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Evaluation of anti proliferative properties of selected species of Caralluma and Boucerosia on skin cancer cell lines

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ABSTRACT

The aim of the present work was designed to study the effects of selected plant extracts of Caralluma and Boucerosia on cancer apoptotic induction. The present study was carried out in four species of Caralluma R.Br. such as Caralluma adscendens (Roxb.) R. Brown var. attenuata (Wight) Grav. & Mayur. (CAA), Caralluma adscendens (Roxb.) R. Brown var. fimbriata (Wall.) Gravely & Mayur. (CAF), Caralluma stalagmifera C.E.C. Fisch. (CS) and Caralluma stalagmifera C.E.C. Fisch. var. longipetala Karupp. & Pull. (CSL) and as well as two species of Boucerosia Wight & Arn. such as Boucerosia lasiantha Wight. (BL) and Boucerosia umbellata (Haw.) Wight & Arn. (BU). The cytotoxicity efficacy was evaluated by MTT assay on A375 human malignant melanoma and A431 human skin cancer cells. Cell viability was demonstrated by the inhibitory concentration 50% (IC_{50}). A dose dependent increase of cell growth inhibition A375 melanoma cells, A431 skin cancer cells was observed, when treated with different concentrations of methanolic extracts (1 µg/ml, 5 µg/ml, 10 µg/ml, 25 µg/ml, 50µg/ml, 75 µg/ml, 100 µg/ml). The plant extracts was shown to be effective for anti-proliferation and induction of apoptosis cell death in skin cancer cells. Therefore, mechanisms underlying the cell death and its potential use for treatment of skin cancer will be further studied.

Key words: cytotoxicity, A431, A375 cell lines, melanoma, carcinoma

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INTRODUCTION

Skin cancer is the most common form of cancer and includes three main types: basal, squamous and malignant cell carcinoma. Annually, 132000 cases of malignant melanoma and more than 3 million cases of other skin cancers are being diagnosed. The most aggressive form of skin cancer is malignant melanoma, formed as a result of malignant transformation of melanocytes, melanin synthesizing cells, responsible for death in 75% of skin cancer patients. The tumor tissue is removed and chemotherapy is practiced only in patients with metastatic disease is not more than 15%. Even though synthetic drugs are available in the market, the plants and their extracts to provide novel products attract researchers in the quest for chemotherapeutics. Plant derived natural products with antitumor properties are alkaloids, phenyl propanoids and terpenoids [1]. Therefore, it is necessary for development of alternative methods for prevention and treatment of skin cancer. In recent years, research is going on naturally occurring substances that can be used in antimelanoma therapies.

Preliminary phytochemical analysis of methanolic extracts of *Caralluma* showed the presence of flavonoids, saponins and pregnane steroids. In nature C_{21} pregnane steroids are available as conjugated as glycosides. *Caralluma* and other genera of the Asclepiadaceae family are rich in esterified polyhydroxypregnane glycosides, exhibit anti-cancer and anti-tumour effects [2, 3, 4], and in future, they play an important role in the development of new drugs. The anti proliferative activity of *Caralluma fimbriata* extract on 3T3 L1 pre-adipocytes is similar to the other compounds such as dehydroepiandrosterone (DHEA) [5] and phenolic acids [6]. Ahmed *et al* (2009) [7] studied effects of different concentrations of *Caralluma tuberculata* crude extract on MCF-7 cell line. Maximum growth inhibition shown by 500 µg/ml of the crude extract was 82% Studies revealed that CAF extract (100 µg/ml) and its isolated compound pregnane glycosides showed anti proliferative activity and cytotoxicity of 3T3L1 pre -adipocytes using MTT assay in dose and duration dependent manner [8]. Two novel acylated steroid glycosides isolated from *Caralluma tuberculata* possess moderate, micromolar cytotoxic activity on the growth and viability of MCF-7 estrogen-dependent, and MDA-MB-468 estrogen-independent breast cancer cells, Caco-2 human colonic cells, HUVECs and U937 cells. Neutral red uptake and MTT assays were used and induce caspase-dependent apoptosis in cancer cells, which may indicate a source of activity in vivo of interest to future drug design [9]. Four acylated pregnane glycosides isolated from *Caralluma quadrangula* were elucidated by NMR and HRESI-MS analysis. Compounds **1**, **2** and **4** showed promising cytotoxic activity against the MRC5 human diploid embryonic cell line [10].

Twenty-seven new pregnane glycosides were isolated from the whole plant of *Caralluma dalzielii*, and their structures elucidated from extensive 2D NMR analysis as well as ESI-MS experiments. All isolated compounds were tested for their antiproliferative activity on J774.A1, HEK-293, and WEHI-164 cell lines. Moderate to high potency of cytotoxicity were found in almost all tested compounds, confirming the significant cytotoxic activity of pregnane glycosides [11]. Many of the medicinal plants rich in flavonoids are reported to reduce disease risk and have therapeutic properties. Flavonoids are rich in fresh vegetables and fruits as well as their consumption can reduce the cancer risk [12, 13]. The present research work on antiproliferative activity of methanolic extracts of *Caralluma* were carried out based on the therapeutic value of flavonoids, since the phytochemical evaluation indicated the presence of flavonoids in the crude methanolic extract. Literature proved that flavonoids are biologically active against different strains of bacteria and many human cancer lines [14]. Research on inhibition of proliferation of human malignant melanoma A375 cells revealed that green tea and its major constituent, epigallo catechin 3-gallate impart chemopreventive and chemotherapeutic effects in non melanoma skin cancers [15]. Matrine, alkaloid from *Sophora japonica* and *Sophora subprostrata* [16], Gambogic acid, a major active ingredient of *Garcinia* [17]. A375 and A431 cell lines were used as a model of skin melanoma malignum cells. The survey provides fundamental data for further studies with isolated active substances from extracts of analysed plant.

MATERIALS AND METHODS

The present study was carried out in four species of *Caralluma* R.Br. such as *Caralluma adscendens* (Roxb.) R. Brown var. *attenuata* (Wight) Grav. & Mayur. (CAA), *Caralluma adscendens* (Roxb.) R. Brown var. *fimbriata* (Wall.) Gravely & Mayur. (CAF), *Caralluma stalagmifera* C.E.C. Fisch. (CS) and *Caralluma stalagmifera* C.E.C. Fisch. var. *longipetala* Karupp. & Pull. (CSL) and as well as two species of *Boucerosia* Wight & Arn. such as *Boucerosia lasiantha* Wight. (BL) and *Boucerosia umbellata* (Haw.) Wight & Arn. (BU). All the selected plant materials four species of *Caralluma* (Asclepiadaceae) such as CAA, CAF, CSL and CS as well as two species of *Boucerosia* like BL and BU were collected from Gooty, Tadipathri and Penukonda areas of Anantapur district and were taxonomically identified by comparing taxonomical literature, voucher specimens i.e. VM 46, VM 47, VM 48, VM 49, VM 50 and VM 51 were deposited in Montessori Mahila Kalasala, Vijayawada

Cytotoxicity Assay

Cell culture reagents

Phosphate buffered saline (PBS), Dimethyl sulfoxide (DMSO) Sigma, St. Louis, USA. Fetal bovine serum (FBS) Dulbecco's Modified Eagles Medium (DMEM) and penicillin were obtained from HyClone, Logan, UT. MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] was purchased from Roche Applied Sciences, Germany.

Equipment: Microplate reader (BioRad, USA).

Cell culture and medium preparation
A375 human malignant melanoma and A431 human skin cancer cells (American type culture collection, Manassas, VA.) were harvested from the logarithmic phase of cultures and resuspended in Dulbecco's modified eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 unit of penicillin. The cell counts were adjusted and equal number of cells were plated into each well of 96-well cell culture plates and allowed to grow overnight at 37 ^{o}C, in presence of 5% CO_2, 95% air and 100% humidity. Cells were plated in 10 cm culture dish and allowed to grow to approximately 90% confluence before experimentation.

Preparation of plant extracts
Stock of CAA, CAF, CS, CSL, BL and BU plant extracts at 100 mg/ml in DMSO were prepared separately and and stored at -0 ^{0}C until used. Ten-fold serial dilutions (20 µl of plant extracts in 180 µl DMEM) were then prepared from the stock solution to obtain working concentrations of extract 1 µg/ml, 5 µg/ml, 10 µg/ml, 25 µg/ml, 50 µg/ml, 75 µg/ml and 100 µg/ml by using DMEM. The plant extract solutions were used immediately for cell cytotoxic assay.

MTT based cell proliferation assay
MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] based cell proliferation assay was first described by [18, 19]. This colorimetric assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. The number of surviving cells is directly proportional to the level of the formazan product created.

Cytotoxicity analysis with MTT Assay
The cytotoxic efficacy of the methanolic extracts of CAA, CAF, CS, CSL, BL and BU as well as isolated compound pregnane steroid were evaluated in A375 human malignant melanoma and A431 human skin cancer cells by MTT cell proliferation assay kit. The assay was carried out according to the instruction provided by the vendor. The A375 human malignant melanoma and A431 human skin cancer cells were plated at a density of 1x104 cells per well in 96-well plates. After 20 h, cells were treated with 100 µl of complete culture medium containing test substances at various concentrations ranging from 1 µg/ml to 100 µg/ml for 72 h. In vehicle control culture wells, a maximum of 0.5% DMSO was added. Culture medium was renewed at every other day with fresh culture medium supplemented with the test substances. Each concentration methanolic extract and pregnane steroid was repeated in four wells. Thereafter, 0.5 mg/ml of MTT in phosphate buffered saline was added to each well and the microplate was incubated further for 4 h at 37 ^{o}C in humidified environment of 5% CO_2. Finally, the cells were solubilized by adding solubilizing solution and allowed to incubate at 37 ^{o}C overnight. After complete solubilization of the formazan crystals the absorbance was read at 540 nm in a microplate reader.

The results (mean OD ± SD) obtained from quadruplicate wells were used in calculation to determine the cytotoxicity 50% of inhibitory concentration (IC_{50}), of the test compounds.

The inhibitory effect of CME of CAA, CAF, CS, CSL, BL and BU on cell growth was assessed and inhibition ratio (I %) was calculated using the following equation [20].

I % = [A540 (control) - A540 (treated)] × 100 / A540 (control)

RESULTS

Cellular cytotoxic activity of plant extracts
All the methanolic extracts of *Caralluma* and *Boucerosia* species i.e., CAA, CAF, CS, CSL, BL and BU showed higher percentage inhibition in cell proliferation of A431 human skin cancer cells compared with A375 human melanoma cells (Fig 1).

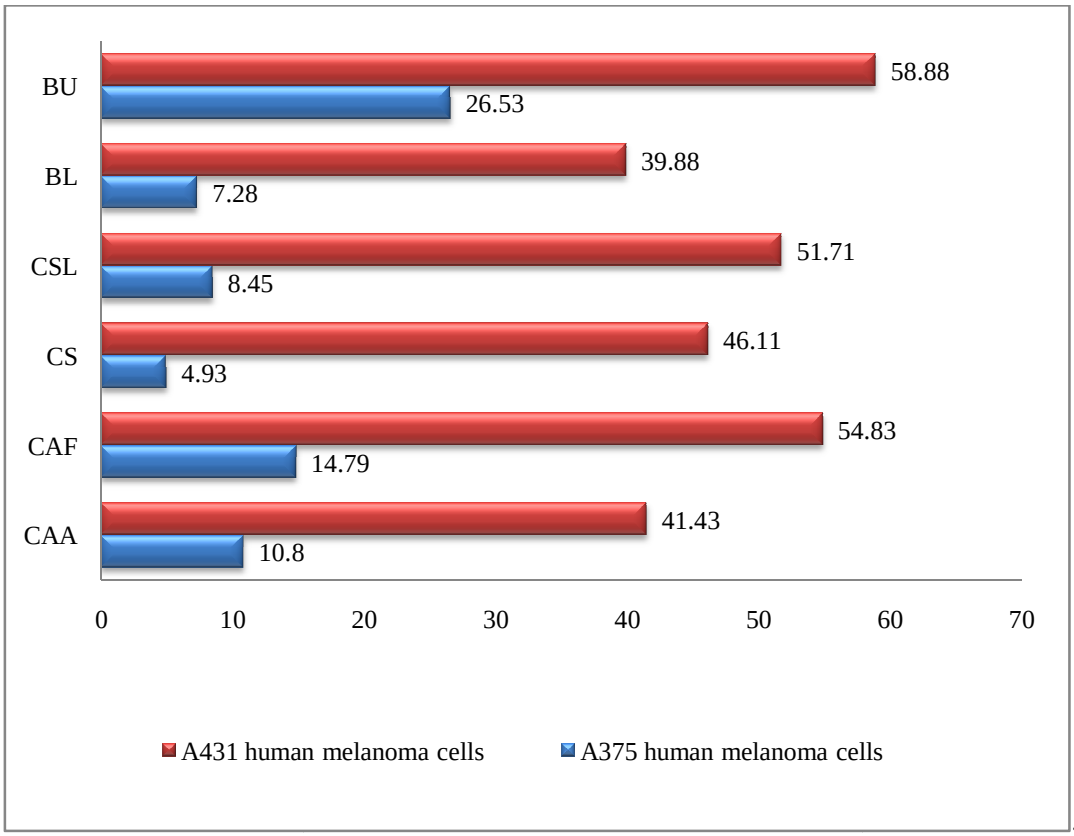
Cellular cytotoxic activity on A375 human melanoma cell line
The effect of crude methanolic extracts of CAA, CAF, CS, CSL, BL and BU on growth of A375 human melanoma cell line was studied by the MTT assay. A range of different concentrations 1 µg/ml, 5 µg/ml, 10 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml of crude extracts of CAA, CAF, CS, CSL, BL and BU were treated against A375 human

melanoma cell line (Table 1). Significant growth inhibition was shown at concentrations above 75 µg/ml (Fig 2). The results showed dose dependent response.

Cellular cytotoxic activity on A431 human melanoma cell line

The methanolic extract of CSL is highly active against A431 human melanoma cells. Crude extracts of CAA, CAF, CS, CSL, BL and BU with a range of different concentrations 1 µg/ml, 5 µg/ml, 10 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml were treated against A431 human melanoma cell line. The results showed dose dependent response (Fig. 3). The % inhibition of A431 cell proliferation, achieved by CAA, CS and BL at 100 µg/ml were 41.43%, 46.11% and 39.88% respectively. For CAA, CS and BL more than 100 µg/ml was needed to achieve IC_{50} value. The half maximum inhibitory concentration (IC_{50}) of CAF, CSL and BU were obtained at 82 µg/ml, 93.8 µg/ml and 78.9 µg/ml respectively. Among the six extracts, BU showed more potent cytotoxic activity and then CAF (82 µg/ml) and CSL (93.8µg/ml) (Fig 4).

Fig 1: Comparative study of cytotoxicity (% inhibition in human cancer cells proliferation) of *Caralluma* and *Boucerosia* on A375 human melanoma cells and A431 human skin cancer cells



**The values represent percentage inhibition at 100 µg/ml concentration of test samples.*

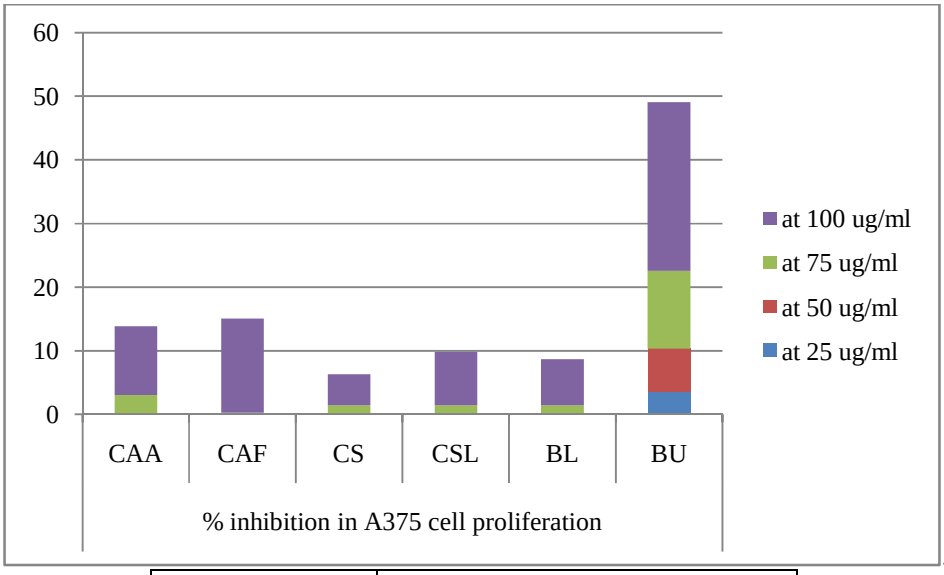
CAA Caralluma adscendens var. attenuata
CAF Caralluma adscendens var. fimbriata
CS Caralluma stalagmifera
CSL Caralluma stalagmifera var. longipetala
BL Boucerosia lasiantha
BU Boucerosia umbellata.

Table 1: Percentage inhibition in A375 cell proliferation by selected species of *Caralluma* and *Boucerosia*

S. No.	*Caralluma* and *Boucerosia* species	% inhibition in cell proliferation (IC_{50} µg/ml). Mean ± S.D
1	*Caralluma adscendens* var. *attenuata* (CAA)	10.8±0.64
2	*Caralluma adscendens* var. *fimbriata* (CAF)	14.79±0.65
3	*Caralluma stalagmifera* var. *longipetala* (CSL)	8.45±0.41
4	*Caralluma stalagmifera* (CS)	4.93±0.14
5	*Boucerosia lasiantha* (BL)	7.28±0.18
6	*Boucerosia umbellata* (BU)	26.53±0.55

All test samples run in triplicates and one way ANOVA test was carried. Values are expressed as mean ± standard deviation (n = 3). The results of single way ANOVA analysis showed significant differences ($p<0.05$) in the means of % inhibition in cell proliferation of A375 human melanoma cells at 100 µg/ml of test sample.

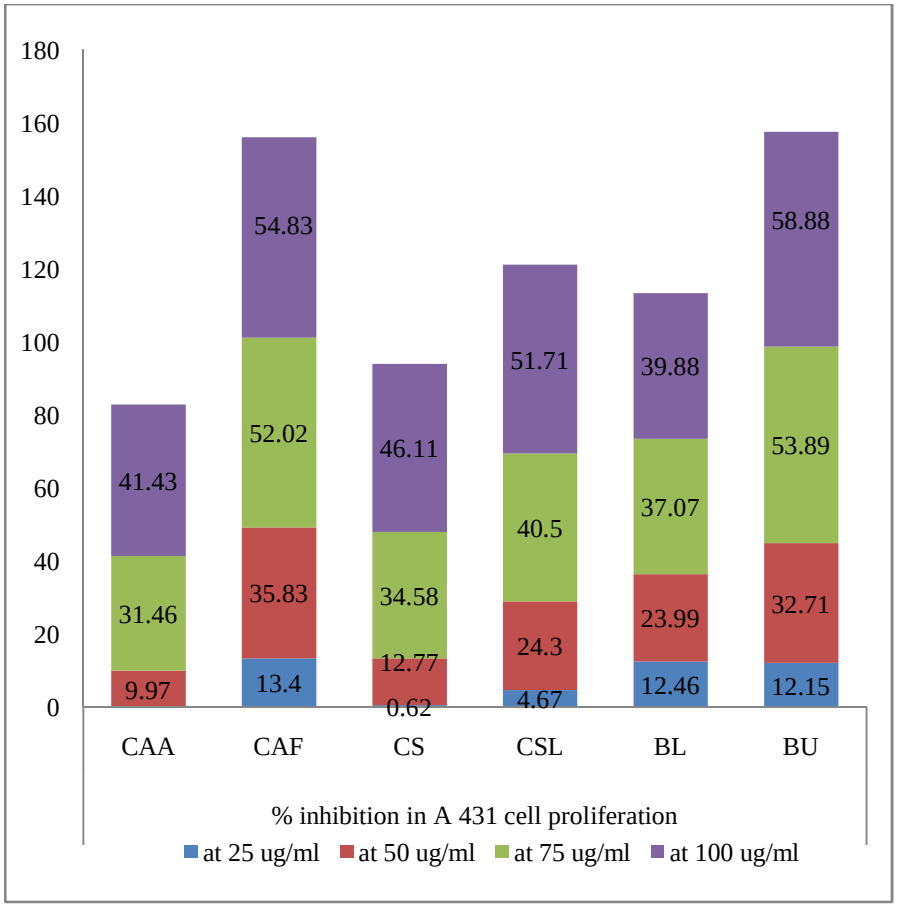
Fig 2: Differentiation of *Caralluma* and *Boucerosia* species based on dose dependent cytotoxicity in A375 cell lines



Concentration (µg/ml)	% inhibition in A375 cell proliferation					
	CAA	CAF	CS	CSL	BL	BU
25						3.52
50						6.81
75	3.05	0.23	1.41	1.41	1.41	12.21
100	10.8	14.79	4.93	8.45	7.28	26.53

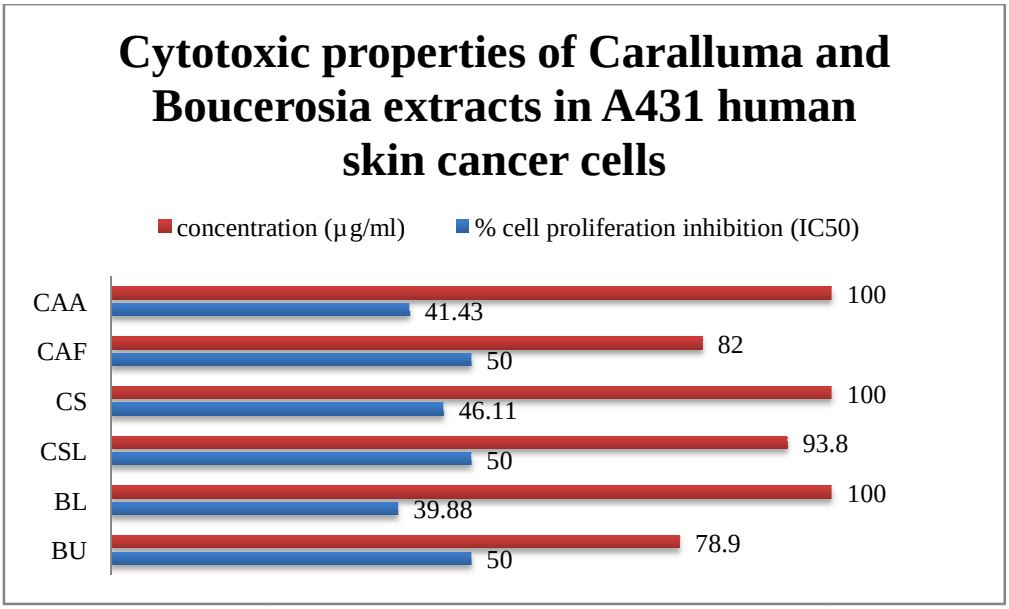
**As the concentration of extract increases, the percentage of A375 inhibition in cell proliferation increases.*

Fig 3: Differentiation of *Caralluma* and *Boucerosia* species based on dose dependent cytotoxicity in A431 cell lines



**As the concentration of extract increases, the percentage inhibition in cell proliferation of A431increases.*

Fig 4: Differentiation of *Caralluma* and *Boucerosia* species based on cell proliferation inhibition (IC_{50} µg/ml) *vs.* concentration (µg/ml)



**The IC_{50} values of CAA, CS and BL were achieved at higher concentrations(>100 µg/ml).*
CAA Caralluma adscendens var. attenuata
CAF Caralluma adscendens var. fimbriata
CS Caralluma stalagmifera
CSL Caralluma stalagmifera var. longipetala
BL Boucerosia lasiantha
BU Boucerosia umbellata.

DISCUSSION

To differentiate selected plants CAA, CAF, CL, CSF, BL and BU based on their anti proliferative activity, screening test was conducted based on their variable cytotoxic activity *in vitro*. The effect of methanolic extracts of selected plants on the growth of A431 cells were examined by the MTT assay. Dose response curves constructed in the range µg/ml express decreasing number of viable cell with increase in concentrations of extracts.

The sensitivity of cells to exposure of extracts is characterized by IC_{50} values. Different cytotoxicity of the tested plant extracts on A431 cells is based on their chemical composition and relative content of biological active substances. The literature data are proving the presence of flavonoids, steroids, saponins and phenolic compounds. Highest toxic effects were observed against A431 cell lines. A375 were sensitive but their sensitivity was less in comparison with A 431 cell line. Further studies are required to determine the detailed and distinguishing features of intracellular pathways involved in the mechanism of cytotoxicity. The study provides information about fundamental data for further research on isolated active substances from extracts of analyzed plant.

CONCLUSION

This research established the fact that *Caralluma* and *Boucerosia* can be used to treat diverse diseases ranging from cancer, atherosclerosis, and obesity related metabolic disorders and hypertension etc. This conclusion is as a result of the abundant of flavonoids and phenolic compounds present in *Caralluma* and *Boucerosia* species. There is no doubt that these plants are reservoir of potentially useful phytoconstituents which serve as medicinally important compounds, provide newer leads and clues for modern drug design. Their antiproliferative effects on some selected cancer lines confirmed usefulness in the treatment of cancer. Further it is concluded that selected species of *Caralluma* and *Boucerosia* possess pharmacological properties which if properly harness can be used in the management cancer. These herbal drugs contain unique constituents which differs from one herb to another, hence the type and extent of their medicinal property also differs.

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