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Novel anti-uterus cancer potential of fruit extract of *Lantana camara* as exhibited through the inhibition of alkaline phosphatase in human endometrial adenocarcinomatic cell line

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Lantana camara **is a reputed poisonous shrub and a notorious weed. Its different parts have exhibited anticancer activity but not considered safe to administer due to its toxicity. In the present work, the fruit extract was studied for its estrogenic and antiestrogenic activity through Ishikawa assay which is an in vitro assay, using human endometrial carcinoma cell line (uterus cancer cells). These results have shown that fraction LC-2 of fruit of L. camara may have interesting compounds with the potential to act as ligands binding the human estrogen receptors, and showing selective estrogen receptor modulator profile through antiestrogenic effect in human uterus cancer cell line. Crude extract had only minute antiestrogenic activity, with no estrogenic and cytotoxic activity observed. Surprisingly, fraction LC-2 which was combination of Vacuum liquid chromatography (VLC) fractions 6 to 13 (hydrophilic in nature) had remarkable anti-estrogenic activity. This fraction showed a dose dependent anti-estrogenic activity. Consequently, this is the first report of the possible antiuterus cancer potential of the fruit of L. camara mediated through antiestrogenic activity. Non significant cytotoxic effects of active fraction LC-2 is the most important aspect of this study which encourages further studies to find some interesting antiestrogenic compounds of natural origin.**

Key words: *Lantana camara*, antiestrogenic activity, Ishikawa assay, cytotoxic effects.

INTRODUCTION

Hormone replacement therapy (HRT), using synthetic estrogens, for the treatment of menopausal symptoms in females is linked with the chances of significant increase in the development of endometrial and breast cancer (Overk et al., 2005). The success of clinical use of antiestrogen tamoxifen for chemopreventive effects in women at high risk of breast cancer strongly encouraged the efforts to find out new antiestrogenic compounds of natural origin (Pisha and Pezzuto, 1997). There are only very few phytochemicals which have shown antiestrogenic activity mediated through the suppression of estrogen induced proliferation/transcription (Collins-

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Burowet al., 2000). The approval of clinical use of antiestrogen tamoxifen by Food and Drug Administration (FDA) gave incentive to the efforts in search of natural products for finding other new chemo-preventive agents of natural origin (Pezzuto et al., 2005).*Lantana camara* L., considered as an ornamental plant and an obnoxious weed, belongs to the family verbenaceae. *L. camara* is used in some folk medicines for cancers and tumors. Extensive work is reported on the phytochemistry of *L. camara*. Several types of mono and sesquiterpenes, triterpenes, iridoid glycosides, furanonaphthoquinones, flavonoids and miscellaneous compounds have been isolated. The toxicity of aerial parts of *L. camara* is well known (Ghisalberti, 2000). Ingestion of leaves by grazing animals can cause hepatotoxicity and even fatality (Ross, 1999). Tritrpenes lantadene A and B are considered to be responsible for this toxicity while some triterpenes from *L. camara* has been found to have anti-inflammatory action (Swarbrick et al., 1998; Safayhi and Sailer, 1997).

The present study was carried out with the objective to explore the estrogenic/antiestrogenic potential of the fruit of *L. camara* using Ishikawa assay which is cell based, and estrogen receptor α (ERα) positive assay, using an endometrial adenocarcinoma cell line which is derived from a glandular epithelial cell line and responds to estrogens and antiestrogens at concentrations near physiological levels (Pisha and Pezzuto, 1997). Induction of alkaline phosphatase indicates estrogenic activity while the prevention of AP (alkaline phosphatase) expression manifested through the inhibition of estrogen induced Activator protein (AP) shows anti-estrogenic activity (Yoo et al*.,* 2005).

MATERIALS AND METHODS

Plant material collection and extraction

The fruit (1 kg) was plucked from *L. camara* L. growing wildly on the campus of Quaid-i-Azam University, Islamabad in 2007. The fresh fruit was macerated in ethyl acetate and methanol sequentially for exhaustive extraction at room temperature (25 ± 2)℃. After two weeks, the mixture was milled and then filtered by ordinary filter paper (Whatmann filter paper No.1). The marc was returned into the mixture while filterate was evaporated through rotary evaporator (Buchi Rota vapor R-200) at 40℃. After repeating the process thrice, the crude extracts obtained were combined into a single extract (30.0 g).

Vacuum liquid chromatography

Crude extract was subjected to Vacuum liquid chromatography (VLC) column. Extract was dissolved in MeOH, mixed with silica gel in 1:1 ratio, and yielded a fine powder after evaporation of the solvent. The size of the column was twenty times the weight of the extract (weight of crude extract × 20 = amount of silica gel used for column). The column was developed by gradient elution with n-hexane/ethyl acetate (EtoAc)/methanol (MeoH) (Table 1) solvent aliquotes in different proportions starting with100% hexane, next with 5% increase of more polar solvent and finally with 100% MeOH thereby obtaining 41 fractions.

Table 1. Solvent system used in vacuum liquid chromatography.

Fraction	Hexane	EtoAc	MeOH
1	100	0	-
2	95	5	-
3	90	10	-
4	85	15	-
5	80	20	-
6	75	25	-
7	70	30	-
8	65	35	-
9	60	40	-
10	55	45	-
11	50	50	-
12	45	55	-
13	40	60	-
14	35	65	-
15	30	70	-
16	25	75	-
17	20	80	-
18	15	85	-
19	10	90	-
20	5	95	-
21	0	100	0
22	-	95	5
23	-	90	10
24	-	85	15
25	-	80	20
26	-	75	25
27	-	70	30
28	-	65	35
29	-	60	40
30	-	55	45
31	-	50	50
32	-	45	55
33	-	40	60
34	-	35	65
35	-	30	70
36	-	25	75
37	-	20	80
38	-	15	85
39	-	10	90
40	-	5	95
41	-	0	100

Values are in percent. The highlighted fractions were combined to form fraction LC-2.

Thin layer chromatography

These fractions were analyzed by Thin layer chromatography (TLC) plates [Hexane: Ethyl acetate (7:3), under ultra violet (UV) light at different wavelengths (254 and 354 nm)] and then sprayed with anisaldehyde reagent. On the basis of similarity of spots, these 41

fractions of *L. camara* were combined into 4 fractions and tested for estrogenic, anti-estrogenic activities, along with their potential cytotoxic effects to ishikawa cells.

Ishikawa assay

The Ishikawa assay was performed following the procedure of Pisha and Pezzuto (1997) at Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, UIC, USA. The Ishikawa cell line was maintained in Dulbecco's modified eagle medium (DMEM/F12) containing sodium pyruvate (1%), non-essential amino acids (NEAA, 1%), glutamax-1 (1%), insulin (0.05%), and heat inactivated fetal bovine serum (FBS, 10%). Ishikawa cells (1.5×10^4 cells/190 µL/well) were preincubated in 96-well plates overnight in estrogen-free medium. This was achieved through washing the cells with sterile phosphate buffered saline (PBS). The objective was to reduce the induction of marker enzyme from estrogens present in the media. At this stage, cells appear to be very transparent when observed under microscope. After washing with PBS, it was removed and trypsin was added, most of which was removed after the cells were exposed to it. Cells were diluted to make the final concentration of cells per well ~50,000 for estrogenic/anti-estrogenic testing. For cytotoxicity test, the concentration per well was maintained at ~1,000 cells. A cytotoxicity plate was made along with each test plate. For cytotoxicity determination, a plate on day 0 was also prepared. Cells were added to three wells of a plate. Each estrogen and anti-estrogen testing plate also had a negative control dimethyl sulphoxide (DMSO) and positive control (Estradiol as estrogen and Tamoxifen as antiestrogen control). Test samples (20 µg/ml final concentrations in DMSO) were added to the cells in a total volume (200 µL media/well) and were incubated at 37°C for 4 days. Cytotoxicity of day 0 plate was harvested at this time.

Harvesting the Induction plates for estrogenic/anti-estrogenic measurements

The induction plates were processed by washing the plates with PBS and adding Triton ×100 (0.01%, 50 µL) in Tris buffer (pH 9.8, 0.1 M). Plates were subjected to a freeze thaw (-80°C) for at least 24 h before warming to 37°C. An aliquot (150 µL) of p-nitrophenylphosphate (phosphatase substrate, 1 mg/mL) in Tris buffer (pH. 9.8, 0.1 M) was added to each well. The enzyme activity was measured by reading the release of p-nitrophenol at 405 nm every 15 s with a 10 s shake between readings for 16 readings, using a Power wave 200 microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, VT). The maximal slopes of the lines generated by the kinetic readings were calculated. Estrogenic induction was calculated using equation 1 and for anti-estrogenic determination, the percent induction as compared with the background induction control was calculated using equation 2.

$$[(slope_{sample} - slope_{DMSO}) / (slope_{estrogen} - slope_{DMSO})] \times 100 = \text{Estrogenic induction (\%)} \quad (1)$$

Antiestrogenic activity was determined by calculating the reduction in percent induction as compared to DMSO control using the equation 2.

$$[1-(slope_{sample} - slope_{cells}) / (slope_{DMSO} - slope_{cells})] \times 100 = \text{Antiestrogenic induction (\%)} \quad (2)$$

Harvesting the plates for cytotoxicity measurements

After removing the previous media from the plated 50 µL of cold 20%, trichloroacetic acid (TCA) was added to each well to fix the cellular proteins to the plates. After that, the tubes were incubated overnight at 4°C. Plates were washed with tap water and oven dried (1 h). To each well, 100 µL sulphurhodamine B (SRB) was added and incubated for 30 min. The plates were then washed with 1% acetic acid, dried and stored in the dark. For cytotoxicity measurement, 200 µL of Tris buffer (0.1 mM) was added to each well. The plates were shaken to completely dissolve the dye. The percent growth was calculated as follows:

$$\text{Growth (\%)} = [(OD_{sample} - OD_{Day\,0}) / (OD_{DMSO} - OD_{Day\,0})] \times 100$$

RESULTS

Crude extract and fractions of fruit of *L. camara* showed interesting results. Marginal anti-estrogenic activity was exhibited by the crude extract without being cytotoxic to human uterus adenocarcinoma cells but one of the fractions, LC-2, showed significant anti-estrogenic activity (Figure 2). This fraction was formed on combining VLC fractions 6 to fraction 13 (Figure 1). Table 1 show the solvent system composition which was used in VLC column to extract these fractions (6 to 13). Furthermore, TLC (Figure 1) shows that these fractions had lipophilic constituents detected as spots on spraying the analytical TLC plate with anisaldehyde reagent. Fraction LC-1 formed on combining VLC fraction 1 to fraction 5 showed only 5% antiestrogenic activity. TLC shows that no compound or spot could be detected in this region. Other two fractions LC-3 and LC-4 comprising of compounds of medium to high polarity (hydrophobic) showed non-significant anti-estrogenic activity. Keeping in view the significance of antiestrogenic activity, the active fraction LC-2 was tested further to establish dose-response curve. Dose dependent antiestrogenic activity was observed (Figure 3). At further lower concentrations (10, 5 and 2.5 µg/ml), cytotoxic activity was absent. Conclusively, this study is important on account of first report of anti-estrogenic potential of fruit extract. Consequently, these results have revealed the possible anti-uterus cancer potential of fruit of *L. camara.*

DISCUSSION

The results obtained in this study are very important because it is an established fact that the number of plants with reported antiestrogenic activity is very limited. Though anti-tumor and anti-inflammatory activities from other parts of *L. camara* are reported (Vijayan et al., 2002), aerial parts are reputed to be poisonous and cytotoxic (Raghu, 2004; Pass, 1979).

In this study, Ishikawa assay was used in employing cultured human endometrial adenocarcinoma cell line. In Ishikawa assay, estrogen controls the expression of enzyme alkaline phosphatase at transcription level, bringing about an increase in its activity while antiestrogens can inhibit this response. This down regulation of enzyme is mostly an estrogen receptor (ER) dependent effect. In

Table 2. Estrogenic activities of crude extract and fractions of fruit of *Lantana camara*.

Fraction	VLC fraction combined	Concentration (µg/ml)	Estrogenic activity
LC-0	Crude	20	-
LC-1	1-5	20	-
LC-2	6-13	20	1±1
LC-3	14-24	20	-
LC-4	25-41	20	-
E2		0.5	100±21

-: shows no activity, E2: estradiol used as positive control.

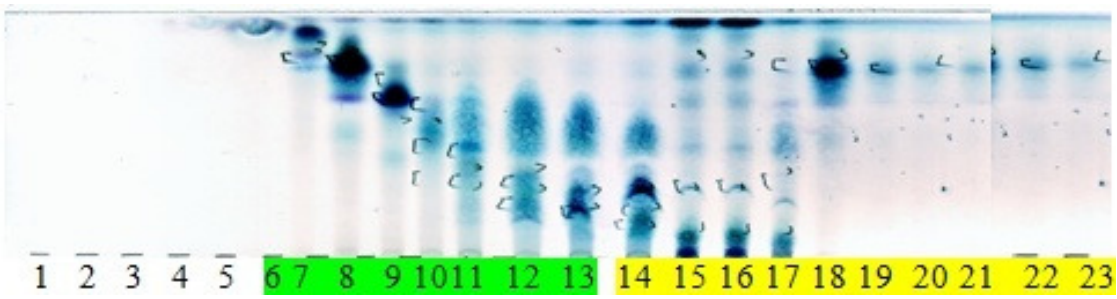

Figure 1. TLC chromatogram of VLC fractions 1 to 23 of fruit of *Lantana camara*.

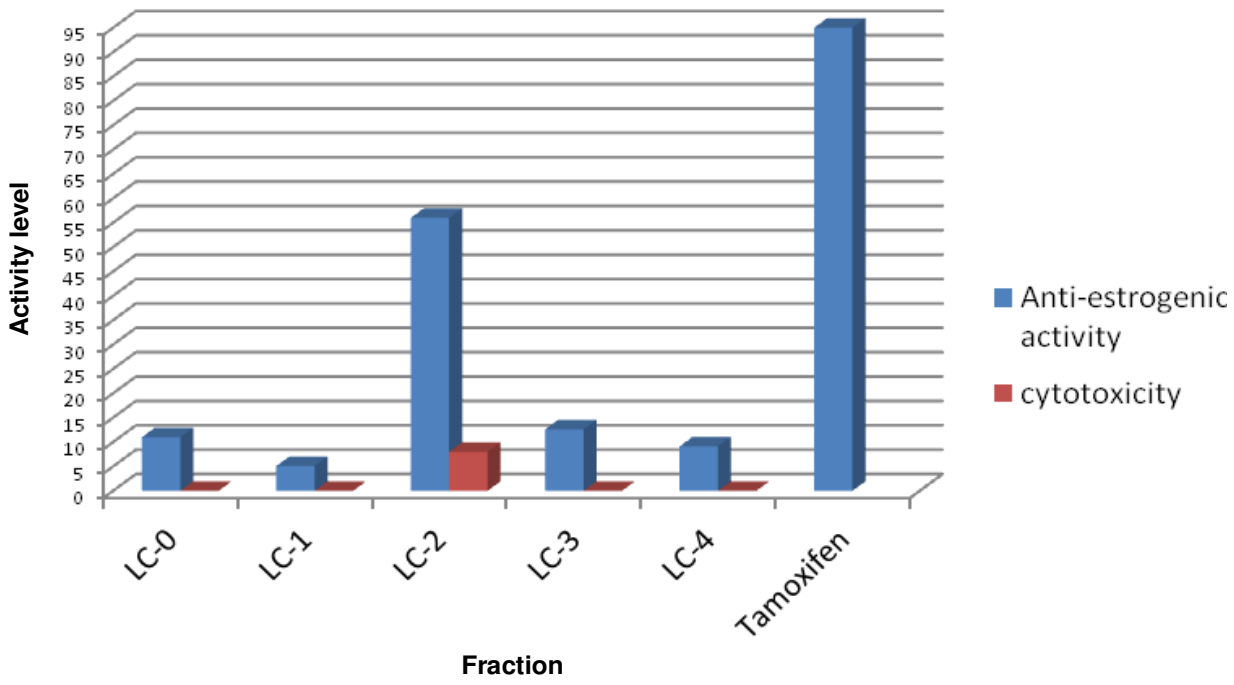

Figure 2. Anti-estrogenic and cytotoxic activities of drude extract (LC-0) and fractions (LC-1 to LC-4) of fruit of *Lantana camara*.Tamoxifen was used as positive control.

this study, estrogenic activity was measured as % induction of alkaline phosphatase while anti-estrogenic activity was measured as % inhibition of estrogen (estradiol) induced induction of alkaline phosphatase while cytotoxicity of all samples was measured as inhibition (%) of growth of cells. The crude extract as well as fractions of fruit of *L. camara* showed no estrogenic activity, as no induction of marker enzyme was observed (Table 2).

As far as anti-estrogenic activity is concerned, fraction LC-2 showed significant inhibition (56%) of estradiol induced stimulation of alkaline phosphatase (Figure 2) with minute cytotoxicity to these cells. It was further confirmed

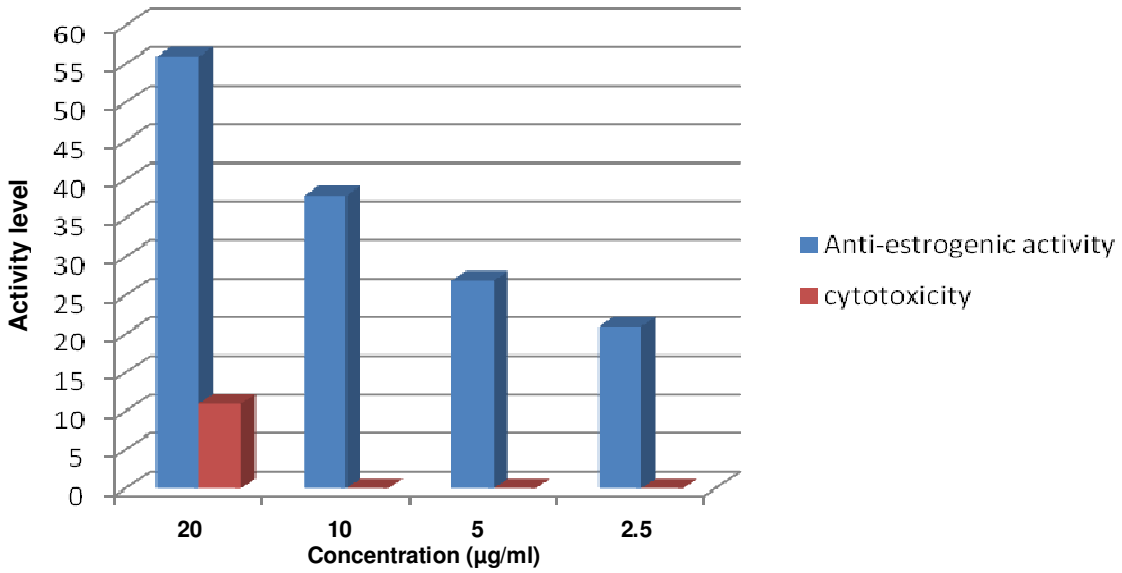

Figure 3. Anti-estrogenic and cytotoxic activities of crude extract and fractions of fruit of *Lantana camara*. Tamoxifen was used as positive control. Data are mean of three values.

by testing the activity at different concentrations, confirming that this inhibition (antiestrogenic activity) is in dose dependent manner (Figure 3). Several mechanisms can be presented to explain this anti-estrogenic activity. Phytoestrogens (phytoconstituents) serving as tissue specific ER ligands may act as estrogen agonists or antagonists, depending upon the tissue type (Vom Saal, 1995). Ahn et al. (2004) described the possible mechanism of estrogenic/anti-estrogenic action, stating that estrogenic action of genistein (phytochemical having estrogenic activity) results from binding of these phytochemicals to estrogen receptor that is somehow associated with the gene transcription and thus the growth and differentiation of the cells.

Anti-estrogens either bind to the estrogen receptors to block them, thus suppressing the estrogen mediated transcription, and subsequent proliferation. There are also other possibilities where anti-estrogens may interfere with other endogenous or exogenous estrogens through some other mechanisms (Vom Saal, 1995). Evidences suggest that most actions of estrogens are estrogen receptor mediated. Estrogen receptor is a protein having some hormone activated transcription factors. Phyto-estrogens act as estrogen analogs and exert agonistic effects on ER. These effects may be mediated either by binding with ER (binding dependent) or by binding independent mechanisms (Strauss et al., 1998). Most of the anti-estrogens function by binding to ERs and are called binding dependent anti-estrogens (Bridgette et al., 2000).

Conclusion

Endometrium cancer is a most common cancer of human female genital tract, so this novel work points at the anti-endometrium (antiuterus) cancer potential of fruit extract of *L. camara*. Further studies involving different other tissues to evaluate estrogenic/antiestrogenic potential of fruit extract of *L. camara* are encouraged as results in one type of tissue may not be sufficient to predict its clinical pharmacology.

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