Potent in vitro Anti-mouth Cancer (KB) and Immunostimulating Activities of the Job’s Tears (Coix lachryma-jobi Linn.) Seed Semi-purified Extract Cocktails Containing Linoleic Acid

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Abstract: The crude methanolic and hexane extracts of non-cooked, steamed and roasted from three Job's Tears cultivars were prepared and further semi-purified by liquid-liquid extraction techniques and silica gel column. The six single semi-purified extracts (F1-F6) were combined as nine cocktails (CT1, CT6, CT8, CT13, CT14, CT21, CT24, CT25 and CT31) according to the IC₅₀ values from the preliminary study and investigated for anti-proliferative and apoptotic induction on mouth cancer cell line (KB) and immunostimulating as well as antioxidative activities. The highest anti-proliferative activity was observed in CT13 showing the IC₅₀ value of 0.53±0.45 µg/mL which was higher than 5-fluorouracil and doxorubicin of 20.34 and 1.60 times, respectively. CT1 which was the combination of F1-F6 and CT13 which was the combination of F4-F6 exhibited significant strong synergistic activity with the combination index value (CI) of 0.28. CT1 at 200 µg/mL showed the highest percentages of apoptotic cells (40.65±10.97%) with no necrotic cells, but lower than cisplatin (100 µg/mL) of 2.18 times. CT14 gave the highest immunostimulating activity with the phagocytosis percentage of 13.0±1.7%, but lower than lipopolysaccharide of 1.08 times. CT31 gave the highest free radical scavenging and metal chelating activities with the SC₅₀ and MC₅₀ values of 0.73±0.07 and 1.99±0.24 µg/mL, but lower than ascorbic acid and EDTA of 18.25 and 4.33 times, respectively. The linoleic acid contents related to anti-cancer activity were also examined by HPLC. This study has demonstrated that CT1 composing of F1-F6 at the percentage ratio of 0.71:2.06:81.38:8.47:2.46 was the potential cocktails of the semi-purified extracts from the Job’s Tears which can be further developed as a novel active compound for oral cancer treatment.

Key words: semi-purified extracts, Job’s Tears, anti-cancer, immunostimulating, linoleic acid

1 Introduction

Oral cancer is a heterogeneous group of cancers arising from different parts of the oral cavity with different predisposing factors, prevalence, and treatment outcomes. It is the sixth most common cancer reported globally with an annual incidence of over 300,000 cases and an increase of 62% has been reported in the developing countries¹. Chemotherapy, being a major treatment modality used for the control of advanced stages of malignancies and as a prophylactic against possible metastasis. The natural components of the diet may serve as chemopreventive agents that suppress the growth and dissemination of neoplastic colon cells⁵. 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) is one of the most widely used food. The low incidence of cancer has been observed in the area of China where people in this area regularly consume Job’s Tears⁶. Several studies have shown that Job’s Tears may have an anti-tumor effect.

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Some bioactive compounds in Job’s Tears, especially cox-
enolide, inhibited tumors, prevented cancer and protected against viral infection. The emulsion of Job’s Tears oil has been approved by the Chinese Ministry of Public Health for anti-cancer activity. This preparation has been used to treat patients for the treatment of various common types of cancer, including lung, breast and liver cancer. Previous study has demonstrated that the steamed whole Thai Black Loei Job’s Tears (W-TBL-S1) crude extract and the semi-purified extracts from butanol fraction showed the highest anti-proliferative activity in mouth epithelial carcinoma cell (KB) at the IC_{50} of 43.61 ± 0.76 and 5.93 ± 0.13 µg/mL, respectively. Some studies have suggested that drug cocktails may exhibit enhanced efficacies with diminished side effects and complications for cancer treatment. Shang et al. have demonstrated that Taxus cuspidata extract and 5-FU, combined as a cocktail, synergistically inhibited the growth of cancer cells in vitro, with the Combination Index values (CI) ranging from 0.90 to 0.26 at the different effect levels from IC_{50} to IC_{90} in MCF-7 cells, CI ranging from 0.93 to 0.13 for IC_{50} to IC_{90} in PC-3M-IE8 cells, and CI<1 in A549 cells. Although several research-es supporting the beneficial effects of combining botanical and chemical medicines have been described, very few studies have been performed on the effects of several botanical extracts combination. This study has aimed to investigate the anti-cancer activities including anti-proliferation and apoptosis induction on mouth cancer cell line (KB) and immunostimulating as well as antioxidative activities of the semi-purified from Job’s Tears cocktails to develop as a potential anti-oral cancer drug.

2 Experimental Procedures

2.1 Materials

Three cultivars of Job’s Tears Thai Black Loei (TBL), Laos Black Loei (LBL) and Laos White Loei (LWL) were purchased from Yongswickapduol Wang Saphung Co., Ltd, Loei in Thailand. A voucher specimen (HFD-183) was deposited in the herbarium of Chiang Mai University (CMU) herbarium and flora database, Department of Biology, Faculty of Science, Chiang Mai University, Thailand. Sul-
forhodamine B (SRB), acridine orange (AO), ethidium bromide (EB), lipopolysaccharide (LPS) from Escherichia coli and EDTA were purchased from Sigma (St. Louis, MO, USA). Basic fuchsin was from Biobasic Inc., NY, USA. Polymorphprep was from Axis-shield, Oslo in Norway. Trypsin was prepared at 0.25% solution in phosphate buffered saline. Complete DMEM medium was prepared from Dul-
becco’s Modified Eagle Medium (GIBCO, Invitrogen 95 Cor-
nor, 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 semi-purified extracts

The Job’s Tears seeds were ground by a mortar. The endosperm and hull (H) were separated manually. The whole seed (W) and hull of Job’s Tears were processed as non-cooked (N), roasted (R) and steamed (S). For roasting, the dried Job’s Tears were heated (300°C) in an iron pan with continuous stirring for 5 min. For steaming, the Job’s Tears were soaked with water for 1 h, steamed in an electric rice cooker for 30 min and dried at 50 ± 2°C in a hot air oven. The processed Job’s Tears (100 g) were soaked in 1 L of solvent for 24 h before extraction by the heating or non-heating process. For the non-heating extraction, the mixture was shaken at 300 rpm in an orbital shaker for 1 h at room temperature (27 ± 2°C). The heating process was performed by refluxing at 70 ± 2°C for 3 h. The crude extract was filtered and the filtrate was concentrated under vacuum by a rotary evaporator (R-124 Büchi, Swit-
zerland). The methanolic crude extract was dispersed in distilled water and then sequential partitioned with sol-
vents of increasing polarities from hexane, ethyl acetate to butanol. The fractionation of hexane crude extract was performed in a silica gel column eluted with hexane, hexane/ethyl acetate (3:1), hexane/ethyl acetate (1:1) and ethyl acetate. Six semi-purified extracts were selected to prepare the cocktail formulations. The descriptions and extract codes of each Job’s Tears sample were presented in Table 1.

2.3 Compositions of semi-purified extract cocktails

The percentage ratios of the semi-purified extract cock-
tails were calculated from the IC_{50} values from the prelimi-
ary study. The compositions and ratios of the semi-puri-
fied extract cocktails were shown in Table 2.

2.4 Cell cultures

The human mouth epithelial carcinoma (KB) cell line provided from Medicinal Microbiology Department, Faculty of Biology, University of Tuebingen, Tuebingen in Germany was cultured in the complete DMEM medium. The cells were maintained in a humidified atmosphere of 5% CO_{2} incu-
bator (Contherm mitre 4000, Contherm Scientific, Hutt city, New Zealand) at 37 ± 2°C. The cells were trypsinized and counted with a haemacytometer.

2.4.1 Anti-proliferative activity by the sulforhodamine B assay

Cells (10^4 cells/well) were plated in 96-well plates and incu-
bated overnight in a humidified atmosphere of 5% CO_{2} incubator at 37 ± 2°C. An amount of 20 µL of the extracts and the four standard anti-cancer drugs (cisplatin, doxorubicin, fluorouracil and vincristine) at various final concentra-
tions 0.1-1000 µg/mL were added to the cells. After 24 h of incubation, the cells were fixed with 50% trichlo-roace-
tic 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 were added to each well to solubilize the dye and were 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 cell growth (G) were determined using the following equation: % Cell growth (G) = (A-C/ B-C) × 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 IC50 values were determined by plotting the percentages of cell growth (G) versus the concentrations of the samples. Extracts which exhibited potential anti-proliferative activity were selected for the apoptotic test.

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

The extracts (200 μg/mL) and the standard anti-cancer drug (100, 10 and 1 μg/mL) were tested on KB cells. Cells without extract were served as the negative control. An amount of 10 μL of the extracts and the standard anti-cancer drug 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) were 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 for 3 times and at least 100 cells of each experiment were counted.

2.5 In vitro immunostimulating activity
2.5.1 Neutrophil separation

Murine neutrophils were separated from the blood obtained by a cardiac puncture under deep anesthesia condition from male Sprague Dawley (SD) rats, weighing between 250 and 300 g purchased from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakhon

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**Table 1** Abbreviations and preparation method of the semi-purified extracts (F1-F6).

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Codes</th>
<th>Solvents</th>
<th>Parts used</th>
<th>Cultivars</th>
<th>Process before solvent extraction</th>
<th>Extraction method</th>
<th>Fractional layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>M-WLWL-R2.E</td>
<td>Methanol</td>
<td>Whole</td>
<td>Laos White Loei</td>
<td>Roasting</td>
<td>Hot extraction</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>F2</td>
<td>H-WLWL-R2.H1/E1</td>
<td>Hexane</td>
<td>Whole</td>
<td>Laos White Loei</td>
<td>Roasting</td>
<td>Hot extraction</td>
<td>Hexane/ethyl acetate (1:1)</td>
</tr>
<tr>
<td>F3</td>
<td>H-WLWL-R2.E</td>
<td>Hexane</td>
<td>Whole</td>
<td>Laos White Loei</td>
<td>Roasting</td>
<td>Hot extraction</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>F4</td>
<td>M-WTBL-S1.E</td>
<td>Methanol</td>
<td>Whole</td>
<td>Thai Black Loei</td>
<td>Steaming</td>
<td>Cold extraction</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>F5</td>
<td>M-HTBL-N1.E</td>
<td>Methanol</td>
<td>Hull</td>
<td>Thai Black Loei</td>
<td>Non-cook</td>
<td>Cold extraction</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>F6</td>
<td>M-HLBL-R1.H</td>
<td>Methanol</td>
<td>Hull</td>
<td>Laos Black Loei</td>
<td>Roasting</td>
<td>Cold extraction</td>
<td>Hexane</td>
</tr>
</tbody>
</table>

**Table 2** The compositions and percentage ratios of the semi-purified extract cocktails.

<table>
<thead>
<tr>
<th>Cocktails</th>
<th>Percentage ratios (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>F1</td>
</tr>
<tr>
<td>CT1</td>
<td>0.71</td>
</tr>
<tr>
<td>CT6</td>
<td>7.16</td>
</tr>
<tr>
<td>CT8</td>
<td>–</td>
</tr>
<tr>
<td>CT13</td>
<td>–</td>
</tr>
<tr>
<td>CT14</td>
<td>6.61</td>
</tr>
<tr>
<td>CT21</td>
<td>–</td>
</tr>
<tr>
<td>CT24</td>
<td>–</td>
</tr>
<tr>
<td>CT25</td>
<td>–</td>
</tr>
<tr>
<td>CT31</td>
<td>–</td>
</tr>
</tbody>
</table>
Pathom in Thailand. The collected blood samples were immediately transferred to the tubes containing EDTA (final concentration 1-2%) as an anticoagulant. Neutrophils were obtained from Polymorphprep centrifugation. Briefly, 5 mL of the blood samples with EDTA was carefully laid over 5 mL of Polymorphprep in a 15 centrifuge tube and centrifuged at 500 g for 30-35 min. Then, the band of the polymorphonuclear (PMN) leukocytes was harvested and mixed with an equal volume of the half-strength saline solution to obtain an iso-osmotic condition. The cell suspension was centrifuged at 400 g for 10 min and resuspended in the phosphate buffer saline (PBS).

2.5.2 Nitroblue tetrazolium (NBT) dye reduction test

The suspension of leukocytes (5 x 10^6/mL) was prepared in 0.2 mL PBS solution in 1.5 mL microcentrifuge tubes. The extracts dissolved in PBS at 10 mg/mL were prepared. An amount of 0.1 mL of the samples was added to the leukocyte suspension. Serum containing LPS at the final concentration of 20 μg/mL and the PBS solution were used as the positive and negative control, respectively. An amount of 0.2 mL of the freshly prepared 0.15% NBT solution was added to each tube before incubation at 37 ± 2℃ for 20 min. After incubation, the leukocytes were harvested by gently centrifugation at 400 rpm for 3-4 min. An amount of 40-50 μL of PBS was added to resuspend the cells. A drop of the cell suspension was put on a microscope slide, dried, fixed with methanol and counter stained with diluted carbol-fuschin (0.3%) for 15 sec. The slides were washed with tap water, dried and observed under the microscope (Olympus Optical Co. Ltd, Tokyo, Japan) using an oil immersion objective. Cells were scored as positive when the cells ingested the particles which were stained with blue-black by the precipitated formazan (the oxygen dependent reduction product of NBT). At least 300 cells were counted for each determination.

2.6 Antioxidation activities

2.6.1 Free radical scavenging assay

Free radical scavenging activities of the extracts and the standard antioxidant (ascorbic acid) were determined by a modified DPPH assay. 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 the ethanol 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 room temperature (27 ± 2℃), 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] x 100, where A was the absorbance of the control and B was the absorbance of the sample. The sample concentrations providing 50% of scavenging (SC50) were calculated from the graph plotted between the percentages of scavenging and the sample concentrations.

2.6.2 Metal chelating activity

The metal ion chelating activity was assayed by the modified ferrous ion chelating method. Briefly, 100 μL of five serial concentrations of the extracts (0.001-10 mg/mL) in 10% v/v DMSO were added to the 2 mM FeCl₂ (50 μL) in distilled water. The reaction was initiated by adding of 50 μL of 5 mM ferrozine and then distilled water to 300 μL. The mixture was incubated at room temperature (27 ± 2℃) for 15 min, and the absorbance at 562 nm was measured by a microplate reader. The negative control was the complex of FeCl₂ and ferrozine. EDTA (0.001 to 10 mg/mL) was used as a positive control. All experiments were performed in triplicate. The inhibition percentages of ferrozine-Fe²⁺ complex formation were calculated as follows: Metal chelating activity (%) = [(A-B)/A] x 100, where A was the absorbance of the control and B was the absorbance of the sample. The sample concentrations providing 50% metal chelating activity (MC50) were calculated from the graph plotted between the percentages of metal chelating activity and the sample concentrations.

2.6.3 Lipid peroxidation inhibition activity

The antioxidant activity of the extracts was assayed by the modified Ferric-thiocyanate method. An amount of 50 μL of five serial concentrations of the extracts (0.001-10 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 NH₄SCN (5mM) and 50 μL of FeCl₂ (2 mM). The mixture was incubated at 37 ± 2℃ in a 96-well microplate for 1 h. During the oxidation of linoleic acid, peroxides were formed leading to the oxidation of Fe²⁺ to Fe³⁺. 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] x 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 (IPC50) were calculated from the graph plotted between the percentages of lipid peroxidation inhibition and the sample concentrations.

2.7 Combination index (CI) for determining synergism, additive or antagonism

The obtained CI values were based on the multiple drug effect equation of the enzyme kinetic models of Chou-Talalay. For mutually exclusive drugs exhibiting similar modes of action, where (D)₁ and (D)₂ were the doses of drug 1 and drug 2 in combination and (Dx)₁ and (Dx)₂ are...
were the doses of drug 1 and drug 2 alone, respectively, causing ×% inhibition, the combination index was described as CI = (D1/(Dx)1 + (D2/(Dx)2 + ... n/((Dn/(Dx)n in this study, (D1), (D2) and (Dn were the IC50 values of the semi-purified fraction 1, semi-purified fraction 2 and semi-purified fraction n in combination (CT formulation) and (Dx)1, (Dx)2 and (Dx)n were the IC50 values of the semi-purified fraction 1, semi-purified fraction 2 and semi-purified fraction n alone, respectively. The resulting combination index (CI) gave a quantitative definition for the additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations. The precise biological significance of various degrees of synergism or antagonism remains to be defined, but it has been proposed that CI values can be interpreted as follows:

- < 0.1: very strong synergism
- 0.1 - 0.3: strong synergism
- 0.3 - 0.7: synergism
- 0.7 - 0.9: moderate to slight synergism
- 0.9 - 1.1: nearly additive
- 1.1 - 1.45: slight to moderate antagonism
- 1.45 - 3.3: antagonism
- > 3.3: strong to very strong antagonism

2.8 HPLC fingerprint of the semi-purified Job’s Tears extract cocktails

The linoleic acid contents of the extracts were determined by HPLC (Luna® C18 10 μm 250 mm × 4.60 mm Phenomenex USA Column, LC1200 UV/VIS Detector and LC1100HPLC Pump) using mixture of 95% (v/v) acetonitrile and 5% (v/v) of 0.1% (v/v) glacial acetic acid as a mobile phase, injection volume of 20 μL, flow rate of 1 mL/min and the UV detector at 205 nm. Linoleic acid contents were determined from the HPLC chromatogram comparing with the standard linoleic acid. The retention time of linoleic acid was 6.577 min. The linoleic acid contents were determined from the standard curve of the standard linoleic acid with r² of 0.9940. The regression equation was y = (1.4 × 10⁷)x + 463164, where y was the area under the curve and x was the concentration of linoleic acid (mg/mL).

3 Results and Discussion

3.1 Anti-proliferative activity on human mouth epidermal carcinoma cells

The anti-proliferative activities (IC50 values) of the single and cocktails of the semi-purified extracts from Job’s Tears and the four standard anti-cancer drugs on human mouth epidermal cancer cells were shown in Table 3. The single semi-purified extracts (F1-F6) gave the IC50 values in the range of 0.46 ± 0.25 to 56.07 ± 30.35 μg/mL, while the cocktails (CT1, CT6, CT8, CT13, CT14, CT21, CT24, CT25 and CT31) of the semi-purified extracts showed the IC50 values in the range of 0.53 ± 0.45 to 24.70 ± 18.39 μg/mL. Among the semi-purified extract cocktails, the highest inhibition was CT13 showing the IC50 value of 0.53 ± 0.45 μg/mL. The anti-proliferative activity of CT13 was higher than 5-fluorouracil (10.78 ± 5.03 μg/mL) and doxorubicin (0.85 ± 0.26 μg/mL) of 20.34 and 1.60 times, respectively, but lower than cisplatin (0.38 ± 0.20 μg/mL) and vincristine (0.05 ± 0.01 μg/mL) of 1.39 and 10.6 times, respectively. The combination of F1-F6 of CT1 and F4-F6 of CT13 exhibited significant strong synergistic interactions with the CI value of 0.28. Moreover, CT13 exhibited higher activity than the single semi-purified fractions F4, F5 and F6 which were the composition in the CT13 of 11.02, 6.40 and 3.19 times, respectively. The HPLC fingerprint of CT13 (Fig. 1) showed the highest linoleic acid content of 31.16%. It has been reported that the anti-cancer action of linoleic acid (LA) is due to the enhancement of ROS generation and the decrease of cell antioxidant capacity resulting in the mitochondrial damage. The main component in CT13 may have an effect on the growth of human mouth epidermal carcinoma cells. F6 was a hexane fraction composed of non-polar or low polar compounds. The antiproliferative active compounds were likely to be non-polar or low polar chemicals since high cytotoxicity has been principally observed in the hexane partitioned fraction. The synergistic effects can be obtained from several compounds such as phenolic acids, lignans, flavonoids, polyphenols, polysaccharides, and phytoestrogens which are the components in the hull of Job’s Tears that was used to prepare F5. These results showed that the anti-proliferative activity in human mouth epidermal carcinoma cells of CT1 and CT13 was strong synergistic with the CI value in the range of 0.1-0.3.

3.2 Apoptotic activity on human mouth epidermal carcinoma cell

The percentages of apoptotic and necrotic cancer cells after induction by the semi-purified extract cocktails from Job’s Tears and cisplatin (the standard anti-cancer drug) on human mouth epidermal carcinoma cells were shown in Table 3. All semi-purified extract cocktails, except CT31, exhibited apoptosis induction activity. The cocktails showed the percentages of apoptotic cells in the range of 3.01 ± 1.70 to 40.65 ± 10.97%. CT1 at 200 μg/mL showed the highest percentage of apoptotic cells (40.65 ± 10.97%) with no necrotic cells indicating of the appropriate anti-cancer properties, but lower than cisplatin at 100 μg/mL (88.65 ± 4.71%) of 2.18 times. The apoptosis without necrosis is the major pathway in the anti-cancer treatment. In necrosis, the cell death process may depend on the treatment time and the sensitivity of cell lines to the extracts. Necrotic cells can induce damage of the surrounding cells by an inflammation, while the apoptotic cells were removed from the surrounding cells without causing inflammation. It was unexpectedly found that CT6, CT21, CT24 and CT31
also showed necrotic cells of $6.79 \pm 2.18$, $23.73 \pm 9.38$, $14.37 \pm 1.59$ and $18.67 \pm 1.83\%$, respectively, while the necrotic cells after induction by cisplatin was not observed. Cisplatin forms inter- and intranad strand crosslinked DNA adducts and its cytotoxicity is mediated by propagation of DNA damage recognition signals to the downstream pathways involving ATR, p53, p73, and mitogen-activated protein kinases, ultimately resulting in apoptosis\(^{19}\). CT1 was composed of many single semi-purified extracts containing several active compounds. F3, the main component

Table 3  Anti-cancer on KB cell line and other related anti-cancer activities of single and cocktails of the semi-purified extracts of the Job’s Tear seeds.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Anti-cancer activities on human mouth epidermal carcinoma (KB) cell line</th>
<th>Immunostimulating activity (%phagocytosis)</th>
<th>Antioxidative activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-proliferation (IC(_{50}); µg/mL)</td>
<td>%apoptotic cells</td>
<td>%necrotic cells</td>
</tr>
<tr>
<td>Single semi-purified extracts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>0.49 ± 0.25</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>F2</td>
<td>1.42 ± 0.42</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>F3</td>
<td>56.07 ± 30.35</td>
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<td>ND</td>
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<tr>
<td>F4</td>
<td>5.84 ± 2.97</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>F5</td>
<td>3.39 ± 1.26</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>F6</td>
<td>1.69 ± 1.03</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cocktails of semi-purified extracts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT1</td>
<td>3.25 ± 1.63</td>
<td>40.65 ± 10.97</td>
<td>0</td>
</tr>
<tr>
<td>CT6</td>
<td>7.92 ± 1.78</td>
<td>34.57 ± 3.34</td>
<td>6.79 ± 2.18</td>
</tr>
<tr>
<td>CT8</td>
<td>7.96 ± 0.74</td>
<td>35.46 ± 9.07</td>
<td>0</td>
</tr>
<tr>
<td>CT13</td>
<td>0.53 ± 0.45</td>
<td>22.90 ± 2.65</td>
<td>0</td>
</tr>
<tr>
<td>CT14</td>
<td>24.70 ± 18.39</td>
<td>31.95 ± 10.44</td>
<td>0</td>
</tr>
<tr>
<td>CT21</td>
<td>5.86 ± 2.18</td>
<td>3.01 ± 1.70</td>
<td>23.73 ± 9.38</td>
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<tr>
<td>CT24</td>
<td>13.65 ± 7.36</td>
<td>3.72 ± 1.48</td>
<td>14.37 ± 1.59</td>
</tr>
<tr>
<td>CT25</td>
<td>13.50 ± 5.01</td>
<td>20.81 ± 4.14</td>
<td>0</td>
</tr>
<tr>
<td>CT31</td>
<td>7.55 ± 3.37</td>
<td>0</td>
<td>18.67 ± 1.83</td>
</tr>
<tr>
<td>The standard anti-cancer drugs and other standards</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0.38 ± 0.20</td>
<td>88.65 ± 4.91 (100 µg/mL)</td>
<td>0 (100 µg/mL)</td>
</tr>
<tr>
<td>5-fluorouracil</td>
<td>10.78 ± 5.03</td>
<td>18.74 ± 6.27 (10 µg/mL)</td>
<td>0 (10 µg/mL)</td>
</tr>
<tr>
<td>Vincristine</td>
<td>0.05 ± 0.01</td>
<td>12.22 ± 1.34 (1 µg/mL)</td>
<td>0 (1 µg/mL)</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.85 ± 0.26</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>EDTA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND= not determine

Fig. 1  HPLC fingerprint of the semi-purified extract cocktails, CT13 using linoleic acid as a marker at the retention time of 6.577 min.
in CT1 may have an effect on the apoptosis induction of human mouth epidermal carcinoma cells. Previous study has indicated that the ethyl acetate fraction of the Job’s Tears methanolic extract has the potential to induce apoptosis in human carcinoma cells. Infact, several compounds in many plants have been shown to have anti-cancer activities with the mechanisms of not only anti-proliferation and apoptosis, but also other complementary and overlapping mechanisms including the regulation of gene expression in cell proliferation, induction of cell-cycle arrest, immunostimulating and antioxidative activities.

3.3 Immunostimulating activity

Immunostimulatory activity in murine neutrophils by the NBT test of the single and cocktails of the semi-purified extracts from Job’s Tears and the lipopolysaccharide (the positive control) was shown in Table 3. All samples gave higher immunostimulatory activity than the negative control in the range of 1.13 to 2.76 times. The intracellular reduction of nitroblue tetrazolium dye to formazan by the neutrophils has confirmed the intracellular killing and preserved the integrity of the neutrophils. The reduction of NBT to insoluble blue formazan was used as a probe for superoxide generation, although it is not entirely specific for the $O_2^-$ radical. The NBT reduction assay estimated the ability of the neutrophils and macrophages to produce oxygen radicals ($O_2^-$, $OH^-$, $O_3$, $H_2O_2$). The ability of macrophages to kill the pathogenic microbes is probably one of the most important mechanisms of protection against diseases. The highest percentage of phagocytosis of the single and cocktails of the semi-purified extracts were F5 (11.5 ± 2.0%) and CT14 (13.0 ± 1.7%), but lower than lipopolysaccharide, the positive control of 1.22 and 1.08 times, respectively. It has been reported that the consumption of the Job’s Tears extracts can increase the activities of cytotoxic $T$ cells and natural killer (NK) cells. The methanolic extract of Job’s Tears seeds inhibited NO and $O_2^-$ production by activated macrophages. The immunostimulatory activity of the extracts from Job’s Tears might be from the fatty acid component in the samples. Palmitic, oleic and linoleic acids were the major fatty acids, accounting for more than 90% of the total fatty acids, whereas stearic, linolenic and arachidic acids were found in lesser amounts. Fatty acids have been activated in order to phagocytose particles, release protein and lipid mediators and produce reactive oxygen and nitrogen species. Neutrophils contain NADPH oxidase, an enzyme that generates superoxide. NADPH oxidase can be directly stimulated by arachidonic acid, leading to free radical production, and can probably stimulate cell microbicidal activity.

3.4 Antioxidative activities

Antioxidative activities including free radical scavenging, metal chelating and lipid peroxidation inhibition of the single and cocktails of the semi-purified extracts from Job’s Tears and the standards (ascorbic acid and EDTA) were shown in Table 3. The highest free radical scavenging activity of the single the semi-purified extract was observed in F1 ($SC_{50}$ value = 0.88 ± 0.44 µg/mL), while the cocktails of the semi-purified extract was observed in CT31 ($SC_{50}$ value = 0.73 ± 0.07 µg/mL), which were lower than ascorbic acid of 22 and 18.25 times, respectively. The highest metal chelating activity of the single and cocktails of the semi-purified extracts were observed in F2 ($MC_{50}$ value = 0.50 ± 0.38 µg/mL) and CT31 ($MC_{50}$ value = 1.99 ± 0.24 µg/mL), which were lower than EDTA of 1.09 and 4.33 times, respectively. All semi-purified extract cocktails showed free radical scavenging and metal chelating activities, while only some single extracts gave these activities. Moreover, only CT6 and CT25 exhibited lipid peroxidation inhibition at the IPC50 values of 29.16 ± 1.48 and 27.02 ± 20.24 µg/mL, but lower than ascorbic acid (0.05 ± 0.01 µg/mL) of 583.2 and 540.4 times, respectively. Thus, the combination of the semi-purified extract in a cocktail formulation can enhance the antioxidative activities. This may be due to the synergistic effects of several compounds in the cocktails.

4 Conclusion

This study has demonstrated that the combination of the semi-purified extract as a cocktail formulation can enhance the anti-cancer activities including anti-proliferation and apoptosis induction on mouth cancer cell line (KB) as well as immunostimulating and antioxidative activities. The anti-proliferative activity of CT1 and CT13 exhibited strong synergistic in human mouth epidermal carcinoma cells. CT1 showed the highest percentages of apoptotic cells (40.65 ± 10.97%) with no necrotic cells demonstrating the appropriate anti-cancer properties. The highest phagocytosis percentage of the semi-purified extract cocktails was observed in CT14 (13.0 ± 1.7%). All semi-purified extract cocktails showed free radical scavenging and metal chelating activities, while only some single extracts gave these activities. Moreover, only CT6 and CT25 exhibited lipid peroxidation inhibition. CT1 cocktails from the combination of F1 to F6 at the percentage ratio of 0.71:2.06:81.38:8.47:4.92:2.46 seemed to be the most potential cocktails because of its high anti-mouth cancer and other related anti-cancer activities.

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