Anti-proliferative and anti-inflammatory activities of entophytic *Penicillium crustosum* from *Phoenix dactylifer*

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Abstract: Natural sources have been and will remain an inspiration source for modern chemistry. The current study investigates the antiproliferative and anti-inflammatory action of the ethyl acetate fraction of *Penicillium crustosum* from *Phoenix dactylifera*. This paper reports the isolation of *P. crustosum* from leaves of *P. dactylifera* and the antiproliferative activities of ethyl acetate fraction on cancer cells. To reach this goal, the anti-proliferation and cytotoxicity effects were evaluated by MTT and LDH assay respectively. The quantitative real time PCR technique was used to investigate IL-6 and IL-8 gene expression. Our results revealed higher anti-proliferative activity against HepG2 (82µg/ml) than MCF7 (126µg/ml) and inhibited the migration of the cell lines. The ethyl acetate fraction significantly altered LDH levels and reduced IL-6 transcript expression on MCF7 cell line but not in HepG2 cell line which could be specific anti-inflammatory drug in breast cancer cell line. These results suggest that *Phoenix dactylifera* extract has a potent anti-proliferative and anti-inflammatory action. Further investigation to isolate the active compounds and mode of action is required.

Keywords: Antiproliferative, Anti-inflammatory, *Penicillium crustosum*, *Phoenix dactylifera*, cell migration.

INTRODUCTION

Natural sources have been and will remain the inexhaustible source of inspiration for the chemists as well as being a remedy for the patients since time immemorial. Plants, animals and microorganisms form the pillars of chemical diversity offering an untold number of lead compounds. Currently, the term endophytes have been frequently used in natural product research. Endophytes are group of microorganism that resides asymptomatically inside the tissues (Krings *et al*., 2007). Endophytes are a huge reservoir of structurally unique bioactive natural products therefore, they were exploited for medicinal uses (Guo *et al*., 2012; Alshatwi, 2011; Strobel and Daisy, 2003). This led to huge efforts worldwide by pharmaceutical companies and research centers to isolate large number of endophytes in order to discover new anticancer agents (Hazalin *et al*., 2012).

In Saudi Arabia, fungal endophytes as potential source of novel biologically active chemical substances are not well explored. The flora of Saudi Arabia has about 2281 species in 853 genera. To date, only a very few reports have been documented on the isolation of endophytes and evaluation of their bioactive extract. From available literature, there is no report on endophytic fungi from *P. dactylifera*. Therefore, the present study aimed to study one of the endophytic fungi associated with the root of *P. dactylifera* to evaluate its anti-proliferative and anti-inflammatory activity against HepG2 and MCF7 cancer cell line.

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Cancer is considered an international burden more than it was before. The annual cost per patient in the Kingdom of Saudi Arabia is about SR 82,500 ($22,000) as estimated by Gulf Cooperation Council and it is expected that the total cost of cancer will exceed SR 2 billion by 2030. Liver and breast cancer is considered to be a leading cause of death in Saudi Arabia (Al-Ahmadi and Al-Zahrani, 2013).

Significant interest is gained for cancer drug targets that act as mediators of inflammatory response. The association between cancer and inflammation was first described 10 years ago (Rizzo *et al*., 2011a; Rizzo *et al*., 2011b; Cario, 2010; Debelec-Butuner *et al*., 2014). Since then, inflammation has been reported to increase proliferation and migration/invasion in types of tumor cells (Debelec-Butuner *et al*., 2014). For this reason, inflammation is considered among the most factors contributing to many cancers development and growth (Coussens and Werb, 2002). Several evidences indicate that the association between chronic inflammation and cancer involve TLRs and their signaling pathways (Akira *et al*., 2001; Akira *et al*., 2001; Karin *et al*., 2006; Takeda and Akira, 2004; Takeda and Akira, 2005; Takeda *et al*., 2003). Tumor micro-environment is regulated by inflammatory cells and by mediators and effectors of inflammation. Among the molecules involved in cancer-related inflammation, transcription factor such as NF-kB, signal transducer, activator of transcription (STAT), COX-2 and cytokines such as TNF-a, IL-1b, IL-23 and IL-6 (Grivennikov *et al*., 2010a). IL-6 is a multifunctional cytokine that plays many biologic roles in different types

of cells including tumor cells. Research demonstrating that IL-6 is associated with tumor progression through drug resistance, inhibition of apoptosis in cancer cells, and angiogenesis stimulation. Studies have shown that the increase in IL-6 concentrations are associated with various cancers tumor stages progression and short survival in cancer patients (Guo *et al*., 2012). Hence, anti-IL-6 therapy is a promising therapeutic strategy for cancer treatment. The development of a variety of human cancers can be attributed to chronic inflammation (Guo *et al*., 2012; Hofseth and Ying, 2006).

Thus, anti-inflammatory agents can be effective in cancer treatment. Therefore, the objectives of this study were to (1) ferment the *P. crustosum* isolated and prepare the extract (2) investigate the antiproliferative activity, cytotoxicity and cell migration assay; (3) determine IL-6 production; (4) standardize the crude extract using HPLC.

MATERIALS AND METHODS

General reagents

The RNA kits were from Qiagen (Hilden, Germany). Primers were obtained from Life technologies/ Invitrogen (Burlington, ON, Canada). The cDNA reverse transcription kit was obtained from Applied Biosystems (Warrington, USA). Sybr Green was purchased from Bio-Rad (Mississauga, ON, Canada).

Isolation, purification and maintenance of the P. crustosum

Endophytic fungi were isolated from the roots of healthy plants of *Phoenix dactylifera* obtained from different areas of Riyadh. Roots were washed with tap water and then followed by distilled water for the removal of debris. Six 1cm measuring pieces were cut from each root. The surface sterilized roots were immersion in 3% sodium hypochlorite for 5 min followed by 70% ethanol for 2 min and then rinsed twice with sterile Milli-Q water. The root segments were placed on the of potato dextrose agar (PDA) surface under aseptic conditions, incubated at 30°C and observed for hyphal appearance. The isolated endophytic fungus, *P. crustosum* was maintained on PDA plates.

Preparation and extraction of fungal broth

For fermentation experiments, Agar plugs were taken from an actively growing culture, that were subcultured on fresh potato dextrose agar plate and incubated for 7 days at pH 7.0 and temperature 30°C until growth of mycelia covered almost 80% of the plate. Two 6mm diameter agar plugs were cut with a sterile cork borer and transferred to 200ml flasks containing potato dextrose broth. At 30°C and 150 rpm flasks were kept for 10 days in an orbital shaker. Mycelia were separated from broth by filtration. The broth was extracted sequentially three times with equal volume of hexane, ethyl acetate and butanol in a separatory funnel. The remaining aqueous phase was evaporated to dryness at 50°C using rotary evaporator and later extracted with methanol (100ml 3X). The organic solvents were dried at 45°C using rotary evaporator and stored at -20°C.

Cell culture and extract treatment

HepG2 and MCF7 cells were cultured in DMEM medium supplemented with 10% FBS, 1 mM sodium pyruvate and 1.5g/l sodium bicarbonate at 5% CO_2 and 37°C. Cells were harvested when they are 85% confluent using 0.25% trypsin and were sub-cultured into 75ml flask, 6-well plate, or 24 according to selection of experiments. After 24h, cells were treated with different concentrations ranging between 25, 50, 75, 100 and 200µg/ml of extract and left for 24h. Selection of LC_{50} of HepG2 (80.80µg/ml) and MCF7 (132µg/ml) was based on the dose-response curve. Cells exposed to methanol were used as control.

MTT assay

The cytotoxic activity of the extract was determined by using MTT assay which measures the mitochondrial ability to reduce MTT into blue formazon product in viable cells. Briefly, Cell were seeded in 24-well plates (7×10^4 cells/well) and subjected to several concentrations of the extracts for 24h. Medium was aspirated and washed twice with PBS (phosphate buffer saline) and replaced with fresh medium of MTT solution (5mg/ml in PBS) and incubated at 37°C for 2h. The resulting formazan was dissolved in HCl-isopropanol, kept in a shaker for 5 minutes. A 200µl was pipetted to 96-well plate and measured at 570 nm in a microtiter plate reader (Biochem Ltd, England).

Wound healing assay

Cells were seeded in 6-well plates until it became confluent. Cells in each well was wounded with a pipette tip and treated with 100µg/mL of ethyl acetate fraction. After incubation (0, 12, 24 hours), the cells were photographed under phase-contrast microscopy.

Cytotoxicity assay

As described by Semlali *et al*. (Semlali *et al*., 2011a), MCF-7 and HepG2 cells at (4×10^5) were cultured for 24 h, The cells were stimulated by ethyl acetate fraction and then incubated in a 5% CO_2 at 37ºC for 24h. Following treatment, cell toxicity was evaluated by measuring the lactate dehydrogenase (LDH) activity by using LDH cytotoxicity kit (Promega, Madison, WI). Briefly, 50µl of supernatant was transferred to a clean 96-well plate and mixed with 50µl reconstituted substrate mix. The plate was incubated in the dark at room temperature for 30 min. To stop the reaction, 50µl of an acid solution was added. Finally, 120µl of supernatants was transferred to a 96-well plate and read at 490nm with multiplate reader (Biochem Ltd, England). 1% of Triton X-100 and Methanol were used as positive and negative control respectively. LDH activity release was plotted using optical density value.

Reverse transcription and gene expression by real-time RT-PCR

As described previously (Semlali *et al*. 2011b; Semlali *et al*. 2008), total RNA was extracted using the RNA Mini kit (Qiagen, Hilden, Germany). The isolated RNA concentration and purity were determined using the Agilent 2100 Bio analyzer system and Agilent Small RNA analysis kit according to the instructions provided by the manufacturer (Agilent technologies, Waldbronn, Germany). 1µg of each sample of RNA was reverse transcribed into cDNA using reverse transcription kit (Applied Biosystems, Warrington, USA). The conditions for the preparation of the cDNA templates for PCR analysis were 10min at 25°C, 2h at 37°C, and 5min at 85°C. Quantitative PCR (qPCR) was performed as previously described (Semlali *et al*., 2011b; Semlali *et al*., 2008). The amounts of the mRNA transcripts were measured using the Applied Biosystems 7500 Fast real-time PCR detection system. Reactions were performed using a PCR Sybr Green Supermix from Applied Biosystems. IL6 and GAPDH primers were (table 1). Each reaction was performed in a 7500 fast real time PCR Thermal Cycler. The thermocycling conditions were established as 5 min at 95°C, followed by 36 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C. Each reaction was done in triplicate. The primer pair specificity was confirmed by the presence of a single melt peak. GAPDH produced an even expression levels changing by less than 0.5 CTs between sample conditions were used as a reference gene. To check that there were no false products amplified during the cycles, the products amplified were run on an agarose gel. The results were quantified using the $2^{-\Delta\Delta Ct}$ (Livak) relative expression method.

High performance liquid chromatography (HPLC) fingerprinting

Sample was filtered through 0.2µm polyvinylidene-difluoride Millipore filter and analysis was achieved using a HPLC (PerkinElmer) equipped with UV/VIS detectors. 10µl of sample was injected and analyzed. Column used was C18 (250X4.6mm) that was maintained at room temperature. Water containing 1% acetic acid (A) and acetonitrile (B) was used as a mobile phase. The gradient program was organized as table 1.

STATISTICAL ANALYSIS

All the data represented in this study are means ± SD of three independent replicate. Statistical significance was determined by T-test. Significance was ascribed at $p<0.05$.

RESULTS

There are no previous reports on the isolation of *P. crustosum* from *P. dactylifera*, antiproliferative activity, cell migration, anti-inflammatory of the *P. crustosum* extract reported here.

Endophyte isolation

From 50 fragments of fresh roots and leaves, 12 strains of endophytic fungi were isolated. However, only one endophytic fungus (NAT001) was used in this study. This strain was identified as belonging to the genus Penicillium using compounds Microscope, based on the morphological structures. Most of the morphological characters of the strain (NAT001) agree with the known features of *Penicillium crustosum***.** The cultural and morphological studies confirmed that the isolate NAT001 belongs to genus Penicillium and species crustosum. However this was further confirmed by sending the sample to Mycological Center, Faculty of Science, Assuit, Egypt. The deposit number is (AUMC10172).

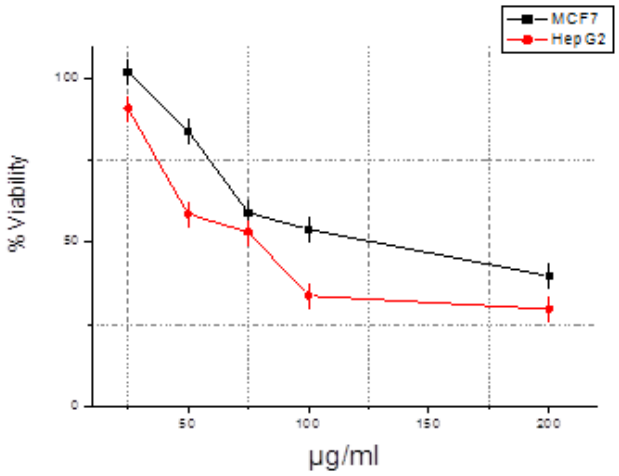

Fig. 1: Cytotoxicity of ethyl acetate fraction on HepG2 and MCF7cell lines. Values are mean of three independent experiments ±SD

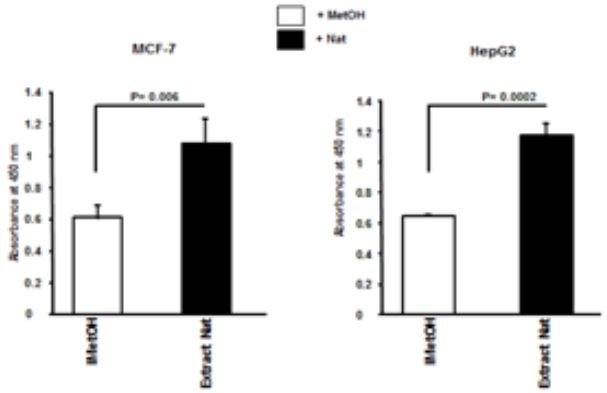

Fig. 2: Cytotoxicity (LDH release). Confluent HepG2 and MCF7 cells exposed to ethyl acetate fraction. Data expressed as averages of three independent experiments. b, Significant ethyl acetate fraction effect within a treatment, $P<0.01$ compared to corresponding medium with methanol exposure

Cell proliferation and LDH leakage assay

In this present study, both MTT assay and LDH leakage assay showed that treatment of cancer cell line with

extract induced cell death in a dose-dependent manner. The *in vitro* cytotoxicity assay indicated that MCF-7 and HepG2 cancer cells were more vulnerable to ethyl acetate than hexane and butanol fraction. Furthermore, Ethyl acetate extract was found to be more effective against HepG2 than MCF-7 cell lines. The extract showed cytotoxic activity with IC_{50} of 82µg/ml against HepG2 cell lines, whereas the IC_{50} of MCF-7 cell lines was 126µg/ml (fig 1). However, we investigated the cytotoxic effect on HepG-2 cells at the concentrations of 82µg/ml after 24h of ethyl acetate fraction treatment and the result showed a significant damage to treated cells (fig. 2).

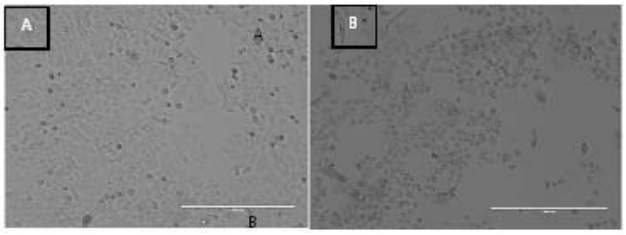

Fig. 3: Alteration of cellular morphology in ethyl acetate fraction treated HepG2 cells. Contraction and shrinkage of cellular morphology of HepG2 cells in the presence of the ethyl acetate fraction. (A) Control; (B) Treated.

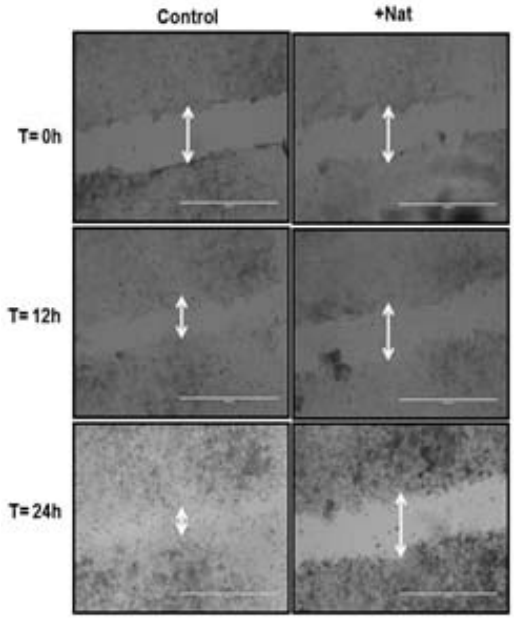

Fig. 4: Effect of ethyl acetate fraction on the migration of HepG2 cells *in vitro*. Cells in 6-well plates were wounded by scratching with a pipette tip and the cells were incubated with 100µg/mL for 0, 12 and 24 hours. The cells were photographed under phase-contrast microscopy

Morphological changes of apoptotic treated with ethyl acetate fraction extract

The morphology of control untreated cells have retained their regular cellular morphologies. However, the normal morphology of cultured HepG2 cells treated with ethyl acetate fraction (100µg/ml) has changed after 24h of incubation. The changes included shrinkage of cytoplasmic materials, detachment and loss of cellular integrity (fig. 3B).

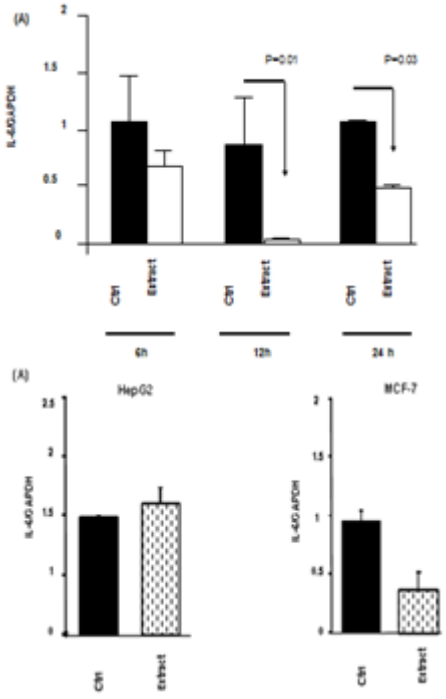

Fig. 5: Effect of *ethyl acetate active* fraction on IL-6 transcription expression on HepG2 cells. Cells were treated with 100µg/ml of the *P. crustosum* active fraction after 24 h. Each bar represents the mean ± SD, n=3.

Scratch motility assay

To further confirm the inhibitory effect of ethyl acetate fraction of *P. crustosum* on HepG2 cell lines migration, a wound healing assay was performed (fig. 4). After 0, 24 and 48 h of incubation, cells gradually covered the space of wound in control while in treated group the ethyl acetate fraction (100µg/ml) didn't cover the space of wound.

Fusarium oxysporum inhibited migration of the metastatic cancer cells lines with a notable anti-angiogenic action (Zhan *et al*., 2007; Matic *et al*., 2013; Lalou *et al*., 2013).

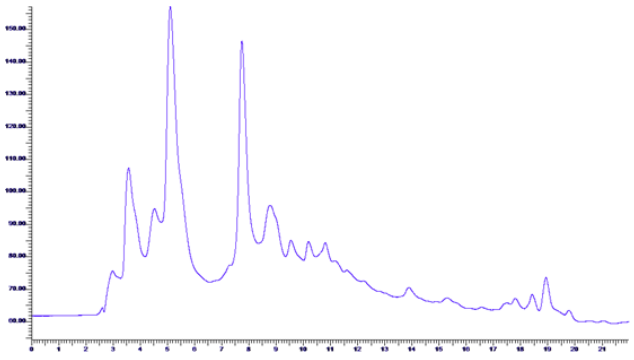

Fig. 6: HPLC chromatogram at 254 nm of the ethyl acetate fraction.

Table 1: Description of primer pairs used in PCR reactionsHPLC analysis

Gene	Primer sequence (5' to 3')	Amp size(bp)
IL-6	F: 5'-TCTCCACAAGCGCCTTCG-3' R: 5'-CTCAGGGCTGAGATGCCG-3'	203
GAPDH	F: 5'-GGTATCGTCGAAGGACTCATGAC-3' R: 5'-ATGCCAGTGAGCTTCCCGTTCAGC-3'	180

Table 2: The gradient program of HPLC mobile phase in profiling of ethyle acetate fraction

Step	Time (min)	Flow (ml/min)	Solvent A	Solvent B	Curve
0	5	0.5	80	20	0
1	15	1	20	80	1
2	5	1	0	100	0
3	2	1	80	20	0

Effect of Ethyl acetate fraction on the expression of IL-2

To study the Il-6 and IL-8 expression brought by *P. crustosum*, a quantitative real-time reverse transcription-PCR was performed. As shown in fig. 5. Ethyl acetate fraction significantly inhibited IL-6 transcript expression ($p=0.025$) at 100µg/mL after 12 and 24h of exposure against MCF7 cell lines. The experiment was further repeated after 12h of exposure to confirm our finding (fig. 5). It was found the extract reduced IL-6 transcript expression on MCF7 cell line but not on HepG2 cell line.

High performance liquid chromatography (HPLC) fingerprinting

HPLC fingerprint was established for the active ethyl acetate fraction that revealed *in vitro* antiproliferative activity against HepG2 and MCF-7 cell lines and anti-inflammatory against MCF-7 only (fig. 6).

DISCUSSION

There are no previous reports on the isolation of *P. crustosum* from *P. dactylifera*, anti-proliferative activity, cell migration, anti-inflammatory of the *P. crustosum* extract. Cancer is multifactorial diseases characterized by out-of-control cell growth and by tumor cells migration associated constantly with increased expression of several genes involved in cell growth and cell motility (Wang *et al*., 2004). Therefore, molecules involved in cancer cell growth and migration could be potential targets for anti-metastasis therapy.

This study proved the anti-proliferative and cytotoxic activity of the P. crustosum extract in liver (HepG2) and breast (MCF-7) cancer cells in a dose dependent manner. The anti-proliferative effects of the ethyl acetate fraction tested showed that this fraction can be used as a potential anticancer treatment for many cancers. The leakage of the cytosolic enzyme mainly LDH is a key indicator of membrane integrity loss. Its release is a typical marker for the late stage of apoptosis and early necrosis (Do *et al*., 2003). Similar results were observed in other endophytic extract in particular the anticancer activity (Zhao *et al*., 2013 ; Hazalin *et al*., 2012). Metastasis is the major threat for a successful cancer treatment (Lu *et al*., 2010) and the prevention of metastasis is one of the main target for improving a patient's health and prognosis. To further confirm the inhibitory effect of ethyl acetate fraction of P. crustosum on HepG2 cell lines migration, a wound healing assay was performed (fig. 4). This is consistent with the effects of other endophyte extracts. Reserachers have demonstrated that the extracts of endophytic Fusarium oxysporum inhibited migration of the metastatic cancer cells lines with a notable anti-angiogenic action (Zhan *et al*., 2007; Matic *et al*., 2013; Lalou *et al*., 2013). Inflammatory responses play significant roles at different stages of tumor development, mainly initiation, invasion and metastasis (Grivennikov *et al*., 2010b). However, the extract showed specific anti-inflammatory activity against breast cancer cell line only therefore; further investigation is required to confirm our finding. HPLC fingerprinting is the best way for chemical characterization (Springfield *et al*., 2005) therefore, in this study, HPLC fingerprint was established for the active ethyl acetate fraction that revealed in vitro anti-proliferative activity against HepG2 and MCF-7 cell lines and anti-inflammatory against MCF-7 only (fig 6).

CONCLUSIONS

This study evaluated the cytotoxicity and anti-inflammatory activity of the ethyl acetate fraction of *P. crustosum*. Further study is required to purify the active compound in the ethyl acetate fraction; therefore, it remains unclear whether the activity shown by this fraction is caused by the action of one single compound, or by the additive and/or synergistic effects of multiple compounds in this fraction. This is the first study to give positive *in vitro* results of anti-inflammatory activity from *P. crustosum* extract. The significant inhibition of IL-6 transcript expression displayed by *P. crustosum* ethyl acetate fraction indicates that fraction possesses at least one anti-inflammatory compound that deserves further studies.

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