Plant Archives Vol. 19 No. 2, 2019 pp. 2693-2700 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

C/EBPA AS A BIOMARKER GENE IN CANCER CELL AND ITS RELATION TO NATURAL ANTICANCER COMPOUNDS

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Abstract

Leukemia is a rapidly progressive disease which is one of the most common causes of death worldwide. Several therapy modalities have been employed in the treatment of leukemia such as natural anticancer compounds which found in plants e.g. *Saussurea Lappa* (*S. lappa*). To determine cytotoxic activity of *S. lappa* extracts in relation to CCAAT/Enhancer Binding Protein Alpha (C/EBPA) gene expression, in addition to phytochemical analysis and antioxidant activity. Successive extraction of *S. lappa* root powder using methanol, ethanol, acetone, ethyl acetate and chloroform solvents with separation of twelve fractions from chloroform extract by thin layer chromatography (TLC). Estimation of total phenolic and total flavonoid were done together with antioxidant, cytotoxic activity of *S. lappa* extracts which has been tested on three leukemia cell cultures by MTT assay. C/EBPA gene expression was detected by quantitative real time PCR (qRT-PCR). The chloroform extract of *S. lappa* showed the highest cytotoxic activity against three cancer cell cultures. Regarding C/EBPA gene expression in leukemic cells after addition of chloroform fractions (C2+C9) from the *S. lappa* plant, we found a statistically significant difference (P value 0.008) with (C9) compound; this denotes the higher cytotoxic activity of (C9) on the leukemia cells. The *S. lappa* roots contain phenol and flavonoid compounds, which have a high cytotoxic effect on leukemic cells and improve CEBPA gene expression which is a known good prognostic marker. Further studies of *S. lappa* for the elucidation of the cytotoxic mechanisms considered as immense importance to deal with leukemia.

Key words : *Saussurea lappa*, phytochemical analysis, antioxidant, cytotoxic activity, CEBPA gene.

Introduction

Cancer is considered the major cause of death worldwide. The prevalence of cancer has actually increased where cancer cells are basically transformed from normal cells which require various genetic mutations (Siegel *et al.,* 2013). One type of cancer is leukemia which is hematological malignancy and has four subtypes: acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL) and chronic myeloid leukemia (CML) (Tubergen *et al.,* 1993). Leukemia is more common among developed countries and higher socioeconomic groups (Gholami *et al.,* 2011). The etiology of leukemia is obscure. Previous studies suggested that genetic, environmental, and occupational factors may contribute to the etiology in some patients and certain populations (Jabbour *et al.,* 2006).

Medicinal plants are used for healing purposes throughout the human history; even in the current era, there are up to 80% of the world population especially the developing countries rely on traditional herbal medicine as a system of health care. Many herbal medicines which prescribed in traditional medicine lacked knowledge and accurate scientific methods (Ekor 2013). *Saussurea lappa* one of those plants it also known as *Saussurea costus*, kuth root or costus. It belongs to Asteraceae family; which includes about 1000 genera and 30,000 species widely distributed in different regions in the world. However, numerous species are found in India and Pakistan and some parts of Himalayas (Pandey *et al.,* 2007 & Shah *et al.,* 2006). *S. lappa* has been used in traditional medicine for the treatment of several ailments and diseases such as asthma, certain bronchitis, ulcer,

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stimulant, antiseptic, carminative, sedative, bronchodilator, astringent agent and stomach problems since ancient times (Chhabra *et al.,* 1998, Lee *et al.,* 1986 and Kim *et al.,* 2001). *S. lappa* has a good cytotoxic activity against cancer (Robinson *et al.,* 2008); In addition, several investigations showed other bioactive properties of *S. lappa* roots such as anti-inflammatory, hepatoprotective, immunomodulator, hypoglycemic, spasmolytic, anticonvulsant, antidiarrheal and antiviral activity (Zahra *et al.,* 2014 & Ghasham *et al.,* 2017).

The main active compounds of *S. lappa* reported as antioxidant compounds are costunolide, dehydrocostus lactone, in addition to some fatty acid such as palmatic, linoleic acids. Also, β-sitosterol, alantolactone and isoalantolactone isolated from *S. lappa* are reported to have medicinal properties (Nandagoapalan *et al.,* 2016).

CCAAT/Enhancer Binding Protein Alpha (C/EBPA) is the myeloid specific transcription factor that can regulate the balance between cell proliferation and differentiation in hematopoietic and non-hematopoietic tissues (McKnight *et al.,* 2001). C/EBPA is mapped to intronless gene on human chromosome 19q13.1. It is belongs to the basic region of leucine zipper (bZIP) class of DNA-binding proteins (Fuchs *et al.,* 2007). C/EBPA plays a major role during the commitment of hematopoietic stem cells towards granulocytic and monocytic differentiation (Dahl *et al.,* 2003). C/EBPA mutations have been observed in AML patients with the approximate frequency of 5-14% (Shih *et al.,* 2005; Sollip Kim *et al.,* 2012).

The purpose of this work to determine the total phenols, total flavonoids of different *S. lappa* root extracts jointly with antioxidant and cytotoxic activities on leukemic cell culture and their relation to C/EBPA gene expression on acute myeloid leukemia cells.

Clinical significance

Phenolic and flavonoid compounds are widely distributed in plants, which have received considerable attention because of its potential antioxidant and cytotoxic activities for human health care. Estimation of total phenolic and total flavonoid were done together with antioxidant, cytotoxic activity of *S. lappa* extracts which has been tested on three leukemia cell cultures by MTT assay. C/EBPA gene expression was detected by quantitative real time PCR (qRT-PCR).

Material and Methods

Plant extracts preparation

The plant materials were collected from local market in Cairo, Egypt and authenticated by El-Orman garden at the taxonomy and flora section. Powdered black root of *S. lappa* (40 g) was extracted successively with 70 ml of chloroform, ethyl acetate, acetone, ethanol and methanol. The extract filtrated by filter papers then evaporated to dryness under reduced pressure by rotary evaporator. The obtained extracts were stored in light-protected brown bottle at -20°C until further use.

Phytochemical screening

Determination of total phenolic content

The amount of total phenolic of the different tested *S. lappa* extracts was determined with the Folin-Ciocalteu reagent (Singleton, 1999). Each sample (50 µl) was added to 2.5 ml diluted Folin-Ciocalteau's reagent (1/100) and 2 ml of Na_2CO_3 (7.5%, w/v) then incubated at 45°C for 15 min. The absorbance of all samples was measured at 765 nm by UV-Vis Spectrophotometry. Gallic acid standards were prepared from 0 to 100 µg, the plot of standard curve was done by using various concentrations of Gallic acid then the results were expressed as Gallic acid equivalent (GAE).

Determination of total flavonoid content

The total flavonoid content was estimated using the Dowd method (Meda, 2005). Briefly, Aluminum trichloride (1 ml 2 %) in methanol was mixed with the same volume of the extract. Mixture was left for 10 min. at room temperature then absorbance was measured at 415 nm. The total flavonoid content was determined using a standard curve with quercetin with concentrations from 0 to 100 µg/l. The total flavonoid was determined as µg of quercetin equivalents (QE)/100 g of *S. lappa* extracts.

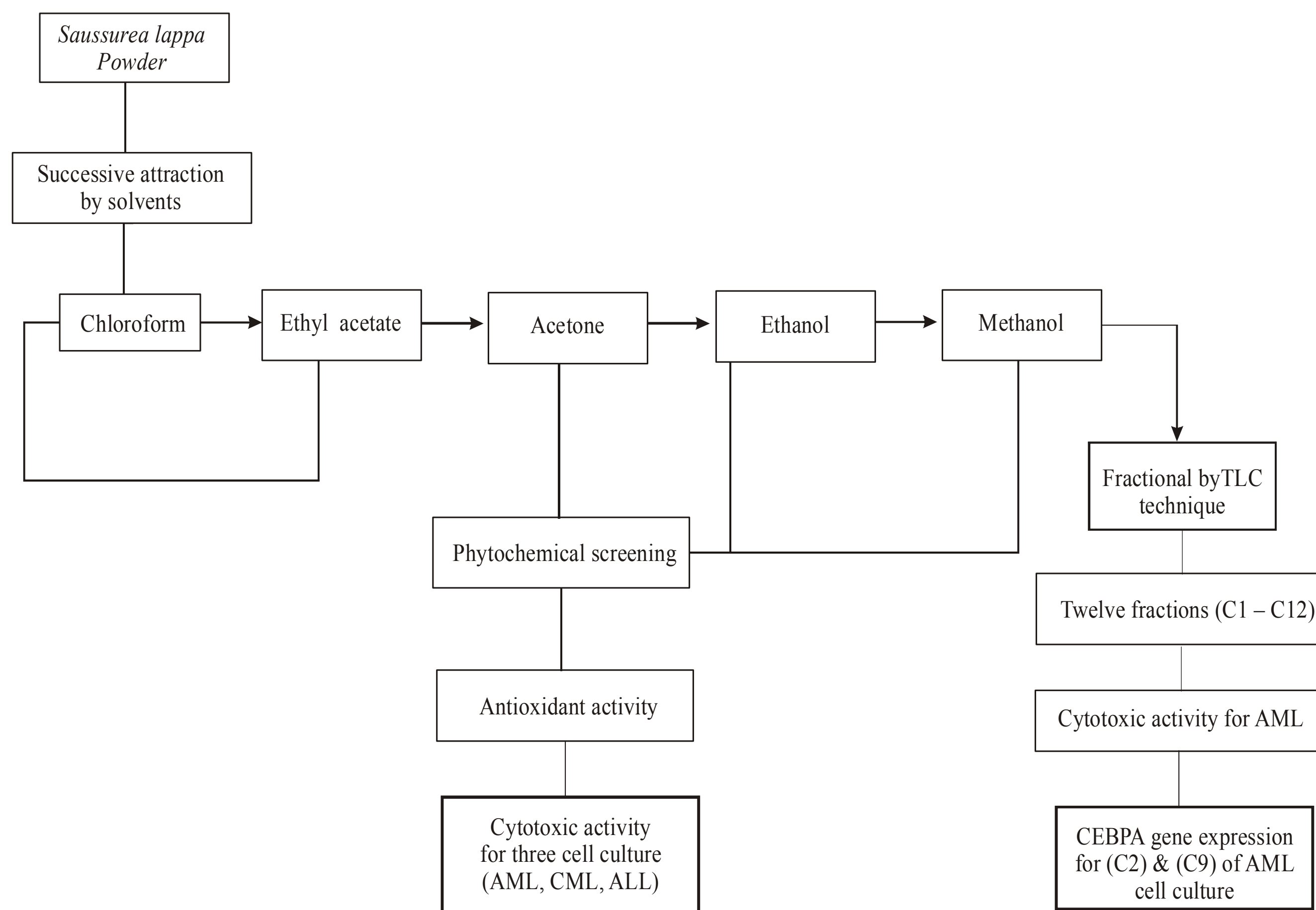
Antioxidant activity

Determination of scavengnig activity

Different consentrations (200, 400, 600, 800 and 1000 ppm) of different *S.lappa* extracts were used. Synthetic antioxidant (BHT, butylated hydroxytoluene) was used as a positive control. Each plant extract (1ml) and positive control was mixed with 1ml DPPH (2,2-diphenyl-1-picrylhydrazyl) reagent [0.002% (w/v)/ methanol solution]. After incubation for 30 min in the dark at room temperature, the absorbance was measured at 517 nm. The radical-scavenging activity was expressed as percentage of inhibition and calculated using the following formula: % inhibition = [(A control – A sample) / (A control)] × 100 (Blois, 1958).

Determination of reducing power

The reducing power was determined using different consentrations (200, 400, 600, 800 and 1000 ppm) of *S.lappa* extracts (Oyaizu, 1986). Gallic acid was used as standard. A volume of 200 µL of each extract as well as the standard at different concentrations were taken separately and mixed with 500 µL of 0.2 mol/L phosphate buffer (pH 6.6) and 500 µL of potassium ferricyanide. The samples were incubated at 50°C for 20 min. Then 500 µL of 10% trichloroacetic acid were added and



centrifuged at 6500 r/min for 16 min. About 700 µL of supernatant was taken and added to 700 µL distilled water and 140 µL of freshly prepared ferric chloride, then left to stand for 10 min at room temperature. Finally, the absorbance was measured at 700 nm. A standard curve for gallic acid was generated and the linear equation was used to calculate the reducing power of each extract as a gallic acid equivalent.

Leukemic cells separation and Trypan blue assay

EDTA peripheral blood samples from AML,ALL and CML patients were obtained from Kasr Al-Ainy Center of Radiation Oncology and Nuclear Medicine, Cairo University hospitals, Egypt. Breifly, one volume of peripheral blood was added to one volume of phosphate buffer saline (pH 7 PBS). then, two volumes of ficoll reagent was added to the previous mixture. Sepration of buffy coat was done by centrifugation for 30 min at 1600 rpm and final cell suspension was made by adding 1 ml PBS. For each examined sample, a new clean, dry test tube was used and 10 µL of cell suspension, 80 µL saline and 10 µL trypan blue (0.4%) were added and mixed (Bennett *et al.,* 1976). Then the number of living cells (non-stained) was calculated using a hemocytometer slide by inverted microscope (Olympus IX70, Japan). Using the following eqution No. of cells/ml = dilution factor $\times 10^4$ × mean of number of cell/ square.

Cytotoxic activity using MTT Assay

The culture medium was prepared using RPMI 1640 media with sodium carbonate and L-glutamine (Lonza, Verviers, Belgium), 10% of fetal bovine serum (Eurolone, Italy, Europe), 100 units/ ml penicillin and 100 mg/ ml streptomycin were added. The anticancer effect of the different five extracts on Leukemic cell culture was determined by the MTT (3-(4, 5-dimethylthiozol-2-yl)-2, 5-diphenyltetrazolium bromide) assay (Denizot *et al.,* 1986). Five thousand cells per well (20 µl) from AML, ALL and CML patients and normal white blood cells (WBC_s) were plated in 96- well plates in the presence of various concentrations of the extracts (100, 200 and 300 ppm) and endoxan as positive control with the same concentrations for 24h at 37°C in 5% CO_2 incubator. Then the media was removed and the plate was incubated for 4 h in the presence of 0.5 mg/ml of MTT. Absorbance that reflects the viable cell number and was measured at 570 nm. % Cell death was calculated using the following formulas % Cell death = (Control OD –Sample OD)/ Control OD × 100.

Thin layer chromatography (TLC) fractionation of the chloroform extract of *S. lappa*

The chloroform extract of *S. lappa* gave the highest

cytotoxic activity so it was fractionated using TLC technique. Garg and Sharma, (2016) The extract was applied on silica gel 60 F254 TLC aluminum sheets (20×20) (Merck, Darmstadt, Germany) at one of extremes to separate the different fractions. Mobile phase was chloroform : ethyl acetate (9:1). Twelve fractions were detected at UV lamp, scratched and numbering as (C1-C12). All the fractions were tested for their cytotoxic activity.

CEBPA gene expression by real time PCR

The buffy coat from 2 ml peripheral blood of AML patients was incubated with 40 µg of (C2) and (C9) fractions of chloroform extracts for 24 hours. This was followed by extraction of total mRNA using QIAamp RNAblood mini kit (Qiagen, Germany), then the amount of extracted mRNA was measured spectrophotometry using Nanodrop ND 1000 instrument (range 200 - 400 ng/µl). The mRNA was converted to cDNA by means of Quantitect Reverse Transcription kit (Qiagen, Germany). cDNA specific CEBPa Taqman primer and probe sets were developed using primer express software.

The nucleotide numbering throughout this study is based on the published sequence available from Applied Biosystems (AB) UK : The forward primer of CEBPa (5'-TCGGTGGACAAGAACAG-3'), the reverse primer (5'GCAGGCGGTCATT-3'), and the probe ([6-FAM]-ACAAGGCCAAGCAGCGC-[TAMRA-6-FAM]). For normalization primers and probe for reference GAPDH gene were used (Applied Biosystems). The probe was labeled with VIC dye and to avoid competition in the PCR reaction tube, the concentrations of primers were adjusted.

All PCR reactions were performed on the Step one AB. In total 10 µg of the reverse transcription volume was used for each PCR reaction in a total volume of 50 µg. Primer and probe concentrations for the CEBPA gene were optimized using manufacturer's procedure. For CEBPa mRNA; 15 pmol for forward and reverse primers and 10 pmol for the probe were used. The conditions of PCR cycles comprised of 10 min at 95°C, 45 cycles of denaturation for 15 s at 95°C and annealing for 1min at 60°C.

Calculation of C/EBPA expression results

To quantify the relative expression of C/EBPA, the Ct (threshold cycle) values were normalized for endogenous reference or housekeeping gene (GAPDH) ($\Delta Ct = Ct_{sample} - Ct_{GAPDH}$) and compared with a calibrator or controllers, using the 'delta–delta Ct method' ($\Delta\Delta Ct = \Delta Ct_{Sample} - \Delta Ct_{average\ control}$). Using the $\Delta\Delta Ct$ value, the relative expression was calculated as ratio ($2^{-\Delta\Delta Ct}$) (Barjesteh, 2003).

Statistical analysis

All analyses were performed in triplicate and data reported as mean ±standard deviation. Data were subjected to analysis of variance (ANOVA) (Duncan Test $P \leq 0.05$). Results were processed by Excel (Microsoft Office 2010) and Assistat Version 5.0.3.32 (Paraiba state, Brazil).

C/EBPA gene statistical analysis

Quantitative data were presented as minimum, maximum, mean and standard deviation (SD) values. Data showed non-parametric distribution so Mann-Whitney U test was used for comparisons between two groups. The significance level was set at $P \leq 0.05$. Statistical analysis was performed with IBM® SPSS® Statistics Version 20 for Windows.

Results and Discussion

Phytochemical constituents

The total phenols, flavonoids content were determined in methanol, ethanol, acetone, ethyl acetate and chloroform extracts. The phenolic and flavonoid contents were calculated using the following equations: Absorbance/Slop. ($Y= 0.0247x$ at $R^2=0.9369$) ($Y= 0.0051x$ at $R^2=0.9718$), respectively. The obtained data were showed in table 1. The methanol extract of *S. lappa* showed highest contents of phenols (69.64±3.46 µg GAE/g DW) followed by acetone extract (57.35±1.02 µg GAE/g DW) and chloroform extract (41.56±2.6 µg GAE/g DW). While, the results of flavonoids can be ordered as acetone, ethanol, methanol, chloroform, ethyl acetate (34.9±0.94µg GAE/g DW), (32.1±1.42 µg QE/g DW), (30.72±1.69 µg QE/g DW), (22.75±2.89 µg QE/g DW), (18.43±0.52 µg QE/g DW) respectively. This is in agreement with Chang *et al*, (2012). Who found that the total phenols ranged from 20.36± 0.14 to 44.54± 0.61 (µg GAE/g DW) and total flavonoid ranged from 15.75±0.13 to 92.15±0.05 (µg QE/g DW). Also, Saha *et al.,* 2013 reported a high content of total phenols 31.59 ± 4.18 (mg GAE/g DW) and a low content of total flavonoid 5.0 ± 0 (mg QE/g DW) for *S. lappa* in ethanol extract. That proved the high polarity of methanol, ethanol and acetone showed high efficient to separate polar phytochemical compounds such as phenols and flavonoids.

Table 1: Total phenols (TP), total flavonoid (TF) in different solvent extracts of *S. lappa* roots.

Extract	TP(µg GAE/g DW)	TF(µg QE/g DW)
Methanol	$69.64^{a}\pm3.46$	$30.72^{b}\pm1.69$
Ethanol	$34.9^{d}\pm0.94$	$32.1^{ab}\pm1.42$
Acetone	$57.35^{b}\pm1.02$	$34.7^{a}\pm0.85$
Ethyl acetate	$24.43^{e}\pm2.23$	$18.43^{d}\pm0.52$
Chloroform	$41.56^{c}\pm2.6$	$22.75^{c}\pm2.89$

GAE: Gallic acid, QE: quercetin.

On the contrary, non-polar solvent such as chloroform and ethyl acetate are unsuitable for separation of polar phenolic and flavonoid compounds. The results were mean values± standard deviations (SD) of duplicate analyses of three replications.

Antioxidant activity

DPPH scavenging activity

Several methods have been developed to measure the efficiency of antioxidants as pure compounds or in extract. These methods focused on different mechanisms of the oxidant defence system that is scavenging active oxygen species and hydroxyl radicals, inhibiting lipid peroxidation, or chelating metal ions (Dorman, 2003). DPPH is a stable free radical that accepts an electron or hydrogen radical and becomes a stable diamagnetic molecule (Garg, 2016). Antioxidant activity by DPPH method for *S. lappa* roots extracts using various solvent systems and synthetic antioxidant BHT; were described in table 2. The methanol extract showed the highest antioxidant activity for all concentrations compared with BHT as a positive control which ranged from 84.77±0.82 % to 88.1±1.39 % followed by acetone extract which ranged between from 56.97±1.05 to 87.87±2.04. Also, the chloroform extract showed high results (83.86b±2.62) and (79.84c±0.47) at 800, 1000 ppm, respectively. While, the ethanol and ethyl acetate extracts showed moderate antioxidant activity. The DPPH activity depended on the concentration of extracts; it increased significantly with increasing concentration of extracts. This is in agreement with Chang *et al.,* 2012 and Garg *et al.,* 2016.

Reducing power activity

The reducing power assay used to evaluate the ability of an antioxidant to donate the electron because the reduction of Fe^{3+} into Fe^{2+} were used as indicator for the reducing power of the compounds. Yildirim *et al.* (2000) In this study, the reducing power of *S. lappa* extracts was determined using Gallic acid as standard, the linear equation $Y= 0.0129x$ used to calculate the reducing power activity table 3. The highest reducing power result was obtained in the methanol extract at 1000 ppm (48.12±1.01 μg/ml GAE), followed by ethyl acetate extract (32.9±0.57 μg/ml GAE) and ethanol extract (20.90±0.71 μg/ml GAE). Ethyl acetate extract recorded the highest results at (200, 400, 600, 800) ppm. These findings indicate that the antioxidant activity is well correlated with the amount of phenolic and flavonoid constituent found in the extracts.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. It was found that in general, the reducing power of the *S. lappa* powder extract gave high effect with methanol and ethyl acetate extracts. While, the reducing power showed moderate result with ethanol extract. The acetone and chloroform extract had a low reducing power of less than 15 μg/mL GAE.

Cytotoxic activity

The cytotoxic activity of the tested five extracts on three leukemic cell cultures AML, CML, ALL at different concentrations (100, 200 and 300 ppm) was determined. The highest percentage of cytotoxic activity in three leukemic cell cultures was found in chloroform extract which ranged from 78.93±2.68 % to 95.58±1.25 % followed by ethyl acetate extract which ranged from 75.0±1.7 to 93.54±0.7. While, the methanol, ethanol and acetone extracts showed the moderate percentage of cytotoxic effect with ranges (60.41b±3.12 - 70.38b±0.87), (58.35 ±4.01- 69.56±2.67) and (41.77±4.48 - 62.63c±1.15), respectively. At all concentrations tested, all extracts exhibited dose - dependent cytotoxic activity. Detailed analysis for the values listed in (tables 4, 6) and the results suggested that extracts obtained by chloroform and ethyl acetate represented good anticancer agents.

The cytotoxic activity of different twelve chloroform fractions which were separated by TLC was tested on AML cells using concentration 100, 200 and 300 ppm and the endoxan was used

Table 2: DPPH assay for different *S. lappa* root extracts.

Extract	DPPH antioxidant activity %				
	200 ppm	400 ppm	600 ppm	800 ppm	1000 ppm
Methanol	83.70^{a}±0.57	84.39^{a}±0.86	84.77^{a}±0.82	87.57^{a}±0.47	88.1^{ab}±1.39
Ethanol	54.61^{b}±3.05	60.1^{c}±0.69	68.78^{bc}±2.66	71.13^{d}±1.14	72.04^{c}±2.08
Acetone	56.97^{b}±1.05	70.9^{b}±4.02	81.58^{a}±2.16	82.88^{bc}±4.66	85.91^{b}±3.99
Ethyl acetate	18.03^{c}±2.51	20.3^{d}±2.5	30.68^{d}±1.86	53.78^{e}±2.2	66.43^{d}±1.89
Chloroform	54.1^{b}±1.2	60.91^{c}±2.5	66.97^{c}±1.73	79.84^{c}±0.47	83.86^{b}±2.62
BHT	57.35^{b}±0.69	68.18^{b}±1.49	71.66^{b}±1.25	84.08^{ab}±0.99	91.12^{a}±1.59

Table 3: Reducing power assay for different concentration of *S. lappa* root extracts.

Extract	DPPH antioxidant activity %				
	200 ppm	400 ppm	600 ppm	800 ppm	1000 ppm
Methanol	5.42^{b}±0.48	7.95^{b}±0.31	11.47^{b}±0.5	18.47^{b}±0.18	48.12^{a}±1.01
Ethanol	6.72^{b}±0.55	7.57^{b}±0.47	11.39^{b}±0.23	12.38^{c}±0.62	20.90^{c}±0.71
Acetone	6.04^{b}±1.4	7.49^{b}±0.68	7.36^{c}±0.28	9.63^{d}±0.24	12.13^{d}±0.58
Ethyl acetate	13.56^{a}±0.27	18.0^{a}±0.23	23.87^{a}±0.41	24.55^{a}±0.39	32.9^{b}±0.57
Chloroform	5.45^{b}±0.16	5.45^{c}±0.32	5.78^{c}±0.93	7.51^{e}±0.28	8.71^{e}±1.09

Table 4: Cytotoxic activity of *S. lappa* root extracts on AML cell culture.

Extract	(Dead cells %) 100 ppm	(Dead cells %) 200 ppm	(Dead cells %) 300 ppm
Methanol	60.41^{b}±3.12	62.82^{b}±3.08	66.55^{c}±2.1
Ethanol	58.35^{b}±4.01	63.71^{b}±4.4	69.56^{c}±2.67
Acetone	41.77^{c}±4.48	50.24^{c}±4.13	52.58^{d}±2.77
Ethyl acetate	75.0^{a}±1.7	79.98^{a}±1.21	87.67^{b}±0.4
Chloroform	78.93^{a}±2.68	82.75^{a}±2.93	91.51^{a}±1.64
Positive controls	77.17^{a}±1.89	81.89^{a}±1.36	90.36^{ab}±0.3

Table 5: Cytotoxic **activity** of *S. lappa* root extracts on **CML** cell culture.

Extract	(Dead cells %) 100 ppm	(Dead cells %) 200 ppm	(Dead cells %) 300 ppm
Methanol	61.25^{b}±3.18	64.77^{b}±6.29	68.52^{b}±4.38
Ethanol	63.5^{b}±2.67	66.14^{b}±3.88	67.9^{b}±2.78
Acetone	53.5^{c}±1.4	54.28^{c}±2.92	56.19^{c}±0.96
Ethyl acetate	77.62^{a}±2.09	81.27^{a}±4.19	83.47^{a}±2.3
Chloroform	81.44^{a}±3.29	83.90^{a}±2.77	86.58^{a}±1.73
Positive controls	80.53^{a}±3.65	84.49^{a}± 5.34	87.66^{a}±3.72

as positive control, table 7. The highest cytotoxic activity was found in the 2nd chloroform (C2) and 9th chloroform (C9) fractions which ranged between (58.23±1.58 - 88.41±0.97) and (74.96±2.14 - 77.53d±2.44) respectively. The other ten fractions came in the second rank which gave moderate cytotoxic activity. The results were mean values± standard deviations (SD) of three replications. This is in accordance with Thara *et al,*. 2012 and Zahara *et al.,* 2014 who reported that the *S.lappa* extracts showed a high cytotoxic effect.

Molecular studies (C/EBPA gene expression)

The cytotoxic effect of twelve fractions (C1 – C12) which were isolated from chloroform extract using TLC technique was tested against AML cells and normal buffy coat cells. The data revealed that (C2) and (C9) fractions recorded the highest percentage of cytotoxic activity. Hence, the effect of these two fractions on C/EBPA gene expression of AML was estimated.

In the present study, C/EBPA gene expression in AML patients compared to the ninth chloroform fraction (C9) table 8 showed statistically significant difference (p value =0.008). However; C/EBPA gene expression of AML leukemic cells compared to the buffy coat cells of second chloroform fraction (C2) - table 9 showed no statistically significant difference (p value =0.421).

Some plants derived products are known to selectively induce apoptosis in cancer cells, which represent the ideal

Table 6: Cytotoxic activity of *S. lappa* root extracts on ALL cell culture.

Extract	(Dead cells %) 100 ppm	(Dead cells %) 200 ppm	(Dead cells %) 300 ppm
Methanol	61.65^{c}±0.61	61.36^{cd}±2.4	70.38^{b}±0.87
Ethanol	59.91^{c}±3.16	62.99^{c}±2.77	62.49^{c}±3.12
Acetone	52.18^{d}±2.78	58.65^{d}±2.07	62.63^{c}±1.15
Ethyl acetate	77.56^{b}±2.95	84.25^{b}±1.34	93.54^{a}±0.7
Chloroform	85.32^{a}±0.91	89.64^{a}±0.9	95.58^{a}±1.25
Positive controls	82.63^{a}±1.92	90.67^{a}±0.94	95.71^{a}±1.06

Table 7: Cytotoxic activity of *S. lappa* root chloroform fractions on AML cell culture.

Fraction	(Dead cells %) 100 ppm	(Dead cells %) 200 ppm	(Dead cells %) 300 ppm
C1	59.07^{bc}±0.93	61.16^{d}±1.59	63.33^{g}±1.24
C2	58.23^{bc}±1.58	77.74^{a}±2.69	88.41^{a}±0.97
C3	52.0d^{e}±3.95	63.45^{d}±4.9	72.49^{f}±0.97
C4	54.78^{cd}±0.68	64.22^{cd}±7.08	73.44^{f}±1.15
C5	45.98^{f}±1.5	66.66^{bc}±4.2	75.70^{ef}±0.92
C6	50.66^{ef}±0.63	65.09^{cd}±4.41	72.98^{f}±3.0
C7	59.23^{bc}±1.18	67.49^{bc}±1.31	79.44^{cd}±2.60
C8	62.15^{b}±1.04	73.67^{ab}±0.55	82.80^{bc}±1.4
C9	74.96^{a}±2.14	75.74^{ab}±2.33	77.53^{de}±2.44
C10	12.31^{g}±4.5	57.43^{d}±4.63	83.20^{b}±3.6
C11	13.93^{g}±6.86	62.71^{d}±12.19	81.78^{bc}±1.5
C12	0.8^{h}±7.79	11.38^{e}±6.88	63.64^{g}±1.57

All values in previous tables are represented as mean ±S.D.
a=highest values followed by b then c then d…

property for successful cytotoxic agents, other researchers reported about the biological and molecule effects of *S.lappa* on cell proliferation, growth and also apoptosis. Regarding C/EBPA gene expression in leukemic cells after addition of chloroform fractions (C2+C9) from the *S. lappa* plant, we found a statistically significant difference (P value 0.008) with (C9) compound; this denotes the higher cytotoxic activity of (C9) on the leukemia cells. According to our best knowledge data regarding this issue were not reported in literature.

The most known important compounds separated from the plant of *S.lappa* are the Costunolide (CE) and dehydrocostuslactone (DE), which are considered natural sesquiterpene lactones. They have been reported for their potential cytotoxic activities and their anticancer mechanisms, including cell cycle arrest, promoting the aggregation of microtubule protein, apoptosis and differentiation, inhibiting the activity of telomerase, reversing multidrug resistance, inhibiting metastasis and invasion of cancer cells. Most studies indicated that the α, β-unsaturated carbonyl group in the α-methylene-γ-

Table 8: C/EBPA expression of AML cell suspension with 9th Chloroform fraction.

C/EBPA	No. of cases	Mean ± SD	Minimum	Maximum	*P*-value
Buffy coat suspension of leukemic cells	5	0.63±0.16	0.41	0.81	0.008*
Buffy coat suspension+(C9)	5	1.26±0.29	0.81	1.62	

*: Significant at $P \leq 0.05$.

Table 9: C/EBPA expression in AML cell suspension with 2nd Chloroform fraction.

C/EBPA	No. of cases	Mean ± SD	Minimum	Maximum	*P*-value
Buffy coat suspension of leukemic cells	5	0.63±0.16	0.406	0.812	0.421
Buffy coat suspension+(C2)	5	0.65±0.16	0.420	0.823	

*: Significant at $P \leq 0.05$.

butyrolactone inside of CE and DE perhaps play some axil roles through conjugation with mercapto (SH)-groups of target proteins to intervene in some key biological processes in cells. Cateni 2006 and Romagnoli 2005 Many studies proved that CE and DE inhibit cell cycle progression through an increase of the G2/M phase combined with a depletion of the G0/G1 phase in various cancer cells. Rasul, 2013 Also, Choi *et al.*, reported that CE and DE can deplete intracellular thiols and lead to the generation of reactive oxygen species (ROS) in cells, which will induce DNA damage and cancer cell apoptosis. Taken together, the activation of p53/p21/p27 is a very common mechanism of anti-cancer activity for secondary metabolites in plants.

Further studies are needed to elaborate on the different applications of anticancer natural compounds in the oncology field to offer patients additional hope.

Conclusion

Determination of the antioxidant and cytotoxic activities of five extracts of *S. lappa* was the main purpose of this study, in addition to the separation of active ingredients of the promising extract. The total phenols, flavonoids content were determined. The methanol extract mentioned the highest phenolic, on the other hand, the greatest flavonoids recorded to acetone extract. The antioxidants of tested extracts were estimated using different assays. The methanol and chloroform extract showed excellent antioxidant across the two assays used and the methanol extract revealed the best reducing power. The Data of The cytotoxic activity on three leukemic cell cultures AML, CML, ALL indicated that the chloroform extract recorded the highest percentage of cytotoxic activity against three leukemic cell cultures and the results suggested chloroform and ethyl acetate extracts represented good anticancer agents. Eleven fractions of promising chloroform extract were separated by TLC and tested on AML cells. The 2nd chloroform (C2) and 9th chloroform (C9) fractions revealed very good cytotoxic activity. Hence, the effect of these two fractions on C/EBPA gene expression of AML was measured. The data summarized that only ninth chloroform fraction (C9) showed a statistically significant difference on that gene expression. The most known important compounds separated from the plant of *S.lappa* are two sesquiterpene lactones : the Costunolide and dehydrocostus lactone so this study suggested that the two effective fractions C2 and C9 may belong to these sesquiterpenes. The chemical identification of the separated fractions with cytotoxic properties must be done. In future further studies of *S. lappa* for the elucidation of the cytotoxic mechanisms considered as immense importance. It is anticipated that *S. lappa* would be a source of useful pharmaceutical materials to deal with leukemia.

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