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Full Length Research Paper

Anti-proliferative effect of *Pleurotus tuberregium* against colon and cervical cancer cells

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Pleurotus tuberregium **is an exotic mushroom species which grows in tropical and subtropical parts of the world. This species is used in Africa for food and as medicine for the treatment of certain diseases. There is limited scientific information on the effects of *P. tuberregium* against cancer cells. This study was carried out to determine the antiproliferative activities of *P. tuberregium* ethanol and water extracts against HCT-116 colon and HeLa cervical cancer cells at concentrations of 0, 100, 200, 400, 600, and 800 μg/ml after 24, 48, and 72 h of exposure using the MTT assay. After 72 h, the ethanol extract of *P. tuberregium* at 800 μg/ml had inhibitory activities of 65 and 47% when tested on HCT 116 and HeLa cells, respectively, while the dried water extract from *P. tuberregium* at the same concentration had inhibitory activities of 45 and 37%. Since both alcohol and water crude extracts of *P. tuberregium* exhibited relatively strong antiproliferative activity, their pharmaceutical applications as chemopreventive supplements or potentially in complementary treatment of cervical and colon cancers need to be further investigated.**

Key words: *Pleurotus tuberregium*, colon and cervical cancer, antiproliferation, complementary, alternative medicine.

INTRODUCTION

Cancer is the second leading cause of death in the U.S. exceeded on only by cardiovascular diseases (ACS, 2009). Current cancer treatments include chemotherapy, radiotherapy, and surgery. However, since conventional methods often have undesirable side effects, alternative treatments referred to as complementary and alternative methods (CAM) are being increasingly used. The most commonly used CAMs include spiritual, chiropractic, homeopathy, acupuncture, and herbal medicine treatments (Cassileth and Deng, 2004; Abenavoli et al., 2008). A major advantage of these alternative therapies is that they have fewer side effects than conventional therapeutic procedures (Deng and Cassileth, 2005). Most CAM products are easily-accessible either over the counter or online. In a recent survey, it was reported that Americans spent $33.9 billion/year on CAM to treat different diseases (Nahin et al., 2009). Plant extracts have been used as CAM in several clinical trials. For example, Taxol® derived from the bark of the Pacific yew tree is used in the treatment of cancers such as head and neck, breast, lung, ovarian, melanoma and lymphomas (Grube et al., 2001). Etoposide, derived from the Mayapple plant, is used to treat bladder, prostate, lung, stomach, and uterine cancers (Baldwin and Osheroff, 2005). *Alstonia scholaris*, commonly known as devil's tree, is an important medicinal plant in Asia, Australia, and Africa. In a recent review, the activity of *A. scholaris* as a radiomodulatory, chemomodulatory, and chemopreventive effects and free-radical scavenging, antioxidant, anti-inflammatory, antimutagenic, and immunomodulatory activities have been described in the treatment and prevention of cancer (Baliga, 2010). Many studies have shown that mushroom extracts inhibit the proliferation of cancer cells and the transformation of normal cells into cancer cells (Zhan et al., 2006; Fang et

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al., 2006). For thousands of years, mushrooms have been used for both edible and medicinal purposes. People from the Far East such as China and Japan have used mushrooms including lingzhi, shiitake, and maitake for medicinal purposes for centuries. In the United States, mushroom consumption has been increasing due to their believed health promoting benefits (Lucier et al., 2003).

Mushrooms contain polysaccharides such as beta-glucans that have antitumor and immune stimulating properties (Wasser, 2002). Borchers et al. (2004) indicated that polysaccharides from mushrooms show antitumor activity by reducing proliferation of HeLa (cervical cancer cells) and HCT-116 (colon cancer) cells. Because they appear to cause no harm to the body and have immunomodulatory, anti-neoplastic, and lipid-reducing activities (Lavi et al., 2006), further exploration of mushrooms for their anticarcinogenic effects is highly warranted. Recently, phytochemical investigations on *Thelephora aurantiotincta,* an edible mushroom*,* revealed that a *T. aurantiotincta* ethanol extract (TAE) was found to decrease cell viability in human hepatocellular carcinoma cells (HepG2) *in vitro* (Norikura et al., 2011).

Pleurotus tuberregium (Fr.) Singer, a mushroom of the Basidomycetes family found in the tropics and subtropics, is commonly called King Tuber Oyster Mushroom (Wong and Cheung, 2008). The sclerotium of this mushroom is important because it contains the mycelial tissue that is harvested for food and medicinal purposes. Studies have evaluated the bioactivities of *P. tuberregium* on leukemia (HL-60) and hepatic (HepG2) cancer cells and have found that the active compound, β-D-glucan, from the cell wall is highly effective in reducing the proliferation of HL-60 and HepG2 cells at 200 µg/ml (Zhang et al., 2001). In a recent study by Tao et al. (2009), it was found that two water soluble extracts from *P. tuberregium* exhibited potent activity against Sarcoma 180 cells *in vitro* at 2 mg/ml. However, there is no information available concerning antiproliferative effects of *P. tuberregium* on colon and cervical cancer cells. Therefore, the objective of this study was to evaluate the antiproliferative effects of *P. tuberregium* extracts against cervical (HeLa) and colon (HCT-116) cancer cells *in vitro*.

MATERIALS AND METHODS

Mushroom material

P. tuberregium was purchased from Fungi Perfecti (Olympia, WA). The sclerotia of *P. tuberregium* were removed from the culture bags, cut into small pieces, and stored at -80°C. The samples were freeze-dried for 72 h in a Labconco free-zone dryer (Kansas City, Missouri). After being freeze-dried the samples were made into a powder using a grinder (Reustch 1640, Germany).

Extraction procedure

The powdered materials (500 g) were divided into two groups, the first group of samples were soaked in 100% sterile deionized water, while the second group of samples was soaked in 80% ethanol (1:5 w/v) overnight with continuous stirring at room temperature. The liquid material was then removed and centrifuged at 10375 × *g* for 20 min at 4°C.

The supernatant was collected in a flask and the final residue was discarded. The water or ethanol in the supernatant was then evaporated under reduced pressure using a Rotovapor (Buchi, Germany) producing crude water and alcohol *P. tuberregium* extracts.

Cell culture

HCT-116 (Human colorectal cancer cell line) and HeLa (Human cervical adenocarcinoma cancer cell line) were purchased from American Type Culture Collection (ATCC, Manassas, VA). HCT-116 cell were cultured in McCoy's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin; and human cervical adenocarcinoma (HeLa) cells cultured in Dulbecco's Modified Eagle medium with 2 mM sodium pyruvate supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cancer cells were maintained at 37°C in a 5% CO_2 incubator with fresh media added every 2 to 3 days for the duration of the study to allow cell growth and proliferation.

Antiproliferative activity assay

The antiproliferative activity of the two *P. tuberregium* crude extracts was evaluated at concentrations of 0, 100, 200, 400, 600, and 800 µg/ml. The extracts were dissolved in a media consisting of 10% fetal bovine serum, 1% streptomycin, along with 89% McCoy's media for HCT-116 cells and 89% Dulbecco's media for HeLa cells. The media was filter sterilized using a 0.22 µm syringe filter. HeLa and HCT- 116 cells, at concentrations 5×10^4 cells/ml, were seeded in a 96-well microplate containing different concentrations of crude extracts. Mitomycin C™ (Sigma-Aldrich, St. Louis, MO) was used as a positive control at 200 µg/ml. The microplates were then incubated for 24, 48, and 72 h at 37°C in a 5% CO_2 atmosphere. After each incubation time period, aliquots of 3-(4,5-dimethythiazol-2-yl)-2,5 diphenyltetrazolium bromide dye, (MTT) (Sigma-Aldrich, St. Louis, MO) were added to each test well at 4:15 ratio and incubated for 3 h. The absorbance was measured at 490 nm using an Elx808 Ultra Microplate Reader (BioTek, Vienna, VA). All assays were performed three times. All results were expressed as the inhibition ratio (φ) of cancer cells proliferation as follows:

$$\Phi = [(A - B) / A] \times 100$$

where A and B are the average number of viable cancer cells of the control group and test group, respectively.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using statistical analysis software (SAS, 2000) with mean treatment at each concentration tested separately by comparing percent reduction. Least significant means was run as a post ANOVA test to determine significance at $p<0.05$.

RESULTS

Table 1 presents a comparison of the antiproliferative activities at 200 µg/ml of the alcohol and water *P.*

Table 1. Percent cell inhibition by *P. tuberregium* extracts and Mitomycin C™ compared at 200 μg/ml during 24, 48 and 72 h exposure.

Parameter	HCT 116 (24 h)	HCT 116 (48 h)	HCT 116 (72 h)	HeLa (24 h)	HeLa (48 h)	HeLa (72 h)
Alcohol	7.74±0.02*	24.17±0.11*	25.12±0.15*	5.84±0.11**	28.84±0.11	34.02±0.09**
Water	7.16±0.09*	17.08±0.18*	23.72±0.01*	0±0.0	18.57±0.14*	19.87±0.08*
Mitomycin C™	17.82±0.05	30.11±0.06	33.66±0.08	2.69±0.09	26.28±0.11	28.37±0.16

* Significantly different (p <0.05) from Mitomycin C™. ** Significant inhibition of *P. tuberregium* extracts-induced activity compared to Mitomycin C™.

tuberregium extracts along with the activity of Mitomycin C™, a known anticancer drug, used as a positive control in this study.

The inhibition of both cancer cell lines was significant at 48 and 72 h of exposure, suggesting that a period of activation is necessary. For HeLa cells, the alcohol extract of *P. tuberregium* showed greater inhibitory activity compared to the purified Mitomycin C™ at all time periods of exposure.

Figures 1 and 2 show dose-dependency of the *P. tuberregium* extracts on each of the cancer cell lines. Figure 1 indicates that both alcohol and water *P. tuberregium* extracts showed inhibitory activity on cell proliferation of HCT-116 cells at all concentrations and time intervals of the study. However, it is apparent that longer activation time is necessary for significant inhibition. The *P. tuberregium* ethanol extract was significantly potent ($p<0.05$) to HCT 116 cells in comparison with the baseline at a concentration of 400 μg/ml and above with a reduction rate reaching 65% at 800 μg/ml at 72 h of exposure. Antiproliferative activity of the alcohol extract does not taper off as the time of exposure approaches 72 h. The activity of water extract from *P. tuberregium* was also significant ($p<0.05$) with close to 50% reduction rate at 800 μg/ml after 72 h of exposure. Both extracts were effective in reducing or inhibiting the growth and proliferation of colon cancer cells.

Figure 2 shows the effect of crude *P. tuberregium* extracts on HeLa cells. The alcohol extract of *P. tuberregium* exhibited greater inhibitory activity than the water extract of *P. tuberregium* at all time intervals. After 24 h of exposure, 25% reduction was observed at the highest concentration, suggesting that the active compounds in this extract did not have sufficient time to be absorbed by HeLa cells.

The alcohol and water extracts from *P. tuberregium* both significantly slowed the growth of HeLa cells at concentrations of 200 μg/ml and higher following a 48 h incubation period. The alcohol extract of *P. tuberregium* had a reduction rate reaching 47% at 800 μg/ml after 72 h of exposure. The activity of water extract from *P. tuberregium* was also significant, although to a lesser degree compared with ethanol extract, reaching 37% at 800 μg/ml after 72 h. Both extracts were effective in reducing the growth and proliferation of cervical cancer cells.

DISCUSSION

The experimental results indicate that crude extracts of *P. tuberregium* significantly reduced the proliferation of viable HCT-116 colon and HeLa cervical cancer cells in a time and dose-dependent manner. Components in mushrooms have previously been shown to inhibit cancer cell proliferation *in vitro* and *in vivo* with effects exerted at different levels including immuno-enhancing activity and direct tumor inhibition (Zhang et al., 2007). The active compounds may be in the form of peptides, polysaccharides, glycoproteins, or phytochemicals. For example, fruiting bodies of the puffball mushroom, *Calvatia caelata*, has been shown to produce a peptide of 8 kDa with an N-terminal sequence that is similar to ubiquitin (Lam et al., 2001). The purified peptide was found to reduce the viability of breast carcinoma cell line MDA-MB-231 by half at a concentration of 0.1 μM. The white button mushroom, *Agaricus bisporus*, produces phytochemicals that are thought to be responsible for the inhibition of MCF7 breast cancer cell proliferation through aromatase inhibition (Grube et al., 2001). Since studies have suggested that β-D-glucan is responsible for the bioacitivity of *P. tuberregium* against breast cancer (Zhang et al., 2007), the antiproliferative effects of *P. tuberregium* toward colon and cervical cancer cells in this study might be likely due to the same compound. More studies are needed to prove this fact.

Compounds that result from the extraction process often vary depending on the extraction methods (Wang and Weller, 2006). For example, using hot water for extraction will yield water-soluble extracts while extraction with alkali solutions results in water-insoluble extracts. Further, chemically fractionating the initial extract often results in a variety of groups that have different functions from one another. For example, the mushroom, *Hypsizygus marmoreus*, has been shown to produce 1,3-α-Glucan and 1,3-β-Glucan from its fruiting bodies (Motoi et al., 2003). Only the purified 1,3-β-Glucan polysaccharide exhibited antitumor activity against Sarcoma 180 tumor in mice while 1,3-α-Glucan did not. In another study, five polysaccharides were derived from the Basidiomycete, *Grifola frondosa*, which have diverse molecular masses with different biological activities (Lee et al., 2003). A few of the fractions resulted in strong free radical and scavenging activity while other fractions increased the proliferation of fibroblasts. These studies

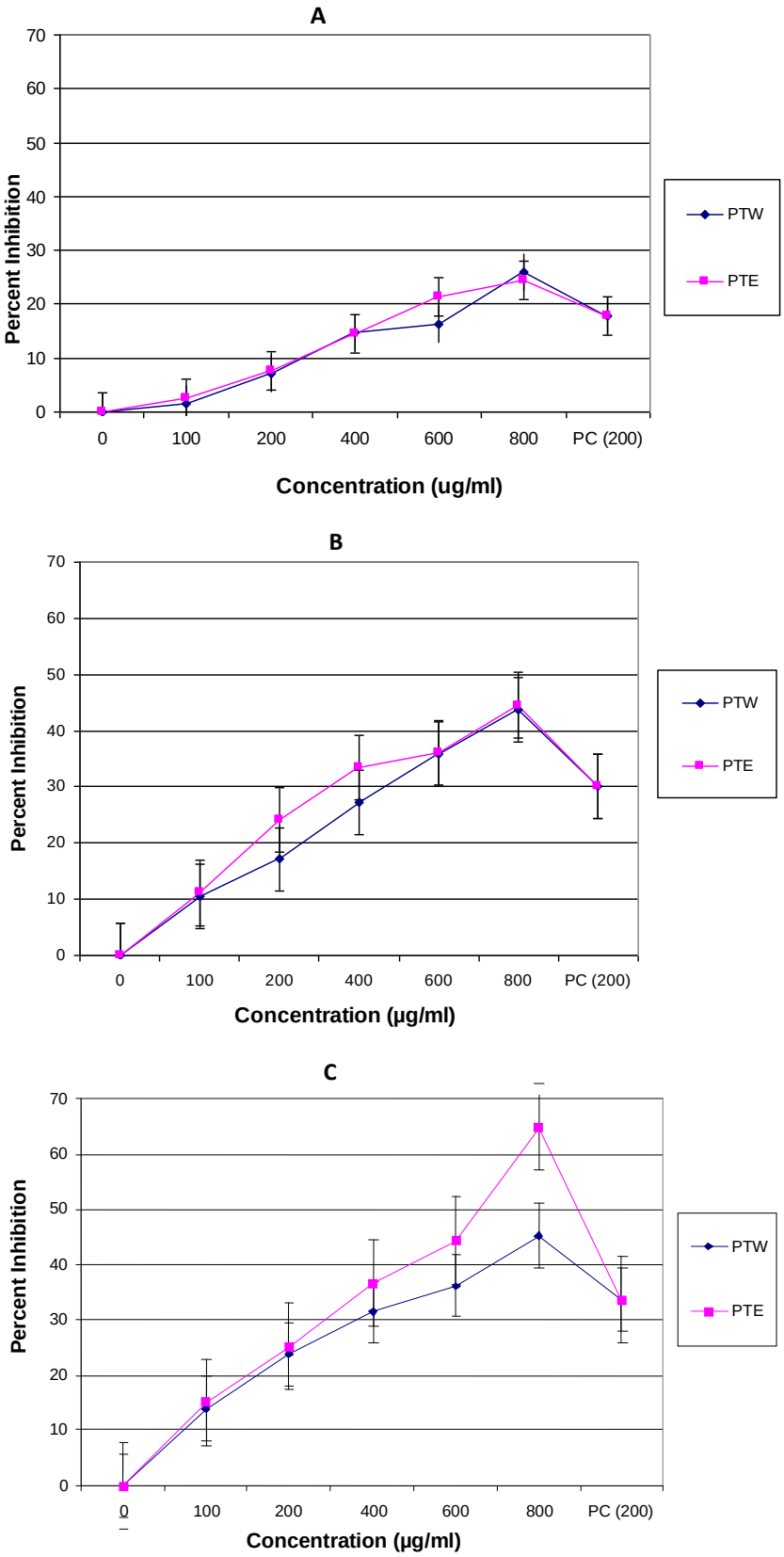

Figure 1. Antiproliferative activity (% Cell inhibition) of the crude extracts from *P. tuberregium* on HCT-116 cells after A) 24 h, B) 48 h, and C) 72 h exposure. PTW = Water extract of *P. tuberregium* and PTE = Alcohol extract of *P. tuberregium*).

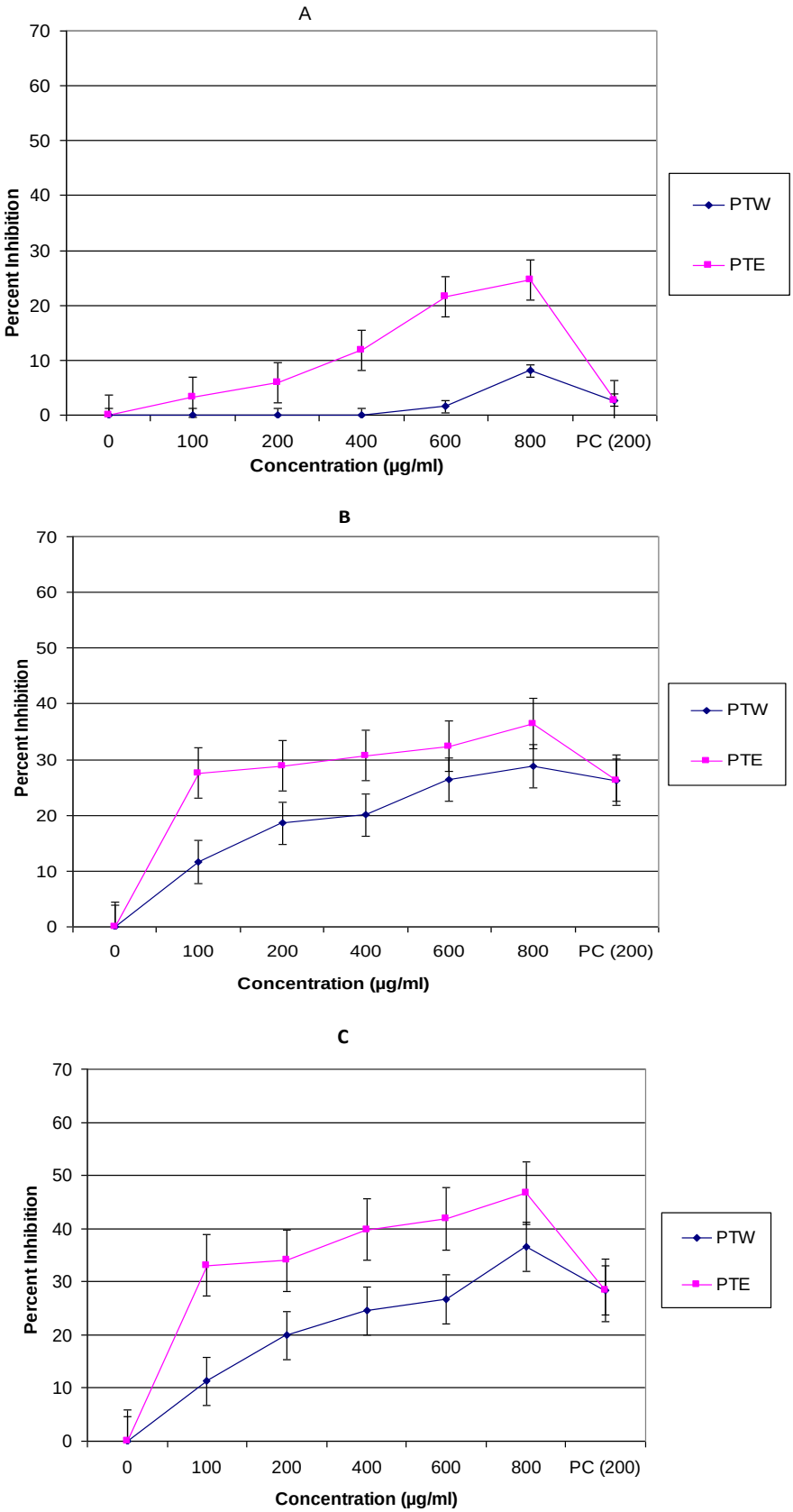

Figure 2. Antiproliferative activity (% Cell inhibition) of the crude extracts from *P. tuberregium* on HeLa cells after A) 24 h, B) 48 h, and C) 72 h exposure. PTW = Water extract of *P. tuberregium* and PTE = Alcohol extract of *P. tuberregium*).

highlight the idea that it may be more precise to use purified fractionated groups of compounds in mushroom extracts when studying their effects since the crude extracts likely contain diverse groups with functions that vary. In our study, however, the crude alcohol extract showed greater inhibition of HeLa cells than the purified mitomycin C used as the positive control. Some crude extracts have compounds that would work synergistically and thus result in weaker anti-carcinogenic activity when separated than when used together in the intact crude extract.

P. tuberregium extracts used in this study demonstrated the ability to inhibit proliferation of cancer cells *in vitro*, with inhibitions reaching 65 and 47% using the ethanol extract on HCT-116 and HeLa cells, respectively. This extract likely contains a number of bioactive compounds with diverse biological activity. Future studies should focus on determining the effect of the active compounds of *P. tuberregium* on gene and protein expression in the cancer cells. Additional research is also necessary to investigate the time and dose-dependent antiproliferative activity *in vitro* of these extracts on other cancer cells. Finally, animal bioassays will be essential in determining the inhibitory activity, acceptable levels of exposure, and side-effects of crude *P. tuberregium* extracts *in vivo.* This will allow comparisons to be made to currently used methods of treatment and potentially offer an alternative for the reduction of undesirable side-effects typically associated with synthetic compounds.

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