

Antioxidant activity and estragole content of ethanolic and methanolic extract of fennel (*Foeniculum vulgare* mill.) and its risk assessment using Margin of Exposure (MOE)

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Abstract

Fennel (*Foeniculum vulgare* Mill.) has been used as traditional medicines, although conversely, it also contains genotoxic and carcinogenic compounds an alkenylbenzene of estragole. Therefore, fennel should be a health risk evaluation. The aims of this study were to analyze the total phenolic content (TPC), total flavonoid (TFC) and the concentration of alkenylbenzene of estragole in ethanolic and methanolic extract of fennel (*Foeniculum vulgare* Mill.). Furthermore, the concentration of estragole in the extract was used to make relation between intakes of extract and the resulted Margin of Exposure (MOEs). The results showed that TPC, TFC and capacity of antioxidant of methanolic extract of fennel were higher than that of ethanolic extract. In simulation, the intake of methanolic and ethanolic extracts of fennel which were > 168-331 and > 49-97 mg/60 kg bw per day for life time, respectively, resulted a MOE < 10,000. Therefore, it was considered as a high priority for risk management actions and would be of high concern from a public health point of view.

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Introduction

The use of herbal medicinal plants extract for health care are widely accepted in the world. Some botanicals are consumed to maintain and promote health or reduce risk of diseases. Plant derived products are generally recognized by consumers as safe, but some botanicals give adverse effects on human health due to naturally occurring genotoxic and carcinogenic compounds (Rietjens *et al.*, 2008; Berg *et al.*, 2011; Moreira *et al.*, 2014). Study Berg *et al.* (2011) showed that there were 30 botanical ingredients that are genotoxic and/or carcinogenic causing a possible risk for human health. Most of these compounds are a member of the group of alkenylbenzenes or unsaturated pyrrolizidine alkaloids.

Fennel (*Foeniculum vulgare* Mill.) has been used as traditional medicines, flavoring agents, food supplement and food preservation for many years. Fennel is used for dyspeptic complaints, bloating and flatulence, infantile colic, primary dysmenorrhea (EMA, 2008) and it is traditionally added in tea consumed in some countries in Europe including

France, Germany, Austria, Czech Republic and Poland (van den Berg *et al.*, 2014). Hard capsules of powdered sweet fennel are permitted used in France since 1990 for treatment of digestive upsets and essential oil of bitter fennel fruit is permitted marketed as a syrup in Germany since 1978 for the relief of symptoms in coughs and colds (EMA, 2008). Fennel contains volatile compounds, phenolic compounds and flavonoid which are related to the prevention of diseases induced by oxidative stress, such as cardiovascular diseases, cancer, and inflammation (Badgular *et al.*, 2014). However, essential oil of fennel contains an alkenylbenzene estragole (1-allyl-4-methoxybenzene) of 5.45% (Anwar *et al.*, 2009) that has already demonstrated to be genotoxic and hepatocarcinogenic in rodents at high doses (SCF, 2001). The metabolism of estragole in the liver produces 1'-hydroxy estragole which is furthermore bioactivated to 1'-sulfooxyestragole mediated by sulfotransferase. The 1' -sulfooxyestragole can bind covalently to DNA resulting to DNA adduct formation (Phillips *et al.*, 1981). Therefore, high intake of fennel containing estragole may pose a potential risk for human. In some countries, the

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using of alkenylbenzenes estragole, methyleugenol, safrole and β -asarone as flavorings in food products is regulated and prohibited, while, some plant food supplements containing these ingredients are not regulated (Berg *et al.*, 2011).

Extraction of botanicals is one step to prepare food supplements (EFSA, 2009). Solvent extraction method was selected to maximize the targeted bioactive compounds but to minimize the unwanted other compounds (Liu, 2008). The chemical composition of the botanical extract can be different due to the different extraction methods (Liu, 2008). Moreover, the extraction solvent may significantly alter the antioxidant activity (Zhou and Yu, 2004). The extracts obtained are preferably to contain more bioactive compounds that are good for health but minimize or even eliminate genotoxic and/or carcinogenic compounds. Methanol is recognized as a good solvent for botanicals extraction giving high extract yield however due to some safety and health issues ethanol is considered as a safer replacement solvent. Some research have been conducted to obtain fennel extract and/or its essential oil using various solvent and methods of extraction which furthermore were measured the total phenol, total flavonoid, antioxidant capacity and the essential oil content (Parejo *et al.*, 2004; Faudale *et al.*, 2008; Anwar *et al.*, 2009; Angelov and Boyadzhieva, 2016). Extract water of distilled fennel contains forty-two phenolic substances in total, 27 compounds had not been reported before including hydroxycinnamic acid derivatives, flavonoid glycosides, and flavonoid aglycons (Parejo *et al.*, 2004). Whereas, nonpolar fraction of fennel contains fatty acids and essential oil of trans-anethole and estragole (Parejo *et al.*, 2002; Burkhardt *et al.*, 2015; Coşge *et al.*, 2008). Extraction of fennel to obtain polar and non-polar compounds was performed by fractionation (Parejo *et al.*, 2002). Total phenolic compounds of non-distilled fennel decreased by following order: crude extract of methanolic > hexane > aqueous (Parejo *et al.*, 2002).

The expert groups of EFSA, the Joint FAO/WHO expert committee on Food Additives (JECFA) and the International Life Sciences Institute (ILSI) recommends a Margin of Exposure (MOE) approach to do risk assessment of genotoxic carcinogens in food (O'Brien *et al.*, 2006). The MOE approach uses a reference point which is taken from an animal experiment and corresponding to a dose that causes a low but measurable response in animals (EFSA, 2005). EFSA (2005) recommends BMDL10 as reference point which is defined as the lower 5% confidence bound on a dose resulting in a 10%

increase in tumour incidence above background, derived by fitting a mathematical model to the experimental potency data. This reference point is divided by estimated daily intake in human to obtain MOE. An MOE of 10,000 or lower is considered as high priority for risk management actions and would be of high concern from public health point of view (EFSA, 2005).

There were many researches of extracting bioactive compounds in fennel using various types of extraction methods and solvents but none of them evaluated the content of genotoxic and carcinogenic of estragole. However, the presence of estragole should be measured to consider safety intake of the fennel extract. The information of this research is necessary to determine the dose of fennel extract administered to the animal by considering genotoxic and carcinogenic compounds. This study used the most utilized solvent of methanol and ethanol as a model of extraction. The aims of this study were to know the total phenolic content (TPC), total flavonoid (TFC), antioxidant capacity and estragole content in ethanolic and methanolic extract of fennel and to do simulation between intake of fennel extract and the MOEs.

Materials and methods

Materials

Dried fennel was obtained from local herb garden unit business. Samples were milled, sieved using 30 mesh, packed in a plastic bag and stored at -20°C prior extraction. Ethanol 95% and methanol for extraction purposes were technical grade. Methanol (pa), estragole, gallic acid, quercetin and *1,1-diphenyl-2-picrylhydrazil* (DPPH) were purchased from Sigma-Aldrich (Singapore).

Preparation of fennel extract

To prepare extract of the herb, sample of 10 g was dissolved in either 100 ml of ethanol 96% or methanol. The mixtures were agitated in a shaker water bath for 24 h. Then, it was filtered through a paper filter. The extraction was repeated two times with the same procedure. The filtrate was pooled and evaporated in a rotary vapor at 40°C. The extract was stored at -20°C until analysis. For analysis of ground fennel, the method was as the same above only methanol was used and the maceration was for 24 h. After filtration, the extract was centrifuged at 4000 rpm for 15 min and stored at 4°C until analysis.

Determination of total phenolic content

Total phenolic content was determined using

Folin-Ciocalteu's reagent (Singleton and Rossi, 1965). The absorbance was measured at 745 nm in a UV-vis spectrophotometer. Results are reported as gallic acid equivalent per g extract (mg GAE/g extract).

Determination of total flavonoid content

Total flavonoid content was determined following the procedure as described by Zhishen *et al.* (1999). The absorbance was measured at 510 nm in a UV-vis spectrophotometer. Results are reported as quercetin equivalent per g extract (mg QE/g extract).

DPPH radical scavenging activity

The antioxidant activity of the extract was measured using DPPH free radical scavenging activity method (Brand-Williams *et al.*, 1995). The sample was measured on the ability for donating hydrogen or scavenging radicals of the stable radical DPPH. Briefly, 2 mL of 0.2 mM DPPH was added 1 mL of extract, at different concentration ranging from 1000-2000 ppm. The mixtures were shaken vigorously and then kept in the dark for 30 min. The absorbance was measured at 517 nm. The scavenging activity was calculated based on DPPH radical scavenged (I%) using formula:

$$I\% = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

Where A_{sample} is the absorbance of an extract solution, and A_{control} is the absorbance of the control solution (containing all of the reagents, except the extract). The percentage of inhibition against concentrations were plotted in the graph and a linear regression was established to calculate the IC_{50} .

Determination of estragole

Estragole in the extract was detected and quantified using LC-MS/MS performed on a UHPLC of AC Cella type 1250 (Thermo Scientific). Sample of 2 μ L was injected on Hypersil Gold (50 mm x 2.1 mm x 1.9 μ m). The mobile phase was 0.1% (v/v) formic acid in methanol (A) and 0.1% formic acid in acetonitrile (B). The flow rate was set 300 μ L/min. A gradient was applied to 20% of B over 0.5 min, after which the eluent of B was brought to 80% in 1.5 min and kept it for 1 min. The initial condition was achieved in 0.5 min. The temperature of the injection was 16°C and temperature of the column was kept at 30°C. The mass spectrometric analysis was done on MS/MS Triple Q (quadrupole) TSQ Quantum Access Max (Thermo Finnigan) with the ESI (Electrospray Ionization) in positive mode controlled by software

TSQ Tune. The electro spray capillary voltage was set at 3 kV, temperature was 250°C, and capillary temperature was at 300°C. Nitrogen was used as sheat gas pressure 40 psi, Aux gas pressure 10 psi with Argon. Sample analysis was carried out using the selected reaction monitoring (SRM) mode and characteristic transitions were recorded. The most intense transition was used as quantifier and subsequent transition as qualifier, for estragole: 163 \rightarrow 91 m/z (quantifier), 163 \rightarrow 121 m/z (qualifier).

Determination of Benchmark Dose Level (BMDL10)

In vivo data of Miller *et al.* (1983) was used to calculate BMDL10 of estragole using BMD software version 2.4. Those data were obtained from animal experiment in female mice administered with estragole via the feed for three times a week for 52 weeks. The duration of experiment was 85 weeks on average (Miller *et al.*, 1983). The dose levels reported in mg/kg diet were converted to adjusted dose levels in mg/kg bw day as also done for safrole (Martati *et al.*, 2014). Adjustment for the length of treatments and the observation period was performed. Adjustment of the doses was to correct the underestimation of the tumor formation that would occur when experiment is finished before the standard life span (Benford *et al.*, 2010). The correction factor for adjustment of the doses were 1) dose given 3 times a week was converted to obtain daily administration (the dose was multiplied by 3/7), 2) duration of the exposure converted to life time exposure (the dose was multiplied by (52/104) x (85/104) and 3) the loss of the compound during the storage as reported by the researcher (Miller *et al.*, 1983) to amount to 5%/day.

Risk assessment of estragole in fennel extract

MOE is a ratio between BMDL10 and intake. In this risk assessment, because real human intake of extract was not known then calculation was performed to know how much consumption of the extract of fennel to have MOE of <10,000 (a high priority of risk management) or >10,000 (a low priority of risk management). The BMDL10 for estragole was calculated using BMD software version 2.4 based on the data (Miller *et al.*, 1983).

Data analysis

The data obtained was expressed as mean \pm standard deviation. The differences of the parameters measured between methanolic and ethanolic extract was tested with T test using Microsoft Excel 2016. P values <0.05 were regarded as significant.

Result and discussion

Table 1 shows that TPC and TFC of methanolic fennel extract are significant ($P < 0.05$) higher than that of ethanolic extract. TPC of this result is in good agreement with Roby *et al.* (2013) who reported that TPC of methanolic fennel extract was higher than that of ethanolic extract. TPC of methanolic and ethanolic of fennel extract were 3.4 and 3.0 mg GAE/ g dry weight, respectively (Roby *et al.*, 2013). The more polar solvent used in extraction resulted the higher TPC extracted from fennel (Roby *et al.*, 2013). Anwar *et al.* (2009) showed different results that TPC and TFC were higher in ethanolic extract than that of methanolic extract of fennel. According to Mohamad *et al.* (2011) methanolic extract of fennel seed contained TPC 29.64 mg GAE/g. TFC of methanolic fennel extract are significant ($P < 0.05$) higher than that of ethanolic extract. The most abundant flavonoid in fennel methanolic extract were quercetin (14.9%) and apigenin (12.5%) (Roby *et al.*, 2013). The antioxidant capacity of methanolic extract of fennel is significant ($P < 0.05$) higher (lower IC_{50}) than that of ethanolic extract. There is a correlation between TPC and TFC and antioxidant activity and both compounds determined antioxidant activity (Do *et al.*, 2014). The free radical scavenging activity of methanolic fennel extract was superior to ethanol solvent. This result was in agreement with Roby *et al.* (2013) which free radical scavenging of the methanol extract of fennel was higher than that of ethanolic extract. The TPC and TFC are higher in extract of fennel than those of ground fennel. The IC_{50} of ground fennel was higher than those of extract. The TPC of ground fennel was 3.13 mg GAE/g (wb) or 3.35

mg GAE/g (db) (6.6% of moisture content) whereas Bi *et al.* (2015) showed TPC of fennel extract was 1.81 mg GAE/g DW. The difference was due to the different extraction method. Bi *et al.* (2015) used water infusion for overnight at room temperature to extract fennel. In this study, the extraction of ground fennel used maceration in methanol for 24 h at room temperature. In pharmaceutical industry, methanol classified class 2 of solvent to be limited (EMA, 2016). According to European Commission (2009), maximum residue limits of methanol in the extracted foodstuff or food ingredient is 10 mg/kg. In this experiment, the residue of methanol in the extract was not measured.

Relation between intake of fennel extract and the MOE

The content of estragole in methanolic and ethanolic extract were 0.011% and 0.037%, respectively. The presence of estragole or other compounds which are carcinogenic and genotoxic in botanicals cannot be avoided resulting in a certain level daily exposure. It is necessary to predict the cancer risk at low dose level representing realistic human dietary intake because estragole is genotoxic and carcinogenic.

The BMDL10 values for estragole were calculated using BMD software version 2.4 based on the data from an animal experiment study in female mice exposed to estragole via the diet administered 3 days a week up to 52 weeks and for 86 weeks of experiment duration (Miller *et al.*, 1983). Table 2 presents an overview of the incidence of hepatocellular carcinomas in female mice with increasing doses of estragole as described by Miller *et al.* (1983) and

Table 1. TPC, TFC, IC_{50} and estragole content of methanolic and ethanolic fennel extract

	Fennel		
	Methanolic extract	Ethanolic Extract	Ground
TPC (mg GAE/g)	13.95 ± 1.34 ^a	5.80 ± 0.13 ^b	3.13 ± 0.04
TFC (mg QE/g)	30.07 ± 2.39 ^a	16.80 ± 1.65 ^b	12.71 ± 0.43
IC_{50} (ppm)	1587.01 ± 204.76 ^a	2409.66 ± 133.14 ^b	6033.14
Estragole (%)	0.011 ^a	0.037 ^b	n.a

Note: n.a: not available

Different superscript letters indicate significant difference ($P < 0.05$) at the same row

Table 2. The incidence of hepatocellular in female mice administered of estragole as reported by (Miller *et al.*, 1983) and the dose levels adjusted

Dose (ppm)	Length of exposure/ sacrifice (weeks)	Time-adjusted dose	No. of animals	No. of animals with hepato- cellular carcinomas incidence
0	52/86	0	50	0
2300	52/86	50	48	27
4600	52/84	98	49	35

Table 3. Results from BMD analysis based on the data of induction of hepatoceluler in mice administered 0, 2300 or 4600 mg/kg bw diet containing estragole (Miller *et al.*, 1983)

Model	No. of parameters	Log Likelihood	p-value	Accepted	BMD ₁₀ (mg/kg bw/ day)	BMDL ₁₀ (mg/kg bw/ day)
Null	1	-100.09				
Full	3	-62.21				
Gamma	1	-62.66	0.63	Yes	7.38	5.96
Logistic	2	-70.66	0	No	21.91	17.37
LogLogistic	1	-62.21	0.99	Yes	4.34	3.03
LogProbit	2	-62.21	1	Yes	0.95	4.68
Multistage	1	-62.67	0.63	Yes	7.38	5.96
Multistage cancer	1	-62.67	0.63	Yes	7.38	5.96
Probit	2	-69.96	0	No	21.20	16.93
Weibull	1	-62.67	0.63	Yes	7.38	5.96
Quantal-linear	1	-62.67	0.63	Yes	7.38	5.96

Table 3 presents results a BMD analysis of these data. The calculated BMDL10 value of estragole is 3.03-5.96 mg/kg bw day. This calculated BMDL10 is a little bit different to the Berg *et al.* (2011) of 3.3 - 6.5 mg/kg bw day. The difference is due to the different results in converted from experimental dose (mg/kg diet) to time-adjusted dose (mg/kg bw day).

Figures 1A and 1B show the simulation of intakes of ethanolic and methanolic extract of fennel and MOEs. It shows that intake of methanolic and ethanolic extracts of fennel which were > 168-331 and > 49-97 mg/60 kg bw per day for a life time resulted an MOE of <10,000 meaning a high priority for risk management actions and would be of high concern from a public health point of view. However, *in vivo* data used to calculate BMDL10 was obtained from animal experiment administered high doses of the pure compound without taking matrix effects into considerations. Martati *et al.* (2014) showed that in

the presence of mace extract (a spice containing an alkenylbenzene of safrole), the formation of DNA adduct was inhibited in an intact cell system of human HepG2 cells and also in *in vivo* experiment. This result showed that others compounds in the extract can reduce the cancer risk due to the presence of inhibitor of sulfotransferase-mediated formation of DNA adduct in the hepato-carcinogenicity in rodents. Sulfotransferase inhibitors in the diet that have been identified were nevadensin, malabaricone C, kaempferol and apigenin (Alhusainy *et al.*, 2010; Martati *et al.*, 2014). Therefore, the adverse effects of genotoxic and carcinogenic of estragole in rodent bioassays will be reduced when estragole is tested in the presence of a matrix containing SULF inhibitors. The risk assessment of extract containing estragole should also take into account other compounds that might reduce the incidence cancer in animal experiment.

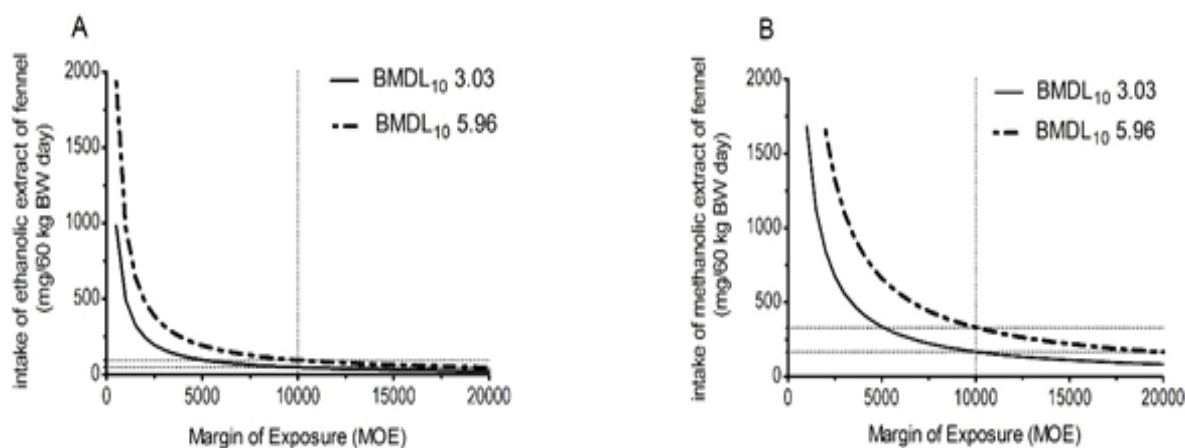


Figure 1. Relation between intake of fennel extract (mg/60 kg BW day) for a life time and MOE, (A) ethanolic extract of fennel and (B) methanolic extract of fennel

Conclusion

The results showed that TPC, TFC and capacity of antioxidant of methanolic extract of fennel were significant higher than that of ethanolic extract. Simulation of intake of methanolic and ethanolic extracts of fennel which was > 168-331 and > 49-97 mg/60 kg bw per day for a life time resulted an MOE of < 10,000. Meaning a high priority for risk management actions and would be of high concern from a public health point of view. Consumption of botanicals and its extract should consider both the health benefit and the risk potential to the health.

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