HOSTED BY
ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Original article http://dx.doi.org/10.1016/j.apjtb.2017.01.025

Plant extracts as natural photosensitizers in photodynamic therapy: *in vitro* activity against human mammary adenocarcinoma MCF-7 cells

Rigo Baluyot Villacorta[1], Kristine Faith Javier Roque[2], Giovanni Alarkon Tapang[2], Sonia Donaldo Jacinto[1*]

[1]*Institute of Biology, University of the Philippines, Diliman, Quezon City, 1101, Philippines*

[2]*National Institute of Physics, University of the Philippines, Diliman, Quezon City, 1101, Philippines*

ARTICLE INFO

Article history:
Received 13 Sep 2016
Received in revised form 27 Oct, 2nd revised form 14 Nov 2016
Accepted 20 Dec 2016
Available online 20 Jan 2017

Keywords:
Photodynamic therapy
MCF-7
Photosensitizer
Lumnitzera racemosa
Albizia procera
Cananga odorata

ABSTRACT

Objective: To examine three plant extracts [*Lumnitzera racemosa* (Combretaceae) (*L. racemosa*), *Albizia procera* (Fabaceae) (*A. procera*) and *Cananga odorata* (Annonaceae)] for their potential as source of photosensitizers in photodynamic therapy.
Methods: Human mammary adenocarcinoma (MCF-7) cells were treated with the plant extracts, which were irradiated with 5.53 mW and 0.553 mW broadband light. Cell viability was assessed using MTT assay and induction of apoptosis was determined using terminal deoxynucleotidyl transferase-dUTP nick end labeling assay.
Results: The crude ethanolic extracts, independently, were nontoxic against cancer and non-cancer cells but when irradiated with 5.53 mW broadband light, *L. racemosa* and *A. procera* extracts were cytotoxic against MCF-7 with IC_{50} of 11.63 μg/mL and 10.73 μg/mL, respectively. With 0.553 mW broadband light, the IC_{50} values were higher at 17.14 μg/mL and 19.59 μg/mL, respectively. Photoactivated *L. racemosa* and *A. procera* extracts were found to be more cytotoxic against MCF-7 than the non-cancer cell line, human dermal fibroblast-neonatal. Moreover, the cytotoxicity of the extracts was mediated by apoptosis.
Conclusions: Two of the plant extracts used, *L. racemosa* and *A. procera* were toxic and induced apoptosis to mammary cell adenocarcinoma, MCF-7 when photoactivated. These extracts were also more toxic to human cancer than non-cancer cell lines.

1. Introduction

The Philippines is very rich in plant biodiversity and yet, there is minimal effort from local scientists to explore their potential for drug development. Previous studies have shown exciting possibilities of using plant extracts in photodynamic therapy (PDT) which has been used to treat a wide variety of diseases including skin diseases, bacterial, viral and fungal infections, and various malignancies [1–5]. This work aimed to explore the potential photosensitizing property of plant extracts from the Philippines. When a photosensitizer is excited by light of a specific wavelength, it produces reactive oxygen species (ROS), which could have devastating effects on living tissue. The advantages of PDT over chemotherapy, radiotherapy and surgery are minimal invasiveness, minimal toxicity (*i.e.* it has no long-term side effects when used properly), short treatment time, and low cost. However, PDT can only treat areas where light can reach and have not been used to treat cancers that have spread to many places [6]. Generally, cells are rapidly ablated by necrosis when high-intensity light is used. Conversely, low-intensity light may lead to a programmed and more orderly death [7]. Several of these photosensitizers, which are usually dyes and porphyrin derivatives, are already being employed in clinical trials and are commercially available. A good photosensitizer should be nontoxic until activated. It should be hydrophilic for easy systemic application. It should be activated by a clinically useful light wavelength. Finally, a good photosensitizer is reliable in the generation of a photodynamic response [8]. Another important guideline for selecting a good sensitizer is selectivity of destruction and localization. *In vivo*, the drug should be able to discriminate

*Corresponding author: Sonia Donaldo Jacinto, Institute of Biology, University of the Philippines, Diliman, Quezon City, 1101, Philippines.
Tels: +63 9162892883 (RB Villacorta); +63 9178383061 (SD Jacinto)
E-mails: villacorta.rigo@gmail.com (RB Villacorta); soniajac2008@gmail.com (SD Jacinto)

Foundation Project: Supported by Institute of Biology, University of the Philippines, Diliman through TA # 9774-362-499-439.
Peer review under responsibility of Hainan Medical University. The journal implements double-blind peer review practiced by specially invited international editorial board members.

between normal tissue cells from target tumor cells and must therefore be localized efficiently [9].

Many of the most effective cancer treatments are either very expensive or unavailable to some countries. In the Philippines, cancer remains one of the leading causes of morbidity and mortality, and even with chemotherapy and radiotherapy, survival rates are relatively low [10]. It would be exciting to discover plant extracts which by themselves have no activity on cancer cells but with exposure to light can turn to photosensitizing agents which are strongly toxic to malignant cells. Hence, the specific objective of screening for plant extracts that possess the ideal features of natural photosensitizers can be used for photodynamic therapy.

2. Materials and methods

2.1. Materials

Cell lines used included human breast adenocarcinoma (MCF-7) and human dermal fibroblast from neonates (HDFn) both purchased from American Type Culture Collection, (ATCC, Manassas, Va, USA). The reagents used were phosphate buffered saline (PBS), minimum essential medium (MEM; Gibco™, Life Technologies™), Dulbecco's modified Eagle's medium (DMEM; Gibco™, Life Technologies™), fetal bovine serum (FBS; Gibco™, Life Technologies™), insulin-transferrin-selenium (ITS-G; Gibco™, Life Technologies™), penicillin-streptomycin (PenStrep; Gibco™, Life Technologies™), trypsin–EDTA (Gibco™, Life Technologies™), absolute ethanol, dimethyl sulfoxide (DMSO; Amresco®), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Amresco®), doxorubicin (DBL™), and Click-iT® TUNEL Alexa Fluor® imaging assay kit. Equipment used were blender (Osterizer®), rotary evaporator (Heidolph Laborota_4001), broadband LED and microscope exposure set-up, 96-well plate reader (Ledetect 96), Axio Observer inverted microscope (Carl Zeiss), and UV–Vis spectrophotometer (SpectroVis® Plus). The plant extracts came from *Lumnitzera racemosa* (Combretaceae) (*L. racemosa*), *Cananga odorata* (Annonaceae) (*C. odorata*), and *Albizia procera* (Fabaceae) (*A. procera*).

2.2. Cell cultures

MCF-7 and HDFn cell lines were purchased from ATCC and maintained at the Mammalian Cell Culture Laboratory (MCCL) at the Institute of Biology, UP Diliman. MCF-7 cells were cultured in medium containing 88% MEM, 10% fetal bovine serum (FBS), 1% insulin and 1% penicillin-streptomycin (PenStrep). HDFn cells were cultured in DMEM with same concentrations of FBS, $NaHCO_3$ and PenStrep. All cell lines were incubated in humidified conditions at 37 °C and 5% carbon dioxide.

2.3. Exposure set-up

For treatment regimens with light, cells were irradiated using a broadband LED as the light source. The cells were seeded in 96-well plates, protected from extraneous light sources and illuminated one well at a time. The well plate was automated to move the light beam from one well to the next and to control the duty cycle of the source. The maximum power output of the LED light source was 5.53 mW.

2.4. Preparation of plant extracts

Aerial parts of *L. racemosa*, *C. odorata* and *A. procera* were collected from the grounds of the University of the Philippines, Diliman, Quezon City on June, 2015. Plant identification was authenticated by staff of the Jose Vera Santos Herbarium of the Institute of Biology, University of the Philippines, Diliman where voucher specimens were deposited with the following voucher specimen numbers: *C. odorata* 5141, *A. procera* 5083 and *L. racemosa* 10434. The specimens were macerated to fine powder using a blender (Osterizer®). The powder was soaked in absolute ethanol for 48 h and filtered using a 25-μm pore sized Whatman filter paper. The filtrate was concentrated in a rotary evaporator (Heidolph Laborota_4001) at 45 °C. The concentrated ethanolic extract was air-dried and dissolved using dimethyl sulfoxide (DMSO) to yield a final concentration of 4 mg/mL (stock concentration).

2.5. Preliminary procedures

The plant extracts were tested on MCF-7 cells using the MTT cell proliferation assay (see below). The three plant extracts were non-toxic to MCF-7 and chosen for the study.

First, an experiment was performed to determine if broadband light activates *L. racemosa* extracts. MCF-7 cells were treated with different concentrations of the extract for 48 h, followed by 5 min of irradiation at 5.53 mW and incubation for 24 h. After it was established that irradiated *L. racemosa* extracts are cytotoxic to cells, the extract was chosen as positive control for photodynamic therapy since controls are not available in the lab. Then, a time-course experiment was performed to determine the least amount of irradiation time needed to elicit maximal response at 50 μg/mL and 5.53 mW using the MTT assay. This exposure time was used for all plant extracts. MCF-7 cells were treated with a combination of *L. racemosa* extracts and 5.53 mW broadband light for 5, 4, 3, 2, 1, 0.5, and 0 min. The cell viability was calculated using cells treated only with DMSO as negative control.

2.6. MTT cell proliferation assay

The assay was conducted after the procedure of Mosmann [11]. MCF-7 cells were seeded at 6×10^4 cells/mL (190 μL per well) in 96-well plates using fresh culture medium, incubated in a humidified incubator at 37 °C at 5% CO_2 for at least 24 h and confirmed viable by microscopic examination. Different treatment regimens (in triplicate) were used for all cell lines studied. This included exposure of the cells to the positive control, doxorubicin (25, 12.5, 6.25, 3.125 μg/mL) for 72 h at 37 °C, to negative control, DMSO (50, 25, 12.5, 6.25 μg/mL) for 72 h at 37 °C, to varying concentrations of plant extract (50, 25, 12.5, 6.25 μg/mL) for 72 h at 37 °C to DMSO for 48 h, followed by irradiation at 5.53 mW for 1 min per well and incubation for 24 h at 37 °C for total treatment time of 72 h, exposure to varying concentrations of plant extract (50, 25, 12.5, 6.25 μg/mL) for 48 h, followed by irradiation at 5.53 mW for 1 min per well and incubation for 24 h at 37 °C for a total treatment time of 72 h, exposure to DMSO for 48 h, followed by irradiation at 0.553 mW for 1 min per well and incubation for 24 h at 37 °C for a total treatment time of 72 h and exposure to varying concentrations of plant extract (50, 25, 12.5, 6.25 μg/mL) for

48 h, followed by irradiation at 0.553 mW for 1 min per well and incubation for 24 h at 37 °C for a total treatment time of 72 h.

Cells under treatment regimens 1, 2 and 3 were without light thus called dark plates. The cells under treatment regimens 4, 5, 6 and 7 were exposed to light and called light plates. All cells for each regimen, were seeded on the first day of the assay in separate 96-well plates. Cell viability was assessed at the termination stage of the MTT cell proliferation assay. After 72 h, 20 µL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added, followed by incubation for 4 h at 37 °C and 5% CO_2. Then, 150 µL of DMSO was added and the absorbance at 570 nm was measured using a 96-well plate reader (Ledetect 96). Cell viability was computed from the toxicity which was determined using the following formula:

$$\text{Cytotoxicity} = \frac{\text{Abs}_{570} \text{ of treated sample}}{\text{Abs}_{570} \text{ of untreated sample}} \quad (1)$$

The procedure was repeated for the HDFn cell line to determine if the cytotoxic effect is specific to cancer cells. HDFn cells were seeded at a density of 4×10^4 cells/mL. The threshold value for toxicity of <30 µg/mL for crude extracts followed that which was determined by the National Cancer Institute [12].

The summary of assay schedule was as follows. Day 1: seeding of cells; Day 2: treatment with extracts and controls; Day 3: incubation; Day 4: irradiation of light plates; Day 5: termination. Three independent experiments were done for all treatment regimens. IC_{50} of extract was calculated from a regression line made of the plot of treatment concentration against cell viability.

2.7. TUNEL assay

To determine if the mechanism of cell death involved apoptosis, terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) assay was performed. MCF-7 cells were seeded at a density of 6×10^4 cells/mL (190 µL per well) in 96-well plates. Cells were confirmed viable then treated, in triplicate, with 3.125 µg/mL doxorubicin (positive control), 50 µg/mL DMSO (negative control), 25 µg/mL ($2 \times IC_{50}$) *L. racemosa* extract, followed by irradiation at 5.53 mW for 1 min per well, and 25 µg/mL *A. procera* extract, followed by irradiation at 5.53 mW for 1 min per well. After 72 h of treatment, the Click-iT® TUNEL Alexa Fluor® imaging assay was conducted following manufacturer's instructions. The cells were stained with Hoechst-33342 and Alexa Fluor® 488 and the plate imaged under Axio Observer inverted microscope (Carl Zeiss) at excitation wavelength of 350 nm and emission wavelength of 460 nm to view Hoechst-33342, and excitation wavelength of 495 nm and 519 nm to view Alexa Fluor® 488.

2.8. Photophysical properties

The 4 mg/mL stock plant extract was diluted to 140 mg/mL using DMSO. The absorption spectrum was evaluated at this concentration [13] and was determined using a standard UV–Vis spectrophotometer (SpectroVis® Plus) at room temperature. DMSO was used to calibrate the spectrophotometer. The wavelength of maximum absorption, λ_{max}, was determined.

2.9. Statistical analyses

Results were subjected to statistical analyses using SPSS (IBM7® SPSS® Version 23). One-way analysis of variance (ANOVA) was used to assess significant difference among the values observed with $P < 0.05$ considered significant. Tukey's honestly significance difference (HSD) test was used as *post hoc* analysis.

3. Results

3.1. Photoactivated L. racemosa extract inhibited proliferation of MCF-7 cells in a time-dependent manner

To determine the irradiation time to be used in the experiment, MTT assay was performed on irradiated *L. racemosa* extract. Preliminary experiments established that *L. racemosa* was not toxic to MCF-7 cells. However, irradiation with high-intensity broad-band light for 5 min at 5.53 mW induced the extract to be cytotoxic to MCF-7 with mean IC_{50} value of 15.05 µg/mL (Figure 1). The mean IC_{50} of the positive control, doxorubicin, was 1.97 µg/mL.

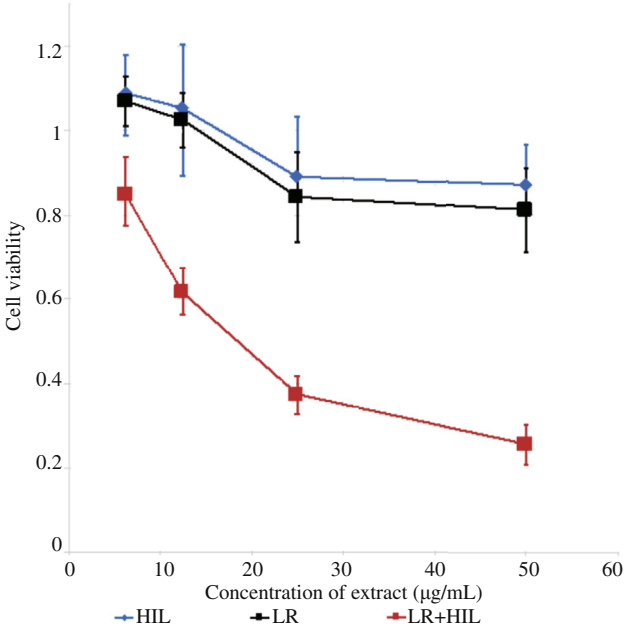

Figure 1. Dose–response curves showing the anti-proliferative effect of irradiated *L. racemosa* extract.
MCF-7 cells were seeded in 96-well plates and treated with different concentrations of *L. racemosa* (LR, black square), DMSO, followed by irradiation at 5.53 mW for 5 min (HIL, blue diamond), and different concentrations of *L. racemosa*, followed by irradiation at 5.53 mW for 5 min (LR + HIL, red square). Values are means ± SD of three trials.

A time-course experiment at 0.5 up to 5 min was performed to determine the least amount of irradiation time needed to produce maximal cytotoxic activity. Figure 2 shows that the toxicity of *L. racemosa* extract varies with length of exposure to light. Statistical analysis using one-way ANOVA and Tukey's HSD test showed that cell viability at 1 min was not significantly different from cell viability at >1 min of irradiation but was significantly different from cell viability at 0.5 min. Hence, the exposure time was set at 1 min.

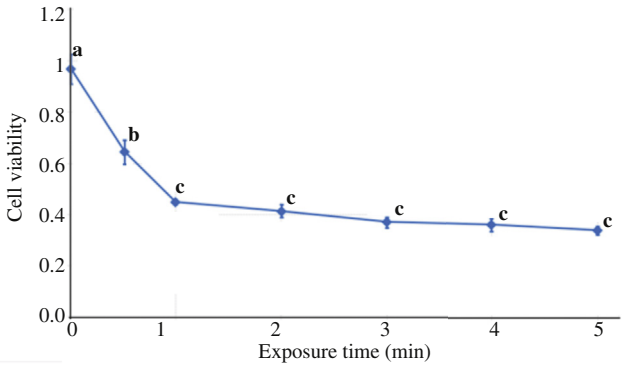

Figure 2. Time-course curve showing the anti-proliferative effect of irradiated *L. racemosa* extract with time.
MCF-7 cells were seeded in 96-well plates and treated with 50 μg/mL *L. racemosa* extract, followed by irradiation at 5.53 mW for 0, 0.5, 1, 2, 3, 4 and 5 min. Letters above points indicate homogenous subsets for alpha = 0.05. Values are means ± SD of three trials. Different letters indicate significant difference at $P < 0.05$ using ANOVA followed by Tukey's post hoc analysis.

3.2. Combining ethanolic extract from L. racemosa and broadband light induced an anti-proliferative effect against MCF-7 cells

MTT assay for *L. racemosa* was repeated for the main experiment using 1 min as irradiation time. Figure 3 shows the dose–response curves of various treatments: *L. racemosa* (LR) alone, high-intensity light (HIL) alone, low-intensity light (LIL) alone, LR with HIL, and LR with LIL. The graphs show that *L. racemosa* extract and broadband light were, independently, non-cytotoxic to MCF-7 cells. However, a combination of the two components induced cytotoxicity. For independent treatments, LR, HIL and LIL, IC_{50} could not be interpolated by linear regression indicating absence of toxicity. The IC_{50} values for treatments LR with HIL and LR with LIL were 11.63 μg/mL and 17.14 μg/mL, respectively. For the positive control, doxorubicin (not shown on Figure 3), the IC_{50} was 2.16 μg/mL.

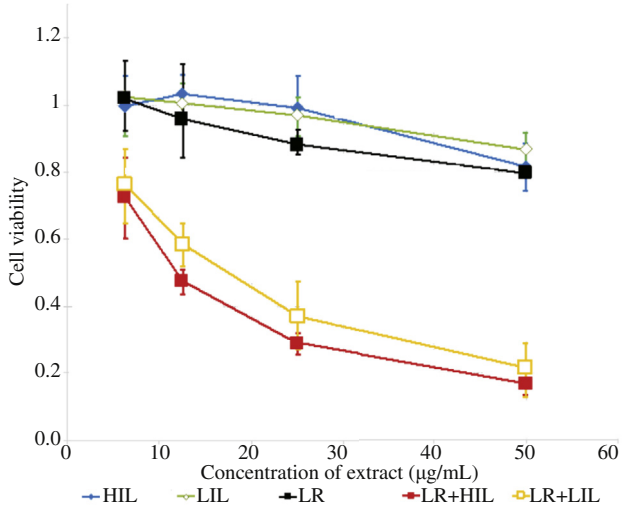

Figure 3. Dose–response curves showing cytotoxicity of irradiated *L. racemosa* (LR) extract on MCF-7 cells in 96-well plates and treated with: different concentrations of DMSO followed by irradiation at 5.53 mW [HIL, blue diamond] or 0.553 mW [LIL, green diamond outline] for 1 min, different concentrations of LR followed by incubation in the dark [LR, black square], and different concentrations of LR followed by irradiation at 5.53 mW [LR + HIL, red square] or 0.553 mW [LR + LIL, yellow square outline] for 1 min. Values are means ± SD of three trials with three replicate wells per concentration.

3.3. Combination of ethanolic extract from C. odorata and broadband light was not cytotoxic to MCF-7 cells

Figure 4 shows the dose–response curves of various treatments of *C. odorata* (CO) alone, high-intensity light (HIL) alone, low-intensity light (LIL) alone, CO with HIL, and CO with LIL. As with *L. racemosa*, *C. odorata* extract and broadband light were, independently, non-cytotoxic to MCF-7 cells. Irradiation of the extract with either HIL or LIL had no effect on its activity against MCF-7 cells. For independent treatments, CO, HIL and LIL, IC_{50} could not be interpolated by linear regression indicating absence of toxicity. The per cent inhibition for treatments CO with HIL and CO with LIL were 35.10 μg/mL and 38.69 μg/mL, respectively. These values are above the threshold value for toxicity of crude extracts of <30 μg/mL set by the National Cancer Institute and are therefore not considered cytotoxic against cancer cells. The IC_{50} of the positive control, doxorubicin, was 2.16 μg/mL.

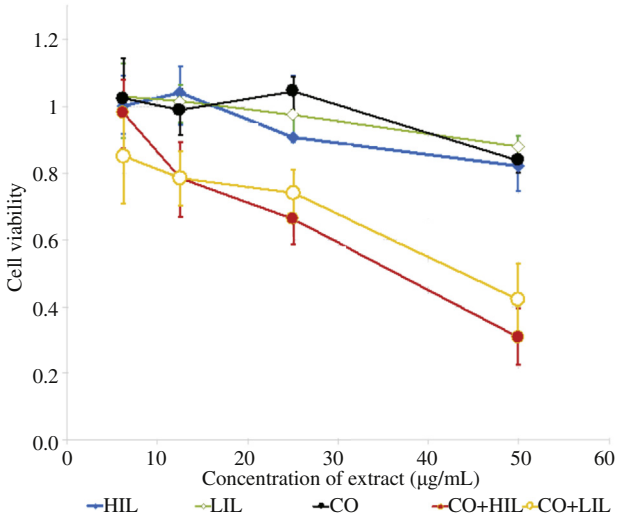

Figure 4. Dose–response curves showing the effect of irradiated *C. odorata* (CO) extract.
MCF-7 cells were seeded in 96-well plates and treated with the following: different concentrations of DMSO followed by irradiation at 5.53 mW [HIL, blue diamond] or 0.553 mW [LIL green diamond outline] for 1 min, different concentrations of CO followed by incubation in the dark [CO, black circle], and different concentrations of CO followed by irradiation at 5.53 mW [CO + HIL red circle] or 0.553 mW [CO + LIL yellow circle outline] for 1 min. Values are means ± SD of three trials with three replicate wells per concentration.

3.4. A combination of ethanolic extract from A. procera and broadband light also produced cytotoxic effect against MCF-7 cells

The same procedure was conducted with *A. procera*. Figure 5 shows that as with *L. racemosa*, *A. procera* extract and broadband light were, independently, non-cytotoxic to MCF-7 cells

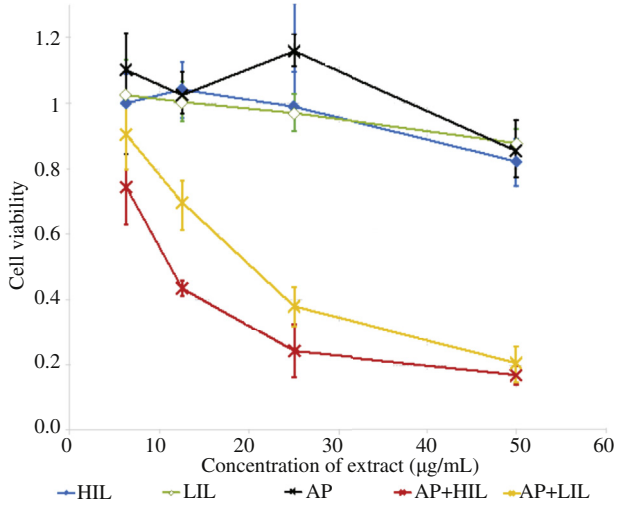

Figure 5. Dose–response curves showing the anti-proliferative effect of irradiated *A. procera* (AP) extract.
MCF-7 cells were seeded in 96-well plates and treated with the following: different concentrations of DMSO followed by irradiation at 5.53 mW [HIL, blue diamond] and 0.553 mW [LIL, green diamond outline] for 1 min, different concentrations of AP followed by incubation in the dark [AP, black X], and different concentrations of AP followed by irradiation at 5.53 mW [AP + HIL, red X] and 0.553 mW [AP + LIL, yellow X] for 1 min. Values are means ± SD of three trials with three replicate wells per concentration.

but, a combination of the two produced an anti-proliferative effect. The IC_{50} values for treatments AP with HIL and AP with LIL were 10.73 µg/mL and 19.59 µg/mL, respectively. The IC_{50} of the doxorubicin was 2.16 µg/mL.

A comparison of IC_{50} values between *L. racemosa* treatments, *C. odorata* treatments and *A. procera* treatments for MCF-7 is shown in the bar graph in Figure 6. A summary of IC_{50} values is shown on Table 1. From an analysis of Figure 6 and Table 1, *A. procera* and *L. racemosa* were the most effective photosensitizers when either HIL or LIL was used as the light component.

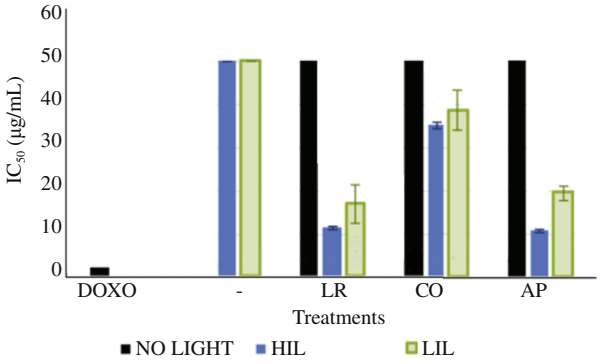

Figure 6. Half maximal inhibitory concentration (IC_{50}) values on MCF-7 for doxorubicin, high intensity light (HIL; 5.53 mW), low intensity light (LIL; 0.553 mW), LR/CO/AP + HIL, and LR/CO/AP + LIL. The value 50 µg/mL was estimated when IC_{50} could not be interpolated by linear regression in treatment regimens involving light only and LR/CO/AP only.

Table 1
Half maximal inhibitory concentration (IC_{50}) values on MCF-7 and HDFn cells for all treatments.

Treatments	Mean IC_{50} (µg/mL)	
	MCF-7 cells	HDFn cells
DOXO	2.16	3.24
HIL	50.00*	50.00*
LIL	50.00*	50.00*
LR	50.00*	50.00*
CO	50.00*	–
AP	50.00*	50.00*
LR + HIL	11.63	28.60
CO + HIL	35.10	–
AP + HIL	10.73	19.39
LR + LIL	17.14	47.24
CO + LIL	38.69	–
AP + LIL	19.59	39.17

*: The value 50 µg/mL was estimated when IC_{50} could not be interpolated by linear regression. –: The value for HDFn was not determined because treatments were not cytotoxic against MCF-7.

3.5. Photoactivated *L. racemosa* and *A. procera* extracts showed less cytotoxicity to non-cancer human dermal fibroblast-neonatal cells

To test the selectivity of the LR and AP treatments, MTT assay was performed on the human non-cancer cell line, HDFn. Table 1 compares the mean IC_{50} values of the treatments on MCF-7 and HDFn cells. For all treatments that showed an effect against MCF-7, the toxicity was greater on cancer cells than non-cancer cells. Moreover, there was no activity against HDFn cells when low-intensity broadband light was used (*i.e.* $IC_{50} > 30$ µg/mL). That is, treatments LR with LIL and AP with LIL were selective to cancer cells. LR with HIL and AP with HIL were inhibitory to both MCF-7 and HDFn cells.

3.6. Photoactivated *L. racemosa* and *A. procera* extracts induced apoptosis in MCF-7 cells

To determine if anti-cancer activity was mediated by apoptosis, TUNEL assay was performed on MCF-7 cells. Figure 7 shows the apoptotic activity of irradiated *L. racemosa* and *A. procera* extracts, similar to the positive control, doxorubicin. Apoptotic cells are characterized by green fluorescence due to Alexa Fluor® 488, which stains fragmented DNA.

3.7. Ethanolic extracts from *L. racemosa* and *A. procera* absorb maximally at 668 nm

To determine whether extracts from *L. racemosa* and *A. procera* are good photosensitizers, their photophysical properties were studied. The wavelengths of maximum absorption, λ_{max}, of both extracts were determined by obtaining the absorption spectra (Figure 8) using a UV–Vis spectrophotometer (SpectroVis® Plus). The reading shows similar absorption spectra with the same λ_{max} at 668 nm, which is within the red region of the visible spectrum. The absorbance of *A. procera* at this wavelength was only slightly higher than that of *L. racemosa*.

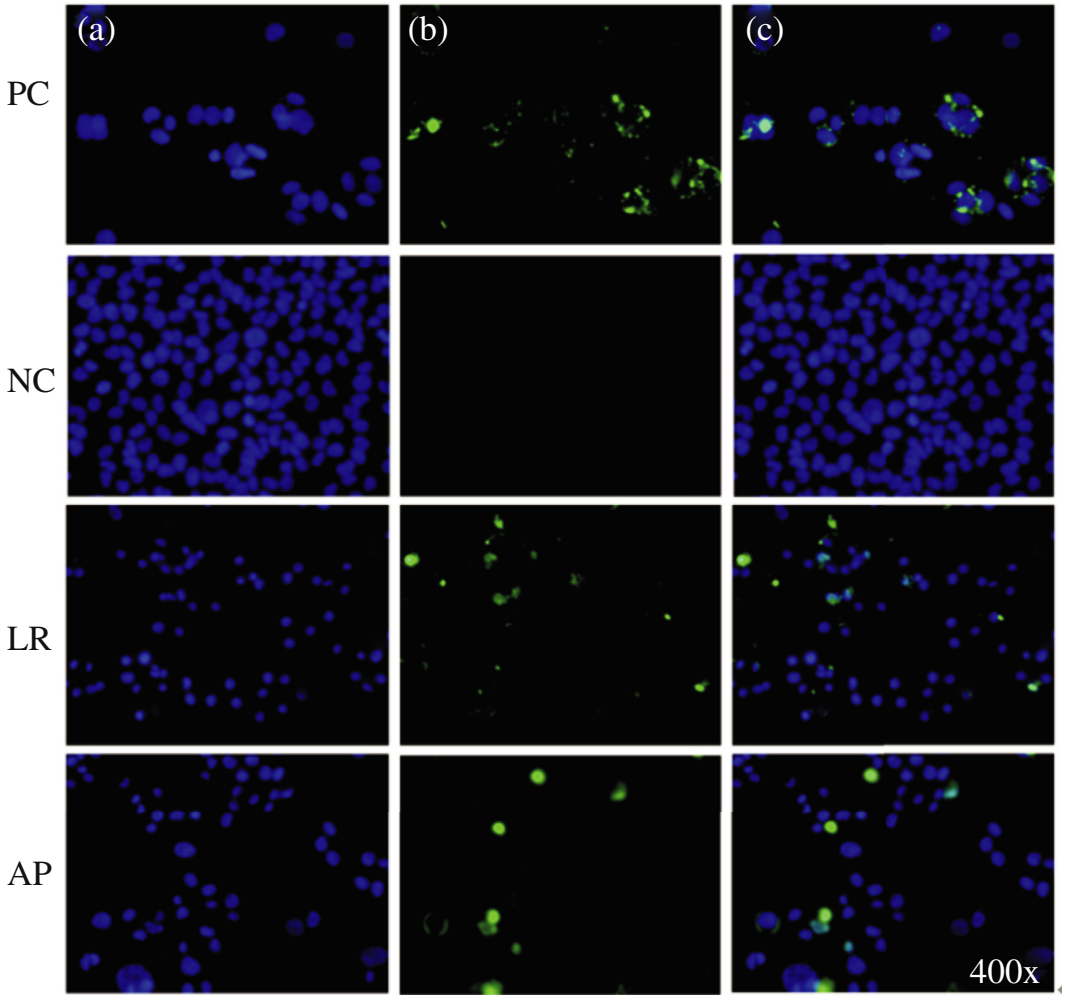

Figure 7. TUNEL assay on cells seeded in 96-well plates and treated with 25 µg/mL (~2 × IC_{50}) *L. racemosa* (LR) with 5.53 mW light for 1 min, and 25 µg/mL (~2 × IC_{50}) *A. procera* (AP) with 5.53 mW light for 1 min. Positive control (PC) is doxorubicin while negative control (NC) is DMSO. Cells were subjected to Click-iT® TUNEL Alexa Fluor® imaging assay (Life Technologies™). Green fluorescence shows apoptotic cells. a: Hoechst-33342, b: Alexa Fluor® 488, c: Overlay.

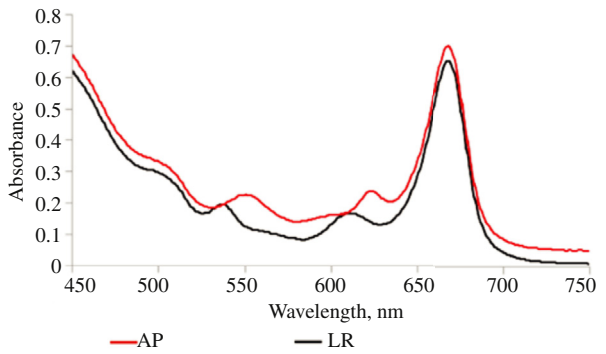

Figure 8. Absorption spectra of ethanolic extracts from *L. racemosa* (black curve) and *A. procera* (gray curve).
Stock solutions (4 mg/mL extract) diluted to 140 µg/mL were read using a UV–Vis spectrophotometer (SpectroVis® Plus). Wavelength of maximum absorption is 668 nm for both extracts.

4. Discussion

A wide variety of plant extracts have been screened for chemotherapeutic properties but their potential as source of photosensitizers in photodynamic therapy has been rarely investigated [14,15]. Looking for potential novel photosensitizers is a crucial first step in PDT studies because, to date, there are only a small number of approved PDT drugs, including Photofrin®, Foscan® and Levulan® which are used mainly for skin, gynecological, gastrointestinal, and head and neck cancers [16]. The present study explored three plant extracts which showed no toxicity to cancer cell lines: *L. racemosa*, *C. odorata* and *A. procera*. However, a purely chemical approach is only one of several ways to treat cancer. Hence, the researchers sought to determine the usefulness of these extracts in PDT. Indeed, toxicity from two of the extracts – *L. racemosa* and *A. procera* – was observed when illuminated by broadband light. The fact that not all plant extracts behaved this way suggests that the presence or abundance of photosensitizing molecules is a distinctive property of some plants such as *L. racemosa* and *A. procera* in this case.

Research showed *L. racemosa* extracts have antibacterial and antihypertensive activity [17,18]. *A. procera* have reported significant antibacterial, analgesic and central nervous system depressant activities [19,20]. No anti-cancer studies have been done on these plants. The present study explored the anti-cancer properties of these plants on MCF-7 cells and moreover, to demonstrate the potential of these plants as photosensitizers in PDT.

The most important property of *L. racemosa* and *A. procera* as most suitable for this study is their lack of toxicity against both cancer and non-cancer cells. A good photosensitizer is

nontoxic unless activated by light of a specific wavelength [8]. Upon irradiation by light, the cytotoxic activity of the extracts dramatically increases, making them good photosensitizing agents. Appropriate controls were used to make sure that it was not light that produced the anti-proliferative effect but the combination of both the light and the extract.

Another important characteristic that makes *L. racemosa* and *A. procera* ideal photosensitizers is their absorption maxima at the red region, specifically at 668 nm, which is within the optical window of biological tissues (between 600 and 800 nm). Below this range, light does not penetrate deep into the tissue. At very long wavelengths (above 800 nm) on the other hand, absorption of photons does not provide enough energy to excite oxygen to its singlet state and to form substantial yield of ROS [21].

Although not tested in the present study, the production of ROS is the known anti-cancer mechanism of PDT. When a photosensitizer absorbs light of a particular wavelength, it is transformed from its ground state to an excited state. This is followed by either one of two types of reactions. In the Type I reaction, the excited photosensitizer reacts directly with an organic molecule in the cellular microenvironment, transferring a hydrogen atom to form radicals. The reduced photosensitizer interacts with oxygen through a redox reaction forming a superoxide anion radical ($O_2^{\bullet-}$). Subsequent one-electron reduction leads to the formation of a virtually indiscriminate oxidant hydroxyl radical ($HO^{\bullet}$). In the Type II reaction, the activated sensitizer transfers its energy directly to molecular oxygen to form singlet oxygen ($^1O_2$). Since the Type II reaction is mechanistically much simpler than Type I, most photosensitizers are believed to operate via the Type II mechanism [21–23]. The ROS generated are capable of causing irreversible damage if generated inside any cell, which means that it cannot discriminate between cancer and non-cancer cells. Surprisingly, based on the results of the present study, HDFn, the non-cancer cells used, are less susceptible to damage than MCF-7 cells for both *L. racemosa* and *A. procera* extracts. When low-intensity light is used, both extracts were not at all cytotoxic to HDFn cells. This selective destruction is another characteristic making *L. racemosa* and *A. procera* ideal photosensitizers.

Selectivity may depend on the extent to which the cells absorb the photosensitizing molecule. This is because $^1O_2$ has a short lifetime in biological systems (approximately 10–320 ns) and a very short radius of action (10–55 nm in cells). Photodynamic damage will occur very close to the intracellular location of the photosensitizer. Therefore, photosensitizers that are not absorbed by the cells, even though they give a high photochemical yield of $^1O_2$, are very inefficient [21,22,24]. Extracts from *L. racemosa* and *A. procera* may have been readily absorbed by MCF-7 cells but not by HDFn cells making the extracts less toxic against HDFn even when activated by light.

Because of the limited migration of $^1O_2$ from the site of its formation, sites of initial cell damage are closely related to the localization of the photosensitizer [25,26]. The localization of a photosensitizer within a cell varies with the type of photosensitizer. Depending on the type used, a photosensitizer may localize on lysosomes, plasma membranes or mitochondria. There are no reports saying that the localization of photosensitizers also varies with cell type so this could not be related to the selectivity of *L. racemosa* and *A. procera* extracts to cancer cells. This is related, nonetheless, to the mechanism of cell death involved. PDT can evoke 3 main cell death pathways: necrotic, apoptotic, and autophagy-associated cell death [21]. Based on the results of the TUNEL assay, the anti-cancer effect of irradiated *L. racemosa* and *A. procera* extracts were mediated by apoptosis. The end result of apoptosis is the fragmentation of nuclear DNA and the dissociation of the cell into membrane-bound particles that are engulfed by adjoining cells, thereby minimizing an inflammatory response. Based on the review by Dougherty *et al.* [22], sensitizers that localize in the plasma membrane are likely to cause necrosis while those that localize in the mitochondria are likely to induce apoptosis. Therefore, it is likely that photosensitizing components from *L. racemosa* and *A. procera* localize in the mitochondria of MCF-7 cells. Apoptosis after PDT is associated with mitochondrial photodamage [27–29]. Some photosensitizing agents, when activated, cause mitochondrial membrane permeability, and this leads to the release of cytochrome *c* and other mitochondrial factors that can trigger an apoptotic response [30]. This apoptotic response is what makes PDT advantageous over other cancer treatment modalities. Malignant cells often exhibit an impaired ability to undergo apoptosis and this is related to their ability to survive chemotherapy [31,32]. Since it is now established that *L. racemosa* and *A. procera* can induce apoptosis, they can be effective against otherwise drug-resistant cell types.

Results of *in vitro* experiments should be translated to clinical application and this involves surmounting some limitations and providing means to overcome them. For most clinical photosensitizers, and theoretically for *L. racemosa* and *A. procera*, red light is used for activation. However, red light has limited ability to penetrate tissue, which is the reason why current photodynamic therapies are used only for skin cancer or lesions in very shallow tissue. Punjabi *et al.* developed a novel strategy that allows photodynamic therapies to access deep-set cancer cells [33]. The design makes use of a biocompatible, low-power, deep-penetrating 980-nm near-infrared light and a new class of up converting nanoparticles (UCNPs). The idea is to convert near-infrared light, which can penetrate more deeply into tissue and can reach deeper set malignant tumors, into visible red light needed in photodynamic therapies to activate photosensitizers. This is achieved by the UCNPs, which are administered together with the PDT drug. UCNP is engineered to have better emissions in the red part of the spectrum and this UCNP was conjugated with the photosensitizer aminolevulinic acid via a hydrazine linkage. This discovery widens the scope of PDT usage and allows non-invasive PDT to treat cancers – including breast, colon, liver and lung cancer – that cannot be accessed and treated using the standard PDT procedures.

Overcoming side effects is an important consideration in clinical PDT. Damage to non-cancer cells can be minimized by using selective PDT drugs and useful photosensitizers that can localize in neoplastic lesions. But photosensitizers are rarely selective given that ROS do not discriminate between cancerous and non-cancerous tissue. Even L. *racemosa* extracts shows little cytotoxicity to HDFn cells. Selectivity could be maximized by using focused lasers as light source or delivery tools such as flexible fiber-optic devices to precisely deliver the light source directly to the tumor region [21].

Deleterious side effects can also be reduced using two-photon excitation of photosensitizer [21,34,35]. Two-photon PDT makes use of short laser pulses with very high peak power so that the photosensitizer absorbs two light photons simultaneously. The first photon excites the molecule from its

ground state to a virtual intermediate excited state while a second photon promotes the molecule from the intermediate state to the singlet excited state. The excited state achieved and the subsequent effects are the same as in one-photon PDT. The difference is that, since the probability of two-photon absorption occurring is very small, two-photon PDT can achieve excitation volumes of a few femtoliters. This extremely confined excitation volume allows for high spatial selectivity and lessens the damage of tissues adjacent to the treated area.

In a novel strategy called metronomic PDT (mPDT), both drug and light are delivered at very low dose over an extended period of time minimizing side effects. In this method, tumor cell-specific apoptosis occurs with minimal tissue necrosis. This minimizes both direct photodynamic damage to normal tissues and secondary damage from the inflammatory response to PDT-induced tumor necrosis [21,36,37].

Controlling the PDT activity of photosensitizers can be achieved through the use of PDT molecular beacons (MBs). The photosensitizer is linked to a singlet oxygen quencher so that its photoactivity is silenced until the linker interacts with a target molecule. With PDT MBs, tumor selectivity no longer depends solely on photosensitizer delivery but also on the tumor specificity of the unquenching interaction and the selectivity of the MB to this interaction [21,37].

Among the three crude ethanolic plant extracts used in this research, none showed cytotoxicity against human mammary adenocarcinoma MCF-7 cells on their own. However, when irradiated with broadband light, two plant extracts, *L. racemosa* and *A. procera*, were cytotoxic against MCF-7. Although not as good as the positive control, doxorubicin, the IC_{50} values obtained were low enough for the two crude extracts to be considered cytotoxic against cancer cells. Based on the results, several characteristics make *L. racemosa* and *A. procera* extracts good photosensitizers in photodynamic therapy. First is that they are chemically inert until activation. Irradiation by light changes the properties of the extracts, increasing their cytotoxic activity against cells. This is the hallmark of any PDT molecule. Moreover, the cytotoxicity is greater in MCF-7 than in non-cancer human dermal fibroblast, neonatal HDFn cells, making the extracts selective to cancer cells. Finally, the extracts absorb maximally at a wavelength that is biologically compatible. This means that, if this study could be translated to clinical application, the appropriate wavelength to be used is long enough to penetrate deeply into tissue but short enough to provide energy to excite oxygen and produce a substantial yield of ROS.

The mechanism of action observed was apoptosis. This study demonstrated anti-cancer activity of crude extracts. Future researchers, should aim to isolate specific compounds from these extracts to be used as drugs in PDT. Therefore, purification and chemical characterization of the pure compound should be performed.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

This study was funded with in-house grant from Institute of Biology, University of the Philippines, Diliman through TA # 9774-362-499-439. GT and KFR acknowledge the support of the UP System Emerging Interdisciplinary Grant C2-001 project for the exposure set-up and control system. The researchers would like to acknowledge research associates Ms. Cielo Marquez, Mr. Carlo Limbo, and Ms. Regina Joyce Ferrer, from the Mammalian Cell Culture Laboratory, Institute of Biology, UP Diliman, for their assistance in the performance of the experiments, as well Mr. Roland Romero, from the Instrumentation Physics Laboratory, National Institute of Physics, UP Diliman, for his assistance in the use of the set-up.

References

[1] Baltazar LM, Ray A, Santos DA, Cisalpino PS, Friedman AJ, Nosanchuk JD. Antimicrobial photodynamic therapy: an effective alternative approach to control fungal infections. *Front Microbiol* 2015; **6**: 202.

[2] Shishkova N, Kuznetsova O, Berezov T. Photodynamic therapy for gynecological diseases and breast cancer. *Cancer Biol Med* 2012; **9**(1): 9-17.

[3] Hayashi N, Kataoka H, Yano S, Tanaka M, Moriwaki K, Akashi H, et al. A novel photodynamic therapy targeting cancer cells and tumor-associated macrophages. *Mol Cancer Ther* 2015; **14**(2): 452-60.

[4] Gasparetto A, Lapinski TF, Zamuner SR, Khouri S, Alves LP, Munin E, et al. Extracts from *Alternanthera maritima* as natural photosensitizers in photodynamic antimicrobial chemotherapy (PACT). *J Photoch Photobio B* 2010; **99**: 15-20.

[5] Abdel-Kader MH. *Photodynamic therapy from theory to applications*. Heidelberg: Springer-Verlag Berlin Heidelberg; 2014.

[6] National Cancer Institute. Photodynamic therapy for cancer reviewed. [Online] Available from: https://www.cancer.gov/about-cancer/treatment/types/surgery/photodynamic-fact-sheet#q4 [Accessed on 12th November, 2016]

[7] Mroz P, Yaroslavsky A, Kharkwal GB, Hamblin MR. Cell death pathways in photodynamic therapy of cancer. *Cancers* 2011; **3**: 2516-39.

[8] Allison R, Moghissi K. Photodynamic therapy (PDT): PDT mechanisms. *Clin Endosc* 2013; **46**(1): 24-9.

[9] Awasthi K, Yamamoto K, Furuya K, Nakabayashi T, Li L, Ohta N. Fluorescence characteristics and lifetime images of photosensitizers of talaporfin sodium and sodium pheophorbide a in normal and cancer cells. *Sensors (Basel)* 2015; **15**(5): 11417-30.

[10] Albano PM, Lumang-Salvador C, Orosa J 3rd, Racelis S, Leano M, Angeles LM, et al. Overall survival of Filipino patients with squamous cell carcinoma of the head and neck: a single-institution experience. *Asian Pac J Cancer Prev* 2013; **14**(8): 4769-74.

[11] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; **65**: 55-63.

[12] Ferraz RPC, Bomfim DS, Carvalho NC, Soares MBP, da Silva TB, Machado WJ, et al. Cytotoxic effect of leaf essential oil of *Lippia gracilis* Schauer (Verbenaceae). *Phytomedicine* 2013; **20**(7): 615-21.

[13] Rodrigues M, Muehlmann L, Longo J, Silva R, Graebner I, Degterev I, et al. Photodynamic therapy based on *Arrabidaea chica* (Crajiru) extract nanoemulsion: *in vitro* activity against monolayers and spheroids of human mammary adenocarcinoma MCF-7 Cells. *Nanomed Nanotechnol* 2015; **6**(3): 1-6.

[14] Marrelli M, Menichini G, Provenzano E, Conforti F. Applications of natural compounds in the photodynamic therapy of skin cancer. *Curr Med Chem* 2014; **21**(12): 1371-90.

[15] Jong W, Tan P, Kamarulzaman F, Mejin M, Lim D, Ang I, et al. Photodynamic activity of plant extracts from Sarawak, Borneo. *Chem Biodivers* 2013; **10**(8): 1475-86.

[16] Dhaneshwar S, Patil K, Bulbule M, Kinjawadekar V, Joshi D, Joshi V. Photodynamic therapy for cancer. *Int J Pharm Sci Rev Res* 2014; **27**(2): 125-41.

[17] Joshi UH, Ganatra TH, Bhalodiya PN, Desai TR, Tirgar PR. Comparative review on harmless herbs with allopathic remedies as antihypertensive. *Res J Pharm Biol Chem Sci* 2012; **3**(2): 673-87.

[18] Abeysinghe PD. Antibacterial activity of aqueous and ethanol extracts of mangrove species collected from Southern Sri Lanka. *Asian J Pharm Biol Res* 2012; **2**(1): 79-83.
[19] Duraipandiyan V, Ayyanar M, Ignacimuthu S. Antimicrobial activity of some ethnomedicinal plants used by Paliyar tribe from Tamil Nadu, India. *BMC Complement Altern Med* 2006; **6**(35): 1-7.
[20] Khatoon M, Khatun H, Islam E, Parvin S. Analgesic, antibacterial and central nervous system depressant activities of *Albizia procera* leaves. *Asian Pac J Trop Biomed* 2014; **4**(4): 279-84.
[21] Agostinis P, Berg K, Cengel K, Foster T, Girotti A, Gollnick S, et al. Photodynamic therapy of cancer: an update. *CA Cancer J Clin* 2011; **6**(1): 250-81.
[22] Dougherty T, Gomer C, Henderson B, Jori G, Kessel D, Korbelik M, et al. Photodynamic therapy. *J Natl Cancer Inst* 1998; **90**: 889-905.
[23] Mehraban N, Freeman H. Developments in PDT sensitizers for increased selectivity and singlet oxygen production. *Materials* 2015; **8**: 4421-56.
[24] Dysart J, Patterson M. Characterization of photofrin photobleaching for singlet oxygen dose estimation during photodynamic therapy of MLL cells *in vitro*. *Phys Med Biol* 2005; **20**: 2597-616.
[25] Moan J, Berg K. The photodegradation of porphyrins in cells can be used to estimate the lifetime of singlet oxygen. *J Photochem Photobiol* 1991; **53**: 549-53.
[26] Peng Q, Moan J, Nesland J. Correlation of subcellular and intratumoral photosensitizer location with ultrastructural features after photodynamic therapy. *Ultrastruct Pathol* 1996; **20**: 109-29.
[27] Kessel D. Reversible effects of photodamage directed toward mitochondria. *J Photochem Photobiol* 2014; **90**(5): 1211-3.
[28] Kessel D, Luo Y, Deng Y, Chang C. The role of subcellular localization in initiation of apoptosis by photodynamic therapy. *J Photochem Photobiol* 1997; **65**: 422-6.
[29] Panzarini E, Inguscio V, Dini L. Overview of cell death mechanisms induced by rose bengal acetate-photodynamic therapy. *Int J Photoenergy* 2011; **2011**: 713726.
[30] Kessel D, Luo Y, Woodburn K, Chang C, Henderson B. Mechanisms of phototoxicity catalyzed by two porphycenes. *Proc SPIE* 1995; **2392**: 122-8.
[31] Su Z, Yang Z, Xu Y, Chen Y, Yu Q. Apoptosis, autophagy, necroptosis, and cancer metastasis. *Mol Cancer* 2015; **14**: 48.
[32] Bai L, Wang S. Targeting apoptosis pathways for new cancer therapeutics. *Annu Rev Med* 2014; **65**: 139-55.
[33] Punjabi A, Wu X, Tokatli-Apollon A, El-Rifai M, Lee H, Zhang Y, et al. Amplifying the red-emission of upconverting nanoparticles for biocompatible clinically used prodrug-induced photodynamic therapy. *ACS Nano* 2014; **8**(10): 10621-30.
[34] Probodh I, Cramb D. Two-photon excitation photodynamic therapy: working toward a new treatment for wet age-related macular degeneration. In: Ying GS, editor. *The recent advances in basic research and clinical care*. Shanghai: InTech; 2012, p. 213-26.
[35] Ogawa K, Kobuke Y. Recent advances in two-photon photodynamic therapy. *Anticancer Agents Med Chem* 2008; **8**(3): 269-79.
[36] Bisland S, Lilge L, Lin A, Rusnov R, Wilson. Metronomic photodynamic therapy as a new paradigm for photodynamic therapy: rationale and preclinical evaluation of technical feasibility for treating malignant brain tumors. *J Photochem Photobiol* 2004; **80**: 22-30.
[37] Zheng G, Chen J, Stefflova K, Jarvi M, Li H, Wilson B. Photodynamic molecular beacon as an activatable photosensitizer based on protease-controlled singlet oxygen quenching and activation. *Proc Natl Acad Sci U. S. A* 2007; **104**(21): 8989-94.