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Research Article

# EVALUATION OF CELLULAR ANTIOXIDANT ACTIVITY OF SELECTED SPECIES OF Caralluma AND Boucerosia ON CELL LINES

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## Abstract

Objective: The aim of the present work was designed to study cellular antioxidant properties of selected plant extracts of *Caralluma* and *Boucerosia* on cell lines. 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). The antioxidant efficacy was evaluated by ability of antioxidant molecules in the extract to scavenge ROS produced by normal metabolism by cells, observed by the oxidation of 2, 7- Dichloro fluorescein diacetate to dichlorofluorescein, followed by decrease in fluorescence intensity. Results: Cellular antioxidant activity was demonstrated by the inhibitory concentration 50% (IC50) of reactive oxygen species (ROS). The percentage ROS inhibition in Raw 264.7 cells when treated with different concentrations of methanolic extracts showed variations among selected species. Conclusion: The plant extracts were shown to be effective for antioxidant potential and can be a promising source of natural antioxidants.

**Key words**: cellular antioxidant activity, *Caralluma*, *Boucerosia*, Reactive oxygen species, Mouse macrophage cell line

## Introduction

Oxygen is primarily used by the cells to generate energy through a process called oxidative phosphorylation, four electrons and four protons are added to oxygen to form water. But a highly reactive oxygen species (ROS) is formed when only a molecule of oxygen gains one electron. Other ROS molecules are $H_2O_2$, $OH^-$ radical, peroxynitrite ($ONOO^-$). In humans, oxidative deterioration of lipids is thought to induce physiological obstruction and cause ageing of the cells and carcinogenesis (Lampart – Szcaapa, 2003). Experimentally, it is proved that lipid oxidation produces free radicals which can harm healthy cells and create harmful molecules and lead into degenerative processes related to ageing of the cells. Controlled production of ROS has an important physiological role but its uncontrolled production causes oxidative damage to biomolecules like nucleic acids, lipids, proteins and carbohydrates resulting pathogenesis of cancer, cardiovascular diseases, atherosclerosis, hypertension, ischemia, diabetes, cirrhosis, cancer, aging, neurodegenerative disorder, Alzheimer's disease, Parkinson disease may occur (Finkel, 2005, Valko *et al.*, 2007).

The term "antioxidant" refers to the compounds that can delay or inhibit oxidation of lipids and other molecules by inhibiting the initiation and elongation of oxidative chain reaction (Velioglu *et al.*, 1998) and which can repair damage done to the body cells. The different ways by which the antioxidants act include reducing agents, free radical scavengers, potential complexes of pro oxidant metals and inhibiting singlet oxygen species (Hudson, 1990). Since plants give protection against free radicals, attention is given to the antioxidant activity of plant extracts. As plants themselves have to counteract stress caused by oxygen, they act as a potential source of natural antioxidants. Hence, preliminary phytochemical screening of medicinal plants for their antioxidant potential is essential, which may lead to development of new drugs. Hence attention has been paid to explore natural substances as substitutes for synthetic antioxidants. Phytoconstituents are good source of natural antioxidants used for promotion, food preservation, food flavoring and cosmetics since they are ecofriendly and harmless than their synthetic counterparts (Shrikumar and

Ravi, 2007). The health protective effect of plants is mainly attributed to their polyphenolics, mainly flavonoids and phenolic acids. Recently, research on natural antioxidants from plant materials has been given a considerable interest and natural products are presumed to be safe since they occur in food crops. Practically it was proved that plants contain a vast variety of constituents called plant chemicals or phytoconstituents that possess antioxidant activity (Pratt, 1992). The cellular antioxidant properties of all phlorotannins (Li *et al*., 2009); *Salvia brachyantha* extract (Esmaeili and Ali Sonboli, 2010), delphinidin and cyanidin (Cvorovic *et al*,. 2010), the main polyphenolic constituent in green tea like epigallocatechin-3-gallate (EGCG) (Suh *et al*., 2010) and methanolic extracts of *Terminalia bellerica* and *Emblica officinalis* (Nampoothiri *et al*., 2011) were assessed by cellular reactive oxygen species (ROS) assay on cell lines using DCFH-DA. Measurement of antioxidant activity using cell lines is important in the screening of medicinal plants for potential health benefits. The cellular antioxidant activity (CAA) assay quantifies antioxidant activity in cell culture and was developed to meet the need for a more biologically representative method than the popular chemistry antioxidant capacity measures.

The species of *Caralluma* (Asclepiadaceae) found in India are eaten raw as well as in cooked form and became a part of traditional system of the country. The medicinal activities of the genus *Caralluma* include carminative, febrifugal, anthelmintic, antirheumatic, antiinflammatory, antinociceptive and antioxidant effects. *Caralluma* spp. acts as appetite suppressant and stimulant of central nervous system. The medicinal properties of *Caralluma* and *Boucerosia* are due to pregnane group of glycosides contained in them. The aim of the study is to evaluate antioxidant activity of 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) as well as two species of *Boucerosia* Wight & Arn. such as *Boucerosia lasiantha* Wight. (BL) and *Boucerosia umbellata* (Haw.) Wight & Arn. (BU) on cell lines. These species show minor variations in their morphological features.

## Materials and Methods

### *Detection of ROS production*

All the selected plant materials i.e., 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 with Gamble flora and other 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.

**Chemicals**: HEPES: 4, 2, hydroxyl ethyl-1-piperazine ethane sulfonic acid (pKa 7.48; Range: 6.8 – 8.2; temperature efficiency 0.014; molecular weight 238.3). HBSS : Hanks balanced salt solution: 1.26 mM $CaCl_2$, 5.37 mM KCl, 0.44 mM $KH_2PO4$, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, 140 mM NaCl, 4.17 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4$.DCFH DA: Reduced 2,7- Dichloro fluorescein diacetate.

### *Cells and cell culture of Raw 264.7 Mouse macrophage cell line*

Mouse macrophage cell line (Raw 264.7) was purchased from National Centre for Cell Science, Pune, India. The cells were cultured in Dulbecco modified Eagle's medium (DMEM) containing 10 % Fetal calf serum, 100 u/ml penicillin, 100 µg/ml streptomycin, 2 mM/L glutamine at $37^0$C in a humidified incubator containing 5% $CO_2$. Cells were plated in 10cm culture dish and allowed to grow to appropriately 40-60% confluency. For all experiments, cells were sub cultured not more than 20 cell passages at 2 x $10^6$ / ml.

### *Detection of ROS by 2, 7- Dichloro fluorescein diacetate (DCFH-DA) method*

The generation process of ROS can be monitored using the fluorescence method. The intracellular formation of ROS can be investigated using oxidation sensitive dye 2, 7-Dichloro fluorescein diacetate (DCFH-DA) as a substrate. Raw 264.7 cells were grown in 96 well micro – titer black plate and were labeled with 5 mM DCFH-DA in 20 mM HEPES – buffered HBSS with 5 mM glucose and incubated at $37^0$C for 15 min. in dark. The nonfluorescent DCFH-DA dye which easily penetrates into cells was then hydrolyzed by intracellular esterase to 2, 7- DCFH and trapped inside the cells. The buffer with DCFH-DA was exchanged with fresh buffer and the cell suspension was mixed gently by centrifugation at 1500 rpm for 5 min, at room temperature. Thereafter, equal number of cells (5 X $10^4$) was transferred to each well of a 96-well micro-titer black plate. Cells were then treated with different concentrations of test samples. 125 µl of HBSS was added in presence or absence of 100 mM $H_2O_2$ alone or in combination with test samples and incubated further for 2 h at $37^0$C. $H_2O_2$ was used for induction of intracellular ROS generation. Vehicle control culture wells received only 0.1% DMSO. The formation of fluorescent dichlorofluorescein (DCF) is due to oxidation of DCFH in the presence of several ROS was recorded at the excitation wavelength of 485 nm and emission wavelength of 530 nm. at an interval of 10 min in a Modulus Microplate reader (Turner Biosystems, Sunnyvale, CA). Results were calculated as area under the curve (AUC) and presented as values relative to control (% of control).

## Results

The effect of methanolic extracts of selected *Caralluma* species CAA, CAF, CL, CSF and *Boucerosia* plants BL and BU on were investigated using oxidation sensitive dye 2,7-Dichloro fluorescein diacetate (DCFH-DA) as a substrate.

The percentage ROS inhibition in Raw 264.7cells by 0.1 µg/ml, 0.5 µg/ml,1 µg/ml, 5 µg/ml,10 µg/ml, 25 µg/ml and 50 µg/ml of CAA were 8%, 17.59%, 28.93%, 41.01%, 46.54%, 57.07% and 68.22% respectively and that of CAF were 5.57%, 8.95%, 12.65%, 25.60%, 40.10%, 45.56% and 53.86% respectively. The percentage inhibition in ROS in Raw 264.7cells 1 µg/ml, 2 µg/ml, 4 µg/ml, 6 µg/ml, 8 µg/ml, 10 µg/ml and 12 µg/ml of CL were 9.07%, 19.31%, 22.82%, 30.37%, 34.29%, 39.11% and 43.05% respectively. The percentage ROS inhibition in Raw 264.7 cells 0.1µg/ml ,0.25 µg/ml ,0.5 µg/ml ,0.75 µg/ml,1 µg/ml, 2 µg/ml and 2.5µg/ml CSF were 4.32%, 7.29%, 15.13%, 24.33%, 27.84%, 35.93% and 34.60% respectively. The percentage ROS inhibition in Raw 264.7cells 0.1 µg/ml, 0.5 µg/ml, 1 µg/ml, 5 µg/ml, 10 µg/ml, 25 µg/ml and 50 µg/ml BL were 7.24%, 7.72%, 30.71%, 28.86%, 38.31%, 38.54% and 40.40% respectively. The percentage ROS inhibition in Raw 264.7 cells of 1 µg/ml, 5 µg/ml,10 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml of BU were 20.82%, 24.68%, 31.73%, 38.22%, 43.42% and 40.45% respectively (Table 1, Fig 1).

**Table 1**: Dose dependent % ROS inhibition by selected species of *Caralluma* and *Boucerosia*.

| **Percentage ROS inhibition shown by *Caralluma* and *Boucerosia* species** | **Test sample concentration (µg/ml)** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0.1** | **0.5** | **1** | **5** | **10** | **25** | **50** |
| *Caralluma adscendens* var. *attenuata* (CAA) | 8 | 17.59 | 28.93 | 41.01 | 46.54 | 57.07 | 68.22 |
| *Caralluma adscendens* var. *fimbriata* (CAF) | 5.57 | 8.95 | 12.65 | 25.6 | 40.1 | 45.56 | 53.86 |
| *Boucerosia lasiantha* (BL) | 7.24 | 7.72 | 30.71 | 28.86 | 38.31 | 38.54 | 40.40 |
| *Boucerosia umbellata* (BU) | Nil | Nil | 20.82 | 24.68 | 31.73 | 38.22 | 43.42 |
| *Caralluma stalagmifera* (CS) | **Test sample concentration (µg/ml)** | | | | | | |
| | 0.1 | 0.25 | 0.5 | 0.75 | 1 | 2 | 2.5 |
| | 4.32 | 7.29 | 15.13 | 24.33 | 27.84 | 35.93 | 34.6 |
| *Caralluma stalagmifera* var. *longipetala* (CSL) | **Test sample concentration (µg/ml)** | | | | | | |
| | 1 | 2 | 4 | 6 | 8 | 10 | 12 |
| | 9.07 | 19.31 | 22.82 | 30.37 | 34.29 | 39.11 | 43.5 |

*As the concentration of extract increases, the percentage inhibition of ROS (Reactive oxygen species) increases

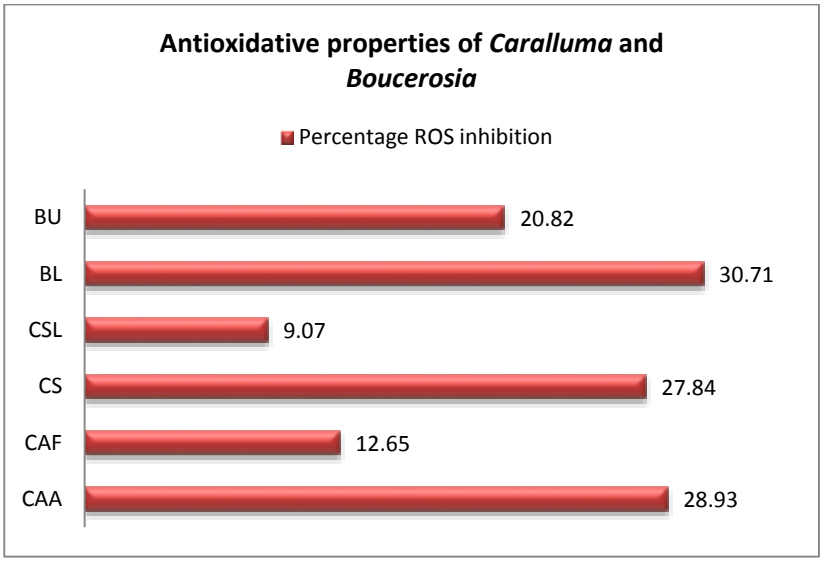


Fig 1: Percentage inhibition of ROS by selected species of *Caralluma* and *Boucerosia*

The % ROS inhibition in Raw 264.7 cells achieved by 50µg/ml of CAA, CAF, BL and BU were 68.22%, 53.86%, 40.40% and 43.42% respectively. The % ROS inhibition in Raw 264.7cells achieved by 12 µg/ml CL was 43.5% and 2.5 µg/ml of CSF was 34.60%. The half maximum ROS inhibitory concentration ($IC_{50}$) CAA and CAF were 17.442 µg/ml and 38.442 µg/ml respectively (Table 2). Among the six selected extracts, CAA shows more potent antioxidant activity and then CAF.

## Discussion

The direct scavenging effect of Methanolic extracts of *Caralluma* CAA, CAF, CL, CSF and *Boucerosia* plants BL and BU were evaluated in live cell system. For that, RAW 264.7 cells were labeled with fluorescent non polar probe DCFH-DA, a specific probe for reactive oxygen species (ROS) (Carini *et al*., 2000) as described in methodology. The underlying principle involved in this assay is that DCFH-DA is hydrolysed by esterase enzyme to become 2,7 dichlorofluorescien (DCFH), which can react with ROS in cells to form a fluorescent product DCF (Bouayed *et al.*, 2007). Whenever the cells labeled with DCFH-DA, get converted into DCFH and trapped inside the cells. A rapid increase in the intensity of DCF fluorescence was observed when the cells were incubated with $H_2O_2$ for 30 min. As the time of incubation increased radical mediated oxidation also increased. However, the pre-treatment with selected extracts decreased the DCF fluorescence in dose dependent manner. All concentrations of selected extracts exhibited the free radical scavenging effect only after 30 min of incubation comparing with control ($H_2O_2$ treatment and non samples) and among the six selected extracts, CAA shows more potent antioxidant activity and then CAF. At 50µg/ml, % ROS inhibition in Raw 264.7 cells were in decreasing order CAA>CAF> BL > BU. However, 2.5 µg/ml of CSF and 12 µg/ml of CL were enough to achieve near value of $IC_{50}$. The half maximum % ROS inhibition ($IC_{50}$) of CAA occurred at lower concentration than CAF (Table 2). Therefore, CSF showed potent scavenging activity when compared with other extracts. All the extracts have antioxidant activity in dose dependent manner and their potent direct free radicals scavenging ability in a cellular environment.

Many of the biological activities originate from antioxidant activity like antimutagensity, antitumour, anticarcinogenicity and antiaging (Sala et al. 2002). Literature revealed that phenolic compounds have potentially beneficial effect on reducing the onset of coronary heart diseases, eye diseases and atherogenic processes (Abu Bakar *et al*., 2009). These phenolic compounds have anti-oxidant activity and anti-free radical properties, play a role to reduce free radicals in the body. The majority of diseases responsible due to oxidative stress are prevented by the anti-oxidants with ROS scavenging ability (Abu Baker *et al*., 2009). These previous findings implicate that dietary polyphenolics from *Caralluma* and *Boucerosia* extracts may supply substantial antioxidants, which may provide health promoting advantage to the consumer. Phenolic compounds have been proposed for a long time, as biological anti-oxidants and their ability depends on the redox properties of their phenolic hydroxyl group which allow them to act as reducing agents, hydrogen donating antioxidants and oxygen quenchers (Rice Evans and Miller, 1996). Further studies for isolation of phenolic compounds to identify which are chiefly particularly responsible for this activity and to further investigate the mechanistic basis of this protection are in prospect

**Table 2**: Differentiation of *Caralluma* and *Boucerosia* species based on ROS inhibition (%) *vs*. concentration (at which inhibition occurred) (µg/ml).

| S.No. | *Caralluma* and *Boucerosia* species | % inhibition in ROS activity | Concentration at which inhibition occurred (µg/ml) Mean±SD |
|---|---|---|---|
| 1 | *Caralluma adscendens* var. *attenuata* (CAA) | 50 | 17.44±1.00 |
| 2 | *Caralluma adscendens* var. *fimbriata* (CAF) | 50 | 38.44±1.00 |
| 3 | *Caralluma stalagmifera* (CS) | 34.6 | 2.5±1.00 |
| 4 | *Caralluma stalagmifera* var. *longipetala* (CSL) | 43.5 | 12±1.00 |
| 5 | *Boucerosia lasiantha* (BL) | 40.4 | 50±2.00 |
| 6 | *Boucerosia umbellata* (BU) | 40.45 | 50±1.00 |

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 ANOVA analysis show significant differences ($p<0.05$) in the means of concentration (µg/ml) at which inhibition occurred.

## Conclusion

The Cellular antioxidant assay is a more biologically relevant method when compared with other antioxidant activity assays because it accounts for some aspects of uptake, metabolism, and location of antioxidant compounds within cells. Based on the results in this study, all the selected plant species could inhibit intracellular free radicals. Collectively, they can be used as potent free radical scavenger in order to maintain the balance between oxidative and anti-oxidative processes to prevent damage of biomolecules in cellular systems. Further detailed studies are needed to elucidate the mechanism of the pro-oxidant effect and to determine its relevance in vivo. Active constituents of these selected plants yet to be isolated to study antioxidant properties.

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