Anticancer Activity of *Dimocarpus longan* Lour. Leaf Extracts in *vitro* and Phytochemical Profile

By

Khaled N. Rashed
Gerda Fouche
Research Article

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**Khaled N. Rashed**<sup>1</sup>*, Gerda Fouche**<sup>2</sup>

<sup>1</sup>National Research Centre, Pharmacognosy Department, Dokki, Giza, Egypt.
<sup>2</sup>Natural Product Chemistry Group, Council for Scientific and Industrial Research, PO Box 395, Pretoria 0001, South Africa.

*Corresponding Author's Email: khalednabih2015@yahoo.co.uk, Tel: 01003642233

**ABSTRACT**

This study deals with the evaluation of anti-cancer effect of *Dimocarpus longan* extracts and also the investigation of the phytoconstituents from the plant extracts. Petroleum ether (40-60°C), Chloroform, ethyl acetate and methanol 80% extracts of *D. longan* leaf were tested for their anti-cancer activity on three cancer cells TK10 (renal), UACC62 (melanoma) and MCF7 (breast) cancer cells using a Sulforhodamine B (SRB) assay. Petroleum ether of *D. longan* at the concentration of 100 µg/ml has shown a significant anticancer effect for UACC62 (melanoma) cancer cell and showed mild anticancer effect for the other two cancer cells while the other extracts have mild anticancer effect on the three cancer cells. Phytochemical profile of the plant extracts proves the presence of triterpenes, flavonoids, tannins and carbohydrates in methanol 80% extract and both chloroform and ethyl acetate have triterpenes and flavonoids while petroleum ether has triterpenes and/or sterols. Chromatographic separation and bioactive fractionation of petroleum ether extract revealed the isolation and identification of β-sitosterol, stigmasterol as the major constituents. The results suggest that petroleum ether extract of *Dimocarpus longan* leaf exhibited a significant anti-cancer effect.

**Keywords:** *Dimocarpus longan*, leaf, anticancer activity, phytoconstituents.

**INTRODUCTION**

For centuries, People have been using plants for their therapeutic values. Plants have shown to be good sources of a variety of drugs for human ailments including cancer. Natural products have played an important role in the discovery of useful antitumor agents especially clinically relevant anticancer drugs; such as taxol, camptothecin, vinblastine and vincristine, which were discovered from higher plants. There is a necessity to explore their uses and to conduct pharmacognostic and pharmacological studies to ascertain their therapeutic properties. Cancer is a leading cause of death worldwide and it is a dreadful disease and combating this disease is of great importance to public health. Phytochemical examination has been making rapid progress and herbal products are becoming popular as sources of plausible anticancer compounds (Parag *et al.*, 2010). Recent Phytochemical examination of plants which have a suitable history of use in folklore for the treatment of cancer has often resulted in the isolation of principles with anti-cancer activity (Afolabi *et al.*, 2007). In our screening program for discovery of new anticancer agents, *Dimocarpus longan* Lour. (soapberry family) is commonly known as longan and it is native to Southeast Asia, such as China, Taiwan and Thailand. It is a tropical tree that produces edible fruit. The fruits of *D. longan* were used as a traditional Chinese medicine for different treatments, such as promoting blood metabolism, soothing nerves, and relieving insomnia (Hsu *et al.*, 1985). Previous phytochemical and pharmacological studies of *Dimocarpus longan* showed that longan pericarp contain high amounts of bioactive compounds, such as phenolic acids, flavonoids, and polysaccharides and exhibit antibacterial, antiviral, antioxidant, anti-inflammatory, and anticarcinogenic properties (Bravo 1998, Pan *et al.*, 2008). The aim of the present research work is to evaluate anti-cancer effect of *D. longan* leaf extracts on three cancer cells TK10 (renal), UACC62 (melanoma) and MCF7 (breast) cancer cells and also determine the phytoconstituents of the plant extracts.

**MATERIALS AND METHODS**

**Experimental**

Spectroscopic data: NMR–Varian. MS (Finnigan MAT SSQ 7000, 70 ev). Silica gel (60-200 mesh, Merck). Thin Layer Chromatography (TLC): pre-coated sheets of silica gel 60 F<sub>254</sub> (Merck). Sephadex LH-20 (Sigma).
Plant Material

*D. longan* leaves were collected from Al-Zohiriya garden, Giza, Egypt in May 2011. The plant was identified by Dr. Mohammed El-Gebaly, Department of Botany, National Research Centre (NRC) and by Mrs. Tereeza Labib Consultant of Plant Taxonomy at the Ministry of Agriculture and director of Orman botanical garden, Giza, Egypt. A voucher specimen is deposited in the herbarium of Al-Zohiriya garden, Giza, Egypt.

Preparation of the extracts

Finely ground leaf of *D. longan* 500 g were extracted with petroleum ether (40-60°C), chloroform, ethyl acetate and methanol 80% solvents by maceration. Each extract was concentrated to dryness to yield, 7 g of petroleum ether, 4.5 g of chloroform, 3.5 g of ethyl acetate and 28.5 g of methanol 80% extracts, respectively. Each extract was tested for the presence of the phytoconstituents according to the following standard tests, Molisch's test for carbohydrates, Shinoda test for flavonoids, forth test for saponins, Salkowski ‘s for terpenes and sterols, FeCl₃ and Mayer’s reagents for detecting of tannins and alkaloids, respectively (Sofowra 1993, Trease and Evans 1989, Harborne 1973).

Phytochemical characterization of Petroleum ether extract

Petroleum ether extract 6 g was subjected to silica gel column chromatography using n-hexane as eluent and gradually increasing amount of ethyl acetate (EtOAc) where two major compounds were isolated. Compound 1 (β-sitosterol) was isolated from n-hexane: EtOAc (80 : 20) elution and compound 2 (stigmasterol) was isolated through elution with n-hexane: EtOAc (75 : 25).

Sulforhodamine B (SRB) assay

The growth inhibitory effects of the extracts were tested in the 3-cell line panel consisting of TK10 (renal), UACC62 (melanoma) and MCF7 (breast) cancer cells using a Sulforhodamine B (SRB) assay. The SRB assay was developed by (Skehan et al., 1990) to measure drug-induced cytotoxicity and cell proliferation. Its principle is based on the ability of the protein dye sulforhodamine B (Acid Red 52) to bind electrostatically in a pH-dependent manner to protein basic amino acid residues of trichloroacetic acid-fixed cells. Under mild acidic conditions, it binds to the fixed cellular protein, while under mild basic conditions it can be extracted from cells and solubilized for measurement. The SRB assay is performed at CSIR in accordance with the protocol of the Drug Evaluation Branch, NCI (National Cancer Institute). The 3-cell line panel used is recommended by the NCI for preliminary screens. The human cell lines TK10, UACC62 and MCF7 were previously obtained from NCI in the framework of a collaborative research program between CSIR and NCI. Cell lines were routinely maintained as monolayer cell cultures in RPMI containing 5% fetal bovine serum, 2 mM L-glutamine and 50µg/ml gentamicin. For the screening experiment, the cells (3-19 passages) were inoculated in 96-well microtiter plates at plating densities of 7-10 000 cells/well and were incubated for 24 h. After 24 h, one plate was fixed with TCA to represent a measurement of the cell population for each cell line at the time of drug addition (T0). The other plates with cells were treated with the experimental samples which were previously dissolved in DMSO as 10000µg/ml stocks and diluted in medium to a final concentration 100µg/ml. Cells without samples served as controls. Blank wells contained complete medium without cells. Emetine was used as a reference standard. The plates were incubated for 48 h after addition of the extracts (100 µg/ml ). At the end of the incubation period, the cells were fixed to the bottom of each well with cold 50% trichloroacetic acid, washed, dried and dyed with SRB. Unbound dye was removed and protein-bound dye was extracted with 10mM Tris base for optical density determination at a wavelength 540 nm using a multwell spectrophotometer. Optical density measurements were used to calculate the net percentage cell growth. The optical density of the test wells after 48-h period of exposure to test compound is Ti, the optical density at time zero is T0, and the control (untreated cells) optical density is C. Percentage cell growth is calculated as:

\[
\frac{[(T_i-T_0)/(C-T_0)] x 100}{[T_{i-T_0}]}\]

for concentrations at which Ti>T0

\[
\frac{[(T_i-T_0)/(T_0)] x 100}{[T_{i-T_0}]}\]

for concentrations at which Ti<T0.

RESULTS AND DISCUSSION

Phytochemical analysis of *D. longan* extracts is shown in table 1, it showed that petroleum ether extract has triterpenes and/or sterols, chloroform and ethyl acetate extracts have triterpenes and flavonoids, while methanol 80% extract has triterpenes, flavonoids, tannins and carbohydrates. The isolated compounds from petroleum ether extract of *D. longan* leaf are shown in Fig. 1.
Table 1: Phytochemical Analysis from the *D. longan* leaf extracts

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Petroleum ether</th>
<th>Chloroform</th>
<th>Ethyl acetate</th>
<th>Methanol 80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triterpenes and /or Sterols</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrates and/or glycosides</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>-</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Coumarins</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alkaloids and/or nitrogenous compounds</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tannins</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(+) presence of constituents, (-) absence of constituents

**Compound 1: β-sitosterol**

**Compound 2: Stigmasterol**

**Fig. 1: Compounds isolated from petroleum ether extract of *D. longan* leaf**

**Structure elucidation of the bioactive compounds isolated of Petroleum ether extract of *D. longan***

β-sitosterol (1): 20 mg, white needles, $^1$H-NMR (400 MHz, CDCl$_3$): $\delta$ 5.37 (IH, m, H-6), 3.52 (IH, m, H-3), 1.09 (3H, s, CH$_3$-19), 0.98 (3H, d, $J = 6.5$, CH$_3$-21), 0.92 (3H, t, $J = 7.4$, CH$_3$-29), 0.85 (3H, d, $J = 6.7$Hz, CH$_3$-26), 0.81 (3H, d, $J = 6.7$Hz, CH$_3$-27), 0.75 (3H, s, CH$_3$-18). $^{13}$C-NMR(100 MHz, CDCl$_3$): $\delta$ 140.4 (C-5), 121.5 (C-6), 71.6 (C-3), 57.2 (C-17), 56.4 (C-14), 50.3 (C-9), 46.3 (C-24), 42.8 (C-13, 4), 39.8 (C-12), 37.6 (C-1), 36.7 (C-10), 35.9 (C-20), 34.2 (C-22), 31.7 (C-8, 7), 31.4 (C-2), 29.2 (C-25), 28.4 (C-16), 26.2 (C-23), 24.5 (C-15), 23.4 (C-28), 21.1 (C-11), 19.8 (C-26), 19.5 (C-19), 19.2 (C-27), 18.6 (C-21).

Stigmasterol (2): 17 mg, white needle crystals, $^1$H-NMR (400 MHz, CDCl$_3$): $\delta$ 5.32 (IH, m, H-6), 5.11 (1H, d, $J = 14.2$, 8.2 Hz, H-22), 5.04 (1H, dd, $J = 14.2$, 8.2 Hz, H-23), 3.54 (IH, m, H-3), 1.04 (3H, s, CH$_3$-10), 0.9 (3H, d, $J = 6.5$, CH$_3$-20), 0.84 (3H, d, $J = 7.4$, CH$_3$-27), 0.82 (3H, d, $J = 7.4$, CH$_3$-26), 0.68 (3H, s, CH$_3$-13). $^{13}$C-NMR(100 MHz, CDCl$_3$): $\delta$ 140.6 (C-5), 138.4 (C-22), 129.1 (C-23), 121.8 (C-6), 71.9 (C-3), 56.7 (C-17), 56.9 (C-14), 50.9 (C-9), 50.7 (C-24), 42.6 (C-13, 4), 39.6 (C-12), 37.4 (C-1), 40.2 (C-20), 36.7 (C-10), 31.4 (C-8, 7), 31.7 (C-2), 30.9 (C-25), 28.8 (C-16), 24.8 (C-15), 24.7 (C-28), 21.5 (C-11), 20.8 (C-26), 20.4 (C-19), 19.7 (C-27), 19.1 (C-21).
Chromatographic separation and purification of the petroleum ether resulted in isolation and identification of compound 1 (β-sitosterol) which gave dark spot under short UV light that changed to violet colour on spraying with vanillin sulphuric and heating in an oven at 110°C for 5min and also compound 2 (stigmasterol) gave a dark spot under short UV light and also gave a violet colour after spraying with vanillin sulphuric and heating in an oven at 110°C for 5min. NMR spectral data has shown signals very close to compound 1 (β-sitosterol), also it is identified by other authors (Pateh et al. 2009). Similarly, a comparison of the NMR spectral data of compound 2 (Pateh et al. 2009; Shirin et al., 2012) with published data allowed the identification of compound 2 as stigmasterol; confirmation of both compounds was done by co-TLC with authentic standards. The extracts of D. longan leaf were tested for their anticancer activities on different cancer cells, TK10 (renal), UACC62 (melanoma) and MCF7 (breast) cancer cells using a Sulfurhodamine B (SRB) assay (Skehan et al., 1990). Petroleum ether (40-60°C), Chloroform, ethyl acetate and methanol 80% extracts of D. longan leaf have a mild anticancer effect for TK10 (renal) and MCF7 (breast) cancer cells while petroleum ether has a significant anticancer effect for UACC62 (melanoma) cancer cells with comparison with a standard drug (Emetine) and the other extracts showed a mild anticancer effect for this cancer cell (table 2). The significant anticancer effect of petroleum ether extract of D. longan is may be due the presence of the bioactive isolated compounds, β-sitosterol, stigmasterol from the extract. A Japanese study reported by Imanaka demonstrated that the oral intake of beta-sitosterol, encapsulated in a liposome, was able to prevent tumor metastasis in rats, although the phytochemical was not absorbed in the serum. The researchers believed that beta-sitosterol works by stimulating the gut immune surveillance systems, as indicated by an increase in natural killer cell activity and production of immune response cytokines (Imanaka et al., 2008). Park and co-workers concluded in their study that "beta-sitosterol potently induces apoptosis in U937 cells (these are leukemia cells) and that beta-sitosterol-induced apoptosis is related to the selective activation of caspase-3 and induction of Bax/Bcl-2 ratio" Beta-sitosterol induced apoptosis in the leukemia cells in a dose-dependent manner (Park et al., 2007). Stigmasterol from Mesua beccariana stem bark displayed strong inhibition on the Raji cell proliferation with IC50 values less than 5 µg/mL and also the proliferation of SK-MEL-28 and HeLa cancer cells were strongly inhibited by pure stigmasterol (Soek, et al., 2012).

Table 2: Anticancer activity of D. longan leaf extracts

<table>
<thead>
<tr>
<th>Extract</th>
<th>Concentration, µg/ml</th>
<th>Growth TK10, %</th>
<th>SD</th>
<th>Growth UACC62, %</th>
<th>SD</th>
<th>Growth MCF7, %</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td>100</td>
<td>-25.92</td>
<td>0.021</td>
<td>-77.40</td>
<td>0.094</td>
<td>24.48</td>
<td>0.088</td>
</tr>
<tr>
<td>Chloroform</td>
<td>100</td>
<td>-4.04</td>
<td>0.046</td>
<td>-21.79</td>
<td>0.002</td>
<td>0.92</td>
<td>0.007</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>100</td>
<td>87.35</td>
<td>0.026</td>
<td>58.38</td>
<td>0.077</td>
<td>66.03</td>
<td>0.073</td>
</tr>
<tr>
<td>Methanol 80%</td>
<td>100</td>
<td>86.12</td>
<td>0.056</td>
<td>52.52</td>
<td>0.007</td>
<td>54.34</td>
<td>0.128</td>
</tr>
<tr>
<td>EMETINE 10µM</td>
<td>100</td>
<td>-61.35</td>
<td>0.007</td>
<td>-86.66</td>
<td>0.006</td>
<td>-46.41</td>
<td>-61.35</td>
</tr>
</tbody>
</table>

SD is standard deviation. % Growth is the net growth of the cells in treated wells compared to untreated controls over the 48h experimental period, i.e. 100% growth means there are the same amount of cells in treated wells as in untreated control wells; 0% growth means the treated wells contain the same number of cells as at the start of the incubation, time 0 (thus no increase in cell number); -100% growth means there are no cells left in the well after 48h.

CONCLUSION

The reported results show that petroleum extract of D. longan leaf has a significant anticancer effect on UACC62 (melanoma) cancer cells. Thus, D. longan leaf could be helpful in cancer prevention and treatment. D. longan could be a natural source of anticancer compounds with anti proliferative and/or apoptotic properties, as cancer chemopreventive and/or therapeutic agents as well, due to its anticancer pharmacological effect, clinical trials are recommended to evaluate the beneficial effects of this plant in human models.

Conflict of interest

There is no conflict of interest associated with the authors of this paper.

REFERENCES


