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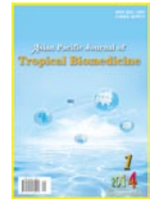

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Cytotoxic activity of kenaf (*Hibiscus cannabinus* L.) seed extract and oil against human cancer cell lines

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PEER REVIEW

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Comments

This is good work in which the author evaluated anticancer activity of KSE and KSO. The results are interesting and suggested that KSE and KSO as promising anti-cancer drugs.
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ABSTRACT

Objective: To examine the cytotoxic properties of both the kenaf (*Hibiscus cannabinus* L.) seed extract and kenaf seed oil on human cervical cancer, human breast cancer, human colon cancer and human lung cancer cell lines.
Methods: The *in vitro* cytotoxic activity of the kenaf (*Hibiscus cannabinus* L.) seed extract and kenaf seed oil on human cancer cell lines was evaluated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and sulforhodamine B assays. Cell morphological changes were observed by using an inverted light microscope.
Results: The kenaf seed extract (KSE) exhibited a lower IC_{50} than kenaf seed oil (KSO) in all of the cancer cell lines. Morphological alterations in the cell lines after KSE and KSO treatment were observed. KSE and KSO possessed effective cytotoxic activities against all the cell lines been selected.
Conclusions: KSE and KSO could be potential sources of natural anti-cancer agents. Further investigations on using kenaf seeds for anti-proliferative properties are warranted.

KEYWORDS
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sulforhodamine B assay, Human cervical cancer, *HeLa*, Human breast cancer, Human colon cancer, Human lung cancer

1. Introduction

There are many plant phenolics that appear to have preventative and treatment potential in combating cancer[1]. Despite the continuing need for effective anti-cancer agents and the association of fruit and vegetable consumption with reduced cancer risk, edible plants are increasingly considered as sources of anticancer drugs[2].

Kenaf (*Hibiscus cannabinus* L.) is a valuable fibre and medicinal plant from the family Malvaceae, which is native to India and Africa[3]. Over the last few years, kenaf plants have received significant attention in Malaysia. The conversion of the National Tobacco Board to the National Kenaf and Tobacco Board shows the Malaysian government's commitment to encouraging the development of the kenaf industry. However, the kenaf

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seed has always been treated by the kenaf industry as an agricultural waste or it has been rendered into animal feed. Members of the genus *Hibiscus* thrive in a variety of climates and produce a diverse array of interesting potential bioactive molecules, such as phenolic compounds, anti-tumour compounds, and phytosterols, with antioxidant, cardio-protective, anti-inflammatory, anti-hypertensive and anti-proliferative activities, which have been pharmacologically investigated[4]. Plants rich in natural antioxidants such as polyphenols, flavonoids are related to reduce the risk of certain types of cancer, which has led to a revival of interest in plant-based foods and drugs[5].

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sulforhodamine B (SRB) colorimetric assays were chosen to determine the cytotoxic effects of a kenaf seed extract and oil. MTT has been demonstrated to be extremely useful and was first used to study the *in vitro* effects of lymphokines and was then developed to measure chemosensitivity in human tumour cell lines[6]. The SRB assay is a popular cytotoxicity assay for addressing *in vitro* diagnostic studies, which were developed by Skehan[7]. SRB is an aminoxanthene dye that binds to basic amino acid residues in proteins through its two sulfonic groups under mild acidic conditions and dissociates under basic conditions[8]. The MTT and SRB assays are common and extensively used methods for *in vitro* anti-cancer drug screening. Both of the assays have their advantages and disadvantages. MTT has been most widely used because of the dyes that yield water-soluble products, offer added flexibility, increase sensitivity, reduce the needed steps and cost[9]. The principle advantages of the SRB assay over the MTT assay are that it is faster, provides a stable endpoint, gives better linearity with cell numbers and is less sensitive to environmental fluctuations[10]. Therefore, to ensure the reliability of the result, both assays were carried out.

The investigations and traditional uses cited above suggested that kenaf seed could be a promising source of anti-cancer agents. Furthermore, there is limited information regarding its anti-cancer effects and thus the present study was undertaken to investigate the *in vitro* anti-cancer properties of the kenaf seed extract (KSE) and oil (KSO) through MTT and SRB assays on human cancer cell lines.

2. Materials and methods

2.1. Sample

Dried kenaf (*Hibiscus cannabinus*) seed was obtained from the Malaysian Agricultural Research and Development Institute (Serdang, Malaysia) and ground into powder with a grinder (SHARP, Japan). The particle size of the kenaf seed powder was less than 1 mm.

2.2. Cancer cell lines

Human cervical cancer (*HeLa* ATCC® CCL-2™), human breast cancer (MCF-7 ATCC® HTB-22™), human colon cancer (HCT-116 ATCC® CCL-247™) and human lung cancer (SK-LU1 ATCC® HTB57™) cell lines were obtained from Aseacyte Sdn Bhd, Malaysia. *HeLa*, MCF-7 and SK-LU1 were cultured in Eagle's minimum essential medium supplemented with 1% L-glutamine, 10% foetal bovine serum, 1% penicillin streptomycin, 1% sodium pyruvate, and 1% non-essential amino acids. HCT-116 was cultured in Eagle's minimum essential medium supplemented with 1% L-glutamine, 10% foetal bovine serum, 1% penicillin streptomycin, and 1% sodium pyruvate. All of the cell lines were stored in a humidified 5% CO_2 incubator.

2.3. Methods

2.3.1. Kenaf seed extract preparation

A 500-mL Scott bottle was prepared and filled with 80% ethanol to a total volume of 500 mL. A total of 50 g of ground kenaf seeds was added to the solvent, followed by ultrasonic extraction (Ultrasonic Homogeniser Labsonic P, 400 W, Sartorius, AG) for 30 min, with a 5-minute pulse duration period and a 5-minute pulse interval period. The extraction was repeated for 3 cycles. The resulting kenaf seed extract was centrifuged at 3 500 r/m for 10 min. The supernatant of the kenaf seed extract was collected and filtered; the pellet was discarded. The filtered supernatant was subjected to rotary evaporation (Rotavapor R-200, Buchi, Switzerland).

2.3.2. Kenaf seed oil preparation

The oils were extracted from the seeds using a Soxhlet extractor with hexane at 60 °C for 8 h, according to methods described by Ng[11]. The oil was then recovered by evaporating the solvent using rotary evaporator Model N-1 (Eyela, Tokyo Rikakikai Co., Ltd., Japan), and the residual solvent was removed by flushing with 99.9% nitrogen.

2.3.3. Cell preparation for assays

Cells were plated in 96-well plates (5×10^4 cells/well) for seeding. After 24 h of cell seeding, a partial monolayer was formed and the optimum cell confluence of 70% was achieved. The 96-well microtiter plates were treated with different concentrations of KSE. About 0.1 mL of well mixed KSE solution (31.25 μg/mL to 2 000 μg/mL) was added into the 96-well plates. Therefore, the final KSE

concentration was 2-fold diluted which range from 15.625 μg/mL to 1 000 μg/mL. Negative control was set as treated the cells with 0.9% of saline. Minimum essential medium was included as the blank control. All of the medium concentration was prepared in duplicate. The plates were incubated at 37 °C for 72 h before the cytotoxicity test is carried out.

2.3.4. MTT

An MTT assay was performed according to a slight modification of the procedure reported by Patel[12]. At the end of the 72 h incubation, the medium in each well was removed, and 50 μL of MTT solution was added. The plates were gently shaken and incubated for 4 h at 37 °C in a 5% incubator. Then, the medium with MTT was removed from the wells. The remaining formazan was dissolved by adding 0.2 mL of dimethylsulfoxide. The solution was pipetted up and down to dissolve the crystals. An enzyme-linked immuno sorbent assay plate reader (Biotek, United State) was used to determine the absorbance at 570 nm with a reference of 630 nm. The percentage growth inhibition was calculated using the formula as follows:

% cell inhibition=100 − [(At − Ab)/Ac−Ab] × 100

Where, At=Absorbance value of the test compound; Ab=Absorbance value of blank; Ac=Absorbance value of control.

The percentage of cell inhibition against the concentration of test compounds was plotted. The half maximal inhibition concentration (IC_{50}) was calculated based on the equation in the plotted graph.

2.3.5. SRB's colorimetric assay

An SRB assay was performed according to a slight modification of the procedure reported by Houghton and his co-workers[13]. Following the 72 h treatment, cytotoxicity was evaluated using the SRB assay. Twenty-five microlitres of 50% trichloroacetic acid was gently added to the wells so that it formed a thin layer over the test compounds to reach an overall concentration of 10%. The plates were incubated at 4 °C for 1 h. The plates were flicked and washed five times with tap water to remove traces of medium, sample and serum and were then air-dried. The air-dried plates were stained with 100 μL SRB and kept for 30 min at room temperature. The unbound dye was rapidly removed by washing four times with 1% acetic acid. The plates were then air-dried; 100 μL of 10 mmol/L Tris base was added to the wells to solubilise the dye. The plates were shaken vigorously for 5 min. The absorbance was measured using an -linked immuno sorbent assay plate reader (Biotek, United State) at a wavelength of 515 nm. The percentage growth inhibition was calculated using the following formula:

% cell inhibition=100 − [(At − Ab)/Ac−Ab] × 100

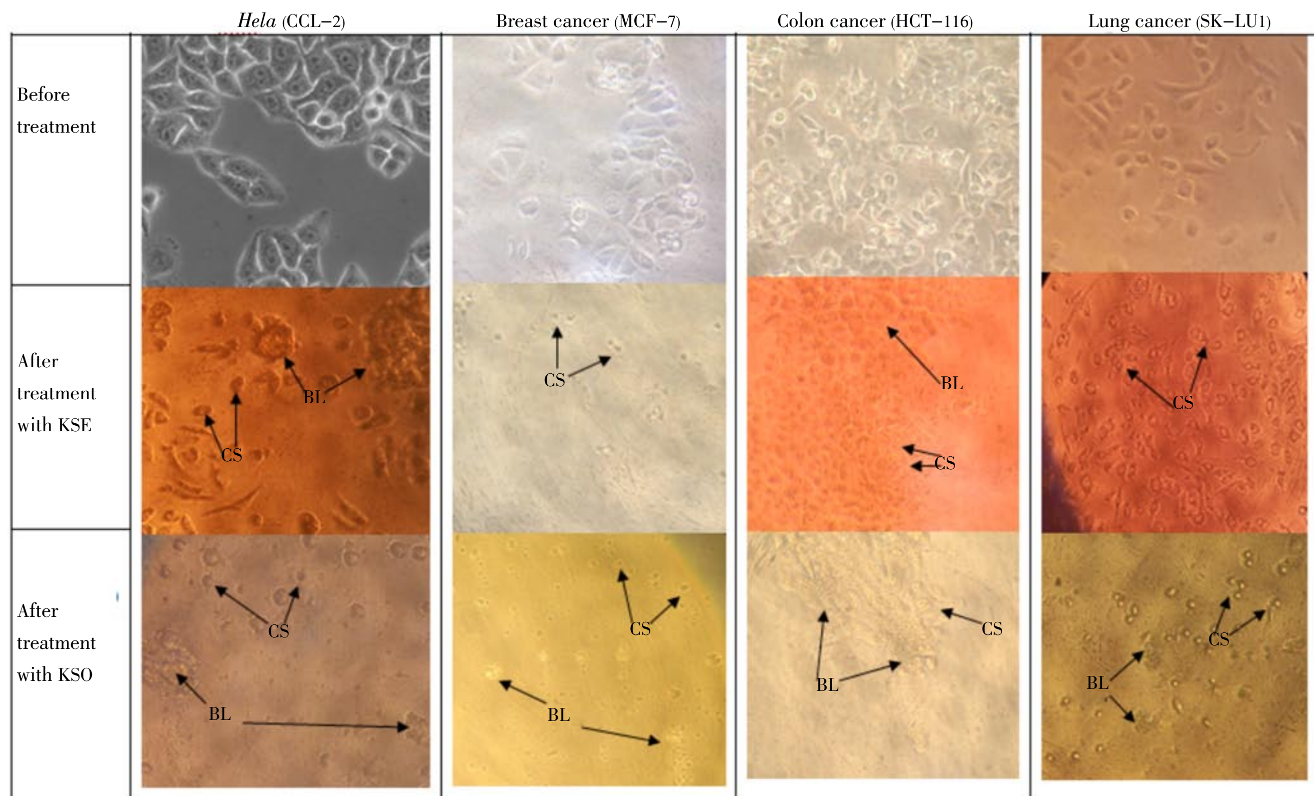

Figure 1. Morphological changes of *HeLa* (CCL-2), breast cancer (MCF-7), colon cancer (HCT-116) and lung cancer (SK-LU1) treated with the KSE and KSO for 72 h.

CS: Cellular shrinkage; BL: Membrane blebbing (Magnification for *Hela* 40×; magnification for MCF-7, HCT-116 and SK-LU1 10×).

Where, At=Absorbance value of the test compound; Ab=Absorbance value of blank; Ac=Absorbance value of control.

The percentage of cell inhibition against the test compound concentration was plotted. The half maximal inhibition concentration (IC_{50}) was calculated based on the equation in the plotted graph.

2.4. Statistical analysis

All experiments were performed in duplicate and measurements were replicated two times (n=4). An analysis of variance was performed, and the average values were compared with Fisher's Multiple Comparison Test. Differences were considered statistically significant at P<0.05. All statistical analyses were performed using Minitab 16 for Windows.

3. Results

Modifications in the morphology of *HeLa*, MCF-7, HCT-116, and SK-LU1 after KSE and KSO treatment at 72 h were observed (Figure 1). *HeLa* had an epithelial-like morphology. Viable round cells might be present in rising numbers as the cell density increases. Similarly, adherent SK-LU1 and HCT-116 cancer cells retained an epithelial-like morphology. This type of lung cancer cells resembled the elongated shape of *HeLa* cancer cells. MCF-7 had a cobblestone-like phenotype with strong cell-cell adhesion. However, when all four cancer cells were exposed to cytotoxic components, two distinct modes of cell death were recognised, namely, apoptosis or necrosis. The majority of cells treated with KSE and KSO showed features of apoptosis such as cellular shrinkage, membrane blebbing and apoptotic body formation as viewed under an inverted light microscope. Figure 1 shows that both KSE and KSO caused the shrinkage and blebbing of cell membranes from *HeLa* and HCT-116 after 72 h of treatment. On the other hand, most of the MCF-7 and SK-LU1 membranes blebbed during shrinkage, and the apoptotic bodies were formed around cells after treating them with KSE and KSO.

From Table 1 and 2, KSE showed strongest cytotoxicity activity toward *Hela* followed by MCF-7, HCT-116 and lastly SK-LU1 while KSO showed strongest cytotoxic activity toward HCT-116, followed by MCF-7, SK-LU1 and lastly *HeLa*. Results in MTT assay were similar with the SRB assay (Figure 2).

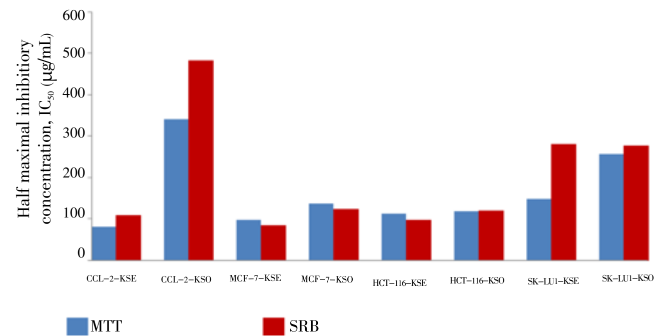

Figure 2. IC_{50} of *HeLa* (CCL-2), breast cancer (MCF-7), colon cancer (HCT-116) and lung cancer (SK-LU1) in the KSE and KSO by MTT and SRB assays.

Table 1

Cell inhibition (%) of *HeLa* (CCL-2), breast cancer (MCF-7), colon cancer (HCT-116) and lung cancer (SK-LU1) in the KSE and KSO by MTT assay.

Concentration	CCL-2 (%)		MCF-7 (%)		HCT-116 (%)		SK-LU1 (%)	
(μg/mL)	KSE	KSO	KSE	KSO	KSE	KSO	KSE	KSO
1 000	80.73±1.16^{b}	73.88±0.99^{b}	100.00±0.00^{a}	97.19±1.08^{b}	94.69±2.60^{a}	98.14±1.54^{a}	89.11±3.52^{a}	76.76±1.48^{b}
500	76.14±3.19^{a}	54.67±0.34^{b}	94.87±2.12^{a}	86.53±4.04^{b}	77.92±2.41^{a}	69.75±1.57^{b}	71.96±3.35^{a}	65.00±0.59^{b}
250	70.96±1.81^{a}	40.84±2.18^{b}	88.99±2.58^{a}	57.86±2.34^{b}	71.01±1.88^{a}	61.46±1.07^{b}	59.24±2.98^{a}	49.12±1.76^{b}
125	58.29±3.19^{a}	32.87±1.17^{b}	68.78±4.21^{a}	37.49±1.85^{b}	65.03±2.12^{a}	50.50±1.92^{b}	43.79±2.31^{a}	38.82±4.80^{b}
62.5	50.64±3.02^{a}	17.30±0.93^{b}	21.82±2.14^{b}	26.83±1.90^{a}	27.95±2.72^{b}	35.78±1.46^{a}	30.16±2.57^{a}	13.82±2.01^{b}
31.25	46.16±2.09^{a}	10.03±1.18^{b}	13.17±2.42^{b}	21.27±1.99^{a}	20.45±1.46^{b}	26.63±1.36^{a}	20.49±1.87^{a}	7.35±1.13^{b}
15.625	11.48±1.39^{a}	4.50±0.90^{b}	8.51±1.26^{a}	8.77±2.22^{a}	8.65±2.00^{b}	15.61±1.17^{a}	12.31±1.26^{a}	2.35±0.96^{b}

Each value is presented in means±standard deviation (n=4). Values within a row with different superscripts for each cancer cell line are significantly different (P<0.05).

Table 2

Cell inhibition (%) of *HeLa* (CCL-2), breast cancer (MCF-7), colon cancer (HCT-116) and lung cancer (SK-LU1) in the KSE and KSO by SRB assay.

Concentration	CCL-2 (%)		MCF-7 (%)		HCT-116 (%)		SK-LU1 (%)	
(μg/mL)	KSE	KSO	KSE	KSO	KSE	KSO	KSE	KSO
1000	84.47±0.60^{a}	66.71±0.71^{b}	96.70±0.89^{b}	98.64±0.91^{a}	99.39±0.24^{a}	97.85±0.80^{b}	78.14±3.91^{a}	69.54±0.30^{b}
500	74.78±2.69^{a}	48.89±1.81^{b}	89.74±1.24^{b}	93.94±1.56^{a}	82.22±0.20^{b}	87.08±1.67^{a}	62.55±2.62^{a}	53.12±0.22^{b}
250	68.54±2.46^{a}	37.30±0.13^{b}	85.92±1.00^{a}	68.03±1.67^{b}	73.33±0.63^{a}	61.81±1.31^{b}	39.30±1.99^{b}	47.70±0.30^{b}
125	61.51±4.53^{a}	30.31±2.75^{b}	76.32±0.62^{a}	45.61±1.81^{b}	52.21±0.65^{a}	53.38±1.54^{a}	36.94±1.82^{a}	34.92±0.36^{a}
62.5	42.44±2.54^{a}	22.05±2.54^{b}	43.87±1.39^{a}	27.12±1.25^{b}	39.27±1.49^{a}	34.14±1.49^{b}	33.37±2.99^{a}	33.32±0.30^{a}
31.25	22.48±1.56^{a}	20.21±2.52^{a}	13.42±1.31^{a}	10.91±1.11^{b}	23.85±1.24^{a}	13.11±0.81^{b}	29.39±1.33^{a}	30.20±0.38^{a}
15.625	13.45±1.38^{b}	18.01±0.22^{a}	7.96±1.33^{a}	7.88±0.86^{a}	14.73±1.69^{a}	9.95±1.51^{b}	27.58±1.39^{a}	28.21±0.17^{a}

Each value is presented in means±standard deviation (n=4). Values within a row with different superscripts for each cancer cell line are significantly different (P<0.05).

4. Discussion

Plants have a nearly unlimited capacity to generate compounds that fascinate researchers in the quest for new and novel chemotherapeutics[14]. The persistent search for new anti-cancer compounds in plant medicines and traditional food is a realistic and promising strategy for its prevention[15]. Therefore, the KSE and KSO were evaluated by using MTT and SRB assays.

Paraskeva indicated that under similar experimental conditions and within the limits of the applied data analyses[16], the MTT and SRB assays generally yielded similar results. Manosroi and his co-workers suggested that sample that had IC_{50} value of less than 125 μg/mL could be a possible candidate for further development to cancer therapeutic agent and a test component with IC_{50} value between 125 and 5 000 μg/mL was considered to have moderate potential to be developed into a cancer therapeutic agent[17]. Thus, KSE and KSO could be a potential source for the treatment components of cancer. To date, there was no reported research on cytotoxic properties of KSE. From Yazan *et al.* research[18], KSO extracted by a supercritical carbon dioxide fluid extractor was believed to possess anti-cancer activities against MCF-7 (MDA-MB-231, 4T1), *HeLa*, lung cancer (A549) and leukaemic (MOLT-4) cell lines. Their KSO had a higher IC_{50} towards MCF-7 and *HeLa* compared to our findings. This result may be attributed to the denaturation of some of the heat sensitive compounds that exist in KSO because of the higher extraction temperature used for the Soxhlet extraction.

KSO contains various active compounds, such as fatty acids, phenolic acids, phytosterols and tocopherols[15,17]. According to Nyam[19], there were seven main phenolic compounds identified in the KSO, namely, gallic acid, *p*-hydroxybenzoic acid, caffeic acid, vanillic acid, syringic acid, and *p*-coumaric and ferulic acids. In addition, a preliminary study on KSE also showed that it contained a relatively high phenolic content. Many studies conducted on cell cultures and animal models indicated that polyphenols are the main phytochemicals with antioxidant and anti-proliferative properties from higher plants[20]. These molecules may act as cancer-blocking agents, preventing the initiation of the carcinogenic process as cancer-suppressing agents and inhibiting cancer promotion and progression[21]. In our preliminary study, KSE contained a higher phenolic content than KSO, which coincides with its cytotoxic activity. This finding showed that phenolic compounds in kenaf seed are responsible for cytotoxic properties.

5. Conclusions

This study provided evidence showing that KSE possessed effective cytotoxic activities against *HeLa*, MCF-7 and HCT-116, while KSO was effective to MCF-7 and HCT-116. KSE demonstrated a greater cytotoxic activity relative to KSO. These properties were likely to be related to the phenolic constituents. This study suggested that KSE and KSO may be used as inexpensive and easily accessible sources of potential natural anti-cancer agents. Further investigations on a larger number of cancer cell lines and *in vivo* studies should be conducted to investigate the possibility of developing the kenaf plants as promising anti-cancer drugs.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

The present study was undertaken to investigate the *in vitro* anti-cancer properties of the KSE and KSO through MTT and SRB assays on human cancer cell lines.

Research frontiers

The reserchers report on anticancer activity of KSE and KSO which will lead to identification of natural products based anticancer agents.

Related reports

Abd Ghafar *et al.* (2013) recently reported on cytotoxic activity of KSO from supercritical carbon dioxide fluid extraction towards human colorectal cancer (HT29) cell lines. They found that KSO-SFE at 600/40 showed the strongest cytotoxicity towards HT29 with IC_{50} of 200 μg/mL. The IC_{50} for NIH/3T3 was not detected even at highest concentration employed.

Innovations and breakthroughs

This study provided evidence showing that KSE possessed effective cytotoxic activities against *HeLa*, MCF-7 and HCT-116, while KSO was effective to MCF-7 and HCT-116. KSE demonstrated a greater cytotoxic activity relative to KSO. This study also suggested that KSE and KSO may be used as inexpensive and easily accessible sources of potential natural anti-cancer agents.

Applications

The finding from this study could lead to the isolation and identification of anticancer phytochemicals from KSE and KSO which could be used to develop anticancer agents.

Peer review

This is good work in which the author evaluated anticancer activity of KSE and KSO. The results are interesting and suggested that KSE and KSO as promising anti-cancer drugs.

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