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Apoptosis-inducing effects of extracts from desert plants in HepG2 human hepatocarcinoma cells

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PEER REVIEW

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Comments

The work is a good standard report on ethnopharmacology on the available plants species from deserts. The work contains new knowledge on the studied local species. The standard investigation technique is used and makes the work to be further referenced. The findings make sense and can be further studied in other following researches.

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ABSTRACT

Objective: To investigat the mechanism of antitumor efficacy of *Origanum dayi* (*O. dayi*) and *Ochradenus baccatus* (*O. baccatus*) extracts by exploring apoptosis-inducing potential.

Methods: The aqueous extracts of aerial parts of aforementioned plants were prepared and used for this study. HepG2 cells were treated with varying concentrations (0, 2 and 5 mg/mL) of each plant extract for 24 or 48 h. Cell apoptosis was measured by annexin V-fluorescein isothiocyanate binding assay and flow cytometry. The expression levels of various apoptosis-related genes were determined by semi-quantitative reverse transcription-polymerase chain reaction.

Results: *O. dayi* and *O. baccatus* extracts exerted apoptotic effects on HepG2 cells for 48 h following treatment. *O. dayi* extract was found to be a better apoptosis-inducing agent than *O. baccatus* extract as the former delivered greater efficacy at a lower concentration. Both extracts manifested upregulation of Bax, Bad, cytochrome c, caspase-3, caspase-7, caspase-9 and poly (adenosine diphosphate-ribose) polymerase.

Conclusions: The aqueous extracts of *O. dayi* and *O. baccatus* are capable of inducing apoptosis in HepG2 cells through modulation of mitochondrial pathway which explains their antitumor activities. These desert plants may serve as useful resources to develop effective remedies for hepatocellular carcinoma and other human malignancies.

KEYWORDS

Ochradenus baccatus, *Origanum dayi*, HepG2 cells, Apoptosis, Gene expression

1. Introduction

Throughout the centuries humans have relied substantially on natural products to treat a variety of diseases and natural agents represent a continuing source for novel drug discovery[1]. According to an estimation made by World Health Organization,

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currently 65% of the World's population relies primarily on traditional medicines derived from plants for primary health care need[2]. Traditional medicinal plants have been used in treating cancer for several millennia in several parts of the globe and herbal medicines alone or in combination with conventional therapeutics are currently being used for cancer treatment worldwide[3-6]. Plant-derived natural products represent a rich source for the discovery and development of anticancer drugs[2,7,8]. Approximately, 80% of all drugs approved by the United States Food and Drug Administration during the last three decades (1981-2010) for cancer therapy are either natural products per se or are based on naturally occurring molecules[9]. With the advent and refinement of new technologies, such as genetic techniques for production of secondary plant metabolites, combinatorial synthesis and high-throughput screening, it is expected that novel compounds from medicinal plants would be identified and developed as safe and effective anticancer drugs.

Plants growing under adverse environmental conditions, such as low nutrient soils, scarcity of water, extreme temperature and strong solar radiation, have developed unique defense system to survive. Phytochemicals (secondary metabolites) endowed with remarkable bioactivities, such as antioxidant, anti-inflammatory, antimutagenic, antiviral, antifungal and antimicrobial properties, represent plant defense systems[10-13]. Desert plant phytochemicals that protect these plants against various stressful environmental conditions may also be effective in preventing and combating adverse pathophysiological anomalies and complex diseases, including cancer. A large number of *in vitro* and *in vivo* studies have provided substantial evidence that botanical extracts as well as isolated phytocompounds derived from plants of desert and semidesert habitats exert potent oncosuppressive and anticancer activities[13-15].

Although numerous desert plants have been studied for their antitumor properties, it is likely that a large number of plants from desert origin are not used for systematic evaluation of their phytoconstituents as potential anticancer agents. Accordingly, we have initiated a comprehensive research program to screen antitumor activities of previously unexplored desert plants. The aqueous extracts of aerial parts of several desert plants, namely *Achillea fragrantissima* (Forssk.) Sch. Bip. (Compositae), *Ochradenus baccatus* Delile (Resedaceae) (*O. baccatus*), *Origanum dayi* Post (Lamiaceae) (*O. dayi*), *Phlomis platystegia* Post (Lamiaceae), and *Varthemia iphionoides* Boiss (Compositae), were prepared and tested against an *in vitro* tumor model utilizing HepG2 human hepatocellular carcinoma cell line. According to our recent communication[16], *O. dayi* extract exhibited a substantial cytotoxic effect against HepG2 cells followed by *O. baccatus* following 24 h exposure with the plant material. Our results also indicated that all plant extracts displayed antiproliferative effect following 48 h exposure. However, a substantial effect was observed with *O. dayi* followed by *O. baccatus*[16]. In spite of encouraging antitumor effects of the aqueous extracts of *O. dayi* and *O. baccatus*, the underlying molecular mechanisms are not understood. Hence, the objective of the present study was to investigate the mechanism of anticancer efficacy of *O. dayi* and *O. baccatus* by exploring apoptosis-inducing potential of these plant extracts using HepG2 human hepatocellular carcinoma cells. To the best of our knowledge and belief, this is the first study investigating the cellular and molecular mechanisms of anticancer effects of *O. dayi* and *O. baccatus*.

2. Materials and methods

2.1. Preparation of plant aqueous extracts

The aqueous extracts of freshly collected aerial parts of *O. baccatus* and *O. dayi* were prepared according to our previously published method[16]. The extracts were stored at 4°C in a refrigerator and subsequently used for various assays as indicated below.

2.2. Cell culture

HepG2 cells were purchased from the American Tissue Culture Collection (Manassas, VA) and grown in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (all purchased from HyClone, Logan, UT). HepG2 cells were incubated in a humidified incubator (NAPCO Series 8000 WJ, ThermoFisher Scientific, Waltham, MA) at 37 °C under an atmosphere containing 5% CO_2. The cells were subcultured at preconfluent densities using 0.25% trypsin-EDTA (Millipore, Phillipsburg, NJ).

2.3. Apoptosis assay by flow cytometry

Apoptosis in HepG2 cells was detected using annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Biolegend, San Diego, CA) following the manufacturer's instruction. Briefly, cells were plated into 6-well culture dishes (3×10^5 cells/well) for 24 h prior to the addition of plant extracts (0, 2 or 5 mg/mL). Following 24 or 48 h incubation with the extract, the percentage of apoptotic cells was determined by the annexin V-FITC/PI assay. The cells were harvested, washed with cold phosphate-buffered saline and resuspended in binding buffer. The cells were treated with annexin V-FITC conjugate and incubated for 15 min at room temperature in the dark condition. The cells were then stained with PI (5 μg/mL) and analyzed by flow cytometry using a BD Accuri C6 software (Accuri® C6 Flow Cytometer, Ann Arbor, MI) within 1 h following the staining. The data acquisition and analysis were performed using BD Accuri C6 Flow software and a minimum of 10000 cells per sample was analyzed and data stored in list mode. Electronic compensation was used to eliminate bleed through fluorescence.

2.4. Gene expression assay

Total RNA from HepG2 cells exposed to each plant extract for indicated time periods was extracted using Quick RNA miniPrep kit (Zymo Research, Irvine, CA) following the vendor's protocol. Briefly, cells were plated into 6-well culture dishes (3×10^5 cells/well) for 24 h prior to the addition of plant extracts (0, 2 or 5 mg/mL). The expression levels of apoptosis-related genes were monitored by semi-quantitative RT-PCR using the cDNA verso kit with a temperature scale of 42 °C for 30 min for RT, and 32 cycles of 94 °C for 30 second, 56 °C for 30 second, and 72 °C for 30 second. The RT-PCR was carried out using the primers: BAX-F – 5'-AGG GGC CTT TTT GTT ACA GG-3', BAX-R – 5'-ACG TCA GCA ATC ATC CTC TG-3'; BAD-F – 5'-GAG CTG ACG TAC AGC GTT GA-3', BAD-R – 5'-GGG TAG GGT GTG TGG AAA AC-3'; CASP3-F – 5'-AGG GTG CTA CGA TCC ACC AGC A-3', CASP3-R – 5'-CCA TGG CTC TGC TCC GGC TC-3'; CASP7-F – 5'-GCC ATG CCC AGG ACA AGC CA-3', CASP7-R – 5'-GCA CGC CGG AGG ACA TGG TT-3'; PARP-F – 5'-CGA CAC GTT AGC GGA GCG

GAC-3', PARP-R – 5'-GCG CCC GCT CTT AGC GTA CT-3'; CYT C-F – 5'-AGA CTC ACC CGT GCT TCA GT-3', CYT C-R – 5'-ACT CCC AAT CAG GCA TGA AC-3' and GAPDH-F – 5'-AGA CAG CCG CAT CTT CTT GT-3'; GAPDH-R – 5'-TAC TCA GCA CCA GCA TCA CC-3'. These primer sequences were designed utilizing the Primer3 online program and synthesized by Eurofins MWG Operon (Huntsville, AL). The PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide staining. The GAPDH was used as the housekeeping gene.

3. Results

In this study, aqueous extracts of aerial parts of two desert plants (*O. baccatus* and *O. dayi*) were prepared and used for *in vitro* experiments. The HepG2 cells were exposed to varying concentrations of each plant extract for either 24 or 48 h and apoptosis was measured by annexin V-FITC/PI assay. The results are displayed in Figures 1 and 2. The addition of *O. baccatus* extract (at 2 or 5 mg/mL) to the cell culture medium marginally induced cell

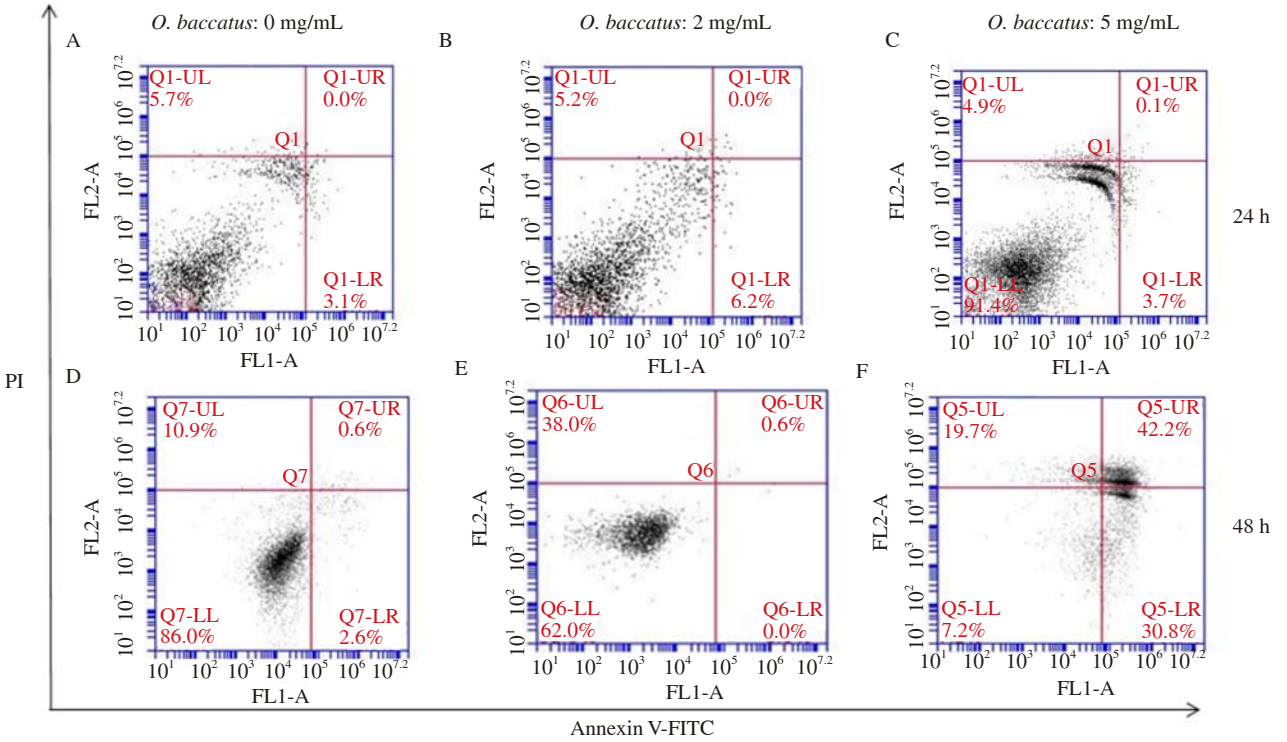

Figure 1. An aqueous extract of *O. baccatus* induces apoptosis of HepG2 cells.

FL1-A: fluorescence 1 area; FL2-A: fluorescence 2 area.

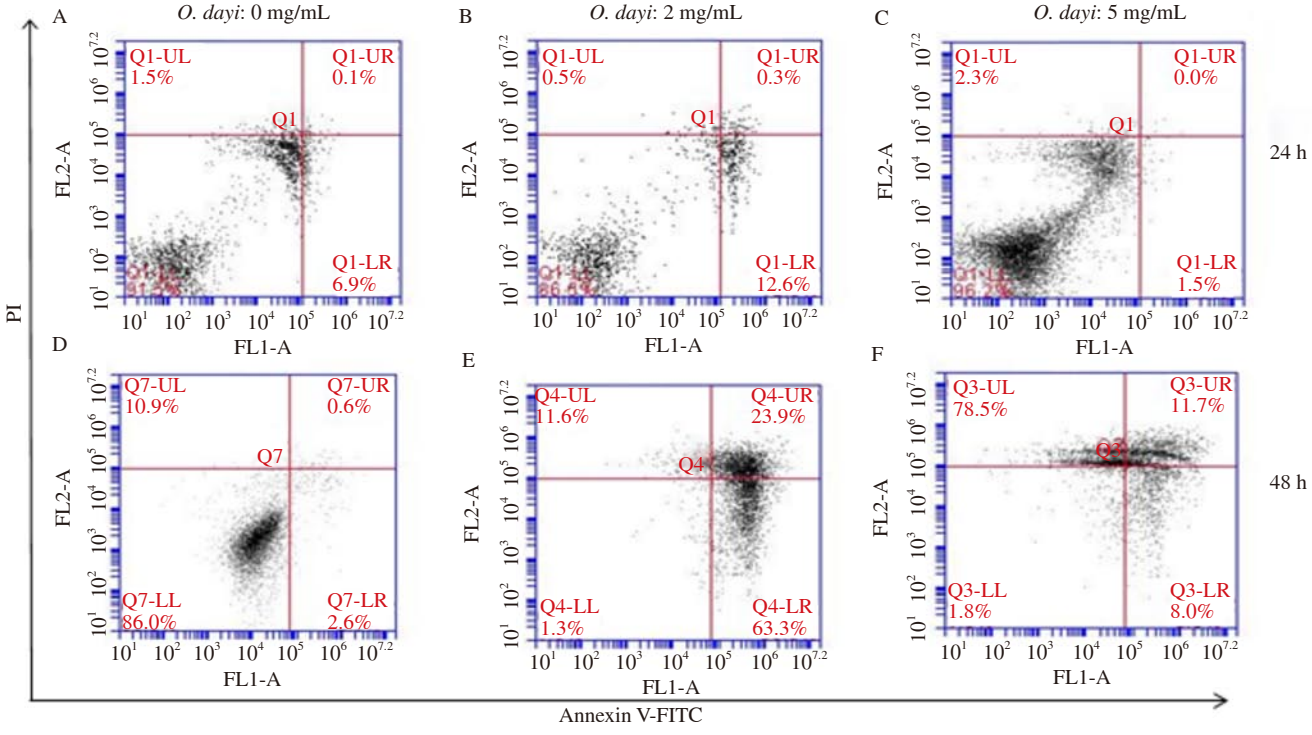

Figure 2. An aqueous extract of *O. dayi* induces apoptosis of HepG2 cells.

FL1-A: fluorescence 1 area; FL2-A: fluorescence 2 area.

killing effect following 24 h as compared to untreated cells (Figures 1A-1C). Although the same extract at 2 mg/mL did not exhibit any apoptotic death following 48 h exposure compared to untreated cells (Figures 1D and 1E), the proportion of apoptotic cells were substantially elevated due to the extract treatment at 5 mg/mL at the same time point (48 h) compared to untreated cells (Figures 1D-1F). In the case of *O. dayi*, a slight increase in the percentage of apoptotic cells was noticed due to the extract treatment at 2 mg/mL as early as 24 h compared to untreated control cells (Figures 2A and 2B). However, the extract at 5 mg/mL was as not effective as the same at a lower concentration for the same time point study (Figure 2C). Interestingly, a marked increase in the proportion of apoptotic cells (63.3%) was observed with *O. dayi* extract at 2 mg/mL at 48 h post treatment in comparison with that of control cells (2.6%) (Figures 2D and 2E). After 48 h, the higher concentration manifested less pronounced effect in terms of apoptosis induction (Figures 2D and 2F).

HepG2 cells exposed to *O. baccatus* and *O. dayi* extracts for various time periods were used to perform RT-PCR to determine expression of several apoptosis-related genes. As depicted in -s 3 and 4, both plant extracts at (2 or 5 mg/mL) elevated the mRNA expression of *BAX, BAD, CASP3, CASP7, CASP9, PARP* and *CYT C*. A better result was noticed for most of the genes 48 h following each extract treatment compared to 24 h post-treatment.

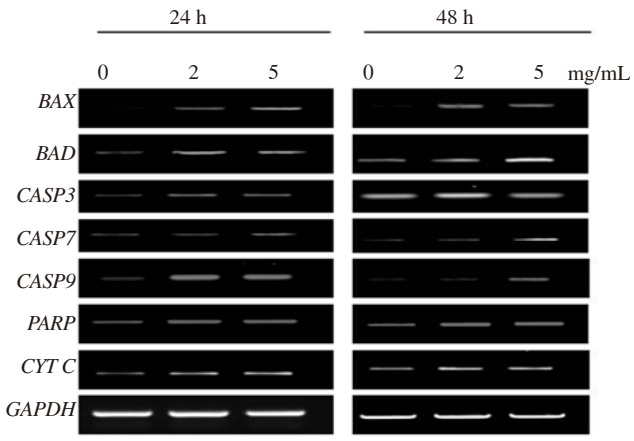

Figure 3. An aqueous extract of *O. baccatus* alters the expression of apoptosis-related genes in HepG2 cells.

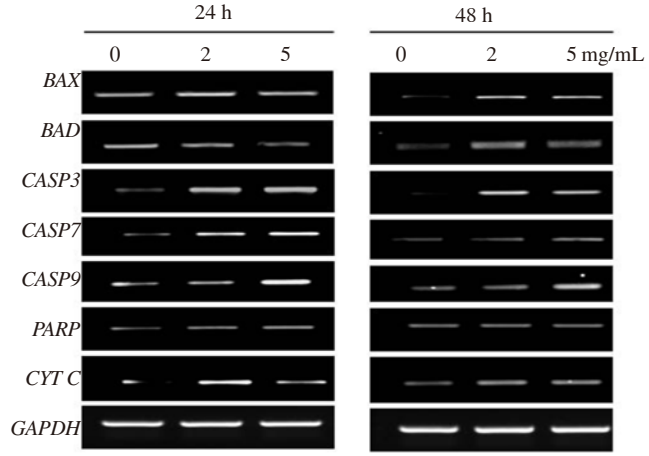

Figure 4. An aqueous extract of *O. dayi* alters the expression of apoptosis-related genes in HepG2 cells.

4. Discussion

Bioactive plant extracts are becoming useful resources to develop less toxic and more effective drugs to manage cancer progression. We have recently reported antitumor effects of extracts from several plants of desert origin. The most promising results were noticed with aqueous extracts of *O. baccatus* and *O. dayi* against HepG2 human liver cancer cells[16]. Nevertheless, the mechanisms of such antitumor activities were not studied. Accordingly, the present work was initiated to probe into possible cellular and molecular mechanism of action of previously reported antiproliferative effects of these two desert plants.

Apoptosis or programmed cell death, a highly structured and orchestrated process, plays an important role in regulating cell number for the growth and homeostasis of tissues by removing aged, damaged and unwanted cells. This self-destructive cellular process is critical for organ development, tissue remodeling, immune regulation and several disease conditions[17]. The homeostasis in eukaryotic cells is subjected to a delicate balance between survival and death signals originated from extracellular domain[18]. One of the salient characteristics of carcinogenesis is that the dividing tumor cells fail to initiate apoptosis following DNA damage[19]. Development of approaches that reinstall the apoptotic machinery selectively within tumor cells could be an effective measure of cancer control[20]. The primary mode of action of most anticancer drugs entails induction of apoptosis in neoplastic cells. Likewise, a large number of natural agents have shown promising anticancer or cancer preventive properties by induction of apoptotic pathways in transformed cells during process of carcinogenesis[21-24].

Various techniques have been developed to detect apoptosis based on several events in the apoptotic process. One of the early events, cells which are undergoing apoptosis reorient phosphatidylserine from the inner side of the plasma membrane to its outer leaflet. Under this condition, cells can bind to annexin V and this process can be used as a marker of apoptosis[25]. In the present study, apoptosis in HepG2 cells exposed to *O. baccatus* and *O. dayi* extracts was determined by annexin V assay. Our results captured using annexin V staining showed that both *O. baccatus* and *O. dayi* plant extracts elicited apoptosis in HepG2 cells 48 h following the treatment with each plant material. The extent of apoptosis induction appears to be greater with *O. dayi* at 2 mg/m/L compared to that of *O. baccatus*. A higher concentration of extract of *O. baccatus* (5 mg/mL) was necessary to visualize any positive effect in terms of apoptosis induction. We previously observed a substantial antiproliferative effects of *O. dayi* followed by *O. baccatus* plant extract against HepG2 cells[16]. Taken together, we suggest that the antitumor effects *O. dayi* and *O. baccatus* plant extracts against HepG2 cells could be achieved, at least in part, by promotion of cell apoptosis.

The mechanisms of apoptosis predominantly involve two signaling pathways, namely mitochondrial (intrinsic) pathway and death receptor (extrinsic) pathway. The death receptor-mediated pathway engages Fas/FasL and other members of the tumor necrosis factor receptor family that activate caspase-8[26,27]. The mitochondrial pathway, implicated in the function of a majority of anticancer drugs, utilizes cytochrome c (cyt. c), apoptotic protease activating factor 1 and caspase-9[28]. The mitochondrial apoptotic pathways is regulated by the Bcl-2 family proteins, including pro-apoptotic proteins, such as Bax, Bad and Bak, and anti-apoptotic proteins, namely Bcl-2, Bcl-xL and Bcl-w[29,30]. The activation of Bax and inhibition of Bcl-2 results in mitochondrial disruption and subsequent release of cyt. c through the outer mitochondrial membrane into the cytosol. Inside the cytosol, cyt. c associates with apoptotic protease activating factor 1 and activates caspase-9 which, in turn, triggers the activation of caspase-3 and/or caspase-7[31,32]. The activated caspase-3 functions

as the key executioner of apoptosis and leads to the cleavage and inactivation of key cellular substrates, including poly (adenosine diphosphate-ribose) polymerase (PARP)[33]. The PARP, a 116 kDa DNA repair enzyme, is mostly activated by DNA strand breaks. The cleavage of PARP has been considered to be a key evidence of caspase-3 activation and apoptosis[34]. In the current study, we measured the expression of various mitochondrial apoptosis-related genes at transcriptional level. Our gene expression study indicates upregulation of Bax, Bad, cyt. c., caspase-3, caspase-7, caspase-9, and PARP. All these results suggest that *O. dayi* and *O. baccatus* bioactive phytoconstituents induce apoptosis in HepG2 cells through mechanisms involving mitochondria-dependent pathway. This is in line with results reported from our laboratory showing crucial role of mitochondrial apoptotic pathway in antitumor efficacy of various natural products in experimental hepatocarcinogenesis[35-37]. Similar tumor cell growth inhibitory and proapoptotic activity via mitochondrial pathway has been observed with phytoconstituents present in an Australian desert tree *Acacia victoriae* (Bentham)[38].

The identity of active phytochemicals responsible for the observed apoptosis-inducing effect is not known at the present time and requires additional experiment. Phytochemical analysis of the whole *O. dayi* plant yielded a large number of volatile constituents, including 1,8-cineole, α-terpineol, (*E*)-sabinene hydrate, (*E*)-sabinene hydrate acetate, terpinen-4-ol and linalyl acetate[39]. Other investigators have identified various phytochemicals, including flavonoids (quercetin 3-*O*-β-glucosyl(1→2)-α-rhamnoside-7-*O*-α-rhamnoside and quercetin 3-*O*-*p*-coumaryl(1→6)-β-glucosyl(1→6)-β-glucoside-7-*O*-α rhamnoside), quercetin glycosides (quercetin 3-gentiobioside, isoquercitrin and quercitrin) and kaempferol glycosides (astragalin and afzelin) in *O. baccatus*[40]. These compounds as well as components yet to be identified may be involved in antiproliferative and proapoptotic activities of two aforementioned desert plans.

In conclusion, the results derived from the present investigation show for the first time that aqueous extracts of two desert plants (*O. dayi* and *O. baccatus*) exert apoptosis (programmed cell death) in HepG2 human hepatocellular carcinoma cells. A treatment period of 48 h seems to be necessary to achieve an apoptosis-inducing effect. *O. dayi* extract emerges as a better apoptosis-inducing agent than *O. baccatus* extract as the former delivers greater efficacy at lower concentration. The mechanism of the proapoptotic effects of both plant materials involves mitochondria- and caspase-3-mediated pathway. All these results provide valuable mechanistic insight to the antitumor effects of *O. dayi* and *O. baccatus* as observed previously[16]. These encouraging preliminary data may facilitate the development of novel chemotherapeutic agents based on desert plant *O. dayi* and *O. baccatus* for the management of hepatocellular carcinoma and other difficult-to-treat and life threatening malignancies.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

The work is an interesting ethnopharmacological report. It provide new information on the property of desert plants and can be further applied for medical usage.

Research frontiers

This is a new experiment on the available species from deserts. This can highlight the advantage from locally available species for further implementation in actual medical usage in the future.

Related reports

This work contains new data. There are no previous reports on this specific topic. But there are some relating reports that have been cited and discussed in the paper.

Innovations and breakthroughs

This work gives new knowledge from experiment on locally available plants. It can be referencing papers in ethnopharmacology field. The work can be further referenced in the future publications.

Applications

The application in real medical usage is possible. The application in oncology and hepatology is possible. The work can be a further referencing work for the other research groups.

Peer review

The work is a good standard report on ethnopharmacology on the available plants species from deserts. The work contains new knowledge on the studied local species. The standard investigation technique is used and makes the work to be further referenced. The findings make sense and can be further studied in other following researches.

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