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ORIGINAL ARTICLE

In vitro anti-cancer activities of Job's tears (*Coix lachryma-jobi* Linn.) extracts on human colon adenocarcinoma

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Abstract The whole seed (W), endosperm (E) and hull (H) of five cultivars of Job's tears (*Coix lachryma-jobi* Linn. var. ma-yuen Stapf) including Thai Black Phayao, Thai Black Loei, Laos Black Loei, Laos White Loei and Laos Black Luang Phra Bang were processed before solvent extraction by non-cooking, roasting, boiling and steaming Each part of the Job's tears was extracted by the cold and hot process by refluxing with methanol and hexane. The total of 330 extracts included 150 methanol extracts and 180 hexane extracts were investigated for anti-proliferative activity on human colon adenocarcinoma cell line (HT-29) by the sulforhodamine B (SRB) assay. The extracts which gave high anti-proliferative activity were tested for apoptotic activity by acridine orange and ethidium bromide double staining and anti-oxidative activities including free radical scavenging and lipid peroxidation inhibition activities. The extract from the hull of Thai Black Loei roasted before extracting by hot methanol (M-HTBL-R2) showed the highest anti-proliferative activity on HT-29 with the IC_{50} values of 11.61 ± 0.95 μg/ml, while the extract from the non-cooked hull of Thai Black Loei by cold methanol extraction (M-HTBL-N1) gave the highest apoptosis (8.17 ± 1.18%) with no necrosis. In addition, M-HTBL-R2 and M-HTBL-N1 indicated

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free radical scavenging activity at the SC_{50} values of 0.48 ± 0.12 and 2.47 ± 1.15 mg/ml, respectively. This study has demonstrated the anti-colorectal cancer potential of the M-HTBL-R2 and M-HTBL-N1 extracts.

1. Introduction

Colorectal cancer is the major cause of morbidity and mortality throughout the world. It accounts for over 9% of all cancer incidences. It is the third most common cancer and the fourth most common cause of death (Haggar and Boushey, 2009). Despite the development of new screening strategies, aggressive surgical and adjuvant therapy, as well as the intensive research, little progress has been made in the successful management of colorectal cancer. This cancer survival is highly dependent on the stage of the disease at diagnosis, and typically ranges from 10 to 90%. In general, the earlier the stage at diagnosis indicated the higher the chance of survival. Diet and lifestyle modifications offer means of reducing the risk of developing colon cancer. The natural components of the diet may serve as chemopreventive agents that suppress the growth and dissemination of neoplastic colon cells (Park, 2008). Because food-derived products exist universally and are expected to be safe, they are highly interesting for the development as chemopreventive agents. Job's tears (*Coix lachryma-jobi* Linn. var. ma-yuen Stapf) which is the native plant of Southeast Asia, exists in the wild and is also cultivated. It is a tall grain-bearing tropical plant of the grass family, Poaceae or Gramineae. Seeds of Job's tears are in oval or egg shape 5 mm in diameter and have a milky white to black outer surface after the dehulling process. From ancient times, this plant has also been widely used as a diuretic, stomachic, analgesic, arthritis and antispasmodic agent. The consumption of the dehulled flour and seeds of Job's tears can improve lipid metabolism, thereby decreasing the risk of heart diseases. In addition, it could reduce liver fat accumulation and protect from the tumor stimulating compounds (Chang et al., 2003). Job's tears is believed to be beneficial to the gastrointestinal tract and may be used as a prebiotic due to its modifying effect on some intestinal bacteria (Chiang et al., 2000). Several studies have shown that Job's tears may have an anti-tumor effect. Some bioactive compounds in Job's tears, especially coixenolide, inhibited tumors, prevented cancer and protected against viral infection (Hung and Chang, 2003). The methanol extract of Job's tears exerted an anti-proliferative effect on A549 lung cancer cells by inducing cell cycle arrest and apoptosis (Chang et al., 2003). Shih et al. have investigated the effect of Job's tears on colon carcinogenesis and COX-2 expression. It was found that Job's tears can suppress the preneoplastic lesion of colon and COX-2 protein expression in colon tumors (Shih et al., 2004). The emulsion of Job's tears oil was approved for anti-cancer activity by the Chinese Ministry of Public Health. This product has been used in patients to treat various common types of cancer, including lung, breast and liver cancer (Normile, 2003). The aim of this present study was to investigate the anti-cancer activities including anti-proliferation and apoptotic activity of the extracts of different parts from different cultivars of Job's tears prepared by various processes.

2. Materials and methods

2.1. Materials

Five cultivars of Job's tears including Thai Black Phayao (TBP) from the Phayao province in Thailand; Thai Black Loei (TBL), Laos Black Loei (LBL) and Laos White Loei (LWL) from the Loei province in Thailand; and Laos Black Luang Phra Bang (LB) from Luang Phra Bang province in Laos were purchased from CCP Northern Co., Ltd., Phayao, Thailand and Yongsawadpudpol Wang Saphung, Co., Ltd., Loei, Thailand. The voucher specimens of the plant samples were authenticated by a botanist at Natural Products Research and Development Center (NPRDC), Science and Technology Research Institute (STRI), Chiang Mai University, Chiang Mai, Thailand. Acridine orange (AO), ethidium bromide (EB) and sulforhodamine B (SRB) were from Sigma (St. Louis, MO, USA). The commercial product which was the emulsion containing the Job's tears oil extract was used as the standard for the anti-cancer activity assays. Trypsin was prepared at 0.25% solution in phosphate buffered saline. Completed DMEM medium was prepared from Dulbecco's Modified Eagle Medium (GIBCO, Invitrogen 95 Corporation, NY, USA) supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml). All other chemicals and reagents were of analytical grade.

2.2. Preparation of the extracts

A portion of Job's tears was subjected to dehulling processing by separating the whole grain into the seed hull and the dehulled grain or endosperm using mortar and hands. The whole seed (W), endosperm (E) and hull (H) of Job's tears were treated before solvent extraction by non-cooking (N), roasting (R), boiling (B) and steaming (S).

2.2.1. Roasting processing

The dried Job's tears were directly placed in the preheated pan (300 °C), and then roasted (dry heated) with continuous stirring for 5 min using a woody scoop.

2.2.2. Boiling processing

The soaked Job's tears (presoaked for 1 h) were placed into the boiling water and cooked for 30 min on an electric hot plate cooker. The leaching solution was collected by centrifugation and filtered through the Whatman® filter paper. The residue was extracted by methanol or hexane whereas the leaching solution was partitioned by liquid–liquid extraction.

2.2.3. Steaming processing

The soaked Job's tears (presoaked for 1 h) were placed separately on a metal dish with gauze and put into a double-layer steamer to be steamed for 30 min on an electric hot plate cooker. The leaching solution was collected by centrifugation and filtered through the Whatman® filter paper. The residue was extracted by methanol or hexane, whereas the leaching solution was partitioned by liquid–liquid extraction.

Each part of the sample was extracted by cold and hot processes by refluxing with methanol and hexane. Briefly, the non-cook and roasted Job's tears or the residue from the boiling and steaming processing were soaked in the solvent for 24 h. For the cold process, the mixture was shaken at 300 rpm on an orbital shaker for 1 h at room temperature (27 ± 2 °C). For the hot process, the sample was refluxed at 70 ± 2 °C for 3 h. After filtration, the filtrate was concentrated under vacuum by a rotary evaporator (R-124 Buchi, Switzerland) and lyophilized (Christ: Alpha 1-2 LD, Germany). The leaching solution from the boiling and the steaming processing was mixed with hexane in the separating funnel, shaken for 10 min and left for 5 min to get the water and hexane layers. Each layer was separated, concentrated and lyophilized. The descriptions and the extract codes of each Job's tears sample were presented in Table 1.

2.3. Cell cultures

The human colon adenocarcinoma (HT-29) cell lines provided from Medicinal Microbiology Department, Faculty of Biology, University of Tübingen, Tübingen, Germany was cultured in the complete medium containing DMEM supplemented with 10%(v/v) fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml). The cells were maintained in a humidified atmosphere of 5% CO_2 incubator (Contherm mitre 4000, Contherm Scientific, Hutt city, New Zealand) at 37 °C. The cells were trypsinized and counted with a hemocytometer.

Table 1 Abbreviations of the Job's tears extracts.

System	Abbreviation	Description
Solvents	H	Hexane extraction
	M	Methanol extraction
Parts use	E	Endosperm
	H	Hull
	W	Whole
Cultivars	TBP	Thai Black Phayao
	TBL	Thai Black Loei
	LBL	Laos Black Loei
	LWL	Laos White Loei
	LB	Laos Black Luang Phra Bang
Process before solvent extraction	N	Non cook
	B	Boiling
	S	Steaming
	R	Roasting
Extraction	1	Cold extraction
	2	Hot extraction
	3	Water part from liquid partition
	4	Hexane part from liquid partition

2.4. Anti-proliferative activity by the sulforhodamine B assay

An amount of 180 µl of cell suspension (10^4 cells/well) was plated in 96-well plates and incubated overnight in a humidified atmosphere of 5% CO_2 incubator at 37 °C. The lyophilized extracts were dissolved by 10%DMSO in DMEM medium and filtrated by 0.2 µm cellulose acetate sterile filter. An amount of 20 µl of the filtrated samples and the four standard anti-cancer drugs (cisplatin, doxorubicin, fluorouracil and vincristine) was added to the cells at the final concentrations range of 0.1–1000 µg/ml per well. After 24 h of incubation, the cells were fixed with 50% trichloroacetic acid solution, incubated at 4 °C for 1 h and washed with distilled water. Excess water was drained off and the plates were air-dried for 24 h. The cells were stained with 50 µl of 0.4% SRB solution in 1% acetic acid for 30 min at room temperature (27 ± 2 °C). After incubation, the SRB solution was poured off and the plates were washed with 1% acetic acid. The plates were air-dried and 100 µl of 10 mM Tris-base solution was added to each well to solubilize the dye and shaken for 30 min at room temperature (27 ± 2 °C). The absorbance at 540 nm was determined by the microplate reader (Bio-Rad, model 680 microplate reader, USA). All experiments were performed in triplicate. The percentages of the cell growth (%G) were determined using the following equation:

$$\%\ \text{Cell growth}(\%G) = (A - C/B - C) \times 100$$

where A was the optical density of the extracts, B was the optical density of the control and C was the optical density at time zero. The IC_{50} values were determined by plotting the percentages of the cell growth (%G) versus the concentrations of the samples (Skehan et al., 1990). Extracts which exhibited potential anti-proliferative activity were selected for the apoptotic test.

2.5. Apoptotic assay by acridine orange (AO) and ethidium bromide (EB) double staining

The selected extracts and the standard anti-cancer drugs were tested at four final concentrations (IC_{50}, 1000, 100 and 10 µg/ml for the extracts, while IC_{50}, 100, 10 and 1 µg/ml for the standard anti-cancer drugs). Cells without extract were served as the negative control. An amount of 10 µl of the extracts and standard anti-cancer drugs at the above concentrations was added to the wells and incubated for 24 h. After that, 10 µl of the AO/EB dye mix (100 µg/ml of AO and 100 µg/ml of EB in PBS) was added to each well. The apoptotic, necrotic and live cells were observed and counted under the fluorescent microscope (Olympus CK40/U-RFLT 50, Olympus, Japan). All experiments were repeated 3 times and at least 100 cells of each experiment were counted (Manosroi et al., 2012).

2.6. Anti-oxidative activities

2.6.1. Free radical scavenging assay

Free radical scavenging activities of the extracts and the standard anti-oxidant (ascorbic acid) were determined by a modified DPPH assay (Manosroi et al., 2010). Briefly, 50 µl of five

serial concentrations of the extracts (at 0.001–10 mg/ml) dissolved in 10%v/v DMSO and 50 µl of ethanolic solution of DPPH were put into each well of a 96-well microplate (Nalge Nunc International, NY, USA). The reaction mixtures were allowed to stand for 30 min at 27 ± 2 °C, and the absorbance was measured at 515 nm by a well reader (Bio-Rad, model 680 microplate reader, USA) against the blank (10%v/v DMSO). Ascorbic acid (0.001–10 mg/ml) was used as a positive control. The experiments were done in triplicate. The percentages of radical scavenging activity were calculated as follows: scavenging (%) = $[(A - B)/A] \times 100$, where A was the absorbance of the control and B was the absorbance of the sample. The sample concentrations providing 50% of scavenging (SC_{50}) were calculated from the graph plotted between the percentages of scavenging and the sample concentrations.

2.6.2. Lipid peroxidation inhibition activity

The anti-oxidative activity of the extracts was assayed by the modified Ferric-thiocyanate method (Manosroi et al., 2010). An amount of 50 µl of five serial concentrations of the extracts (0.01–100 mg/ml) dissolved in 10%v/v DMSO was added to 50 µl of linoleic acid in 50% (v/v) DMSO. The reaction was initiated by the addition of 50 µl of NH_4SCN (5 mM) and 50 µl of $FeCl_2$ (2 mM). The mixture was incubated at 37 ± 2 °C in a 96-well microplate for 1 h. During the oxidation of linoleic acid, peroxides were formed leading to the oxidation of Fe^{2+} to Fe^{3+}. The latter ions forming a complex with thiocyanate can be detected at 490 nm. The solution without the sample was used as a negative control. Ascorbic acid (0.001–10 mg/ml) was used as a positive control. All determinations were performed in triplicate. The inhibition percentages of lipid peroxidation of linoleic acid were calculated by the following equation: inhibition of lipid peroxidation (%) = $[(A - B)/A] \times 100$, where A was the absorbance of the control and B was the absorbance of the sample. The sample concentrations providing 50% inhibition of lipid peroxidation (IPC_{50}) were calculated from the graph plotted between the percentages of lipid peroxidation inhibition and the sample concentrations.

2.7. Statistical analysis

All assays were performed in triplicate of three independent experiments. Data were calculated and presented as mean ± standard deviation (SD) using the SPSS version 17 program.

3. Results and discussion

3.1. Physical characteristics and percentage yields

Abbreviations of the Job's tears extracts of five cultivars prepared by various extraction processes are shown in Table 1.

Table 2 The highest percentage yields of the Job's tears extracts of the five cultivars (total 330 extracts).

Cultivars	Part use	Solvents	Code	Extract appearance and color	Percentage yields (%)
Thai Black Phayao (TBP)	Whole	Methanol	M-WTBPB3	Solid in white	11.83
		Hexane	H-WTBPB3	Solid in white	5.14
	Endosperm	Methanol	M-ETBPB3	Solid in white	16.02
		Hexane	H-ETBPB3	Solid in white	12.48
	Hull	Methanol	M-HTBPB3	Solid in dark brown	1.12
		Hexane	H-HTBPB3	Solid in dark brown	1.26
Thai Black Loei (TBL)	Whole	Methanol	M-WTBLB3	Solid in light yellow	10.85
		Hexane	H-WTBLN1	Oily liquid in yellow	3.42
	Endosperm	Methanol	M-ETBLS3	Solid in light brown	6.82
		Hexane	H-ETBLR2	Oily liquid in yellow	5.54
	Hull	Methanol	M-HTBLR1	Solid in dark brown	2.94
		Hexane	H-HTBLB3	Solid in light brown	4.90
Laos Black Loei (LBL)	Whole	Methanol	M-WLBLB3	Solid in white	18.13
		Hexane	H-WLBLB3	Solid in white	7.18
	Endosperm	Methanol	M-ELBLB3	Solid in white	16.82
		Hexane	H-ELBLB3	Solid in light brown	15.81
	Hull	Methanol	M-HLBLB3	Solid in light brown	4.66
		Hexane	H-HLBLB3	Solid in dark brown	5.39
Laos White Loei (LWL)	Whole	Methanol	M-WLWLB3	Solid in light brown	22.24
		Hexane	H-WLWLB3	Solid in white	9.09
	Endosperm	Methanol	M-ELWLB3	Solid in white	19.96
		Hexane	H-ELWLB3	Solid in white	14.70
	Hull	Methanol	M-HLWLB3	Solid in light brown	4.48
		Hexane	H-HLWLB3	Solid in dark brown	6.88
Laos Black Luang phra bang (LB)	Whole	Methanol	M-WLBB3	Solid in light brown	5.61
		Hexane	H-WLBB3	Solid in white	7.29
	Endosperm	Methanol	M-ELBB3	Solid in white	17.33
		Hexane	H-ELBB3	Solid in dark brown	17.00
	Hull	Methanol	M-HLBB3	Solid in dark brown	3.31
		Hexane	H-HLBB3	Solid in dark brown	4.12

Note: Percentage yields (%) = [Dried extract weigh (g)/dried Job's tears weigh (g)] × 100.

A total of 330 extracts included 150 methanol extracts and 180 hexane extracts. The extracts prepared by the cold extraction (1), hot extraction (2) and the hexane layer of the liquid partition (4) were in the form of oily liquid yellow–brown in color, whereas the extracts from the water layer of the liquid partition (3) were in solid form white-brown in color. Most methanol extracts were in solid form. These extracts may contain both polar and non-polar compounds which can be extracted better by methanol than hexane. The highest percentage yields of the Job's tears extracts of the five cultivars are summarized in Table 2. The endosperm extracts gave high yields because of the starch which was the main constituent found in the grain of the Job's tears (Chaisiricharoenkul et al., 2011). It has been indicated that there are at least six classes of chemical constituents in the hull of Job's tears including phenolic acids, lignans, flavonoids, polyphenols, polysaccharides, and phytosterols (Hsia et al., 2007). The extracts which gave the highest yield was the water (boiling) layer of the liquid partition (B3). In fact, the heated solvents have been reported to be able to release the cell wall bioactives by breaking down the cellular constituents resulting in the high percentage yields. Previous study has also shown that the proximate composition of Job's tears of the four countries including Laos, Thailand, Vietnam and Taiwan appeared not to be different (Wu et al., 2007).

3.2. Anti-proliferative activity on human colon adenocarcinoma cell line (HT-29)

The anti-proliferative activity expressed by the IC_{50} values of the selected Job's tears extracts, the four standard anti-cancer drugs and the commercial product on HT-29 are shown in Table 3. Only 34 extracts (10.3% of the 330 extracts) exhibited

Table 3 Anti-proliferative activity (IC_{50} values) of the selected Job's tears extracts on human colon adenocarcinoma cell line (HT-29) after a 24 h incubation.

Solvents	Cultivars	Parts use	Code	IC_{50} (μg/ml)
Methanol	Thai Black Phayao (TBP)	Hull	M-HTBP-N1	203.98 ± 2.21
			M-HTBP-N2	18.75 ± 0.92
			M-HTBP-B1	337.66 ± 0.39
			M-HTBP-B2	19.24 ± 0.67
			M-HTBP-R1	54.39 ± 0.46
			M-HTBP-R2	290.76 ± 0.21
			M-HTBP-S1	208.94 ± 0.51
			M-HTBP-S2	67.55 ± 1.57
		Whole	M-WTBP-B2	136.71 ± 1.02
			M-WTBP-S2	301.51 ± 1.99
	Thai Black Loei (TBL)	Hull	M-HTBL-N1	53.05 ± 2.61
			M-HTBL-B1	239.96 ± 1.21
			M-HTBL-B2	49.54 ± 0.31
			M-HTBL-R1	31.02 ± 1.18
			M-HTBL-R2	11.61 ± 0.95
			M-HTBL-S1	16.65 ± 0.75
			M-HLBL-N1	108.11 ± 0.71
			M-HLBL-B2	297.45 ± 1.72
			M-HLBL-R1	348.12 ± 0.22
			M-HLBL-R2	206.49 ± 1.23
			M-HLBL-S2	216.87 ± 1.23
	Laos White Loei (LWL)	Hull	M-HLWL-R1	120.19 ± 0.13
			M-HLWL-R2	53.71 ± 0.85
			M-HLWL-S1	171.81 ± 1.87
		Whole	M-WLWL-N2	74.94 ± 1.63
	Laos Black Luang phra bang (LB)	Hull	M-HLB-R1	53.81 ± 2.34
Hexane	Thai Black Phayao (TBP)	Hull	H-HTBP-B2	39.95 ± 1.34
			H-HTBP-B4	36.86 ± 1.42
			H-HTBP-S4	184.48 ± 0.28
		Whole	H-WTBP-S4	112.50 ± 2.89
	Laos White Loei (LWL)	Hull	H-HLWL-S4	116.54 ± 1.64
		Whole	H-WLWL-R2	13.36 ± 0.67
	Laos Black Luang phra bang (LB)	Hull	H-HLB-S4	56.98 ± 0.83
		Whole	H-WLB-S4	38.83 ± 1.32
Cisplatin				1.94 ± 0.29
Doxorubicin				8.21 ± 0.72
Vincristine				2.11 ± 2.88
Fluorouracil				0.12 ± 0.05
The commercial product				ND

Note: ND = not detected.

anti-proliferative activity. The anti-proliferative activity of the commercial product was not detected. The highest inhibition on HT-29 was the extract from the hull of Thai Black Loei roasted before the hot methanol extraction (M-HTBL-R2) with the IC_{50} values of 11.61 ± 0.95 μg/ml, but less effective than doxorubicin (8.21 ± 0.72 μg/ml) of 1.42 times. Most extracts from the hull part showed anti-proliferative activity, while the endosperm part showed no activity. Phytosterols such as campesterol, stigmasterol and β-Sitosterol in the hull of Job's tears may have an effect on the growth of human colon adenocarcinoma cell lines (Bhandari et al., 2014). β-Sitosterol can inhibit the HT-29 human colon cancer cell growth by mediating through the influence of signal transduction pathways that involve the membrane phospholipids (Awad et al., 1996). Phytosterols have been shown to inhibit colon cancer development. As known, Job's tears bran has a higher content of phytosterols than the hull or the polished Job's tears (Wu et al., 2007). In fact, the hull parts used in this study included the bran. Fatty acids and their metabolites have been previously reported to suppress tumor cell growth. Gamma-linolenic acid (GLA), arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were the most effective fatty acids in tumor cell growth inhibition (Das, 1991). Anti-cancer action of linoleic acid (LA) is due to the enhancement of ROS generation and the decrease of cell anti-oxidant capacity resulting in the mitochondrial damage (Lu et al., 2010). Oleic acid (OA) is a potent inhibitor of store-operated Ca^{2+} entry (SOCE) in which the Ca^{2+} influx

Table 4 The percentages of apoptotic, necrotic and live cells induced by the selected Job's tears extracts on human colon adenocarcinoma cell line (HT-29) after a 24 h incubation.

Samples	Final conc. (μg/ml)	Apoptotic cells (%)	Necrotic cells (%)	Live cells (%)
Negative control (10% DMSO in DMEM medium)	–	6.82 ± 1.76	5.77 ± 2.69	87.42 ± 2.96
M-HTBP-B2	1000	7.77 ± 2.45	0.00 ± 0.00	92.23 ± 2.45
	100	5.74 ± 0.25	1.62 ± 0.77	92.65 ± 0.57
	10	5.04 ± 1.25	2.45 ± 0.51	92.51 ± 1.50
	IC_{50}	6.04 ± 1.21	3.60 ± 0.26	90.36 ± 1.20
M-HTBL-R1	1000	5.66 ± 1.90	5.74 ± 1.45	88.60 ± 0.99
	100	11.25 ± 1.32	11.25 ± 4.07	77.50 ± 4.11
	10	7.03 ± 3.60	5.45 ± 3.40	87.52 ± 6.52
	IC_{50}	6.65 ± 3.51	8.40 ± 2.92	84.95 ± 6.21
M-WLWL-N2	1000	5.43 ± 2.47	0.00 ± 0.00	94.57 ± 2.47
	100	7.83 ± 0.96	1.82 ± 0.60	90.36 ± 1.37
	10	5.77 ± 2.39	1.98 ± 0.51	92.26 ± 1.86
	IC_{50}	6.86 ± 2.52	1.61 ± 0.77	91.53 ± 2.38
M-HTBL-R2	1000	6.14 ± 2.14	3.14 ± 1.41	90.72 ± 1.61
	100	5.03 ± 1.59	4.25 ± 1.44	90.72 ± 1.55
	10	3.06 ± 0.70	3.88 ± 1.49	93.06 ± 2.22
	IC_{50}	6.35 ± 2.42	4.57 ± 2.32	89.09 ± 1.32
H-WLWL-R2	1000	6.06 ± 0.61	2.61 ± 1.60	91.33 ± 1.32
	100	6.03 ± 0.67	6.39 ± 1.09	87.58 ± 1.70
	10	7.33 ± 3.86	6.08 ± 1.75	86.59 ± 3.56
	IC_{50}	7.78 ± 4.60	5.59 ± 1.75	86.63 ± 8.26
M-HTBL-S1	1000	4.99 ± 1.37	2.27 ± 0.63	92.74 ± 0.76
	100	6.60 ± 0.56	2.88 ± 0.65	90.52 ± 1.62
	10	5.44 ± 4.31	3.10 ± 1.81	91.46 ± 6.07
	IC_{50}	3.62 ± 1.60	0.62 ± 0.18	95.76 ± 1.99
M-HTBP-N2	1000	3.42 ± 0.56	2.16 ± 0.93	94.42 ± 1.56
	100	3.77 ± 1.67	2.26 ± 1.03	93.97 ± 0.64
	10	2.92 ± 1.97	3.95 ± 0.65	93.13 ± 2.44
	IC_{50}	6.02 ± 1.30	3.93 ± 1.90	90.05 ± 2.67
M-HTBL-N1	1000	8.17 ± 1.18	0.00 ± 0.00	91.83 ± 1.18
	100	6.46 ± 3.39	0.00 ± 0.00	93.54 ± 3.39
	10	5.20 ± 3.04	0.00 ± 0.00	94.80 ± 3.04
	IC_{50}	7.04 ± 0.95	0.00 ± 0.00	92.96 ± 0.95
Doxorubicin	100	21.84 ± 4.71	0.00 ± 0.00	78.16 ± 4.71
	10	13.43 ± 5.62	0.00 ± 0.00	86.57 ± 5.62
	1	5.74 ± 3.26	0.00 ± 0.00	94.26 ± 3.26
	IC_{50}	8.36 ± 2.55	0.00 ± 0.00	91.64 ± 2.55
The commercial product	1000	2.50 ± 0.93	0.00 ± 0.00	97.50 ± 0.93
	100	2.72 ± 1.04	0.00 ± 0.00	97.28 ± 1.04
	10	2.48 ± 0.82	0.00 ± 0.00	97.52 ± 0.82

pathway involved the control of multiple cellular and physiological processes including cell proliferation (Carrillo et al., 2012).

Most methanolic extracts showed anti-proliferative activity. This may be due to various polar and non-polar compounds in the extracts which can be dissolved by methanol. The roasted extracts (R) gave higher anti-proliferative activity than the non-cooked, boiled and streamed extracts, may be due to the high content of phenolic compounds. The bound phenolics that are usually bound to the proteins inside the Job's tears can be released during the roasting processing in short-time at high temperature. As there was no leachate produced by roasting, phenolics loss was limited (Xu and Chen, 2013). Huang et al. have demonstrated that phenolic compounds in the Job's tears test possess an anti-inflammatory effect, suggesting that phenolic acids may be some of the active compounds in Job's tears (Huang et al., 2009). The ethyl acetate fraction of ethanolic Job's tears bran extract has been reported to suppress DMH-induced preneoplastic lesions of the colon in F344 rats. Phenolic acid may be one of the active compounds (Chung et al., 2010). However, it is still unknown for the active compounds in Job's tears which have anti-proliferative activity on HT-29. This activity may be the result of the synergistic effects of several compounds. Selection of the extracts for the further apoptosis induction and anti-oxidative activity assays was based on the high percentage yields, ease of Job's tears part preparation, environment friendliness of the solvent used, ease of processing before the extraction and the high anti-proliferative activity.

3.3. Apoptosis induction activity on HT-29

The percentages of the apoptotic and necrotic cells of HT-29 induced by the selected Job's tears extracts in comparing with doxorubicin and the commercial product are shown in Table 4. Apoptotic cells can be also observed in the control group (the cells treated with 10% DMSO in DMEM medium). DMSO can induce apoptosis in a number of cell lines via mitochondrial dysfunction. The extracts from the hull of Thai Black Loei roasted before extracting by cold methanol (M-HTBL-R1) and the non-cooked hull of Thai Black Loei extracting by cold methanol (M-HTBL-N1) were the two extracts which showed high apoptosis induction activity. The commercial product was less effective than these two extracts of 4.13 and 3.00 times, respectively. M-HTBL-R1 at 100 μg/ml gave the highest percentages of apoptotic cells at 11.25 ± 1.32%, but less effective than doxorubicin (21.84 ± 4.71%) of 1.94 times. However, it was unexpectedly found that M-HTBL-R1 also indicated the highest percentages of necrotic cells at 11.25 ± 4.07%. However, M-HTBL-N1 at 100 μg/ml gave high apoptosis (8.17 ± 1.18%) but was less effective than doxorubicin of 2.67 times with no necrosis which was the appropriate anti-cancer properties. Doxorubicin can induce apoptosis through topoisomerase II inhibition and free radical generation (Hurley, 2002). The anti-cancer mechanisms of the Job's tears extracts may be different from the standard anti-cancer drugs. Interestingly, both extracts which showed high apoptosis induction activity were from the hull parts of Job's tears. Phytosterols in hull may be responsible for this effect. β-Sitosterol which is a phytosterol has been reported to induce apoptosis by inducing caspase-3 and caspase-9 activation accompanied by proteolytic cleavage of poly(ADP-ribose)-polymerase. In addition, β-Sitosterol-induced apoptosis in HT116 cells was associated with a decreased expression of the anti-apoptotic Bcl-2 protein and mRNA and a concomitant increase of the pro-apoptotic Bax protein and mRNA as well as with the release of cytochrome c from the mitochondria into the cytosol. β-Sitosterol also inhibited the expression of cIAP-1 without significant changes in the level of cIAP-2 (Choi et al., 2003). In addition, linoleic acid (LA) has been reported to induce cancer cell apoptosis by enhancing the cellular oxidant status and inducing mitochondrial dysfunction (Lu et al., 2010). The polysaccharide of Job's tears has been shown to inhibit A549 cell proliferation and induce apoptosis via a mechanism primarily involving the activation of the intrinsic mitochondrial pathway (Lu et al., 2013). The extracted apple polysaccharides (APs) demonstrated apoptosis-inducing effects on two human colorectal cancer cells (HT-29 and SW620), increased expressions of Bax, nuclear p65 and cytoplasmic pI-κBα, and decreased expressions of Bcl-2, Bcl-xl and cytoplasmic I-κBα. In addition, APs induced apoptosis by slightly activating the NF-κB pathway (Zhang et al., 2012).Thus, polysaccharide contained in the Job's tears extracts may be one of the compounds that can induce apoptosis on HT-29 cells. However, the mechanism of polysaccharide induced apoptosis on HT-29 cells is still unknown. Nevertheless, the results from this study agreed with the work of Lee et al. They have shown that the ethyl acetate fraction of adlay bran methanol extract (ABM-Ea) exhibited anti-proliferative effects against the HT-29 and COLO 205 human colon cancer cell lines via apoptosis (Lee et al., 2008). In fact, there may also be other compounds in Job's tears extracts which have the anti-cancer activity, but not by the mechanism of anti-proliferation and apoptosis. These compounds may have other complementary and overlapping mechanisms including the regulation of gene expression in cell proliferation, induction of cell-cycle arrest and anti-oxidative activities (Sun et al., 2002).

3.4. Anti-oxidative activities

Anti-oxidative activities including free radical scavenging and lipid peroxidation inhibition of the selected extracts are shown in Table 5. The commercial product gave SC_{50} values more

Table 5 Antioxidant activities of the selected Job's tears extracts.

Extracts	SC_{50} (mg/ml)	IPC_{50} (mg/ml)
M-HTBP-B2	1.48 ± 0.48	–
M-HTBL-R1	2.70 ± 1.28	–
M-WLWL-N2	4.91 ± 2.45	–
M-HTBL-R2	0.48 ± 0.12	–
H-WLWL-R2	> 1000	1.67 ± 1.10
M-HTBL-S1	0.40 ± 0.07	–
M-HTBP-N2	1.07 ± 0.05	–
M-HTBL-N1	2.47 ± 1.15	359.87 ± 4.16
The commercial product	> 1000	–
Ascorbic acid	0.20 ± 0.02	0.02 ± 0.01

Note: SC_{50} = The sample concentrations providing 50% of scavenging; IPC_{50} = The sample concentrations providing 50% inhibition of lipid peroxidation.

than 1000 mg/ml and did not indicate any lipid peroxidation inhibition activity. The cold methanol extract from the streamed hull of Thai Black Loei (M-HTBL-S1) exhibited the highest free radical scavenging activity (SC_{50} values of 0.40 ± 0.07 mg/ml), but was less effective than ascorbic acid (0.20 ± 0.02 mg/ml) by 2 times. M-HTBL-R2 and M-HTBL-N1 which exhibited high anti-proliferation and apoptosis induction indicated the SC_{50} values of 0.48 ± 0.12 and 2.47 ± 1.15 mg/ml, but was less effective than ascorbic acid by 2.4 and 12.4 times, respectively. The phenolic compounds including flavonoids, anthraquinones, anthocyanidins, xanthones and tannins have been reported to scavenge DPPH. They also scavenged superoxide and hydroxyl radicals by the single electron transfer (Choi et al., 2002). The extract from the whole seed of Laos White Loei roasted before hot hexane extraction (H-WLWL-R2) gave the highest lipid peroxidation inhibition at the IPC_{50} values of 1.67 ± 1.10 mg/ml, but was less effective than ascorbic acid (0.02 ± 0.01 mg/ml) by 83.5 times. This may be due to the phenolic compounds in the Job's tears seeds including coniferyl alcohol, syringic acid, ferulic acid, syringaresinol, 4-ketopinoresinol and mayuenolide (Kuo et al., 2001). Obviously, the black hull cultivar gave a slightly higher DPPH activity than the white one. This may be due to the high total phenolic content of the black hull cultivar (Chaisiricharoenkul et al., 2011). In fact, free radicals are found to be involved both in initiation and promotion of the multi-stage carcinogenesis (Sun, 1990). Anti-oxidants can prevent damage to cells by supplying extra electron molecules to stabilize free radicals (Markham, 2005). Hence, the high free radical scavenging activity of the Job's tears extracts may be the synergistic effect of the anti-cancer activity.

4. Conclusions

This study has demonstrated the *in vitro* anti-cancer activities of Job's tears extracts on HT-29. The extract from the hull of Thai Black Loei roasted before hot methanol extraction (M-HTBL-R2) exhibited the highest anti-proliferative activity with the IC_{50} value of 11.61 ± 0.95 μg/ml, but was less effective than doxorubicin of 1.42 times. The extract from the hull of Thai Black Loei roasted before the cold methanol extraction (M-HTBL-R1) showed the highest apoptosis induction activity at 11.25 ± 1.32%, but was less effective than doxorubicin (21.84 ± 4.71%) by 1.94 times. However, it also gave the highest percentages of necrotic cells at 11.25 ± 4.07%. But, the hull of Thai Black Loei extracted by the cold methanol extraction (M-HTBL-N1) indicated high apoptosis (8.17 ± 1.18%) with no necrosis. M-HTBL-R2 and M-HTBL-N1 exhibited the anti-oxidant activity with the SC_{50} values of 0.48 ± 0.12 and 2.47 ± 1.15 mg/ml, but was less effective than ascorbic acid by 2.4 and 12.4 times, respectively. Hence, M-HTBL-N1 appeared to have appropriate anti-cancer properties than M-HTBL-R1. This study has suggested the anti-colon cancer potential of the non-cooked hull Job's tears extract from Thai Black Loei cultivar prepared by the cold methanol extraction.

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