent conditions for extraction the phenolic compounds from *C. gigantea*. However, total phenolic content within *C. gigantea* may be subjected to change depending on their growth conditions (Kasote et al., 2011).

3.3 Conclusions

This study probed the total phenolic compounds in *C. gigantea* both tuber and leaf. The total phenolic content in tuber part was less than leaf (using methanol-water binary mixture as a solvent for extraction) excepted methanol-water binary mixture solvent at ratio 80:20.

Furthermore, this study also propose an idea or guideline in the design of solvent system for extraction the interested bioactive compounds in plant. In the design of the optimal solvent system, this study showed that at the constant temperature, the efficiency of the total phenolic extraction process depend on the selected solvent species and the ratio of each solvent compositions. Each extraction process have the optimal solvent system for extraction the specific bioactive compounds. The optimal solvent system from each process can be obtained by experimental and calculated the suitable solvent’s solubility parameter.

Chapter IV

Antimicrobial activity of *Colocasia gigantea*

C. gigantea has been used as a minor food crop and traditional folk medicine in Thailand. Up to now, there have had many articles to present the antimicrobial activity of *Colocasia esulenta* which is the same genus of *C. gigantea*. However, there has not a report to show antimicrobial activity of *C. gigantea*.

The aim of this study was to evaluate the potential of *C. gigantea* extract as an antimicrobial agent. Hence, this study was discussion about relation between phenolic compounds in *C. gigantea* with antimicrobial activity both gram-positive and gram-negative pathogenic bacteria.

4.1 Materials and Methods

The extracts from both tuber and leaf of *C. gigantea* in the total phenolic content's investigation were used for testing the antimicrobial activity. Tested pathogenic bacteria comprised *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 25923. The bacteria were maintained by subculturing periodically on nutrient agar and were preserved at 4°C prior to use.

4.1.1 Minimum inhibitory concentration (MIC)

Based on the method suggested by Satirapipathkul and Leela (2011), MICs of the extracts were determined by the broth dilution method using serial dilution of the plant extracts. Briefly, the test bacteria were prepared in nutrient broth and incubated at room temperature for 24 hour. After that, the cultures's concentrations were adjusted with sterilized saline to bring the optical density at 660 nm to 0.05. Serial two-fold dilutions of the extracts were prepared in nutrient broth with concentrations ranging from 500 to 1.95 μg/ml. The 1 ml of each serially diluted extract was separately added to the tubes containing an equal volume of the inoculum (1 ml). All the tubes (total volume of 2 ml) were kept at 37◦C for overnight.

The MIC values were implied as the highest dilution (lowest concentration) of the sample, which showed clear fluid with no development of

turbidity. Dettol was used as positive control. The active ingredient in dettol is chloroxylenol (4-chloro-3,5-dimethylphenol (C_8H_9ClO) an aromatic chemical compound (Wells, 1984). Negative control (nutrient broth and microorganisms) was also included.

4.1.2 Disc diffusion method

Based on the method of Satirapipathkul and Leela, 2011; antimicrobial activity was tested by the disc diffusion method. Small discs of filter paper (diameter 6.0 mm) were impregnated with 15 μl of different extracts which gave no development of turbidity in MICs test and placed on top of the seeded media of each bacterial strains. The antibacterial assay plates were kept at 37◦C for overnight and the diameters of the clear zones were noted. In this experiment, the diameter of the zone of inhibition around each disc (disc diameter included) was taken as a measure of the antibacterial activity. Each experiment was carried out in duplicate and the mean diameter of the inhibition zones was recorded.

4.2 Results and Discussions

4.2.1 Minimum inhibitory concentration (MIC)

Table 2 indicates the minimum inhibitory concentrations (MIC) of the methanol-water extracts which required to completely inhibit the growth of the three bacterial pathogens. The relative growth of each microorganism overnight of incubation in the presence of different concentrations of *Colocasia gigantea* extracts was compared to the control. From Table 2, the extracts from leaf of *C. gigantea* were suppresser inhibition than the extracts from tuber in *Bacillus subtilis*. 80% of methanol extract in leaf of *C. gigantea* gave the MIC of 125 mg/ml in *Escherichia coli* while the growth of *Staphylococcus aureus* was affected at the MIC of 62.5 mg/ml of aqueous extracts both tuber and leaf. 100% of methanol extract from tuber and all of the extracts form leaf gave the same MIC value of 250 mg/ml for inhibited the growth of *S. aureus*. From the MIC results, the concentration at 250 mg/ml was selected to used further as a sample concentration in disc diffusion method because at concentration 250 mg/ml, all of methanol-water extracts inhibited the tested three bacterial pathogens.

Table 2: MICs of extracts of *Colocasia gigantea* using different methanol fraction as a solvent; (a) tuber; (b) leaf.

(a)

Methanol ratio	**MICs of extracts in tuber (mg/ml)**				
	Positive control	**Negative control**	***E. coli***	***B. subtilis***	***S. aureus***
0	+	-	+	+	- (62.5)
20	+	-	+	+	- (250)
40	+	-	+	+	- (250)
60	+	-	+	+	- (250)
80	+	-	- (250)	+	- (250)
100	+	-	- (250)	- (250)	- (250)

(b)

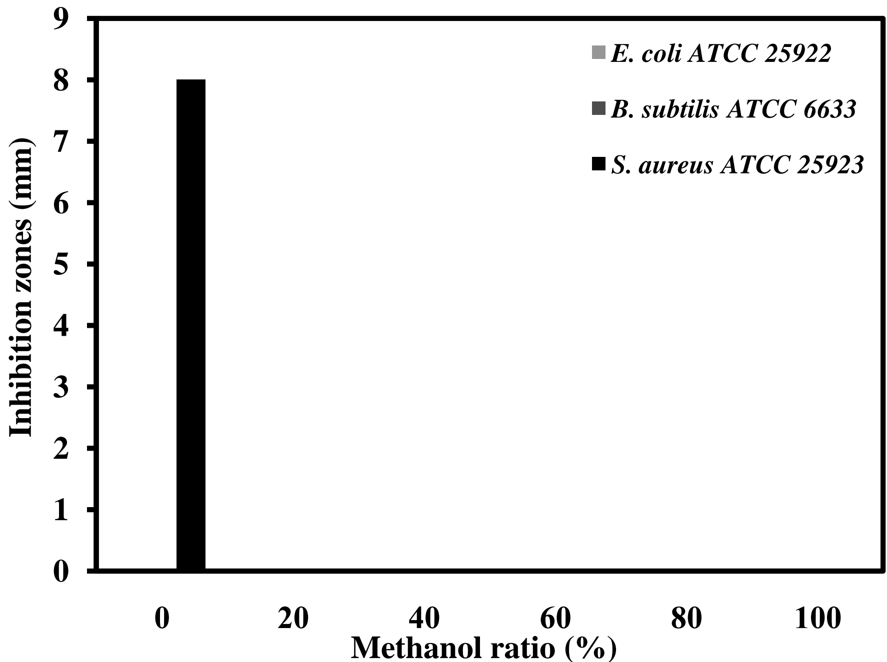
Methanol ratio	**MICs of extracts in leaf (mg/ml)**				
	Positive control	**Negative control**	***E. coli***	***B. subtilis***	***S. aureus***
0	+	-	+	- (250)	- (62.5)
20	+	-	+	- (250)	- (250)
40	+	-	+	- (250)	- (250)
60	+	-	+	- (250)	- (250)
80	+	-	- (125)	- (250)	- (250)
100	+	-	- (250)	- (250)	- (250)

4.2.2 Antimicrobial assay

The antimicrobial activity of methanol-water solvent extracts of *Colocasia gigantea* (250 mg/ml) was studied by the disc diffusion method. As show in Figure 11, all extracts of leaf of *C. gigantea* were found to be more effective than extracts from tuber against all microbial strains in this experiment. The extract from leaf using 100% of methanol as a solvent exhibited the maximum inhibition against *Escherichia coli* resulting in the inhibition zone of 14 mm. The highest inhibition zone of *Bacillus subtilis* was observed in treatment of extract from leaf using 40 and 60% of methanol as a solvent produced the inhibition zone of 16 mm. All leaf's extracts from 0-100% of methanol fraction and tuber's extract from aqueous solution were effective against *Staphylococcus aureus* but they gave the maximum inhibition zone of 13 mm using 20 and 100% of methanol as a solvent.

The experimental result shows a significant activity inhibitory of *C. gigantea* extract against all pathogenic bacterial strains. Extracts from leaf of *C. gigantea* displayed an excellent activity against gram-positive *B. subtilis*, *S. aureus*. From the chapter 3, the highest total phenolic content in leaf of *C. gigantea* was obtained by using 60% methanol as a solvent and the highest inhibition zone of *B. subtilis* was also found at 60% methanol. This may implied that phenolic compounds in leaf of *C. gigantea* were the major compounds of antimicrobial activity against *B. subtilis*. Satirapathkul and Leela, 2011 observed that the bioactive compounds responsible for the antimicrobial action should be more hydrophilic in nature. In addition, a higher antibacterial activity was also observed against gram-positive bacteria. These results confirm that the gram-positive bacteria *B. subtilis* and *S. aureus* are more sensitive to the leaf's extracts than gram-negative bacteria *E. coli*. Gram-negative bacteria is resister to bioactive compounds than gram-positive bacteria because the bacteria's cell wall structure of gram-negative bacteria has the lipopolysaccharide (LPS) in outer membrane layer which protects cell from antibiotic, detergents, heavy metals, dyes and digestive enzymes (Baron, 1996). Ikigai et al., 1993 reported that the resistance of gram-negative bacteria is actually partially related to the highly negative charges on the LPS layer even though its exact mechanism is not yet understood. Singh et al., 2011 and Wei et al., 2008 reported that antibacterial activity of *Colocasia esculenta* which is the same family as *Colocasia gigantea*. *C. esculenta* was found that its aqueous extract from leaf can inhibit the growth of *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Citrobacter freundii*, *Streptococcus* mutans MTCC-890, *Bacillus subtillis* MTCC-121, *Klebsiella pneumoniae* MTCC-109, *Pseudomonas fragi* MTCC-2458, *Escherichia coli* MTCC-483. Kubde et al., 2010 and Nair et al., 2005 reported that 100% of methanol extract from *C. esculenta*'s leaf were effective against *Klebsiella pneumonia* NCIM 2719, *Staphylococcus aureus* NCIM 2079, *Bacillus subtilis* NCIM 2063, *Escherichia coli* NCIM 2065 and *Pseudomonas aerugenosa* NCIM 2036.

(a)



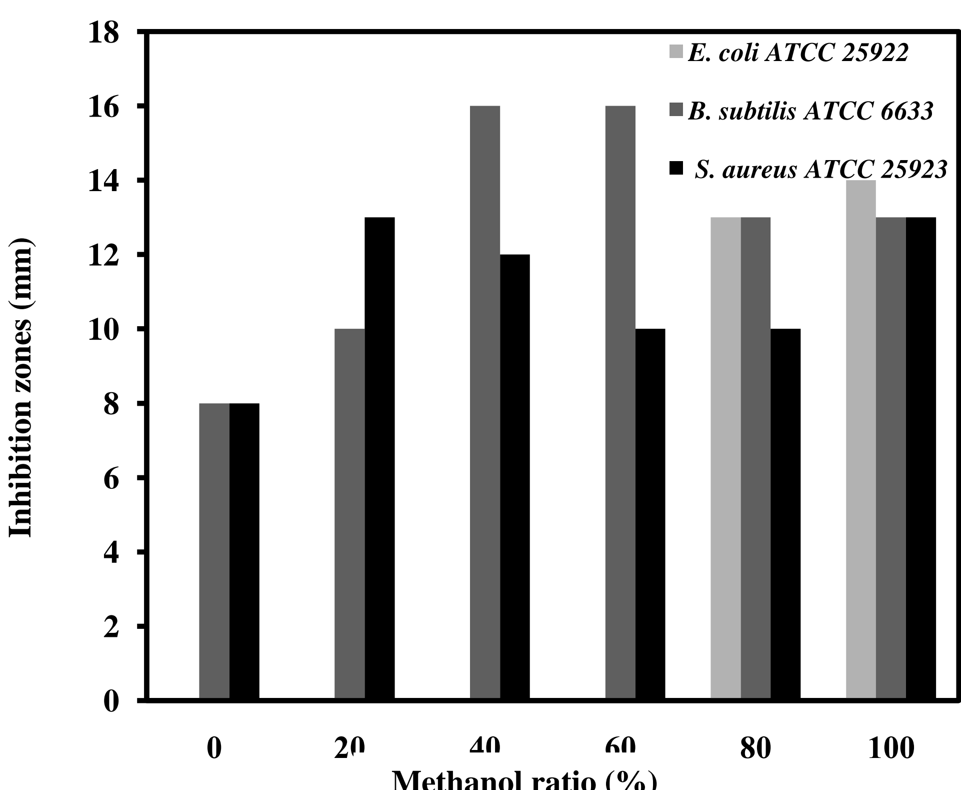


Figure 11: Antibacterial activity of *Colocasia gigantea* against *E. coli*, *B. subtilis* and *S. aureus*; (a) tuber; (b) leaf.

Wei et al., 2011 reported that octadecadienoic acid, acetic acid, propanoic acid and formic acid were the major bioactive compounds which exhibited the antimicrobial activity of *Colocasia esculenta*. These compounds could be classified to free fatty acids (FFAs).

The mechanism of action of FFAs against pathogenic bacteria was reviewed by Desbois and Smith, 2010. They displayed that FFAs play an important role in prevention and killing of various pathogenic bacteria in many organisms. The main target of FFAs acting on bacteria cell is a cell membrane. FFAs bind the electron carriers in the electron transport chain directly or enter into the inner membrane, cause disorder in electron carriers in the bacterial cell membrane (Galbraith and Miller, 1973; Peters and Chin, 2003). Furthermore, the proton gradient and membrane potential are also reduced because the electron transport chain is disrupted by FFAs. This outcome is decreased in bacteria's ATP production and out of energy (Greenway and Dyke, 1979; Chamberlain et al., 1991; Stulnig et al., 2001). FFAs may inhibit bacteria's ATP production. They may bind directly to ATP or prevent the enzyme functioning correctly (Sheu and Freese, 1972; Greenway and Dyke, 1979; Beck et al., 2007; Borst et al., 1962; Boyaval et al., 1995). FFAs also cause the bacterial death by another mechanism such as cell lysis (Greenway and Dyke, 1979; Chamberlain et al., 1991), inhibition of enzyme activity (Kurihara et al., 1999; Zheng et al., 2005; Won et al., 2007; Hamel, 2009; Sado-Kamdem et al., 2009), impairment of nutrient uptake (Galbraith and Miller, 1973; Shibasaki and Kato 1978), peroxidation and auto-oxidation (Knapp and Melly, 1986; Hazell and Graham, 1990; Wang and Johnson, 1992; Schönfeld and Wojtczak, 2008). In order to inhibited the gram-positive bacteria in this experiment. In the figure 11b, *Bacillus subtilis* was more sensitive to leaf part extract of *Colocasia gigantea* than *Staphylococcus aureus*. Kenny et al., 2009 explained that *S. aureus* has thick cell wall to prevent or make more difficult for bioactive compounds or antibiotics to penetrate into the cell membrane. The thick cell wall of *S. aureus* is produced by genes encoding proteins involved in the synthesis of the cell wall upon exposure to unsaturated FFAs. Chamberlain et al., 1991; Xiong and Kapral ,1992 also explained the resistance of *S. aureus* to the FFAs that *S. aureus* contains high levels of carotenoids which are an antioxidant substance in their cell membrane. Carotenoids may protect *S. aureus* from the leaf part extract of *C. gigantea* from inhitbition or killing. The various mechanisms of FFAs for antimicrobial activity are illustrated in the figure 12.

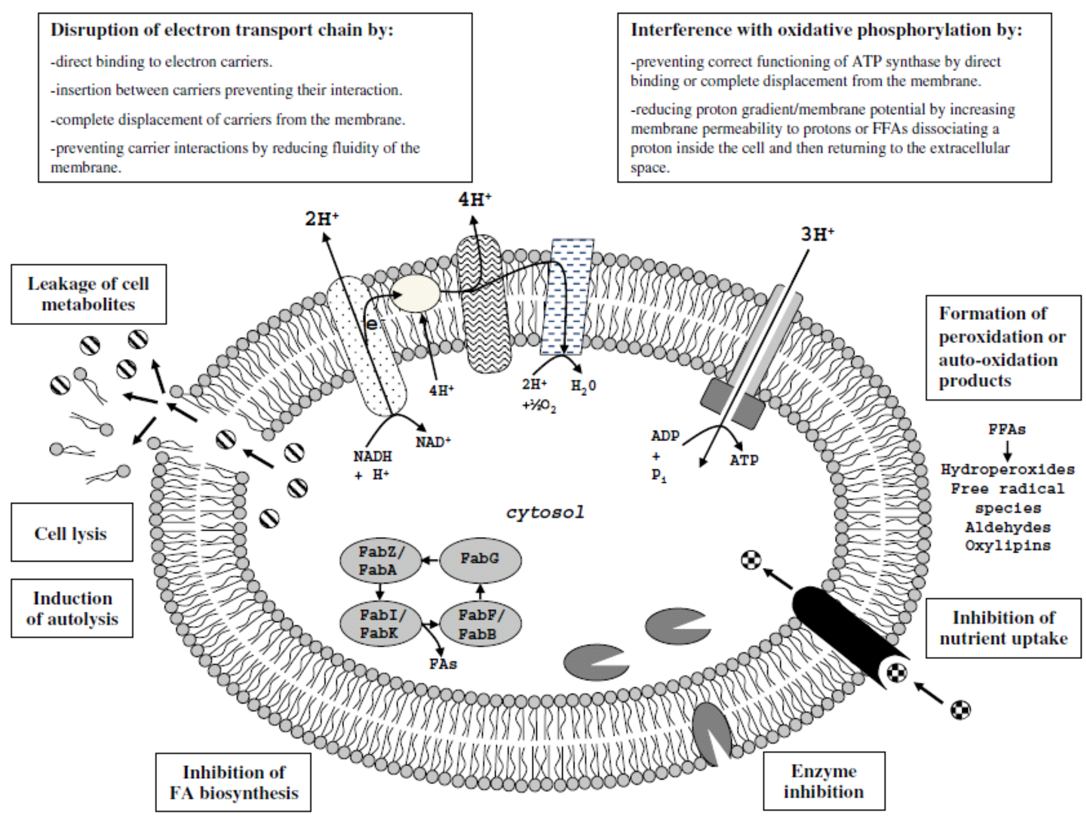


Figure 12: Mechanisms of action of free fatty acids act on pathogenic bacteria (Desbois and Smith, 2010)

4.3 Conclusions

The extracts from leaf of *Colocasia gigantea* (using methanol-water at various ratio as a solvent) had an ability to inhibit *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*. The extracts from the leaf of *C. gigantea* were found to be more effective than extracts from tuber against all microbial strains in this experiment. This study also showed that the extracts from the leaf of *C. gigantea* were more effective to the gram-positive bacteria (*B. subtilis* and *S. aureus*) than gram-negative bacteria (*E. coli*).

Chapter V

Cytotoxic activity of *Colocasia gigantea*

The purpose of this chapter was to explore the cytotoxicity of *Colocasia gigantea*. Bioassay-guided fractionation method was used for investigation the active fraction that had cytotoxic activity to the tested cells *in vitro*. Henceforth, the active fraction was identified the bioactive compounds which probably had cytotoxic activity by GC-MS spectroscopy.

5.1 Materials and Methods

5.1.1 Extraction and fractionation method

The samples were extracted by maceration with n-hexane (n-C_6H_{14}) for 3 days at room temperature (3 times each) and then filtered with vacuum filter. The filtrate was concentrated to remove n-hexane under reduced pressure by rotary vacuum evaporator to obtained crude n-hexane extract. The extract was kept at room temperature in glass bottle covered with aluminium foil. The marc after had finished n-hexane extraction, marc was extracted with dichloromethane (CH_2Cl_2) and methanol (CH_3OH) (increase polarity) with the same extraction process like n-hexane respectively. These extracts were subjected to cytotoxic activities investigation for chose the most active extract to column chromatography for further purification. The extraction process of *C. gigantea* is summarized in Figure 13.

In the fractionation process, all solvents were redistilled prior to use. Chromatographic techniques were used for fractionation the bioactive compounds in this study. The details of material and detection in Chromatographic Technique were described in page 39.

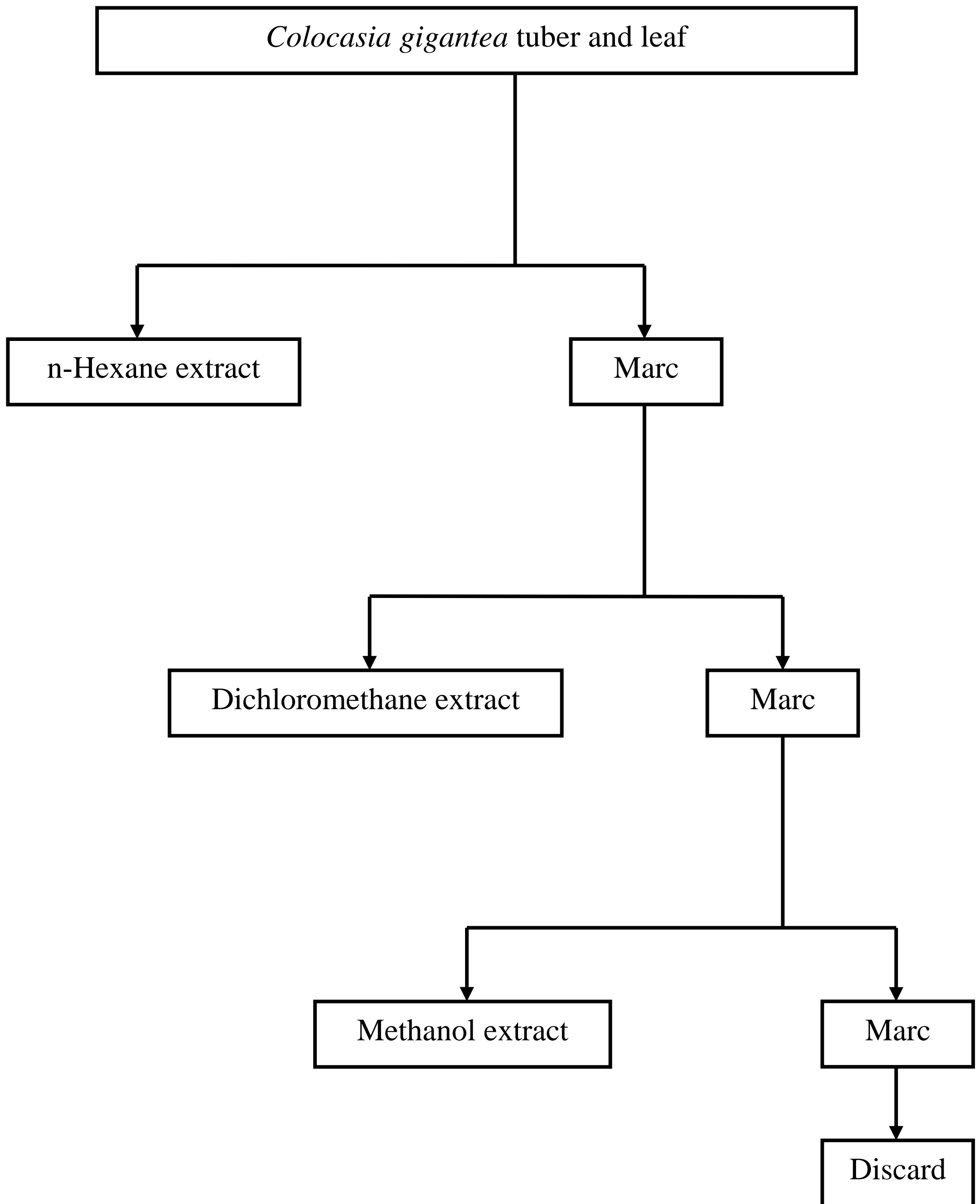


Figure 13: The extraction process of *C. gigantea*

5.1.1 Fractionation by Chromatographic Techniques

1. Thin layer chromatography (TLC)

Technique	:	one way ascending
Stationary phase	:	TLC aluminium sheet silica gel 60F 254, Layer thickness 0.2 mm (E. Merck)
Distance	:	6.5 cm
Temperature	:	room temperature (approximate 28-37°C)
Detection	:	1. UV light at the wavelengths of 254 and 365 nm 2. 5% anisaldehyde in sulfuric acid heating at 110°C
Solvent system	:	Various solvent systems depending on samples

2. Column chromatography (CC)

2.1 Quick column chromatography

Column	:	Sintered glass funnel with diameter 100 mm
Stationary phase	:	Silica gel 60 (No. 9385, E. Merck) particle size 0.040-0.063 mm (230-400 mesh ASTM)
Packing method	:	Dry packing
Sample loading	:	In dry packing, the sample was dissolved in a small amount of most appropriate solvent and then mixed with a small quantity of adsorbent dried and loaded on the top of the column.
Solvent system	:	Various solvent systems depending on samples
Detection	:	Fractions were examined by TLC observing under UV light at the wavelengths of 254 and 365 nm and then sprayed with 5% anisaldehyde in sulfuric acid heating at 110°C . The similar TLC pattern fractions were combined.

2.2 Flash column chromatography

Column	:	Flash bottom glass column
Stationary phase	:	Silica gel 60 (No. 9385, E. Merck)
		particle size 0.040-0.063 mm (230-400 mesh ASTM)
Packing method	:	Wet packing
Sample loading	:	In wet packing, the sample was dissolved in a small amount of solvent system and then loaded on the top of the column.
Solvent system	:	Various solvent systems depending on samples
Detection	:	Fractions were examined by TLC observing under UV light at the wavelengths of 254 and 365 nm and then sprayed with 5% anisaldehyde in sulfuric acid heating at 110°C . The similar TLC pattern fractions were combined.

5.1.2 Identification of fractions

Modification of Mitova et al., 2003; Briefly, Leco gas chromatography (GC)-MS was used for identification. The column HP5-MS (30 m x 0.25 mm, 0.25 µm film thickness) was used. The injector port was at 300°C. The injection volume was 0.2 µl and injections were carried out under split/splitless: split split ratio: 1:20. Helium was used as a carrier gas with a constant flow 1ml/min. The temperature was programmed from 40°C (2 min) to 300°C at a rate of 6°C min^{-1} with 10 min hold. The fractions of the test solution were identified by comparing the spectra with known compounds stored in internal library.

5.1.3 Cytotoxicity assay

5.1.3.1 Cancer Cell Lines and Culture

Melanoma (A375) and cervical cancer (Hela) cells were obtained from the Department of Anatomy, Faculty of Medicine, Chulalongkorn University (Figure 14). White blood cells (WBC) were received from the volunteers blood

in Nanomedicine laboratory, Department of Anatomy, Faculty of Medicine, Chulalongkorn University (Figure 14). A375 and Hela cells were grown in modified Eagle's medium (DMEM) and Roswell Park Memorial Institute Medium (RPMI) for WBC supplemented with 10% (v/v) fetal bovine serum, 1% penicillin/streptomycin and 3.7 g/L sodium bicarbonate (Na_2CO_3) at 37°C in an incubator containing 5% CO_2.

5.1.3.2 Cytotoxic investigation method

1. Cancer Cell Lines

A375 and Hela were washed with two times of PBS and then were collected by trypsinization and stained with trypan blue. Cell counts were adjusted to 10^3 cell/well and seeded in 96-well plates containing a DMEM medium incubated (24 hr) at 37°C in an incubator containing 5% CO_2.

2. White Blood Cell

WBC was stained with trypan blue. Cell counts were adjusted to 10^3 cell/well and seeded in 96-well plates containing a RPMI medium incubated (24 hr) at 37°C in an incubator containing 5% CO_2.

After 24 hr incubation, A375, Hela and WBC were treated with various concentrations of *Colocasia gigantea* samples and then incubated for 48 hr. Samples-free (DMEM for cancer cell lines and RPMI for WBC) was used as negative control. Doxorubicin was used as the positive control which was an effective anticancer drug

The PrestoBlue^{TM} Cell Viability Reagent was used for quantitative cells viability after treated (48 hr incubation). 10 µl of PrestoBlue^{TM} Cell Viability Reagent was added to each well and then incubated for 30 minutes. The cytotoxicity was determined by measurement the fluorescence of the converted PrestoBlue^{TM} Cell Viability Reagent at a wavelength of 590 nm in a microplate reader (BioTeK, *Synergy HT*). The example of IC_{50} calculation are shown in appendix B. Percent (%) of cell viability was calculated from:

$$\text{Cell viability (\%)} = \frac{\text{Treated cells}}{\text{Negative control}} \times 100$$

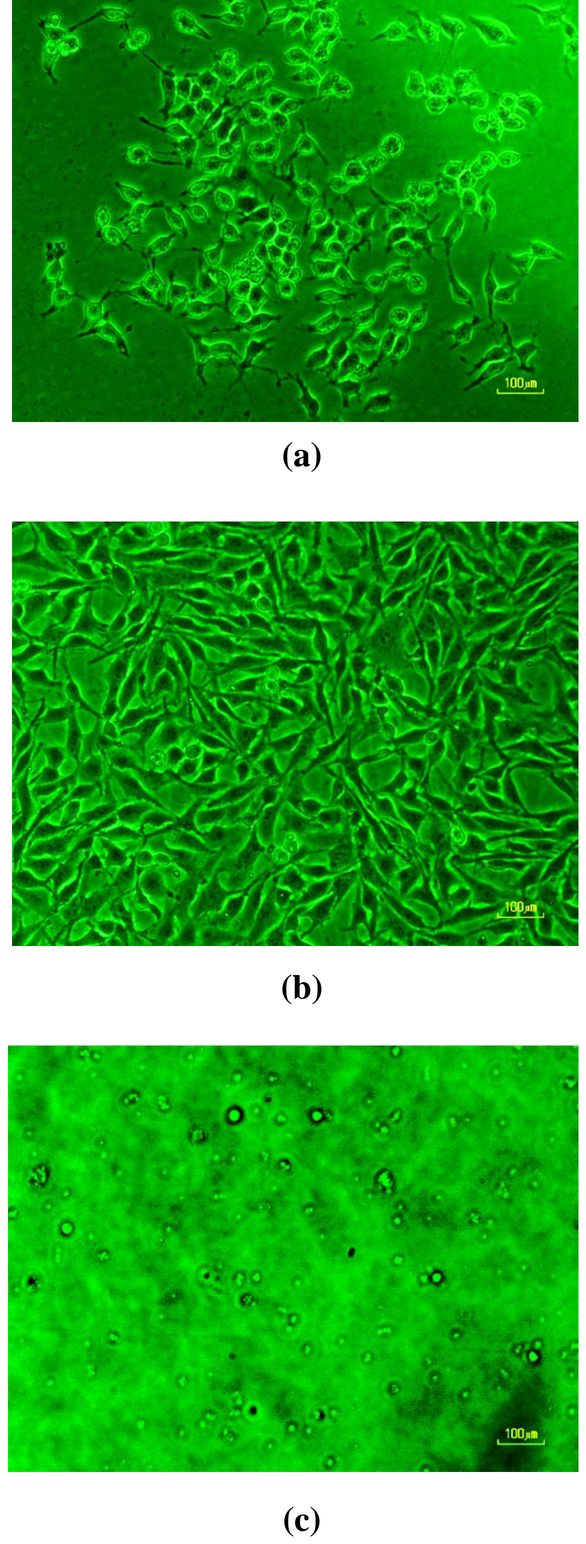


Figure 14: (a) Cervical cancer cells (Hela); (b) Melanoma cancer cells (A375) and (c) White blood cells (WBC)

5.2 Results and Discussions

5.2.1 Screening of the crude extracts for anticancer activity (n-C_6H_{14}, CH_2Cl_2 and CH_3OH extracts)

The objective of initial experiment was to screen the crude n-C_6H_{14}, CH_2Cl_2 and CH_3OH extracts both tuber and leaf of *Colocasia gigantea* for anticancer activity *in vitro*. The concentration of the extracts was 62.5, 125, 250, 500 and 1000 µg/ml. As show in Figure 16, only the fraction 1T from tuber had cytotoxicity effect on Hela and A375 cells. The IC_{50} values were 585 and 351 µg/ml for Hela and A375 cells respectively. Increasing concentration of fraction 1T was result in very significantly decreasing in Hela and A375 cells viability. The IC_{50} values of doxorubicin on Hela and A375 cells were 7.03 and 1.93 µM (equivalent to 3.82 and 1.05 µg/ml respectively). Fraction 1T from tuber not only had cytotoxicity to Hela and A375 cells but also significantly stimulated WBC cells proliferation. Perversely, fraction 2L from leaf enhanced both Hela and A375 cells proliferation very significantly. Hela cells's proliferation was dependent on fraction 2L concentration which the highest proliferation occurred at 250 µg/ml while A375 cells's proliferation was dependent on both fraction 2T, 3T and 2L. The IC_{50} values of all extract samples and doxorubicin on WBC cells were more than 1000 µg/ml and 10 µM (equivalent to 5.43 µg/ml) respectively. From the experimental results, the fraction 1T was selected for further investigation.

The most active extract fraction was loaded in quick column chromatography which packed with silica gel and was eluted sequentially with n-C_6H_{14} (100) followed by CH_2Cl_2:$C_4H_8O_2$ (100:0, 90:10, 80:20, 70:30, 60:40, 50:50 and 0:100), to yield four fractions (4T, 5T, 6T and 7T). Fraction 5T (CH_2Cl_2:$C_4H_8O_2$, 80:20) gave minimum of the half maximal inhibitory concentration value (IC_{50}, µg/ml) and also significantly stimulated WBC cells proliferation. It was chosen for further fractionation after that fraction 5T was passed through flat column chromatography (FCC) on silica gel; n-C_6H_{14}:CH_2Cl_2:$C_4H_8O_2$ (30:65:5), to yield four fractions (8T, 9T, 10T and 11T). Yield and outline of the fractionation and isolation processes are illustrated in Figure 15.

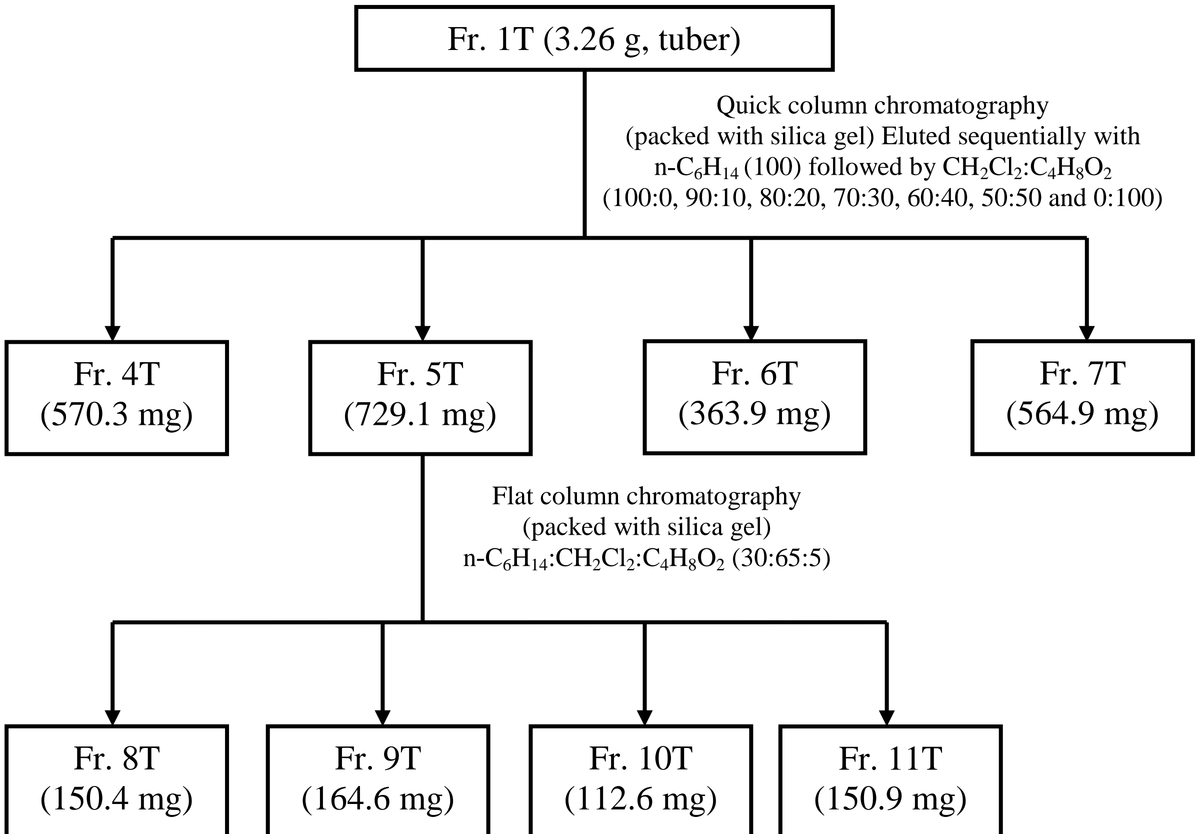


Figure 15: Yield of fractions from fractionation of the bioactive compound for cytotoxic activity of *Colocasia gigantea*

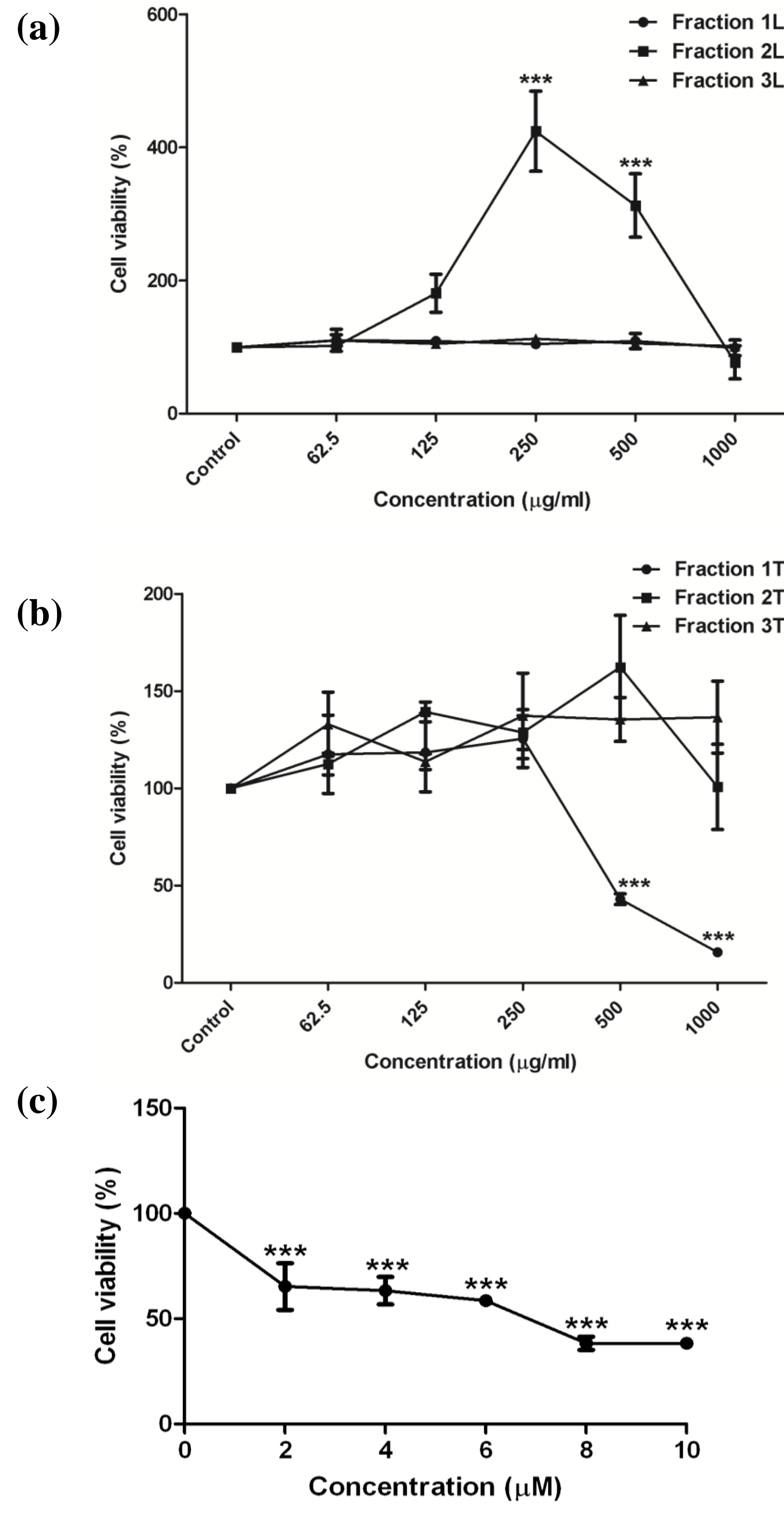


Figure 16: Screening of fraction 1L, 2L, 3L, 1T, 2T and 3T for cytotoxicity on Hela cells *in vitro*, (a) growth inhibition of leaf's fractions (1L, 2L and 3L); (b) growth inhibition of tuber's fractions (1T, 2T and 3T); (c) growth inhibition of doxorubicin; the triple (***) asterisk indicates a significant difference from the control $p<0.001$, one-way ANOVA, Dunett's test. Results are mean values ± SD of independent experiments performed in triplicate.

(d)

(e)

(f)

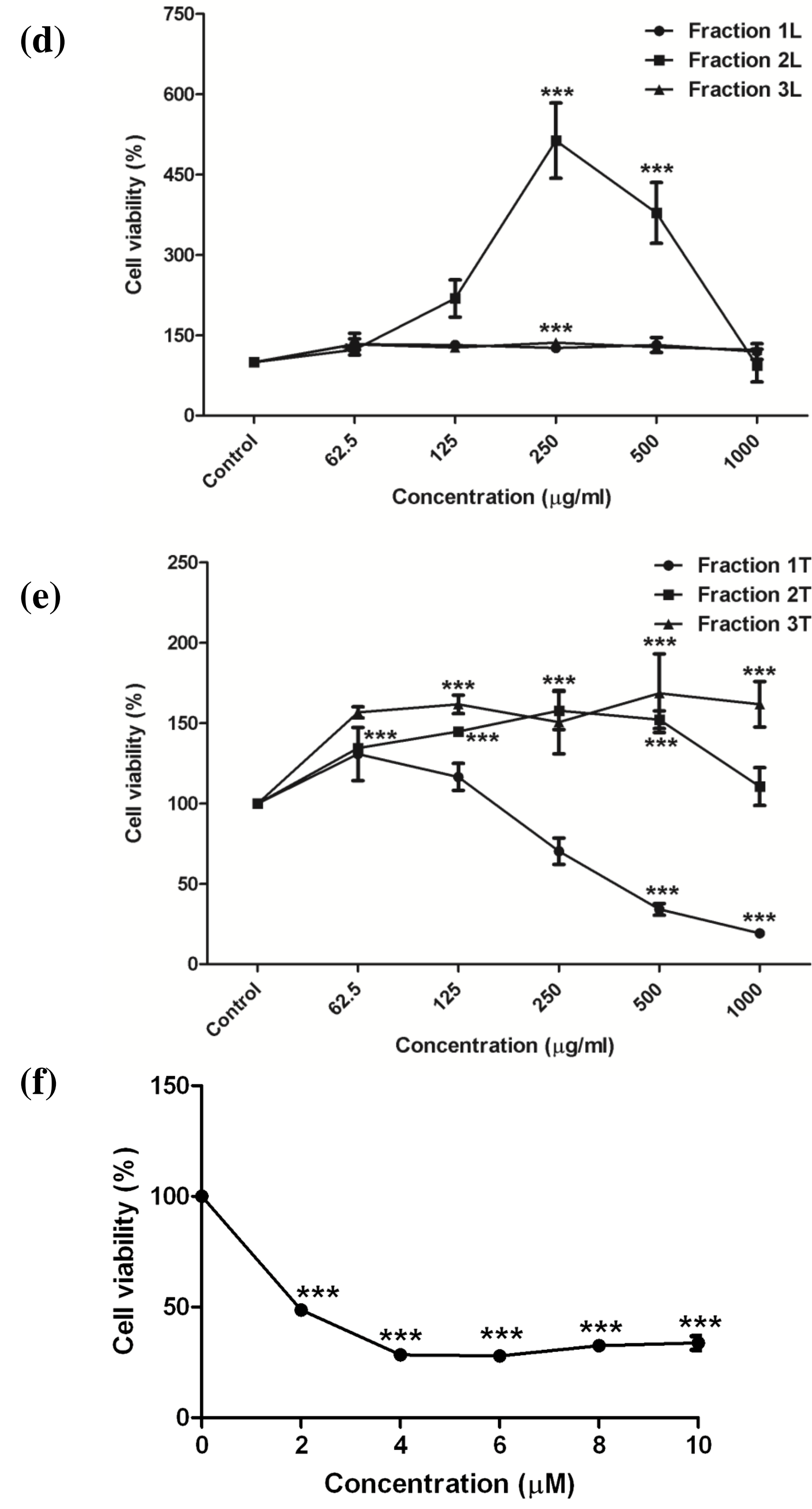


Figure 16 (cont.): Screening of fraction 1L, 2L, 3L, 1T, 2T and 3T for cytotoxicity on A375 cells *in vitro*, (d) growth inhibition of leaf 's fractions (1L, 2L and 3L); (e) growth inhibition of tuber's fractions (1T, 2T and 3T); (f) growth inhibition of doxorubicin; the triple (***) asterisk indicates a significant difference from the control $p<0.001$, one-way ANOVA, Dunett's test. Results are mean values ± SD of independent experiments performed in triplicate.

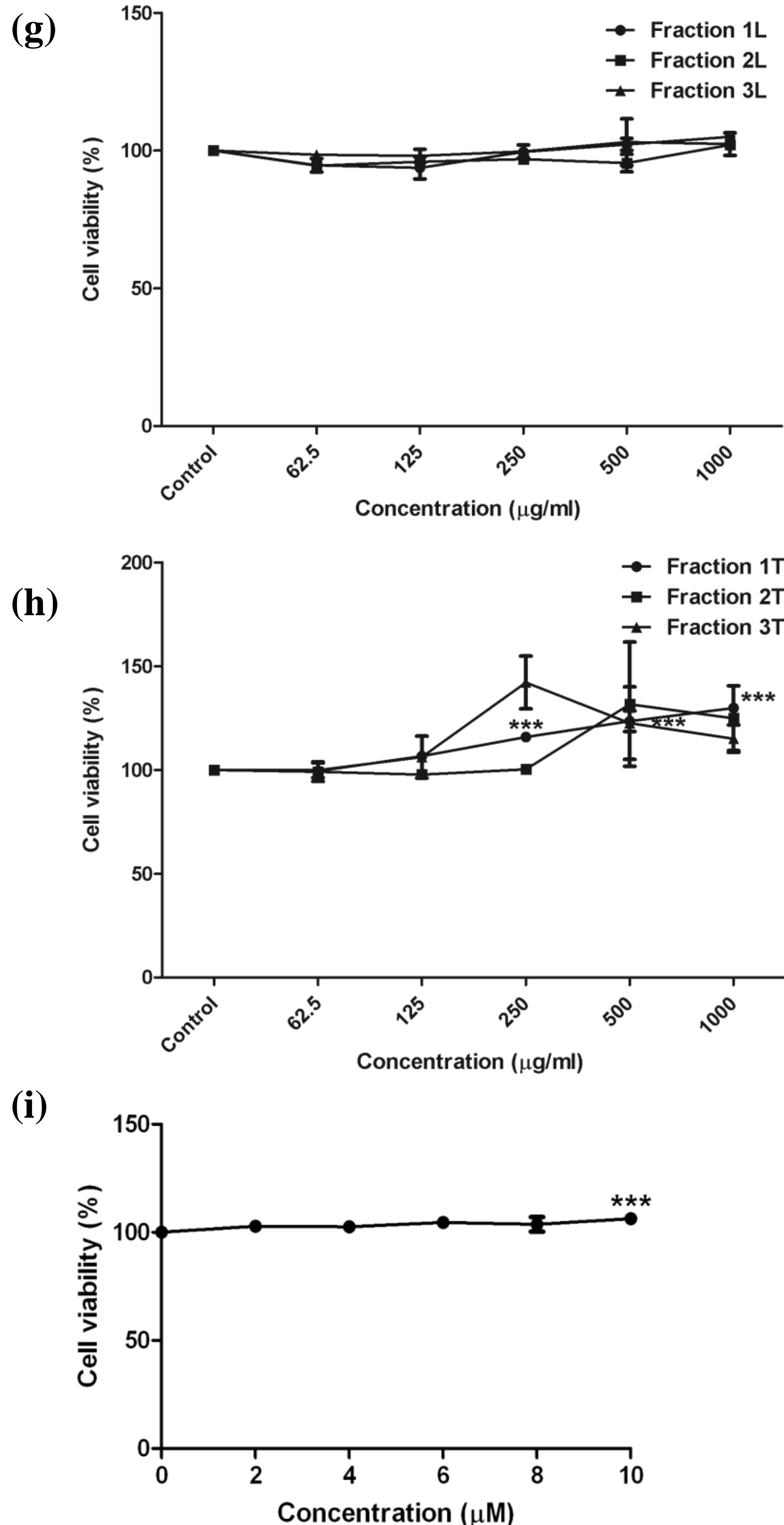


Figure 16 (cont.): Screening of fraction 1L, 2L, 3L, 1T, 2T and 3T for cytotoxicity on WBC cells *in vitro*, (g) growth inhibition of leaf's fractions (1L, 2L and 3L); (h) growth inhibition of tuber's fractions (1T, 2T and 3T); (i) growth inhibition of doxorubicin; the triple (***) asterisk indicates a significant difference from the control $p<0.001$, one-way ANOVA, Dunett's test. Results are mean values ± SD of independent experiments performed in triplicate.

5.2.2 Bioassay-guided fractionation of the fraction 1T from tuber

Since the fractions were collected from quick column chromatography (4T, 5T, 6T and 7T). The TLC pattern of each fraction are shown in appendix C. Each fraction was further cytotoxic testing and results are shown in Table 3. Table 3 show that all of fractions had cytotoxicity effect on Hela and A375 cells. IC_{50} values of all fractions were higher than 500 µg/ml and fraction 5T enhanced very significantly on WBC cells proliferation at concentration 500 µg/ml. Figure 17 show that Hela and A375 cells viability were depended on fraction's concentration. After cytotoxicity testing, fraction 05 was chosen for further isolation because it was not only to inhibit Hela and A375 cells but also enhanced WBC cells proliferation. Increasing concentration of fraction 5T decreased Hela and A375 cells viability. Figure 18 show Hela and A375 cells morphology changing when treated with fraction. It was to confirm fraction 5T ability in term of anticancer activity.

8T, 9T, 10T and 11T fractions were fractionated from fraction 5T which exhibited cytotoxic effect on Hela and A375 cells and leaded WBC cells proliferation. The bioactive ingredients of them after fractionation were analyzed by GC-MS spectroscopy and are shown in Table 4. Analysis from the chromatograms (appendix D) showed the expected bioactive ingredients in fractions 8T, 9T, 10T and 11T which belonged to 4,22-stigmastadiene-3-one, diazoprogesterone, 9-octadecenoic acid (Z)-, hexyl ester and oleic acid respectively.

Colocasia esulenta has been reported to possess several biologically active ingredients and has been used as foods and traditional medicinal plant. Although, there are many reports highlighting biological activities of the phytochemicals in *C. esculenta*. On the contrary, there are practically few reports on *Colocasia gigantea*'s biological activities and its bioactive ingredients. This was the first time to report cytotoxic activity of *C. gigantea* extract to Hela, A375 and WBC cells and the expected bioactive ingredients. In this study, it was shown that not all part of *C. gigantea* have cytotoxic effect on Hela and A375 cells whereas some of *C. gigantea* leaf fraction can promote Hela and A375 cells proliferation significantly. In the same manner, Wei et al., 2011 reported that *C. esculenta*'s leaf extract also encourage human breast adenocarcinoma (MCF-7) proliferation. I suggested that this effect should be considered when using *C. gigantea* leaf as an ingredient in cancer patients's food. *C. gigantea* extract fraction 1T from tuber can have two different path way for cancer cells inhibition. At first, it can directly restrain cancer cells proliferation by its bioactive ingredients. Secondly, it also encourages the immune system by performing like a mitogen (Brown et al., 2005). Ohno et al., 1994 reported that mitogen activated lymphocytes can kill numerous types of colon cancer cells, both in humans and in rodents.

Table 3: The half maximal inhibitory concentration (IC_{50}, µg/ml) of fraction 4T, 5T, 6T and 7T on Hela, A375 and WBC cells *in vitro*

Fractions	**The half maximal inhibitory concentration (IC_{50}, µg/ml)**		
	Hela	**A375**	**WBC**
4T	104.1	24.27	> 500
5T	93.03	26.02	> 500
6T	166.1	30.04	> 500
7T	210.1	54.88	> 500
Doxorubicin	3.82	1.05	> 500

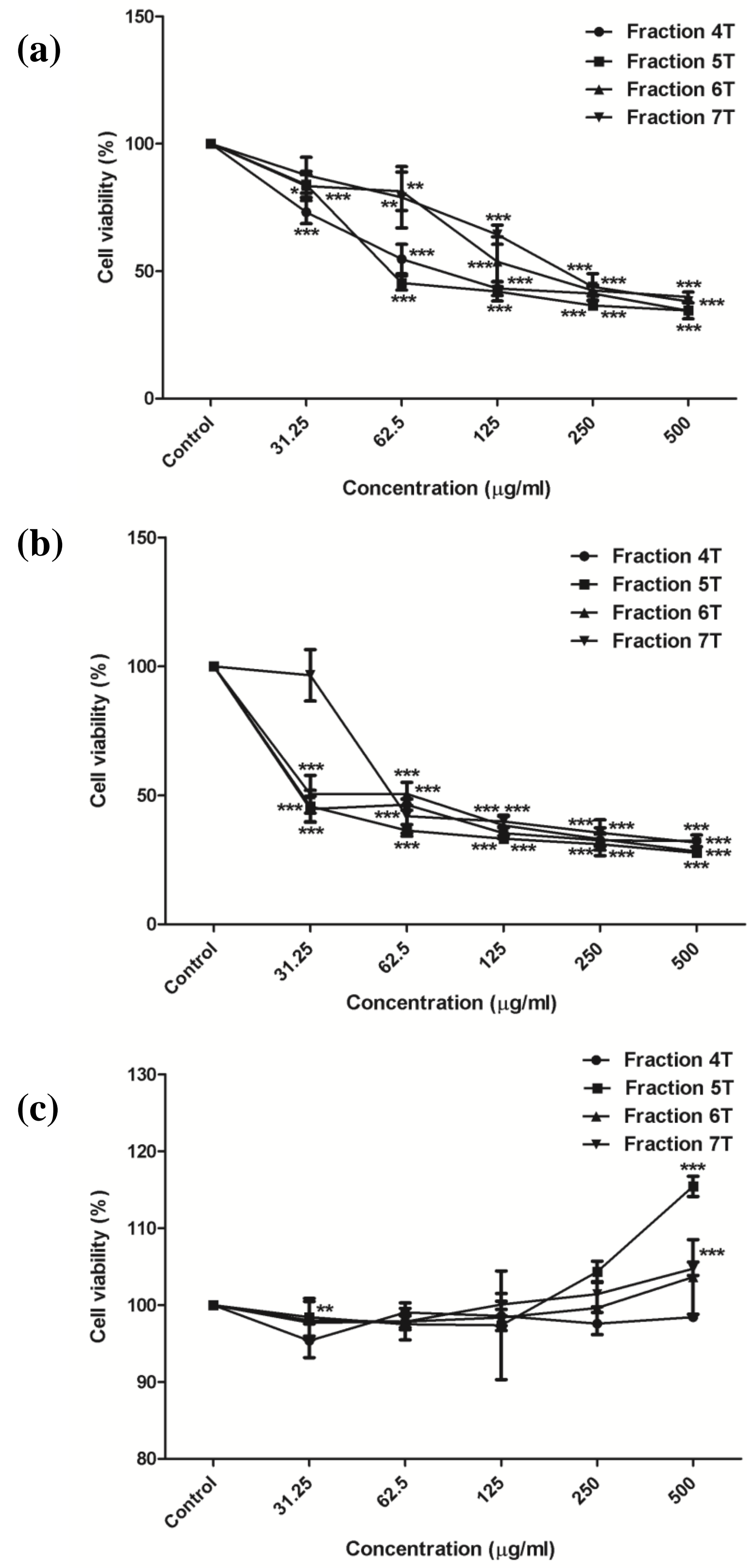


Figure 17: The cytotoxic activity of fraction 4T, 5T, 6T and 7T *in vitro*;(a) The effect of the fractions on Hela cells; (b) The effect of the fractions on A375 cells; (c) The effect of the fractions on WBC Cells; the triple (***) asterisk indicates a significant difference from the control $p<0.001$, one-way ANOVA, Dunett's test. Results are mean values ± SD of independent experiments performed in triplicate.

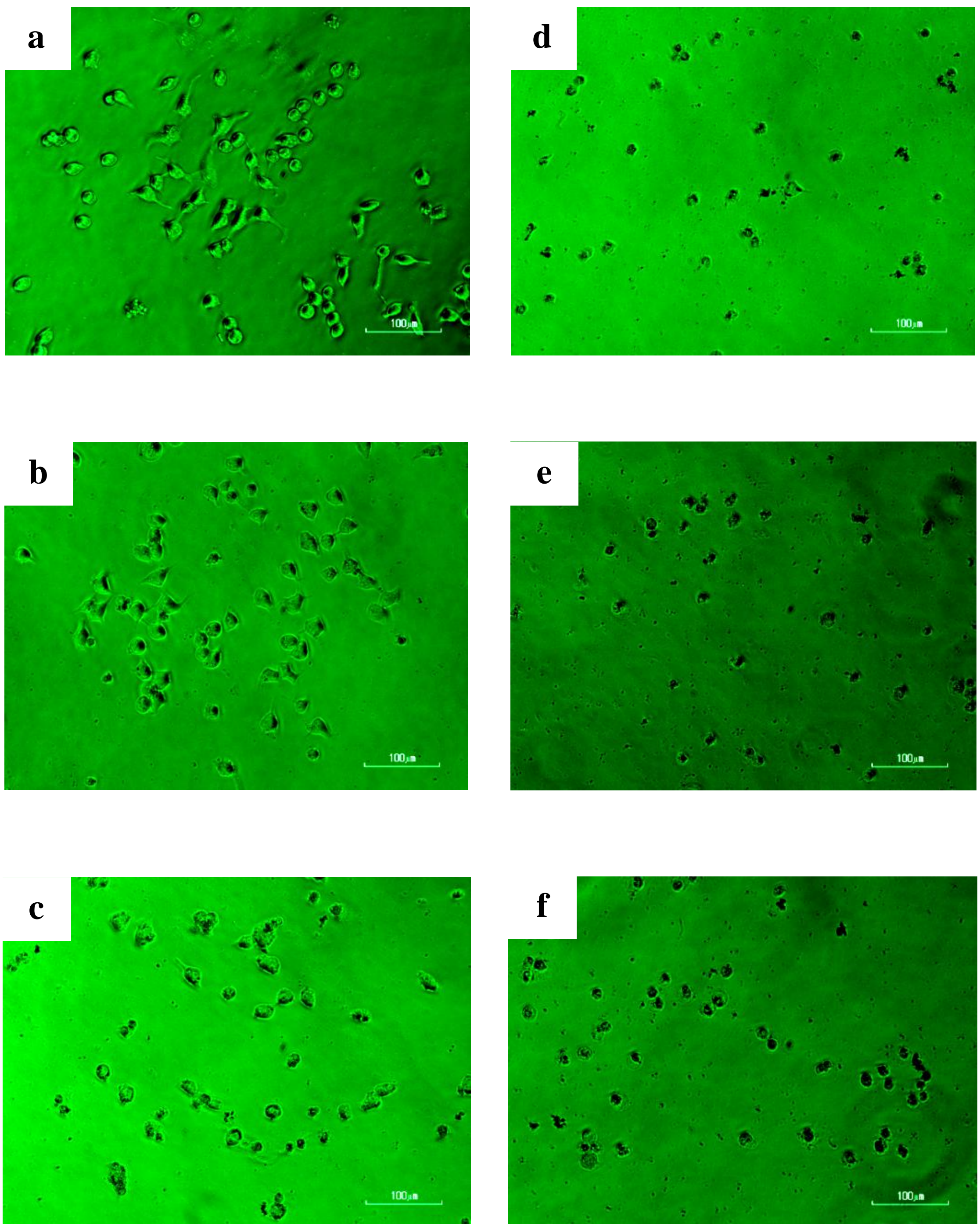


Figure 18: The Hela cell's morphology after treated fraction 5T; (a) Control; (b-f) Hela cells were treated with fraction 5T at concentration 32.25, 61.5, 125, 250 and 500 μg/ml respectively.

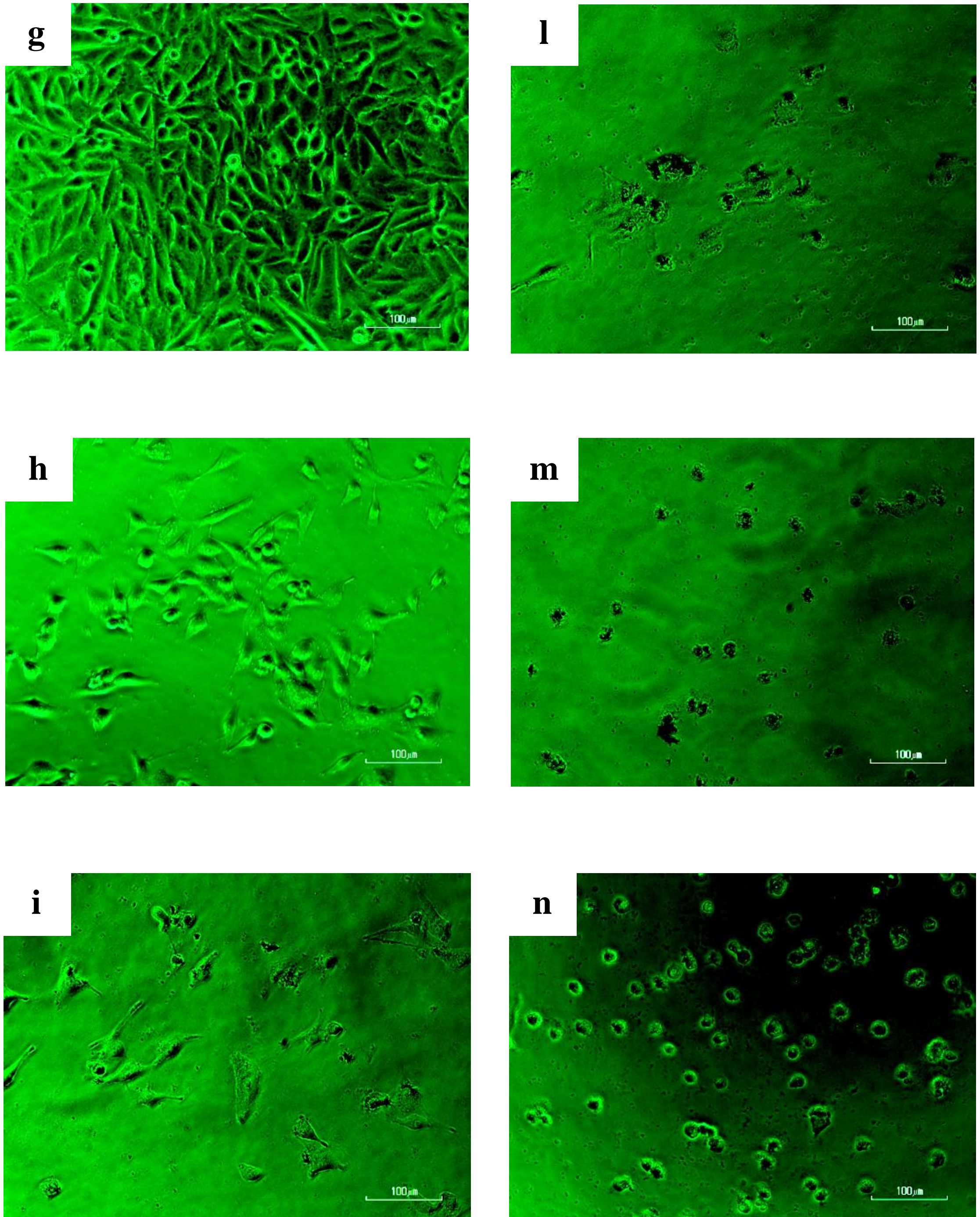


Figure 18 (cont.): The A375 cell's morphology after treated fraction 5T; (g) Control; (h-n) A375 cells were treated with fraction 5T concentration 32.25, 61.5, 125, 250 and 500 μg/ml respectively.

Table 4: The chemical ingredients of the fraction 8T and 9T which were fractionated from fraction 5T and analyzed by GC-MS spectroscopy.

No.	Rt (sec)	Mol. Formula	%	Name
Fraction 8T				
1	150.9	$C_6H_{12}O$	1.34	Furan, tetrahydro-2,5-dimethyl-
2	183.75	$C_3H_8O_2$	39.08	(S)-(+)-1,2-Propanediol
3	226.95	$C_5H_8O_2$	1.23	1-Propen-2-ol, acetate
4	240.75	$C_6H_{12}O$	3.49	Hexanal
5	1160.75	$C_{10}H_{18}O_3$	1.46	Nonanoic acid, 9-oxo-, methyl ester
6	1627.7	$C_6H_{12}O$	1.31	Pentanal, 2-methyl-
7	1868.8	$C_7H_{17}NO$	0.74	Dimethylamine, N-(neopentyloxy)-
8	2022.7	$C_{18}H_{34}O_2$	3.90	Oleic Acid
9	2037.1	$C_{19}H_{37}MoNO_3P_2$	0.95	Molybdenum, tricarbonyl-[N-butyl-bis[2-(butylphosphino)ethyl] amine]
10	2099.8	C_{11}H19ClO	4.74	10-Undecenoyl chloride
11	2240.1	$C_{28}H_{46}O_4$	0.98	Phthalic acid, dodecyl 2-ethylhexyl ester
12	2276.3	$C_{12}H_{22}N_2O_4$	6.63	N,N'-Bis(2-methyl-2-nitrosopentan-4-one)
13	2464.75	$C_{10}H_{16}O_2$	3.04	4,8-Dioxatricyclo[5.1.0.0(3,5)]octane, 1-methyl-5-(1-methylethyl)-, (1à,3á,5á,7à)-
14	2804.05	$C_{26}H_{36}O_8$	2.41	1H-Cyclopropa[3,4]benz[1,2-e]azulene-5,7b,9,9a-tetrol, 1a,1b,4,4a,5,7a,8,9-octahydro-3-(hydroxymethyl)-1,1,6,8-9a-triacetate, [1aR-(1aà,1bá,4aá,5á,7aà,7bà,8à,9á,9aà)]-tetramethyl-, 5,9,
15	2824.4	$C_{29}H_{46}O$	10.66	4,22-Stigmastadiene-3-one
16	2874.1	$C_{21}H_{30}N_4$	5.15	Diazoprogesterone
Fraction 9T				
1	239.55	C_7H_{16}	4.18	Hexane, 3-methyl-
2	241.05	$C_4H_{10}N_2$	4.12	(2-Aziridinylethyl)amine
3	2022.45	$C_{18}H_{34}O_2$	13.65	Oleic Acid
4	2718.2	$C_{27}H_{38}O_4S_2$	18.17	2á,4a-Epoxymethylphenanthrene-7-methanol, 1,1-dimethyl-2-methoxy-8-(1,3-dithiin-2-ylidene)methyl-acetate1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-,
5	2736.7	$C_{21}H_{30}N_4$	48.42	Diazoprogesterone

Table 4 (cont.): The chemical ingredients of the fraction 10T and 11T which were fractionated from fraction 5T and analyzed by GC-MS spectroscopy.

No.	Rt (sec)	Mol. Formula	%	Name
Fraction 10T				
1	150.7	$C_6H_{12}O$	2.66	Furan, tetrahydro-2,5-dimethyl-
2	226.45	$C_5H_8O_2$	4.44	1-Propen-2-ol, acetate
3	239.55	$C_{32}H_{45}NO_3$	10.36	3'H-Cycloprop(1,2) cholesta-1,4,6-trien-3- one, 1'-carboethoxy-1' -cyano-1á,2á-dihydro-
4	240.45	$C_6H_{12}O$	16.27	Hexanal
5	573.45	$C_6H_{12}O_2$	0.65	Pentanoic acid, 2-methyl-
6	700.65	$C_{12}H_{20}O_4$	0.76	Oxalic acid, allyl heptyl ester
7	728.75	$C_9H_{18}O_2$	1.69	Octanoic acid, methyl ester
8	932.95	$C_7H_{12}O$	0.94	4-Pentenal, 2-ethyl-
9	1026	$C_9H_{16}O_3$	1.42	Methyl 8-oxooctanoate
10	1040.35	$C_{11}H_{20}O$	1.01	2-n-Hexylcyclopentanone
11	1116	$C_9H_{16}O_2$	4.78	4-Octenoic acid, methyl ester, (Z)-
12	1160.9	$C_{10}H_{18}O_3$	9.82	Nonanoic acid, 9-oxo-, methyl ester
13	1627.7	$C_8H_{16}O$	1.71	2-Heptanone, 5-methyl-
14	1712.2	$C_8H_{16}O_2$	0.96	2-Methylheptanoic acid
15	1869.95	$C_{13}H_{26}O_2$	2.69	Decanoic acid, propyl ester
16	2023.7	$C_{24}H_{46}O_2$	18.08	9-Octadecenoic acid (Z)-, hexyl ester
17	2047	$C_7H_{17}NO$	0.77	Dimethylamine, N-(neopentyloxy)-
18	2051.7	$C_9H_{17}ClO$	2.92	Nonanoyl chloride
19	2095.35	$C_{10}H_{16}O_2$	1.44	Tetrahydrofuran-2-one, 3-[2-pentenyl]-4-methyl-
Fraction 11T				
1	151.15	$C_6H_{12}O$	1.40	Furan, tetrahydro-2,5-dimethyl-
2	536.55	$C_5H_{10}O_2$	13.02	Pentanoic acid
3	1115.75	$C_7H_{14}O_2$	2.69	Hexanoic acid, methyl ester
4	2022.7	$C_{18}H_{34}O_2$	23.58	Oleic Acid
5	2050.4	$C_{11}H_{20}O_3$	2.43	Decanoic acid, 2-oxo-, methyl ester
6	2240.15	$C_{27}H_{38}O_4S_2$	17.86	2á,4a-Epoxymethylphenanthrene-7-methanol, 1,1-dimethyl-2-methoxy-8-(1,3-dithiin-2-ylidene)methyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-, acetate

To demonstrate that the cytotoxicity of bioactive ingredients must specify to the cancer cells and safety to the consumers, human normal white blood cells were used. Fraction 1T from tuber significantly stimulated WBC cells proliferation. The experimental result on WBC cells was resembled to Brown et al., 2005 experimental result when they treated *Colocasia esculenta* extract on normal mouse splenocyte cells. They suggested that *C. esculenta* has an endogenous mitogen, and a mannose-binding lectin similar to Phasleus vulgaris (PHA-P) that activates lymphocytes (Bezerra et al., 1995; Shewry et al., 2003). Some of lectins can induce lymphocyte proliferation by interleukin-2 production. Lymphocytes incubated 1-2 days with high doses of interleukin-2, could induced normal lymphocytes to lymphokine activated killer cells (LAK). LAK cells have a non-specific tumoricidal activity that kill various types of cancer cells (Ohno et al., 1994).

The main idea of bioactive ingredients anti-cancer property *in vitro* has concentrated on non-nutritive plant compounds (Brown et al., 2005) . Bioassay-guided fractionation was a method for identification of the active ingredients. Column chromatography was a tool for fractionation of bioactive ingredients in a study. TLC and GC-MS spectroscopy were the equipments to observe the various components in each fraction. 4,22-Stigmastadiene-3-one and Diazoprogesterone can be classified to phytosterol. There have been many reports about anticancer activities of phytosterol (Woyengo et al., 2009; Awad and Fink , 2000). Phytosterol can against carcinogenesis by various mechanisms as show in Figure 19. According to Figure 19, phytosterols in *Colocasia gigantea* may are bioactive ingredients that play an important role in an anticancer compound. In 2004, Awad et al. reported the mechanism of phytosterol on cancer cells that phytosterol alleviated cancer development by reducing the production of carcinogens and In 2005, Vivancos and Moreno reported that phytosterol increased the activities of antioxidant enzymes, superoxide dismutase and glutathione peroxidase in cultured macrophage cells. They suggested that phytosterol prevented cells from damage by reactive oxygen species. Phytosterol also has induced apoptosis in cancer cells (an important mechanism in the inhibition of carcinogenesis (Woyengo et al., 2009). Park et al. 2007, Moon et al. 2007 and Rubis et al., 2008 published the apoptosis mechanism of β-sitosterol that It increased activity of caspase-3 and Bcl-2 pathway was inactivated. Furthermore, Moon et al., 2007 explained the activation of caspase that phytosterol could be mediate by extracellular signals that were complemented by mitochondrial pathways. Moreover, phytosterol also inhibited angiogenesis and metastasis in cancer cell proliferation (Prescott, 2000). Choi et al., 2007 found out that campesterol induced angiogenesis in endothelial cells isolated from human umbilical vein and reduced proliferation of the cells by campesterol treatment. The anticancer property mechanism of oleic acid was proposed by Menendez et al., 2005. They observed that oleic acid supports trastuzumab (Herceptin, Anticancer medicine) when used against cancer cell cultures. They suggested that oleic acid up-regulates polyomavirus enhancer activator 3 (PEA3) which represses the expression of Human Epidermal Growth Factor Receptor 2

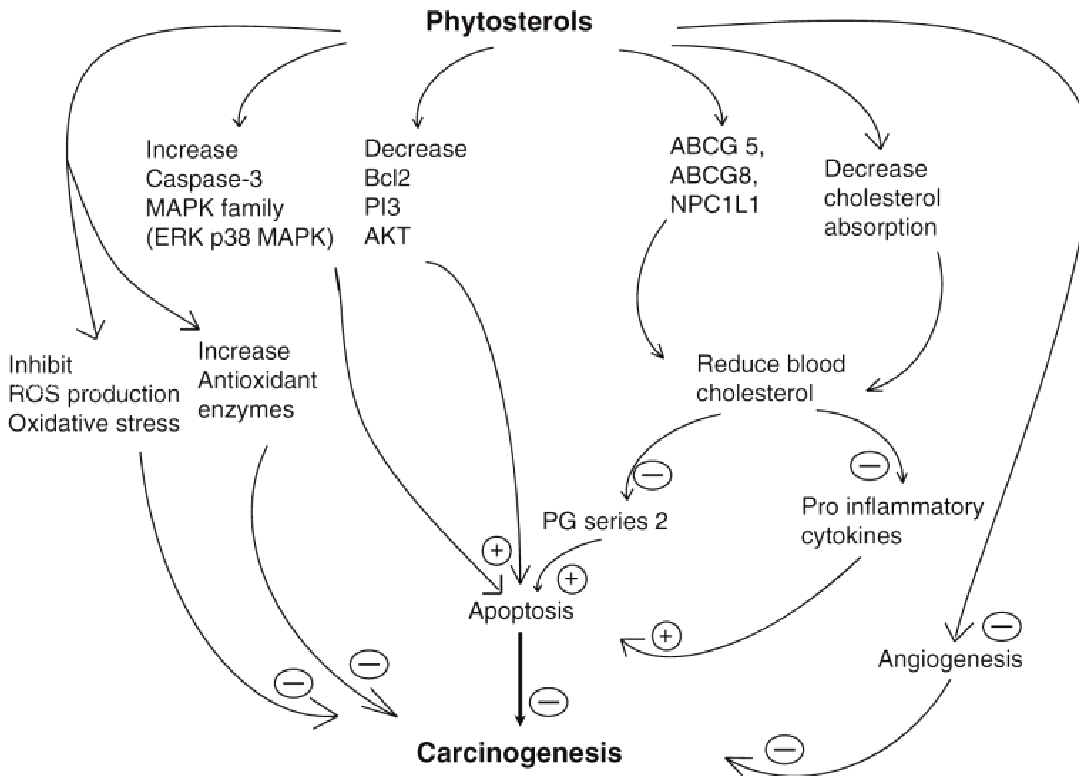


Figure 19: Various phytosterol's mechanisms on carcinogenesis (Woyengo et al., 2009)

(HER2/*neu*), a causing of breast cancers. Therefore, high levels of PEA3 result in low levels of HER2/*neu* that prevented the risk of cancers occurring by HER2/*neu* over-expression (Menendez et al., 2005). Valko et al., 2007 and Sakthivel et al., 2010 reported that Diazoprogesterone, 9-Octadecenoic acid (Z)-, hexyl ester and Oleic Acid not only have an anticancer activity but also have antimicrobial, hypercholesterolaemic and anti-ulcerogenic activities.

5.3 Conclusions

The extract from the tuber of *Colocasia gigantea* (using n-hexane as a solvent) has a cytotoxic activity on Hela and A375 cells. It also enhances WBC cells proliferation.

On the other hand, the extract from the leaf of *C. gigantea* (using dichloromethane as a solvent) has not a cytotoxic activity on Hela and A375 cells and promotes Hela and A375 cells proliferation significantly.

Chapter VI

Conclusions

This study evaluated the phenolic compounds which are the powerful natural antioxidant compounds and also explored the biological activities of *Colocasia gigantea*'s extract in term of antimicrobial and cytotoxic activities.

The opitimal solvent condition for the extraction of phenolic compounds was obtained by varying the solvent species and compositions. The optimal solvent polarity for extraction phenolic compounds from *C. gigantea* was predicted as a solubility parameter approximately 33.6 and 42.5 $MPa^{0.5}$ for tuber and leaf respectively. This method can apply for designed solvent in many extraction processes.

The antimicrobial activity of *C. gigantea* was studied against gram positive and negative bacterial strains (*Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*). The extracts from leaf of *C. gigantea* were found to be more effective than extracts from tuber against all microbial strains in this experiment.

In the experimental result of cytotoxic study showed that not all part of *C. gigantea* had cytotoxicity effect to cervical cancer and melanoma cells whereas some of *C. gigantea* leaf fraction could promote Hela and A375 cells proliferation significantly. The potential anticancer property of *C. gigantea* was found in the extract from tuber (using n-C_6H_{14} as a solvent) against Hela and A375 cells *in vitro* and stimulate WBC cells proliferation. *C. gigantea* extract from tuber by using n-C_6H_{14} as a solvent can be developed to novel cancer chemotherapy agent in the treatment or prevention of cervical cancer and melanoma and deserve to be investigated in the future.

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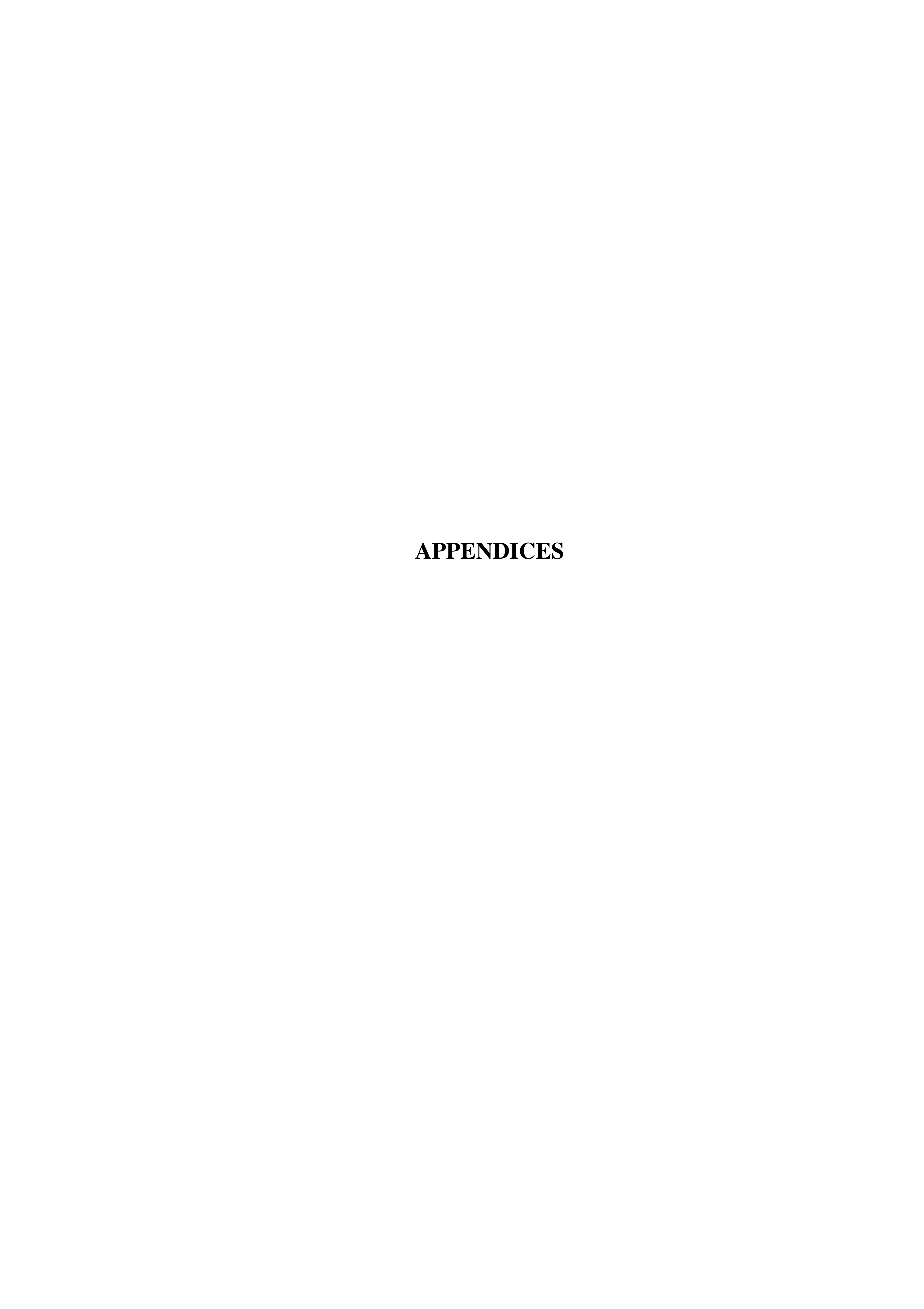

APPENDICES

APPENDIX A

Total phenolic content and design of optimal solvent

Table A1: Raw data of total phenolic content determination from *Colocasia gigantea* both tuber and leaf

Equation (Standard curve); x= ((y+0.0078)/0.7253)

y: ABS, x: gallic acid concentration (mg/ml)

Methanol ratio (%) / Experiment	**Tuber mg/g (dry weight)**		
	1st	**2nd**	**3rd**
0	88	90	76
20	66	75	61
40	59	56	45
60	62	63	58
80	196	199	181
100	43	41	37
	Leaf mg/g (dry weight)		
0	144	146	149
20	149	151	153
40	146	140	140
60	153	153	158
80	118	116	120
100	98	94	97

* Absorbance at 765 nm

Raw material : Solvent; 1 g : 30 ml

Design of optimal solvent for extraction of phenolic compounds from *Colocasia gigantea*

The concept of solubility parameter of pure specie *i* (δ_i) represent the relation between the extraction efficiency of phenolic compounds and solvent's polarity (Kim et al., 2007). The solubility parameter can write in term of the cohesive energy ($E_{coh,i}$) which is a linear combination of hydrogen bonding interaction ($E_{h,i}$), polar interaction ($E_{p,i}$) and dispersion interaction ($E_{d,i}$) (Kim et al., 2007). The relation of solubility parameter and the cohesive energy is shown in equation 1 (Kim et al., 2007; Hertz, 1989).

$$\delta_i = \sqrt{\frac{\Delta H_{v,i} - RT}{V_i}} = \sqrt{\frac{E_{coh,i}}{V_i}} = \sqrt{\frac{E_{h,i} + E_{p,i} + E_{d,i}}{V_i}} \quad (1)$$

This equation concept was derived by Hildebrand and Scott (Hertz, 1989). They derived the equation from ideal gas law (nonpolar fluids (nonelectrolytes)) where $H_{v,i}$ is a heat of vaporization of specie *i* , R is a gas constant, T is a absolute temperature and V_i is a mole volume of pure specie *i*. The value of δ_i is reported in $MPa^{0.5}$ unit. This equation is only suitable for vapors obeying the ideal gas law such as nonpolar fluids (nonelectrolytes) (Hertz, 1989).

For the polar solvent, the equation 1 can be rearranged by Hansen modification. In the Hansen modification, there are three major forces that must be considered: electrostatic force (dipole moment), hydrogen bonding interaction and dispersion force (London force). Therefore, the solubility parameter can be changed as:

$$\delta_i = \sqrt{\delta_{d,i}^2 + \delta_{p,i}^2 + \delta_{h,i}^2} \quad (2)$$

where $\delta_{d,i}$ is the dispersion interaction's solubility parameter of species *i*, $\delta_{p,i}$ is the electrostatic force (polar) interaction's solubility parameter of species *i* and $\delta_{h,i}$ is the hydrogen bonding interaction's solubility parameter of species *i*. For the pure species *i*, the solubility parameter is summarized in table A2. Kim et al., 2007 introduced that the solubility parameters of alcohol-water binary mixtures could be approximated by simple mixing rule as show in equation 3.

$$\delta_m = \sum_i x_i \delta_i \quad (3)$$

where δ_m is a solubility parameter of the binary mixtures and x_i is a volume fraction of pure species *i*. Furthermore, Barton described the effect of temperature for the solubility parameter when the temperature is changing (Kim et al., 2007).

$$\left(\frac{\delta_1}{\delta_2}\right)^2 = \frac{T_2}{T_1} \quad (4)$$

where T_i is a extraction temperature (K).

Table A2: Solubility parameters of solvent (Kim et al., 2007)

Solvent	**Molecular weight ($g/mole^{-1}$)**	**Molecular volume ($cm^3 mole^{-1}$)**	**δ ($MPa^{0.5}$)**		
			δ_h	**δ_p**	**δ_d**
Water	18.02	18.1	40.4	22.8	12.2
Methanol	32.0	40.7	24.0	13.0	11.6
Ethanol	46.1	58.7	20.0	11.2	12.6
n-Propanol	60.1	75.2	17.7	10.5	14.1
iso-propanol	60.1	76.8	16.0	9.8	14.0
Ethylene glycol	62.1	55.9	29.8	15.1	10.1
Acetone	58.1	74.0	11.0	9.8	13.0

Solvent	**Molecular weight ($g/mole^{-1}$)**	**Molecular volume ($cm^3 mole^{-1}$)**	**δ ($MPa^{0.5}$)**	
			δ (25^oC)	**δ (80^oC)**
Water	18.02	18.1	48.0	44.1
Methanol	32.0	40.7	29.7	27.3
Ethanol	46.1	58.7	26.1	24.0
n-Propanol	60.1	75.2	24.9	22.9
iso-propanol	60.1	76.8	23.4	21.5
Ethylene glycol	62.1	55.9	34.9	32.1
Acetone	58.1	74.0	19.7	18.1

Example I (for solubility parameter's calculation)

Calculated the solubility parameter for a binary mixture methanol-water at ratio 20:80 at 25°C (room temperature)

Solution

Assumptions

1. The mixture followed the simple mixing rule.
2. Constant temperature at 25°C
3. Liquid phase mixture (In liquid phase mixture, the solubility parameter would be followed Hansen modification.)

For simple mixing rule: $\delta_m = \sum_i x_i \delta_i$

At table A2: the solubility parameter for methanol and water at 25°C is 48.0 and 29.7 respectively.

For the volume fraction of water and methanol at $x_i = 0.5$;

$$\delta_m = \sum_2 0.5(48.0) + 0.5(29.7)\ \mathrm{MPa}^{0.5}$$

$$\delta_m = 38.85\ \mathrm{MPa}^{0.5}\ \underline{\text{Ans}}$$

Note: For the another water-methanol fractions, they are same method and assumptions for calculated solubility parameters.

Example II (for estimated the optimal solubility parameter)

In the Figure 17b show the correlations of content of phenolics from *Colocasia gigantea* in the leaf with polarity of solvent. The fitting equation for correlation was obtained from Microsoft excel 2007 software.

The fitting equation received from Microsoft excel 2007 software:

$$y = -0.3325x^2 + 28.282x - 449.16, \quad R^2 = 0.9131$$

where y is a content of phenolics (mg/g) and x is a polarity (MPa$^{0.5}$)

The optimal condition could received from mathematics solving (differential equation).

$$\frac{dy}{dx} = -0.3325(2)x + 28.282 = 0$$

$$x = 42.5293 \approx 42.53\ \mathrm{MPa}^{0.5}\ \underline{\text{Ans}}$$

APPENDIX B

Cytotoxicity assay

Example for cell viability's and IC_{50} calculation

In this example show only the Hela cells viability and IC_{50}'s calculation when were treated with fraction 1T from tuber of *Colocasia gigantea*.

Step 1 Normalization of the raw data

Table B1: Hela cells's viability raw data

Sample concentration (µg/ml)	Absorbance at 590 nm		
	First experiment	Second experiment	Third experiment
0	10068	9938	10470
62.5	9531	12433	13891
125	9609	13266	13238
250	11144	13948	13155
500	4020	4372	4727
1000	1725	1614	1446

Table B2: Hela cells's viability after normalization

Sample concentration (µg/ml)	Absorbance at 590 nm		
	First experiment	Second experiment	Third experiment
0	100.0	100.0	100.00
62.5	94.66	125.1	132.7
125	95.44	133.5	126.4
250	110.7	140.4	125.6
500	39.93	43.99	45.15
1000	17.13	16.24	13.81

Step 2 Calculate the mean value of the triplicate experiment and plot a graph for finding a IC_{50} value

Table B3: Mean and standard deviation value of the triplicate experiment

Sample concentration (µg/ml)	Statistics value	
	Mean (%)	Standard deviation (%)
0	100	0
62.5	117.5	20.12
125	118.5	20.24
250	125.6	14.84
500	43.02	2.74
1000	15.73	1.72

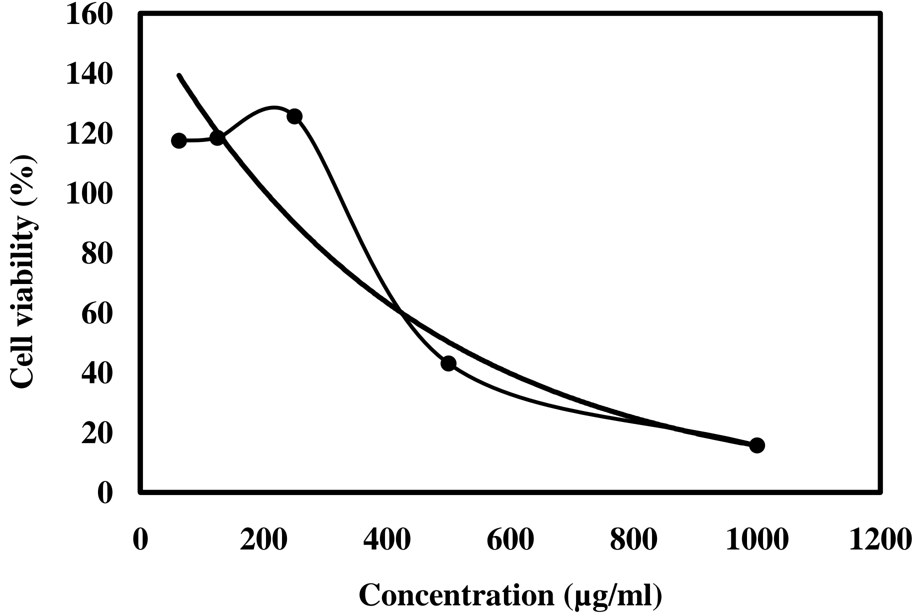

Figure B1: Relation between sample concentration (µg/ml) and Hela cells's viability (%) after were treated with fraction 1T *Colocasia gigantea* tuber.

From the Figure B1, the relation between sample concentration and cell viability was obtained from Microsoft excel 2007 software.

The fitting equation received from Microsoft excel 2007 software:

$$y = 161.12e^{-0.002x}, \quad R^2 = 0.9506$$

where y is a cell viability (%) and x is a sample concentration (µg/ml)

Finding the IC_{50} value, y should be set at 50% cell viability and solving for x value;

$$50 = 161.12e^{-0.002x}$$

$$x = 585.06 \approx 585 \frac{\mu g}{ml} \quad \underline{\text{Ans}}$$

APPENDIX C

Chromatographic patterns (TLC)

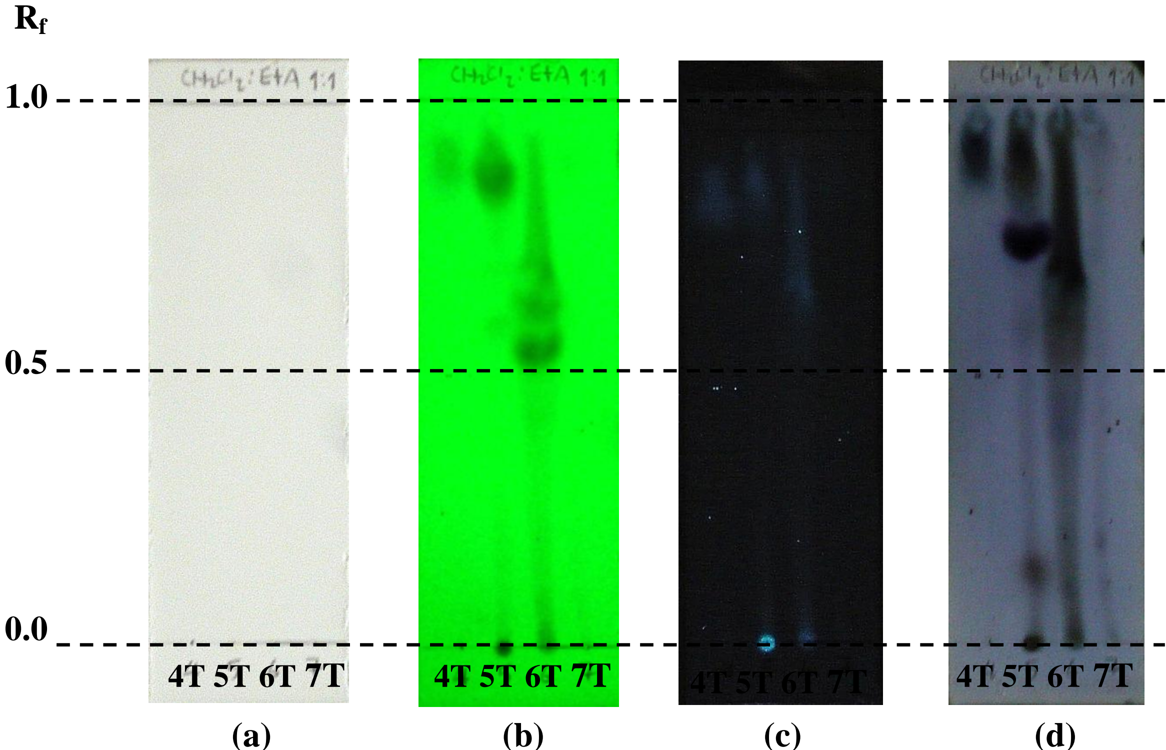

Figure C1: TLC profiles of the fraction 4T, 5T, 6T and 7T of *Colocasia gigantea*'s tuber. The solvent system was dichloromethane:ethyl acetate (1:1) and the plates were viewed under, (a) visible light; (b) UV light at 254 nm; (c) UV light at 354 nm and (d) visible light after being sprayed with 5% anisaldehyde.

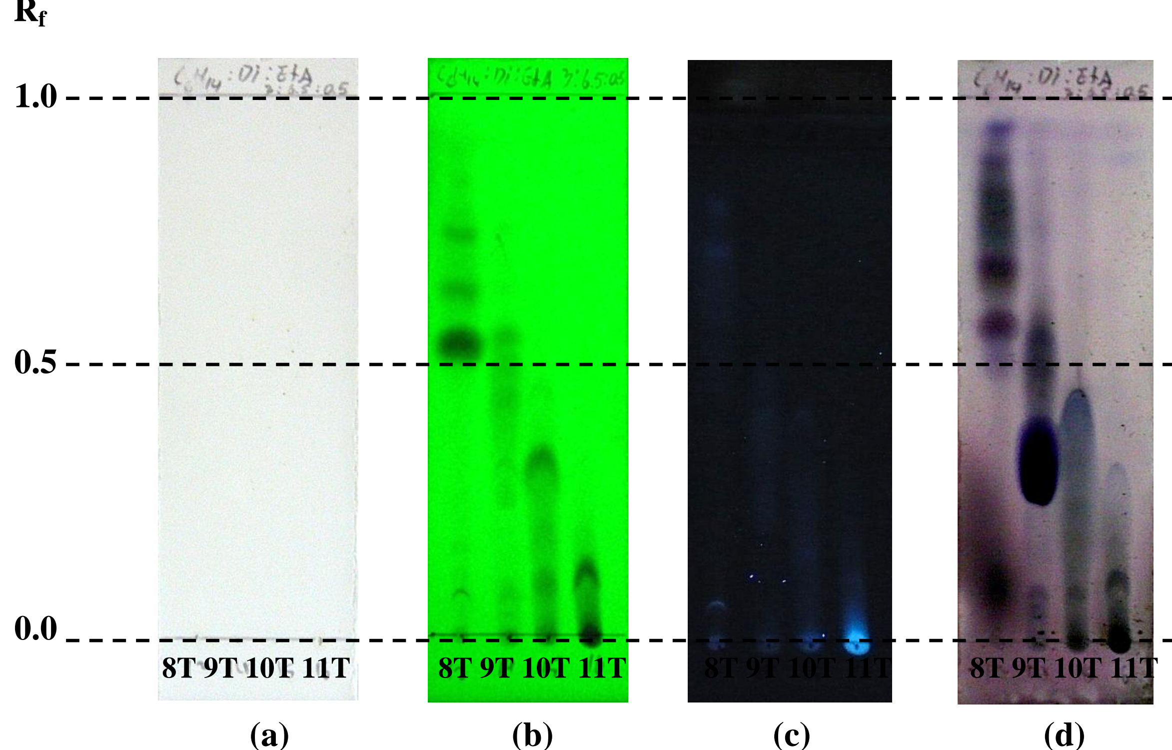

Figure C2: TLC profiles of the fraction 8T, 9T, 10T and 11T of *Colocasia gigantea*'s tuber. The solvent system was n-hexane:dichloromethane:ethyl acetate (3:6.5:0.5) and the plates were viewed under, (a) visible light; (b) UV light at 254 nm; (c) UV light at 354 nm and (d) visible light after being sprayed with 5% anisaldehyde.

APPENDIX D

GC-MS spectroscopy data

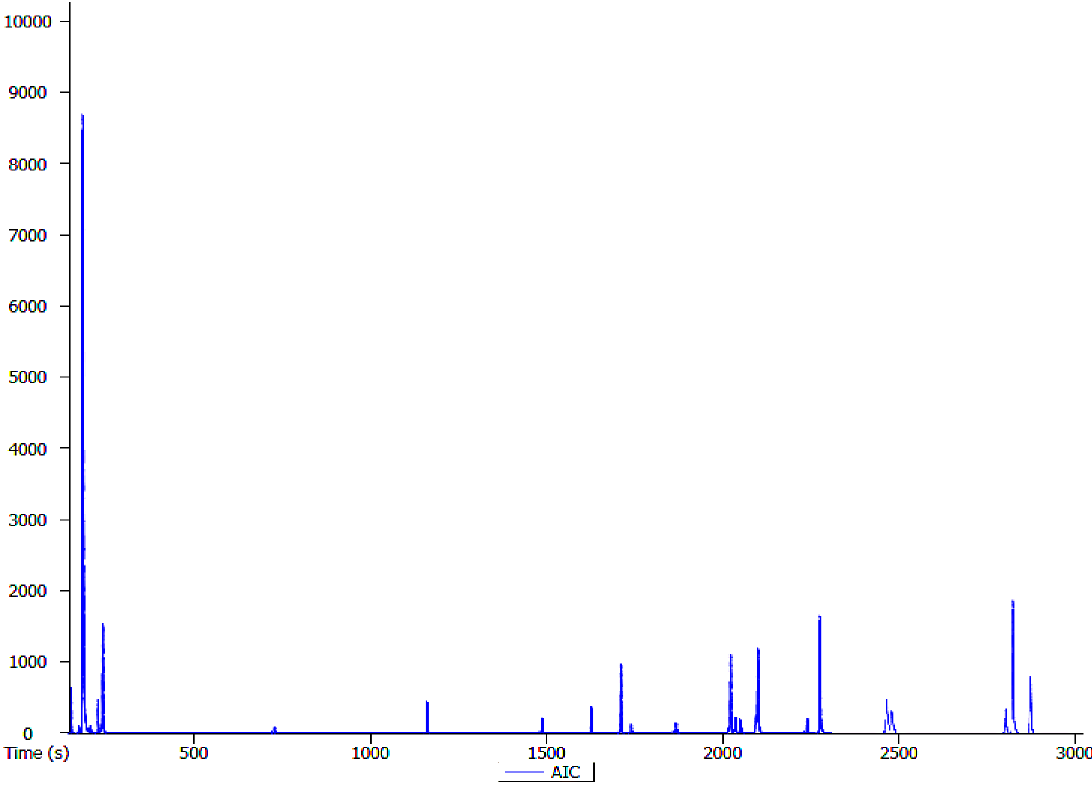

Figure D1: GC-MS chromatogram of fraction 8T

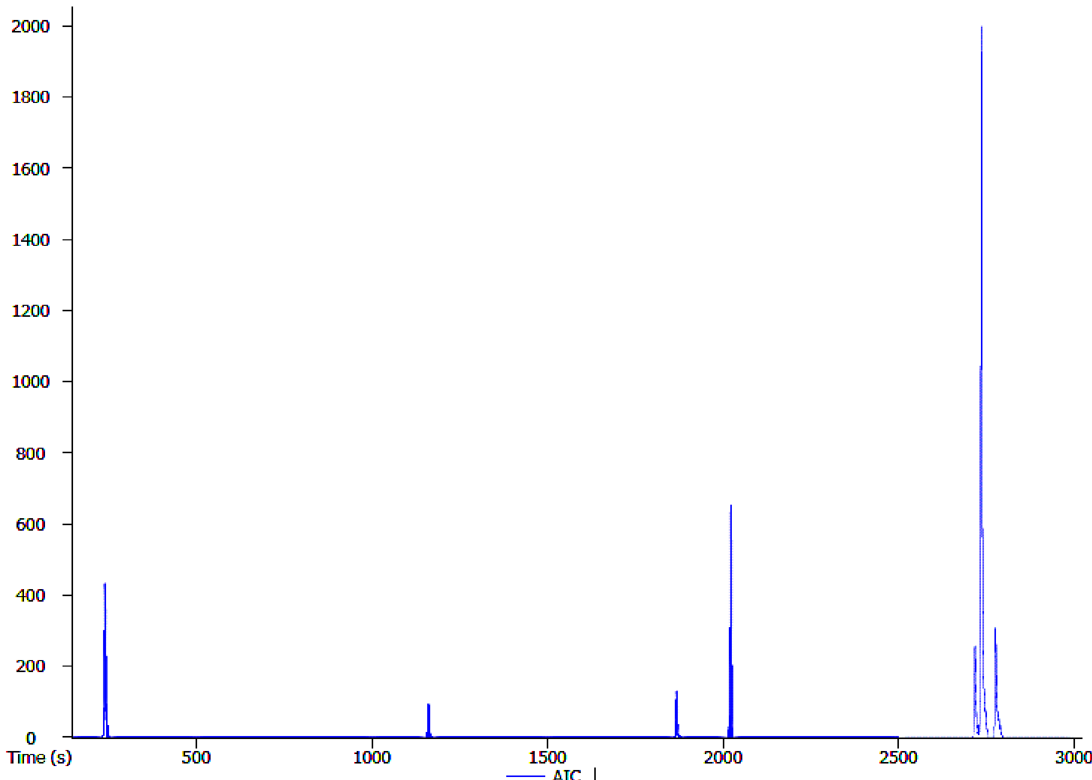

Figure D2: GC-MS chromatogram of fraction 9T

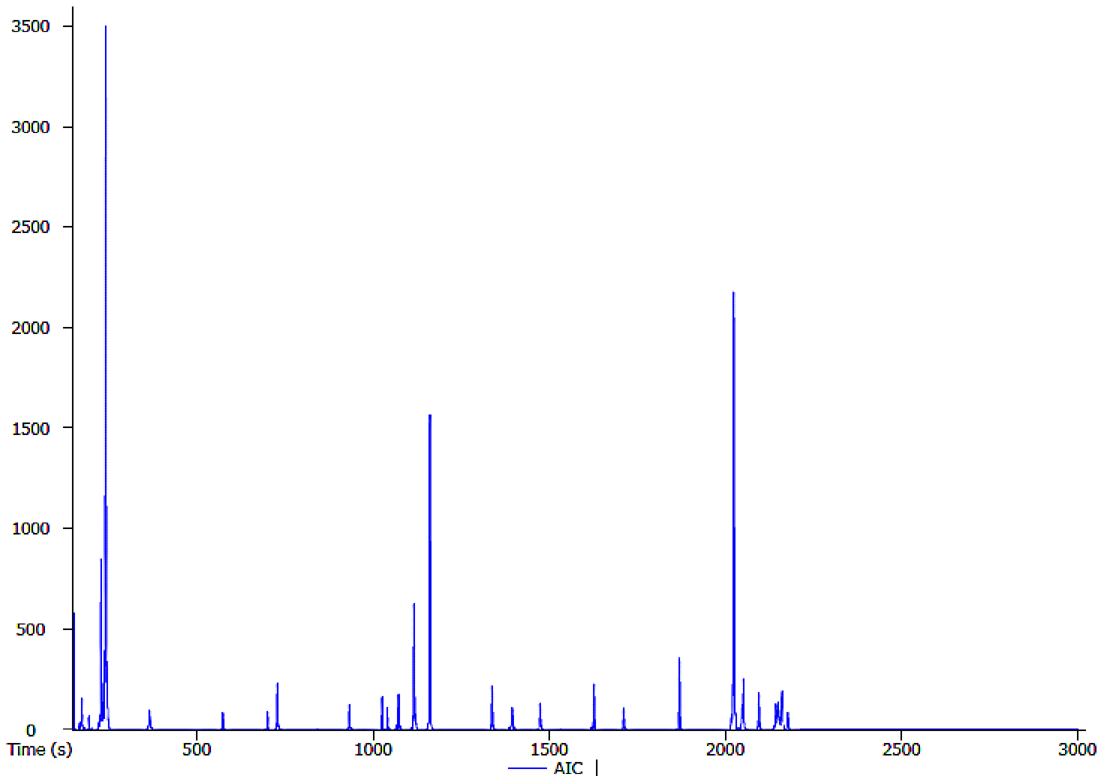

Figure D3: GC-MS chromatogram of fraction 10T

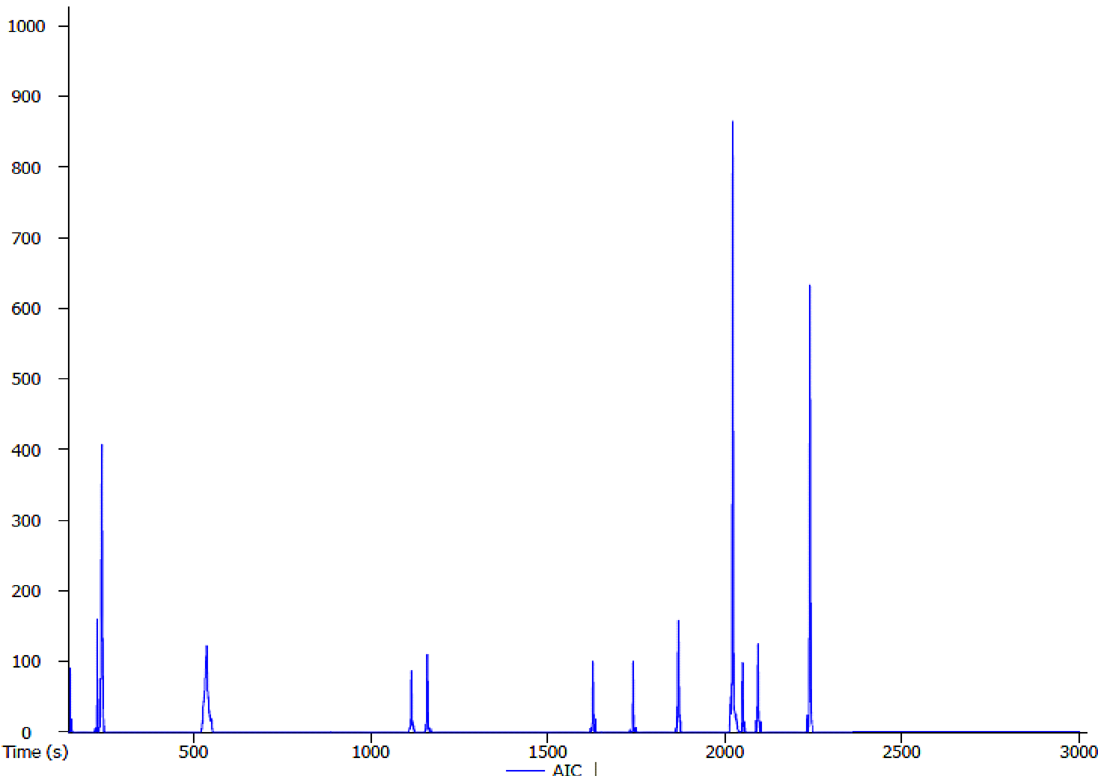

Figure D4: GC-MS chromatogram of fraction 11T

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