Antineoplastic activity of *Holoptelea integrifolia* (Roxb.) Planch bark extracts (*in vitro*)

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Abstract: Cancer remains the major public health concern with a number of cancer patients relying on chemotherapy as a treatment option. Although, advances in biomedical research have led to increased anticancer agents in recent years, the treatment is not always effective due to resistance, toxicity or other factors. Phytochemicals and their active components isolated from plants have provided diversified effective drugs many of them are currently used against cancer and other diseases. *Holoptelea integrifolia* (Roxb.) Planch (Ulmaceae) is a widely distributed plant in many parts of the world, also grown in gardens of Pakistan. It is an ornamental plant with certain medicinal characteristics due to many valuable and active phytoconstituents in various parts of the plant. We looked at *in vitro* antineoplastic effects of four different extracts, in butanol (BMBU), hexane (BMHx), ethyl acetate (BMET) and chloroform (BMCHF), from bark of *Holoptelea integrifolia* on small cell lung cancer, breast, prostate, colorectal and hepatocellular cancer cell lines. Plant extracts BMHx and BMET showed significant cytotoxic effects on breast and prostate cancer cells. These preliminary studies are encouraging to proceed further this research in future, regarding the isolation of active phytoconstituents in these extracts as well as its mechanism in chemoprevention and combination anticancer therapy.

Keywords: *Holoptelea integrifolia*, natural anticancer agents, jungle cork tree, phytomedicine.

INTRODUCTION

Neoplastic diseases are major public health concern worldwide and the main modalities for treatment are chemo and/or radiotherapy leading to surgery. These attempts are commonly tried in conjunction for controlling and treating neoplastic conditions. Despite of major advances in the biomedical sciences the incidence of cancer remains to be stagnantly high in both developing and developed countries. Cancer remains the second most leading cause of disease associated death and so is one of the biggest burdens on public health. Treatment primarily involves surgery, radiation and chemotherapy or combination of these. Unfortunately, successes of these modalities are limited either due to ineffectiveness of the drug or patient stops responding over a period of time. There is a continuous need of isolating more potent novel anticancer agents and least toxicity. Natural products presents a huge reservoir of bioactive compounds in variety of plant species, however, only a fractional percentage of these have been examined and used as chemo-adjuvants or as anticancer agents. There is a global interest in identifying new anticancer compounds from plants and from traditional sources.

(Wang et al., 2012).

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recommended by local healers to relieve inflammation in edema, rheumatic swellings, whereas the paste of stem bark has shown to relieve common fever, scabies and inflammation of the lymph glands and eyes and bark paste is used externally to relieve rheumatic swelling, hemorrhoids, and dysmenorrhea. It is also employed in dyspepsia, piles, analgesic and diabetes mellitus whereas leaves, seeds, and stem bark are topically used against wide range of skin diseases (Rizwani et al., 2012; Sharma et al., 2009; Subash and Augustine, 2013; Sharma et al., 2005).

Presence of alkaloids, tannins, glycosides, flavonoids, phenols, saponins, and reducing sugars were found in the preliminary phytochemical investigations in ethanolic stem bark extract of *Holoptelea integrifolia*. In addition, the bark also contained the holoptelin-A (epifriedelinolpalmitate), holoptelin-B (epi-friedelinol stearate), tri-terpenoid fatty acid esters, friedelin and epifriedelinol which has shown beneficial effects in certain bladder cancer, rheumatic inflammation, fever, dysentery, convulsions, inflammation, and ulcers treatment (Kumar et al., 2012). The purpose of this study was to look at the cytotoxicity of the 4 different bark extracts of *Holoptelea integrifolia* (n-Hexane, EtOAc, n-ButOH and CHCl₃) on variety of different cancer cells i.e. BEL-7404, H460, KB-3-1, DU-145, MDA-MB-435 and HCT-116 cancer cells and primary HEK293 cells.

**MATERIAL AND METHODS**

**Plant material and chemicals**
The bark of the *Holoptelea integrifolia* plant was collected from the premises of the University of Karachi, Pakistan during January-April 2009 and identified by an expert of the University of Karachi. A herbarium voucher specimen No. 0045 was deposited in the museum of the Department of Pharmacognosy at University of Karachi, Pakistan. The purified chemicals used during the research work were commercially purchased from Oxoide (England) and Merck (Germany). All other reagents and solvents were purchased from VWR (West Chester, PA, USA).

**Fractionation and isolation**
*Holoptelea integrifolia* plant bark (10 kg) were cleaned and then chopped into small pieces. These pieces were percolated in 80% methanol at room temperature for 15 days. The percolate was filtered thrice separately by using Whatman filter paper No.1. Thereafter, under reduced pressure and controlled temperature 40°C, the filtrate was evaporated to dryness and the methanolic extract was lyophilized to a powdered form. Lyophilized powder (300g) was partitioned with an equal quantity of distilled water (450ml) and n-hexane (450ml) 1: 1, n-Hexane layer was evaporated under reduced pressure and temperature 40°C to obtain n-hexane extract (BMHx). The same aqueous layer along equal volume of ethyl acetate was added in the separating funnel and shaken well, layers were separated out and the Ethyl acetate layer was evaporated to get the ethylacetate extract (BMET). This extract was divided into two portions; one portion of the ethyl acetate extract was acidified with 0.5 N HCl and treated with chloroform to get a chloroform extract (BMCHF) while the other was kept for performing biopharmaceutical studies. Whereas, the partition procedure was then followed by the addition of pre-saturated n-butane (n-butanol) to the water portion and allowed to separate layers. Both the layers were evaporated to get n-butanol extract (BMBU) and Aqueous extract.

**Cell lines and cell culture**
The hepatocellular carcinoma cell line BEL-7404, lung cancer line H460, human epidermoid carcinoma cell line KB-3-1, prostatic cancer cell line DU-145, breast carcinoma cell line MDA-MB-435, colon cancer cell line HCT-116, human primary embryonic kidney cell line HEK293 (control, non-cancer cell line) were grown as adherent monolayers in flasks with Dulbecco's Modified Eagle Medium (DMEM) culture medium supplemented with 10% fetal bovine serum in a humidified incubator containing of 5% CO₂ at 37°C.

**Cell cytotoxicity using MTT assay**
The MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl
tetrazolium bromide) assay (Carmichael 1970) was used to determine cytotoxicity of all four extracts (n-Hexane, EtOAc, n-ButOH and CHCl₃) on BEL-7404, H460, KB-3-1, DU-145, MDA-MB-435 and HCT-116 cancer cells and primary HEK293 cells as control. Briefly, the cells were harvested with trypsin and resuspended in a final concentration of 5x10⁵ cells/well. Cells were seeded evenly into (180 µl/well) 96-well multiplates. Different concentrations of plant extract (n-Hexane, EtOAc, n-ButOH, CHCl₃) were added (10 µl/well) into designated wells. After 72 h of incubation, 20µl of MTT solution (4 mg/ml) was added to each well, and the plate was further incubated for 4 h, allowing viable cells to convert the yellow-colored MTT into dark-blue formazan crystals. Subsequently, the medium was discarded, and 100 µl of dimethylsulfoxide (DMSO) was added into each well to
dissolve the formazan crystals. The absorbance was determined at 570 nm by an OPSYS microplate Reader from DYNEX Technologies, Inc. (Chantilly, VA, USA). The mean±SD concentration was calculated from at least three experiments performed in triplicate each time. The IC50 (concentrations required to inhibit growth by 50%) were calculated from survival curves using the Bliss method.

**RESULTS**

Natural products have historically been used for combating human ailments. There is global interest in the biomedical community to look at natural products as chemoadjuvants for cancer treatment. The cytotoxic effect of *Holoptelea integrifolia* (Roxb) Planch extracts (n-Hexane (BMHx), EtOAC (BMET), nButOH (BMBU) and CHCl3 (BMCHF)) were determined by MTT assay in the hepatocellular carcinoma cell line BEL-7404, lung cancer cell line H460, human epidermoid carcinoma cell line KB-3-1, prostatic cancer cell line DU-145, colon cancer cell line HCT-116, breast carcinoma cell line MDA-MB-435 and human primary embryonic kidney cell line HEK293 and is shown in (Figs. 1A-G, Fig. 2 and Table 1).

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>Cell Survival (%)</th>
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<tbody>
<tr>
<td>0.00</td>
<td>150</td>
</tr>
<tr>
<td>0.30</td>
<td>100</td>
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<tr>
<td>1.00</td>
<td>50</td>
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<td>3.00</td>
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</tr>
<tr>
<td>30.00</td>
<td>0</td>
</tr>
<tr>
<td>100.00</td>
<td>0</td>
</tr>
<tr>
<td>300.00</td>
<td>0</td>
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</tbody>
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**Fig. 1:** The cytotoxic effects of various extracts BMHx, BMBU, BMET, and BMCHF from the bark of *Holoptelea integrifolia* in the (A) BEL-7404, (B) KB-3-1, (C) H460, (D) DU145, (E) HCT-116, (F) MDA-MB-435 and (G) HEK293 cell lines, as determined by MTT assay, are shown. The mean±SD were calculated from at least three experiments performed in triplicate at each time.
Antineoplastic activity of Holoptelea integrifolia (Roxb) Planch bark extracts (in vitro)

Table 1: The cytotoxic effects (IC$_{50}$) of various plant extracts (BMBU, BMHx, BMET, BMCHF) in the BEL-7404, H460, KB-3-1, DU145, MDA-MB-435, HCT-116 and HEK293 cell lines

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>IC$_{50}$ ± SEM (µg/mL)</th>
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<tbody>
<tr>
<td>BEL-7404</td>
<td>217.64 ± 5.85</td>
</tr>
<tr>
<td>H460</td>
<td>295.03 ± 11.18</td>
</tr>
<tr>
<td>KB-3-1</td>
<td>205.40 ± 2.87</td>
</tr>
<tr>
<td>DU145</td>
<td>91.31 ± 1.35*</td>
</tr>
<tr>
<td>MDA-MB-435</td>
<td>141.82 ± 3.09</td>
</tr>
<tr>
<td>HCT-116</td>
<td>292.06 ± 1.85</td>
</tr>
<tr>
<td>HEK293</td>
<td>165.38 ± 9.80</td>
</tr>
</tbody>
</table>

IC$_{50}$: concentration that inhibited cell survival by 50%. Data are means ± SEM of at least three independent experiments performed in triplicate. ** P < 0.01; Statistical difference (lower) were calculated by comparing the IC$_{50}$ of the compound with that of HEK293.

Fig. 2: The IC$_{50}$ values of various extracts BMHx, BMBU, BMET and BMCHF from the bark of Holoptelea integrifolia on the HEK293 as compared with their IC$_{50}$ values on (A) BEL-7404, (B) KB-3-1, (C) DU145, (D) HCT-116 (E) MDA-MB-435, and (F) H460 cell lines are shown. The IC$_{50}$ (concentrations required to inhibit growth by 50%) were calculated from survival curves using the Bliss method as shown in materials and methods.
DISCUSSION

Most of the herbal drugs act on cell cycle and they control the initiation, progression and development of cancer cells as herbs (Nigella sativa and Cuminum cyminum) has ability to suppress the cancerous process. Ideally, any drug to be considered as an anticancer agent should be selectively toxic to the specific cancer cells and non-toxic to the normal non-cancerous cells. Preferably, primary cells of a specific cancer should be considered as a control to compare these effects of potential anticancer candidates. However, due to unavailability of such controls, HEK293 was considered to be a control in this study. The IC₅₀ values BMBU, BMCHF, BMHx, and BMET extracts from the bark of Holoptelea integrifolia (Roxb) Planch are shown in table 1 on various cancer cells and non-cancerous HEK293 cells. The IC₅₀ values of BMBU extracts were either equal or more in cancer cells than that of control HEK293 cells, with an exception on prostate cancer cells DU-145 with almost 1.8 folds difference in IC₅₀ values (table 1 and fig. 2). Also, the BMCHF extracts were toxic to HEK293 cells and the IC₅₀ values were not different in cancer or non-cancer cell lines (table 1 and fig. 2). However, interestingly, BMHx extracts were selectively over approx. 2-fold more toxic in prostate cancer cells DU-145, over 2.5-fold more toxic in colon cancer cells (fig. 2D) and over 5-fold more toxic in breast cancer cells MDA-MB-435 (fig. 2E) compared to HEK293. Similarly, BMET extracts were selectively approx. 2- to 3-fold more toxic in all the cancer cells compared to non-cancer HEK293 cells (fig. 2A-F).

Specifically, the IC₅₀ values of only 29.77±1.17 mg/ml of only BMET in prostate cancer cells DU-145 was over 3-fold highly toxic than HEK293 (fig. 2C). These results suggest that differential and selective cytotoxicity were observed from different extracts of the bark of Holoptelea integrifolia (Roxb) Planch which are required for pharmacological effect.

Owing to such medicinal importance in variety of disease condition, several groups are focused to isolate the active phytochemicals from the bark of Holoptelea integrifolia plant. Some phytochemicals previously isolated such as 2,3-dihydroxy-olean-12-en-28-acid, hederagenin, friedelan-3β-ol, friedelin (Misra et al., 1975, 1977), Epifriedelinol, 2 aminonaphthaquinon, β-sitosterol, β-D-glucose and triterpenoids fatty acid esters as Holoptelin A and B (Saraswathy et al., 2008) has been studied for their role in inflammatory and other diseases. While betulin and betulinic acid, isolated from the methanolic bark extracts of Holoptelea integrifolia, have shown antioxidant properties (Maryam et al., 2013; Saraswathy et al., 2008). Only one recent report (Lakshmi et al., 2010) evaluated the antitumor activity of EtOH extracts of leaves of Holoptelea integrifolia against Dalton's ascitic lymphoma (DAL) in mice. They found that Holoptelea integrifolia leaf extracts showed significant antitumour activity, increased restored hematological parameters and significantly increased the survival of DAL bearing mouse in a dose-dependent manner (Lakshmi et al., 2010). For anti-cancer activity numbers of bioactive constituents have been reported, including polysaccharides, alkaloids, saponins and organic acids. An increasing number of triterpenoids have been reported to exhibit cytotoxicity against a variety of cancer cells without manifesting any toxicity in normal cells (Setzer et al., 2003).

CONCLUSION

In human beings rich health had been able due to the contribution of medicinal plants (Food and medicines). The bioactive constituents present in plant extracts are responsible for anticancer activity and has led to more screening in the recent era for their valuable beneficial information. To the best of our knowledge, this is the first study to report the anticancer activity of BMBU, BMCHF, BMHx, and BMET extracts from the bark of Holoptelea integrifolia on six different cancer cells, compared to one non-cancer cell line HEK 293. This should be only considered a preliminary study as this gives a new direction to isolate the active phytochemicals present in the BMHx and BMET that were seen to be selectively toxic in specific cancer cells. Although, BMCHF possesses cytotoxic potential, it is not selective and has similar toxicity in non-cancer cells. However, we cannot rule out the possibility of a mixture of phyto-constituents that may be antagonising or masking the effect on other beneficial components in BMCHF. Identifying these behaviors may be expensive and laborious and so BMCHF extracts will not be pursued in our immediate goals. More importantly, the BMHx possess over 5-fold more selective toxicity in breast cancer cells while BMET extracts are over 3-fold more toxic in a prostate cancer cell line compared to HEK293 cells. This study will be further expanded to a panel of breast cancer cells and prostate cancer cells with different phytoconstituents of BMHx and BMET, respectively. The antineoplastic mechanisms of the isolated phytoconstituents will be further explored using a more pharmacokinetic and pharmacodynamic driven approach to develop a safe and site specific anticancer agent which would hopefully have higher therapeutic potential to eradicate various types of cancer.

ABBREVIATIONS

BMBU as bark of Mughsi (plant name in Urdu) Butanol extract, BMHx as bark of Mughsi (plant name in Urdu) Hexan extract, BMET as bark of Mughsi (plant name in Urdu) Ethyl acetate extract, BMCHF as bark of Mughsi (plant name in Urdu) chloroform extract.

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REFERENCES


