TOTAL PHENOLIC CONTENT, ANTIOXIDANT AND CYTOTOXIC ACTIVITIES OF *Citrus hystrix* LEAF EXTRACTS

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I would like to dedicate this thesis to my dearest uncle, MR Ravichandran Ramasamy. Without him, none of this would have happened. Thank you for everything.

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ABSTRACT

Citrus hystrix (C.hystrix) locally known as *Limau purut* is a culinary flavouring that is common in Malaysia and South East Asian cuisine as well as in herbal preparations. The aim of this study is to evaluate the antiproliferative, antioxidant and total phenolic content of ethanolic and aqueous extract of *C.hystrix* leaves. Qualitative phytochemical screening was also conducted to detect the presence of phytochemical compounds. The cytotoxic effect of the extracts on colon cancer cells (HT29) were determined by MTT assay. For the antioxidant activity, 2, 2, diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay was used. Total phenolic content (TPC) was evaluated by using Folin-Ciocalteu method. Qualitative phytochemical screening was carried using standard procedures. The ethanolic extract of *C.hystrix* exhibited higher percentage yield of 22.05 % compared to 19.22 % of aqueous during the solvent extraction method. The cytotoxic activity of the extracts showed IC_{50} values of 0.25 and 3.25 mg/ mL for ethanolic and aqueous extract respectively indicating stronger cytotoxic effects on HT29 cells in ethanolic extract. The TPC was also higher in ethanolic extract compared to aqueous which was 0.12 and 0.104 mg of GA/ g of extract respectively. For the antioxidant activity, both the ethanolic and aqueous extract showed IC_{50} values of > 8 mg/mL in comparison of 0.03 mg/mL of the standard ascorbic acid, indicating low antioxidant activity. Both the ethanolic and aqueous extract of *C.hystrix* exhibited a weak, negative correlation between TPC and percentage inhibition of DPPH with R^2 value of 0.2278 and 0.434 respectively. The phytochemical screening of the extracts reveals the presence of several phytochemical compounds namely alkaloid, saponin, flavonoid and fixed oil and fats with maximum presence of phytochemicals in ethanolic compared to aqueous extract of *C.hystrix.* Relatively, the present result shows that the ethanolic extract of *C.hystrix,* which exhibited higher percentage yield, antiproliferative activity, TPC content and maximum presence of phytochemicals can be used as a potential source of anticancer agent but not a good source of natural antioxidants.

ABSTRAK

Citrus hystrix (C.hystrix) atau dikenali sebagai *Limau purut* digunakan dalam masakan Malaysia dan Asia Tenggara serta dalam perubatan tradisional. Tujuan kajian ini dilakukan adalah untuk mengesan aktiviti anti- kanser, anti- oksida, jumlah kandungan fenolik serta penyaringan sebatian fitokimia di dalam ekstrak ethanol dan aqueous daun *C.hystrix*. Aktiviti anti-kanser ekstrak daun limau purut di atas sel kanser kolon (HT29) dianalisis menggunakan kaedah MTT. Aktiviti anti-oksida dianalisis menggunakan kaedah DPPH. Jumlah kandungan fenolik ditentukan menggunakan kaedah Folin- Ciocalteu. Kesemua kehadiran bahan fitokimia ditentukan dengan menggunakan ujian penyaringan bahan fitokimia.Hasil kajian mendapati ekstrak ethanol daun limau purut mempunyai jumlah berat yang lebih tinggi iaitu 22.05 % berbanding ekstrak aqueous iaitu 19.22 %. Ekstrak ethanol juga menunjukkan nilai IC_{50} yang lebih rendah iaitu 0.25 mg/ mL berbanding ekstrak aqueous iaitu 3.25 mg/ mL dalam ujian anti- kanser terhadap sel kanser kolon .Bagi ujian jumlah kandungan fenolik, ekstrak ethanol menunjukkan nilai lebih tinggi iaitu 0.12 mg GA/ g dibandingkan dengan ekstrak aqueous iaitu 0.104 mg GA/g. Bagi aktiviti anti – oksida kedua- dua ekstrak ethanol dan aqueous menunjukkan nilai IC_{50} > 8 mg/mL dibandingkan dengan nilai peratus inhibisi ascorbic acid iaitu 0.03 mg/ mL yang menunjukkan aktiviti anti- oksida kedua-dua ekstrak daun limau purut adalah rendah.Ujian penyaringan fitokimia telah mengesan beberapa bahan fitokimia iaitu alkaloid, saponin, flavonoid, dan minyak/lemak. Berbanding dengan ekstrak aqueous, lebih banyak bahan fitokimia dikesan di dalam ekstrak ethanol. Kedua- dua ekstrak ethanol dan aqueous daun limau purut menunjukkan polarisasi negatif di antara jumlah kandungan fenolik dan peratus (%) inhibisi DPPH di mana nilai R^2 adalah 0.2278 bagi ekstrak ethanol dan 0.4344 bagi ekstrak aqueous. Secara keseluruhan, didapati ekstrak ethanol *C.hystrix* mempunyai jumlah ekstrak tinggi serta mempunyai aktiviti anti – kanser, kandungan fitokimia dan fenolik yang lebih tinggi. *C.hystrix* berpotensi untuk digunakkan sebagai agen anti-kanser namun bukan sebagai anti-oksida semula jadi.

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LIST OF ABBREVIATIONS/ SYMBOLS

ANOVA	-	analysis of variance
ATC	-	animal tissue culture
BHA	-	butylated hydroxyl anisole
BHT	-	butylated hydroxyl toluene
$CHCl_3$	-	chloroform
CO_2	-	carbon dioxide
DPPH	-	2, 2-diphenyl-1-picrylhydrazyl
H_2SO_4	-	sulphuric acid
GAE	-	gallic acid equivalent
HCl	-	hydrochloric acid
HT-29	-	human colorectal adenocarcinoma cell line
IC	-	inhibitory concentration
K	-	potassium
K562	-	human erythromyeloblastoid leukemia cell line
KB	-	human mouth epidermal carcinoma
L	-	litre
Ml	-	milliliter
Mg	-	milligram
MTT	-	3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
Molt4	-	human leukemia cells
µg	-	microgram
NCI	-	National Cancer Institute
OD	-	optical density
PBS	-	phosphate buffer saline
PG	-	propyl gallate
P388	-	murine leukemic cell lines

RPM1- 1640	-	Roswell Park Memorial Institute
SPSS	-	Statistical Package for the Social Sciences
TPC	-	total phenolic content
TAC	-	total antioxidant capacity
TPA	-	12-Otetradecanoylphorbol 13-acetate
TBHQ	-	tertiarybutyl hydroquinone
USA	-	United States of America
USDA	-	United States of Department griculture
UV	-	ultraviolet
U937	-	human macrophage cell line
WHO	-	World Health Organization
%	-	percentage
$^{\circ}$C	-	degree Celsius
EW	-	extract weight
DW	-	dry weight

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Colon cancer, also known as the colorectal cancer is the fourth leading cause of cancer death worldwide (American Cancer Society, 2014). In Malaysia, a total of 2 866 cases were registered nationwide with the Malaysian National Cancer Registry in 2006 and represent 13.2% of all cancer cases registered (Kong *et al.,* 2010). Patients diagnosed with colorectal cancer often undergoes treatments such as chemotherapy, radiation therapy and surgery (American Cancer Society, 2014).However, chemotherapy works by killing abnormally fast dividing cells and thus effects cells that divide rapidly under normal circumstances, which leads to unwanted side effects (Chueahongthong *et al.,* 2011). Hence, naturally occurring plant compounds that have cancer inhibitory effects but have fewer side effects are a valuable alternative treatment for cancer (Jusoh *et al*., 2012).

Medicinal plants have played an important role in the treatment of various types of cancers (Valiyari *et al*., 2012). Numerous plant-derived compounds including taxol from *Taxus brevifolia* , camptothecin from *Camptotheca acuminate*, Decne, vinca alkaloids from *Catharanthus roseus*. Don and podophyllotoxin from

Podophyllum peltuturn are used in clinic all over the world (Valiyari *et al.*, 2012; Chueahongthong *et al.*, 2011).

In this project, the cytotoxic effects of ethanolic and aqueous crude extract of *Citrus hystrix* leaves on colon cancer cells (HT29) were examined. *Citrus hystrix* which is locally known as *Limau purut* or Kaffir lime is one of the traditional medicinal plant that has gain the interest of many researches over the decades due to their potential source of natural antioxidants (Almey *et al*., 2010; Jamilah *et al*., 1998; Nurain *et al*., 2013; Wong *et al.*, 2006; Idris *et al.*, 2008). Besides its leaves, the crude extract of *C.hystrix* such as peels, stems, and juice have shown potential antioxidant activity as well (Ghafar *et al.*, 2010; Chowdhury *et al.*, 2009). *C.hystrix* also exhibits anti-microbial (Chowdhury *et al*., 2012; Suri *et al*., 2002) activities.

In regard to cancer research, *Citrus hystrix* extracts have been shown to have anti-proliferative activity on KB (cervical cancer) and P388 (mouse leukemia) cell lines (Manosroi *et al.*, 2006).Glyceroglycolipids in *Citrus hystrix* leaves could inhibit 12-Otetradecanoylphorbol 13-acetate (TPA) and skin carcinogen activities in mice (Murakami *et al.*, 1995). According to research conducted by Ampasavate *et al*. 2010, *C.hystrix* leaves had strong cytotoxic effects on four leukemic cell lines (U937, K562, HL60, Molt 4).

The leaves of *C.hystrix* are used in many Malaysian and South- East Asian regions cuisines, for example in the famous local dishes such as Tom yam, stews, curries, and sauces (Almey *et al*., 2010; Nurain *et al*., 2013). It is also well known for its medicinal properties in treating skin disorders (Aziman *et al.*, 2012). Besides, it has also been used in aromatheraphy, nutraceutical, and personal care products (Almey *et al*., 2010). Due to its various ethnomedical properties, this plant was selected for this study (Almey *et al*., 2010; Aziman *et al*., 2012). Also, as the leaves of this plant have been used as a spice in many Thai and Malaysian cuisines, the safety of *C.hystrix* leaves are highly acceptable (Chueahongthong *et al.*, 2011). Till date, little is known concerning the effect of crude extract of *Citrus hystrix* leaves on colon cancer (HT29) cells.

The aim of this project was to determine the cytotoxic effect of ethanolic and aqueous crude extracts of *C.hystrix* leaves on colon cancer cells (HT29), to determine the antioxidant activity and total phenolic content (TPC) of the crude extracts, as well as to determine the phytochemical compounds present in each extract by performing phytochemical screening.

1.2 Statements of Problem

The current medical treatment for colorectal cancer includes polypectomy (removing the polyp) or colon resection (colectomy) through surgery, chemotherapy and radiation therapy (American Cancer Society, 2014). However, some of these methods cause undesired side effects by the non-specific targeting of both normal and cancer cells (Chueahongthong *et al.,* 2011). Thus, research for cancer treatment by using natural products has been increasing rapidly (Valiyari, 2012). Among natural sources, medicinal plants have played an important role in the treatment of many forms of cancer. Numerous studies have identified medicinal plant extracts which not only displayed antioxidant activity, but also cytotoxicity to many forms of cancer (Valiyari, 2012; Chueahongthong *et al.,* 2011). Such compounds include curcumin (Sandur *et al*., 2007), guava extract, and basil leaf extract (Manosroi *et al.,* 2006).

Morever, synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiarybutyl hydroquinone (TBHQ) and propyl gallate (PG) are conventional food antioxidants which are being added to foods to prevent the oxidation process of foods during the exposure to environments and increase the shelf life (Jamilah *et al.,* 2011; Wong *et al.,* 2006). Due to increasing regulatory scrutiny, consumer concerns and safety issues concerning synthetic antioxidants, the possibility of natural antioxidants from plants, as an alternative to the synthetic antioxidants are also being actively studied (Almey *et al.,* 2010; Nurain *et al*., 2013; Jamilah *et al*., 2011). Due to their natural origin, antioxidant from plants,

does not exhibit side effects and safer for consumption in comparison to synthetic antioxidants (Nurain *et al.*, 2013).

Citrus hystrix leaves have various medical and culinary uses in South East Asia (Almey *et al*., 2010; Aziman *et al*., 2012). As the plant is used mostly for cuisine purposes, the cytotoxic effect of the *Citrus hystrix* leaf crude extract on colon cancer cells are studied.

1.3 Research Objectives

Followings are the objectives of this research:

1.3.1 To investigate the cytotoxic effect of *C.hystrix* leaf extract on human colon cancer cells (HT29).

1.3.2 To determine the antioxidant properties of *C.hystrix* leaf extract.

1.3.3 To investigate the total phenolic content of *C.hystrix* leaf extract.

1.3.4 To identify the presence of phytochemical compounds present in *C.hystrix* leaf extract by performing phytochemical screening.

1.4 Scope of Research

This project was aimed to determine the cytotoxic effect of *Citrus hystrix* crude extracts on colon cancer cells, to determine the antioxidant and total phenolic content of C. *hystrix* leaves extracts as well as to identify phytochemical compounds present in the crude extract of the leaves. The leaves were extracted by using two

polar solvents namely aqueous and ethanol. Colon cancer cells, (HT-29) were used to test the cytotoxic effect of *C. hystrix* by using the MTT assay. 50% inhibition concentration (IC_{50}) of the active substances was determined as the lowest concentration which reduced cell growth by 50%. The antioxidant activity was measured by using the DPPH free radical scavenging assay and expressed as percentage of DPPH radical inhibition and IC_{50} values. The total phenolic content was determined by using the Folin- Ciocalteu method and is expressed as mg of GA/g of extract. Following that, phytochemical screening was conducted to determine the presence of phytochemicals such as phenol, tannin, saponin, flavonoid, alkaloid, terpenoids and fixed oils.

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