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Antioxidant, toxic and cytotoxic activities of *Butea superba* Roxb

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Abstract

This study investigated total phenolics content (TPC), total flavonoids content (TFC), antioxidant, toxicity, and cytotoxicity of *B. superba* ethanolic extract (BSE), which the plant is used in Thai traditional medicine. The toxicity was evaluated using a brine shrimp lethality assay and the cytotoxicity was done through Michigan Cancer Foundation (MCF-7) and prostate cancer (PC-3) cancer cell lines, particularly nuclear morphological changes and Deoxyribonucleic acid (DNA) fragmentation. The result found that BSE had low TPC and TFC (87.16 µg gallic acid equivalent (GAE)/mg and 38.29 µg CE/mg). The effective concentrations at 50% (EC_{50}) and 99% (EC_{99}) through 2,2-diphenyl-l-picrylhydrazyl (DPPH) and Ferric thiocyanate (FTC) assays were 3,610.10 ($EC_{50/DPPH}$), 215.11 ($EC_{50/FTC}$), 7,304.05 ($EC_{99/DPPH}$), and 401.88 ($EC_{99/FTC}$) µg/mL, respectively. Lethal concentration at 1% (LC_1) and 50% (LC_{50}) were 54.63 and 184.24 µg/mL Therapeutic index (TI) and margin of safety (MOS) assessment of BSE by DPPH and FTC assay were 0.051 (TI_{DPPH}), 0.856 (TI_{FTC}), 0.007 (MOS_{DPPH}), and 0.136 (MOS_{FTC}). BSE demonstrated an antiproliferative effects on MCF-7 and PC-3 cells in a dose-dependent manner, and induced apoptosis and DNA fragmentation in both cancer cells. In conclusion, BSE proposed to develop anticancer agents that could contribute to medicinal benefits.

Keywords: *Butea superba*, Antioxidant activity, Toxicity, Cytotoxicity, Apoptosis

1. Introduction

Various environmental stresses can induce the generation of free radicals and reactive oxygen species (ROS). ROS can cause various disorders and diseases such as tissue damage, allergy, inflammation, atherosclerosis, diabetes mellitus, neurodegenerative diseases, and cancers. Many plants have been reported to contain high levels of antioxidants, which have the potential to be used against chronic and degenerative diseases, including plants with antifungal, antibacterial, antiviral, antioxidant, anti-allergy, anti-inflammatory, and anticancer properties [1]; conversely, the studies should also take into consideration phytochemical toxicity.

Breast and prostate cancers are the most common two forms of cancer which continue to cause mortality for Thai people. Conventional cancer treatments comprise surgery, radiation, and chemotherapy, but these do not always prevent metastasis and mortality. Therefore, the development of natural products still exists as one of the alternative treatments which might be employed as a cancer cure [2].

Butea superba Roxb. belonging to the family Fabaceae is a herb distributed in the deciduous forests of Thailand. *B. superba* has been used in Thai traditional medicine to remedy several symptoms; for example, the bark and flowers are used for antibacterial and antifungal activities, and the root is used to improve physical strength, male sexual performance, and erectile dysfunction [3,4]. The tuberous root has a long cylindrical shape. The root was found to contain flavonoids, flavonoid glycosides, isoflavonoids, and sterol compounds [5,6]. Flavonoids and flavonoid glycosides from *B. superba* showed cyclic adenosine monophosphate (AMP) phosphodiesterase inhibitor activity [5]. *B. superba* contained genistin, genistein, and daidzein that possessed potent antioxidant properties [7] and showed strong antimicrobial ability [3]. *B. superba* exhibited the ability to

improve erectile function in erectile dysfunction patients without apparent toxicity [4] and showed inhibition activity of acetylcholinesterase in the brains of mice [8]. Alone and in combination, genistein and daidzein from *B. superba* could improve sperm numbers and motility, cholesterol, and testosterone levels [9]. However, in the traditional remedy pamphlet of Kwao Krua Tuber, it was reported that the daily dosage of *B. superba* powder should not exceed two-thirds of pepper seeds [10]. Consistently, a significant difference in the changes in hematological, biochemical, and histopathological parameters was found in male and female rats receiving *B. superba* powder at the dose of 250 and 1,000 mg/kg body weight (BW)/day [11]. Recently, some phytochemicals from *B. superba* were found to inhibit the proliferation of human epidermoid carcinoma and breast cancer cell lines [6]. Moreover, phytosterols from *B. superba* could reduce cholesterol levels and the benign prostatic hyperplasia of prostate cancer appearance [12]. In this family, many plant species have effects on treatment such as *B. monosperma* (Lam.) Taubert. The bioactive compounds of this plant offer antioxidation, antimicrobial, anti-inflammation, and antitumor properties, as well as acting against breast and hepatic cancers [13].

The antioxidant activity of *B. superba* is based on the phytochemicals; however, few documents reported on the toxicological profiles and anticancer treatment. Thus, this study aimed to measure the total phenolic content (TPC) and total flavonoid content (TFC) of *B. superba* extract (BSE). The antioxidant activity was investigated using two assays and correlation analysis was carried out on the TPC and TFC of BSE and its antioxidant activity. We determined the toxicity of BSE on brine shrimp using a lethality assay along with therapeutic index (TI) and margin of safety (MOS) analysis on the lethal concentration of toxicity and its effective concentration of antioxidant activity. The anticancer properties were evaluated by analyzing cytotoxicity, morphological change, and Deoxyribonucleic acid (DNA) fragmentation in Michigan Cancer Foundation (MCF-7) human breast cancer and prostate cancer (PC-3) human prostate cancer cell lines. The results of this study were expected to reveal the potential of BSE as pharmacological development and anticancer agent, confirming safe consumption and increasing value-added to *B. superba* products as well.

2. Materials and methods

2.1 Chemicals

Folin-Ciocalteu's phenol reagent, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and linoleic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM/F-12), Roswell Park Memorial Institute 1640 Medium (RPMI-1640), fetal bovine serum (FBS), penicillin/streptomycin, and resazurin were from GIBCO brand, Invitrogen, Thermo Fisher Scientific Corporation (Waltham, MA, USA). Hoechst 33342 and 3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were the Molecular Probes brand, Thermo Fisher Scientific Corporation (Waltham, MA, USA). Genomic DNA Extraction Kit and DNA ladder were bought from RBC Bioscience Corporation, Taiwan. Chemicals and reagents in this study were of analytical grade.

2.2 Plant material and extract preparation

Tuberous roots of *B. superba* were collected from Sung Men, Phrae province, Thailand on May 2019. The plant sample under the specimen voucher code no. MFLU-310 was identified by Assoc. Prof. Yuthana Smittasiri. *B. superba* gathering and utilization were allowed by the Plant Varieties Protection Office, Department of Agriculture (Project code: 0207/2562). As for plant preparation, roots were peeled, cleaned, sliced, dried, ground to powder, and collected in a dry container until extraction. The extraction started from the *B. superba* root powder was extracted with 80% ethanol overnight by using Soxhlet extractor. The extract was vaporized under reduced pressure at 45°C, lyophilized, and stored at -70°C until use. When using, the extract was dissolved in Dimethyl sulphoxide (DMSO) and diluted according to the desired concentrations.

2.3 Total phenolic content determination

The TPC of BSE was measured by the Folin-Ciocalteu method [14]. Briefly, one hundred µl of the extract solution was added with 400 µl of distilled water and 100 µl of Folin-Ciocalteu reagent (Folin-Ciocalteu's phenol reagent: methanol, 1:1, v/v), and mixed them on a vortex mixer. The mixed sample was added 1.4 mL of 2% sodium carbonate solution and incubated for 45 min. The mixture was measured optical density at 760 nm. The reading value was declared as micrograms of gallic acid equivalent per milligram of dry basis (GAE/mg). The equation calculated from the gallic acid standard curve was expressed as the following.

$$Y = 0.0048x + 0.0629;\ R^2 = 0.9999 \quad (1)$$

2.4 Total flavonoid content determination

TFC was measured by the colorimetric method [15]. Briefly, one hundred and twenty-five µl of the extract solution was mixed with 625 µl of distilled water and 37.5 µl of 5% sodium nitrite. Seventy-five µl of 10% aluminum chloride was added and incubated for 10 min, and then 250 µl of 1 M sodium hydroxide was added and adjusted to 1.25 mL with distilled water. The optical density was identified at 510 nm. TFC was declared as micrograms of catechin equivalent per milligram of dry basis (CE/mg). The equation calculated from the catechin standard curve was expressed as the following.

$$Y = 0.0028x + 0.0353;\ R^2 = 0.9995 \quad (2)$$

2.5 Measurement of free radical scavenging assay

Free radical scavenging activity was investigated by DPPH assay [16]. Fifty µl of the extract solution was mixed with 1.95 mL of DPPH reagent solution and incubated in the dark condition for 60 min. The optical density was identified at 515 nm. Radical scavenging activity was computed using the below formula and declared as the effective concentration at 25%, 50%, 75%, and 99% (EC_{25}, EC_{50}, EC_{75}, and EC_{99}).

$$\text{Scavenging activity (\%)} = \left[1 - \frac{(OD_1 - OD_2)}{OD_0}\right] \text{ x } 100 \quad (3)$$

Where OD_0 was the optical density of the control, OD_1 was the optical density of the DPPH solution present in the sample, and OD_2 was the optical density without the DPPH solution.

2.6 Measurement of ferric thiocyanate assay

Ferric thiocyanate (FTC) assay was performed as reported by Huang et al. [17]. One mL of the extract solution was mixed with 1.5 mL of 2.51% linoleic acid solution, 2.5 ml of 0.05 M phosphate buffer, pH 7.0, and then incubated in the dark at 40°C. Five hundred ml of the mixed sample, 4.9 mL of 75% ethanol, and 50 µl of 30% ammonium thiocyanate were mixed and allowed to stand for 3 min. Fifty µl of 20 mm ferrous chloride in 3.5% hydrochloric acid was added. The optical density was measured at 500 nm every 24 h until the optical density of control reached its maximum. Inhibition of lipid peroxidation was computed using the below formula and declared as the effective concentration at 25%, 50%, 75%, and 99% (EC_{25}, EC_{50}, EC_{75}, and EC_{99}).

$$\text{Lipid peroxidation inhibition (\%)} = \left[1 - \frac{(OD_1 - OD_2)}{OD_0}\right] \text{ x } 100 \quad (4)$$

Where OD_0 was the optical density of the control, OD_1 was the optical density present in the sample, and OD_2 was the optical density of the sample without the thiocyanate solution.

2.7 Measurement of brine shrimp lethality assay

Toxicity was conducted with brine shrimp lethality assay (BSLA). The procedure for BSLA was modified from the method described by Solis et al. [18] and Finney [19]. Brine shrimp (*Artemia salina* Linn.) eggs were obtained from an ornamental fish shop. After larval hatching, they were incubated in artificial seawater (Mariscience Int'I Co., Ltd., Thailand), pH 8.5 ± 0.5, under continuous light, at 25 ± 3 ˚C for 24 - 36 h. Twenty nauplii were transferred onto a 24-well plate containing extracts solution at various concentrations for 24 h. The dead larvae were counted. The percentage of mortality was calculated as follows:

$$\text{Mortality (\%)} = \left[1 - \frac{(A_1 - A_2)}{A_1}\right] \text{ x } 100 \quad (5)$$

Where A_1 was the live control (the medium without the sample), A_2 was the deaths present in the samples.

The lethal concentration at 1%, 25%, 50% and 75% (LC_1, LC_{25}, LC_{50} and LC_{75} values) was determined at 24 h using Probit analysis [19].

2.8 Determination of toxicological profile

The toxicological profile was calculated using the concentration-response relationship between the lethal concentration of brine shrimp and the effective concentration of antioxidant activity. Therapeutic index (TI) and MOS were determined, described by Tohsakulkaew et al. [20] as the following formula:

$$\text{Therapeutic index} = \frac{LC_{50}}{EC_{50}} \quad (6)$$

$$\text{Margin of safety} = \frac{LC_1}{EC_{99}} \quad (7)$$

2.9 Cell lines

MCF-7 (human breast adenocarcinoma cell line) and PC-3 (human prostate cancer cell line) were obtained from American Type Culture Collection (ATCC: Manassas, USA). MCF-7 cells were cultured DMEM/F-12, while and PC-3 cells were cultured in RPMI-1640. Both cell lines were grown at 37 ˚C, 5% CO_2 supplemented with 10% FBS and 1% penicillin/streptomycin.

2.10 Cytotoxic activity by MTT assay

Cytotoxicity of MCF-7 and PC-3 cell lines was determined by MTT assay. This colorimetric assay based on the potential of mitochondrial succinate dehydrogenase of viable cells to reduce MTT (yellow) to formazan derivatives (dark purple) [21]. Briefly, MCF-7 and PC-3 cells seeded in 96-well plates (1 x 10^4 cells/well) and allowed to adhere for 24 h. After incubation, the cultured cells were treated with different BSE concentrations and incubated for 24 h. The treated medium was discarded and added in each well with 100 µl of MTT dye solution (5 mg in 1 mL of phosphate-buffered saline, pH 7.4). The plates were incubated for 4 h. The formazan crystals dissolved in 150 µl of dimethyl sulfoxide. The optical density was determined at 570 nm. A decrease in the optical density exhibited a reduction in cell existence [22]. Antiproliferative activity was calculated to percentages according to the following formula and plotted against the concentrations of samples. The median inhibitory concentration (IC_{50}) was derived from the best fit line obtained by linear regression measurement.

$$\text{Antiproliferation (\%)} = \left[1 - \frac{(OD_1 - OD_2)}{(OD_0 - OD_2)}\right] \text{ x } 100 \quad (8)$$

Where OD_0 was the optical density of the control, OD_1 was the optical density of the treated sample, and OD_2 was the optical density of the treated sample without cells.

2.11 Cytotoxic activity by resazurin assay

Cytotoxic activity was determined by resazurin assay. This assay is a colorimetric assay based on the conversion of resazurin solution (purple) to resorufin solution (red) via decrease reactions of metabolic cells. Briefly, MCF-7 and PC-3 cells were seeded in 96-well plates (1 x 10^4 cells/well) and cultured for 24 h. After incubation, the cells were treated with different BSE concentrations and incubated for 24 h. The treated medium was discarded and incubated with 100 µl of resazurin dye solution in each medium without FBS for 2 h. The optical density was measured at 570 nm. A decrease in the optical density displayed a reduction in cell existence [22,23]. The percentage of cell death was calculated using the following formula Equation 8 and the IC_{50} value was analyzed.

2.12 Detection of nuclear condensation by Hoechst assay

Hoechst assay was used to identify the nuclear condensation, conducted as a morphological marker of apoptosis (fragmented and shrunken or condensed nuclei). MCF-7 and PC-3 cells were seeded in each 6-well plates (3 x 10^5 cells/well) and incubated for 24 h. Both cell lines were treated with different BSE concentrations for 24 h and then washed 2 times with ice-cold phosphate-buffered saline, pH 7.4. After that, cells were fixed with 500 µl 10% formaldehyde for 5 min, washed with phosphate-buffered saline, and stained with 1 µg/µl of Hoechst 33342 stain for 15 min in the dark. Changes in the nuclear morphology in the cells were investigated under an inverted fluorescence microscope Nikon Eclipse Ts2-FL and photographed with Nikon DS-Fi3 microscope camera (Nikon Corporation, Tokyo, Japan).

2.13 DNA fragmentation

MCF-7 and PC-3 cells were seeded in each 75 cm^2 culture flask (2.4 x 10^6 cells/flask) and incubated for 24 h. After incubation, the cells were treated with different concentrations of BSE and continued to incubate for 24 h. After treatment, the cells were collected and centrifuged. The cell pellets were then washed with phosphate-buffered saline and centrifuged. DNA was extracted from the cell pellets using a genomic DNA purification kit. RNase A was added to sample lysate and incubated at room temperature for 30 min. The isolated DNA was centrifuged at 13,000 rpm for 3 min and eluted with elution buffer. The purified DNA was resuspended in Tris-HCl EDTA buffer and quantified using a Nanodrop. DNA samples (2 µg/wells) were electrophoresed at 60 V for 2 h by 1% agarose gel electrophoresis. Then the DNA fragment was visualized under ultraviolet light.

2.14 Statistical analysis

Each result was expressed as the mean ± standard error of the mean (mean ± SEM) of four independent experiments. All statistical significances were analyzed using one-way analysis of variance with the least significant difference test to identify the level of significance at $p < 0.01$. The statistical tests for correlation analysis were analyzed using the linear correlation coefficient. The SPSS statistics version 20 software program was used for the analyses.

3. Results and discussion

3.1 Total phenolic and flavonoid contents

Phytochemicals and antioxidant properties have generally positive relationships. In this study, the TPC of BSE was investigated using the Folin-Ciocalteu method and was presented as a gallic acid equivalent (standard curve equation: $y = 0.0048x + 0.0629$, $R^2 = 0.9999$). The TFC of BSE which was evaluated using the colorimetric method was displayed in a catechin equivalent (standard curve equation: $y = 0.0028x + 0.0353$, $R^2 = 0.9995$). The results indicated that the TPC and TFC of BSE were 87.16 ± 0.55 µg GAE/mg and 38.29 ± 0.93 µg CE/mg, respectively (Table 1). Normally, the amount of TPC and TFC could indicate the potential antioxidant activity of extracts.

Polyphenolic compounds and their constituents in plants are mostly capable of extraction by ethanol, while compounds importantly point out the antioxidant property and cytotoxicity of the medicinal plant. *B. superba* was reported to contain various phytochemicals. Flavonoids, one of the most diverse and widespread groups of natural compounds, are the major subclass of polyphenols and antioxidants in plants. BSE was also reported as a rich source of the flavonoid (3,7,3'-trihydroxy-4'-methoxyflavone) and the flavonoid glycoside (3,5'-dihydroxy-4'-methoxyflavone-7-O-ß-D-glucopyranoside) [5]. Furthermore, some documents showed that BSE contained four isoflavones (7,4'dimethoxyisoflavone, 7-hydroxy-6,4'-dimethoxyisoflavone, Formononetin, and Prunetin), a derivative isoflavonoid (Medicarpin) [6], and four isoflavones (Biochanin A, Genistin, Daidzein, and Genistein) [7,9].

Table 1 Total phenolic content (TPC), total flavonoid content (TFC) and EC_{50} and EC_{99} values in free radical scavenging assay and ferric thiocyanate assay of BSE.

Phytochemicals		Radical scavenging activity		Inhibition of lipid peroxidation	
TPC (µg GAE/mg)	TFC (µg CE/mg)	$EC_{50/DPPH}$ (µg/mL)	$EC_{99/DPPH}$ (µg/mL)	$EC_{50/FTC}$ (µg/mL)	$EC_{99/FTC}$ (µg/mL)
87.16 ± 0.55	38.29 ± 0.93	3,610.10 ± 36.43	7,304.05 ± 79.30	215.11 ± 2.85	401.88 ± 10.80

Each value represents the mean ± SEM of four independent experiments; EC_{50}, median effective concentration; EC_{99}, the ninety-nine percent of the effective concentration.

3.2 Antioxidant activities

Antioxidant activity via DPPH free radical scavenging assay refers to the proton-donating ability of the substance exhibited via $EC_{25/DPPH}$, $EC_{50/DPPH}$, $EC_{75/DPPH}$, and $EC_{99/DPPH}$ values. By definition, a low EC value means a high capacity for free radical scavenging. In this study, BSE was able to scavenge oxidative species in a concentration-dependent manner. BSE at 1,000 µg/mL could inhibit DPPH radicals up to 16% (data not shown). The $EC_{25/DPPH}$, $EC_{50/DPPH}$, $EC_{75/DPPH}$ and $EC_{99/DPPH}$ values of BSE were approximately 1,725.44 ± 37.07, 3,610.10 ± 36.43, 5,494.77 ± 54.98 and 7,304.05 ± 79.30 µg/mL, respectively (Table 1 and Figure 1A). Also, the antioxidant property can be determined by lipid peroxidation inhibition via ferric thiocyanate (FTC) assay and is expressed as $EC_{25/FTC}$, $EC_{50/FTC}$, $EC_{75/FTC}$, and $EC_{99/FTC}$ values. By definition, a low EC value means a high

ability in lipid peroxidation inhibition. The results showed that BSE at 100 µg/mL could inhibit lipid peroxidation up to 22% (data not shown). The $EC_{25/FTC}$, $EC_{50/FTC}$, $EC_{75/FTC}$, and $EC_{99/FTC}$ values of BSE were approximately 119.82 ± 1.55, 215.11 ± 2.85, 310.40 ± 6.89, and 401.88 ± 10.80 µg/mL, respectively (Table 1 and Figure 1B).

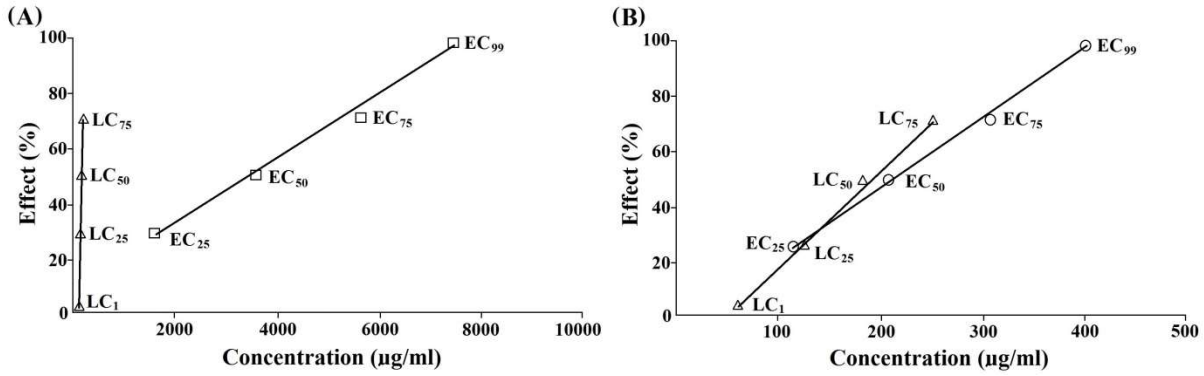

Figure 1 *In vitro* concentration-response relationships of LC of cytotoxicity evaluated by brine shrimp assay and EC of free radical scavenging (A) and EC of lipid peroxidation inhibition (B).

Phenolic compounds play an important role in antioxidant biochemical pathways. Phenolic and flavonoid compounds could act against free radicals and lipid peroxidation. *B. superba* was reported to contain various phytochemicals, mainly phenolic hydroxyl groups, flavonoid, flavonoid glycoside, anthocyanin, and steroids with bioactivities [5-9]. *B. superba* extract exhibited a pivotal role in antioxidant capacities via scavenging DPPH and ABTS, and inhibiting low-density lipoprotein (LDL) oxidation in dose-dependent manners [24]. This was in agreement with the tuberous roots of *B. superba* methanolic [5-7] and the ethanolic extracts [25,26]. Corresponding to Table 2, the correlation coefficients between TPC and antioxidant activities (using DPPH and FTC assays) of BSE concentrations are positively correlated for DPPH assay (0.9793) and FTC assay (0.9063). Similarly, the correlation coefficients between TFC and antioxidant activities show good positive correlation for the DPPH assay (0.9774) and FTC assay (0.9067). Other plants such as *T. ornata*, *P. tetrastromatica*, *T. decurrens*, and *C. racemosa* also were documented with positive correlations among phytochemicals and antioxidant activities [27]. Therefore, it could confirm that BSE produces a variety of constituents and bioactivities.

Table 2 Correlation between phytochemicals and bioactivities (antioxidant and toxic activities) of BSE.

Assay	Correlation coefficient (R)	
	Total phenolic content (TPC)	Total flavonoid content (TFC)
DPPH radical scavenging assay	0.9793	0.9774
Ferric thiocyanate assay	0.9063	0.9067
Brine shrimp lethality assay	0.9783	0.9782

3.3 Toxic activity on brine shrimp

The toxicity of the plant extract can be monitored by the brine shrimp lethality assay (BSLA). After 24 h of BSE treatment, brine shrimp mortality was counted and calculated to percentages, LC_1 and LC_{50} determination. In this study, the percentage of brine shrimp mortality increased in a dose-dependent manner (Table 3). The LC_1, LC_{25}, LC_{50}, and LC_{75} values of BSE were approximately 54.63 ± 8.43, 140.69 ± 7.32, 184.24 ± 6.49, and 235.25 ± 2.05 µg/mL, respectively (Table 3 and Figures 1A - 1B).

The BSLA represents a rapid, inexpensive, and simple bioassay for preliminary plant extract assessment, which in most cases correlates reasonably well with cytotoxicity. This means that a lower LC_{50} value than 1,000 µg/mL is considered bioactive [28,29]. In our study, the LC_{50} value of BSE was less than 1,000 µg/mL; therefore, the BSE was more toxic on brine shrimps after 24 h of treatment. Consistently, *B. monosperma* showed significant cytotoxicity via LC_{50} 290 µg/mL [30]. Similarly, other plant species such as *Aloe vera* (L.) Burm., *Citrus aurantium* L., *Ocimum basilicum* L., *Pimenta dioica* (L.) Marr., and *Justicia pectoralis* Jacq. had LC_{50} values lower than 1,000 µg/mL (3.59, 3.99, 9.92, 32.78, and 60.14 µg/mL, respectively), which were highly correlated *in vitro* (LC_{50} values of brine shrimp) and *in vivo* lethal dose (LD_{50} values of mice) [29]. LC_{50} values of methanolic and ethanolic mangosteen hull extracts via BSLA were also documented at approximately

153.88 and 161.75 µg/mL, which correlated to median inhibitory concentration (IC_{50}) values of PC-3 cells (233.33 and 239.20 µg/mL) and MCF-7 cells (437.54 and 466.41 µg/mL) [22]. Also, in Table 2, the correlation coefficients between phytochemicals and the toxicity of BSE concentrations were more positively correlated for TPC (0.9783) and TFC (0.9782).

Table 3 Toxicity evaluated by brine shrimp lethality and toxicological profile (TI and MOS) derived from mortality and antioxidant activity of BSE.

Conc.	Mortality	LC_1	LC_{50}	TI		MOS	
(µg/mL)	(%)	(µg/mL)	(µg/mL)	TI_{DPPH}	TI_{FTC}	MOS_{DPPH}	MOS_{FTC}
50	2.50 ± 1.44^{d}	54.63 ± 8.43	184.24 ± 6.49	0.051	0.856	0.007	0.136
100	6.25 ± 1.25^{d}						
150	26.25 ± 1.25^{c}						
200	60.00 ± 4.08^{b}						
250	83.75 ± 2.39^{a}						
300	91.25 ± 2.39^{a}						

Each value represents the mean ± SEM of four independent experiments. Different letters within the same column are significantly different at $p < 0.01$. LC_1, the one percent of lethal concentration; LC_{50}, median lethal concentration; TI, therapeutic index; MOS, the margin of safety.

3.4 Concentration-response activities for toxicological profile determination of BSE

The TI and MOS by BSLA can be used in the context of the toxicological pharmacology of substances. In Table 3, BSE showed TI (TI_{DPPH}; 0.051 and TI_{FTC} 0.856) and MOS (MOS_{DPPH}; 0.007 and MOS_{FTC}; 0.136), based on DPPH radical scavenging and inhibition of lipid peroxidation.

In this study, BSE exhibited toxicity. The LC_{50} value of brine shrimps was lower than 1,000 µg/mL, which corresponds to other reports [22,28-30]. Toxic and antioxidant studies of BSE are useful to predict the risk, especially through the TI and MOS studies. In this study, the acute toxicity of BSE was first estimated via statistical TI and MOS prediction. In this case, high values of TI and MOS indicated pharmacological safety. Manasathien et al. [31] reported that TI_{DPPH} of pomegranate peel water (PPW) and ethanolic (PPE) extracts measured 11.49 and 9.92, while TI_{FTC} of the same extracts were 78.04 and 66.91. Also, MOS_{DPPH} and MOS_{FTC} values of PPW and PPE were similar at 250 and 150, respectively. These values indicate greater effectiveness in animal treatments and would preferably be accepted as an effective activity. However, the TI and MOS values of our study were very low. It was likely indicated that BSE had low effectiveness, or might be a side effect. A further study by micronucleus test demonstrated that *B. superba* extract could induce acute micronucleus formation. *In vitro*, mutagenic and antimutagenic assays confirmed that the safe consumption level of *B. superba* extract should be at a low dose [32].

3.5 Antiproliferative effects of BSE

The antiproliferative effect of BSE on two different human cancer cell lines, MCF-7 and PC-3 cells was investigated by *in vitro* cytotoxicity MTT and resazurin assay. Both cancer cells showed similar susceptibility to BSE in a dose-dependent manner (Table 4). Based on the MTT and resazurin assays in Table 4, the IC_{50} values of MCF-7 cells were 1,405.82 ± 34.01 and 1,299.96 ± 17.90 µg/mL, and the IC_{50} values of PC-3 cells were 1,618.72 ± 14.06 and 1,676.17 ± 7.60 µg/mL.

For both MTT and resazurin assays, the antiproliferative capacity of MCF-7 and PC-3 cells by the BSE was significant in a dose-dependent manner. The IC_{50} of MCF-7 is significantly lower than that of PC-3 ($p < 0.01$); therefore, BSE had a cytotoxic effect on MCF-7 cells greater than the effect on PC-3 cells. BSE obtained some bioactive compounds, genistin, daidzein, genistein, prunetin, medicarpin, formononetin, 7,4'-dimethoxyisoflavone, and 7-hydroxy-6,4'-dimethoxyisoflavone as well as sterol compounds, including ß-sitosterol, campesterol and stigmasterol [5-7,25,26]. Moreover, the correlation coefficient between phytochemicals (TPC and TFC) and both antiproliferation of cancer cells (using MTT and resazurin assays) of BSE concentrations showed good positive correlation (data not shown). Many flavonoids and sterols are phytoestrogens that can be considered for cancer treatment. In the YES-hERα+hTIF2 based assay, 7-hydroxy-6,4'-dimethoxyisoflavone showed highly potent ERα-estrogenic activity, and prunetin, medicarpin, formononetin and 7,4'-dimethoxyisoflavone showed much weaker ERα-estrogenic activity. However, *B. superba* ethanolic extract did not exhibit estrogenic activity in the YES-hERα+hTIF2 based assay but exhibited anti-estrogenic activity in the incubation assay [26]. BSE could delay cell proliferation in human epidermoid carcinoma of the cavity, breast cancer cell lines, and human small cell lung carcinoma [6]. BSE could induce an antiproliferative effect on the MCF-7 cell growth at 10, 100, and 1,000 µg/mL after 4 days incubation by the

IC_{50} value of 370.91 µg/mL [33]. Therefore, BSE could exhibit antiproliferative effects on MCF-7 and PC-3 cells concerning the possible anti-estrogenic mechanism on estrogen receptor cells.

Table 4 Cytotoxic activity of BSE in MCF-7 and PC-3 cells, investigated via MTT and resazurin assays.

Conc.	MCF-7 antiproliferation (%)		PC-3 antiproliferation (%)	
(µg/mL)	MTT	Resazurin	MTT	Resazurin
1,300	39.88 ± 3.44^{d}	50.21 ± 0.79^{d}	10.32 ± 2.77^{d}	11.65 ± 1.41^{e}
1,400	$49.25 \pm 3.53^{c,d}$	51.80 ± 1.29^{d}	19.13 ± 4.88^{d}	21.81 ± 0.65^{d}
1,500	54.98 ± 2.78^{c}	60.39 ± 0.36^{c}	33.65 ± 3.31^{c}	23.70 ± 1.11^{d}
1,600	69.40 ± 1.74^{b}	60.79 ± 0.43^{c}	$46.42 \pm 3.80^{b,c}$	32.38 ± 1.30^{c}
1,700	$74.12 \pm 0.89^{a,b}$	64.21 ± 0.62^{b}	56.90 ± 1.50^{b}	48.96 ± 0.67^{b}
1,800	82.86 ± 2.97^{a}	69.26 ± 1.03^{a}	77.48 ± 2.05^{a}	74.18 ± 2.21^{a}
IC_{50} (µg/mL)	1,405.82 ± 34.01	1,299.96 ± 17.90	1,618.72 ± 14.06	1,676.17 ± 7.60

Each value represents the mean ± SEM of four independent experiments. Different letters within the same column are significantly different at $p < 0.01$. IC_{50}, median inhibition concentration.

3.6 Apoptotic induction by Hoechst 33342 staining

Evaluation of the changes in MCF-7 and PC-3 cellular morphology by BSE was monitored by both unstained and stained analysis using Hoechst 33342. The apoptotic cells are characterized by cell shrinkage, membrane blebbing, nuclear condensation, and chromosomal DNA fragmentation (Figures 2 and 3). The untreated MCF-7 cells are illustrated in Figures 2A and 2D. Based on information of the antiproliferation effect, the optimal concentrations for *in vitro* apoptosis investigation of MCF-7 cells were 1,400 µg BSE/mL (Figures 2B and 2E) and 1,600 µg BSE/mL (Figures 2C and 2F) for 24 h. The untreated PC-3 cells are displayed in Figures 3A and 3D. The optimal concentrations for the apoptosis study of PC-3 cells were at 1,600 µg BSE/mL (Figures 3B and 3E) and 1,800 µg BSE/mL (Figures 3C and 3F) for 24 h.

These results indicated that BSE could induce observable changes illustrated via the percentages of apoptotic cells in MCF-7 and PC-3 cells in a dose-dependent fashion. Thus, total apoptotic cells were BSE concentration-dependent. The remarkable changes of the cells were the cytoplasmic membrane shrinkage, loss of contact with the neighbouring cells, and membrane blebbing (Figures 2B-C and 3B-C). Moreover, with BSE treatment and staining with Hoechst 33342, the cellular morphological changes could be indicated through condensed nuclei and nuclear fragmentation (Figures 2E-F and 3E-F). These recognized that BSE could induce apoptosis of MCF-7 and PC-3 cells.

3.7 DNA fragmentation in human cancer cell lines

A distinctive biochemical feature of apoptosis is DNA fragmentation, which is the cleavage of chromosomal DNA into internucleosomal fragments that appears on agarose gel electrophoresis. In Figure 4, DNA degradation in BSE-treated cell lines for 24 h was displayed, whereas the untreated control cells exhibited intact genomic DNA when observed on agarose gel under a UV transilluminator. The smearing pattern was observed for 1,400 and 1,600 µg/mL (DNA from BSE-treated MCF-7 cells) and 1,600 and 1,800 µg/mL (DNA from BSE-treated PC-3 cells), and the result showed DNA fragmentation caused by BSE treatments in both cancer cells (Figure 4).

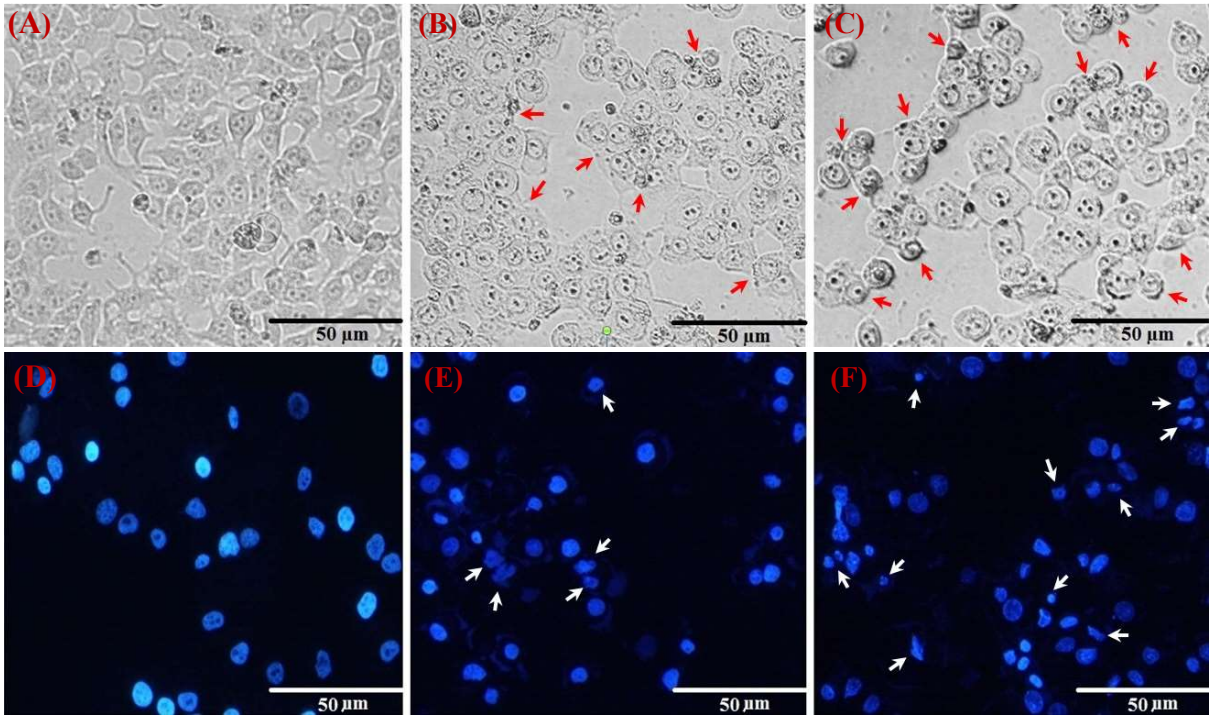

Figure 2 Morphological alterations in MCF-7 cells observed under inverted microscopy after treatment by A-C were unstained and D-F stained with Hoechst 33342. A and D, untreated control MCF-7 cells; B and E, BSE-treated cells with 1,400 µg/mL; C and F BSE-treated cells with 1,600 µg/mL. The red arrow referred to cell shrinkage and membrane blebbing, while the white arrow indicated nuclear condensation and chromosomal DNA fragmentation.

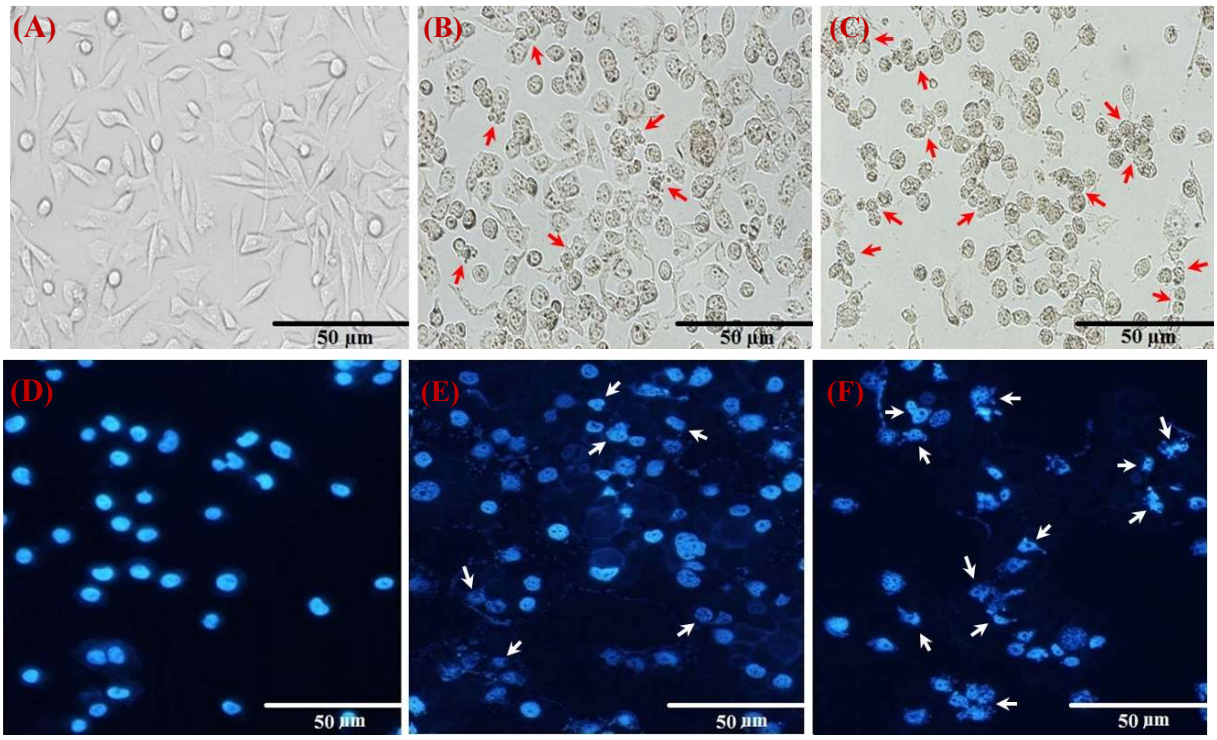

Figure 3 Morphological alterations in PC-3 cells observed under inverted microscopy after treatment by A-C were unstained and D-F stained with Hoechst 33342. A and D, untreated control PC-3 cells; B and E, BSE-treated cells with 1,600 µg/mL; C and F BSE-treated cells with 1,800 µg/mL. The red arrow referred to cell shrinkage and membrane blebbing, while the white arrow indicated nuclear condensation and chromosomal DNA fragmentation.

DNA fragmentation is one of the hallmarks of apoptosis. In particular, apoptosis can take two directions: (*i*) the extrinsic direction concerning the death receptor signalling, and (*ii*) the intrinsic direction concerning the mitochondrial cascades [34]. The extrinsic direction is related to caspase-8, while the intrinsic direction is related to caspase-9 [35]. However, activation of effector caspases (caspase-3 and -7) is associated with both directions. The activity of caspase-3 and -7 is involved in the initiation of DNA fragmentation. Therefore, these findings indicated that BSE may work by several mechanisms. In particular, BSE could induce apoptotic DNA fragments toward MCF-7 and PC-3 cells corresponding to the high percentages of condensed and fragmented nuclei detected by Hoechst staining.

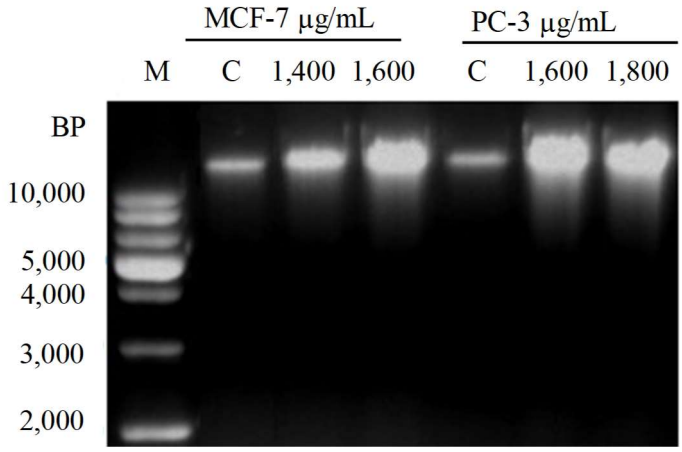

Figure 4 DNA fragmentation of cancer cells: MCF-7 (1,400 and 1,600 µg/mL) and PC-3 (1,600 and 1,800 µg/mL). The cells are treated with BSE at the indicated concentrations. Lane M: DNA maker, Lane C: untreated control MCF-7 and PC-3 cells.

4. Conclusion

This study found that BSE has low levels of phenolic and flavonoid compounds and low antioxidant properties. Furthermore, BSE showed toxicity on brine shrimps when considering the toxicological profile. According to concentration levels, however, BSE exhibited specific cytotoxicity against cancerous MCF-7 and PC-3 cells by inducing apoptosis. The antiproliferative effects on both cells were due to apoptotic induction, proofing via apoptotic body formation, and chromosomal DNA fragmentation. This study proposed that BSE was worthy of a further cytotoxic study on human cancer cells. BSE constituents not only serve as novel powerful cytotoxic agents but also affect other cell lines. It is thus suggested that BSE toxicity should be further explored in normal cells, animals, and humans, and in terms of the toxicological risk assessments TI and MOS. Greater awareness of BSE utilization is necessary, and further study should be carried out in its pharmacology.

5. Ethical approval

The experimental protocol was approved by the ethics committee on animal experimentation, Nakhon Ratchasima Rajabhat University (Project code: NRRU-AE01-2562).

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7. References

[1] Lansky EP, Newman RA. Punica granatum (pomegranate) and its potential for prevention and treatment of inflammation and cancer. J Ethnopharmacol. 2007;109(2):177-206.

[2] Pandey G, Madhuri S. Some medicinal plants as natural anticancer agents. Phcog Rev. 2009;3(6):259-263.

[3] Marandi RR, Britto SJ, Ignace K. Antimicrobial and phytochemical analyses of bioactive compounds of *Butea monosperma* (Lam.). Taub. and *Butea superba* Roxb. from Jharkhand. Am J PharmTech Res. 2015;5(6):411-424.

[4] Cherdshewasart W, Nimsakul N. Clinical trial of *Butea superba*, an alternative herbal treatment for erectile dysfunction. Asian J Androl. 2003;5(3):243-246.

[5] Roengsumran S, Petsom A, Ngamrojanavanich N, Rugsilp T, Sittiwicheanwong P, Khorphueng P, et al. Flavonoid and flavonoid glycoside from *Butea superba* Roxb. and their cAMP phosphodiesterase inhibitory activity. J Sci Res Chula Univ. 2000;25(1):169-176.

[6] Ngamrojanavanich N, Loontaisong A, Pengpreecha S, Cherdshewasart W, Pornpakakul S, Pudhom K, et al. Cytotoxic constituents from *Butea superba* Roxb. J Ethnopharmacol. 2007;109(2):354-358.

[7] Chukeatirote E, Saisavoey T. Antimicrobial property and antioxidant composition of crude extracts of *Pueraria mirifica*, *Butea superba* and *Mucuna macrocarpa*. Maejo Int J Sci Technol. 2009;3(1):212-221.

[8] Mizuki D, Qi Z, Tanaka K, Fujiwara H, Ishikawa T, Higuchi Y, et al. *Butea superba*-induced amelioration of cognitive and emotional deficits in olfactory bulbectomized mice and putative mechanisms underlying its actions. J Pharmacol Sci. 2014;124(4):457-467.

[9] Eumkeb G, Tanphonkrang S, Sirichaiwetchakoon K, Hengpratom T, Naknarong W. The synergy effect of daidzein and genistein isolated from *Butea superba* Roxb. on the reproductive system of male mice. Nat Prod Res. 2017;31(6):672-675.

[10] Sunhee C, Kitisri P. The remedy pamphlet of Kwao Krua Tuber of Luang Anusarn Suntatakromkarnphiset. Chiang Mai:Chiangmai Upatipongsa Press;1931.

[11] Chavalittumrong P, Chivapat S, Punyamong S, Rattanajarasroj S, Butraporn R. Chronic toxicity of *Butea superba* Roxb. Bull Dept Med Sci. 2001;43(3):182-195.

[12] Field FJ, Born E, Mathur SN. Effect of micellar ß-sitosterol on cholesterol metabolism in CaCo-2 cells. J Lipid Res. 1997;38(2):348-360.

[13] Vashishtha A, Jehan T, Lakhanpaul S. Genetic diversity and population structure of *Butea monosperma* (Lam.) Taub. a potential medicinal legume tree. Physiol Mol Biol Plants. 2013;19(3):389-397.

[14] Singleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Meth Enzymol. 1999;299:152-178.

[15] Jia Z, Tang M, Wu J. The determination of flavonoid content in mulberry and their scavenging effects on superoxide radicals. Food Chem. 1999;64(4):555-559.

[16] Moreno CS, Plaza L, Ancos B, Cano MP. Quantitative bioactive compounds assessment and their relative contribution to the antioxidant capacity of commercial orange juices. J Sci Food Agric. 2003;83(5):430-439.

[17] Huang DJ, Chen HJ, Hou WC, Lin CD, Lin YH. Sweet potato (*Ipomoea batatas* [L.] Lam 'Tainong 57') storage root mucilage with antioxidant activities *in vitro*. Food Chem. 2006;98(4):774-781.

[18] Solis NP, Wright WC, Anderson MM, Gupta PM, Phillipson DJ. A microwell cytotoxicity assay using Artemia salina (brine shrimp). Planta Med. 1993;59(3):250-253.

[19] Finney DJ. Probits analysis. 3rd ed. Cambridge University Press; 1971.

[20] Tohsakulkaew C, Khlinsukhon T, Temcharoen P. Principles of Toxicology. 3rd ed. Faculty of Science, Mahidol University, Bangkok, Thailand. 1996. p. 21.

[21] Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods. 1989;119(2):203-210.

[22] Manasathien J, Effects of mangosteen hull extracts on bioefficacy and antiproliferation of human breast and prostate carcinoma cell lines. Suranaree J Sci Technol. 2017;24(4):475-488.

[23] Goegan P, Johnson G, Vincent R. Effects of serum protein and colloid on the alamar Blue assay in cell cultures. Toxicol in Vitro. 1995;9(3):257-266.

[24] Manakasem Y. Antioxidation in the red kwao krua (*Butea superba* Roxb.). Planta Med. 2009;75(09):75PA9.

[25] Eumkeb G, Naknarong W, Sirichaiwetchakoon K. The effects of red kwao krue (*Butea superba* Roxb.) extract on sperm quality and testosterone level in mice. Thai J Pharm Sci. 2013;38 Suppl:120-123.

[26] Cherdshewasart W, Mahapanichkul T, Boonchird C. Estrogenic and anti-estrogenic activities of the Thai traditional herbs, *Butea superba* Roxb. Biosci Biotechnol Biochem. 2010;74(11):2176-2182.

[27] Chia YY, Kanthimathi MS, Khoo KS, Rajarajeswaran J, Cheng HM, Yap WS. Antioxidant and cytotoxic activities of three species of tropical seaweeds. BMC Complement Altern Med. 2015;15(1):339-352.

[28] Meyer BN, Ferrigni NR, Putnum JE, Jacobson LB, Nichols DE, McLauglin JL. Brine shrimp: a convenient general bioassay for active plant constituents. Planta Med. 1982;45(5):31-34.

[29] Parra AL, Yhebra RS, Sardinas IG, Buela LI. Comparative study of the assay of *Artemia salina* L. and the estimate of the medium lethal dose (LD50 value) in mice, to determine oral acute toxicity of plant extracts. Phytomedicine. 2001;8(5):395-400.

[30] Krishnaraju AV, Rao TVN, Sandararaju D, Vanisree M, Tsay H, Subbaraju GV. Assessment of bioactivity of indian medicinal plants using brine shrimp (*Artemia salina*) lethality assay. Int J Appl Sci Eng. 2005;3(2):125-134.

[31] Manasathien J, Indrapichate K, Intarapichet K. Antioxidant activity and bioefficacy of pomegranate *Punica granatum* Linn. peel and seed extracts. Global J Pharmacol. 2012;6(2):131-141.

[32] Cherdshewasart W, Sutjit W, Pulcharoen K, Chulasiri M. Mutagenic and antimutagenic effects of the traditional herb used for treating erectile dysfunction, *Butea superba* Roxb. Biosci Biotechnol Biochem. 2010;74(5):923-927.

[33] Cherdshewasart W, Cheewasopit W, Picha P. The differential anti-proliferation effect of white (*Pueraria mirifica*), red (*Butea superba*), and black (*Mucuna collettii*) Kwao Krua plants on the growth of MCF-7 cells. J Ethnopharmacol. 2004;93:255-260.

[34] Mowatt JH, Dive C, Martinou JC, James D. Role of mitochondrial membrane permeabilization in apoptosis and cancer. Oncogene. 2004;23(16):2850-2860.

[35] Ashkenazi A, Amundson SA, Myers TG, Scudiero D, Kitada S, Reed JC, et al. An informatics approach identifying markers of chemosensitivity in human cancer cell lines. Cancer Res. 2000;60(21):6101-6110.