

Original Article

Antiproliferative, apoptotic induction, and antiinvasive effects of *Leersia hexandra* (L.) Sw., *Panicum repens* Linn., and *Brachiaria mutica* (Forsk.) Stapf extracts on human cancer cells

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Abstract

In vitro study of antiproliferation and cytotoxicity of the hexane and butanolic extracts of the three grass species, namely *Leersia hexandra* (L.) Sw. (“Yaa sai”), *Panicum repens* Linn. (“Yaa channakaat”), and *Brachiaria mutica* (Forsk.) Stapf (“Yaa khon”) demonstrated selective antiproliferative properties in both human lung cancer A549 and cervical cancer HeLa cell lines relative to normal human lung fibroblasts MRC-5, with significant differences in IC₅₀ values ($p<0.05$). The butanolic extract of *P. repens* Linn. displayed the strongest growth inhibition in A549 and HeLa cells (IC₅₀ 2 and 1.1 mg/ml, respectively), whereas all other extracts exhibited only moderate to low antiproliferative effects (IC₅₀ values, 90-980 mg/ml). Most grass extracts induced cell lethality at higher concentrations, suggesting their cytotoxic effects. The ELISA-based apoptosis assay showed that the hexane extract of these grasses triggered a significant increase in the level of apoptosis ($p<0.05$) in treated A549 cells. However, all the extracts induced ladder-like DNA fragmentation in both tumor cell lines in a dose- and time-dependent manner, suggesting that these extracts exhibit cytotoxicity through apoptotic induction. The study of anti-invasive effects of the three grass species revealed that the hexane extract of *L. hexandra* (L.) Sw. (50-100 µg/ml) and butanolic extract of *P. repens* Linn. (1 µg/ml) effectively reduced the invasive capacity of MDA-MB-231 cells.

Keywords: *Leersia hexandra* (L.) Sw., *Panicum repens* Linn., *Brachiaria mutica* (Forsk.) Stapf, antiproliferative, apoptosis, antiinvasive

1. Introduction

Cancer is one of the leading causes of death in the world. Lung and cervical cancers are among the most commonly diagnosed cancers in men and women, respectively in Thailand (Vatanasapt *et al.*, 2002). Currently, chemotherapy

is still the standard treatment method for lung and advanced cervical cancers together with radiation and surgery for advanced cervical cancer, but these treatments provide only a limited increase in survival rate (Bradley, 2005; Le Chevallier and Lynch, 2004; Moore, 2006). Due to the low efficacy and severe side effects of these current treatment options, an increasing number of cancer patients resort to alternative medicines, including herbal therapies.

Due to the geographic location in the tropical region, Thailand is naturally abundant in species of medicinal plants

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and herbs. Interestingly, several plants in the Poaceae (Gramineae) family such as *Bambusa arundinacea* Willd. (wild bamboo), *Coxi lachryma* (adlay seed), *Lolium multiflorum* Lam. (ryegrass) have been shown to possess anticancer activities. For instance, the methanolic extract prepared from bamboo leaves was reported to contain the bioactive compounds of methyl diester that induced rapid apoptosis in the human leukemia CMK-7 cells (Kim *et al.*, 1995). The methanolic extract of the adlay seed inhibited the growth of human lung cancer A549 cells by inducing cell cycle arrest and apoptosis (Chang *et al.*, 2003). In addition, two bioactive compounds such as sodium 1-monolinolenin (SML) (Kyohkon *et al.*, 2001) and phytol (Komiya *et al.*, 1999), isolated from the leaves of Italian ryegrass, showed growth inhibition and apoptotic induction on human lymphoid leukemia Molt 4B cells in a dose- and time-dependent manner. Also belonging to the Poaceae family, the three grass species: *Leersia hexandra* (L.) Sw. ("Yaa sai"), *Panicum repens* Linn. ("Yaa channakaat"), and *Bracharia mutica* (Forsk.) Stapf ("Yaa khon"), are Thai herbal medicines, which have long been prescribed for treating various diseases. Some parts of these plants are commonly made for herbal medicines by soaking in alcohol. For instance, leaves, stems, and other unindicated parts of *L. hexandra* (L.) Sw., roots and rhizomes of *P. repens* Linn. as well as *B. mutica* (Forsk.) Stapf are used in the treatment of dysuria, fever, gonorrhea, menopausal symptoms, heart, kidney, and liver diseases (Boonyaprapashara and Chokchaichareonporn, 2000).

However, the antitumor activity of these three grass species has not yet been investigated. Moreover, since the current therapeutic modalities for advanced lung and cervical cancers are limited, continual search for novel anticancer agents with highly therapeutic efficacy and less side effects is necessary. Therefore, in this study, we examined the growth inhibitory and apoptosis-inducing effects of the grass extracts on two human cancer cell lines: non-small lung cancer A549 and cervical cancer HeLa cells versus the normal human lung fibroblast cell line MRC-5. Also, we investigated the antiinvasive effect of the extracts on MDA-MB-231, a highly invasive breast adenocarcinoma cell line.

2. Materials and Methods

2.1 Plant material

The whole plants of *Leersia hexandra* (L.) Sw. ("Yaa sai"), *Panicum repens* Linn. ("Yaa channakaat"), *Bracharia mutica* (Forsk.) Stapf ("Yaa khon") were collected from Phitsanulok, Thailand, in April 2005. The voucher specimens of these three plants are Totium 010, 007, and 006, respectively, are kept at the Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok. The plant material was identified by Assoc. Prof. Dr. Kornkanok Ingkaninan, Pharmaceutical Sciences, Naresuan University, Phitsanulok, Thailand.

2.2 Preparation of plant extracts

The collected plant materials were completely dried, ground, and macerated in 95% ethanol for 3 days and filtered. The marc was remacerated in 95% ethanol for another 3 days and filtered. The 2 sets of the filtrate were pooled and evaporated to give crude extract, which was then dissolved in mixed solvent of methanol and water (9:1). The dissolved crude extract was re-extracted in succession with the equal volume of hexane, dichrolomethane, and butanol, each of which was used 3-4 times. The extracts obtained from each solvent were combined and concentrated to dryness under reduced pressure. The percentage yield is shown in the order of hexane, dichrolomethane, and butanol as follows: *L. hexandra* (L.) Sw. (28.34, 6.19, 13.11), *P. repens* Linn. (13.65, 7.01, 20.24), *B. mutica* (Forsk.) Stapf (18.49, 16.92, 19.33). These extracts were dissolved in DMSO to make a stock solution and sterilized by filtration (pore size as 0.45 μ m).

2.3 Cell culture

Three cancer cell lines were used in this study: A549 (human non-small cell lung cancer), HeLa (human cervical cancer), and MDA-MB-231 (human breast cancer), all of which were cultured at 37°C and 5% CO₂ in RPMI-1640 supplemented with 10% FBS and 1% antibiotic-antimycotics. A549 was kindly provided by Dr. Chantragan Phiphobmongkol (Chulabhorn Research Institute). The normal human lung fibroblast cell line MRC-5, which was cultured in MEM supplemented with 10% FBS, 1 mM nonessential amino acid, 1% sodium pyruvate, and 1% antibiotic-antimycotics, was also tested in this study.

2.4 Antiproliferation assay

HeLa, A549, and MRC-5 cells were seeded at 1x10⁴ cells per well in 100 μ l of media containing each grass extract at 31.25, 62.5, 125, 250, 500, 1000, 2000 μ g/ml. This concentration range is not applicable to the butanol extract of *P. repens* Linn. because all cell lines tested could be totally killed with this range. Consequently, the cell survival curve could not be determined, resulting in unobtainable IC₅₀ values. The optimal range for this extract is 0.392 to 6.25 μ g/ml. Taxol was used as positive control at 0.0256, 0.128, 0.64, 3.2, 16, 32 nM. After the cells were incubated with each extract for 3 days, the effect of these extracts on cell growth was determined by SRB assay as described previously (Fricker and Buckley, 1996; Skehan *et al.*, 1990). Cell survival was measured as the percentage absorbance at 492 nm obtained with treated cells compared to untreated cells. The IC₅₀ values were calculated from the Prism program by plotting the percentage survival versus the concentrations, interpolated by cubic spine.

2.5 Cell death detection by enzyme-linked immunosorbent assay (ELISA)

Apoptosis was assayed using Cell Death Detection ELISA^{PLUS} Assay (Roche, Indianapolis, IN) that quantitatively measures cytoplasmic histones associated DNA fragments (mono and oligonucleosomes) produced as a result of apoptosis in cells. For determination of apoptosis by ELISA, A549 and HeLa cells (1×10^4 cells per well) were incubated in the absence (negative control) or presence of each grass extract in the range of 100-1000 $\mu\text{g}/\text{ml}$ for 48 h, except the butanol extract of *P. repens* Linn. used at 1.0 $\mu\text{g}/\text{ml}$. Cell lysates were placed into the streptavidin coated microplate for analysis according to the manufacturer's instructions (Kikuchi *et al.*, 1997). The apoptotic level was expressed as an enrichment factor of nucleosomes in cytoplasm and was calculated as follows: Enrichment factor = [absorbance of the sample (treated)] / [absorbance of the corresponding negative control]

2.6 Analysis of DNA fragmentation

1×10^6 A549 and HeLa cells were incubated overnight and treated separately with grass extract for 24, 48, and 72 h in 6-well flat bottom plates. Cells were collected and DNA was extracted and resuspended in hydration buffer (10 mM Tris.Cl, pH 8.5) according to the manufacturer's instructions (FlexiGene DNA Kits, Qiagen). The collected DNA (10 mg) was electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

2.7 Invasion Assay

The tumor invasion assay was performed using MultiScreen-MIC filter plates (Millipore) precoated with Matrigel according to the manufacturer's instructions (Ngamkitidechakul *et al.*, 2003). MDA-MB-231 cells were seeded at 5×10^4 cells per well into the upper well of the MultiScreen-MIC filter plate and incubated in the absence (the control group) or presence of plant extract at desired concentration (the test group) in serum-free medium. The lower well of the MultiScreen-MIC receiver plate was filled with 10% serum-containing medium as a chemoattractant. After 48 h of incubation at 37°C and 5% CO_2 , the invaded cells in the bottom well were labeled with Calcein AM and fluorescence was read in a fluorescence plate reader at excitation/emission wavelengths of 485/530 nm. Cells in duplicate wells without Matrigel coated filter served as controls for cell proliferation and/or death during the incubation period. Relative invasion was calculated by dividing the fluorescence value of invading cells by that of total cells plated in duplicate wells without coated filter. Percent stimulated invasion in presence of grass extract is calculated as follows: % Cell invasion = [Relative invasion_(test) / Relative invasion_(control)] $\times 100$

3. Results and Discussion

3.1 Antiproliferative and cytotoxic effects of the grass extracts on cell growth

The three grass extracts namely *L. hexandra* (L.) Sw., *P. repens* Linn, and *B. mutica* (Forsk.) Staph displayed a dose-dependent reduction in cell survival of all cell lines tested and induced cell lethality at higher concentrations, suggesting their cytotoxic effects (data not shown). Interestingly, these extracts revealed selective antiproliferation against both tumor cell lines relative to normal fibroblast MRC-5 with significant differences in IC_{50} values (Figure 1A and 1B). The butanolic extract of *P. repens* Linn. showed the highest growth inhibitory activity against A549 and HeLa tumor cells ($\text{IC}_{50} = 2$ and 1.1 mg/ml, respectively). On the contrary, its hexane extract was much less potent to these tumor cells, which is similar to the hexane and butanolic extracts of *L. hexandra* (L.) Sw. and *B. mutica* (Forsk.) Staph

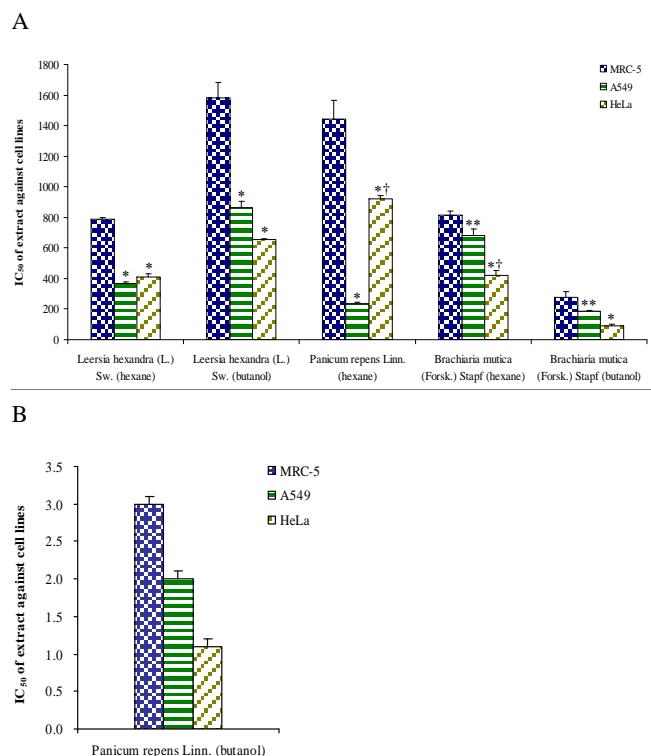


Figure 1. IC_{50} ($\mu\text{g}/\text{ml}$) of active grass extracts on A549 and HeLa cancer cells versus MRC-5 fibroblasts; these cells were treated with (A) the hexane and butanol extracts of *L. hexandra* (L.) Sw., *B. mutica* (Forsk.) Staph, the hexane extract of *P. repens* Linn., or with (B) butanolic extract of *P. repens* Linn. Values are presented as mean \pm SEM ($n = 3-5$). * Significantly different from control at $p < 0.01$ ** Significantly different from control at $p < 0.05$ † Significantly different from A549 at $p < 0.01$ IC_{50} (nM) of the anticancer drug paclitaxel (Taxol) on A549, HeLa, and MRC-5 is 8.5, 2.1, and 1.1 nM, respectively (not shown in bar graph).

that exhibited moderate to low antitumor activities (IC_{50} = 90-980 mg/ml). These results suggest that some highly toxic bioactive compound(s) from *P. repens* Linn. were exclusively partitioned into the more polar solvent butanol. In the aspect of selective antitumor activities of grass extracts, the butanolic extract of *L. hexandra* (L.) Sw. and *P. repens* Linn. was more active against HeLa than against A549, whereas their hexane extract was more active against A549 than against HeLa. Unlike *L. hexandra* (L.) Sw. and *P. repens* Linn. extracts, the hexane and butanol extracts of *B. mutica* (Forsk.) Staph showed the same selective antitumor effects as both extracts were more potent to HeLa than to A549.

3.2 Apoptotic effects of the grass extracts on cancer cells.

Since apoptosis is involved in the cytotoxic effect of many anticancer agents isolated from plants (Iwasaki *et al.*, 2006; Kim *et al.*, 1995; Peng *et al.*, 2008), we next examined whether the grass extracts exert this effect through induction of apoptosis. An ELISA-based apoptosis assay demonstrated that the butanol extract of *P. repens* Linn. (1 mg/ml) and all other extracts tested (250-1,000 mg/ml) induced a range of 1.8- to 11.2-fold increase of the relative-apoptotic values of treated versus untreated A549 cells (Figure 2). In contrast, the apoptotic level in HeLa cells treated with all extracts was not significantly different from that in the control (Figure 2). Because an apoptotic index is defined as the relative absorbance of treated versus untreated cells, the very high absorbance of untreated HeLa cells (20 times higher than untreated A549 cells) resulted in substantially decreased

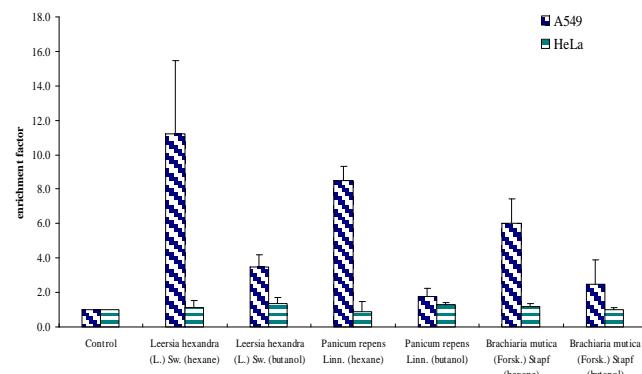


Figure 2. Effects of the hexane (250 μ g/ml) and butanolic extracts (A549: 1,000 μ g/ml, HeLa: 500 μ g/ml) of *L. hexandra* (L.) Sw. ("Yaa sai"), the hexane (A549: 250 μ g/ml, HeLa: 800 μ g/ml) and butanolic extracts (1 μ g/ml) of *P. repens* Linn. ("Yaa channakaat"), as well as the hexane (A549: 1,000 μ g/ml, HeLa: 300 μ g/ml) and butanolic extracts (A549: 250 μ g/ml, HeLa: 100 μ g/ml) of *B. mutica* (Forsk.) Staph ("Yaa khon") on apoptosis in A549 and HeLa tumor cells. Values are presented as the mean values of the enrichment factor \pm standard deviation (S.D.) ($n = 3$). * Significantly different from control at $p < 0.05$.

apoptotic values of extract-treated HeLa cells. This problem could be due to a lot of dead cells or too many cells used. Therefore, higher induction of apoptotic level for HeLa cells might be obtained with appropriately optimized culturing conditions or cell concentration.

Despite some low or undetectable apoptotic levels obtained by ELISA assay, the classical DNA ladder assay apparently revealed classic DNA ladders, a biochemical hallmark of apoptosis, in both cancer cell lines treated with all grass extracts. In this assay, the hexane and butanolic extracts of the three grass species were used to treat cancer cells at 0.5, 1.0, or 2.0 mg/ml for 12, 24, and 36 h, except the butanol extract of *P. repens* Linn. used at 1.0, 10, or 20 mg/ml. Ladder-like DNA fragmentation was observed in a dose- and time-dependent manner. Only the classical ladders induced by the minimum effective dose of each grass extract at the minimum incubation time for A549 and HeLa cells are shown in Figure 3 (A,B) and 3(C,D), respectively. Also, both cell lines were separately treated with the anticancer drug paclitaxel (Taxol), and were run in parallel as positive controls.

3.3 Antinvasive effects of the grass extracts on MDA-MB-231 breast adenocarcinoma cells.

We investigated the antiinvasive effects using the *in vitro* Matrigel assay in which MDA-MB-231 cells were treated with two different non-cytotoxic doses of each extract (low and high doses) for 48 h (Figure 4) and were evaluated for their invasive capacity through a Matrigel-coated filter plate toward a chemoattractant (10% serum). As shown in Figure 4, the grass extracts reduced the invasion of MDA-MB-231 cells in a dose-dependent manner. The high dose of all plant extracts resulted in a lower percent of cell invasion when compared to the respective low dose although the difference was not statistically significant ($p > 0.05$). In addition, the hexane extract of *L. hexandra* (L.) Sw. in the range of 50-100 μ g/ml reduced the invasive capacity of MDA-MB-231 cells by about 50% ($p < 0.05$). At the lowest concentration of 1 μ g/ml, the butanolic extract of *P. repens* Linn. was also found to suppress tumor cell invasion by 30% ($p > 0.05$).

4. Conclusion

This study is the first report that reveals the growth inhibitory apoptotic induction, and antiinvasive effects of *L. hexandra* (L.) Sw., *P. repens* Linn., and *B. mutica* (Forsk.) Staph extracts on human cancer cells. The butanolic extract of *P. repens* Linn. exhibits strong and selective cytotoxicity, which is mediated through apoptosis. Further analyses demonstrate that this grass extract also exerts strong antiinvasive effects against invasive breast cancer cells. All together, the butanolic extract of *P. repens* Linn. has potent anticancer properties and might be beneficially evaluated as a potential chemotherapeutic agent to treat invasive cancers.

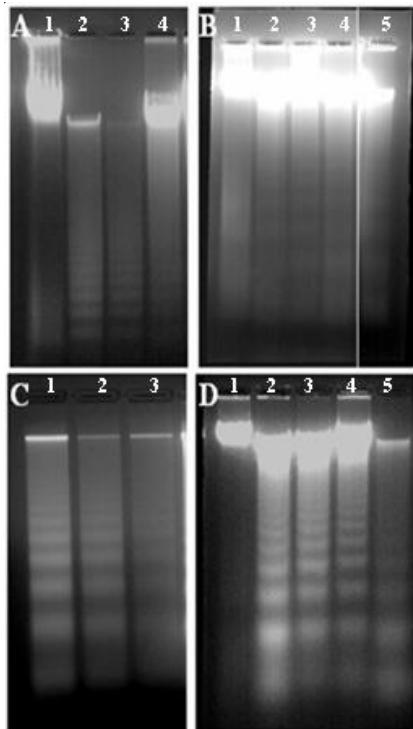


Figure 3. DNA ladder of A549 (A, B) and HeLa (C, D) cells treated with the grass extracts. A549 cells were treated with the culture medium only (lane A1, B1), the hexane extracts (A) of *L. hexandra* (L.) Sw. (lane 2), *P. repens* Linn. (lane 3), and *B. mutica* (Forsk.) Staph (lane 4) at 2 mg/ml for 24 h, the butanolic extracts (B) of *L. hexandra* (L.) Sw. at 2 mg/ml for 48 h (lane 2), *P. repens* Linn. at 20 µg/ml for 24 h (lane 3), *B. mutica* (Forsk.) Staph at 2 mg/ml for 48 h (lane 4), and 12 µM Taxol for 24 h (lane 5). HeLa cells were treated with the culture medium only (lane D1), the hexane extracts (C) of *L. hexandra* (L.) Sw. (lane 1) as well as *P. repens* Linn. (lane 2) at 1 mg/ml for 24 h, and *B. mutica* (Forsk.) Staph at 1 mg/ml for 48 h (lane 3), 100 nM Taxol for 48 h (lane D2), the butanolic extracts (D) of *L. hexandra* (L.) Sw. at 2 mg/ml for 48 h (lane 3), *P. repens* Linn. at 10 µg/ml for 48 h (lane 4), and *B. mutica* (Forsk.) Staph at 1 mg/ml for 48 h (lane 5). DNA was isolated, subjected to agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light.

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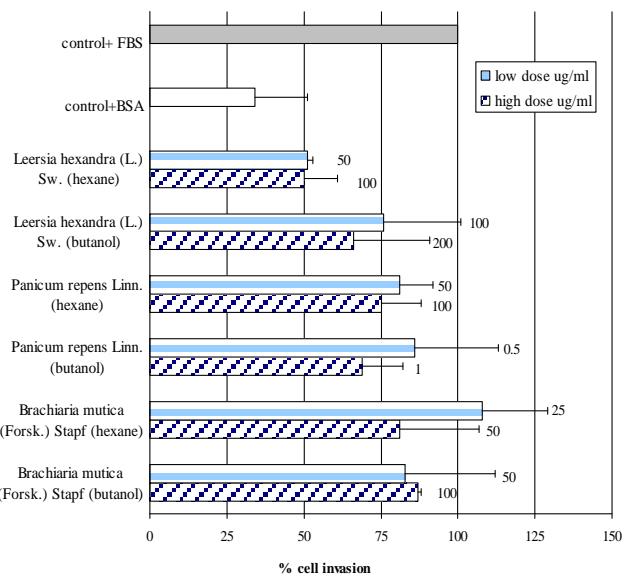


Figure 4. Effects of the hexane and butanolic extracts of *L. hexandra* (L.) Sw., *P. repens* Linn. and *B. mutica* (Forsk.) Staph on the invasion of MDA-MB-231 cells through Matrigel. Low and high doses of each extract (listed above the bar of each barchart) were used to treat tumor cells for 48 h. An invasive rate of 100% is defined by control experiments of highly invasive MDA-MB-231 cells tested in the absence of grass extracts. Each point and bar represents a mean \pm SD (n = 3). * Significantly different from control at $p < 0.05$.

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