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IN VITRO CYTOTOXIC ACTIVITY OF *Ferula assafoetida* ON OSTEOSARCOMA CELL LINE (HOS CRL)

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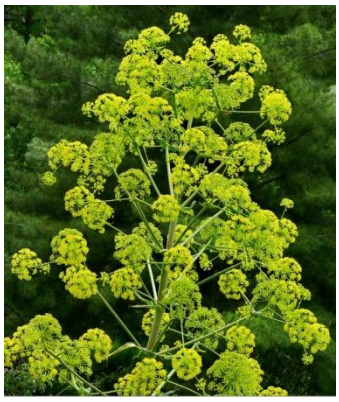
Graphical abstract



Abstract

Ferula assafoetida is a herb that has been used traditionally to treat rheumatism, broken bones and stomach illness. The aim of this research is to study the cytotoxicity of *F. assafoetida* as an anti-cancer agent. Briefly, resin of *F. assafoetida* was macerated with ethanol or methanol for 3 days. The mixtures were then filtered, concentrated and dried. The cytotoxicity of extracts on an osteosarcoma cell line, HOS CRL, was determined using MTT assay method. The results were analysed with Tukey's test ($p<0.05$) showed that F. *assafoetida extract* has cytotoxic effect which is dependent on the type of solvent (methanol > ethanol) and concentration (higher methanolic content > lower methanolic content). In conclusion, extracts of *Ferula assafoetida* might possess cytotoxic activity which could be useful to be developed for an anti-cancer treatment.

Keywords: *Ferula assafoetida*, cytotoxicity, anti-cancer

Abstrak

Ferula assafoetida adalah herba yang telah digunakan secara tradisi untuk merawat penyakit sendi, patah tulang dan sakit perut. Matlamat kajian ini adalah untuk mengkaji ketosikan *F. assafoetida* sebagai agen anti-kanser. Secara ringkas, resin daripada *F. assafoetida* telah direndam dengan etanol atau methanol selama 3 hari. Campuran kemudiannya dituras, dipekatkan dan dikeringkan. Ketoksikan setiap ekstrak pada sel osteosarcoma, HOS CRL, telah ditentukan menggunakan asai MTT. Keputusan yang dianalisis dengan ujian Tukey ($p<0.05$) menunjukkan bahawa ekstrak *F. assafoetida* mempunyai kesan sitotoksik yang dipengaruhi oleh jenis pelarut (methanol >etanol) dan kepekatan (kandungan methanol lebihtinggi > kandungan methanol lebih rendah). Kesimpulannya, ekstrak *F. assafoetida* mungkin mempunyai aktiviti sitotoksik yang amat berguna untuk dibangunkan sebagai rawatan anti-kanser.

Kata kunci: *Ferula assafoetida*, sitotoksik, anti-kanser

1.0 INTRODUCTION

The increased use of naturally occurring sources of medicine is generally due to perceived benefits and the assumption that it carries less side-effect [1]. Locally, active screening for new drug candidates with better efficacy, such that were carried out by various agencies and institutions in Malaysia on the widely used medicinal plants, further accentuates the image of natural products. At the same time, some of the synthetic medicines were shown to be of lesser potency than they were originally thought of or less intolerable by the body and caused significant side-effects, and this drives further the need to find a better alternative. On the international scene, influential bodies such as U.S. National Cancer Institute had been active in screening new anticancer agents from nature, in transcontinental collaboration with agencies in Asia and Africa [2].

Ferula assafoetida is an example of plant that has been used as medicine through many centuries and across many cultures. It is widely cultivated in India, Afghanistan and Iran where it is used in various traditional foods as a spice and also as a traditional healing agent [3]. In Iranian traditional medicine, *F. assafoetida* is used to treat abdominal pain, constipation, diarrhea, parasitic infection, and upper respiratory disease especially asthma and cough [4, 5]. In Malaysia, its gum is chewed by females for amenorrhea while in China the decoction of the plant is taken orally as a vermifuge [6]. In Saudi Arabia, its dried gum is used medicinally for whooping cough, asthma and bronchitis [6]. The use of *F. assafoetida* even reaches the countries of the South American continents such as Brazil, where hot water extract of the dried gum is taken orally by males as an aphrodisiac [7]. Thus far, *F. assafoetida* has been shown to contain several medicinal properties including anticonvulsant, antispasmodic, aromatic carminative, digestive, diuretic, expectorant, tonic, alterative, antiperiodic, deobstruent, deodorant, circulatory stimulant, treatment of stomach ache and indigestion, bronchitis, bronchial asthma, whooping cough, laxative, sedative, nervine, analgesic, antihelminthic, aphrodisiac and antiseptic properties [8, 9].

The possibility of *F. assafoetida* as an anti-cancer effect could be seen in an epidemiological study which shows a correlation between the use of *F. assafoetida* and the incidence of cancer in selected countries [3]. The data showed that the rate of cancer is quite high in countries like Japan, Russia, China and Indonesia where the *F. assafoetida* usage in not common rather than in countries which cultivate *F. assafoetida* such as Pakistan, Afghanistan and Iran [3].

Many studies were conducted on the cytotoxic activity of *F. assafoetida* on cancer cell lines such as human breast adenocarcinoma (MCF-7), human hepatocellular carcinoma (HePG-2), non-small cell lung carcinoma (A-549) human colorectal adenocarcinoma (HT-29) and human cervical carcinoma (HeLa) [9,10]. However, no study had been performed on bone cancers such as osteosarcoma.

Thus, the purpose of this study is to evaluate cytotoxic activity of *F. asafoetida*extracts in HOS ATCC® CRL-1543™ cell line using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay method and to compare the differences in cytotoxic activity between absolute ethanol and absolute methanol extracts of *F. assafoetida*.

2.0 EXPERIMENTAL

2.1 Sample Collection

The resin of *F. assafoetida* was purchased from an Indian shop in Kuantan, Pahang. The osteosarcoma cell line, HOS ATCC® CRL-1543™ was obtained from a female Caucasian bone tissue with osteosarcoma. The cell line was purchased from American Type Cell Collection (ATCC), USA.

2.2 Preparation of Extract

The resin was cut with a knife and crushed into small pieces by using a blender. Then, the resin (516.1 g) was soaked in absolute ethanol (550 mL) and extracted by cold maceration method. The resin was macerated in ethanol for 3 days, left on the shaker at room temperature. Afterwards, the ethanolic resin was filtered using 101 Double Rings filter paper. The dilute liquid extract was concentrated with a rotary-evaporator to obtain a viscous liquid. This liquid was finally dried in the dryer for one day to remove the remaining traces of solvent. These procedures were also employed for methanolic extraction using the resin (558.6 g) and absolute methanol (550 mL). The yields of ethanolic and methanolic extracts were 0.752% w/w and 2.390% w/w respectively.

2.3 Preparation of Medium

The medium used to culture HOS ATCC® CRL-1543™ was Eagle's Minimum Essential Medium (EMEM), supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin. This medium is known as complete medium and was pre-warmed at 37°C prior to use. The preparation and cells handling was carried out in a sterile condition.

2.4 Culturing Cells

The cells were cultivated according to method outlined by Nemati *et al*. [11]. Briefly, cryopreserved cells were thawed rapidly and plated at high density to optimize recovery. The cryopreserved vial was removed from the liquid nitrogen freezer and left to agitate continuously for one or two minutes. The thawed cell suspension was transferred into sterile centrifuge tubes containing 2 mL warm complete medium and centrifuged for 10 minutes at 1500 rpm. The supernatant was removed and the cell pellet was

re-suspended in complete medium and before being transferred into 25 cm^2 tissue culture flask. The culture medium was changed every 48 hours.

2.5 Subculturing Cells

The method by Nemati *et al.* [11] with slight modification was used in subculturing the cells. HOS ATCC® CRL-1543™ was grown in a monolayer culture in 25 cm^2 tissue culture flasks with 5 mL of Eagle's Minimal Essential Medium (EMEM) and supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin. The cell was maintained at 37°C (RH 95%), with 5% CO_2. The culture medium was changed every 48 hours. For routine weekly maintenance, the cells were sub-cultured at confluence. The medium was removed and cells were detached from the wall of the flask using enzymatic degradation using 1 minute incubation at 37°C in trypsin-EDTA. Then the suspension of the cells was poured into a sterile 20 mL tube and cells were pelleted at 1500 rpm for 5 minutes in a centrifuge. The supernatant was poured out and the cells were re-suspended in 5 mL of fresh medium. After separating the cells by passage, the appropriate volume of cells were pipetted into fresh medium in a flask.

2.6 Cells Treatment

The method explained by Freshney [12] was modified for cell treatment. The cells were seeded into 96-well plates for 24 hours or until confluence. The cell line was treated with sets of extract concentrates at 0.5, 1, 1.5, 2, 2.5, 5, 10 and 20 mg/mL.

These concentrations were prepared for ethanol and methanol extracts by serial dilution. The concentration of stock solution was prepared at 10% (w/v). Dimethyl sulfoxide (DMSO) 0.5% was used as negative control. A well containing cells and media-only is used as neutral control and another media-containing well is used as a blank. The cells were observed for the following 24 and 48 hours under the inverted microscope and changes in the behaviour and morphology of the cells were noted and compared with established data.

2.7 Cells Viability and Proliferation

Cell viability and proliferation was assessed with 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bro- mide (MTT) assay. The method outlined by Hamzeloomoghadam *et al.*, [8] with slight modification was used. After 24 hours of incubation of the cells at 37°C, the medium was replaced with fresh medium containing different concentrations of the extracts. The cells were exposed to each sample for 24 hours and 48 hours at 37°C. Then, the medium was replaced with MTT and incubated for another 4 hours in a CO_2 incubator at 37°C. At the end of the incubation period, the medium containing MTT was removed and remaining formazan crystals were dissolved with DMSO. The formation of formazan crystals is indicative of cell viability and this was determined by reading the absorbance using Tecan microtitre plate reader at 570 nm wavelength with reference at 630 nm.

The relative cell viability of neutral control was calculated by the following formula:

$$\frac{[E-B]}{[N-B]} \times 100\%$$

where:
E = the average absorbance of extract
B = the average absorbance of blank
N = the average absorbance of negative control (DMSO)

3.0 RESULTS AND DISCUSSION

The study evaluates *in vitro* cytotoxic activity of *F. assafoetida* on HOS ATCC® CRL-1543™ cell line; and compare the differences between absolute ethanol and methanol extracts of *F. assafoetida* in terms of their cytotoxic effects at different time-points, 24 and 48 hours. The percentage of HOS cell viability with concentrations of ethanol and methanol extracts at different time-point, 24 and 48 hours were tabulated in Table 1. It shows that in the methanol extract, cell viability does not change much over time, and the higher concentration shows stronger cytotoxic property (Figure 1). In comparison, in the ethanol the viability of cells in general, is much higher than the methanol extract (Table 1), with the higher concentrations showed better cytotoxic property than the lower concentrations (Figure 2).

Table 1 Percentage of HOS cell viability with concentrations of ethanol(EtOH) and methanol (MeOH) extracts at time-points 24- and 48-hours. (+) denotes neutral control

Concentration (mg/mL)	Cell viability (%)			
	24 hours		48 hours	
	EtOH 24 hrs	MeOH 24 hrs	EtOH 48 hrs	MeOH 48 hrs
0+	100	100	100	100
0.5	913	131.3	276.1	137.6
1	556.6	130.6	253.1	142.7
1.5	454.1	124.9	234.2	139.2
2	167.1	108.2	187.7	116.3
2.5	93.15	87.2	69.64	40.56
5	79.9	39.9	66.4	9.3
10	40.9	32	53.4	20.7
20	38.8	63.3	29.5	35.2

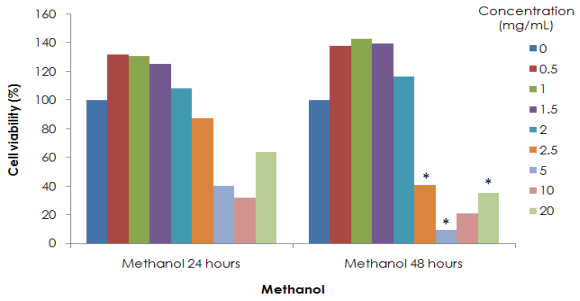


Figure 1 Percentange of cell viability (%) with different concentration of methanol extract (mg/mL). * indicates significant difference at 48 hours in comparison to 24 hours reading, p<0.05 using Tukey's test

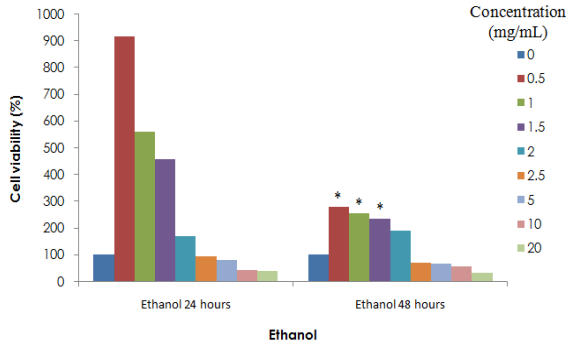


Figure 2 Percentange of cell viability (%) with different concentration of ethanol extract (mg/mL). * indicates significant difference at 48 hours in comparison to 24 hours reading, p<0.05 using Tukey's test

These findings demonstrate that *F. assafoetida* methanol extracts have cytotoxic effect against the tumor cell line. The cytotoxicity of *F. assafoetida* methanol extract depends on the concentration of the extract, whereby high concentration of extract from 5 mg/mL and above can cause lower percentage of cell viability (Figure 1).

Reviewing the literature, we found that other studies also found differences in the cytotoxic property of *Ferula* spp. extract depending on the type and concentration of the extracts. Mishra and Behal [14] who used 2.5, 5, 10 and 20 mg/mL concentrations of *F. assafoetida* extract on human breast adenocarcinoma cell line (MCF) and HEP-G2 cancer lines found a significant decrease in both cells population as the concentration is increased. Other factors may also come into play, such as the type of sampling from the plant may influence cytotoxic level. For instance, Sahranavard *et al.*,[15] who used methanolic extract of *F. szowitsiana* root and tested for the cytotoxic activities against three cancer cell lines (MCF7, HepG2, WEHI164) and one normal cell line (MDBK) found them cytotoxic in all cell lines.

The cytotoxicity of *F. assafoetida* may be due to the high amount of essential oils as essential oils are usually toxic for biological systems [8]. The study done by Kuete *et al.*, Mazzio and Soliman [16,17] supported the claim that the essential oils of different *Ferula* species exhibited cytotoxic effect against human tumor cell lines. *F. assafoetida* is also known for its bioactive secondary metabolites like coumarins, phenylpropanoids, sesquiterpenes and disulphide compounds. The cytotoxic activities of stylosin (a monosterpene extracted from *F. ovina*) and mogoltacin (a sesquiterpene coumarin from *F. badrakema*) showed that they also might have cytotoxic properties against tumor cell line by inducing DNA lesions and increasing apoptosis of cells [18]. This toxicity is probably related to the presence of sesquiterpene prenylated coumarin derivative ferulenol, which showed antibacterial properties and cytotoxicity toward human tumor cell lines [8]. The resin was obtained from the roots part, where most of the sesquiterpene coumarin were stored [19]. These sesquiterpene coumarins may be considered as potential natural compounds for the treatment of malignancies [20]. *F. assafoetida* also have been reported to contain ferulic acid and farnesiferols which can prevent vascular endothelial growth factor-initiated processes, angiogenesis and the progression of mouse Lewis lung cancer in mice [17].

In this study, the methanol extract is more cytotoxic compared to the ethanol extract. The yields of extract of *F. assafoetida* resin using ethanol (516.1 g) and methanol (558.6 g) extractions were 0.752% and 2.390% w/w, respectively. These results were in line with previous study by Sultana *et al.,* [21] in which the data showed that the absolute methanol have greater yield than ethanol. The research done by Unnikrishnan and Kuttan [22] which used both aqueous and alcoholic extracts tested for *in vitro* cytotoxicity study. The result showed that alcoholic extracts of the species were found to be more cytotoxic to these cells than their aqueous extracts. In addition, alcoholic extracts have been shown to enhance inhibitory action than aqueous extract, and this could be due to the polyphenolic and flavonoid contents which are known to have antioxidant and chemopreventive actions [12].

4.0 CONCLUSION

This is the first study where *F. assafoetida* has been used to evaluate for the cytotoxic activity on osteosarcoma cell line (HOS). From the findings, it is suggested that *F. assafoetida* has a dose- and extraction-dependent cytotoxic activity. The methanol extract possess better cytotoxic effect than the ethanol extract, and extraction with higher concentration of methanol has better cytotoxicity than the lower concentration. However, further studies should be focused on identification of the suitable concentration and bioactive compound that is responsible for the cytotoxic activity. Other extracting methods can also be used for optimization of

cytotoxic effect of *F. assafoetida,* possibly in comparison to the methanol extract.

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