

## RESEARCH ARTICLE

## Anticancer and antioxidant activity of *Curcuma zedoaria* and *Curcuma amada* rhizome extracts

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### Abstract

*Curcuma zedoaria* and *Curcuma amada* rhizome solvent extracts were evaluated for their anticancer and antioxidant activity. The isopropyl extract of *C. zedoaria* exhibited high anticancer activity compared to acetone extract of *C. amada*. Crude protein of *C. zedoaria* showed good anticancer activity when compared to crude protein of *C. amada*. Acetone extract of *C. zedoaria* showed high radical scavenging activity of 88.7% and superoxide scavenging activity recording 83.15%. Acetone extract of *C. zedoaria* showed 82.5% hydroxyl radical scavenging activity.

**Keywords:** *Curcuma zedoaria*, *Curcuma amada*, isopropyl, anticancer, antioxidant, superoxide.

### Introduction

*Curcuma zedoaria* is a perennial herb belongs to the family Zingiberaceae. *Curcuma zedoaria* is used in traditional eastern medicines for digestion and an agent for purifying the blood. It is also used as antivenom for the cobra bite (Lakshmi *et al.*, 2011). *Curcuma zedoaria* has been used to treat coronary heart disease, liver cancer, anaemia, chronic, pelvic inflammation and helps prevent leukopenia due to cancer therapies (Dharmananda *et al.*, 2010). *Curcuma amada* (mango ginger) is a plant of the ginger family Zingiberaceae and is closely related to turmeric. The rhizomes are very similar to ginger but have a raw mango taste (Srinivasa Rao *et al.*, 1989). Ayurveda and Unani medicinal systems have given much importance to mango ginger as an appetizer, alexteric, antipyretic, aphrodisiac, diuretic, emollient, expectorant and laxative to cure biliousness, itching, skin diseases, bronchitis, asthma, hicough and inflammation due to injuries (Nadkarni, 1999).

The biological activities of mango ginger include antioxidant activity, antibacterial activity, antifungal activity, anti-inflammatory activity, platelet aggregation inhibitory activity, cytotoxicity, antiallergic activity, hypotriglyceridemic activity, brine-shrimp lethal activity, enterokinase inhibitory activity, CNS depressant and analgesic activity (Warrier *et al.*, 1994; Nadkarni, 1999; Joshi, 2000; Saji and Sasikumar, 2004). Against these backdrops, this study was aimed to assess the anticancer and antioxidant potential of the rhizome solvent extracts *Curcuma zedoaria* and *Curcuma amada*.

### Materials and methods

#### Chemicals

1% PBS (Phosphate buffer saline), Fluorescent probe, 2',7'-dichlorofluorescein-diacetate (DCFH-DA), Hexane, acetone, Isopropyl alcohol, Coomassie Brilliant Blue G 250 (CBB-G250), ethanol, ortho-phosphoric acid, Bovine serum albumin (BSA), Mono and dibasic sodium phosphate, phosphate buffer 6.8, 7.2. Analytical grade chemicals supplied by Loba, Hi-Media, S.D.Fine Chemicals, E.Mer ck, Qualigens and Sigma Chemicals (U.S.A) were used.

#### Source of plant material

Fresh aerial parts of *C. zedoaria* and *C. amada* were collected during June 2011 to may 2012. Subsequently, the rhizome is collected and washed thoroughly in running tap water followed by distilled water. It was then dried at room temperature and powdered using mechanical pulveriser and subjected for extraction.

#### Preparation of rhizome solvent extracts

Acetone extracts were extracted by soaking 300 g powder in 900 mL of acetone for 72 h. The extract was filtered through Whatman No. 40. The above preparation was repeated thrice. Isopropyl alcohol extracts were prepared by soaking 250 g powder in 750 mL of IPA for 72 h. The extract was filtered through Whatman No.40, The above preparation were repeated thrice. Hexane extracts were prepared by soaking 300 g powder in 750 mL of hexane for 72 h. The extract was filtered through Whatman No. 42, the above preparation were repeated thrice.

Acetone, isopropyl alcohol and hexane extracts was condensed in rotary evaporator with constant temperature at 45°C at 200 rpm and the residue was used for further analysis.

#### Extraction and estimation of protein

Protein was extracted used by GD water and phosphate buffer (pH 6.8 and 7.2). One gram of sample was grinded with 10 mL of respective solvent. Homogenates and sample was filtered and the filtrate was centrifuged at 5000 rpm for 5 min. supernatant was treated with ice cold acetone at 4°C, centrifuged at 10000 rpm for 10-15 min. supernatant was discarded and pellet was dissolved in respective solvent. The dissolved protein was estimated (Bradford, 1976). The samples were prepared in 100 mL of PBS. Dye binding solution (5 mL) was added in each tube, mixed for 5 min where red dye turns into blue due to protein binding and absorbance was read at 595 nm.

#### Anti-cancer activity

DMEM was prepared using double distilled water, and pH is adjusted to 7.2. Penicillin (100 U/mL dissolved in PBS) is added and sterilized by filtering through 0.2 µm filter in laminar flow chamber. Complete growth medium is prepared with supplements, glutamine; 2 mM, Fetal Calf Serum (FCS) (certified and heat inactivated); 10%, amphotericin; 1 mg/mL, Gentamycin; 100 µg/mL, Streptomycin; 250 U/mL, Penicillin; 250 U/mL in a 5% CO<sub>2</sub> incubator. The amount of FCS may vary depending upon the cell line used. Inhibition percentage was calculated using the following formula:

Inhibition percentage =  $(1 - T/C) \times 100$ , where, T- average absorbance of sample, C-average absorbance of control.

#### Measurement of intracellular reactive oxygen species by Fluorescent probe, 2',7'-dichlorofluorescein-diacetate

Cell suspension (0.2 mL) was taken ( $1 \times 10^6$ ) and incubated with the test sample. Cells (200 ML) were taken after treatment and diluted in 3 mL of PBS and added 300 µL DCFH-DA (1 µg/mL). Reading was taken immediately without any delay using spectrofluorometer (Excitation-485 nm, emission-530 nm) and incubated for 30 min in dark conditions (Bhosle *et al.*, 2005).

#### Fluorescence microscopy for ROS measurement

Test compound was added to suspend cells (approx.  $1 \times 10^6$  cells/mL) in 1 mL warm medium in small petri dishes. After the treatment, add DCFH-DA dye (1 µg/mL) and incubate cells at 37°C, for 30 min. Then, the cells were washed once by adding 2 mL of warm PBS and finally the cells were viewed under fluorescence microscope using blue filter.

#### Assessment of mitochondrial membrane potential

Approximately  $1 \times 10^6$  cells/mL was added in 1 mL medium in dark test tubes and the treated sample was added and incubated at different time intervals (24/48/72 h). 10 µL Rhodamine-123 dye (10 µg/mL) was added and cells were incubated at 37°C in 5% CO<sub>2</sub> for 30 min

and centrifuged at 1200 rpm for 10 min. Supernatant was discarded and 2 mL of warm PBS was added in pellet. Finally it was read at 480 and 550 nm using spectrofluorometer.

#### Fluorescence microscopy method

Approximately  $1 \times 10^6$  cells/mL was added in 1 mL medium in petri dishes, treated sample was added and incubated. 10 µL Rhodamine-123 dye (10 µg/mL) was added and incubated at 37°C with 5% CO<sub>2</sub> for 30 min and washed with 2 mL of warm PBS. Finally the cells were observed under fluorescence microscope (510-590 nm)

#### Analysis of apoptotic morphological changes

Approximately  $1 \times 10^6$  cells/mL was added in 1 mL medium in petri dishes, treated sample was added and incubated. 50 µL/mL EB/AO was added and incubated at 37°C with 5% CO<sub>2</sub> for 30 min and washed with 2 mL of warm PBS. Finally the cells were observed under fluorescence microscope (510-590 nm) (Coligan, *et al.*, 1995).

$$\% \text{ apoptotic cells} = \frac{\text{Total No. of red cells}}{\text{Total No. of red and green cells}} \times 100$$

#### Dot plot rapid assay

The rapid screening assay was performed by the method proposed by Soler-Rivas *et al.* (2000). Aliquots of plant extracts (3 µL) were spotted carefully on TLC plates and dried for 3 min. The sheets bearing the dry spots were placed upside down for 10 sec in 0.4 mM DPPH solution and the layer was dried. The stained silica layer revealed a purple background with yellow spots, which showed radical scavenging capacity. The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and is purple in color. The color turns from purple to yellow as the molar absorptivity of the DPPH radical at 517 nm reduces from 9660 to 1640 when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H.

The resulting decolorization is stoichiometric with respect to number of electrons captured. Antioxidant compounds may be water-soluble lipid soluble, insoluble, or bound to cell walls. Hence, extraction efficiency is an important factor in quantification of antioxidant activity of foods. The extracts (20 µL) were added to 0.5 mL of methanolic solution of DPPH and 0.48 mL of methanol. The mixture was allowed to react at room temperature for 30 min. Methanol served as the blank and DPPH in methanol, without the extracts, served as the positive control. After 30 min of incubation, the discoloration of the purple colour was measured at 518 nm in a spectrophotometer (Genesys 10-S, USA). The radical scavenging activity was calculated as follows:

$$\% \text{ Inhibition} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100$$

### Superoxide radical scavenging activity

Measurement of superoxide anion scavenging activity was performed based on the method described by Winterbourne *et al.* (1975). The assay mixture contained sample with 0.1 mL of Nitro blue tetrazolium (1.5 mM NBT) solution, 0.2 mL of EDTA (0.1M EDTA), 0.05 mL riboflavin (0.12 mM) and 2.55 mL of phosphate buffer (0.067 M phosphate buffer). The control tubes were also set up where in DMSO was added instead of sample. The reaction mixture was illuminated for 30 min and the absorbance at 560 nm was measured against the control samples. Ascorbate was used as the reference compound. All the tests were performed in triplicate and the results averaged. The percentage inhibition was calculated by comparing the results of control and test samples.

### Hydroxyl radical scavenging activity

The scavenging capacity for hydroxyl radical was assayed according to the method of Yu *et al.*, (2002). About 60 µL of FeSO<sub>4</sub>.7H<sub>2</sub>O (1 mM) was added to 90 µL of aqueous 1, 10-phenanthroline (1 mM). About 2.4 mL of 0.2 M Na<sub>2</sub>HPO<sub>4</sub> (pH 7.8) was added to the mixture, followed by the addition of 150 µL of H<sub>2</sub>O<sub>2</sub> (0.17 M) and 1.5 mL of different concentrations of extract (250-1000 µL) in sequence. The mixture was incubated for 5 min at room temperature. The absorbance of the mixture was read at 560 nm after 5 mm on the UV/visible spectrophotometer against blank (distilled water).

$$\% \text{ scavenging activity} = \frac{\text{Abs. of control} - \text{Abs. of test}}{\text{Abs. of control}} \times 100$$

### Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) to evaluate the significant of difference of means of various treatment groups using SPSS statistical software package (Version: 10). The values are presented as mean ± S.D and P<0.05.

## Results and discussion

The cell viability and cytotoxicity activity were determined by MTT assay and measurement of intracellular ROS is performed by DCFH-DA. Assessment of mitochondrial membrane potential and analysis of apoptotic morphological changes in cultured cells is evaluated by acridine orange/ethidium bromide (AO/EB) staining. Different concentrations of *C. zedoaria* and *C. amada* extracts were added to human lung cancer cell line (NCI-H460) and anticancer activity was detected. Free radicals are chemical species containing one or more unpaired electrons that makes them highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability. Free radicals contribute to more than 100 disorders in humans including atherosclerosis, arthritis and ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis and cancer (Nagarajan *et al.*, 2012).

Compared to all extracts, crude proteins of *C. zedoaria* and *C. amada* were studied against cell cytotoxicity (24 h) by MTT assay. The percentage of cytotoxicity was high in *C. zedoaria* crude protein compared to *C. amada* crude protein. Effect of different extracts and crude proteins on percentage cell viability (24 h) in human lung cancer cells (NCI-H460) is shown in figures 1 to 3.

Fig. 1. Fluorescence microscopy showing apoptotic cell death.

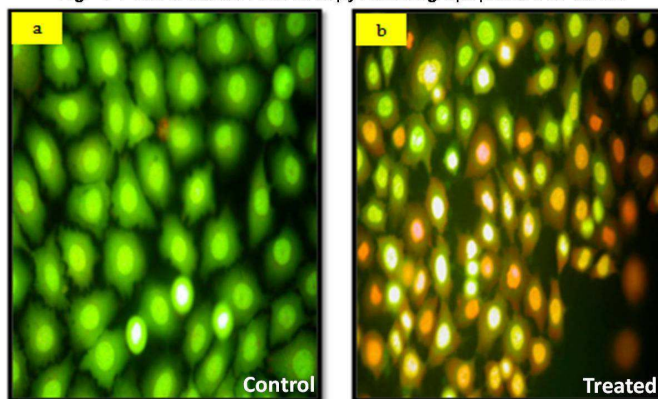


Fig. 2. Cell viability of extracts and crude proteins of *C. zedoaria* and *C. amada*.

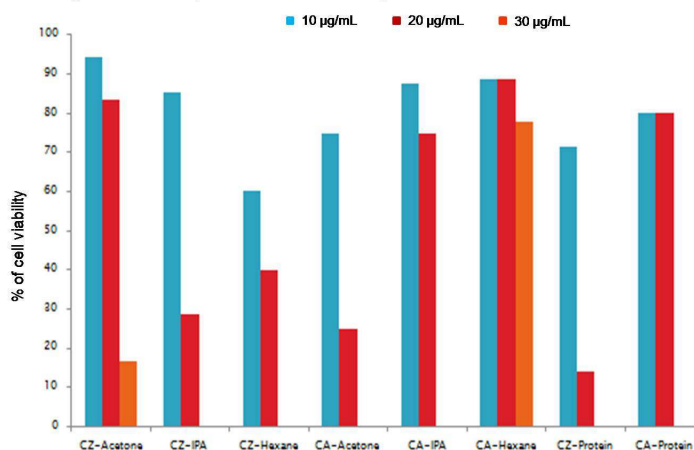
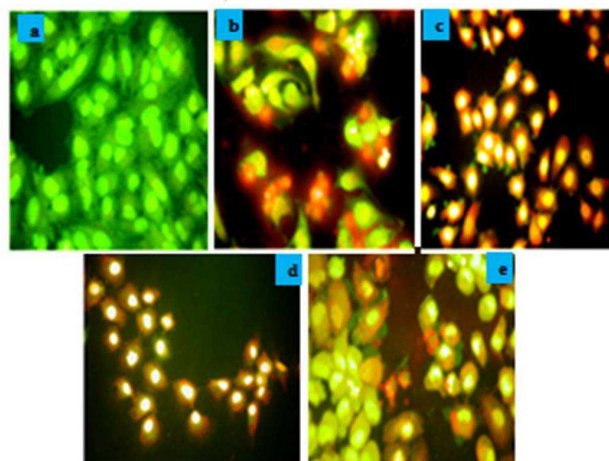


Fig. 3. Apoptotic morphological changes in selected extracts and crude proteins on NCI-H460 cells.



a. Control, b. *C. zedoaria* IPA; 30 µg/mL, c. *C. amada* acetone; 25 µg/mL, d. *C. amada* crude protein; 20 µg/mL, e. *C. zedoaria* crude protein; 35 µg/mL).

Table 1. DPPH rapid scavenging activity of various extracts of *C. zedoaria* and *C. amada*.

Conc. ( $\mu\text{g/mL}$ )	% of Inhibition					
	Acetone		IPA		Hexane	
	<i>C. zedoaria</i>	<i>C. amada</i>	<i>C. zedoaria</i>	<i>C. amada</i>	<i>C. zedoaria</i>	<i>C. amada</i>
10	19.5	2.81	7.54	3.84	2.43	4.46
20	35.29	5.82	18.49	6.92	6.55	5.26
30	58.06	19.27	30.75	17.5	16.1	19.92
40	68.75	25.7	33.01	25.5	22.09	20.67
50	79.44	40.16	48.67	42.6	23.09	24.2
60	88.71	58.63	60.37	50.76	38.76	31.47
IC 50 value ( $\mu\text{g/mL}$ )	25.83	51.16	49.69	59.10	77.39	93.32

Values are means of three replicates and the values in the columns with same letter are not significantly different at  $p < 0.05$  level.

Table 2. Superoxide radical scavenging activity of various extracts of *C. zedoaria* and *C. amada*.

Conc. ( $\mu\text{g/mL}$ )	% of Inhibition					
	Acetone		IPA		Hexane	
	<i>C. zedoaria</i>	<i>C. amada</i>	<i>C. zedoaria</i>	<i>C. amada</i>	<i>C. zedoaria</i>	<i>C. amada</i>
10	47.36	36.84	42.1	5.26	10.52	0
20	55.78	47.36	52.63	26.31	21.05	15.78
30	58.94	57.89	57.89	31.5	31.5	26.31
40	76.31	63.15	68.42	42.1	42.1	42.1
50	83.15	68.42	73.68	57.89	52.6	42.1
IC 50 value ( $\mu\text{g/mL}$ )	17.92	25.911	19	43.18	47.52	59.38

Table 3. Hydroxyl radical scavenging activity of various extracts of *C. zedoaria* and *C. amada*.

Conc. ( $\mu\text{g/mL}$ )	% of Inhibition					
	Acetone		IPA		Hexane	
	<i>C. zedoaria</i>	<i>C. amada</i>	<i>C. zedoaria</i>	<i>C. amada</i>	<i>C. zedoaria</i>	<i>C. amada</i>
10	23.08	19.04	25.39	17.46	14.28	9.52
20	41.26	23.8	31.7	20.63	22.2	20.63
30	55.55	26.98	46.03	28.57	26.98	23.8
40	63.49	46.03	55.5	39.57	34.9	28.57
50	82.5	66.6	71.42	50.7	53.96	44.44
IC 50 value ( $\mu\text{g/mL}$ )	27	37.53	36.03	49.30	46.33	56.25

#### Measurement of intracellular ROS by DCFH-DA

The effect of antioxidant or free radical compounds on DCFH-DA was measured against the fluorescence of the provided DCF standard (Brandt *et al.*, 1965; Bass *et al.*, 1983). Effect of selected extracts on ROS level generation in NCI-H460 cells is shown in figure 4. Results showed that crude proteins of both the plants exhibited better activity when compared to extracts. The selected IPA extract of *C. zedoaria* showed better activity when compared to acetone extract of *C. amada* and in case of crude protein; *C. zedoaria* exhibited high activity compared to crude protein of *C. amada*.

#### Assessment of mitochondrial membrane potential

In this study, the best extract and crude proteins in each species is selected for mitochondrial membrane potential. Effect of selected extracts and crude protein on mitochondrial membrane potential alterations in NCI-H460 cells are shown in figure 5. The present study showed that crude proteins of both the plants exhibited better mitochondrial membrane potential compared to organic extracts.

The selected IPA extract of *C. zedoaria* showed high mitochondrial membrane potential when compared to acetone extract of *C. amada* and in the case of crude protein; *C. zedoaria* showed high mitochondrial membrane potential when compared to crude protein of *C. amada*.

#### Analysis of apoptotic morphological changes in cultured cells by AO/EB staining

Results showed that crude proteins of both the plants exhibited a better anticancer activity when compared to extracts. The selected IPA extract of *C. zedoaria* showed better anticancer activity when compared to acetone extract of *C. amada* and in the case of crude protein *C. zedoaria* has good anticancer activity when compared to crude protein of *C. amada* (Fig. 3). At low concentrations, a majority of AO+, EB- cells were detected, indicating apoptotic granulation and an intact cell membrane. As the concentration increases late-apoptotic (AO+, EB+) and necrotic (AO-, EB+), cell populations appeared (Réthy *et al.*, 2006).

Fig. 4. Effect of selected extracts and crude proteins against Reactive Oxygen Species.

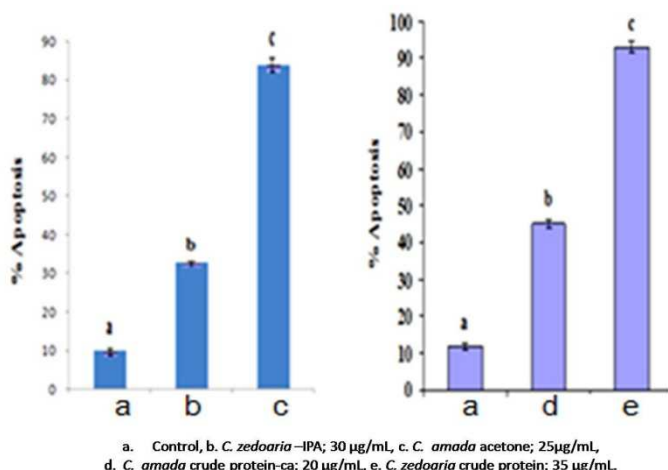
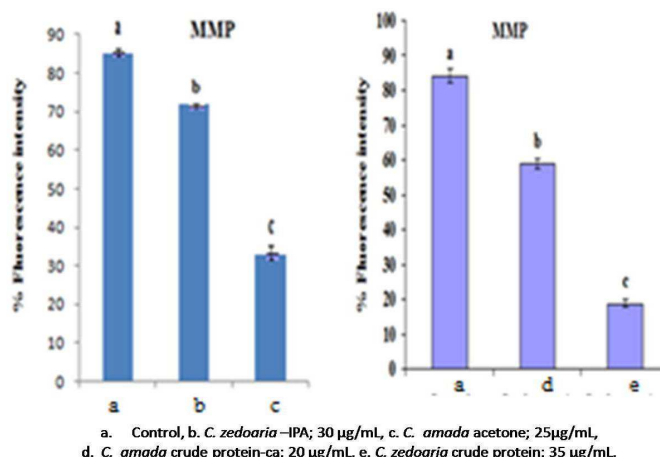


Fig. 5. Effect of selected extracts and crude proteins on mitochondrial membrane potential alterations in NCI-H460 cells.



### Dot plot rapid assay with DPPH

To make a semi-quantitative visualization, different extract fractions from *C. zedoaria* and *C. amada* were detected in TLC plates by DPPH staining method which is shown in figure 6. For the rapid screening each diluted sample was applied as a dot on a TLC plate that was then stained with DPPH solution.

The appearance of white colour in the spots reveal a potential value for the indirect evaluation of the different extracts from *C. zedoaria* and *C. amada* in the dot plot (Soler-Rivas *et al.*, 2000; Chang *et al.*, 2002). The method is typically based on the inhibition of the accumulation of oxidized products since the generation of free radicals is inhibited by the addition of antioxidants.

### DPPH rapid scavenging activity

It was found that acetone extract (60 µg/mL) of *C. zedoaria* recorded high radical scavenging activity of 88.7±0.57%, followed by IPA extract of *C. zedoaria* (60.3 ±0.90%), acetone extract of *C. amada* (58.6 ± 1.04%). IPA extract of *C. amada* (50.76±2.9%) showed less radical scavenging activity in comparison with *C. zedoaria*, with hexane extracts of *C. zedoaria* (38.7± 0.61%), *C. amada* (31.4 ± 0.62%) had the lowest radical scavenging activity (Table 1). According to Dhal and Sahu (2012) DPPH free radical scavenging activity of the enzymatic extracts in *C. zedoaria*, was high recording 39.7±0.2% at the concentration of 200 µg/mL.

### Superoxide radical scavenging activity

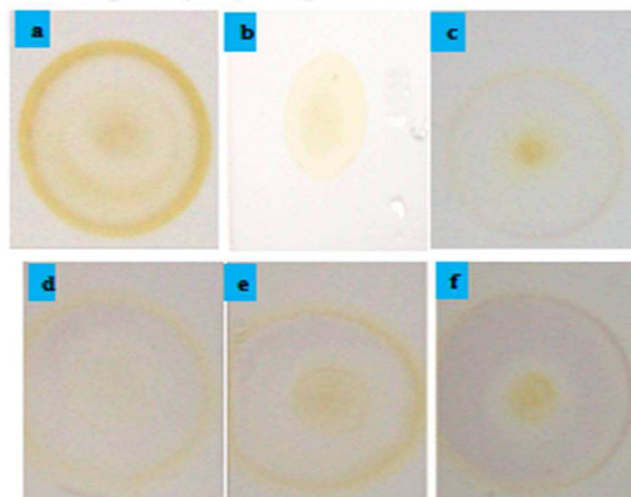
When compared to all the extracts, *C. zedoaria* acetone extract (5 mg/mL) recorded 83.15±3.03% scavenging activity. This value was slightly lower than the extent of scavenging activity induced by *C. amada* acetone extract 68.42±0.72% (Table 2).

### Hydroxyl radical scavenging activity

Different extracts of *C. zedoaria* and *C. amada* showed a concentration-dependent scavenging effect towards FeSO<sub>4</sub> generated-hydroxyl radicals.

At a concentration of 5 mg/mL, the scavenging activity of *C. zedoaria* acetone extract (82.5 ±3.01%) was slightly higher than the scavenging activity of *C. amada* acetone extract (66.6±0.99%). The percentage of inhibition and IC<sub>50</sub> values of hydroxyl radical scavenging activities of different extracts of *C. zedoaria* and *C. amada* were evaluated. Different extracts of *C. amada* acted as a weak scavenger of hydrogen peroxide when compared to different extracts of *C. amada* (Table 3). The findings are in accordance with Dhal and Sahu (2012) who reported hydroxyl radical scavenging activity was around 48.23±0.05% in the enzymatic extracts as compared to ascorbic acid (standard), which was found to be 60.33±0.40% at the concentration of 50 µL.

Fig. 6. Dot plot rapid assay of selected extracts in DPPH.



a. *C. zedoaria* acetone extract, b. *C. zedoaria* IPA extract, c. *C. zedoaria* hexane extract, d. *C. amada* acetone extract, e. *C. amada* IPA extract, f. *C. amada* hexane extract.

### Conclusion

Based on this study both *C. zedoaria* and *C. amada* have potential anticancer and antioxidant activity. To conclude, further investigations are necessary to find out the active compounds responsible for mechanisms and pathways for its anticancer and antioxidant activity.

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