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CHALEPIN AND RUTAMARIN ISOLATED FROM *RUTA ANGUSTIFOLIA* INHIBIT CELL GROWTH IN SELECTED CANCER CELL LINES (MCF7, MDA-MB-231, HT29, AND HCT116)

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Abstract: *Many anticancer drugs have their origin in natural sources like plants. In this study, extracts from Ruta angustifolia were screened against four (4) selected cancer cell lines and a normal lung fibroblast cell line (MRC-5). The chloroform extract showed higher cytotoxic activity against MCF7, HT29, HCT116, but mild cytotoxicity against MDA-MB231 and no activity against MCR5 (IC_{50} > 100μg/mL). Chalepin and rutamarin were then isolated from the active chloroform extract. Chalepin displayed remarkable cytotoxicity against all tested cancer cell lines but no activity against MCR5. Rutamarin, on the other hand, showed remarkable cytotoxicity activity against MCF7, HT29, and HCT116, whereas no activity against MDA-MB-231 and MRC5 was observed. The present study therefore revealed that chalepin and rutamarin inhibit cell growth against cancer cell lines in a dose and time-dependent manner. These initial results, therefore, suggests that chalepin and rutamarin may serve as potential source of anticancer agents.*

Keywords*: Cell Growth, Chalepin, Rutamarin*

Introduction

Cancer has been reported as a source of morbidity and mortality worldwide (Sylla & Wild, 2012). In 2008 new cancer cases and cancer-related deaths of about 12.7 and 7.6 million respectively has been reported (Ferlay et al., 2010). About 56% of these new cases were reported to occurred in developing countries; conversely by 2030, 70% of all new case has been projected to be present in developing countries (Boyle & Levin, 2008). The incidence mostly increases due to population growth and rise in life expectancy (Lyerly et al., 2011). In fact, projection of the global cancer burden will be doubled in the next two decades and hence, raising investment in health system, thereby posing a real medical problem (Vineis & Wild, 2014).

Complementary and alternative medicine now covers a large spectrum of old and new strategies which offers options to prevent and treat diseases such as cancer and are now been considered in oncology management (Harvey et al., 2015; Wang et al., 2012).

However, current researches are geared toward finding small molecules that can serve as potential anticancer agents which are effective, less cost and less toxic from natural plant products. Natural products have been important source of therapeutic agents (Hait, 2009); it has been reported that, 80% of the approved drugs with chemotherapeutic agents and their sources are derived from natural compounds (Cragg et al., 2009).

Medicinal plants contain various bioactive compounds that posed therapeutics properties. Over a long period of time, therapeutic potential in plant has been explored and several others are still under investigation. Anti-inflammatory, antimalarial, antiviral, analgesic, and antitumor are among the collection of therapeutic effects of medicinal plants (Raina et al., 2014).

Rutaceae family contains several plant species of medicinal interest; which has ecological, economic and therapeutic values (Januário et al., 2009). Rutaceae family belongs to the order of sapindales and has over 1600 species with about 150 genders. These species are widely distributed all over tropical and temperate region throughout the world. However, the plants are more abundant in countries like South Africa, Australia and tropical America (Albarici et al., 2010).

Ruta species is one of the common genuses used in the current Italian traditional medicine, economic botany and folk life (Pollio et al., 2008). *Ruta* species are known to have various classes of natural products; these include alkaloids, coumarins, flavonoids, lignans, saponins, and triterpenes. Many of these compounds exhibited a broad range of biological activities such as abortive, antidotal, antifungal, anti-inflammatory, antioxidant, depressant and phytotoxic (Amar et al., 2012).

Literature survey revealed little or no detailed biological pharmacological investigation on *Ruta angustifolia* as well as its constituents; consequently, there is limited published information on the bioactivity of *Ruta angustifolia*, particularly on cytotoxicity. Therefore, it is imperative to investigate cytotoxic potential of the plant and its isolated compounds against some cancer cells.

Methodology

Plant material and extraction

Ruta angustifolia was collected at a Nursery in Sungai Buloh, Selangor, Malaysia. Identification of the plant sample was done at Institute of Biological Sciences, Faculty of Science, University of Malaya. Plant sample deposited with Herbarium no. KLU48128 was kept at Rimba Ilmu, University Malaya, Kuala Lumpur, Malaysia.
The aerial parts of *Ruta angustifolia* Pers. were dried and grounded into powder (176.34 g). 90% aqueous methanol was used to extract the powdered sample at room temperature for 72 hours, obtaining a methanol extract (63.29 g, 35.89%). The methanol extract (63.29 g) was then extracted using hexane resulting in hexane-soluble fraction (1.63 g; 2.58%) and hexane insoluble scum. The hexane-insoluble scum was then partitioned in chloroform-water in 1:1

yielding chloroform-soluble extract (14.58 g; 23.04%). The water layer was further partitioned using ethyl acetate-water in 1:1 ratio yielding an ethyl acetate-soluble extract (1.58 g; 2.50%) and water extract (45.49 g; 71.88%). The methanol, hexane, chloroform and ethyl acetate extract fractions were constituted with DMSO to form 40 mg/ml as stock solutions.

Isolation of chalepin and rutamarin

Chalepin and rutamarin were isolated from the chlorophyll free chloroform extract. This was prepared by addition of activated charcoal into the chloroform fraction and then filtered using filter paper. The solvent was evaporated from the filtrate using rotary evaporator at reduced temperature and pressure. Methanol was then used to prepare 5 mg/ml concentration and filtered using membrane filter before injecting 5 µl of the extract into the analytical (XDB-C-18, 4.6 × 250, 5µm) Agilent HPLC column at through which numerous peaks were then detected and monitored at 200 nm UV absorbance. The extract was subsequently, prepared to 50 mg/ml concentration using methanol, filtered and later 50 µl injected into semi-prep column (Agilent XDB-C-18, 9.6 × 250 mm, 5 µm) at flow rate of 5 ml/min.

The analysis was done on Agilent system HPLC 1260 infinity comprising of a quaternary pump with autosampler (ASL 1260), the system also consists of a thermostat, thermostatted column (TCC), a fraction collector (FC-AS), a 1260 diode array detector (DAD VL+) and an LC software from Agilent OpenLAB CDS Chemostation. Binary eluent system was used for the analysis using chromatographic grade acetonitrile and ultrapure water following specific gradient conditions. The gradient conditions used was isocratic gradient from 30% ACN for 0 – 20 min; linear gradient from 30 – 60% ACN for 20 – 25 min; linear gradient 60% - 100% for 25 – 35 min; and isocratic 100% for 35 – 40 mins at a flow rate of 1 ml/min. The column was set to 30^{o}C temperature for running of the analysis.

Though many peaks were detected, correspond to chalepin and rutamarin were continuously collected from the fraction collector. The collected fractions were subjected to TLC, based their similarity on TLC against the pre-isolated and identified compounds of chalepin and rutamarin; the fractions were then pooled to two fractions. The mobile phase used for the analysis was removed by evaporation through rotary evaporator at 40^{o}C. The dried compounds expected to be chalepin and rutamarin were weighed and then reconstituted in methanol. The two fractions were subjected to analytical HPLC for confirmation of their purity. However, to obtain and confirm their structures, gas chromatography (GC-MS) and nuclear magnetic resonance (NMR) analysis was employed to determined mass spectral and NMR data respectively.

A gas chromatography (Agilent Technologies 6980 N) equipped with 5979 Mass Selective Detector (70eV direct inlet) was used for GC-MS analysis. A HP-5 ms capillary column was used (5% phenyl methyl siloxane) (30.0 m × 25 mm × 25 µm) with temperature set first at 100^{o}C, and later raised to 300^{o}C at 5^{o}C per minute. Helium as carrier gas was used to held the system for 10 minutes at a flow rate of I ml/min. ChemStation was used to auto integrate the total ion chromatogram obtained and identified components through comparison with accompanying spectral database (Wiley, Mass Spectral Library, USA.

Cell lines

Human hormone-dependent breast adenocarcinoma cancer cell (MCF7), Human non-hormone-dependent breast adenocarcinoma cancer cell (MDA-MB-231), human colon adenocarcinoma cancer cell (HT29), human colon carcinoma cancer cell (HCT116), normal colon fibroblast cell (CCD-112CoN) and normal lung fibroblast cell (MRC-5) were obtained from American Tissue Culture Collection (ATCC, USA). MDA-MB-231 and MCF7 were cultured in DMEM complemented using 10% v/v FBS, 2% v/v penicillin/streptomycin, 1% v/v amphotericin B, HT29 and HCT116 were cultured in RPMI 1640 complemented using 10% v/v FBS, 2% v/v penicillin/streptomycin, 1% v/v amphotericin B, while CCD-112CoN and MRC-5 were cultured in EMEM complemented using 20% v/v FBS, 2% v/v penicillin/streptomycin, 1% v/v amphotericin B.

In vitro cytotoxicity

The cytotoxicity assay was performed by adopting an SRB assay as described by Houghton with modifications (Houghton et al., 2007). Cells density of 40,000/ml for HT29 and HCT116; and 50,000/ml for MCF7, MDA-MB-231 and MRC-5 were plated onto sterile 96-well flat bottom plates. The cells were incubated for 24 hours to allow cells adherence; after which media was removed and fresh media containing different concentrations of extract fractions and isolated compounds at 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 µg/ml added to the cells. The plates treated with extract were incubated for 72 hrs, while plates treated with isolated compounds were incubated for 24, 48 and 72 hrs at 37^{o}C and 5% CO_2. In contrast, media in the control cells was replaced with fresh media contains neither extract fractions nor chalepin and rutamarin. After various incubation periods, 50 µl of 40% ice-cold TCA was added to the cells and then incubated at 4^{o}C for 1 hour. Following incubation, the media containing TCA then aspirated, and cells washed using deionized water, followed by addition of 50 µl of 0.4% SRB in 1% acetic to each well. The cells were then incubated for 30 minutes at room temperature. SRB solution was aspirated, and cells washed using 1% v/v acetic acid before solubilizing the stain by adding 10 mM Tris base. Finally, the plates were further incubated for 5 minutes at 500 rpm on a microtiter plate shaker and absorbance recorded at 492 nm and 620 nm as a background with microplate reader (Biotek Synergy H1 Hybrid). The percentage inhibition from each sample test was calculated via the expression below:

$$\textbf{\% inhibition} = \frac{\textbf{Absorbance}_{control} - \textbf{Absorbance}_{sample}}{\textbf{Absorbance}_{control}} \times \textbf{100}$$

The IC_{50} (i.e. the agent concentration that causes 50% cell inhibition or death) was determined from the dose-dependent response curves of each cell line. The experiment performed in triplicates.

Result and Discussion

Isolation, identification and confirmation of pure compounds

The extraction**,** isolation and identification of the pure compounds have been previously described. Chalepin and rutamarin from Ariel part of *Ruta angustifolia* (Richardson et al., 2016). Although many other compounds were isolated and identified from the plant, but only peaks correspond to chalepin and rutamarin were isolated in this study. Therefore, peak **C**

(retention time = 28.890**)** correspond to chalepin compound whereas peak **R** (retention time = 32.342) correspond to rutamarin compound (Figure 1). The isolated peaks; chalepin and rutamarin show single spot when spotted against their pre-isolated and identified standards (Figure 2). This indicates that the compounds were the actual compounds isolated in this study. However, structure of the compounds; chalepin and rutamarin were identified and confirmed by their spectral and NMR data, in addition to co-TLC with the authentic samples; and were consistency with the published data (Del Castillo et al., 1984; Orlita et al., 2008; Richardson et al., 2016; Wu et al., 2003; Yang et al., 2007).

Preliminary cytotoxic activity of crude methanol, chloroform, ethyl acetate and hexane extracts

Crude methanol, chloroform, ethyl acetate and hexane extract fractions cytotoxic effect of *Ruta angustifolia* were presented in Table 1; the data are presented as IC_{50}, obtained from three different experiments. A plant extract is considered cytotoxic active as reported by United State National Cancer Institute plant screening program, if the IC_{50} value of the plant extract is $\leq$ 20 µg/ml, following incubation between 48 to 72 hours (Geran, 1972; Lee & Houghton, 2005). The ethyl acetate extract was found to be inactive against all the cancer and normal cells tested in this study with IC_{50} of >100 µg/ml. In contrast chloroform extraction shows remarkable cytotoxicity against all the tested cancer cells. Importantly, all extracts did not present any toxicity against the normal cell line (IC_{50} >100 µg/ml) (Table 1). This result is consistence with report by (Richardson et al., 2016).

Cytotoxicity activity of chalepin and rutamarin

Chalepin and rutamarin were tested against HT29, HCT116, MCF7, MDA-MB-231 and MRC-5. The result revealed that chalepin has strong cytotoxic activity against HT29, HCT116 and MCF7; whereas the compound has mild effect on MDA-MB-231 cells. Similarly, rutamarin shows a remarkable cytotoxicity against HT29, HCT116, MCF7 and no activity on MDA-MB-231 cells. Importantly, both compounds did not present any toxicity against the normal cell line (IC_{50} >100 µg/ml) (Table 1). It has been reported that a pure compound is considered cytotoxic active as reported by US NCI plant screening program, if the IC_{50} value is $\leq$4 µg/ml, following 48 to 72 hours incubation (Geran, 1972; Lee & Houghton, 2005).

These results, therefore, indicates that chalepin and rutamarin actively contributed to the cytotoxic effect of the chloroform fraction from *Ruta angustifolia* (Pers.). Furthermore, the cytotoxicity activity of these compounds was observed to be a dose- and time dependent fashion. The result of the present study is similar as recently reported (Richardson et al., 2016).

Extract fractions and isolated compounds inhibits cell growth in dose-dependent manner

Inhibition of the percentage cell growth is a preliminary indication of cytotoxic effect and anti-proliferative effect of any plant extracts and its active constituents. However, the lower the IC_{50} of any extract or its active components, the higher the percentage of cell growth

inhibition and the more effective it is in exerting cytotoxicity activity. In this study, extract fractions from *Ruta angustifolia* shows different percentage cell growth inhibition against different cancer cell tested and therefore presented different strength of inhibition. Hence, from the results chloroform fraction has higher percentage of inhibition on cell growth against the tested cancer cell. Whereas ethyl acetate fraction exhibited lowest percentage of inhibition on cell growth against the tested cancer cells (Figure 3a, 3b, 3c and 3d). The result, therefore, suggested that chloroform fraction has higher potency of anti-proliferative against all the cancer cells and ethyl acetate fraction has the lowest potency to that effect.

Similarly, chalepin and rutamarin isolated from chloroform fraction of *Ruta angustifolia* exert a significant cell growth inhibition among the tested cancer cells. In this study, chalepin and rutamarin shows various degree of inhibition against the tested cancer cells (Figure 4a and 4b). The results indicate that both chalepin and rutamarin has higher percentage of cell growth inhibition against HT29, whereas the two compounds show lowest cell growth inhibition against MDA-MB231. The percentage inhibition shows by the two compounds among the different cancer cells further supported the various degree of IC_{50} obtained for both compounds on difference cancer cells. Importantly, the results shows significant increased inhibition of cell growth by both extract fractions and the isolated compound was a dose-dependent manner. This study is consistence with report by (Ho et al., 2013; Phang et al., 2016; Richardson et al., 2016).

Conclusion

The results of this study demonstrated cytotoxic potential of the *Ruta angustifolia* extract fractions and its isolated compounds; chalepin and rutamarin. The extract fractions as well as chalepin and rutamarin were found to significantly inhibited growth of cancer cells and therefore can serves as potential source of anticancer agents. However, studies are ongoing to evaluate possible mechanisms by which this plant's compounds exert their anti-proliferative activity against cancer cells.

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Table 1: Cytotoxic activity of extract fractions (methanol, chloroform, ethyl acetate and hexane) and isolated compounds (chalepin and rutamarin) against selected cancer cells and normal cell lines after 72 hrs of incubation

	IC_{50} (µg/ml)				
Extracts	HT29	HCT116	MCF7	MDA-MB-231	MRC-5
Methanol	11.6 ± 0.3	61.0 ± 2.4	33.7 ± 2.4	>100	>100
Hexane	25.3 ± 1.2	32.6 ± 1.3	20.3 ± 1.6	>100	>100
Ethyl acetate	>100	>100	>100	>100	>100
Chloroform	7.5 ± 0.4	17.2 ± 1.0	12.0 ± 0.8	39.9 ± 1.7	>100
Chalepin	5.6 ± 0.3	7.0 ± 0.9	8.5 ± 1.4	19.8 ± 0.8	>100
Rutamarin	2.6 ± 0.3	3.1 ± 0.6	4.3 ± 0.3	>100	>100
Doxorubicin	0.18 ± 0.01	0.28 ± 0.02	0.10 ± 0.00	0.07 ± 0.01	0.35 ± 0.01

Data are expressed as mean ± SD of 3 experiments

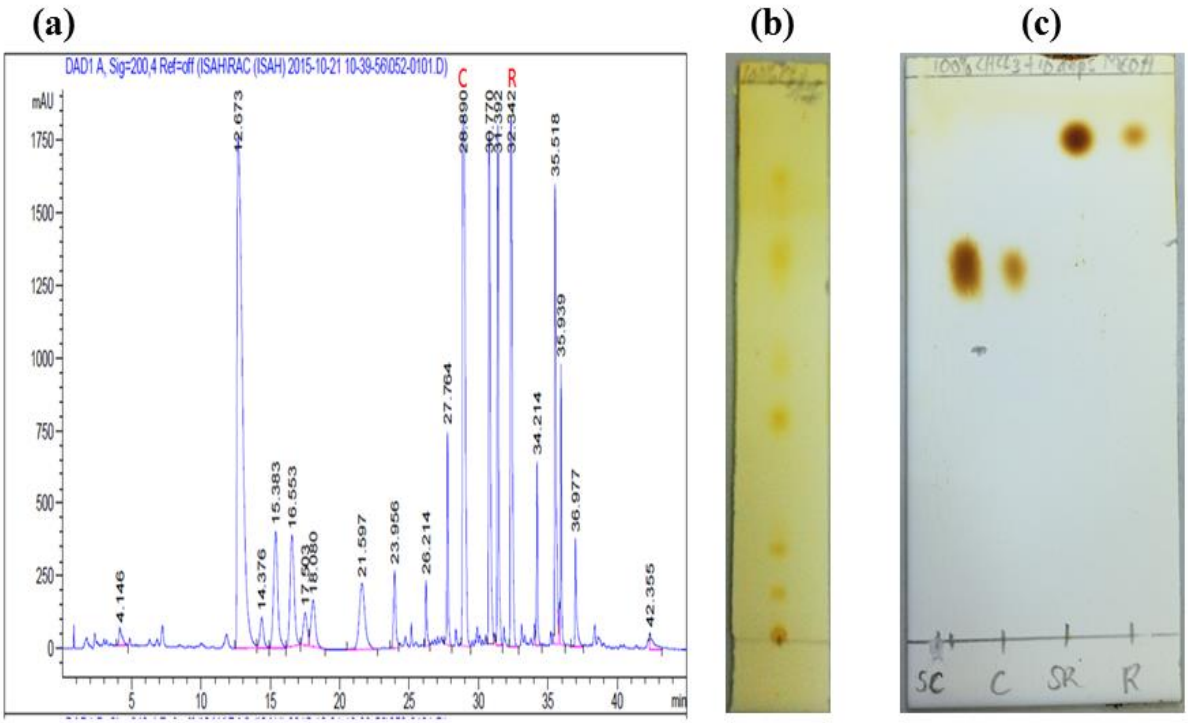

Figure 1: (a) HPLC profile indicating peaks corresponding to **chalepin (C)** and **rutamarin (R),** Tin layer chromatography of **(b)** chloroform extract after charcoaled and **(c)** chalepin and rutamarin spotted against their pre-isolated standards.

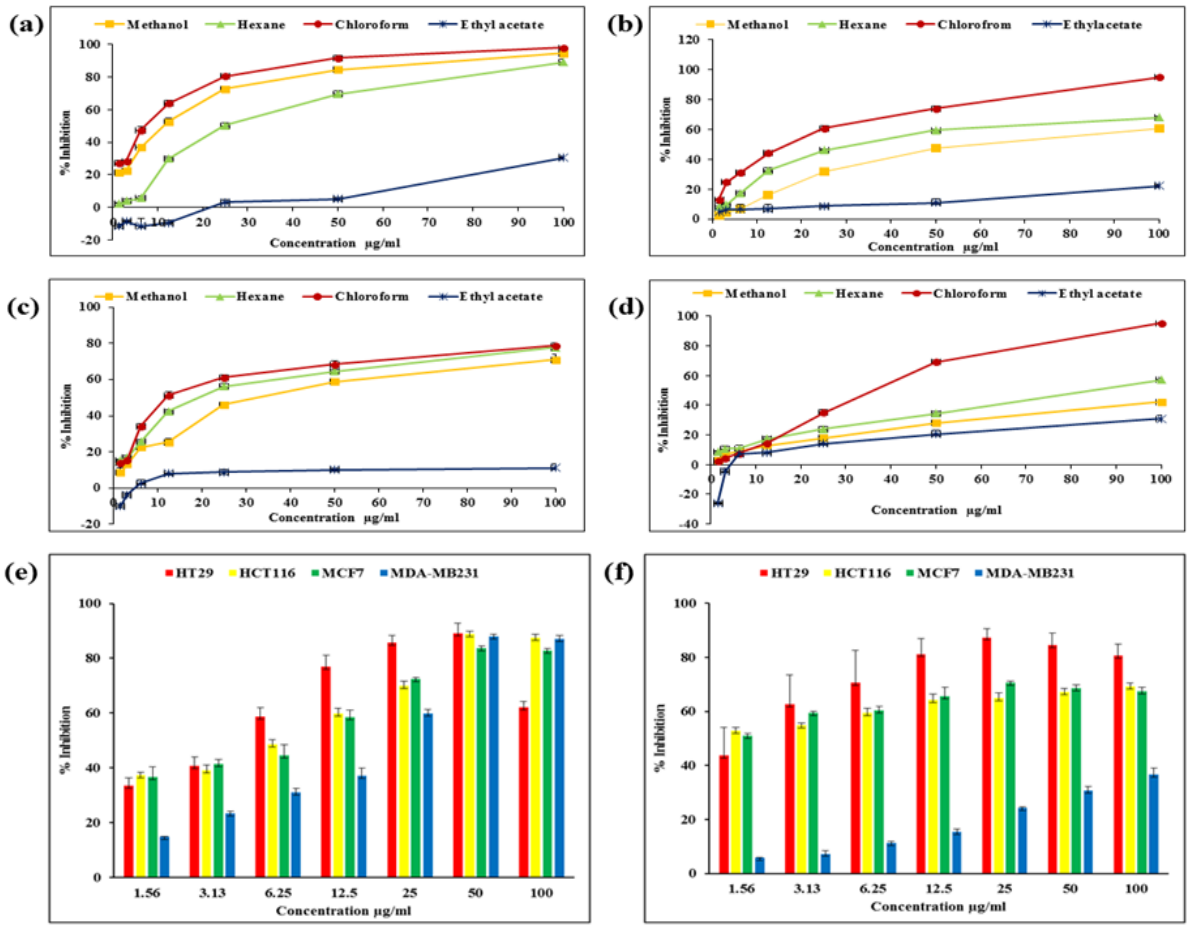

Figure 2: Percentage inhibition of extract fractions against **(a)** HT29 **(b)** HCT116 **(c)** MCF7 and **(d)** MDA-MB231; while (**e**) chalepin **(f)** rutamarin against HT29, HCT116, MCF7 and MDA-MB231 after 72 hours treatment. Data represent mean ± SD (n = 3).