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A Study on Anti-Cancer Properties of *Saussurea lappa* (*Asteraceae*) Against Breast and Colonic Cancer Cell Lines

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Abstract

The cytotoxicity of *Saussurea lappa* (*Asteraceae*) aqueous extract was examined on five different cell lines; Human Lung Fibroblast Cells (MRC5), Human Dermal Fibroblast adult (HDFa), Breast Cancer Cells (MCF7), Human Colonic Cancer (Caco2) and Canine Kidney cells (MDCK). *Saussurea lappa* extract showed cytotoxic activities with IC50 values ranging from 2.5 mg/ml to 0.85 mg/ml and it was observed that the Human Lung Fibroblast cells were the most sensitive cells (IC50 values; 0.8). The genotoxic potentialities of extract were observed as regulatory mechanism of gene expression with the finding that *Saussurea lappa* extract could regulate cell apoptosis. In MCF7 treated cells, treatments had the ability to down regulate the expression of both P53 and Bcl2 genes, whereas TGF, BAX, IKaB were up regulated. On the other hand, the treated Caco2 cells showed down regulation for P53, Bcl2 and IKaB genes.

Aim: The aim of the present study were primarily to check the possible use of *Saussurea lappa* species as a natural anticancer remedy, secondly; the study investigated the action of extract on some cancer marker gene expression in the treated cells compared with the untreated ones.

Keywords: *Saussurea lappa*; Colon cancer; Breast cancer; Dermal fibroblasts; Genotoxic potentialities

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Introduction

Cancer is the second leading cause of death worldwide and is considered to calculate for 9.6 million deaths in 2018 (second to cardiovascular diseases). Although times of moving ahead in the management of cancer, significant lack and need for refinement remains. Oncogenes is described by changes at the cellular, genetic, and epigenetic levels and abnormal cell cycles involves increased cell proliferation, disorder cell cycle regulation, decreased apoptosis, arrest of cell differentiation, angiogenesis, cell invasion, and metastasis that are induced by altered expression of oncogenes, transcriptional factors included in these processes and decreasing of tumor suppressor genes [1].

The use of plant-derived products in cancer treatment may decrease the complications of chemotherapeutic drugs. For thousands of years, medicinal plants have been used as a treatment in cancer indifferent parts of the world especially in ancient Egypt, India, China, and the Arab world. It has been reported that more than 3,000 natural derived species were used as anticancer therapies globally [2]. Some of these natural products have recently been tested and may have potency in anticancer therapies but the possible mechanism of action of such plan-derived products is also discussed to be potential nominee as anti-cancer agents [3].

The genus *Saussurea* DC of the family *Asteraceae* comprises about 300 species in the world of which about 61 species present in India. *Saussurea costus*, commonly known as *costus* or *kuth*, is a species of thistle in the genus *Saussurea* native to India. Essential oils extracted from the root of this plant have been used in traditional medicine and in perfumes since ancient times. This plant derived product may act as a potential anticancer properties against several types of malignancies such as; leukemia's, liver, breast, ovarian, prostatic, colonic and bladder cancers [4-7]. *Saussurea lappa* dried roots (known as *costus* root) have been used in traditional medicine in India, China, Japan, and Pakistan [8].

The active compounds present in *Saussurea lappa*; among the hydrocarbon, β-elemene, which belongs to the sesquiterpenes group, is announced to inhibit mouse pancreatic cancer and neo plastic metastasis, and have antitumor effect, (costunolide and dehydrocostus lactone),both showed high anticancer activity, through inhibition of cancer cell proliferation [6], inducement of cancer cell apoptosis and differentiation [9].

Choi and Ahn [10] reported that the *Saussurea lappa* extract contains compounds which induce G2/M phase arrest in the ovarian cancer (SK-OV-3) cells through up-regulation of p21, down-regulation of Cdk1 [10]. However, Kuo et al. [11] and Kretschmer et al. [13] showed that these compounds are eligible of blocking the S-phase progression through Cdk inhibitor up-regulation and cyclin inhibition pathways [11-13].

The activation or suppression of p53/p21/p27 is a very concerted mechanism of anti-cancer activity for secondary metabolites in *Saussurea lappa* plants. Choi and Ahn [10], Lee et al. [14] reported that *Saussurea lappa* extract can induce DNA damage and cancer cell apoptosis and can suppress the expression of p53 and the p53 targets, the p21 and p27 Cdk inhibitors as well [10,14]. Studies by Degterev et al. [15] and Bocca et al. [16] had explained a dose-dependent anti proliferative activity in human breast cancer MCF-7 cells as microtubule-interacting agents [15,16].

The BCL-2 family of proteins, including the anti-apoptotic proteins, BCL-2, BCL-X, BCL-XL, and the pro-apoptotic proteins, BAX, BAK, BID, BAD, monitor apoptotic mitochondrial pathways by regulating mitochondrial membrane permeability [17,18]. Resistance to apoptosis may be the pathogenesis of cancer [19]. Choi and Ahn [10] reported that there was a marked increase in the expression of the apoptotic protein BAX that down streamed target p53, causing the release of cytochrome C from the mitochondria, and in turn, eliciting the intrinsic signaling pathways of apoptosis in DE-treated SK-OV-3 ovarian cancer cells [10]. Oh et al. [20] demonstrated that DE inhibited nuclear transcription factor-κB (NF-κB) activation and enhanced caspase-8 and caspase-3 activities to deliver HL-60 cells susceptible to Tumor Necrosis Factor-α (TNF-α) -induced apoptosis [20]. It was also reported that DE induced apoptosis in human leukemia HL-60 cells by activating caspase-3 after a reduction in mitochondrial membrane potential [21]. In addition, DE inhibited survival signaling through the Janus Tyrosine Kinase (JAK) -Signal Transducer and Activator of Transcription-3 (STAT3) signaling and induced apoptosis in breast cancer MDA-MB-231 cells by up-regulation of BAX and BAD, down-regulation of BCL-2 and BCL-XL, and nuclear relocation of the mitochondrial factors apoptosis-inducing factor and Endo [11].

Materials and Methods

Plants

Saussurea lappa were collected from a local herbal store and the plant was identified and authenticated by the taxonomists of Biology Department, Faculty of Science, King Khalid University, Abha, Kingdom of Saudi Arabia.

Preparation of extracts

The fruits of *Saussurea lappa* were extracted by maceration technique as has been described by Harbone protocols [22]. About 150 g were macerated at room temperature with continuous shaking in 1 L distilled H_2O. The supernatant was filtered and subjected to evaporation at 60°C for 16 h and residue was weighed, dried in oven and stored at 4°C until used.

Table 1: The list of primers used in the gene expression analysis.

Primers	The nucleotide sequence 5' to 3'	Annealing Temp
GPDHF	ATTGACCACTACCTGGGCAA	60°C
GPDHR	GAGATACACTTCAACACTTTGACCT	
Bcl2-forward	TATAAGCTGTCGCAGAGGGGCTA	60°C
Bcl2-reverse	GTACTCAGTCATCCACAGGGCGAT	
P53-forward	AACGGTACTCCGCCACC	60°C
P53-reverse	CGTGTCACCGTCGTGGA	
Survivin-forward	TGCCCCGACGTTGCC	56°C
Survivin-reverse	CAGTTCTTGAATGTAGAGATGCGGT	
IkappaB-α F	CATGAAGAGAAGACACTGACCATGGAA	56°C
IkappaB-α R	TGGATAGAGGCTAAGTGTAGACACG	
BAX Forward	CCTGTGCACCAAGGTGCCGGAACT	55°C
BAX Reverse	CCACCCTGGTCTTGGATCCAGCCC	
MMP-7 F	GATGGTAGCAGTCTAGGGATTAACTTC	53°C
MMP-7 R	GGAATGTCCCATACCCAAAGAA	
TNFF	TCTCTAATCAGCCCTCTGGCC	53°C
TNFR	TGGGCTACAGGCTTGTCACTC	

Determination of phytochemical compounds in *Saussurea lappa* extract

The phytochemical compounds such as tannins, phenols, flavonoids, alkaloids, reducing sugars, volatile oils, glycosides, amino acids, proteins, saponins and terpenoids were determined in the licorice extract according to procedure as described by Harbone and Baxter [22].

Determination of total phenolic compounds and antioxidant activity in *Saussurea lappa* extract

The concentration of total soluble phenolics in *Saussurea lappa* extract was estimated according to the method described by Malick and Singh [23] using Gallic acid as standard. The ability of licorice extract to scavenge DPPH free radicals was evaluated according to the method described by Braca et al. [24] and Kumarasamy et al. [25].

Preparation of mammalian cell lines

Human Primary Dermal Fibroblasts adult (HDFa), Canine Kidney Cells (MDCK), Breast Cancer Cells (MCF7) and Human Colonic Cancer (Caco2) cells were cultured in DMEM media. Media were supplemented with 200MM l-glutamine and 10% fetal bovine serum (Gibco-BRL, Germany).

Assay of cytotoxicity in *Saussurea lappa* extract

The non-toxic doses of *Saussurea lappa* extract were tested on three different normal cell lines as human dermal fibroblast, human kidney cells (MRC5) and Madin-Darby canine kidney cells MDCK according to the method as described by Borenfreund and Puerner [26].

Mode of action of *Saussurea lappa* as anticancer

The cell proliferation assay in *Saussurea lappa* extract was performed according to the manufacturer's protocol of cell proliferation ELISA BrdU Kit (Roche Applied Science), cell proliferation in response to treatments was assayed using the

Table 2: Chemical analysis of *Saussurea lappa* extract.

Tests	*Saussurea lappa* aqueous extract
Tannins	-
Reducing sugars	+
Glycosides	+
Alkaloids	++
Flavonoids	+++
Volatile oils	+
Terpenoids	+++
Protein + amino acids	+
Saponins	+

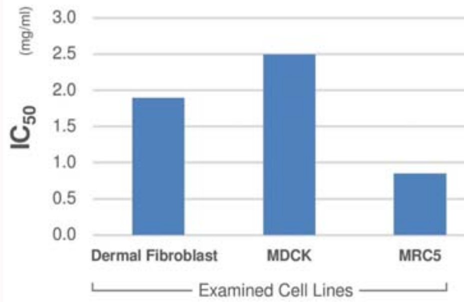

Figure 1: Cytotoxicity of *Saussurea lappa* aqueous extract on normal cells.

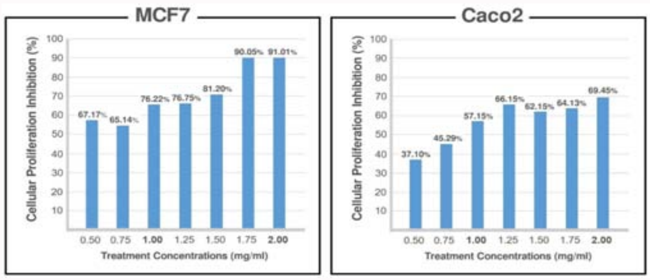

Figure 2: Anti-proliferation activities of *Saussurea lappa* aqueous extract on MCF7cells (on the left), and Caco2 cells (on the right).

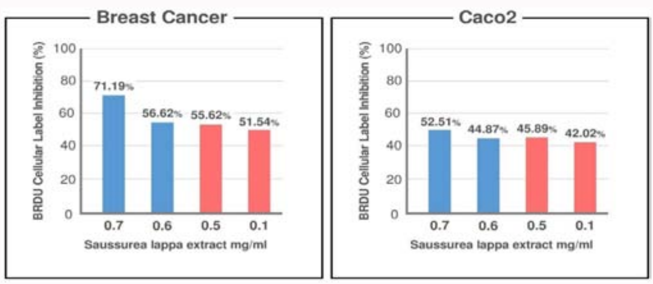

Figure 3: Inhibition of cellular BRDU label during treatment of MCF7 and Caco2 cells.

measurements of 5-bromo-2-deoxyuridine (BrdU) incorporated into cellular DNA.

Determination of gene expression by using RT-qPCR

The anticancer activity of *Saussurea lappa* extract was examined using real time PCR for several genes in the treated breast cancer and human colonic cancer cells compared with the non-treated cells. The cells were treated with the resultant nontoxic concentration of *Saussurea lappa* extract for 48 h as previously described by Yang et al. [27]. The RNA extraction from the treated and non-treated cells using RNA extraction kit (QiaGene, Germany) and RT-qPCR (for quantification of gene expression) was carried out. The first cDNA strand was synthesized using oligo-dT primer (Thermo scientific) and Master Mix (Qiagen, Germany). GAPDH gene was used as an internal control reference and the RT-PCR was performed using Syber Green master mix (Qiagen, Germany). The primers used in this study are listed in Table 1.

Results

Phytochemical analysis *Saussurea lappa*

The analysis indicated that *Saussurea lappa* aqueous extract contained flavonoids, terpenoids, alkaloids, glycosides and saponins as important compounds (Table 2). Phenolic and flavonoid contents were found in concentrations of 6.5 mg/g of catechol-equivalent phenolics and 13.0 mg/g of gallic acid-equivalent flavonoid.

Cytotoxicity effect of *Saussurea lappa* aqueous extract on normal cells

The safety levels of *Saussurea lappa* extract were tested on three different normal cells. The selected cells were fibroblast cells, MRC5 and MDCK. The data presented in Figure 1 revealed that the effect of treatment half maximal Inhibitory Concentration (IC50) on the three examined cells ranged from 2.5 mg/ml to 0.85 mg/ml. It was observed that both the human cells were sensitive to the extract but the MDCK was the tolerant one. The highest IC50 values of 2.5 mg/ml and 1.9 mg/ml were for MDCK and in the human cell the extract treatment with IC50 exhibited a very low value of 0.85 mg/ml.

Anti-proliferation activities of *Saussurea lappa* aqueous extract on breast cancer and Caco2 cells

The anti-proliferative activities of *Saussurea lappa* extract against cancer cells (breast cancer and colon cancer) were quantitatively estimated and the represented in Figure 2. Different plant extract concentrations were used on the breast cancer and colon cancer cells to examine the anti-proliferation activities. The results detected that the highest anti-proliferation activity was 91.2% and 63.4% respectively. On the other hand, MCF7 cells showed inhibition in proliferation with activity of 91.2% to 57.1%. However, Caco2 cells exhibited inhibition in proliferation ranging from 69% to 37%. This latter finding specified that breast cancer cells were the more sensitive to this extract treatment.

BrdU proliferation assay using *Saussurea lappa* extract

In order to explain the mode of action of *Saussurea lappa* extract on cell proliferation, a colorimetric cell proliferation assay by using 5-bromo-2’-deoxyuridine (BrdU) was utilized. As detected by the BrdU assay, the anti proliferative activities of extract on both MCF7 and Caco2 cells were more potent. In addition, BrdU cellular label inhibition in MCF7 treatment was greater (71.9%) but inhibition was 52% only in case of Caco2 cells (Figure 3).

The molecular activities of *Saussurea lappa* extract and on MCF7and Caco2

In order to c the possible mode of action of the treatments at the molecular level, the expression levels of the genes; survivin, TFG, BAX, BCL2, B21, IKaB and P53 in treated cancer cells were measured using RT-qPCR. Data presented in Figure 4 revealed that *Saussurea lappa* extract could regulate cell apoptosis. In MCF7 treated cells, treatments had the ability to down regulate the expression both of

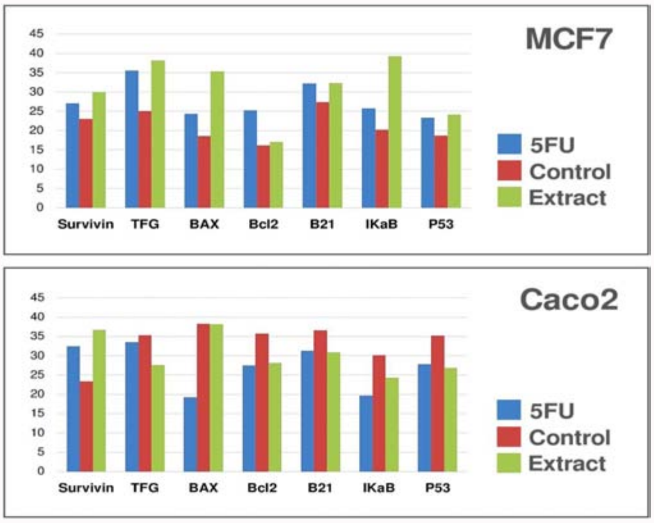

Figure 4: Gene expression pattern of *Saussurea lappa* extract on breast cancer and caco2 cell lines.

P53 and Bcl2 genes, whereas surviving TFG, BAX, IKaB were up regulated. On the other hand, the treated Caco2 cells showed down regulation for P53, Bcl2 and IKaB (Figure 4).

Discussion

Saussurea lappa is mainly used clinically to treat cancer and inflammatory and digestive tract diseases. To date, many active ingredients have been extracted from *Saussurea lappa*. Our analysis indicated that *Saussurea lappa* aqueous extract contained flavonoids, terpenoids, alkaloids, glycosides and saponins as important compounds. Both phenolic and flavonoid contents were determined in the extract, concentration of 6.5 mg/g of catechol-equivalent phenolics and 13.0 mg/g of gallic acid-equivalent flavonoid respectively. Previous studies reported that ingredients of *Saussurea lappa* like costunolide, dehydrocostus lactone, and cynaropicrin have explained their exceptional pharmacologic properties. Some active ingredients had potency to be developed into new drugs to treat diseases. Cynaropicrin, lappadilactone, iso-dihydrocostunolide, costunolide, and dehydrocostus lactone could be used to inhibit angiogenesis and treat cancers [28,29].

In evaluation of the anticancer activity of *Saussurea lappa* extract, we had first observed cell proliferation in three different normal cell lines; Human Dermal Fibroblast adult (HDFa), human kidney cells (MRC5) and Madin-Darby Canine Kidney cells (MDCK) exposed to non-toxic doses (2.5 mg/ml to 0.85 mg/ml) of *Saussurea lappa* extract for 48 hours. The extract exhibited significant dose-dependent antiproliferative activity. The antiproliferative effect of the extract peaked at 1.9 mg/ml for human dermal fibroblast adult, whereas for MRC5 cells it was 0.85 mg/ml and for MDCK it was 2.5 mg/ml. The breast cancer cells were more sensitive to the extract than the colon cancer cells. This was a hopeful result, since breast cancer is particularly chemo sensitive [30,31]. Although surgery was important in the initial therapy of patients with breast or colon cancer, most patients required chemotherapy to clear any residual microscopic or macroscopic peritoneal implants. Human breast cancer cell lines appeared to be sensitive to the extract and suggested its anticancer activity [32-34]. We also found that dehydrocostus lactone exerted antiproliferative effects in several breast cancer cell lines by altering the cell cycle. In cells exposed to dehydrocostus lactone, G2/M phase arrest was induced. Dehydrocostus lactone had been shown to induce cell cycle arrest at the G2/M phase in several cancer cell lines *in vitro* [35,36]. This was consistent with our results, which indicated that exposure to dehydrocostus lactone induced significant cell cycle arrest. In this study, the inhibitory effect of dehydrocostus lactone on cancer cell proliferation resulted in cell cycle arrest and apoptosis. Under the same conditions, exposure to dehydrocostus lactone resulted in significant apoptosis in all the cell lines examined. This was consistent with reports that dehydrocostus lactone is an effective anticancer agent capable of inducing apoptosis [35-38]. Apoptosis was an important series of events that led to programmed cell death, and was essential for development and tissue homeostasis. The potential mechanisms underlying the apoptotic process include factors regulating the balance between the induction and inhibition of apoptosis. Lately, the regulation of apoptosis had been proposed as a promising target for cancer chemotherapy [39-42]. Thus, apoptosis induced by dehydrocostus lactone may have a significant potential anticancer effect.

In our study, we have found that *Saussurea lappa* extracts *viz.* Dehydrocostus Lactone (DHE) and costunolide effectively inhibited breast and colon cancer growth concomitant with induction of apoptosis *in vitro* and *in vivo*. Extract treatment resulted in a significant decrease of Bcl-2 and P53 genes, suggesting that changes in the ratio of proapoptotic and antiapoptotic Bcl-2 family proteins might have contributed to the apoptosis-promotion activity of DHE. However TGF, BAX, IKaB were up-regulated genes.

An increasing body of literature evidences underlined the critical involvement of intracellular redox state in cancer and in inflammation-associated diseases. Under normal conditions, mammalian cells contained 1 mM to 10 mM cytosolic GSH, depending on cell type and metabolic factors. GSH represented approximately 95% of total non-proteinthiols and was the main modulator of the cellular redox environment. The cytoplasmic high ratio between the reduced and oxidized glutathione (GSH/GSSG) was a main factor in keeping the cysteine residues of intracellular proteins in the reduced form. The decrease in GSH content, leading to the drop in the cellular redox potential, was often induced by oxidative stress. In the present study we supposed that, in line with above-described notion, in MCF7 and Caco2 cells, GSH content must be far higher than GSSG and that the active constituents in the *Saussurea lappa* extract *viz.* DCE and CS dose-dependent induced the consistent drop in intracellular GSH level without significantly affecting GSSG content. The decrease in GSH concentration may be due to their ability both to generate oxygen species (ROS) [43,44] and to interact with GSH. The drop in GSH content induced by these two compounds may be due, at least in part, to their capacity to increase ROS production. This was in line with literature evidences. Since this interaction was shown to be highly efficient, it was assumed that two lactones elicit the rapid drop in the intracellular GSH content mainly through their capacity to interact with it.

The disturbance in the GSH/GSSG homeostasis was implicated in the induction of reversible S-glutathionylation of cysteine residues of sensitive proteins [45,46]. Recently, STAT3 has been shown to be S-glutathionylated with concomitant loss of its phosphorylation in HepG2 cells treated with diamide, a strong oxidant compound, indicating that this signal transcription factor is susceptible to redox regulation [47].

Taken together, the results of this study suggest that dehydrocostus lactone in the extract possessed significant antiproliferative activity via cell cycle arrest and apoptosis, particularly in breast cancer. Due to these features, dehydrocostus lactone may serve as a beneficial anticancer drug. However, further studies are needed to clarify its exact mechanisms of action.

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