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ORIGINAL CONTRIBUTIONS

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EVALUATING THE ROLE OF DITHIOLANE RICH FRACTION OF *FERULA ASAFOETIDA* (APIACEAE) FOR ITS ANTIPROLIFERATIVE AND APOPTOTIC PROPERTIES: *IN VITRO* STUDIES

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Asafoetida resin has been reported for various biological activities but its use has been widely restricted owing to its pungent smell and pool water solubility. *Aim: In vitro* study of the anticancer potential of microwave-extracted essential oil (EO) of *Ferula asafoetida*. *Materials and Methods:* The phytochemical investigation and *in vitro* cytotoxicity assessment was carried out in two human liver cancer cell lines. The expression of *NFKB1*, *TGFB1*, *TNF*, *CASP3* was analyzed by reverse transcription polymerase chain reaction. *Results: Ferula asafoetida* EO contains high concentrations of dithiolane, which possess antiproliferative activity in human liver carcinoma cell lines (HepG2 and SK-Hep1) in a dose-dependent manner. The bioactive compounds in *F. asafoetida* are capable of induction of apoptosis and altered NF-kB and TGF-β signalling with increase in caspase-3 and TNF-α expression. *Conclusion:* Further elucidation of bioactive molecules and underlying mechanisms could lead to potential intervention in liver cancer in animal models. The safety and efficacy as well as the mode of EO action in animal models would be highly crucial.

***Key Words*: *F. asafoetida*, essential oil, phytochemicals, hepatocellular carcinoma, dithiolane, HepG2, SK-Hep1.**

In Ayurveda, asafoetida (the aromatic resin from the roots of *Ferula asafetida*) is considered as one of the best condiments for balancing "vata dosha" being helpful for treating digestive and respiratory disorders such as whooping cough, asthma, ulcer, epilepsy, stomachache, flatulence, bronchitis, weak digestion and influenza [1]. Among various activities of asafetida demonstrated in the recent pharmacological and biological studies are antioxidant [2], antimicrobial [3], antiviral [4], antifungal [5], cancer chemopreventive [6], antidiabetic [7], anticarcinogenic [8], antispasmodic and hypotensive [9], relaxant [10] and neuroprotective [11] properties.

The essential oils (EOs) are the secondary metabolites present in lower amounts in various plant parts. The composition and other biological properties of the EOs depend on their constituents. EOs are among the most valuable plant products used in the medicine and complementary treatment strategies. Exploration of EOs and their constituents toward their beneficial role in different cancers is currently under lens. EOs of *Hypericum hircinum* L. subsp. *Majus, Pinus wallichiana, Solanum erianthum, Thymus vulgaris* L., *Mentha arvensis, Guatteria pogonopus, Ageratum conyzoides* Linnæus and *Lippia multiflora* Moldenke revealed antiproliferative activity on human prostatic adenocarcinoma [12]. Bayala *et al*. [13] also demonstrated antiproliferative activity of EOs in prostate cancer and glioblastoma cell lines. Generally, biological activity of an EO is related to its chemical composition, to the major functional groups of compounds (alcohols, phenols, terpene compounds and ketone). However, some of the minor compounds could also be of importance as the various molecules could synergistically act with the major compounds of Eos.

The current study was carried out with the aim of determining the pro-apoptotic and antiproliferative efficacy of microwave-extracted EO of *Ferula asafetida* in hepatocellular carcinoma (HCC) cell lines. We also analyzed the expression of several genes that could be involved in mediating such activities.

MATERIALS AND METHODS

Plant material and EO preparation. Resin of *Ferula asafoetida* was obtained from National Foods (Hing Manufacturer Company, Vadodara) and the sample was authenticated by Dr. Vasant A. Patel, Department of Botany, Smt. S.M.P. Science College, Hemchandracharya North Gujarat University, Gujarat, India. Specimen sample of *Ferula asafoetida* has been submitted at the Institute of Science, Nirma University, Ahmedabad, Gujarat, India with the voucher no. ISNU/FA/CN-120421/01.

The EO was obtained by microwave-assisted hydro-distillation of resin at 750 W at 100 °C for 30 min. The EO was collected and then stored in refrigerator. Extraction efficiency was found to be 8.3 ± 0.2%.

Gas chromatographic — mass spectrometric analysis. The phytochemical investigation of EO of *Ferula asafoetida* was performed by a gas chromatography — mass spectrometry (GC-MS) equipment Agilent 7890A GCMS Single Quad (Agilent, USA) along with headspace and flavor library with flame ionization detector (FID) and an HP-5 capillary column (30 m, 0.25 mm i.d., film thickness 250 nm). The GC oven temperature was kept at 60 °C for 10 min, increased to 220 °C at a rate of 4 °C/min, kept constant at 220 °C for 10 min, and then increased to 240 °C at a rate of 1 °C/min. The sample was injected in a volume of 0.1 µl. The split ratio was 40:1 [14].

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Abbreviations used: DMSO – dimethyl sulfoxide; EO – essential oil; FBS – fetal bovine serum; HCC – hepatocellular carcinoma.

Cell culture. Human liver cancer cell lines HepG2 and SK-Hep1 were obtained from NCCS, Pune, India. The cells were cultured in Eagle's minimal essential media with 10% FBS at 37 °C in the atmosphere of 5% CO_2. The cells were subcultured by trypsinization followed by splitting the cell suspension into fresh flasks and supplementing with fresh culture medium.

The isolated EO was diluted in ethanol to obtain a stock solution corresponding to 0.5% (v/v). The above stock solution was then used for preparation of serial dilutions. Doxorubicin hydrochloride used as a positive control was dissolved in DMSO to obtain the primary stock solution of 20 mM that was further used for preparation of serial dilutions.

Cytotoxicity assay. The cells were trypsinised and counted using hemocytometer and then plated in a 96-well plate at the density of $5 \cdot 10^3$ cells/well. The cells were incubated overnight under desired growth conditions to allow the exponential growth. The cells were then treated with increasing concentrations of EO and doxorubicin as a positive control, respectively, followed by incubation for 48 h. Following incubation, the plates were taken out and 20 µl of 5 mg/ml of MTT 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution were added to all the wells followed by additional incubation for 3 h at 37 °C. The supernatant was aspirated and 150 µl of DMSO was added to each well to dissolve formazan crystals. The absorbance of each well was then read at 540 nm [15].

The percentage cytotoxicity corresponding to each treatment was calculated using the following formula:

$$\% \ Cytotoxicity = (1\text{-}X/R) \cdot 100,$$

where X = absorbance of treated cells; R = absorbance of untreated cells.

Gene expression studies. Total RNA was isolated using TRI reagent (Sigma-Aldrich, USA), according to the manufacturer's protocols. The quantification RNA was determined at 260 and 280 nm (UV-2450, Spectrophotometer, Shimadzu, Japan). Gene expression study was carried out for *NFKB1*, *TGFB1*, *TNF*, *CASP3*, and was assessed by reverse transcription polymerase chain reaction. Reverse transcription was carried out using a First strand cDNA synthesis Kit (Thermo Scientific, USA). β-Actin was used as the normalization control.

Polymerase chain reaction was carried out as per the protocol of Jena *et al.* [16]. Relative quantities of different mRNA expressions were analyzed by TotalLab 1.0 software (Magnitec Ltd., Israel), normalized with β-actin expression.

Statistical analysis. GraphPad Prism V.6 was used to perform statistical analysis. One way ANOVA followed by Tukey's multiple comparison test was used to determine significance level at 95% significant interval between experimental groups. All values are expressed as mean ± SD.

RESULTS

EO composition. The yield of EO isolated from Kabuli was around 16%. The GC analysis of EO of asafoetida, showed presence of 1,2-dithiolane (87.4%) and n-propyl sec-butyl disulfide (10.6%) which comprised of 98%. Other constituents included sec-butanethiol (0.26%), propyl disulfide (0.35%), sec-butyl disulfide (0.32%), 3,6-dimethyl-1,4-dithiane-2,5-dione (0.30%), 1,3-dithiane (0.51%) and allyl sulfone (0.22%) comprising the remaining 2% (Fig. 1).

Effect of Ferula asafoetida EO on HCC cell lines. Dithiolane-rich EO was evaluated for its cytotoxic potentials by performing the MTT assay. The oil exhibited profound cytotoxic potentials on both the cell lines in a dose dependent manner. IC_{50} value of the oil was calculated through the growth curve derived from the MTT assay (Fig. 2) and was found to be 7.21 ± 0.29 µg/ml and 8.0 ± 0.36 µg/ml for HepG2 and SK-Hep1, respectively. The isolated EO was found to be equally effective against both cell lines in a dose dependent manner.

Mode of action determination of Ferula asafoetida. Following treatment with EO, both *NFKB1* and

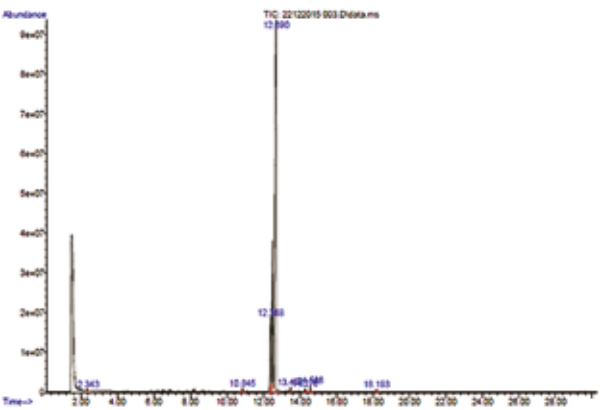

Fig. 1. GC-MS analysis of EO showing peaks of maximum value of 1,2-diothiolane

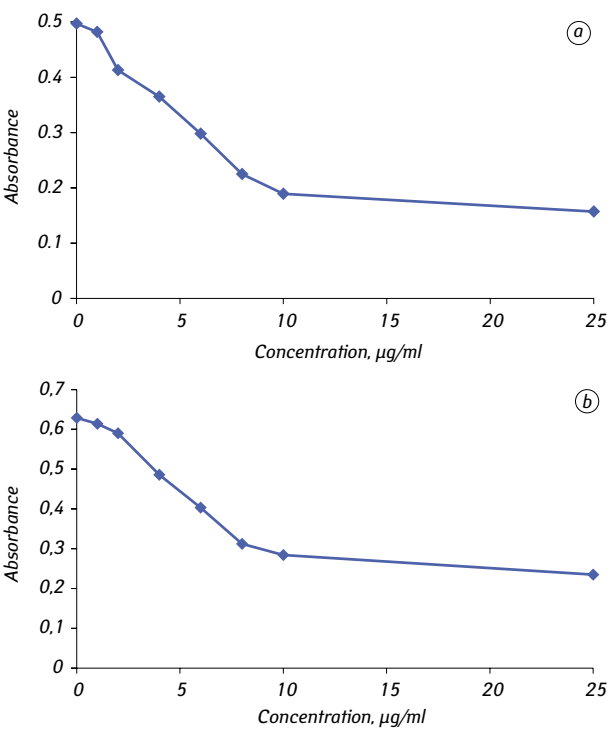

Fig. 2. MTT assay of HepG2 (*a*) and SK-Hep1 (*b*) following treatment with isolated asafoetida EO

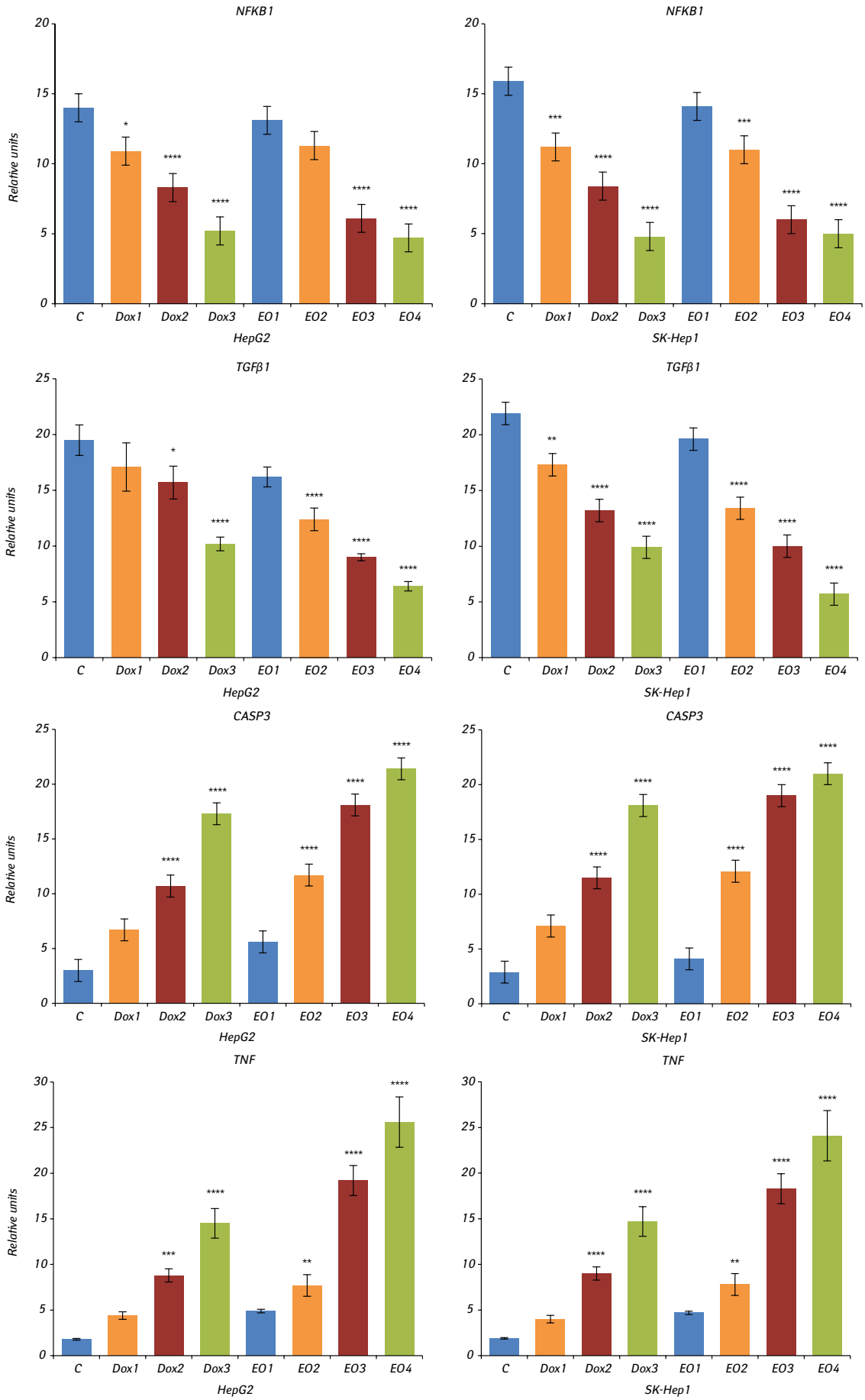

Fig. 3. *NFKB1, TGFB1, CASP3* and *TNF* expression in cell lines following treatment with isolated asafoetida EO. D1, D2, D3 — cells treated with 0.1, 0.2 and 0.5 μM doxorubicin, respectively. EO1, EO2, EO3, EO4 — cells treated with 1, 10, 25 and 50 μg/ml EO, respectively. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

TGFB1 were downregulated as shown in Fig. 3. Simultaneously, *CASP3* and *TNF* showed significant elevation after treatment with EO (see Fig. 3).

DISCUSSION

Indian traditional medicine emphasizes the role of *Ferula asafoetida* as a potential drug for cancer treatment. The studies have showed that asafoetida has potent antitumor and antimetastatic effects in experimental breast cancer model [17]. There is a study exploring the chemopreventive potential of asafoetida against dimethylhydrazine-induced rat colon cancer [18]. Asafoetida extract inhibited two stage chemical carcinogenesis induced by 7,12-dimethylbenzanthracene and croton oil on mice skin with significant reduction in papilloma formation [19].

There have been many reports regarding the efficacy of EO from different plants on HCC cell lines. The antihepatoma activity was found in the IC_{50} of 55 μg/ml of *A. squamosa* L. pericarps [20]. *S. intermedia* volatile oil was cytotoxic for HepG2 and MCF-7 cells [21]. *Siegesbeckia pubescens* volatile oil activated apoptotic proteins in HepG2 [22].

Umbelliferone (7-hydroxycoumarin), a widespread natural product of the coumarin family, has been reported to exhibit antitumor and immunomodulatory effects against sarcoma 180 in mice, inhibiting tumor growth and increasing survival time of tumor bearing animals [23]. Umbelliferone induced apoptosis in HepG2 cells, which shows the potential to be used in the management and treatment of liver cancer [24].

The present study was a preliminary effort to evaluate the effects of EO of *Ferula asafoetida* in HCC cell lines. The oil inhibited the proliferation of HepG2 and SK-Hep1 cells in a dose dependent manner. Moreover, in the present study, the *Ferula asafoetida* isolated oil significantly reduced the expression of *NFKB1* and *TGFB1*. It should be mentioned that NF-κB plays important roles in processes, including development, cell growth and survival, and proliferation and are involved in many pathological conditions. An increase in NF-κB expression in the nucleus correlates with the development of metastases [25].

The significance of caspase activation in hepatocytes undergoing apoptosis in the liver with permeabilization of the mitochondrial external membrane and release of different proteins from the mitochondrial intermembrane space into the cytosol has been demonstrated [26]. Targeting caspase activity has gained significant attention for developing novel strategies to combat cancer [27].

TNF-α has been reported to be involved in inducing pro-apoptotic activities through both caspase dependent as well as caspase independent pathways [28]. In liver fibrosis, TNF-α plays an important role in the activation of hepatic stellate cells and extracellular matrix deposition [29].

In the present study, both *CASP3* and *TNF-α* show the significant increase following treatment with EO, thus controlling the carcinogenesis.

From the present investigation, it would be concluded that the dithiolane-rich EO displayed pro-apoptotic and antiproliferative potential against human liver cancer. The safety and efficacy on the oil needs to be ascertained on *in vivo* models.

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REFERENCES

1. Al-Jafari AH, Vila R, Freixa B, *et al.* Antifungal compounds from the rhizome and roots of *F. hermonis*. Phytother Res 2013; **27**: 911–5.

2. Kavoosi G, Rowshan V. Chemical composition, antioxidant and antimicrobial activities of essential oil obtained from *Ferula asafoetida* oleo-gum-resin: effect of collection time. Food Chem 2013; **138**: 2180–7.

3. Bhatnager R, Rani R, Dang AS. Antibacterial activity of *Ferula asafoetida*: a comparison of red and white type. J Appl Biol Biotechnol 2015; **3**: 18–21.

4. Mahendra P, Bisht S. *Ferula asafoetida*: traditional uses and pharmacological activity. Pharmacogn Rev 2012; **6**: 141–6.

5. Mostafa Z, Soheil P, Mahdi J, *et al.* Antifungal effects of asafoetida seed essential oil on *in vitro* growth of five species of plant pathogenic fungi. Int Res J Appl Basic Sci 2013; **4**: 1159–62.

6. Saleem M, Alam A, Sultana S. Asafoetida inhibits early events of carcinogenesis:a chemopreventive study. Life Sci 2001; **68**: 1913–21.

7. Abu-Zaiton AS. Anti-diabetic activity of *Ferula asafetida* extract in normal and alloxan-induced diabetic rats. Pak J Biol Sci 2010; **13**: 97–100.

8. Mallikarjuna GU, Dhanalakshmi S, Raisuddin S, *et al.* Chemomodulatory influence of *Ferula asafoetida* on mammary epithelial differentiation, hepatic drug metabolizing enzymes, antioxidant profiles and N-methyl-N-nitrosourea- induced mammary carcinogenesis in rats. Breast Cancer Res Treat 2003; **81**: 1–10.

9. Fatehi M, Farifteh F, Fatehi-Hassanabad Z. Antispasmodic and hypotensive effects of *Ferula asafoetida* gum extract. J Ethnopharmacol 2004; **91**: 321–4.

10. Khazdair MR, Boskabady MH. The relaxant effect of *Ferula asafoetida* on smooth muscles and the possible mechanisms. J Herb Med Pharmacol 2015; **4**: 40–4.

11. Moghadam FH, Dehghan M, Zarepur E, *et al*. Oleo gum resin of *Ferula assa-foetida* L. ameliorates peripheral neuropathy in mice. J Ethnopharmacol 2014; **154**: 183–9.

12. Bayala B, Bassole IHN, Scifo R, *et al*. Anticancer activity of essential oils and their chemical components — a review. Am J Cancer Res 2014; **4**: 591–607.

13. Bayala B, Bassole IH, Gnoula C, *et al.* Chemical composition, antioxidant, anti-inflammatory and anti-proliferative activities of essential oils of plants from Burkina Faso. PLoS One 2014; **9**: e92122.

14. Kameli Z, Alizadeh A, Aboutalebi A. Bitter and Sweet *Ferula assa-foetida* L. essential oil composition from Iran and its comparison with *Ferula assa-foetida* L. used in India. Intl Res J Appl Basic Sci 2012; **3:** 919–23.

15. Chaudhary H, Jena PK, Seshadri S. Evaluation of hydroalcoholic extract of *Eclipta alba* for its multidrug resistance reversal potential: an *in vitro* study. Nutrition and Cancer 2013; **65**: 775–80.

16. Jena PK, Prajapati B, Mishra PK, *et al.* Influence of gut microbiota on inflammation and pathogenesis of sugar rich diet induced diabetes. Immunome Res 2016; **12**: 109–19.

17. Bagheri SM, Abdian-Asl A, Moghadam MT, *et al*. Antitumor effect of *Ferula assa foetida* oleo gum resin against breast cancer induced by 4T1 cells in BALB/c mice. J Ayurveda Integ Med 2017; **8**: 152–8.

18. Panwar R, Rana PS, Dhawan DK, *et al.* Chemopreventive efficacy of different doses of *Ferula asafoetida* oleo-gum-resin against 1, 2-dimethylhydrazine (DMH) induced rat colon carcinogenesis. J Phytopharm 2015; **4**: 282–6.

19. Unnikrishnan MC, Kuttan R. Tumour reducing and anticarcinogenic activity of selected spices. Cancer Lett 1990; **51**: 85–9.

20. Chen YY, Peng CX, Hu Y, *et al.* Studies on chemical constituents and anti-hepatoma effects of essential oil from *Annona squamosa* L. pericarps. Nat Prod Res 2017; **31**: 1305–8.

21. Sharifi-Rad J, Sharifi-Rad M, Hoseini-Alfatemi SM, *et al.* Composition, cytotoxic and antimicrobial activities of *Satureja intermedia* CA Mey essential oil. Int J Mol Sci 2015; **16**: 17812–25.

22. Lv D, Guo KW, Xu C, *et al.* Essential oil from *Siegesbeckia pubescens* induces apoptosis through the mitochondrial pathway in human HepG2 cells. J Huazhong Uni Sci Technol Med Sci 2017; **37**: 87–92.

23. Stefanova TH, Nikolova NJ, Toshkova RA, *et al*. Antitumor and immunomodulatory effect of coumarin and 7-hydroxycoumarin against Sarcoma 180 in mice. J Exp Ther Oncol 2007; **6**: 107–15.

24. Yu SM, Hu DH, Zhang JJ. Umbelliferone exhibits anticancer activity via the induction of apoptosis and cell cycle arrest in HepG2 hepatocellular carcinoma cells. Mol Med Rep 2015; **12**: 3869–73.

25. Aggarwal BB. Nuclear factor-kappa B: the enemy within. Cancer Cell 2004; **6**: 203–8.

26. Li Z, Berk M, McIntyre TM, *et al*. The lysosomal-mitochondrial axis in free fatty acid-induced hepatic lipotoxicity. Hepatol 2007; **47**: 1495–503.

27. Green DR. Apoptotic pathways: ten minutes to dead. Cell 2005; **121**: 671–4.

28. Lai W, Fenghe D, Xiaodong W. TNF-α induces two distinct caspase-8 activation pathways. Cell 2008; **133**: 693–703.

29. Connolly MK, Bedrosian AS, Mallen-St Clair J, *et al.* In liver fibrosis, dendritic cells govern hepatic inflammation in mice via TNF-alpha. J Clin Invest 2009; **119**: 3213–25.