Evaluation of the Cytotoxic Effect of New Extract of Camel Urine Fractional on Normal and Cancer Cell Lines

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Abstract

Aim of the work: In this study, we evaluated the anti-cancer activity of new purified fractions from camel urine previously named as (PMF) in various cancer cell lines (A549, HCT116, HEpG2, MCF-7, U251, Hela), compared with that in normal cell lines. It has been reported that extracts from animal urine including those from pregnant mare urine can be used as a drug regimen for the treatment of different types of human disease.

Methodology: Studies have shown repeatedly that the extract PMF could be used in cancer treatment on the basis of its capacity to selectively attack various cancer cell lines.

Results: The current investigation revealed that new and more purified fraction of PMF named as (newPMF) possesses effective and selective anti-cancer properties against several types of cancer cell lines.

Conclusion: The anti-cancer properties of two new fractions of PMF (formed by separation of the small molecules from the macromolecules using 3kD and 50kD membrane filters) were evaluated.

Key words: Camel, urine, cancer, PMF, A549, HCT116, HEpG2, MCF-7, U251, Hela and NMR spectroscopy.

Introduction

Use of animal urine in the traditional treatment of human diseases has a long history but it is also the source of several drugs used in modern medicine. Premarin, for example, is derived from the urine of a pregnant mare and is a common source of estrogen for the treatment of symptoms experienced by pre-menopausal and menopausal women [1-5]. As one of the most prescribed drugs in the United States, Premarin has been marketed since 1942, its name being derived from the words “pregnant mares’ urine” (PMU) [6].
The successful use of Cisplatin, a platinum complex drug, as a potent anti-tumor agent has triggered interest in other metal-containing compounds as potential anticancer drugs [7]. In our previous studies, we fractionated camel urine and found that one fraction (PMF) induced apoptosis and inhibited cell proliferation in various cancer cell lines [8-11]. Further investigation identified nanoparticles of the alkali metals cesium and rubidium in the PMF fraction[12].

Further investigation of PMF compounds is essential to gain a better understanding of their bioactivity and to identify potential anti-cancer compounds that may be used in cancer therapy. The sub-fractionation of PMF according to molecular size using a filtering membrane is one approach for identifying the active components and establishing their efficiency as candidates as cancer drug. In this study, we compared the anti-cancer activity of different PMF extracts, both with and without the presence of protein macromolecules. As in previous studies, the PMF extract was filtered using a 3KD filter tube [9]. For purposes of comparison, the bio-activity tests of the filtrate were carried out under the same experimental conditions as used originally on PMF. The results obtained show that the new PMF extract has effective and selective anti-cancer activity in a range of different cancer cells.

**Materials & methods**

**Sample ultra-filtration**

To remove glycerol from the filter membrane, the 3 KD centrifugal tube filters were washed several times with 0.5 ml water then centrifuged at 3500 g and 37 °C until no nuclear magnetic resonance (NMR) signal was obtained from the filtrate. The filters were kept moist at 4 °C prior to use, then 0.5 ml samples of urine extract (PMF) were filtered by centrifugation at 10,000 g with the temperature maintained at 4 °C throughout. A volume of 450 µl of the filtrate was transferred to a 5 mm NMR tube and 50 µl of D2O then added.

**PMF sub-fractionation and cytotoxicity experiment**

**Reagents**

The following reagents were used: DMEM (Gibco, cat# 12800-116), fetal calf serum (Gibco, cat# 12484-028), Trypsin 1: 250 (Gibco, cat# 27250-018), phosphate buffer saline, 50%
(w/v) TCA, 1% (v/v) acetic acid, 0.057% (w/v) SRB IN 1% (v/v) acetic acid, 10mM Tris base solution.

**Preparation of PMF sub-fractions**

The PMF fraction was prepared following Khorshid (2009) [9], and then filtered with ethanol using 0.1 or 0.2 µml nylon filters in order to separate the larger from the smaller molecules as sub-fractions of PMF.

A high and a low concentration (100 and 10 µg/ml media) stock solution of the filtered PMF were used. Finally, the PMF was sub-fractionated into large and small fractions using a 3 kD centrifugeand 50 kD tubes (Millipore, USA). The extracts were separated into two major fractions on the basis of molecular weight this being greater than 50 kD for the high molecular weight fraction, or less than 3 kD (low molecular weight fraction). NMR spectrometry was used to identify the small and large molecules in the PMF fraction prior to evaluation of their cytotoxicity in cancer cell lines.

**NMR spectroscopy**

Nuclear magnetic resonance (NMR) spectroscopy is a powerful tool allowing investigation of the chemical composition of a given sample [13-16]. Furthermore, NMR can be used for structural elucidation and to study molecular dynamics [17-22]. Consequently, NMR is generally used as a complementary technique to mass spectrometry (MS) for the identification and quantitative analysis of the chemical composition of natural products and biological samples, such as urine [7-9, 23-26]. In fact, both NMR and MS have been used intensively for drug discovery from natural products [27-28], for drug assessment [29], for characterizing newly designed drugs [30-31], and for evaluating drug toxicity [32]. Wishart et al., for example, employed NMR spectroscopy in conjunction with different MS techniques to establish that some 209 different metabolites can be identified in human urine using NMR [33]. However, the inherent limitation of NMR spectroscopy is a low sensitivity meaning that compounds present at a concentration lower than micromolar cannot be detected. However, developments in NMR machinery, such as the use of cryogenic probes [34], micro-probes [35] and increased magnetic field strength [36], as well as the invention of dynamic nuclear polarization (DNP) [37-40], continue to enhance the applicability of NMR approaches.

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Procedure

NMR spectrometry
The sample was prepared by dissolving around 20 mg of camel urine extract in 500 µl of deuterated water (D$_2$O). The solution was vortexed for 30 s, and then transferred to 5 mm NMR tubes. The NMR spectra were obtained using a Bruker 600 MHz spectrometer equipped with a Bruker 5-mm broadband observe (BBO) multinuclear probe (BrukerBioSpin, Rheinstetten, Germany). All NMR spectra were recorded in D$_2$O at 298 K using excitation sculpting with the gradient water suppression pulse sequence (zgesgp) in the standard Bruker pulse sequence library [41]. The spectra were recorded by collecting 128 free induction decays (FIDs) and digitized into 64 K complex data points with 3 s recycle delay time. Before Fourier transform, the FID values were multiplied by an exponential function equivalent to a 1 Hz line broadening factor. Bruker Topspin 2.1 (BrukerBioSpin, Rheinstetten, Germany) software was used in all NMR experiments to collect and analyze the data [41].

Cell lines
A549, MCF-7, HCT116, U521, Hela and HepG2 cancer cell lines, and a normal HFS cell line were obtained from the cell bank of the Tissue Culture Unit, KFMRC, KAU, Jeddah, KSA. All cell lines were cultured in DMEM media supplemented with 10% FCS, 100 µg mL$^{-1}$ Penicillin/ streptomycin. All cells were grown in 25 ml tissue culture flasks.

SRB cytotoxicity assay:
A549, MCF-7, HCT116, Hela, HepG2, U251, Vero and normal HFS cell lines were plated with densities of 1x10$^5$ cells per well, in separate 96-well plates, and incubated at 37°C in a humidified incubator with 5% CO$_2$ for 24 hr. The 180 µl medium was replaced with different concentrations of fractions (10, 7.5, 5, 2.5 and 1 µg/ml) without disturbing the cells. Each concentration for each fraction was repeated 6 times. Plates were then incubated for 48 or 72 hr at 37°C in a humidified incubator with 5% CO$_2$. Next, 50 µl of cold 50% (w/v) TCA was added to each well without removing the medium and the plates were incubated at 4°C for 1 hr to fix the cells on the plates. The plates were washed four times with water and dried at room temperature. Then, 100 µl of 0.57% (w/v) SRB solution was added and the plates were incubated at room temperature for 30 min. The plates were re-washed four times with 1% (v/v) acetic acid to remove the unbound SRB dye and dried at room temperature. Finally, 200
μl of 10mM (w/v) Tris base solution (pH 10.5) was added to the plates which were then incubated for 30 min.

The optical density (OD) was measured at 510 nm in a microplate reader, and survival fractions were plotted between the OD and the drug concentration using a GraphPad statistical program.

The data were expressed as mean of the optical density obtained from three independents experiments (each experiment was performed in three replicate wells).

**Results**

In this study we used proton NMR spectroscopy to evaluate the removal of protein and other macromolecules from fraction of PMF because proton NMR signals of macromolecules are broad compared to the corresponding signals of small molecules. Figure (1) shows the 600 MHz proton NMR spectrum of camel urine extract and only a few molecules can be identified. Figure (2) shows the difference between the proton NMR spectra of the extract samples with proteins present and the spectra after protein separation using a 3KD filter tube. It can be seen in Figure (3) that all the aromatic region signals were identical in both spectra, which suggests that these signals came from small molecules and, furthermore, that the filtration process was an effective method to separate macromolecules from small molecules.

Using a number of different cancer cell lines the cytotoxic effects of original PMF (old), the new PMF filtrate (mixture), and its separated parts comprising small molecules (MW less than 3 KD) and the high molecular weight fraction (MW greater than 50 kDa) were tested. The results show that the small molecules fraction of PMF had cytotoxic effects on various cancer cell lines. However, the new PMF mixture was found to have greater cytotoxic effects on various cancer cell lines than did its fractions, small or large molecules. These results suggest that the smaller molecules are the ones with the anti-cancer properties but that these
molecules may depend on macromolecules to increase and facilitate these anti-cancer activities, or possibly to interact with cancer cell receptors.

II- Cytotoxicity assay:

1. The effect of PMF on normal cells

1. The effect of PMF fraction on normal skin cells (HFS):

Investigation of the effects of PMF on normal skin cells (HFS) revealed that, even at very high concentrations, PMF (or its fractions) had no significant cytotoxic effect on HFS compared with cancer cells (MCF7, A549, HCT116, U251, HepG2). The results showed slight decrease in the number of cells at the beginning of experiments (<0.025) due to the high concentration of drugs (Figure 4). Similar to normal skin cells, the results showed that the nourishing effect of the PMF fraction on normal skin cell HFS with the IC$_{50}$ concentration of cancer cells as illustrated in Figure 5.

1. The effect of PMF fraction on Vero cell lines:

As with normal skin cells (HFS), the results showed that, even at very high concentrations, neither PMF nor its fractions had any significant cytotoxic effect on normal Vero cells as compared with cancer cells (MCF7, A549, HCT116, U251, HepG2). There was a slight decrease (<0.25) in the number of the cells at the beginning of the experiments, this being due to the high concentration of drugs present (Figure 6).

1. The Effect of different PMF fractions on cancer cells

Several experiments were conducted to compare the cytotoxic effects of the purified PMF extracts on cancer cell lines with those of the parent extracts. The results obtained indicated that the effect of the new fraction with extra purification (new PMF, which consists of both large and small molecules) is greater than that of the parent extracts, as shown in Figures 7-12.

1. The effect on lung cancer cells (A549)

Several experiments were conducted to compare the effects of the old PMF fraction (G), the new PMF fraction (mix), and the parent extracts of PMF, comprising only small (S) or only large molecules (L) on different cancer cell lines. Lung cancer cells (A549) of the old PMF fraction (G), the new PMF fraction (mix), and the parent extracts of PMF, comprising only small (S) or only large molecules (L). The results showed that the new PMF fraction (mix)
had a stronger cytotoxic effect on A549, MCF7, Hela cells, glial, colon and liver cancer cell lines than did the old PMF fraction (Figure 7-12).

2. The effect on breast cancer cells (MCF7)
These experiments were designed to compare the effects of the old PMF fraction (G), new PMF (mix), and the separated large (L) and small molecules (S) of PMF on breast cancer cells (MCF-7). The results obtained showed that the new PMF fraction (mix) had a more cytotoxic effect on the MCF-7 cells than the old fraction (G), or its component parts, the small (S) and large (L) molecule fractions (Figure 8).

1. The effect on cervical cancer (Hela) cells
The results from this experiment indicated that the new PMF fraction had a greater cytotoxic effect on Hela cells than its component small (S) and large (L) molecule sub-fractions (Figure 9).

1. The effect on human glioma cell lines U251 (glial)
The results showed that the new PMF fraction (mix) had a greater cytotoxic effect on glial cancer cells than did the separated parts of it, the small (S) and large molecule (L) sub-fractions (Figure 10).

2. The effect on colon cancer cell line (HCT116)
The results indicated that the new PMF fraction (mix) has greater cytotoxic effects on colon cancer cells than the separated fractions of it, the small (S) and large (L) molecules (Figure 11).

3. The effect on Human hepatocellular liver carcinoma cell line (HepG2)
The results showed similarly that the cytotoxic effect of the new PMF fraction (mix) on liver cancer cells was greater than the effects of its separated component sub-fractions, the small (S) and large (L) molecules (Figure 12).

Discussion
The cytotoxic properties of the bioactive fraction (G) of camel urine (PMF) against different types of cancer cells have been studied previously (Khorshid et al., 2005-2011) [42-43]. It was found that although highly cytotoxic against A549 human lung cancer cells and leukemic L1210 cells, PMF is not cytotoxic to normal human cells indicating that PMF can selectively target cancer cells. In the current study, of the cytotoxic effects of purified fractions of PMF on different types of cancer cell lines, the results were found to accord well with those of previous studies [43-46] reporting the anti-cancer effect of PM701 on several cancer cell lines that included (A549, L1210, HCT116, HepG2, MCF-7 and U251). Moreover, other fractions of camel urine, such as PM701 and its bioactive fraction PMF and sub-fraction PMFK, were shown to have cytotoxic properties against different cancer cell lines. In another study, Raouf et al. [47] reported that the addition of PMF to A549 cells for different periods of treatment caused a series of changes in the A549 cells; live images of these cells showed that the severe lethal effects of PM701 on them began immediately after addition of the substrate. It was established that the nuclei of cancer cells incubated in PM701 is attacked by the substrate, this leading to an irreversible degradation of the cancer cells.

The results from the current study are consistent with those of Khorshid (2011) [43], who found that PM701, its bioactive fraction (PMF), and its sub-fraction (PMFK) all exhibit anti-cancer activity. Korshid’s earlier study was directed at the examination of lyophilized PM701 and its bioactive fractions on the growth of breast cancer cells (MCF-7). PM701, PMF and PMFK were found to inhibit significantly the proliferation of MCF-7 cells, demonstrating the apoptotic effect of PM701 and its fractions on breast cancer cells, through its direct effect on the cell nuclei. In an earlier study [48], we reported that PM70 is natural, easily available, cheap, sterile, non-toxic, and causes selective cell death of cancer cells. Furthermore, there is no requirement for high concentrations in chemotherapy. In fact, PM701 has been found to have nourishing effects on normal skin fibroblasts in a tissue culture medium. The effect of PM701 on A549, L1210 and on other cancer cell lines is well documented (41-43).

The results obtained here are consistent with preclinical studies [46] demonstrating that PM701 has no hepatotoxic, nephrotoxic effects, nor any hematological toxicity. Histopathological preclinical studies showed that PM701 has no effects on the gastric mucosa and that it does not alter the parenchymatous architecture of the liver and kidneys, which display a preserved hepatocellular outline and no signs of necrosis. Moreover, there was
found to be an enlargement of the germinal centers of the white pulp lymph nodes in the spleen, indicating the activation of the immune system without any concomitant effect on the vital body organs.

Finally, under all experimental conditions used, camel urine extract (PMF) has been established to be non-toxic and no significant adverse effects have been observed. This natural product, which has been used for many years for the treatment of a range of human diseases and without any reported harmful effects, is of considerable interest on the basis of its anticancer activity. In fact, early Phase I clinical trials using capsules and syrup containing PMF have been carried out on healthy volunteers to confirm that no harmful absence of side effects were caused [45]. Further work on PMF is required and this agent remains under intensive investigation.

**Conclusion**

In this study, the anti-cancer properties of two fractions of PMF (formed by separation of the small molecules from the macromolecules using 3kD and 50kD membrane filters) were evaluated.

It was established that the optimum anti-cancer effects of PMF are obtained using the mixture of both small and large molecules. These results suggest that the bioactive molecules are the smaller ones, while the large molecules may work as receptors and transport the bioactive molecules into the cancer cells. These results combined with previous reports indicate that PM701, and its bioactive fraction PMF, are safe as anti-cancer agents, having negligible effects on normal tissues such as those of the vital organs including the liver and kidney. It is strongly recommended that PMF be subjected to clinical trials.

**Declaration**

"Some part of this manuscript was previously presented and published in the following conference.

Conference name: 2nd International Conference on Bioprocess and Engineering

Dates: June 26-27, 2014

Location: Valencia, Spain."

References


2- Zhu BT. Ting is it necessary to control the level of estrogen receptor α and β activation in postmenopausal hormone replacement therapy in order to achieve the optimal outcome? Molecular Medicine Reports. 2008; 1(1): 15-20.


6- Seibert D S, Poole C F. A general model for the optimization of sample processing conditions by solid-phase extraction applied to the isolation of estrogens from urine. Journal of High Resolution Chromatography. 1998; 21(9): 481-490.


Figure 1: 600MHz $^1$HNMR spectrum of original camel urine extract in D$_2$O at 298K; the figure shows that the extract contains both small molecules and macromolecules, mainly proteins; broadened peaks (black arrows) indicate typical protein signals.
Figure 2 Stack plot of 600MHz NMR spectra of camel urine extract in D$_2$O at 298K NMR spectrum before (blue) and after (red) filtration with 3KD membrane filter tube. The figure shows that no broadened signals were observed in the filtrated sample (red) spectrum indicating the removal of all proteins and other macromolecules from the filtrated sample.

Figure 3 Extension of proteins region of Figure 2 600 MHz NMR spectrum. The figure show that protein region signals are absent from the filtered sample. This suggest that the filtration is an effective method to separate the macromolecules from small molecules.
SRB cytotoxicity of different PMF fractions on HFS

![Graph showing survival fraction of HFS cells with different PMF concentrations.](image)

**Figure 4:** The cytotoxic effect of different three batches of PMF fraction (PMFG, PMFM and PMFE) against normal cell line, HFS cells.

![Graph showing survival fraction of HFS cells with new PMF concentration.](image)

**Figure 5:** Cytotoxic effect of new PMF fraction compared with smaller and larger fractions, where the new PMF (more purification) fraction has a nourishing effect on normal skin cell HFS with the IC_{50} concentration of cancer cells.
Figure 6: Cytotoxic effect of new PMF fraction compared with smaller and larger fractions, where the PMF fractions (as a mixture or with its separated parts) have a non significant effect on normal Vero cells even at very high concentrations.

Figure 7: Cytotoxic effect of the three different batches against cancer cell line A549, where the effect of new PMF is stronger than the effect of old PMF or of the fractions of new PMF on A549 cancer cells.

Figure 8: Cytotoxic effect of the three different batches against cancer cell line MCF7, where the new PMF fraction (mix) has more cytotoxic effect on MCF7 than old fraction (G) or than the separated parts of it small (S) and large (L) molecules.
Figure 9: Cytotoxic effect of new PMF fraction compared with smaller and larger fractions, where new PMF fraction (mix) has better cytotoxic effect on Hela cells than the separated parts of it small and large molecules.

Figure 10: Cytotoxic effect of new PMF fraction compared with smaller and larger fractions, where the new PMF fraction (mix) has better cytotoxic effect on glial cancer cells than the separated parts of it small and large molecules.

Figure 11: Cytotoxic effect of new PMF fraction compared with smaller and larger fractions, where the new PMF fraction (mix) has more cytotoxic effect on colon cancer cells than the separated parts of it small and large molecules.
Figure 12: Cytotoxic effect of new PMF fraction compared with smaller and larger fractions, where new PMF fraction (mix) has more cytotoxic effect on liver cancer cells than the separated parts of it small and large molecules.