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Original Article

Evaluation of cytotoxic activity of *Fomes fomentarius* extracts against brine shrimp larvae and colon adenocarcinoma cell line (SW742)

Running Title: Cytotoxic activity of fomes fomentarius

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Abstract

Aim: Cancer is one of the leading worldwide public health problems. Natural product has provided a valuable source biological active compound for maintaining humans health even after years of introduction of synthetic drugs. Fomes fomentarius (F. fomentarius), a fungus belongs to Polyporaceae family, is widely used for different medicinal purposes. Considering inadequate reports on the fungus, cytotoxic effect of its extracts on brine shrimp larvae and colon adenocarcinoma cell line (SW742) was investigated in this study.

Methods: The fruiting body of the fungus was chopped and extracted with water, ethanol, methanol, and ethanol-methanol (50-50) solvents. Regarding the results of brine shrimp lethality assay (BSLA), ethanol extract showed the highest toxicity, so subjected to fractionation with chloroform, ethyl acetate, and water. The fractions were tested for cytotoxic activity in human colon adenocarcinoma (SW742) cells by MTT assay. Ethyl acetate fraction exhibited more cytotoxic activity against SW742 cell line. Therefore, it was chosen to be purified using silica gel column chromatography. Subfractions of ethyl acetate were tested for their cytotoxicity by MTT assay. The content of these subfractions were also analyzed using gas chromatography-mass spectrometry (GC-MS).

Results: According to the results, ethanol extract had the greatest potency in BSLA, and ethyl acetate fraction showed the highest cell cytotoxicity. Based on GC-MS chromatogram, palmitic acid was the compound with the largest quantity in the three selected subfractions of ethyl acetate.

Conclusion: It was found that palmitic acid with other compounds in F. fomentarius had cytotoxic activity against colon adenocarcinoma cells. This feature may provide a biological basis for further clinical evaluation.

Keywords: Colorectal cancer; Column chromatography; Cytotoxicity; Fomes fomentarius; MTT assay; Palmitic acid

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Introduction

In many studies, mushrooms were indicated as an important natural source for biological active compounds with anticancer and immunomodulatory properties (1). Fomes fomentarius (F. fomentarius), belonging to Polyporaceae family, is a woody fungus with large size that grows as saprophyte on deciduous trees (2). The fungus was traditionally used as a styptic by surgeons and dentists, or a remedy in gastroenteric disorders, oral ulcers, and cancers (3, 4). Anti-nociceptive, hypoglycemic, anti-inflammatory, anti-bacterial, anti-diabetic, and anti-tumor properties of F. fomentarius were reported in some recent studies (5- 8). Besides, different classes of metabolites were reported, including proteins, polysaccharides, triterpenes, steroids, coumarins, and volatile compounds (3).

Among other nutritive and bioactive compounds of mushrooms, glucans have been suggested to possess antimicrobial and cytotoxic activity by direct activation of leukocytes through stimulating their phagocytic effect as well as production of nitrogen intermediates and reactive oxygen (9). Intra- and extracellular polysaccharides of F. fomentarius showed anti-proliferative activity against human gastric cancer cell lines SGC-7901 and MKN-45 in a dose-dependent manner (10). Isolated polysaccharides from the fungus displayed strong anti-tumor effect against human lung carcinoma A549 cells by induction of single-stranded DNA breakage and apoptosis (11).

The objective of the present study was evaluation of cytotoxic activity of different extracts of F. fomentarius against brine shrimp larvae and human colon adenocarcinoma (SW742) cells. To identify active components of the fractions, they were analyzed using GC-MS method.

Methods and materials

Preparation of the extracts

The fruiting bodies of F. fomentarius were collected from Neka forest, Mazandaran province in the north of Iran on host tree Carpinus betulus in the summer of 2017. For preparation of extracts of the fungus, methanol, ethanol, and mixture of ethanol:methanol (50:50) were applied to extract 50 g of the powder of the fungus separately (4×48 h). Aqueous extract of the fungus was prepared by boiling the 50 g of the fungus powders in a closed container with distilled water for 4 h. The obtained extracts were filtered using Wattman paper No. 1, concentrated using rotary evaporator (Heidolph, Germany), and freeze drying was done (Christ, UK) (12).

According to the preliminary brine shrimp lethality test, ethanol extract was chosen to prepare sub-fractions to find the more potent fraction or compounds. The fungus powder (1100 g) was extracted with ethanol, and the extract was fractioned using chloroform, ethyl acetate, and distilled water by liquid-liquid extraction method.

Cytotoxicity tests

Brine shrimp lethality assay (BSLA)

Cytotoxic activities of the fungus extracts were assessed using Artemia salina according to the previous studies to calculate 50% of lethal dose (LD50) of each extract (13, 14). Eggs of the shrimp were placed in a glass flask containing 400 mL artificial seawater with temperature of 29-30 °C, and aerated under continuous illumination of fluorescence lamp. Ten to 20 freshly hatched free-

swimming nauplii were used for the bioassay and treated with various concentrations (100, 250, and 500 µg/mL) of each extract in 24 well chamber slides. Numbers of alive nauplii were determined after 24 h by control of forward motion of them in 30 s. Artificial seawater was used as negative control.

Cytotoxic assay

The human colon adenocarcinoma (SW742) cell line was obtained from National Cell Bank of Iran (NCBI, Pasteur Institute, Tehran, Iran). Cells were cultured in RPMI-1640 cell culture medium (PAA, Germany) supplemented with 10 µL fatal bovine serum (FBS; Gibco, USA), penicillin (100 U/mL), and streptomycin (100 µg/mL) (Roche, Germany) at 37 °C in air/carbon dioxide (95:5) atmosphere. Cytotoxic activity of fractions was evaluated against SW742 cells by MTT assay (15, 16). Cells (1×104 cells/well) were plated in 96-well plates (Nunc, Denmark). They were then incubated before addition of fractions for 24 h. Cells were treated with different concentrations of chloroform, ethyl acetate, and aqueous fractions (62.5, 125, 250, and 500 µg/mL). Twenty µL of MTT reagent (Merck, Germany) in phosphate buffered saline was added to each well, and the plate stored at 37 °C for 4 h. The medium was then discharged and formazan blue dissolved in 100 µL dimethyl sulphoxide (DMSO). Absorbance of dissolved solutions was detected using a micro plate reader (Anthos, Austria) after incubation at 37 °C for 10 min. Cytotoxicity was expressed as the concentration of extract inhibiting cell growth with 50%, IC50 (SD). All tests and analysis were oerfomed three times.

Purification of ethyl acetate fraction

Based on cytotoxicity results, ethyl acetate fraction was more cytotoxic and applied to analyze more to find responsible compounds for cytotoxic activity. Ethyl acetate fraction (5.57 g) was chromatographed on glass column (3.5×30 cm), filled with silica gel (70-230 mesh, Merck, Germany), and eluted with ethyl acetate: hexane (30:70-100:0). Obtained fractions were evaluated by pre-coated silica gel 60 F254 (Merck, Germany) thin layer chromatography (TLC) under UV chamber (254 and 366 nm) and anisaldehyde reagent followed by heating for 5 min. Three main subfractions (E1, E2, E3) prepared, and their cytotoxic activities were assessed using MTT test. According to the inhibition activity of the three subfractions of ethyl acetate portion against SW742 cell line, E1 was purified on silica gel column and eluted with ethyl acetate: hexane (50:50-100:0) yielding compound 1 (10 mg).

Nuclear Magnetic Resonance (NMR) spectra

Compound 1 was dissolved in CDCl3 to obtain NMR spectra on Bruker (Billerica, MA, USA) DRX 500 instrument (500 MHz for 1H-NMR) with tetramethylsilane (TMS) as an internal standard. The compound was identified by comparison of chemical shifts of protons with previous reported data (17).

Gas chromatography-mass spectroscopy (GC-MS)

According to the MTT assay results, another sub-fraction of ethyl acetate extract (E3, 11.4 mg) was subjected to analyze using GC-MS method. The analysis was carried out on Hewlett-Packard 6840

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gas chromatograph with selective mass detector MS 5973 series C (EI, 70 eV) equipped with capillary column HP-5 (30 m × 0.25 mm; film thickness: 0.25 µm). The carrier gas was helium at flow rate of 1 mL/min. Temperature scheduled as 50 °C for 3 min and raised to 280 °C with rate of 5 °C/min. Using Wiley library, the mass spectra of compounds was identified and compared .

Statistical analysis

Lethality percentage was calculated based on Probit Analysis (SPSS 11.5, USA) with 95% of confidence interval (CI). The median growth inhibitory concentration (IC50) values were calculated using the IC50 of dose-response curve in the Sigma plot 11 software. Data of three independent experiments with similar results were presented as mean (standard deviation, SD). The values compared using one-way Anova test and p value less than 0.05 was considered as statistically significant. Statistical analyses and methods in the published literature (SAMPL) guideline were followed in the present study for statistical reporting.

Results

BSLA data

The larva of A. salina were treated with extracts of fruiting body of F. fomentarius with different concentrations. The respective LD50 values of aqueous, methanol, ethanol, and ethanol: methanol (50:50) extracts against larva were 4492.89, 743.56, 76.79, and 197.45 µg/mL, indicating that solvents with various polarity could extract the compounds with different potency of toxicity. The ethanol extract with concentration of 500 µg/mL showed the significant cytotoxicity on larva with mortality rate of 100% (**Figure 1**). Results clearly highlighted the cytotoxicity of the ethanol extract in comparison with others, leading to further analysis and evaluation of cytotoxicity of the extract in more details.

Cytotoxicity against SW742 cells

Based on the BSLA, ethanol extract of the fungi exhibited higher toxicity against A. salina larvae; therefore, it was selected for further analysis. The main fraction of ethanol was subsequently divided into three subfractions by chloroform, ethyl acetate, and distilled water (**Figure 1**).

After 24 h, The viability of SW742 cell line treated with different concentrations of the fractions was evaluated (**Figure 2**). Ethyl acetate fraction with IC50 value of 61.1 µg/mL caused more toxicity against the cell line after 24 h in comparison with other fractions. The respective IC50 values of aqueous and chloroform fractions were calculated 289.8 and 158.8 µg/mL after 24 h. Ethyl acetate fraction with concentrations of 125, 250, and 500 µg/mL provided the highest cytotoxicity toward the cell line comparing to other fractions (**Figure 2**). In contrast with aqueous fraction, the chloroform fraction showed more growth inhibition activity against SW742 cell line. According to the cytotoxicity results, ethyl acetate subfractions (E1- E3) were applied for more cytotoxic analysis to identify more potent cytotoxic part. Among ethyl acetate subfractions, E1 (IC50: 81.45 µg/mL) inhibited growth of SW742 cell line higher than E2 (IC50: 2440.60 µg/mL) and E3 (IC50: 314.19 µg/mL)

subfractions (**Table 3**). therefore, E1 was applied for THE purification of the components. However, E1 with different concentrations (15.12-125 µg/mL) showed similar growth inhibition against the cancer cell line (**Figure 3**).

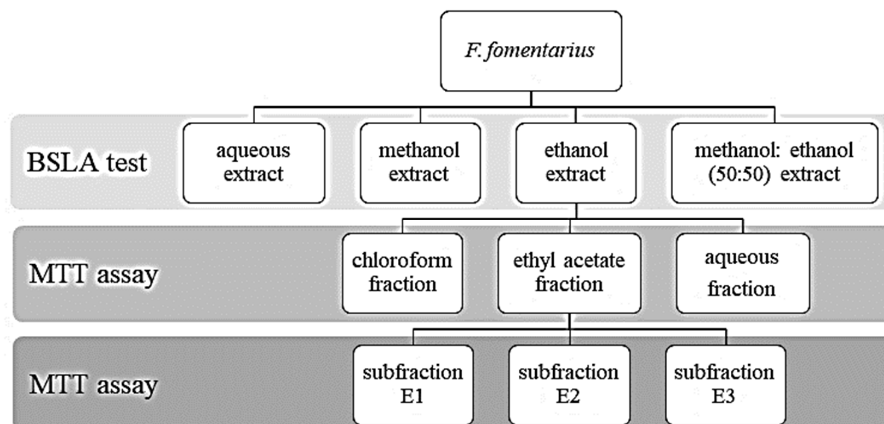

Figure 1. The process of *F. fomentarius* extraction and fractionation.

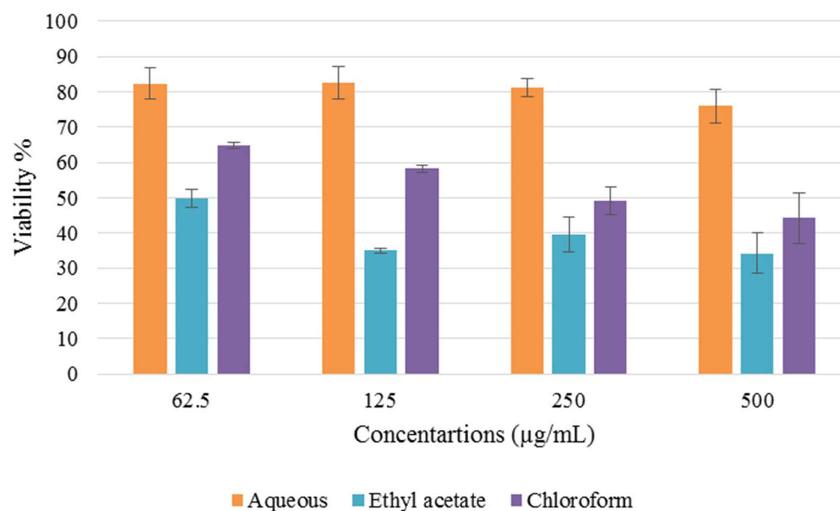

Figure 2. Cytotoxicity evaluation of different fractions of *F. fomentarius* ethanol extract against SW742 cell lines after 24 h.

Identification of compound

The isolated compound from ethyl acetate subfraction (E1) identified hexadecenoic acid (palmitic acid) using 1H-NMR by comparison with previous data (17), which WERE explained as follows: 1H-NMR data (500 MHz, CDCl3), δ H (ppm): 0.89 (3H, t, J=5 Hz, CH3), 1.27 (24H, m, CH2×12), 1.27 (24H, m, CH2×12), 1.57 (2H, m, CH2), 2.19 (2H, m, CH2).

The other subfraction (E3) was subjected to analysis with GC-MS (**Figure 4**). Eleven compounds compromising 97.77% of the subfraction E3 were identified, including some alkane hydrocarbons and saturated fatty acids.

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The identified compounds comprised of tetradecane (2.22%), hexadecane (3.49%), octadecane (3.40%), n-hexadecanoic acid (29.46%), eicosane (2.50%), (E)-9-octadecanoic acid (10.25%), octadecanoic acid (15.08%), docosane (1.99%), eicosane (1.55%), tetracosane (13.26%), and heptacosane (14.57%). n-Hexadecenoic acid or palmitic acid with octadecanoic acid or stearic acid were the major part of the other ethyl acetate subfraction, E3.

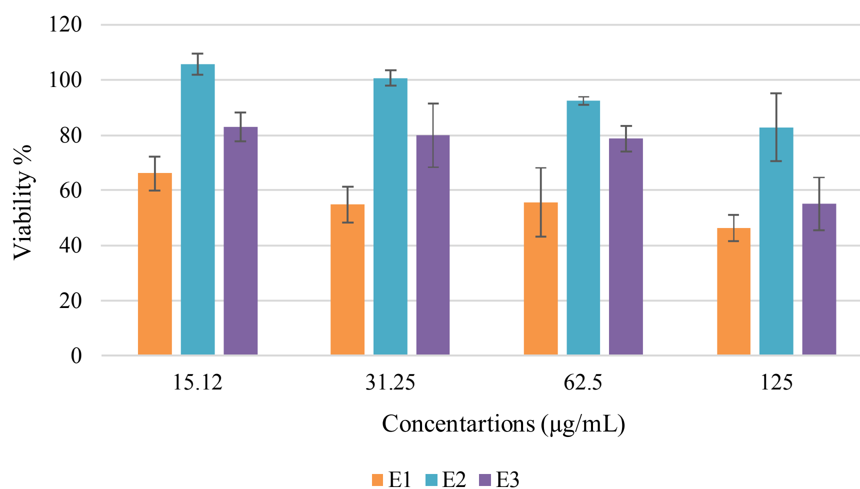

Figure 3. Cytotoxicity evaluation of different ethyl acetate subfractions of *F. fomentarius* against SW742 cell lines after 24 h

Discussion

In the present study, cytotoxicity of F. fomentarius extracts were demonstrated against human colon adenocarcinoma (SW742) cells by using MTT assay. Ethanol extracted more toxic compounds from the mushroom against brine shrimp larva with LC50 of 76.79 µg/mL. In this method, brine shrimp larvae was utilized and proposed as simple bioassay method for analysis of natural products cytotoxicity. As mentioned by Meyer et al., LC50 less than 100 µg/mL could be a potent which caused death of the nauplii attributing to the bioactive compounds presented in the extract. While LC50 values greater than 1000 µg/mL are not toxic (18). Therefore, aqueous extract of the mushroom can be considered as non-toxic. However, water-extractable polysaccharide of fruiting body of F. fomentarius with molecular weight of 12 kDa exhibited cell growth inhibition and apoptosis induction against lung cancer cell line A549 dose dependently (11). The results of another study also revealed selective cytotoxic activity of F. fomentarius aqueous extract against Hela and N87 cells with no activity against normal cell line of MRC5 (8). Similarly, extra and intracellular polysaccharides of the fungi inhibited growth of human gastric cell line SGC 7091 (10). The results of our study was not consistent with the previous reports and aqueous extract of the fungus was not cytotoxic against SW742 cell line, which may indicate diversity in content of the

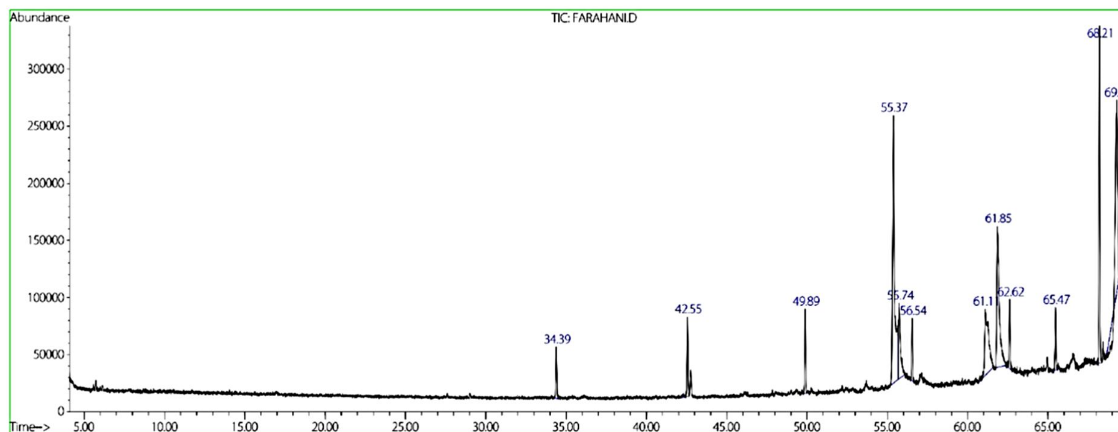

Figure 4. Chromatogram of GC-MS analysis related to ethyl acetate subfraction E3 of *F. fomentarius*.

extract and biological differences among cancer cell lines.

For the detection and isolation of more potent constituents, ethanol extract was fractioned to three more fractions of aqueous, ethyl acetate, and chloroform fractions in the present study. Ethyl acetate fraction was the most potent fraction in inhibition of growth of SW742 cells based on MTT assay results. Previous studies also reported that anti-proliferative and cytotoxic activities of F. fomentarius were related to the presence of bioactive chemical compounds, including botulin, daphnetin, and triterpenoids, in ethanol extract of the fungi. Apoptosis caused by botulin via decreasing PARP and Bcl-2 with induction of cleaved PARP, caspase-9 and caspase-3 (19). Betulin induced apoptosis and inhibited cell migration in HBL-60 and MDA-MB-231 cells (20, 21).

Hexadecenoic acid (palmitic acid) was purified and identified from ethyl acetate subfraction E1, which was more toxic against SW742 cell lines and comprised the main part of other subfraction E3. The result of the present study were in agreement with a previous study reporting the major fatty acids in F. fomentarius as palmitic acid and stearic acid (22). In addition, high concentrations of palmitic acid can induce cell death (23). Palmitic acid showed selective cytotoxicity toward human leukemic cells MOLT-4 through induction of apoptosis with no toxicity against HDF, a normal cell line. Since molecular target of palmitic acid is DNA topoisomerase I in tumor cells with no effect on DNA topoisomerase II, it seems that the compound can be a lead compound for anticancer drugs (24). Apoptosis and autophagy were induced by palmitic acid through mitochondrial dysfunction and endoplasmic reticulum stress induced following oxidative stress in Chang liver cells (25).

Conclusion

In summary, we demonstrated that ethyl acetate fraction of F. fomentarius had cytotoxicity against SW742 colon adenocarcinoma cells, while other

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extracts of the fungi showed no considerable cytotoxic activity against the cancer cell line. Further investigation is needed on the preventive role of the F. fomentarius extract in vivo.

Conflict of interests: The authors declare that there is no conflict of interest.

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