



## Pharmaceutical and Cosmeceutical Biological Activities of Hemp (*Cannabis sativa* L var. *sativa*) Leaf and Seed Extracts

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### ABSTRACT

The fresh Hemp (*Cannabis sativa* L var. *sativa*) leaves and seeds were extracted by maceration in 95% ethanol and investigated for their biological activities. The percentage yields were  $3.77 \pm 0.31$  and  $1.58 \pm 0.20\%$ , respectively. For cosmeceutical activities, the leaf extract exhibited higher DPPH scavenging, metal chelating and tyrosinase inhibition activity than the seed extract. The leaf extract exhibited pro MMP-2 inhibition ( $56.27 \pm 5.98\%$ ) and active MMP-2 inhibition (100%) with the pro MMP-2 inhibition lower than ascorbic acid of 1.11 times. The melanin induction in B16F10 melanoma cells of the leaf extract was 1.06 and 2.22 times of the control and theophylline, respectively. Moreover, it showed  $5\alpha$ -reductase inhibition ( $13.57 \pm 3.21\%$ ), but lower than dutasteride of 3.78 times. For pharmaceutical activities, the leaf extract showed anti-bacterial activity on *S. mutans* which was lower than erythromycin of 17 times and anti-proliferation on HepG2 and KB cell lines ( $IC_{50}$  values= $13.17 \pm 1.53$  and  $5.16 \pm 1.66$  mg/ml), which were lower than doxorubicin of 2,800 and 3,000 time, respectively. The high apoptotic on HeLa cells of the leaf extract was observed at  $13.26 \pm 7.40\%$ . The seed extract showed lipid peroxidation inhibition and anti-bacterial activity on *S. aureus* higher than the leaf extract. This study has demonstrated the potential of the Hemp leaf extract which can be further developed for pharmaceutical and cosmeceutical application.

**Keywords:** *Cannabis sativa* L, Hemp, antioxidant, tyrosinase, cytotoxicity, MMP-2, melanin,  $5\alpha$ -reductase, antibacterial, antiproliferative, apoptosis

## 1. INTRODUCTION

Traditionally, Hemp (*Cannabis sativa* L. var. *sativa*) was cultivated as a multiuse crop, serving as the source of fibers, foods and medicinal products [1]. Despite the utility of this crop, hemp farming in many countries was banned starting in the years from 1930s, due to the presence of the psychoactive compound namely delta-9 tetrahydrocannabinol (THC) in the plant flowers and leaves. The hemp plant provides three products including fibre from the stems, oil from the seeds and narcotic from the leaves and flowers. The chemical compositions of hemp are very complex, with almost hundreds of compounds such as flavonoids, fatty acids, phenolic spiroindans, dihydrostilbenes, nitrate substances (amines, ammonium salts, spermidine-derived alkaloids). The hemp flavour is due to the volatile terpenic compounds such as monoterpenes at 47.9-92.1% and sesquiterpenes at 52-48.6% of the total terpenes. Compounds like friedeline, epifriedelinol,  $\beta$ -sitosterol, carvone and dihydrocarvone were isolated from the roots of Hemp. Seeds contain the high content of oils, while flowers have richer in oils than leaves [2]. Several studies have investigated various aspects of Hemp such as constituents, biological activities and industrial application. Leizer et al. have reported that Hemp seed oil contained linoleic acid (52-62%w/w),  $\alpha$ -linolenic acid(12-23%w/w),  $\beta$ -caryophyllene (740 mg/l), myrcene (160 mg/l),  $\beta$ -sitosterol (100-148 g/l) and trace amounts of methyl salicylate determined by GC-MS and LC-MS [3]. The protein hydrolysates of the defatted Hemp seeds exhibited varying DPPH radical scavenging (with the lowest  $IC_{50}$  of 2.3 mg/ml) and  $Fe^{2+}$  chelating (with the lowest  $IC_{50}$  of 1.6-1.7 mg/ml) abilities and reducing power (with the highest absorbance at 700 nm of 0.31-0.35), depending on their  $Y_{sp}$  (TCA-soluble peptides) and  $H_o$  (surface hydrophobicity) values [4]. The crude extracts and acidic fractions of the Hemp leaves have been evaluated for antimicrobial

activity. The ethanolic, petroleum ether extract and the acidic fraction gave activity both against gram-positive (*Bacillus subtilis*, *Bacillus pumilus* and *Staphylococcus aureus*) and gram-negative (*Myotis flavus* and *Proteus vulgaris*) bacteria and also fungi (*Aspergillus niger* and *Candida albicans*) [5]. The leaf extracts of Hemp have been demonstrated the potential for the control of both hospital- and community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) [6]. The combination of essential oils of the plants with antibiotics may be useful in fighting against emerging microbial drug resistance [7]. The aim of this present study was to compare the pharmaceutical and cosmeceutical biological activities of the Hemp leaf and seed extracts including antioxidative activity, tyrosinase inhibition, cytotoxicity on human skin fibroblasts, gelatinolytic activity on MMP-2, melanogenesis in B16F10 melanoma cells,  $5\alpha$ -reductase inhibition, anti-bacterial activity, anti-proliferative and apoptosis induction activities. The results from this study can be used to evaluate the potential of the leaf and seed Hemp extracts for pharmaceutical and cosmeceutical applications.

## 2. MATERIALS AND METHODS

### 2.1 Materials

Vitamin E ( $\alpha$ -tocopherol), vitamin C (l-(+)-ascorbic acid, 99.5%), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), ethylenediaminetetraacetic acid (EDTA), kojic acid (99.0%), ammonium thiocyanate ( $NH_4SCN$ ), Folin-Ciocalteu reagent, ferrozine and ferric chloride ( $FeCl_2$ ), acrylamide (minimum 99%), theophylline, synthetic melanin, acridine orange (AO), ethidium bromide (EB) and glycerol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ammonium sulfate and glycine from BDH Limited Poole, England and trifluoroacetic acid and sodium chloride from Merck, Germany were used. Mushroom tyrosinase (4276 U/mg), l-tyrosine, N,N'-methylenebisacrylamide, TEMED (N,N,N',N'-tetramethyl ethylenediamine) and linoleic acid

(99.0%) were from Fluka (Switzerland). Tris (hydroxymethyl) methylamine was purchased from Fisher Scientific UK Limited, UK, sodium dodecyl sulfate and coomassie brilliant blue R-250 from Bio-Rad Laboratories, UK, and bromophenol blue dye and ammonium persulfate from Amersco Inc., USA were used. All other chemicals and reagents were of analytical grade.

## 2.2 Preparation of the Hemp Leaf and Seed Extracts

The Hemp leaves were collected from Chiang Mai and Chiang Rai province in Thailand during February to March and Hemp seeds were obtained from Tak province in Thailand during February to March in 2012. The specimen was authenticated by a botanist at Thai-China Flavours and Fragrances Industry Co., Ltd., Phra Nakhon Si Ayutthaya in Thailand (the voucher specimen no. is TCFF2012HE1-2). They were ground to powder. The fresh leaf and seed powder was macerated with 95% (v/v) ethanol at room temperature ( $27 \pm 2$  °C) for 48 and 72 h, respectively. The extract was filtered through the Whatman no.1 filter paper connected with a vacuum pump. The residues were re-extracted by the same procedure for 2 times. All filtrates were collected, pooled and dried by a rotary evaporator ( $50 \pm 2$  °C) until all solvents were evaporated. The percentage yields were calculated on the dry weight basis. The dried extracts were kept in an amber vial until use.

## 2.3 Antioxidant Activities

### 2.3.1 DPPH radical scavenging assay

Free radical scavenging activity of the Hemp leaf and seed extracts were determined by a modified DPPH assay. Briefly, 50  $\mu$ l of five serial concentrations of the extracts (0.01-100 mg/ml) dissolved in ethyl alcohol, and 50  $\mu$ l of DPPH in ethanol solution were put in each well of a 96-well microplate (Nalge Nunc International, NY). The reaction mixtures

were allowed to stand for 30 min at room temperature ( $30 \pm 2$  °C), and the absorbance was measured at 515 nm by a well reader (Model 680 microplate reader, Bio-Rad Laboratories Ltd., Corston, UK) against the negative control (DMSO). Ascorbic acid (0.001-10 mg/ml) was used as a positive control. The experiments were done in triplicate. The percentages of free radical scavenging activity were calculated as the following: Scavenging (%) =  $[(A-B)/A] \times 100$ , where A was the absorbance of the negative control and B was the absorbance of the sample. The sample concentration providing 50% of scavenging ( $SC_{50}$ ) activity was calculated from the graph plotted between the percentages of the scavenging activity and the sample concentrations.

### 2.3.2 Lipid peroxidation inhibition activity

The antioxidant activity of the Hemp leaf and seed extracts were assayed by the modified ferric-thiocyanate method. An amount of 50  $\mu$ l of five serial concentrations of the extracts (0.01-100 mg/ml) dissolved in ethyl alcohol was added to 50  $\mu$ l of linoleic acid in 50% (v/v) DMSO. The reaction was initiated by the addition of 50  $\mu$ l of  $NH_4SCN$  (5mM) and 50  $\mu$ l of  $FeCl_2$  (2mM). The mixture was incubated at  $37 \pm 2$  °C in a 96-well microplate for 1 h and measured at 490 nm. The solution without the sample was used as a negative control. The  $\alpha$ -tocopherol (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 concentration providing 50% inhibition of lipid peroxidation ( $IPC_{50}$ ) was calculated from the graph plotted between the percentages of lipid peroxidation inhibition and the sample concentrations.

### 2.3.3 Metal ion chelating assay

The metal ion chelating activity of the Hemp leaf and seed extracts were assayed by the modified ferrous ion chelating method. Briefly, 100  $\mu$ l of five serial concentrations of the extracts (0.01-100 mg/ml) dissolved in ethyl alcohol were added to the solution of 2 mM FeCl<sub>2</sub> (50  $\mu$ l) in distilled water. The reaction was initiated by the addition of 5 mM ferrozine (50  $\mu$ l) and the total volume was adjusted to 300  $\mu$ l by distilled water. Then, the mixture was left at room temperature (30 $\pm$ 2 $^{\circ}$ C) for 15 min. Absorbance of the resulting solution was then measured at 570 nm by a microplate reader. EDTA (0.001-10 mg/ml) was used as a positive control. The negative control contained FeCl<sub>2</sub> and ferrozine which were the complex formation molecules. All experiments were performed in triplicate. The inhibition percentages of ferrozine-Fe<sup>2+</sup> complex formation were calculated by the following equation: Metal chelating activity (%) = [(A-B)/A]  $\times$  100, where A was the absorbance of the negative control and B was the absorbance of the sample. The sample concentration providing 50% metal chelating activity (CC<sub>50</sub>) was calculated from the graph plotted between the percentages of metal chelating activity and the sample concentrations.

### 2.4 Tyrosinase Inhibition Assay

The tyrosinase inhibition activity of the Hemp leaf and seed extracts were assayed by the modified dopachrome method using tyrosine as a substrate. Briefly, 50  $\mu$ l of five serial concentrations of the extracts (0.001-10 mg/ml) dissolved in DMSO, and 50  $\mu$ l of 100 units mushroom tyrosinase solution in 0.1 M phosphate buffer, and 50  $\mu$ l of 1 mg/ml tyrosine solution in 0.1 M phosphate buffer, and 50  $\mu$ l of 0.1 M phosphate buffer were added into each well of a 96-well plate. The mixture was incubated at 37 $\pm$ 2 $^{\circ}$ C for 60 min and the absorbance at 450 nm was measured. Kojic acid (0.001-10 mg/ml) was used as a positive

control. The solution without the extracts was used as a negative control. All experiments were performed in triplicate. The percentages of tyrosinase inhibition were calculated according to the following equation: Tyrosinase inhibition activity (%) = [(A-B)-(C-D)/(A-B)] $\times$ 100, where A was the absorbance of the blank after incubation, B was the absorbance of the blank before incubation, C was the absorbance of the sample after incubation, and D was the absorbance of the sample before incubation. The sample concentration providing 50% inhibition (IC<sub>50</sub>) was calculated from the graph plotted between the percentages of tyrosinase inhibition activity and the sample concentrations.

### 2.5 Cytotoxicity on Human Skin Fibroblast of the Extracts by SRB Assay

The human skin fibroblasts at the 10<sup>th</sup> passage from Faculty of Dentistry, Chiang Mai University in Thailand were used. The cells were seeded in 96-well plates at an amount of 10,000 cells/well and allowed to attach overnight. Then, the cells were exposed to various concentrations (0.0625-5 mg/ml) of the Hemp leaf and seed extracts for 24 h. After incubation, the adherent cells were fixed by adding cold 50%w/v trichloroacetic acid and further incubated for 1 h at 4 $^{\circ}$ C. Then, the cells were rinsed with distilled water, air-dried and stained with 0.4%SRB in 1%glacial acetic acid for 30 min at room temperature (27 $\pm$ 2 $^{\circ}$ C). The unbound SRB was removed by washing with 1% glacial acetic acid solution for four times. After air-drying, 100  $\mu$ l per well of 10 mM Tris base were added to dissolve the bound stain. After mixing, the absorbance was measured at 540 nm with a microplate reader (Biorad, Milan, Italy). The untreated cells were used as a negative control. Cell viability (%) was calculated by the following equation:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance}_{\text{treated cells}}}{\text{Absorbance}_{\text{untreated cells}}} \times 100$$

## 2.6 Gelatinolytic Activity on MMP-2 Inhibition Zymography

The human skin fibroblasts at the 10<sup>th</sup> passage were seeded in 6-well plates at an amount of  $5 \times 10^5$  cells/well. The monolayer of the cells was maintained in the culture medium without FBS for 24 h, treated with the extracts and incubated for 48 h. The culture supernatants were collected to assess for the gelatinolytic activities of MMP-2 in the culture media. The SDS-PAGE zymography using gelatin as a substrate was performed. Briefly, 20  $\mu$ L of the cell culture supernatant were suspended in the loading buffer (0.125M Tris (pH 6.8), 4% SDS and 0.04% bromophenol blue) and without prior denaturation and run on the 10% SDS polyacrylamide gel containing gelatin (1 mg/ml). After electrophoresis, gels were washed to remove SDS and incubated for 20 min in the renaturing buffer (50 mM Tris, 5 mM CaCl<sub>2</sub>, 0.02% NaN<sub>3</sub>, 2.5% Triton X-100). The gels were then incubated for 24 h at 27 °C in the developing buffer [50 mM Tris (pH 7.5), 5 mM CaCl<sub>2</sub>, 0.02% NaN<sub>3</sub> and 1% Triton X-100]. Gels were subsequently stained with 0.5% Coomassie brilliant blue G-250 and destained in 30% methanol and 10% acetic acid (v/v) to detect gelatinolytic activity. The gel was documented by a gel documentation system (Bio-Rad Laboratories, UK) and analyzed by the Quantity 1-D analysis software. The area multiplied by intensity (mm<sup>2</sup>) of the bands on the gel was determined as the relative MMP-2 content. The percentages of MMP-2 inhibition in comparing to the control (the untreated systems) were calculated using the following equation:

$$\text{MMP-2 inhibition (\%)} = 100 - \left[ \frac{\text{MMP-2 content of sample}}{\text{MMP-2 content of control}} \times 100 \right]$$

The assays were done in three independent separate experiments. The potency of MMP-2 inhibition of the extracts was compared with

the positive control (ascorbic acid).

## 2.7 Melanogenesis in B16F10 Melanoma Cells

Cytotoxicity assay of the extracts at various concentrations (0.01, 0.1, 1 and 10 mg/ml) in B16F10 melanoma cells (ATCC, Virginia, USA) was determined by the SRB assay which was the same method as in section 2.5. The cytotoxicity assay of the extracts was performed to evaluate for the appropriate concentration that gave more than 80% cell viability in order to use in the further melanogenesis assay.

### 2.7.1 Melanin content measurement

The melanin content was measured according to the previously described method with some slight modifications [8]. Briefly, cells at the density of  $10 \times 10^4$  cells/well were plated in 6-well plates and incubated overnight for cell adhesion. The extracts were then added and incubated for 72 h. The cells were then washed with phosphate-buffer saline (PBS) and dissolved in 500  $\mu$ L of 2 N NaOH at 60°C for 1 h. The absorbance was measured at 450 nm using a microplate reader and the melanin amount was determined in comparing to the standard melanin. The total protein content was measured by the Bradford dye-binding method using bovine serum albumin as the standard [9]. For the determination of the actual melanin formation from the same cell numbers, the melanin content of each treatment was divided by the total protein content. The percentages of the relative ratio of melanin content were calculated as the following:

$$\begin{aligned} \% \text{ Relative ratio of the melanin content} \\ = (\text{Mt/Mc}) \times 100 \end{aligned}$$

Where Mt was the melanin content of the extracts divided by the total protein content of the extract treated system and Mc was the melanin content of the control divided by the total protein content of the control.



### 2.7.2 Tyrosinase activity measurement

Tyrosinase activity was analyzed by the method described by Ohguchi et al. with some slight modifications [10]. Briefly, the cells treated with different extracts were washed with ice-cold PBS and then lysed by incubation at 4°C for 30 min in the lysis reagent (Fermentas, EU) containing protease inhibitors (Roche, Germany). The lysates were centrifuged at 15,000 rpm for 10 min. The obtained supernatants were collected, mixed with the mixture containing 50 mM phosphate buffer (pH 6.8) and 0.05% l-dopa, and then incubated at 37°C for 2 h. After incubation, the dopachrome formation was measured at 450 nm using a microplate reader. The enzyme activity was calculated in comparing to the standard mushroom tyrosinase. The total protein content of the extract treated system was also evaluated. Enzyme activity of the extract treated system was compared to that of the control system and calculated as the percentages of the relative ratio of the tyrosinase activity as the following equation:

$$\begin{aligned} \% \text{ Relative ratio of tyrosinase activity} \\ = (T_t/T_c) \times 100 \end{aligned}$$

Where  $T_t$  was the tyrosinase activity from the extracts divided by the total protein content from the extracts and  $T_c$  was the tyrosinase activity of the control divided by the total protein content from the control.

### 2.7.3 TRP-2 activity measurement

The assay of the TRP-2 activity was performed by the method described by Barber et al. with some slight modifications [11]. Briefly, the supernatant obtained from the lysis of the treated cells was mixed with the mixture consisting of 1 mM phenylthiourea, 2 mM EDTA and 10 mM sodium phosphate buffer (pH 6.8). Dopachrome solution, separately prepared by mixing an equal volume of 1 mM Dopa and 2 mM  $\text{NaIO}_4$ , was added into the

cell lysate mixture and incubated at 37°C for 2 h. The decreased absorbance based on the consumption of the dopachrome was measured at 450 nm. The reaction mixture with bovine serum albumin instead of the cell lysate was used as a negative control. The total protein content of the treated cells was also evaluated. The TRP-2 activity from the extracts was compared with that from the control and calculated as the percentages of the relative ratio of the TRP-2 activity according to the following equation:

$$\begin{aligned} \% \text{ Relative ratio of TRP-2 activity} \\ = (D_t/D_c) \times 100 \end{aligned}$$

Where  $D_t$  was the TRP-2 activity of the extract treated system divided by the total protein content of the extract treated system and  $D_c$  was the TRP-2 activity from the control divided by the total protein content from the control.

## 2.8 5 $\alpha$ -reductase Inhibition Activity on DU-145

The cytotoxicity assay of the extracts at various concentrations (0.0001, 0.001, 0.01, 0.1 and 1 mg/ml) in human prostate carcinoma cell line (DU-145) provided by Prof. Dr. Toshihiro Akihisa at the College of Science and Technology, Nihon University in Tokyo, Japan was determined by the SRB assay the same method as in section 2.5. The concentrations of the samples which gave % cell viability of more than 90% were used.

### 2.8.1 Cultivation of cells

The pellets of human DU-145 cells were plated onto the 6-well plates separately at the density of  $8.0 \times 10^5$  cells/well, incubated with 10% (v/v) FBS-RPMI medium containing penicillin (100 U/ml) and streptomycin (100 mg/ml) in a 5%  $\text{CO}_2$  incubator (Shel Lab, model 2123TC, U.S.A.) at 37°C. Cells were then exposed for 24 hours to the extracts and the

standard dutasteride at the final concentration of 5 mg/ml. The medium were removed, and the cells were washed with PBS, trypsinized with 0.25% trypsin solution for 2 min and suspended in PBS.

*Total RNA extraction:* The total RNA from the cell pellets was extracted by the RNA extraction kit (NucleoSpin<sup>®</sup>, Macherey-Nagel, CA, U.S.A.) according to the instructions of the manufacturer. The concentration of the total RNA was quantified by Qubit Fluorometer and Quant-iT<sup>™</sup> RNA BR assay kit (Invitrogen, CA, U.S.A.). The total RNA solution was kept at -20°C until used.

### 2.8.2 Reverse transcription-polymerase chain reaction (RT-PCR)

The 5 $\alpha$ -reductase type 1 and 2 genes were amplified from the extracted RNA by SuperScrip<sup>™</sup> One-Step RT-PCR with Platinum<sup>®</sup> Taq kit (Invitrogen, CA, U.S.A.) according to the manufacturer's protocol. Briefly, five micrograms of the total RNAs were reverse transcribed with RT/ Platinum Taq<sup>®</sup> mix and subjected to PCR cycles with the primers for human 5 $\alpha$ -reductase type 1 and 2 (SRD5A1 and 2) as follows: 94°C for 15 s, 55°C for 30 s, 72°C for 45 sec for 35 cycles. The human 5 $\alpha$ -reductase type 1 primers were designed based on GenBank accession no. NM\_001047.2 and NM\_000348, respectively, with a forward (5'- CCA TGT TCC TCG TCC ACT AC-3') and reverse (5'- TTC AAC CTC CAT TTC AGC GT -3'), produced 707 bp amplicon and human 5 $\alpha$ -reductase type 2 (SRD5A2) forward (5'- GGG TGG TAC ACA GAC ATA CG-3') and reverse (5'- TCA CGA CTA TGA GGA GAG GG-3'), produced 938 bp amplicon [12]. The RT-PCR products were loaded on 1% agarose gel in the 1X tris-acetate-EDTA (TAE) buffer chamber at 100 V for 30 min. The human 5 $\alpha$ -reductase type 1 and 2 dsDNA samples were quantified by the Qubit fluorometer and Quant-iT<sup>™</sup> dsDNA assay kit (Invitrogen, CA, U.S.A.).

### 2.9 Anti-bacterial Activity of The Extracts

*Streptococcus mutans*, *Staphylococcus aureus* and *Propionibacterium acne* from Department of Odontology and Oral Pathology, Faculty of Dentistry, Chiang Mai University; Department of Microbiology, Faculty of Medicine, Chulalongkorn University and Department of Tropical Hygiene, Faculty of Tropical Medicine, Mahidol University, respectively were used in this study. Ten milligrams of the extracts were dissolved with 1 ml of absolute ethanol. The final concentration was 10 mg/ml. The bacteria from the inoculated flask were swabbed onto the BHI agar plates by the cotton buds. An amount of 20  $\mu$ l of the extracts was pipetted and put on the 0.9 mm sterile paper disc. The sterile distilled water and erythromycin (15  $\mu$ g) paper disc were used as the negative and positive controls, respectively. The discs were placed onto the BHI agar plates and incubated at 37°C for 48 hr. For *P. acne*, the plates were put in an air tighted box with an anaerobic condition. The clear zones around the discs of the extracts were measured and compared to that of the erythromycin disc.

### 2.10 Anti-proliferative Activity on Human Cancer Cell Lines

Four human cancer cell lines including human cervical adenocarcinoma (HeLa) (ATCC CCL-2) and human mouth epidermal carcinoma (KB) (ATCC CCL-17) from American Type Culture Collection, USA, human hepatocellular carcinoma (HepG2) (ATCC HB-8065) from Faculty of Tropical Medicine, Mahidol University, and human colon adenocarcinoma (HT-29) (ATCC HTB-38) from Department of Medical Biology, Faculty of Biology, University of Tuebingen, Germany, were used. The extracts were investigated for anti-proliferative activity by the SRB assay as previous described in section 2.5. The anti-cancer drug (doxorubicin) was used as a positive control. The absorbance was measured at 570 nm by a microplate reader.

The percentages of growth inhibition (G) were determined by the following equation:

$$G (\%) = (T_{\text{treat}} - T_0 / C - T_0) \times 100$$

where  $T_{\text{treat}}$  was the absorbance of the treated plate,  $T_0$  was the absorbance of the reference plate (the incubated plate at the first day before the treatment) and  $C$  was the absorbance of the control plate. The concentration providing 50% of growth inhibition ( $IC_{50}$  mg/ml) of the extracts was calculated from the graph plotted between the percentages of growth inhibition [%G] and the extract concentrations.

### 2.11 Apoptotic Assay by AO/EB Staining

The Hemp leaf and seed extracts were investigated for apoptotic activity in the 4 cancer cell lines the same as in section 2.10 by acridine orange (AO) and ethidium bromide (EB) double staining. After incubation, the cells were treated with the extracts at the 3 final concentrations (0.005, 0.5 and 5 mg/ml) and incubated for 24 h. To stain the apoptotic cells, 10  $\mu$ l of the AO/EB dye mix (100  $\mu$ g/ml of AO and 100  $\mu$ g/ml of EB in PBS) were

added to each well, and the apoptotic and necrotic cells were viewed and counted under the fluorescent microscope with the total of 100 cells [13]. The experiments were done in triplicate.

## 3. RESULTS AND DISCUSSION

### 3.1 Preparation of the Hemp Leaf and Seed Extracts

The average percentage yields of the Hemp leaf and seed extracts were  $3.77 \pm 0.31$  and  $1.58 \pm 0.20\%$ , respectively. The appearance of the extracts was viscous fluid in dark brown and brown green color, respectively with the specific odors.

### 3.2 Antioxidant Activities

The antioxidant activities of the Hemp leaf and seed extracts were shown in Table 1. The leaf extract ( $SC_{50}$  value of  $2.73 \pm 0.42$  mg/ml) gave higher DPPH scavenging activity than the seed extract ( $SC_{50}$  value of  $14.39 \pm 2.27$  mg/ml) of 5.27 times, but lower than ascorbic acid ( $SC_{50}$  value of  $0.012 \pm 0.002$  mg/ml) and  $\alpha$ -tocopherol ( $SC_{50}$  value of  $0.038 \pm 0.01$  mg/ml) of 227.5 and 71.8 times, respectively. For chelating activity,

**Table 1.** Comparison of antioxidative and tyrosinase inhibition activities of the Hemp leaf and seed extracts.

| Extracts             | $SC_{50}$ of DPPH radical scavenging assay (mg/ml) | $CC_{50}$ of chelating assay (mg/ml) | $IPC_{50}$ of lipid peroxidation inhibition assay (mg/ml) | $IC_{50}$ of tyrosinase inhibition assay (mg/ml) |
|----------------------|--|--------------------------------------|---|--|
| Leaf                 | $2.73 \pm 0.42$                                    | $0.93 \pm 0.20$                      | $246.32 \pm 69.38$  | $0.049 \pm 0.02$                                 |
| Seed                 | $14.39 \pm 2.27$                                   | $1.92 \pm 1.05$                      | $92.68 \pm 30.77$   | $0.07 \pm 0.06$                                  |
| Ascorbic acid        | $0.012 \pm 0.002$                                  | NA                                   | NA  | NA   |
| $\alpha$ -tocopherol | $0.038 \pm 0.01$                                   | NA                                   | $0.045 \pm 0.02$  | NA   |
| EDTA                 | NA   | $0.15 \pm 0.002$                     | NA  | NA   |
| Kojic acid           | NA   | NA                                   | NA  | $0.005 \pm 0.004$                                |

Note: NA represented not applicable.  $SC_{50}$  value (mg/ml) was the concentration of the sample that scavenged 50% of the DPPH radicals.  $IPC_{50}$  value (mg/ml) was the concentration of the sample that inhibited 50% of the lipid peroxidation.  $CC_{50}$  value (mg/ml) was the concentration of the sample that chelated 50% of the metal ion.  $IC_{50}$  value (mg/ml) was the concentration of the sample that inhibited 50% of the tyrosinase enzyme.



the leaf extract ( $CC_{50}$  value of  $0.93 \pm 0.20$  mg/ml) gave higher chelating activity than the seed extract ( $CC_{50}$  value of  $1.92 \pm 1.05$  mg/ml) of 2.06 times, but lower than EDTA ( $CC_{50}$  value of  $0.15 \pm 0.002$  mg/ml) of 6.13 times. However, the seed extract ( $IPC_{50}$  value of  $92.68 \pm 30.77$  mg/ml) showed higher lipid peroxidation inhibition activity than the leaf extract ( $IPC_{50}$  value of  $246.32 \pm 69.38$  mg/ml) of 2.66 times, but lower than  $\alpha$ -tocopherol ( $IPC_{50}$  value of  $0.045 \pm 0.02$  mg/ml) of 2059.56 times.

The antioxidant activities from the two Hemp extracts might be due to tetrahydrocannabinol (THC) as well as other cannabinoids that have a phenolic group which possesses mild sufficient antioxidant activity to protect oxidative stress [14]. The content of total phenolic compounds at the level of 0.44 mg/g as gallic equivalents in Hemp oil has been reported [15]. Siger et al. reported that the total phenolic content in the cold-pressed Hemp seed oil was at 2.4 mg/100 g as caffeic acid equivalents and the amount of the Hemp oil needed to decrease the initial DPPH concentration by 50% ( $EC_{50}$ ) was 8.7  $\mu$ g [16]. In fact, Hemp seed is a rich source of high quality oil and protein. Its oil is rich in polyunsaturated fatty acids, especially linoleic ( $\omega$ -6) and  $\alpha$ -linolenic ( $\omega$ -3) acids [17-19]. Audu et al. have demonstrated that Hemp leaf contained cannabinol, tetrahydrocannabinol, cannabidiol, cannabidiol acid, tetrahydrocannabivarin, cannabivarin and cannabichromene [20]. These compounds are potent antioxidants [21].

### 3.3 Tyrosinase Inhibition Activity

Tyrosinase inhibition activity of the Hemp extract has not been previously reported. The leaf extract ( $IC_{50}$  value of  $0.049 \pm 0.02$  mg/ml) exhibited more potent tyrosinase inhibition activity than the seed extract ( $IC_{50}$  value of  $0.07 \pm 0.06$  mg/ml) but gave lower activity than kojic acid ( $IC_{50}$  value of  $0.005 \pm 0.004$  mg/ml) of about 9.8 and 14 times, respectively (Table 1). In fact, the Hemp extracts contain a wide range of diversity bioactive compounds, such as terpenoids and flavonoids which may be responsible for the tyrosinase inhibition activity [22].

### 3.4 Cytotoxicity of the Extracts on Human Skin Fibroblast

The percentages of human skin fibroblast viability treated with the Hemp leaf and seed extracts were shown in Table 2. When the concentrations of the extracts increased from 0.0625 to 5 mg/ml, the cell viability decreased linearly with the  $r^2$  of 0.95. At high concentration (5 mg/ml), the leaf and seed extracts gave %cell viability at  $57.66 \pm 6.49$  and  $32.37 \pm 4.30$ , respectively. Both leaf and seed extracts at the concentration range of 0.0625-1 mg/ml gave no cytotoxicity with the cell viability of more than 70%, except 1 mg/ml seed extract. The cytotoxic effects of the seed extract were more than those from the leaf extract. This was possibly from the different compositions in the extracts. The seed extract which composed of non-polar

**Table 2.** The percentages of cell viability on human skin fibroblast at the 10<sup>th</sup> passage by the SRB assay of the Hemp leaf and seed extracts.

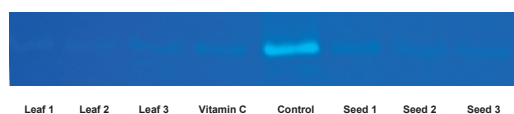
| Extracts | Concentration of the extracts (mg/ml) |                   |                   |                  |                  |
|----------|---------------------------------------|-------------------|-------------------|------------------|------------------|
|          | 0.0625                                | 0.125             | 0.25              | 0.5              | 1                |
| Leaf     | 122.12 $\pm$ 5.76                     | 115.22 $\pm$ 9.77 | 105.42 $\pm$ 5.04 | 88.77 $\pm$ 7.01 | 73.02 $\pm$ 3.57 |
| Seed     | 95.67 $\pm$ 6.01                      | 93.25 $\pm$ 5.06  | 85.32 $\pm$ 5.57  | 78.77 $\pm$ 8.78 | 66.12 $\pm$ 7.63 |

Note : Cell viability (%) = (absorbance<sub>test cell</sub> / absorbance<sub>control cell</sub>) x100

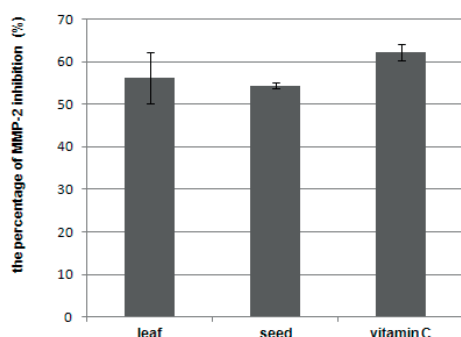
compounds including terpenoid compounds such as phytocannabinoids, plant sterols and polyunsaturated fatty acids [23], may increase cell membrane permeability resulting in the increased cytotoxicity in comparison to the leaf extract which composed of mostly the polar compounds such as polyphenols, flavones and polyholozides [2]. The highest concentration at 1 mg/ml of the Hemp leaf and seed extracts that exhibited low cytotoxicity with %cell viability of  $73.02 \pm 3.57$  and  $66.12 \pm 7.63$ , respectively was observed. Thus, this concentration of the extracts was selected for the further gelatinolytic activity of the MMP-2 inhibition on human skin fibroblasts.

### 3.5 Gelatinolytic Activity on MMP-2 Inhibition Zymography

Figures 1 and 2 presented the gelatinolytic



**Figure 1.** Zymograms of MMP-2 inhibition on human skin fibroblast of Hemp leaf and seed extracts at 1 mg/ml and ascorbic acid (positive control), (n=3).



**Figure 2.** Comparison of the gelatinolytic activity of MMP-2 inhibition on human skin fibroblast between Hemp leaf and seed extracts (at 1 mg/ml) and ascorbic acid (the positive control).

activity of MMP-2 inhibition on human skin fibroblast at the passage 10<sup>th</sup> of the leaf and seed extracts at 1 mg/ml in comparing to the positive control (vitamin C). Cells treated with leaf and seed extracts indicated the pro MMP-2 inhibition of  $56.27 \pm 5.98$  and  $54.40 \pm 0.66\%$ , while the active MMP-2 inhibition were 100 and 100%, respectively. However, the pro MMP-2 inhibition of both extracts was lower than that of vitamin C ( $62.21 \pm 1.93\%$ ) of 1.11 and 1.14 times, respectively. In fact, it is still unknown for the mechanism of the extracts on the MMP-2 inhibition. One possible mechanism is the antioxidant activity. It has been reported that the exogenous hydrogen peroxide and endogenous ROS can induce MMP expression in the endothelial cells, cardiac fibroblasts, macrophages and breast cancer cells [24]. Because MMPs are upregulated by the increased formation of the reactive oxygen species (ROS), antioxidant approaches can thus decrease the MMP-2 upregulation [25]. The leaf extract showed higher MMP-2 inhibition than the seed extract. The inhibition of MMP-2 expression of the extract appeared to relate to its DPPH radical scavenging activity. The leaf extract that composed of mostly the polar compounds [2] which have numerous double bonds and hydroxyl groups in their structures may be able to donate the electrons through resonance to stabilize the free radicals [26]. In addition, ROS suppression is a bypass way to inhibit MMP-2 activation. The extracts may inhibit the MMP-2 synthesis and secretion steps or interrupt the activation processes by converting the latent form of MMP-2 (pro MMP-2) to an active form (active MMP-2), resulting in the decrease of the area and intensity of the active MMP-2 on the zymogram.

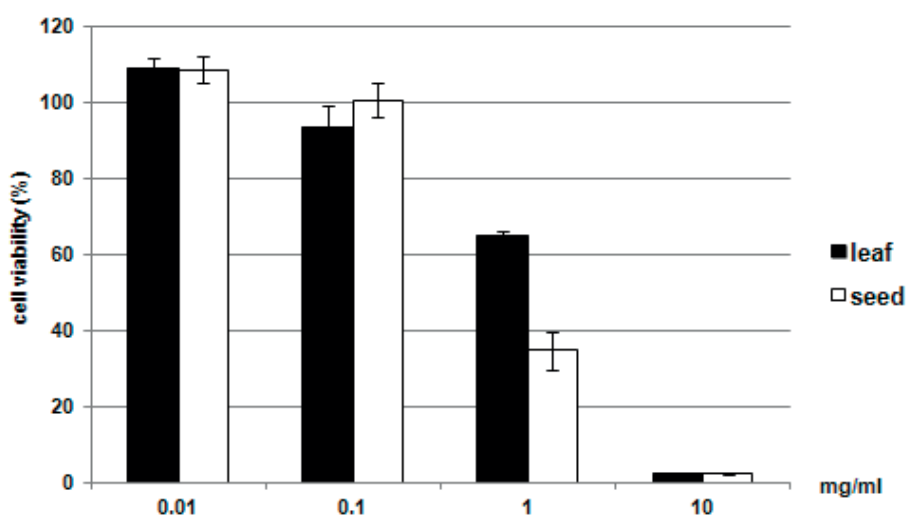
### 3.6 Melanogenesis in B16F10 Melanoma Cells

The cytotoxicity assay in B16F10 melanoma cells of Hemp seed and leaf extracts was performed to evaluate for the appropriate

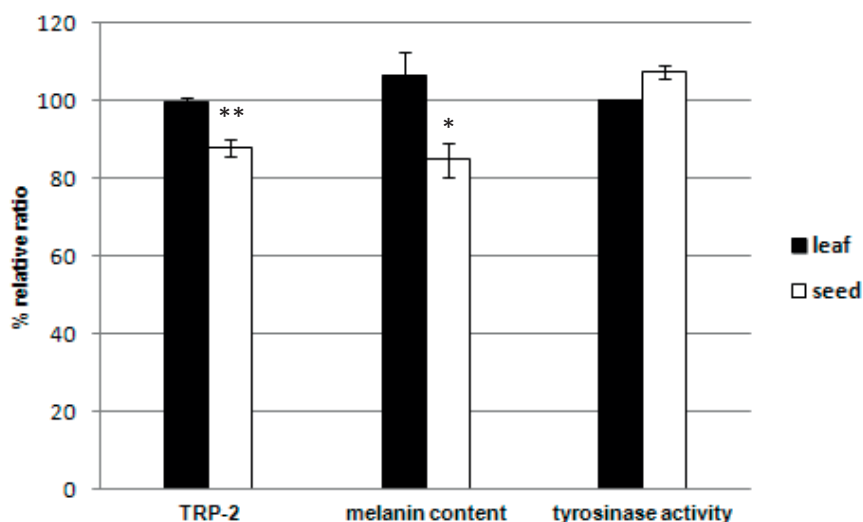
concentration to be used in the melanogenesis assay. Figure 3 demonstrated the cytotoxic effects of the extracts at 0.01- 10 mg/ml. The appropriate concentration which gave more than 80% cell viability was at 0.1 mg/ml for both seed and leaf extracts. Melanogenesis assay of the extracts was performed in comparing to the positive control (theophylline) at 0.05 mg/ml. Theophylline significantly demonstrated melanin stimulation with the tyrosinase and TRP-2 activities of 2.38, 3.34 and 1.04 times, respectively, higher than the control. The pigment biosynthesis of theophylline regulates through cAMP pathway as well as the increases of the gamma-glutamyl transpeptidase- and tyrosinase-reactive cells, resulting in the induction of melanin production with the increased level of tyrosinase activity. The melanogenesis effects of Hemp seed and leaf extracts were shown in Figures 4. The seed extract decreased the melanin formation of 0.15 times of the control. Tyrosinase activity of the seed extract was 1.07 times higher than the control, while its TRP-2 activity was 0.88 times lower than the control.

Meanwhile, the leaf extract stimulated melanin production higher than the control of 1.06 times. Activities of tyrosinase and TRP-2 of the leaf extract were not different in comparing to the control.

Although both seed and leaf extracts were obtained from the same plant, the active compounds found in different parts of the plant were different. The unsaturated fatty acids, such as linoleic acid,  $\alpha$ -linolenic acid and oleic acid, have been reported as the major compositions in the Hemp seed oil, while the active substance reported in the Hemp leaf is tetrahydrocannabinol [3]. The different constituents of seed and leaf extracts may affect melanogenesis in the different manner. The unsaturated fatty acids found in the Hemp seed extract have been reported on melanogenesis inhibition activity [27]. Meanwhile, Hemp leaf extract increased the melanin content without having any effect on the activities of the two enzymes, tyrosinase, and TRP-2. This non-correlation effect of melanogenic enzymes and melanin content caused by the



**Figure 3.** Cytotoxicity assay of Hemp leaf and seed extracts at 0.01-10 mg/ml on B16F10 melanoma cells.



**Figure 4.** Percentage relative ratio of TRP-2 activity, melanin content and tyrosinase activity of B16F10 melanoma cells treated with Hemp leaf and seed extract at 0.1 mg/ml. \* $p < 0.05$  and \*\* $p < 0.01$  were considered significant difference from the control.

complicated mechanisms of action of the active compounds in the leaf extract which is needed in the further investigation.

### 3.7 5 $\alpha$ -reductase Inhibition Activity on DU-145

The cytotoxicity test of the Hemp leaf and seed extracts on DU-145 showed the  $IC_{50}$  values at  $24.87 \pm 2.56$  and  $15.38 \pm 2.19$  mg/ml, respectively. The concentrations of the extracts used for the investigation of 5 $\alpha$ -reductase inhibition activity were 5 mg/ml. The leaf extract ( $13.57 \pm 3.21$  % of the control) showed higher 5 $\alpha$ -reductase inhibition activity than that of the seed extract ( $5.78 \pm 1.34$  % of the control), but lower than the standard dutasteride ( $51.31 \pm 5.84$  % of control) (Figure 5). This low activity might be from the low content of 5 $\alpha$ -reductase inhibitors containing in the extracts such as unsaturated fatty acids, linoleic acid, gamma-linolenic acid and oleic acids [28].

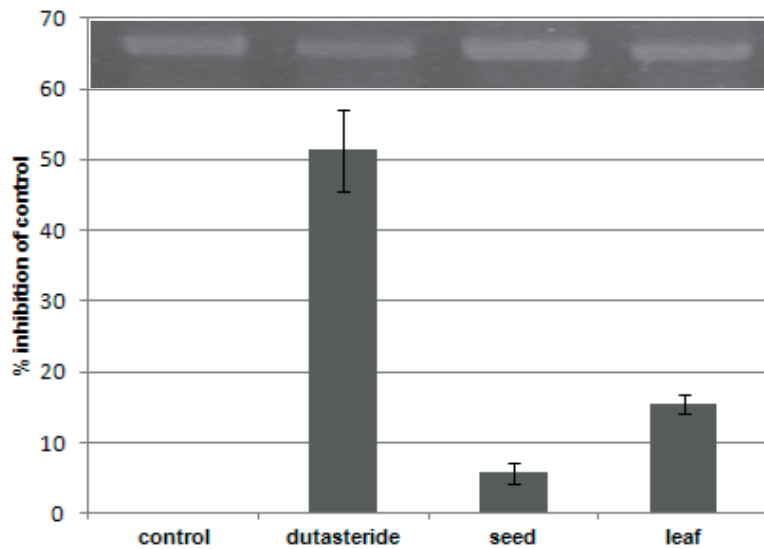
### 3.8 Anti-bacterial Activity of the Extracts

Table 3 showed the clear zones (mm) indicating of the anti-bacterial activity of the

Hemp extracts. The seed extracts exhibited the anti-bacterial activity on *S. aureus* with the clear inhibition zone of  $1.00 \pm 0.00$  mm, but lower than erythromycin of 26 times, while the leaf extracts gave the anti-bacterial activity on *S. mutans* with the clear inhibition zone of  $1.33 \pm 0.58$  mm, but lower than erythromycin of 17 times. The seed and leaf extracts did not give any anti-bacterial activity on *P. acnes*.

### 3.9 Anti-proliferative Activity on Human Cancer Cell Lines

Table 4 indicated the anti-proliferative activity of the extracts on 4 human cancer cell lines by the SRB assay. The seed extracts exhibited the anti-proliferative activity on HepG2 cell line with the  $IC_{50}$  value of  $12.07 \pm 1.18$  mg/ml, which was lower than doxorubicin of about 200 times. The leaf extracts gave the  $IC_{50}$  values of  $13.17 \pm 1.53$  and  $5.16 \pm 1.66$  mg/ml on HepG2 and KB cell lines, which were lower than doxorubicin of 2,800 and 3,000 times, respectively. However, the Hemp seed and leaf extracts did not show any anti-proliferative activity on HT-29 and HeLa cell lines.



**Figure 5.**  $5\alpha$ -reductase (type 1) inhibition on DU-145 cells at 5 mg/ml of the Hemp seed and leaf extracts in comparing to the standard dutasteride (5 mg/ml). (A) agarose gel electrophoresis of dsDNA of  $5\alpha$ -reductase (type 1) enzyme after the inhibition of the samples and (B) the percentages of  $5\alpha$ -reductase (type 1) inhibition. % inhibition = [(control-sample)/control] x 100.

**Table 3.** Anti-bacterial activity of the Hemp leaf and seed extract on *S. mutans*, *S. aureus* and *P. acnes*.

| Samples                   | Clear Inhibition Zone (mm.) |                  |                 |
|---------------------------|-----------------------------|------------------|-----------------|
|                           | <i>S. mutans</i>            | <i>S. aureus</i> | <i>P. acnes</i> |
| Erythromycin (15 $\mu$ g) | 23.5 $\pm$ 0.71             | 26.67 $\pm$ 1.15 | 31.5 $\pm$ 3.54 |
| Seed extract              | ND                          | 1.00 $\pm$ 0.00  | ND              |
| Leaf extract              | 1.33 $\pm$ 0.58             | ND               | ND              |
| Distilled water           | ND                          | ND               | ND              |

Note: ND = Not detected (no activity)

**Table 4.** Anti-proliferative activity of the Hemp leaf and seed extracts on four human cancer cell lines.

| Extracts    | IC <sub>50</sub> (mg/ml) |                     |                     |                     |
|-------------|--------------------------|---------------------|---------------------|---------------------|
|             | HT-29                    | KB                  | HeLa                | HepG2               |
| Seed        | ND                       | ND                  | ND                  | 12.07 $\pm$ 1.18    |
| Leaf        | ND                       | 5.16 $\pm$ 1.66     | ND                  | 13.17 $\pm$ 1.53    |
| Doxorubicin | 0.0043 $\pm$ 0.0002      | 0.0274 $\pm$ 0.0015 | 0.0113 $\pm$ 0.0024 | 0.0042 $\pm$ 0.0025 |

Note: ND = Not detected (no activity)



### 3.10 Apoptotic Assay by AO/EB Staining

The percentages of apoptotic and necrotic cells of the Hemp extracts on the 4 cancer cell lines were demonstrated in Table 5. The Hemp leaf and seed extracts exhibited the highest apoptotic cells of  $13.26 \pm 7.40$  and  $9.25 \pm 2.55\%$  at 5 mg/ml, respectively in HeLa cell line. For necrosis, the seed extract showed the highest necrotic effect against KB and HepG2 with 100% at 5 mg/ml. The leaf extract gave the highest necrotic cells of  $19.43 \pm 13.01\%$  at 5 mg/ml in HepG2 cell line. This result has indicated that the leaf extract showed potent apoptotic induction in all cancer cell lines, while the seed extract can also induce apoptosis, but less than the leaf extract. This might be from the lower content of cannabinoid in the Hemp seed that can induce apoptosis in cancer cells than that in the leaf extract [29, 30].

### 4. CONCLUSIONS

This study has demonstrated that the leaf extract exhibited higher DPPH scavenging, metal chelating and tyrosinase inhibition activity than the seed extract of 5.27, 2.06 and 1.43 times, respectively. Both leaf and seed extracts at the concentration range of 0.625-1 mg/ml gave low cytotoxicity in human skin fibroblast with the cell viability of more than 70%. The cells treated with the leaf and seed extracts indicated the pro MMP-2 inhibition of  $56.27 \pm 5.98$  and  $54.40 \pm 0.66\%$ , while the active MMP-2 inhibition was 100 and 100%, respectively. The seed extract gave the melanin

inhibition, tyrosinase activity and TRP-2 activity in B16F10 melanoma cells of 0.15 1.07 and 0.88 times of the control, while the leaf extract showed the melanin induction of 1.06 times of the control. The leaf extract showed higher 5 $\alpha$ -reductase inhibition activity than that of the seed extract. The seed extracts exhibited antibacterial activity on *S. aureus*, whereas the leaf extracts gave anti-bacterial activity on *S. mutans*. The seed extracts exhibited anti-proliferative activity on HepG2 cell line with the IC<sub>50</sub> value of  $12.07 \pm 1.18$  mg/ml. The leaf extracts gave the IC<sub>50</sub> values of  $13.17 \pm 1.53$  and  $5.16 \pm 1.66$  mg/ml on HepG2 and KB cell lines, respectively. The Hemp leaf and seed extracts demonstrated the highest apoptotic cells in HeLa cell line. Table 6 summarized the biological activities of the Hemp leaf and seed extracts. Therefore, the Hemp leaf extract can be further developed as pharmaceutical and cosmeceutical products as anti-cancer, anti-bacterial (*S. mutans*), anti-aging, whitening, anti-hair loss and canities treatment.

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### DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

**Table 5.** The percentages of the apoptotic and necrotic cell numbers in four cancer cells induced by the Hemp leaf and seed extracts.

| Sample    | Conc. (mg/ml) | KB cell line |           | HeLa cell line |           | HT29 cell line |           | HepG2 cell line |             |
|-----------|---------------|--------------|-----------|----------------|-----------|----------------|-----------|-----------------|-------------|
|           |               | %Apoptosis   | %Necrosis | %Apoptosis     | %Necrosis | %Apoptosis     | %Necrosis | %Apoptosis      | %Necrosis   |
| seed      | 5             | 0            | 100       | 9.25±2.55      | 6.76±1.29 | 1.59±1.43      | 0         | 0               | 100         |
|           | 0.5           | 0            | 0         | 0              | 0         | 0              | 0         | 0               | 0           |
|           | 0.005         | 0            | 0         | 0              | 0         | 0              | 0         | 0               | 0           |
| leaf      | 5             | 6.87±1.77    | 0         | 13.26±7.40     | 0         | 2.40±1.35      | 0         | 4.04±3.03       | 19.43±13.01 |
|           | 0.5           | 0            | 0         | 9.03±3.14      | 0         | 0              | 0         | 0               | 0           |
|           | 0.005         | 0            | 0         | 0              | 0         | 0              | 0         | 0               | 0           |
| Control   | -             | 0            | 0         | 0              | 0         | 0              | 0         | 0               | 0           |
| Cisplatin | 0.1           | 44.85±11.18  | 1.69±1.55 | 88.68±4.79     | 0         | 12.16±3.54     | 0.57±0.99 | 28.87±5.76      | 4.95±2.90   |

**Table 6.** Summarization of the highest biological activities of the Hemp leaf and seed extracts for pharmaceutical and cosmeceutical applications.

| Extract | Pharmaceutical                 |      |                |       |                  |                  | Cosmeceutical                     |                  |  |                   |                          |       |
|---------|--------------------------------|------|----------------|-------|------------------|------------------|-----------------------------------|------------------|--|-------------------|--------------------------|-------|
|         | Anti-proliferation / Apoptosis |      | Anti-bacterial |       | Anti-aging       |                  | Whitening (Tyrosinase inhibition) |                  | Anti-hair loss (5α-reductase inhibition) |                   | Canities (melanogenesis) |       |
|         | KB                             | HeLa | HT29           | HepG2 | <i>S. mutans</i> | <i>S. aureus</i> | <i>P. acne</i>                    | SC <sub>50</sub> | CC <sub>50</sub>                         | IPC <sub>50</sub> |                          | MMP-2 |
| Leaf    | ✓                              | ✓    | ✓              | ✓     | -                | -                | ✓                                 | ✓                | ✓  | ✓                 | ✓                        | ✓     |
| Seed    | -                              | -    | -              | -     | ✓                | -                | -                                 | -                | -  | -                 | -                        | -     |

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