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Evaluation of some *in vitro* Anti-Carcinogenic Activities of Polysaccharides Extracted from Ascomata of the Desert Truffle *Terfezia claveryi* Chatin

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ABSTRACT

This research study carried to evaluate, *in vitro*, anticancer activities of polysaccharides extracted from desert truffle *Terfezia claveryi* Chatin. Ehrlich's ascites carcinoma (EAC) cell line was used to determine, *in vitro*, anticancer activities of this extract *in vitro*. MTT assay was used to quantify the half maximal inhibitory concentration (IC_{50}). Further, flow cytometry was used to determine apoptosis and cell cycle analysis. The obtained results indicated that the IC_{50} of *T. claveryi* polysaccharides extract was 77.6 µg/mL and 47.6 µg/mL after 24 and 48 hrs of treatment, respectively. Flow cytometric analysis demonstrated that the apoptotic effect of *T. claveryi* polysaccharides extract on EAC cells was dose and time dependent. The treatment of EAC cells either with cisplatin (20 µg/mL) or with *T. claveryi* polysaccharides extract (IC_{50}) for 24 or 48 hrs decreased their percentage of G0/G1phase while increased the arrest of G2 phase in cell cycle. In conclusion, these results showed a considerable *in vitro* anti-carcinogenic effect of *T. claveryi* polysaccharides extract, suggesting its potential application as anticancer agent.
KEY WORDS: *Terfezia claveryi*, Polysaccharides, Ehrlich's ascites carcinoma, IC_{50}, Apoptosis, Cell cycle.

INTRODUCTION

Although there are many therapeutic strategies including chemotherapy, radiation and surgery to treat cancer, treatment with chemotherapy cause severe side effects to different organs of the human body [1, 2]. Accordingly, new therapeutic strategies to treat cancer without harming the host organs is crucial to avoid the toxic effects of chemicotherapies [3, 4, 5]. Many studies have been reported that the natural products could be a new approach to ameliorate chemotherapy side effects or to treat cancer [6, 7, 8]. Furthermore, several studies have been reported that mushrooms have several biological activities such as anti-oncogenesis, anti-metastasis, provide a synergistic antitumor activity together with conventional chemotherapy, also have direct antitumor activity [9, 10, 11, 12]. *Terfezia claveryi* is known as edible desert truffles distributed all over world and especially in the Mediterranean Basin [13, 14, 15]. These hypogeous fungi have been documented as medicinal food in different civilization such as Chinese, Greek and Egyptian [16, 17]. It has a unique nutritional profile of unsaturated fatty acid, vitamins, minerals, and protein [18]. Different chemical constituents were found in *T. claveryi* species such as protein (17.6 2%), linoleic acid (62%), carbohydrates, ergosterins and sterol glycosides [19, 20].
Asian black truffle has been used in traditional folk medicine as adjuvant therapy of gastric cancer, while desert truffle (*Terfezia boudieri*) elicited antimicrobial and antioxidant activities [21, 22]. Black and white truffles also showed anti-inflammatory and cytotoxic activities [23]. In addition, polysaccharides isolated from Tuber truffles exhibited high antioxidant and antitumor activities [15, 24]. Although the desert truffle *T. claveryi* showed important medicinal activities as mentioned above, including antioxidant, antibacterial and antifungal activities [25, 26, 27, 28, 29]. On the other hand, there is a very little information available on its antitumor effects at the level of preclinical and clinical studies. Therefore, the present study is conducted to evaluate, *in vitro* the anti-carcinogenic activities of polysaccharides extract isolated from desert truffle *T. claveryi* by using EAC cells.

MATERIAL AND METHODS

Collection of *T. claveryi* ascomata and polysaccharides extraction.

Fresh ascomata of *T. claveryi* were collected during the maturing stage, at the end of winter, from the sub-soil near the roots of *Helianthemum sp.* at north-eastern region near Arar city in Saudi Arabia. They were identified by Prof.

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Bawadekji from Northern Border University. These ascomata were chopped into small pieces, lyophilized, and then ground into fine powder, 100 g. of this powder was soaked in 2 L of 95% (vol/vol) ethanol at 70ºC for 2 hours in order to remove phenols. This step was repeated twice, ethanol was evaporated; and then the residue passed for ulterior extraction with 2 L of distilled water at 80°C (3 times/6 hours). The gathered filtrate solution was centrifuged at 8400 g for 10 minutes and then concentrated and dialyzed [30]. The protein liberated from the extract is removed by Sevag reagent, then, the extract was precipitated by washing four times with ethanol [31]. Precipitate was collected by centrifugation for 10 minutes at 8400 g and then lyophilized to obtain the crude polysaccharide extract.

Maintenance and expansion of the tumor cell line.
Ehrlich ascites carcinoma (EAC) cell line was obtained from the Centre of Excellence of Cancer Research, Tanta University, Tanta, Egypt. EAC cells were maintained in female Swiss albino mice by weekly intraperitoneal (i.p.) inoculation of viable $2x10^6$ cells/mouse at the Cancer Institute, Cairo, Egypt. Tumor cell suspensions were prepared in balanced salt solution at pH 7.4 to a final concentration of 5×10^6 viable cells/ mL [32].

Assessment the antitumor activity of *T. claveryi* polysaccharides extract.
EAC cell count using trypan blue assay:
EAC cells were seeded into 6-well culture plates (Falcon, Oxnard, CA) at a density of 1×10^6 cells/well in RPMI-1640 medium supplemented with 5% fetal calf serum (Gibco, Grand Island, N.Y.), 50 µM β-mercaptoethanol (Sigma Chemical Co.) and antibiotics. Sets of subconfluent cells in plates were treated with different concentrations of *T. claveryi* polysaccharides extract, and subsequently incubated in a humidified 5% CO_2 environment. After 24 and 48 hrs, cells were harvested by trypsinization using trypsin-EDTA. Cells were washed with sterile saline (0.9%) and 0.5-1 mL of 10% trypsin-EDTA solution was added to the cell monolayer. After incubation for 3-5 min, cells were collected, stained with trypan blue (Sigma Chemical Co. USA) according to [33], and counted using a hemocytometer under light microscope. Viable and dead cells were counted separately for each condition.

Determination of EAC cell proliferation using MTT assay:
The cytotoxic effect of *T. claveryi* polysaccharides on EAC cell proliferation was measured using MTT assay according to the method developed by [34]. EAC cells were seeded in 96-well culture plates at a density of 1×10^6 cells/well in RPMI-1640 medium supplemented with fetal calf serum, β-mercaptoethanol and antibiotics. Then, cells were treated with different concentrations of *T. claveryi* polysaccharides extract, and later incubated in a humidified 5% CO_2 environment. 150 µl of the medium were removed After 24 and 48 hrs, from each well. Tetrazolium salt MTT (3[4,5-dimethylthiozol-2-yl]-2,5-diphenyl tetrazolium bromide, Sigma Chemical Co.) has been used to determine the extent of EAC cell proliferation, which sticks to active mitochondria and turns into blue formazan product. MTT (5 µl of 20 mg/ mL) was added to each well and incubated for 4 hours at 37ºC. The dark blue crystals were dissolved by the addition of 150 µl of 0.04 M HCl/ isopropanol, the plates were inserted into a Dynatech MR580 micro-elisa spectrophotometer; after an incubation in the dark for overnight and a test wavelength of 570 nm and a reference wavelength of 630 nm were used to have the optical densities.

Determination of EAC cell apoptosis:
Measurement of apoptosis in EAC-untreated cells and EAC-treated cells with different concentrations of *T. claveryi* polysaccharides extract was performed using Annexin V, apoptosis Detection kit II (Cat. No 556570) as proposed by [35]. Briefly, EAC cells were washed twice with cold PBS and then re-suspended in 1X binding buffer at a concentration of 5×10^6 cells/mL. 100 µl of the solution (5×10^5 cells) were shifted to a 5 mL culture tube. 5 µl of Annexin-V and 5 µl propidium iodide (PI) were added. The cells were gently vortexed and incubated for 15 min at RT (25°C) in the dark. 400 µl of 1X binding buffer were added to each tube, and then analysed by BD FACSCanto™ II flow cytometer. In this case, changing of cell stain is an indicator; positive stain for both Annexin-V and PI are either in the end stage of apoptosis, exhibiting necrosis, or are dead cells. While negative stained cells for both Annexin-V and PI are alive and not exhibiting determinable apoptosis.

Cell cycle analysis of EAC cells after treatment for 24 and 48 hrs with extract:
In order to evaluate the effect of *T. claveryi* polysaccharides on the distribution of tumor cells in G1, S and G2/M phases of the cell cycle, Cell cycle analysis was achieved by using flow cytometry after DNA staining to detect the total amount of DNA as described by [36]. Approximately, 2×10^6 of EAC cells cultured in the presence of the IC_{50} (77.6 µg/ml) of *T. claveryi* polysaccharides extract. Cells were collected after 24 and 48 hrs of incubation, and then washed with PBS and proceeds to be fixed with cold 70% ethanol and kept at -20°C for twelve hours. After washing

cells twice by adding 2 mL cold PBS (1800 rpm, 5 min), the supernatant was eliminated, and cells were stained with a solution containing 300 µg/mL of PI/ triton X 100 staining solution (1000 µl of 0.1% triton + 40 µl PI + 20 µl RNAse). The analysis of samples was done using BD FACSCanto™ II flow cytometer and data were analyzed using BD FACS Diva software.

Statistical analyses

Data are expressed as means ± SD. Excel (Microsoft Corporation, USA) and Minitab (version 18.1, Minitab, Inc., USA) were utilized. One-way ANOVA and Tukey pairwise comparisons were used to test the differences of means among different groups, Equal variances were assumed for the analysis with significance level $\alpha = 0.05$. P values < 0.05 are considered significant. Grouping information using the Tukey method was used, means that do not share a letter are considered significantly different.

RESULTS

EAC cells viability decrease with an increase of *T. claveryi* polysaccharide concentrations

To test the impact of *T. claveryi* polysaccharides extract on the viability of EAC cells, different concentrations of the extract were used to determine the percentage of the viable cells after 24 and 48 hrs post treatment. The results showed that by increasing the concentration of the extract. The percentage of dead cells was significantly increased while percentage of the viable cells was significantly decreased. Except for the concentration of 25 µg/mL (Fig. 1). By increasing the time of the exposure to the *T. claveryi* polysaccharides extract, more increase in the percentage of the dead cells and more decrease in the percentage of the viable cells (Fig. 2).

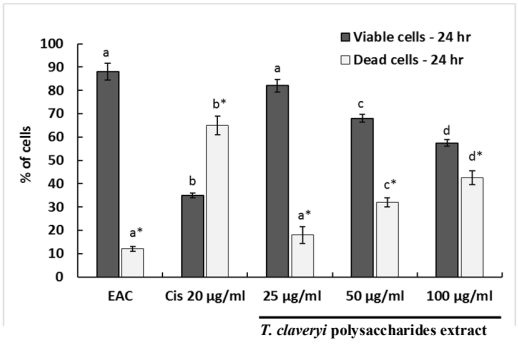

Figure 1. Treatment with different concentration of *T. claveryi* polysaccharide extract for 24 hrs decreased the viability of cells with the increase of the extract concentrations.

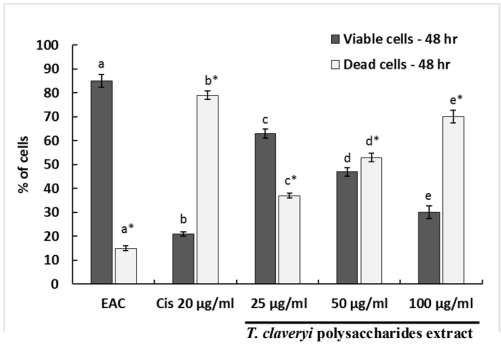

Figure 2. Treatment with *T. claveryi* polysaccharides for 48 hrs showed more decrease in the percentage of the viable cells.

All these increases and decreases were significantly different ($p < 0.05$) in comparison to EAC cells without *T. claveryi* polysaccharides extract.

***T. claveryi* polysaccharides extract showed a moderate anticancer activity**

To evaluate the anticancer activity of *T. claveryi* polysaccharides extract, MTT assay was performed, the half maximal inhibit concentration (IC_{50}) was determined. Briefly, EAC cells (1×10^6 /well) were seeded and let overnight to adhere in the plate. Different concentrations of *T. claveryi* polysaccharides extract range between 25 to 150 µg/mL were added in accordance with experimental design. Cells were incubated for 24 and 48 hrs in 5% CO_2 incubator. The result showed that the (IC_{50}) were 77.6 and 47.6 µg/mL after 24 and 48 hrs, respectively (Fig. 3).

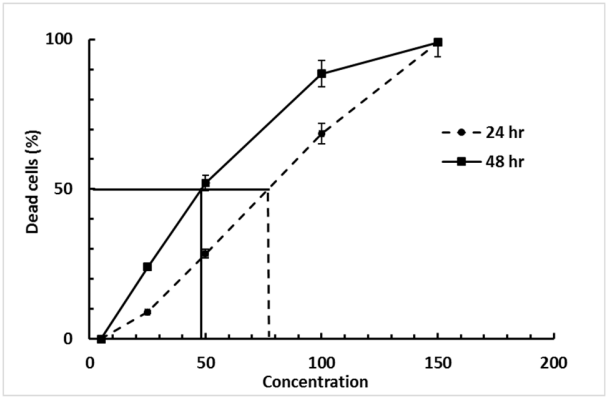

Figure 3. Half maximal inhibitory concentration (IC_{50}) of *T. claveryi* polysaccharides extract on EAC-cells after 24 and 48 hrs of post treatment.

Anticancer activity of *T. claveryi* polysaccharides extract was dose and time dependent

To detect the percentage of the apoptotic cells (early and late apoptotic) after the treatment with *T. claveryi* polysaccharides extract for 24 or 48 hrs. By using sterilized 6-well plate, 5×10^{6}/ EAC-cells/well were seeded and incubated for overnight to adhere to the plate surface as mentioned above. Different concentrations of *T. claveryi* polysaccharides extract were applied. Cells incubated again for 24 or 48 hrs. Cells harvested and stained with Annexin-V and propodimiodied (PI) based on a certain protocol for flow cytometry. Flow cytometry analysis showed after 24 hrs of treatments, that *T. claveryi* polysaccharides extract induced apoptosis of EAC cells in dose dependent pattern. Treatment with 25, 50 and 100 µg/mL of *T. claveryi* polysaccharides extract for 24 hrs, the apoptotic percentage were 14, 25 and 35%, respectively (Fig. 3). Treatment with the same concentrations of the extract for 48 hrs showed apoptotic percentage 25, 35 and 54%, respectively (Fig. 4). The results showed that the increase of the apoptotic percentages was dose and time dependent.

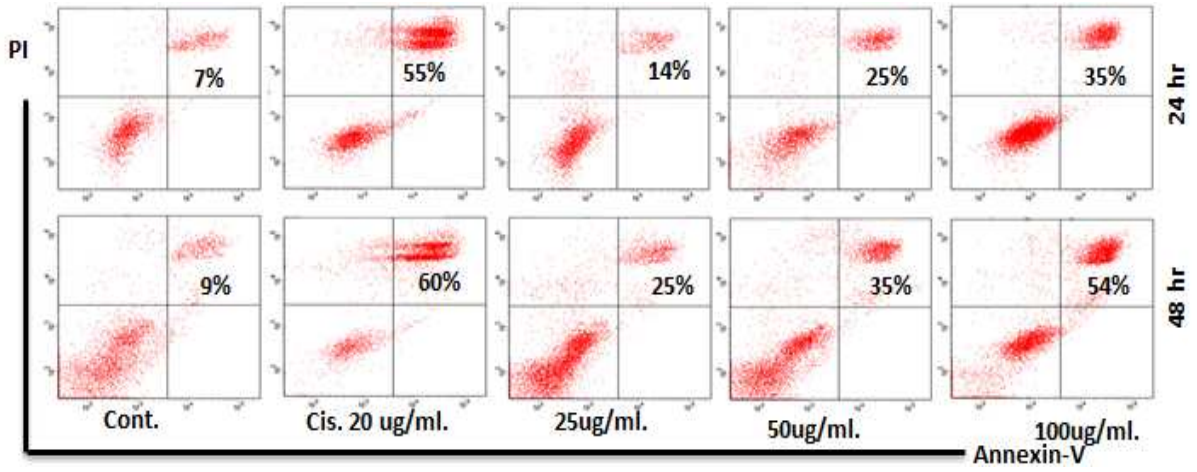

Figure 4. Treatment with *T. claveryi* polysaccharides extract showed an increase as concentration and time dependent pattern.

The percentage of viable cells, early apoptotic, and late apoptotic EAC cells after 24 hrs with no treatment (-ve control) was 89, 2.4 and 7%, respectively. EAC cells treated with 25, 50 and 100 µg/mL of *T. claveryi* extract for 24 hrs, the percentage of viable cells were 80.9, 67.8 and 55.6%, respectively (Fig. 5). EAC cells treated with the same concentrations of *T. claveryi* polysaccharides extract for the same time showed percentage of early apoptosis as 4.7, 6.8 and 9.4%, respectively, and for late apoptosis were 14, 25 and 35%, respectively (Fig. 5).

The percentages of viable cells, early apoptotic, and late apoptotic EAC cells after 48 hrs with no treatment (-ve control) were 87, 3.1 and 9.1%. After the treatments for 48 hrs by *T. claveryi* extract concentrations 25, 50 and 100

µg/mL the percentage of viable cells were 68, 56 and 33.5%, respectively (Fig. 6). EAC cells treated with the same concentrations of *T. claveryi* extract for the same time showed percentage of early apoptosis as 6.7, 8.4 and 11.1%, respectively. While, the percentages of late apoptosis were 25, 35 and 54%, respectively (Fig. 5). Of note, treatment with 20 µg/mL of cisplatin (reference drug) for 24 hrs, showed a percentage of viable cells (33.6%), early apoptotic (10.5%) and late apoptotic (55%). While the treatment with the same dose of cisplatin for 48 hrs showed a percentage of viable cells (23.4%), early apoptotic (16.4%) and late apoptotic (60%) (Figs. 5, 6).

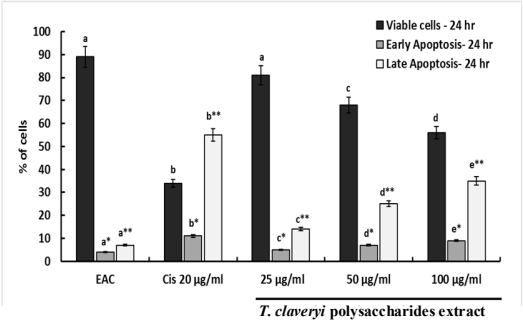

Figure 5. Treatment with *T. claveryi* polysaccharides extract for 24 hrs increased the apoptotic percentages of EAC-cells in dose dependent pattern.

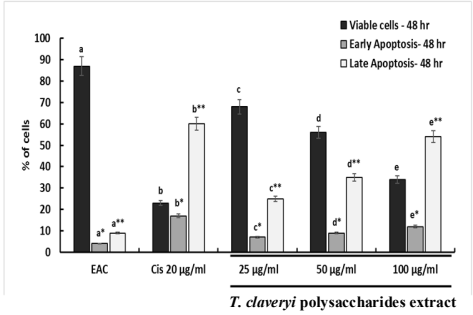

Figure 6. Treatment with *T. claveryi* polysaccharides extract for 48 hrs showed increase in early and late apoptostic EAC-cells.

Treatment with IC_{50} of *T. claveryi* polysaccharides extract for 24 and 48 hrs increased the percentage of G2 and decreased the percentage of G0/G1.

To evaluate the effect of *T. claveryi* polysaccharides extract on the distribution of tumor cells in G1, S and G2/M phases of the cell cycle. Approximately, 2×10^6 of EAC cells cultured in the presence of IC_{50} of *T. claveryi* polysaccharides extract for 24 and 48 hrs. The results showed that the selected IC_{50} of the extract increased the percentage of G2 phase and decreased the percentage of G0/G1 phase of the exposure to the extract for 24 or 48 hrs. as shown in Figure 7.

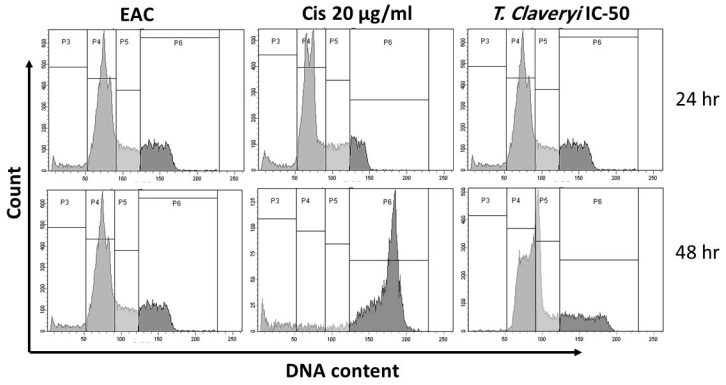

Figure 7. Treatment with IC_{50} of *T. claveryi* polysaccharides extract for 24 and 48 hrs increased the percentage of G2 and decreased the percentage of G0/G1.

DISCUSSION and CONCLUSION

Several medical applications of desert truffle *T. claveryi* were reported. It has reported that *T. claveryi* had a potent antibacterial and antifungal activities [25, 26, 27, 28, 37, 38]. Fungal polysaccharides extract especially *T. claveryi* exhibit anti-inflammatory activity that might contribute to the prevention of inflammatory diseases [39]. Further, it has been reported that the methanol extract of *Terfezia* sp. had antimicrobial activity against a wide range of both Gram-positive and Gram-negative bacteria [40]. Furthermore, it was reported that a higher oxidative inhibition on

lipid peroxidation after the treatment with *T. claveryi* [24, 41]. In addition, it was reported that the aqueous extract of *T. claveryi* has a potent hepatoprotective activity against $CC1_4$ [38]. Our results showed that the IC_{50} of *T. claveryi* polysaccharides extract on EAC cells were 77.6 and 47.6 µg/mL after 24 and 48 hrs of exposure, respectively, these findings showed that the polysaccharide extract of *T. claveryi* has a moderate anticancer activity *in vitro*. While other study showed that *T. claveryi* polysaccharides extract inhibited the human brain carcinoma cell line [42]. Similarly, to our findings, *T. claveryi* polysaccharides extract showed inhibition activity against PC3 and MCF7 cell lines [43]. The inhibition of cell growth by *T. claveryi* extracts might be due to its antioxidant properties [17]. After we obtained IC_{50} of *T. claveryi* polysaccharides extract, the percentage of the apoptotic cells was determined at different concentrations of the extract by incubation of the treated cells for 24 and 48 hrs. According to our findings, *T. claveryi* polysaccharides extract induced apoptosis in dose and time dependent pattern. Therefore, the treatment with the extract for 48 hrs induced significant increase in apoptotic percentages more than its effect for 24 hrs. Consistent with our finding, recent study showed that *T. claveryi* hexane extract significantly promoted cell apoptosis through the mitochondrial pathway and DNA fragmentation [42]. From the previous points of view, it can be speculated that anticancer effect, and apoptotic activity of *T. claveryi* extract is due to the presence of main chemical constituents and due to the antitumor activity of the polysaccharides. Regarding to the effect of the *T. claveryi* polysaccharide extract on cell cycle of EAC cells. Cells were cultured in 6-well plates at a confluence of 2×10^6 cells/well. Cells were treated with extracts for 24 and 48 hrs with respective IC_{50} values. Then, cell cycle phases and DNA content were analyzed by flow cytometry. We found that the half maximal concentrations (IC_{50}/24hrs and IC_{50}/48 hrs) of *T. claveryi* polysaccharide extract could inhibit cell proliferation and arrest cell cycle in G1 phase. These results were in agreement with other study which showed that polysaccharides from the fungus *Pleurotus abalonus* induced the cell-cycle arrest [44, 45]. In conclusion, these results showed a considerable effect of *T. claveryi* polysaccharides extract *in vitro* as anti-carcinogenic agent. Further studies are required to assess the antioxidant capacity of the *T. claveryi* polysaccharides extract and its anticancer effect using tumor bearing mouse model.

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