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Cytotoxic potentials of the culture extract of the endophytic *Aspergillus niger* strain *karmali* isolated from *Punica granatum* against SKOV3 and A549 cancer cell lines

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Research

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Ibrahim KARIDIO DIORI[1*] and Senay HAMARAT SANLIER [1,2,*]

[1]*Department of Biochemistry, Faculty of Science, Block E, Ege University, Erzene Mahallesi, Bornova/Izmir 35040, Turkey.*
[2]*ARGEFAR, Faculty of Medicine, Ege University, Bornova/ Izmir 35040, Turkey*

CORRESPONDENCE AUTHOR

KD Ibrahim (Email: kardio_ib@outlook.fr)
HS Senay (Email: senay.sanlier@gmail.com)

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ABSTRACT

Endophytes encompass the widespread *taxa* of yet under-explored endosymbionts that are believed to bestow, to the benefit of their host plants bioactive compounds enabling the later to withstand biotic and/ or abiotic stresses. Some of these molecules are pharmacologically valuable compounds. Herein, we do report the isolation of a newly identified endophytic strain of *Aspergillus niger* named k*armali* from roots and twigs of *Punica granatum. Aspergillus niger* strain *karmali* has been used in a solid-state fermentation on rice medium enriched with peptone and artificial seawater. The polar phase of the culture extract was fractionated using Dry Column Vacuum Chromatography (DCVC). The cancer treatment potential of the major fraction of the endophytic compounds was determined *in vitro* as its cytotoxicity against ovarian cancer cell line SKOV-3 and lung cancer cell line A549. As a result of cell culture experiments, IC_{50} values were determined to be 1.08 µg / ml for SKOV3 cells and 11.7 µg / ml for A549 cells at 24 hours. The cancer treatment potential of the purified endophytic compound was found to be more active on SKOV-3 cell. Biocompatibility tests were performed in order to determine its effectiveness *in vivo* conditions, and it was determined to be biocompatible.

Keywords:*Aspergillus niger* strain *karmali*, cytotoxic, endophyte, *Punica granatum*, A459, SKOV3, ovarian cancer, lung cancer

1.INTRODUCTION

Etymologically thought, endophyte is derived from the Greek words "*Endon"* meaning "*inside"* and *"phyto"* meaning *"*plant". So literally, endophyte means "*inside the plant."* The term was first introduced by De Bary (1, 2). Broadly, endophyte refers to the *endosymbiotic microbiota* that comprises bacteria and fungi that asymptomatically live within and/ or between plant cells (3,5). Many *species* of the same or different *genera* could be hosted by the same or distinct plant tissues. Endophytes are widely spread among and within plant *species* with remarkable diversity. To date, all the plants investigated have been individually proven to be associated with at least one kind of endophytic microbe either in its *phyllosphere* and/or *rhizosphere* (4,6-9). Endophytes are mostly located in relatively privileged niches. Therein, they establish an interrelationship with their host that could range from commensalism to mutualism. Endophytes are broad *taxa* of unexplored; thus, poorly known functional bacterial and fungal *communities* that, depending on their genotype, the environmental conditions, and the genotype of their host plant, from within, alter its biochemistry, physiology and evolutionary ecology.

Sometimes endophytes produce among others, secondary metabolites that favor plant growth, increase yield and resistance against abiotic stresses (10-16). Moreover, the widely spread endophytes could also produce secondary metabolites with antimicrobial activities against pathogens, as well as toxic and noxious compounds against insect and vertebrate herbivores thereby reflecting a "barrier effect" against biotic stressors. Some of these bioactive compounds are as well rare as valuable novel compounds that could have medical significance (17-20).

Considering that plants threating factors such as global warming, desertification, recurrent episodes of floods and droughts, in parallel with the rapid global population growth with its associated increasing food demands, medicines (mostly derived from plant sources), favour the widespread nowadays phenomenon of biodiversity scarce; it is emergent to overcome frontiers of current knowledge about the accurate and efficient use of endophytes for agricultural, industrial and pharmaceutical purposes to avoid the permanent disappearance of many *species* even before scientists explore their scope of usefulness(21-24). Following the production of *Taxol* from the endophytic fungi *Taxomyces andreanae* isolated from the inner bark of *Taxus brevifolia*, scientists developed interests in the yet-poorly known diversified *genera* of the endophytic microbiota of plants (23).

Our interest firstly, focused on the research on endophytes from *Punica granatum* (*nar ağacı*), at the Faculty of Science of Ege University, then the anticancer potential of their eventually produced metabolites. The historical use of *P. granatum* for its natural and holistic medical significance dates far back to ancient times as noted in Egyptian mythology and art. The plant represents an enormous treasure of pharmacophoric compounds. Extracts from different parts of *P. granatum* have been proven useful for relieving from various ailments such as throat inflammation, coughs, periodontal disease, urinary infection, bronchitis, diarrhoea, tapeworm parasitism, arthritis, diabetes, cancer, cardiopathy *etc.* (25-31).

Research data evidenced the high content of phytochemicals in *P. granatum*, mainly polyphenols such as ellagitannins/punicalagins, delphinidin, cyaniding, pelargonidin glycosides, cathechins, gallocatechins, prodelphinidins *etc*. (32-35).

Despite the tremendous interests' researchers manifest towards pomegranate for its nutraceutical potentials, there are very few reports about its endophytic microbiota. Of recent, antimicrobial producing fungal endophytes named as IPG3-1 and IPG3-3 were isolated from *P. granatum* (36). (37) reported, the production of novel styrylpyrones from the endophytic fungi *Penicillium glabrum* isolated from *P. granatum*. In late 2017, *Q. cyanescens* was reported for the first time as isolated endophytes from the *Lythraceae* family (24). Considering the significance of phytochemical reported from *P. granatum* and the fewer availability of data about its eventual endophytic microbiota, focusing on the later would tell us more about their eventual potentials with regard to bioactive compounds production.

Every year 18.1 million new cases of cancer are diagnosed, and 9.6 million people die due to cancer. In 2015, according to estimations of the World Health Organization (W.H.O.), cancer is the first or second cause of premature deaths in 91 countries out of 172. Additionally, cancer is the third or fourth cause of premature deaths in 22 countries. One type of cancer, the most commonly diagnosed and major cause of cancer deaths, i.e. lung cancer, alone is the fourth global deadly disease after ischemic heart disease, stroke, and diabetes mellitus. The global maps of cancer types, incidence, prevalence, and mortality appear as variegated pictures linked to the geographical region, socioeconomic development and/ or life-style of the population. For instance, ovarian cancer, the eighth commonest female cancer, and the third gynaecological cancer is common in developed and transitioning countries while it is rare in low income-African countries (38-41).

Herein, we do report the isolation of a newly identified endophytic strain of *Aspergillus niger* named k*armali* from roots and twigs of *P. granatum. Aspergillus niger* strain *karmali* has been used in a solid-state fermentation on rice medium enriched with peptone and artificial seawater. The polar phase of the culture extract was fractionated using Dry Column Vacuum Chromatography (DCVC). The cancer treatment potential of the major fraction of the endophytic compounds was determined *in vitro* as its cytotoxicity against ovarian cancer cell line SKOV3 and lung cancer cell line A549.

2. MATERIAL AND METHODS

Isolation and purification procedures

Collection and surface sterilization of plant samples

Samples from fresh roots, twigs, leaves, flowers, and unripe fruits were collected from a *P. granatum* tree at the Faculty of Science of Ege University (38° 27'34.2"N 27°13'51.8"E), and brought to the laboratory of Biotechnology (Department of Biochemistry), in sterile plastic bags. Samples were chosen based on their appearance of being healthy, i.e. not showing disease symptoms, brought into the laboratory and processed within an hour to lower risks of contamination by ambient microorganisms. The collected samples were exhaustively washed by means of running tap water so as to remove eventually, sand and dust on their surface. Roots and twigs' samples were cut into small segments with sterile bistoury. Next, the plant materials were then processed to remove epiphytes through surface sterilization i.e. a combination of a sequential immersion of the sample into a different solution at each step. The flower, leaves and fruits' samples were immersed in 70% EtOH for 30 seconds, then in 2% NaOCl for 10-15 seconds, then in 70% EtOH again for 30 seconds and finally in sterile-distilled water (sdH_2O) for 15 seconds twice. Roots and twigs' samples were passed through the same process, but with relatively long periods i.e. 3 min in 70% EtOH, 1 min in NaOCl, 1 min in EtOH, and 1 min in sdH2O twice. The surface-sterilized samples were dried under aseptic conditions in the folds of sterile tissue papers. The roots and twigs' samples were sliced longitudinally while others were cut into more or less forms and placed into media (PDA + chloramphenicol, LB-agar), containing Petri dishes so that the freshly cut edges were in direct contact with the agar surface. To check for the efficiency of the procedure, 0.1 ml of the final rinsing water was inoculated on LB-agar, PDA + chloramphenicol, and incubated under the same conditions as the samples (16, 42-47).

Isolation and purification of the endophytes

The controls were monitored for up three weeks without showing any organismal growth. Many fungal strains grew from the same samples. Each individual strain was transferred into a new Petri according to the hyphae tips method. Observation of the cultures' morphologies, colours, and down-side culture image uniformity helped in the purification process. Many cultures, media were used, some served, as well for purification as for the screening process for the eventual potentiality to produce specific valuable secondary metabolites. In the course of the conduct of the present study, an endophytic fungal *species* isolated from the roots of *P. granatum* have been selected.

Identification of the endophytic fungal strain

The genomic DNA of the endophytic fungi was extracted and isolated using the *QuickDNA Fungal/*

Bacterial kit purchased from ZYMO RESEARCH. The ITS region of the extracted genomic DNA was amplified using the primers ITS1 and ITS4 and sequenced for molecular biological identification purpose. The sequenced ITS region was submitted to the National Centre for Biotechnology Information (NCBI) GenBank database and was attributed to the accession number MH886512.1

The phylogenic tree from the internal spacer's sequence of *Aspergillus niger* strain *karmali* is presented in **figure 1** below.

Fermentation and extraction

Prior to the solid-state fermentation, the endophytic fungus was cultured on Potato Dextrose Agar (PDA), for one week. The culture surfaces of the Petri dishes were cut into small pieces and transferred into Erlenmeyer flasks containing sterilized solid rice medium. The rice medium contained 100g (per 1000 ml flask), of commercially available rice enriched with 5.5g peptone, 100ml artificial seawater, and 900 ml distilled water in 1000ml Erlenmeyer flasks. Prior to sterilization, the so prepared medium was kept at room temperature, overnight under static conditions. The inoculated prepared rice media (cultures), were kept at room temperature, under static conditions in a closed cupboard for 40 days. The fermentation process was brought into an end by adding 250ml of EtOAc into each culture flask. The cultures, so-submerged with EtOAc, were hermetically covered with an adhesive plastic and incubated at 40º C, 150 rpm overnight. The culture was again exhaustively extracted again with EtOAc, at 40ºC, 150 rpm for 2 hours. The concentrated crude extract was exhaustively extracted with MeOH: *n-hexane* solvent system, and the polar phase concentrated for purification.

Purification

Dry column vacuum chromatography (DCVC) was used for the purification process coupled with silica gel TLC plates that served as to determine the eventual fractions under UVfluorescent detector (48). The solvent system used as eluent/ mobile phase for the DCVC was nhexane: EtOAc. After loading the celite-bound-sample on the column, it was then eluted with *nhexane* thrice, then *n-hexane* -EtOAc with increasing polarity, i.e. 19:1; 18:2; until 0:20 was achieved. At this point, the 0:20 hexane-EtOAc was repeated four to five times, before the EtOAc- 90% MeOH system was introduced. The later system was also eluted with an increasing polarity,
i.e. EtOAc- 90% MeOH 19:1; 18:2 and so on (48,49).

MTT assay

The cytotoxicity of the sample was assessed according to the MTT assay developed by Hansen (50). The cell viability was determined by comparing the spectroscopic analysis data of the treated and untreated samples. The cytotoxicity was tested against two cancer cell lines, namely A549 and Sköv3. The tests were performed in triplicates and the averages of the separate experiments served on the statistical analysis of the data to determine the significance of the drug's eventual effect against the cancer cell lines.

3. RESULTS & DISCUSSION

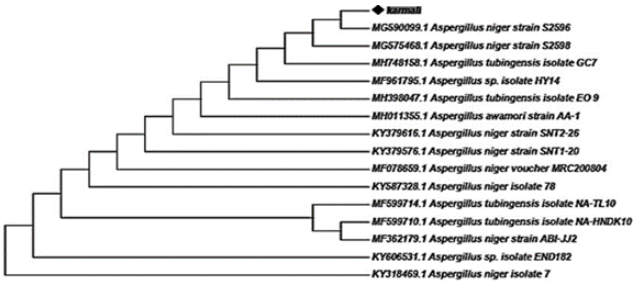

Figure 1 The phylogenic tree from the internal spacer's sequence of *Aspergillus niger* strain *karmali*

Cancer Treatment Potential of Isolated Endophytic culture's extract *in vitro*

Research

Cytotoxicity Studies

The MTT test was used for cytotoxicity analysis and cell viability was measured at 24 h, 48 h and 72 h. The amount of viability detected in the cells incubated in the medium was accepted as 100% and the calculation was made in the cells where the drug samples were applied. Cell viability graphs were plotted with Excel.

The cytotoxicity produced by the secondary metabolite in the concentration range (400.625 µg / mL)

against A459 cell lines is shown for 24 hours in Figure 2, for 48 hours in Figure 3 and for 72 hours in Figure 4, respectively. Likewise, the cytotoxicity produced by the same endophytic metabolic against SKOV3 is shown by figure 5, figure 6 and figure 7 for 24, 48 and 72 incubation time after the sample is added.

It was found that the secondary metabolite increased cytotoxicity with increasing drug concentration in 24th hour results. The results recorded after 48 hours and 72 hours firstly reflected a proportional increment of the cytotoxicity of the drug with respect to its concentration before the concentration of 10 µg/ml was reached. For 10 µg /ml and above the cytotoxicity was inversely proportional to the concentration of the drug.

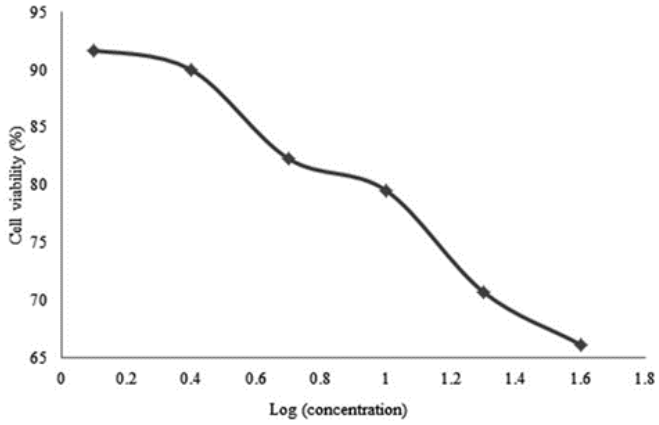

Figure 2: Percentage of cell viability versus drug's (secondary metabolite) doses administered to A549 cells for 24 hours

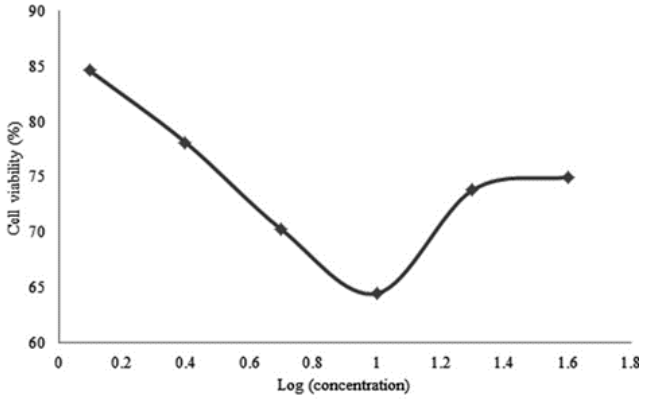

Figure 3: Cell Viability percentages versus secondary metabolite doses administered to A549 cells for 48 hours

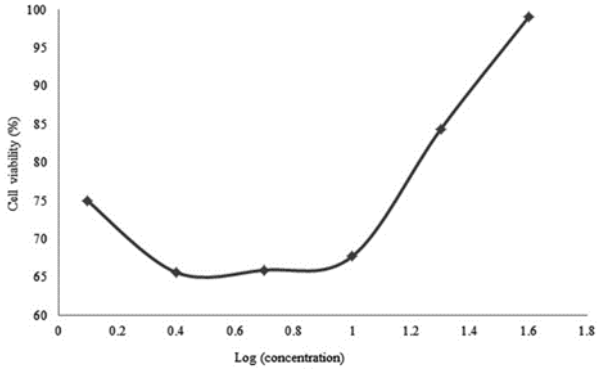

Figure 4: Percentages of cell viability versus secondary metabolite doses administered to A549 cells for 72 hours.

For the SKOV-3 cell lines, 24 hours incubation effects are shown in Figure 5, 48 hours in Figure 6 and 72 hours in Figure 7. It was found that the metabolite expressed a proportional cytotoxicity with respect to its concentration in 24th hour results. At the 48th and 72nd hour results, the concentration of the drug was proportional to its cytotoxicity below 10 µg / ml. For concentration equal or above 10µg/ml, the concentrations seemed inversely proportional to the toxicity of the drug.

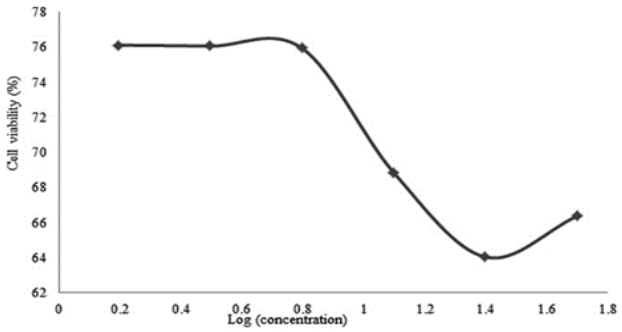

Figure 5: Percentage of viability versus secondary metabolite doses administered to SKOV-3 cells for 24 hours.

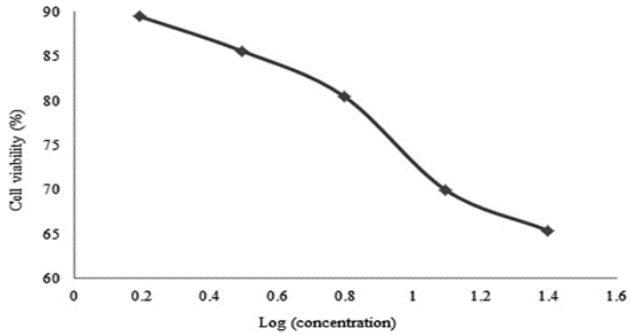

Figure 6: Cell Viability percentages versus secondary metabolite doses administered to SKOV-3 cells for 48 hours

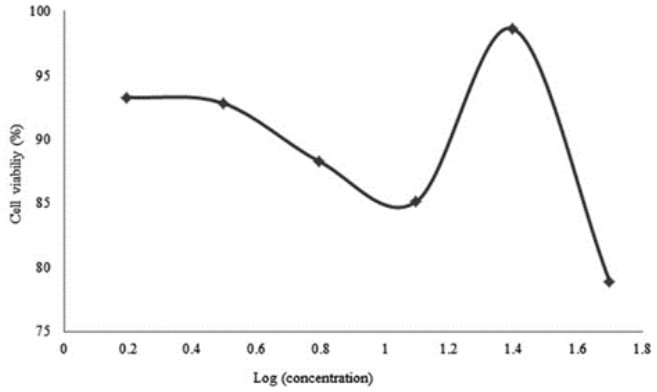

Figure 7: Cell Viability percentages versus secondary metabolite doses administered to SKOV-3 cells for 48 hours

IC_{50} values of the drugs on A549 and SKOV-3 cells at 24, 48 and 72 hours were calculated by Graphpad Prism 5.0 program using percent viability of the cells against the log (concentration) of the drugs. The values obtained are shown in Table I below

Table 1: A549 and SKOV-3 on 24, 48hours and 72 hours after incubation of free drug groups and drug IC50 values

Cell lines	24 hr(µg/ml)	48 hr(µg/ml)	72 hr(µg/ml)
A549	11.7	9.604	20,1±*0,04*
SKOV-3	1,08±0,003	7.968	1,85 ±0,007

Determination of Biocompatibility of Isolated Endophytic metabolic (drug sample)

Protein Binding

It is known that adsorption to plasma proteins significantly determines the *in vivo* behaviour of particles (51). Phagocytosis is facilitated by proteins adsorbed to the drug's surface, and the drug's surface chemistry affects the amount and type of binding proteins (52). For example, adsorption of opsonins facilitates phagocytosis and causes the removal of drugs from the systemic circulation by the mononuclear phagocytic system.

Conversely, the binding of disopsonins such as albumin increases the circulation time in the blood (51). In the study, the amount of protein binding in fetal bovine serum of the secondary metabolite was determined. Since the amount of plasma protein may vary from one person to another, experiments were performed at different concentrations of substance and serum. In **Table II**, protein binding amounts and binding percentages are given.

Table 2: Quantity and percentage of binding of endophyte compound to serum proteins.

Serum: drug ratio	mg protein / mg drug	Protein Binding Percentage
10:90	-	-
20:80	0,121	83,740
40:60	0,214	74,259
60:40	0,355	82,129

Hemolysis

Hemolysis experiments were performed by incubating the secondary metabolite with erythrocytes at different concentrations. **Figure 8** shows the photographs of the experiment. PBS was used as the negative control and Triton X, which was known to cause lysis of the cells, was used as the positive control. According to the results, the secondary metabolite did not cause hemolysis compared to the positive and negative controls. When all of the protein binding and hemolysis results are considered, it can be said that the drug sample was biocompatible.

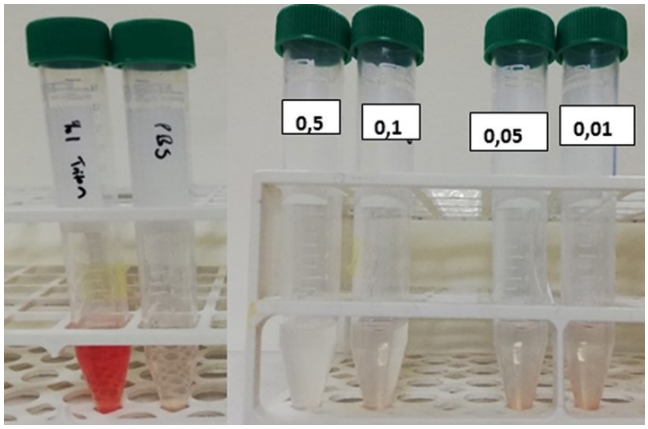

Figure 8: Image of tubes after hemolysis assay centrifugation (left to right respectively): Positive control, negative control, drug concentrations of 0.5; 0.1; 0.05; 0.01 mg/ml

CONCLUSION

The cancer treatment potential of the endophytic compound produced by *A. niger karmali,* in *vitro*, was determined as its cytotoxicity against ovarian cancer cell SKOV3 and lung cancer cell A549. As a result of cell culture experiments, IC_{50} values were determined as 1.08 µg / ml for SKOV3 cell and 11.7 µg / ml for A549 cell at 24 hours. The cancer treatment potential of the purified endophyte compound was found to be more effective on SKOV3 cell. Biocompatibility tests were performed in order to determine its effectiveness *in vivo* conditions and it was determined to be biocompatible.

Author contributions: This study has been conceived, designed, the experiments performed, the data analysis and the writing of the manuscript were done by Dr Ibrahim KARIDIO DIORI under the supervision of Prof.Dr. HAMARAT SANLIER Senay.

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