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Ferula gummosa gum induces apoptosis via ROS mechanism in human leukemic cells

Maliheh Moradzadeh[1], Hamid Reza Sadeghnia[1-3], Seyed Hadi Mousavi[4], Merat Mahmoodi[5], Azar Hosseini[2*]

[1] Golestan Rheumatology Research Center, Golestan University of Medical Sciences, Gorgan, Iran
[2] Pharmacological Research Center of Medicinal Plants, Mashhad University of Medical Sciences, Mashhad, Iran
[3] Neurocognitive Research Center, Mashhad University of Medical Sciences, Mashhad, Iran
[4] Medical Toxicology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran
[5] Immuno-Biochemistry lab, Immunology Research Center, Mashhad University of medical Sciences, Mashhad, Iran

Correspondence to: hoseiniAZ@mums.ac.ir

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Abstract: Ferula species known for its oleo-resins that are recognized valuable industrial crops and food products. In this study, we examined the level of cellular oxidants, cytotoxicity, apoptosis and differentiation induced by oleo resin gum from *Ferula gummosa* (30-250 µg/mL), as well as Arsenic trioxide (50 µM, as positive control), in leukemic (NB4 and HL-60 cells) and normal polymorph nuclear cells during 72 h. Resazurin assay was used to determine cell viability following treatment with *F. gummosa* (30-250 µg/mL). Intracellular reactive oxygen species was measured by fluorimetry using carboxy 2′, 7′-dichlorofluorescein diacetate. Apoptotic cells were evaluated using PI staining of DNA fragmentation by flow cytometry (sub-G1 peak). Differentiation of cells evaluated by Giemsa staining and Nitro Blue Tetrazolium reduction. *F. gummosa* showed a concentration-dependent suppression in cell survival with IC_{50} values of 41.8 µg/mL for HL60 and 59.2 µg/mL for NB4 cells after 72 h treatment. ROS formation and apoptotic cells were concentration-dependently increased following treatment with *F. gummosa*, similar to As_2O_3. *F. gummosa* did not induce differentiation of leukemic cells towards granulocytic pattern. The resin did not have toxic effect on PMN cells (<800 µg/mL). In conclusion, the present study demonstrated that *F. gummosa* induced apoptosis through ROS mechanism on leukemic cells as a concentration and time dependent manner. The precise signaling pathway by which *F. gummosa* induce apoptosis needs further research.

Key words: *Ferula gummosa*; Leukemia; Apoptosis; Differentiation; ROS.

Introduction

Acute promyelocytic leukemia (APL) is one of the most threatening hematological malignant cancers. Promyelocytes exhibits a failure of myeloid differentiation (1-3). Arsenic trioxide (As_2O_3) is used for treatment of APL because of induction of apoptosis, inhibition of growth, and promotion of differentiation. As_2O_3 as other chemotherapy drugs as causes various side effects (4). There could be significant value to patents if it is possible to develop combination therapies that required lower doses of As_2O_3 in APL patients. In recent years, natural compounds are major subjects for antineoplastic agents increasingly used in the treatment of leukemia (5). Natural products have always been good sources for development of new therapeutics for management of several diseases. Also recent studies have shown relationship between consumption of fruit and vegetable with cancer. The studies have shown some of herbal medicine such as *Hibiscus cannabinus* (*Kenaf*) (6), *Ginseng root* (7), *Euphorbia formosana* (8), *Allium sativum* (9), and *Vernonia amygdalina* (10) have cytotoxic effects on leukaemia cell lines. Ferula species is known for its oleo-resins that are recognized valuable industrial crops. *Ferula gummosa* Boiss. (baridje in Persian), is a monocarpic, perennial herbaceous wild plant of 0.8–3 m height, indigenous to Iran, growing in the northern and western parts of the country at higher than 2000 m a.s.l. and is a resinous plant with a strong odor (11). The root of *F. gummosa* is glandular and rich in oleo-gum-resin that can be exuded naturally or manually during vegetative period of the plant. The oleo-gum-resin slowly exudes as a viscous liquid, collects in a drop and hardens. The oleo-gum-resin and remaining root are important export products and contribute to regional economic profit. Oleo-resins are traditionally used in high quality paints and varnishes (12), as additives in soaps, detergents and perfumery (13, 14). Integral oleo-gum-resins, or their individual components, are used as flavors or emulsifiers in food products and beverages (15-17). Essential oils and oleo-resins are also known-for their sedative, anti-spasmodic, anti-microbial, anti-rheumaticand anti-diabetic properties (11, 18, 19). In the present study, we investigated the cytotoxic and differentiation effects of *F. gummosa* on leukemic (HL60 and NB4) cells compared to Arsenic trioxide (As_2O_3) (as positive control).

Materials and Methods

Oleo-gum-resin Preparation

The oleo-gum-resin from *F. gummosa* (Boiss.) was collected from Sabzevar, Khorasan Razavi province, Iran, during September 2015. After removal the soil around the herb, a scrap was made on the surface near root and exudates were received in stainless steel

containers. The oleo-gum-resin was collected during 8 days from about 30 randomly selected healthy plants of 4–6 years old. The united exudates were stored in double layer tied enclosed plastic container in refrigerator (4°C).

Cell lines and reagents

HL60 and NB4 cells were purchased from cell bank of Pasteur Institute (Tehran, Iran). Resazurin reagent [300 µM resazurin, 78 µM methylene blue, 1 mM potassium hexacyanoferrate III and 1 mM potassium hexacyanoferrate II], 2,7-dichlorofluorescin diacetate (DCFH-DA), propidium iodide (PI), nitroblue tetrazolium (NBT), phorbol-myristate-acetate (PMA) and As_2O_3 (stock concentration of 1mM) were bought from Sigma (St Louis, MO). High-glucose Roswell Park Memorial Institute medium (RPMI1640), penicillin-streptomycin and fetal bovine serum were obtained from Gibco BRL Life Technologies (Grand Island, NY). Giemsa stain was purchased from MERK (Darmstadt, Germany).

Human normal cells isolation and Cell culture

Polymorphonuclear cells (PMNs) from healthy volunteers were isolated under sterile conditions by two consecutive Ficoll-Hypaque (Pharmacia) density gradient centrifugations(20). PMN purified to more than 97%, as judged by morphological examination of Wright-stained smears.After washing with sterile phosphate-buffered saline (PBS), the cells were resuspended in RPMI medium.HL60, NB4 and PMN cells were maintained at 37° C in a humidified atmosphere (90%) containing 5% CO_2. RPMIwith 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. The cells were incubated with various concentrations of *F. gummosa gum* (30-250 µg/mL) or As_2O_3 (50µM) for 24, 48 and 72 h. For each concentration and time course study, there was a control sample which remained untreated and received the equal volume of medium. All different treatments were carried out in triplicate.

Cell viability

The cell viability was determined by resazurin reagent. 100µl of the NB4, HL60 and PMN cells containing 1×10^5 cells were added to each well in 96-well tissue culture plates, then cells were treated with *F. gummosa gum* (30-250 µg/mL) or As_2O_3 (50µM) for 24, 48 and 72 h. Then 20µl of resazurin reagent were added into each well and the plates were incubated for 4 h. Fluorescence intensity of the product resorufin, proportional to the number of viable cells per well, was measured by a fluorescence Victor X5 2030 Multilabel Plate Reader (PerkinElmer, Shelton, Connecticut) with excitation at 530 nm and emission at 590 nm (21).

Measurement of Reactive Oxygen Species

In brief, HL60 and NB4 cells (10^5/well) incubated with DCF-DA (20 µM) for 30 min in the dark and then treated with *F. gummosa gum* (30-250 µg/mL) or As_2O_3 (50µM) for 2 h. After that, the cells were transfered to a 75-mm falcon polystyrene tube. After washing twice with PBS, The DCF fluorescence intensity was detected using a FLUO-star galaxy fluorescence plate reader (Perkin Elmer 2030, multilabel reader, Finland) with excitation wavelength set at 485 nm and emission wavelength set at 530 nm (22).

Apoptosis

Apoptotic cells were detected by using PI staining of the treated cells followed by flow cytometry to detect the so-called sub-G1 peak. Briefly, HL60 and NB4 cells were treated with *F. gummosa gum* (60-250 µg/mL) or As_2O_3 (50µM) for 48 h. The cells were then incubated at 4 °C overnight in the dark with 500 µl of a PI hypotonic buffer (50µg/mL). Samples were then analyzed by flow cytometry. A total of 10,000 events per sample were obtained and the data was analyzed using WINMDI software (23).

Morphological and differentiation assay

Diagnosis and morphological typing were determined by standard techniques using Giemsa-stained smears and differentiation test was performed by Nitro Blue Tetrazolium reduction. The NBT reaction provides a sensitive and easily quantitated leukemic cells differentiation marker, eliminating observer subjectivity associated with morphological assessment alone. Cells (1×10^6 cells/ mL in 6-well plate) were treated with *F. gummosa gum*(30 and 60 µg/mL) or As_2O_3 (0.5µM) (24) for 3 days and then, washed twice with PBS and the pellets were resuspended in equal volume of 0.2% NBT dissolved in Dulbecco's phosphate-buffered saline containing 200 ng/mL of freshly diluted phorbol myristate acetate (PMA). After 25 min incubation at 37°C in the dark, cytospin in slides were prepared and stained with Giemsa and 300 cells were scored for the presence of blue-black formazan granules (25).

Statistics

Statistical analysis were made using Graph Pad PRISM (Version 6, Graph Pad Software, Inc., San Diego, CA) software with one-way analysis of variance and Tukey's multiple comparison post-tests to determine significant differences between several treatment groups. P-value less than 0.05 were considered as statistically significant. The values are presented as the mean± SEM of three independent experiments performed in triplicate.

Results

***F. gummosa gum* decreased cell viability in a concentration-dependent manner**

To evaluation the toxic effects of *F. gummosa gum*, HL60 and NB4 cells were incubated with diverse concentration of *F. gummosa gum* (30-250 µg/mL) and As_2O_3 (50µM) as a positive control for up to 72 h and the cell viability was measured by resazurin reagent (Fig. 1).The PMNcells were incubated with *F. gummosa gum* (200-800 µg/mL) for 24 h. As compared with PMN cells, *F. gummosa gum*-mediated toxicity was significantly higher in HL60 and NB4 cells. The results are graphically shown in Figure 1 and summarized in Table 1. As shown in figure 1, treatment with *F. gummosa* gum decreased cell viability as dose and time-dependently in both of cell lines.The IC_{50} (concentration of 50% inhibition) values of *F. gummosa* gum in HL60 cells were

Table 1. IC_{50} (concentration of 50% inhibition) values of *F. gummosa*in NB4 and HL60 cells at 24-72 h.

Treatment \ Cell lines	HL60 cells			NB4 cells		
	24 h	48 h	72 h	24 h	48 h	72 h
F. gummosa (µg/mL)	91.29±0.17	81.38±0.15	41.86±0.08	109.30±0.15	89.46±0.13	59.24±0.11

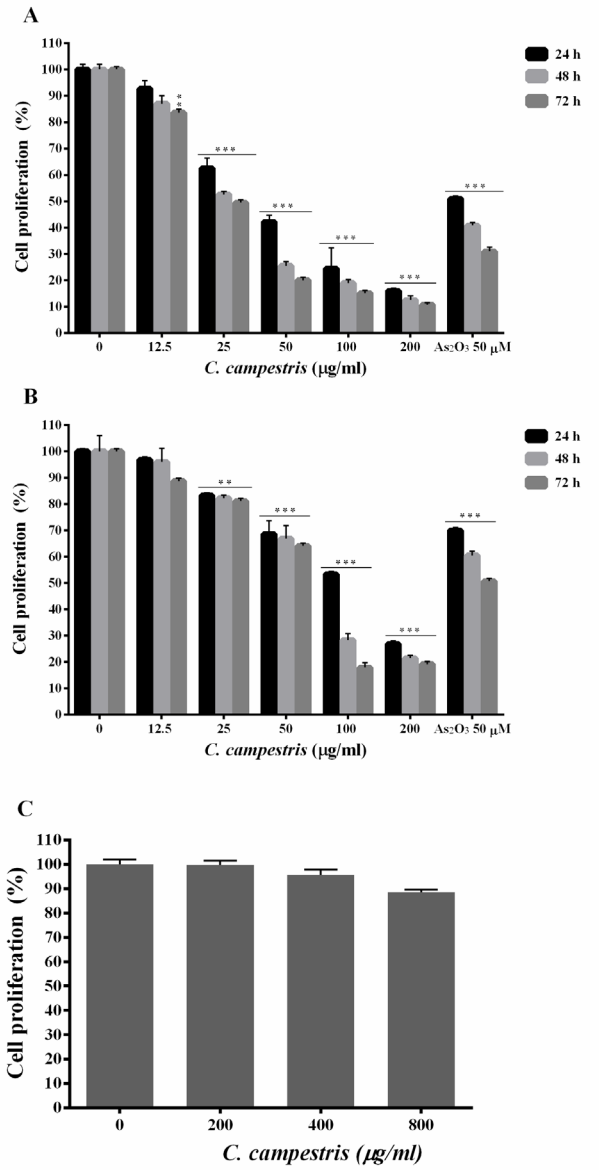

Figure 1. Anti-proliferative effects of *F. gummosa* gum on leukemic cells.(A) HL60 and (B) NB4cells treated with different concentrations of *F. gummosa* gum (30-250 µg/mL) or As_2O_3 (50µM) for 24-72h. (C) Normal polymorph nuclear (PMN) cells treated with *F. gummosa* gum (200-800 µg/mL) for 24. The percentage cell viability (quantitated by resazurin assay) was normalized against the negative controls for each cell type. Data are expressed as the mean ± SEM of three separate experiments. $^{*}p< 0.05$, $^{***}p< 0.001$ as compared with control value.

91.29, 81.38 and 41.86 µg/mL for 24, 48 and 72 h, respectively. On the other hand, After 24, 48 and 72 h treatment, the IC_{50} values of *F. gummosa* gum in NB4 cells were found to be 109.30, 89.46 and 59.24 µg/mL, respectively (Table 1). Treatment of HL60 and NB4 cells with >125 µg/mL *F. gummosa* gum for 72 h significantly reduced cell viability more than As_2O_3 ($p<0.001$). The viability of *F. gummosa* gum treated-HL60 cells at 125 µg/mL significantly reduced to 7.5, 6.8 and 5% after 24, 48 and 72 h, respectively (Fig. 1, $p<0.001$). In concentration 250 µg/mL *F. gummosa* gum, viability of HL60 cells reduced to 6.7, 6.5 and 4% against 50.9, 40.8 and 30.9% in As_2O_3 group after 24, 48 and 72 h, respectively (Fig 1, $p<0.001$). While viability of *F. gummosa* gum treated-NB4 cells at 125 µg/mL significantly reduced to 19.0, 13.6 and 7.6% after 24, 48 and 72 h, respectively (Fig 1, $p<0.001$). In concentration 250 µg/mL*F. gummosa* gum, viability of NB4 cells reduced to 13.9, 10.5 and 6.5% against 70.0, 60.5 and 50.7% in As_2O_3 group after 24, 48 and 72 h, respectively (Fig 1, $p<0.001$). In contrast, the resin did not have cytotoxic affect up to 800 µg/mLin PMN cells after 24 h (Fig 1).

F. gummosa gum concentration-dependently increased formation of ROS

Our findings showed a regular statistically time- and concentration-dependent increase in ROS generation in HL60 and NB4 cells, 2 h after treatment with *F. gummosa gum* compared to control group (Fig. 2). On comparing with control HL60 cells, 125 and 250 µg/mL*F. gummosa gum* induced the formation of ROS during 2 h of incubation (648± 15.1 and 784±18.2%, against 601± 14.1% in As_2O_3 group, $p< 0.001$). There is also a significant increase in ROS content in NB4 cells,2 h after treatment with 250 µg/mL*F. gummosa gum* compared to control group (333±17.2% against 285± 18.1% in As_2O_3 group, $p< 0.001$).

F. gummosa gum concentration-dependently induced apoptotic cell death

Figure 3 shows that *F. gummosa gum* at high concentration induces apoptosis in NB4 and HL60 cells, significantly. After 48 h treatment, *F. gummosa gum*-treated HL60 cells showed 89.0% (125µg/mL) and 89.4%

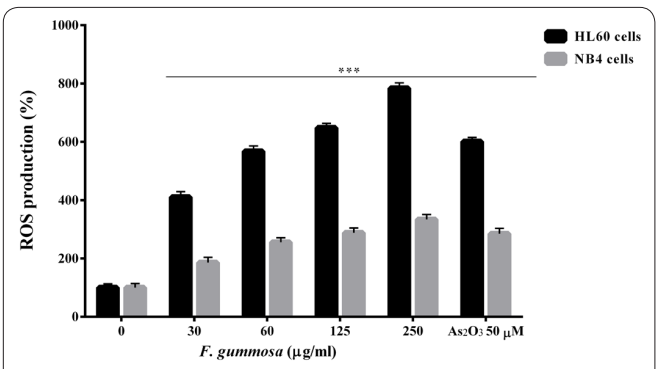

Figure 2. Effect of *F. gummosa* gum on intracellular reactive oxygen species (ROS) in leukemic cells. NB4 and HL60 cells treated with different concentrations of *F. gummosa* gum (30-250 µg/mL) or As_2O_3 (50µM) for 2 h. Data are expressed as the mean ± SEM of three separate experiments. $^{***}p< 0.001$as compared with control value.

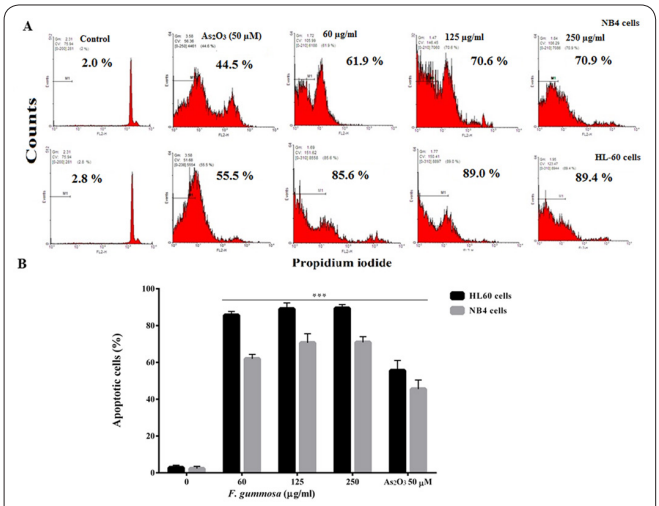

Figure 3. Apoptotic cell death induced by *F. gummosa* gum inleukemic cells. (A) NB4 and HL60 cells were incubated with different concentrations of *F. gummosa* gum (60-250 µg/mL) or As_2O_3 (50 µM) for 48 h. Apoptosis was assayed by PI staining and analysed by flow cytometry. (B) Apoptosis rate shown by bar graph. The data shown are the means ± SEM from three independent experiments. $^{***}p< 0.001$ as compared with control value.

(250 µg/mL) against 55.5% in As_2O_3 (50µM) group ($p< 0.001$). *F. gummosa gum*-treated NB4 cells showed 70.9% (250 µg/mL) against 45.5% in As_2O_3 group ($p< 0.001$). These results showed that the number of apoptotic cells increased concomitantly with concentration, as compared with control cells. The resin increased apoptosis in HL60 more than NB4 cells ($p< 0.001$, Figure 3).

F. gummosa gum did not induce differentiation of leukemic cells toward granulocyte pattern

The Gimsa and NBT tests showed, *F. gummosa gum* did not induce maturation of cells to neutrophils. In morphological Gimsa assay, cells showed promyelocytes characteristic with cytoplasmic granules as well as control. In Functional NBT assay, cells did not have any of intracellular blue-black formazan deposits (Figure 4).

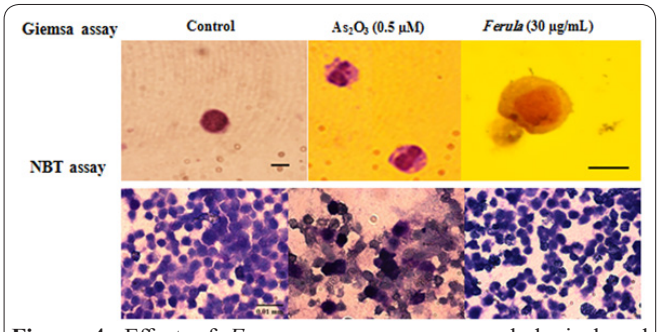

Figure 4. Effect of *F. gummosa* gum on morphological and functional properties of granulocyte in leukemic cells. NB4 cells were treated with *F. gummosa* gum (30µM) orAs_2O_3 (0.5 µM) for 72 h. Morphologic and functional properties of granulocyte determined by Gimsa and NBT assays, respectively. In Giemsa-stained slides, As_2O_3-treated cells (as positive control) showed polymorphonuclear morphology of granulocyte and *F.gummosa*-treated cells showed promyelocytes characteristic, same as control. On NBT slides,As_2O_3-treated cells (as positive control) show intracellular blue-black formazan deposits and *F.gummosa*-treated cells did not have any of intracellular blue-black formazan deposits, (bar represents 0.01 mm).

Discussion

In recent years, the use of natural products such as fruits, vegetables and herbs has been particularly considered because of having active ingredients. It is believed that many of natural products have the potential to act as anticancer agents in human. Many patients use natural products as alternative therapies for cancer or other conditions (26). Several studies have demonstrated herbal medicine are containing different ingredients such as antioxidant compounds which have positive effects against different diseases, such as cancer, coronary diseases, inflammatory disorders, neurologic degeneration, and aging (27, 28). Whereas oxidative stress play role in cancer disease, as result herbal medicine can be have positive effects in cancer disease. The anti-cancer properties of medicinal herbs are mediated through different mechanisms including altered carcinogen metabolism, induction of DNA repair systems, immune activation and suppression of cell cycle progression/induction of apoptosis (29). Herbal phytochemicals are used as promising resources for anticancer remedies or adjuvant for chemotherapeutic drugs to elevate their efficiency and reduce their side effects (30). Nowadays herbal medicines are considered because of cheap and available. This study is the first to investigate the anti-neoplastic and differentiation effects of *F. gummosa* in leukemic cells. Our findings showed *F. gummosa* has significant cytotoxicity in the cancer cells at selective concentrations. The cytotoxic effect of *F. gummosa* was more pronounced against the neoplastic cells than human PMN cells. Our findings showed oleo-resin gum increased cell death through ROS production in cell lines. It is noteworthy to mention that these effects are comparable with standard anti-leukemic drug, As_2O_3. The results in this work indicate that *F. gummosa* causes concentration-dependent proliferation inhibition and ROS dependent apoptosis. The results also were shown that apoptosis induced in HL-60 (AML M2) cells more than NB4 (AML M3) cells. These may relate to inherent metabolic properties of the cancer cells and previous study indicated that NB4 cells showed highly ‘‘glycolytic’’ properties (31, 32).

There have been numerous studies on the effects of different *Ferula* species extracts on cancer cells. Gudarzi et al. investigated the cytotoxic effects of the ethanolic, acetonic and aqueous extracts obtained from the seeds and gum of *F. gummosa* Boiss against three cancer cell lines: MCF-7 (human breast adenocarcinoma), BHY (human oral squamous cancer) and SKMEL-3 (human malignant melanoma) or HGF (human gingival fibroblast) cell line as a control. They found that gum of *F. gummosa* Boiss exerts an anti-cancer effect (33). Phytochemical studies have shown *F. gummosa* gum is containing umbelliprenin, terpenoids and sesquiterpene which have cytotoxic effects on cancer cell lines. Umbelliprenin is synthesized by various Ferula species, and it is a constituent of a number of plant species consumed as food such as; *celery*, *Angelica archangelica*, *Coriandrum sativum*, and *Citrus limon* (34). This compound has cytotoxic effect via 4 pathways including matrix metalloproteinase (MMP) inhibitory effect (35), in vitro and in vivo models have provided evidence of antilipoxygenase, antioxidants; and anti-inflammatory pro-

perties for umbelliprenin (36), it delayed the formation of papilloma in a mouse model (37), induce apoptosis (38). Sesquiterpene lactones are the active constituents of a variety of medicinal plants used in traditional medicine for the treatment of inflammatory diseases (39). Probably, the cytotoxic effect of *F. gummosa* is related to active compounds. In conclusion, the present study demonstrated that *F. gummosa* inhibited the growth and proliferation of leukemic cells without differential effect on leukemic cells. The marked difference in cytotoxicity between cancer and normal cells suggests an exciting potential for *F. gummosa* as novel alternatives to cancer therapy. The IC_{50} values reported in our study may be considerably lower if the pharmacological active compounds become pure. *F. gummosa* not only increased levels of ROS, but also induced concomitant increase of apoptosis. The precise signaling pathway by which *F. gummosa* induce apoptosis needs further research.

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Conflict of interest: none.

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