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Antioxidant and antimicrobial activities of callus culture and leaf extracts of wild *Crotalaria retusa* (rattlepod)

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Abstract
The present study describes the *in vitro* antimicrobial and antioxidant activity of methanol extracts of callus culture and leaves of wild plants of *Crotalaria retusa* Linn. Callus cultures from leaves were initiated on MS media supplemented with various combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-Benzyloaminopurine (BAP). Optimum callus regeneration was obtained on MS medium supplemented with 0.5 mg/L BAP and 1.0 mg/L 2,4-D with 50% cultures response at 20 days. The wild plant extracts (WPE) showed the highest antibacterial activity with zone of inhibition (ZI) of 13.83±0.33 mm on *Pseudomonas aeruginosa* (Gram negative), as the most affected organism. The callus culture extract (CCE) gave the lowest MIC value of 0.78 mg/ mL for most of the bacteria and fungi and the lowest MBC values of 0.78 mg/ mL and 1.56 mg/ mL against bacteria and fungi, respectively. The WPE showed a higher antioxidant activity (91%) than the CCE (58%). Comparable total phenol content was observed in the WPE and CCE, whereas the WPE showed a higher flavonoids content (6.56±1.52 mg equivalent of quercetin/g of extract) than the CCE (3.22±1.01 mg equivalent of quercetin/g of extract). The *in vitro* propagation protocol employed in this study supported the rapid development of calluses from *Crotalaria retusa* seed explants which may offer an alternative to the exploitation of this valuable medicinal plant for its antimicrobial and antioxidant properties via optimization of the tissue culture technique.

Keywords: *Crotalaria retusa*; *In vitro* propagation; Callus culture; Antimicrobial activity; Antioxidant activity

INTRODUCTION

The rattlepod, *Crotalaria retusa* Linn., is a medicinal plant belonging to the old family Fabaceae (Daimon *et al.*, 2002; Andreia *et al.*, 2008). However, according to the APG (Angiosperm Phylogeny Grouping), it now belongs to the upgraded family Papilionaceae. The common name rattle-pod is derived from the fact that the seeds become loose in the pod as they mature, and rattle when the pod is shaken (Thomas, 2003). The Genus *Crotalaria* is a group of herbaceous plants and woody shrubs, with some 600 or more species of *Crotalaria* described worldwide, mostly from the tropics and with at least 500 species from Africa. *Crotalaria retusa* has been reported to have therapeutic properties which include: antileukemic, antitumour, antispasmodic, antineoplastic, cardio-depressant and hypotensive properties (Morris, 1999; Sridhar and Bhagya, 2007; Nuhu *et al.*, 2009). The plant is known for its broad spectrum antimicrobial activity and antioxidant potentials (Ayoola *et al.*, 2008; Bellary *et al.*, 2012). Antioxidants are important in the prevention of human

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diseases. Antioxidant compounds may function as free radical scavengers, complexing agents for pro-oxidant metals, reducing agents and quenchers of singlet oxygen formation (Bell, 1980; Constable *et al.*, 1974; Rice-Evans *et al.*, 1997). Compounds that scavenge free radicals have great potential in ameliorating these diseases (Kirakosyan *et al.*, 2003). It is reported that phenol compounds in plants possess strong antioxidant activity and could help to protect cells against oxidative damage caused by free radicals (Kahkohen *et al.*, 1999). Plant-derived polyphenols are of great importance because of their potential antioxidant and antimicrobial properties. Phenol compounds exhibit a considerable free-radical scavenging (antioxidant) activity, which is determined by their reactivity as hydrogen- or electron-donating agents, the stability of the resulting antioxidant-derived radicals, their reactivity with other antioxidants and finally their metal chelating properties (Ang-Lee, 2001; Wojdylo *et al.*, 2007). However, no report exist in literature on comparative antimicrobial and antioxidant properties of wild and callus culture of *Crotalaria retusa*.

Thus, the objective of the present study was to determine the antimicrobial and antioxidant activities of the methanol extract of the wild plant and callus cultures of *Crotalaria retusa*.

EXPERIMENTAL

Plant collection and preparation. The plant (*Crotalaria retusa*) was collected during the month of December 2012 from the Botanical Garden, University of Ibadan, Ibadan. Nigeria. Identification and authentication of the plant was done at the Forest Herbarium, Ibadan by Mr. O. S. Shosanya, where voucher specimen was deposited in with FHI number, 109847. Healthy whole plants were collected into sacks. The samples were then transported to the laboratory where the leaf samples were separated and kept at room temperature until processing. The whole leaf samples of *Crotalaria retusa* were made clean of contaminants and dried under shade at room temperature. The dried leaf samples were finely milled into powder using a mechanical grinder. The powdered samples of *Crotalaria retusa* were then packed in a sealed plastic container until extraction.

***In vitro* propagation.** The ex-plant (seed) underwent series of disinfection using ethanol, sodium hypochlorite, Tween 20 and distilled water, to clean and clear off any possible contamination or to minimize the rate of contamination. The properly disinfected ex-plant was inoculated into the MS medium (Murashige and Skoog, 1962) and supplemented with growth regulators, such as the Benzyl amino-purine (*BAP*) and 2,4-Dichlorophenoxyacetic acid (2,4-D), for multiplication and regeneration into callus. The *in vitro* callus of this plant species was then used for the comparative studies.

Extraction and solvent partitioning. The dried powder samples of *Crotalaria retusa* (500 g) were extracted with methanol (4 L) for 72 h until complete extraction. After extraction, it was filtered with defatted cotton wool and evaporated *in vacuo*. The extraction procedures were followed thrice and the concentrated extracts were combined (42.09 g). Also, about 1 g of the dried and milled callus of *in vitro* cultivated *Crotalaria retusa* was extracted with 55 mL of methanol, to obtain the crude methanol callus extract (0.35 g). The extracts were refrigerated until when needed for bioassays. The percentage yield of the various extracts was calculated.

Sources of test micro-organisms and preparation of standard inocula. Pure cultures of bacteria: *Staphylococcus aureus* (UCH 7580), *Escherichia coli* (UCH 6504), *Klebsiella pneumonia* (UCH 7545), *Pseudomonas aeruginosa* (UCH 7450), *Bacillus subtilis* (UCH 8536), and *Proteus vulgaris* (UCH 7206), were maintained on

Mueller-Hinton agar slants at 4^{o}C, while the fungi: *Candida albicans* (UCH 8128) and *Aspergillus flavus* (UCH 9892) were maintained on Sabourauds' Dextrose Agar at 4^{o}C. The microbes were obtained from the Medical Microbiology Department, College of Medicine, University College Hospital (U.C.H.), Ibadan, Nigeria.

Antimicrobial susceptibility tests. The antimicrobial tests were based on agar diffusion method (Bauer *et al.*, 1966) using bacterial and fungal cell suspension whose concentration was equilibrated to a 0.5 McFarland standard, however with slight modifications. Inoculum of each bacterial and fungal suspension was spread on a Mueller-Hinton agar plate and Sabouraud Dextrose agar (SDA) plate, respectively. Plates were incubated at room temperature for five minutes. A Sterile cork borer was used to bore holes (6 mm diameter) and filled with the extracts which were dissolved in Di-methyl sulfoxide (DMSO) at 10 mg/ mL. They were left on the bench for 30 min to ensure adequate diffusion of the extracts. Agar plates with DMSO only were used as negative control, the antibiotic disc ring was used as the positive control for bacteria while fluconazole was used as the positive control for fungi. The inoculated plates were incubated at 37°C for 24 h for clinical bacterial strains and at 25°C for 48 h for fungi. Antimicrobial activity was evaluated by measuring the inhibition zone against test microorganisms to the nearest mm along two axes and the mean of the two readings was then obtained. The experiment was performed in triplicate.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) were also determined for the bacterial and the fungal strains that were sensitive to the extracts in the agar diffusion assay. A broth macro dilution and agar diffusion methods were used for the study. Serial two-fold dilutions of each extract were prepared in 10% (v/v) di-methyl sulfoxide (DMSO), to obtain the following concentrations (mg/mL): 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 and 0.39. A volume of 0.5 mL of each dilution was added to 0.5 mL of Mueller-Hinton broth. These were inoculated with 0.5 mL of culture of the test bacterial and fungal strains. After incubation of the cultures at 37^{o}C (for bacteria) and 25^{o}C (for fungus) the MIC values were determined as the lowest concentration of the extract that demonstrated no visible growth. The MBC was determined using the agar diffusion method from the MIC by plating out the concentration as determined for the extracts (Thitilerdecha *et al.*, 2008).

Determination of total phenolic content. The total phenolic compound in each was determined by using the Folin-Ciocalteau reagent method as developed by Velioglu *et al.* (1998) with slight modifications. Briefly, 0.75 mL of 10-fold diluted Folin-Ciocalteu reagent and100 µg/ mL of methanol extracts were placed in a test tube. The mixture was mixed and allowed to stand at room temperature for 5 min. A volume of 0.75 mL of a 6% (w/v) Na_2CO_3 solution was added. The mixture was homogenized and all the test tubes were allowed to stand at room temperature for 90 min. The absorbance was measured for all solutions using a 725s UV-VIS spectrophotometer at constant wavelength 725 nm. Gallic acid was used as the standard for a calibration curve. The total phenolic content was expressed as gallic acid equivalent (GAE) mg/g edible portion using the following equation derived from the calibration curve: $y = 14.289x + 0.1042$, $R^2 = 0.9831$, where y is the absorbance and x is the gallic acid equivalent (mg).

Determination of total flavonoids content. The total flavonoid content of the extracts was estimated by the aluminium chloride colorimetric method of Hossain et al. (2011)

with minor modifications. Briefly, 0.5 mL solution of each plant extract (at 100 µg/ mL) was separately mixed in a tube containing 1.5 mL of methanol followed by 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. The mixture was left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm wavelength with a 725s UV-VIS spectrophotometer. Quercetin at concentrations of 6.25 to 100 µg/mL was used as a standard to quantify the total flavonoid content from the calibration curve with the equation: $y = 0.0157x + 0.0451$, $R^2 = 0.9973$, where *y* is the absorbance and x is the quercetin equivalent (mg). Results were expressed in microgram quercetin equivalents (QE)/gram. The estimation of total flavonoid content in the extract was carried out in triplicate and the results averaged.

DPPH radical-scavenging activity. The free radical scavenging activity was determined by previously reported methods (Shimada *et al.*, 1992; Yang *et al.*, 2006) with slight modifications. The methanol extracts were dissolved with methanol to prepare various sample solutions at 100, 80, 60, 40, 20, 10, and 5 µg/ mL. Each extract solution (2 mL) was mixed with 1 mL of methanol solution containing DPPH radicals, with a final concentration of 0.2 mM DPPH. Quercetin and Gallic acid were used as standard reference. The mixture was shaken vigorously and maintained for 30 min in the dark and absorbance was measured at 517 nm. A decreasing absorbance of the DPPH solution indicates increase in DPPH scavenging activity. The absorbance of the control was obtained by replacing the sample with methanol. The percentage scavenging activity was calculated using the formula; Scavenging activity (%) = [(A_{517} of control - A_{517} of sample) / A_{517} of control] ×100. DPPH radical scavenging activity was measured in duplicate, and the values are reported as the average.

Statistical analysis. All experimental results were expressed as mean ± standard error of the mean of obtained measurements. The results with $P < 0.05$ were regarded to be statistically significant. The IC_{50} values were calculated by linear regression analysis. It was defined as the effective concentration of sample to obtain 50% antioxidant or metal chelating activity.

RESULTS AND DISCUSSION

Callus induction. In this study, callus development was initially tested using seed and nodal explants obtained from naturally growing wild plant of *Crotalaria retusa*. The explants were cultured on MS medium supplemented with BAP and 2,4-D (Table 1). Among all the treatments, T2 recorded a significantly moderate level of callus growth response to the seed-explant (Table 2). Treatment 1 showed no callus growth. Callus formation in other treatments was observed as friable and yellow callus (Figure 1). The nodal explants exhibited notorious contamination whereas the seed explants, produced from the fifth week of culture with average and maximum development observed after eight and eleven weeks, respectively (Table 2). Observations from the callusing of *C. retusa* indicated that BAP and 2,4-D played crucial roles in callus growth. The BAP plays a role in promoting shoot proliferation whereas 2,4-D supports root proliferation, necessitating a balance between both plant growth promoting hormones (PGPHs) to induce callus growth. The seed-explants cultured on MS medium supplemented with BAP (0.5 mg/L) and 2, 4–D (1.0 mg/L) treatment gave rise to moderately flourishing friable yellow callus of *C. retusa* with a response of 55.55±0.18% (Figure 1).

Extraction yield. The dried masses of the crude methanol extracts were obtained by evaporating the solvent in the extracts and calculating the percentage yield. The

percentage yield of the wild plant extract (WPE) and callus culture extract (CCE) of *Crotalaria retusa* were 8.42% and 46.67%, respectively.

Antimicrobial activity. The *in vitro* antimicrobial activity of *C. retusa* wild plant and callus culture extracts were tested against clinical isolates of different gram positive and gram negative bacteria as well as fungi pathogens. Results obtained indicated that the two extracts were effective against gram negative, gram positive, and against *Candida albicans*. The WPE and CCE showed antimicrobial activity against all the microorganisms used with the exception of *Aspergillus flavus*. The inhibition zones of the microorganisms sensitive to the WPE and CCE ranged from 10.20±0.17 to 13.83±0.33 mm, respectively (Table 3). The highest inhibition zone (13.83±0.33 mm) was observed in WPE against *Pseudomonas aeruginosa* (Table 3). However, there were no significant differences in the results obtained for the WPE and CCE with respect to diameter of zone of inhibition. These results are of interest since they have been obtained with methanol crude extracts and are not pure products. Thus, the WPE and CCE of *C. retusa* could be considered to have a good potency level. The CCE had the lowest MIC value of 0.78 mg/ mL for three out of the six bacteria (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*) strains tested and one fungus (*Candida albicans*). Also, the CCE had the lowest MBC value of 0.78 mg/ mL for *Pseudomonas aeruginosa*, a gram-negative bacterium.

The antimicrobial activity may be due to the phytochemical constituents of the plant. These are known to be responsible for different activities such as antioxidant, antimicrobial, antifungal and anticancer activity (Hossain and Nagooru, 2011; Kokate, 1997). Saponins are reported to be involved in plant disease resistance because of their antimicrobial activities (Anyasor *et al.*, 2010). Several authors have already reported on poly-phenols exhibiting a wide range of biological activities such as antioxidant, anti-inflammatory, antimicrobial, anti-angiogenic, anticancer and anti-allergic activities (Chao *et al.*, 2002; Barile *et al.*, 2007; Ayoola *et al.*, 2008; Igbinosa *et al.*, 2009). The antimicrobial activity of these extracts could be due to these bioactive compounds. The results that were obtained from the preliminary assay (zone of inhibition evaluation) for both the CCE and WPE were comparable, whereas the MIC and MBC revealed that the CCE exhibited better antimicrobial activity with lower MIC and MBC values than the WPE.

Antioxidant activity. The total phenol and flavonoid contents of the extracts were estimated from the calibration curves for Gallic acid and for quercetin, respectively. The total phenolic and flavonoid contents of crude plant extracts are shown in Table 4. The differences in total phenolic and flavonoid contents between the wild plant extract and the callus culture extract were significant. The TPC and TFC of the WPE and CCE were in the range of 7.6±0.84 to 8.7±0.59 mg GAE/g and 6.6±1.52 to 3.2±1.01 mg QE/g, respectively. The radical scavenging activities of the extracts based on DPPH assay are presented in Figure 2. The scavenging effects of crude methanolic extracts of the wild plant and callus cultures on DPPH radicals increased with concentration. The WPE showed higher free radical scavenging activity (IC_{50}: 13.4±0.09 µg/mL) than the CCE which showed moderate antioxidant capacity (IC_{50}: 60.9±1.80 µg/mL). The antioxidant capacity of standards quercetin and gallic acid evaluated by their IC_{50} values were 3.0±0.0043 and 3.1±0.0029 µg/mL, respectively (Table 4).

Table 1. List of MS media supplemented with different growth regulators used for *Crotalaria retusa* callus cultures

No.	Media Codes	Growth regulators concentration (mg/L)
1	T1	MS + 0.5 BAP + 0.5 2,4-D
2	T2	MS + 0.5 BAP + 1.0 2,4-D
3	T3	MS + 0.5 BAP + 1.5 2,4-D
4	T4	MS + 0.5 BAP + 2.0 2,4-D
5	T5	MS + 0.5 BAP + 2.5 2,4-D

Table 2. Callusing frequency of seed-explant of *Crotalaria retusa* cultured on MS media supplemented with different combinations of growth regulators

Media codes	Callus initiation in days	% Response in Callus Development	Callus type
T1	-	0.00±0.00	-
T2	20	55.55±0.18	Fragile
T3	12	33.33±0.17	Fragile
T4	15	33.33±0.17	Fragile
T5	12	22.22±0.15	Fragile

Data are presented as means of 10 replicates per treatment (± S.E. M).

Table 3 Antimicrobial activity of the extracts of *Crotalaria retusa* leaves (10 mg/mL) against tested microorganisms.

Microorganisms	Zone of Inhibition (mm)		MIC (mg/mL)		MBC (mg/mL)	
	WPE	CCE	WPE	CCE	WPE	CCE
Escherichia coli	12.17±0.44	11.50±0.29	3.13	0.78	6.25	1.56
Klebsiella pneumonia	11.17±0.44	10.50±0.29	6.25	1.56	12.5	3.13
Pseudomonas aeruginosa	13.83±0.33	11.80±0.33	3.13	0.78	6.25	0.78
Proteus vulgaris	11.17±0.17	10.20±0.17	1.56	6.25	3.13	12.5
Gram-positive bacteria						
Staphylococcus aureus	10.83±0.17	12.30±0.60	6.25	0.78	12.5	1.56
Bacillus subtilis	12.33±0.88	10.80±0.17	3.13	1.56	6.25	3.13
Fungi						
Aspergillus flavus	0.00±0.00	0.00±0.00	ND	ND	ND	ND
Candida albicans	10.50±0.29	11.70±0.50	3.13	0.78	6.25	1.56

WPE: wild plant extract, CCE: callus culture extract, DMSO- Dimethyl-sulfoxide. Data are presented as means of three replicates ± S.E.M., 0.00-No inhibition zone.

TABLE 4. IC_{50} value, Total Phenolic and Flavonoid Contents of *Crotalaria retusa* Plant

Samples	IC_{50} (µg/mL)	TPC (mg GAE/g)	TFC (mg QE/g)
WPE	13.4±0.09	7.6±0.84	6.6±1.52
CCE	60.9±1.80	8.7±0.59	3.2±1.01
Quercetin	3.0±0.0043	-	-
Gallic Acid	3.1±0.0029	-	-

WPE: wild plant extract, CCE: callus culture extract. Data are presented as means ± S.E.M (n=3).

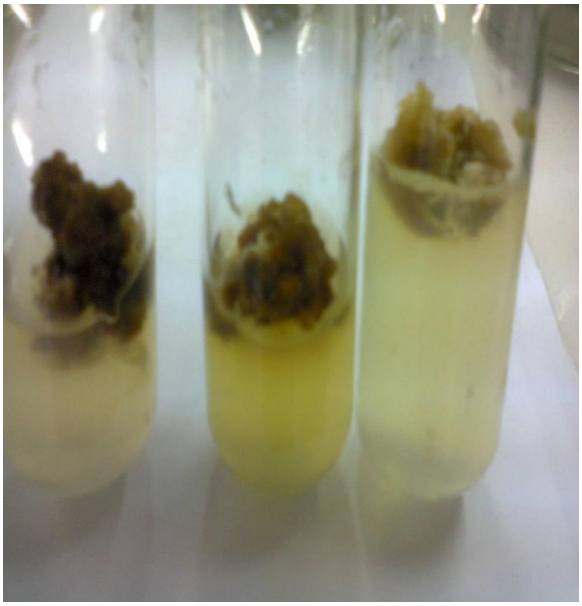

Figure 1 Yellow friable callus of *Crotalaria retusa* showing maximum response at 11 weeks

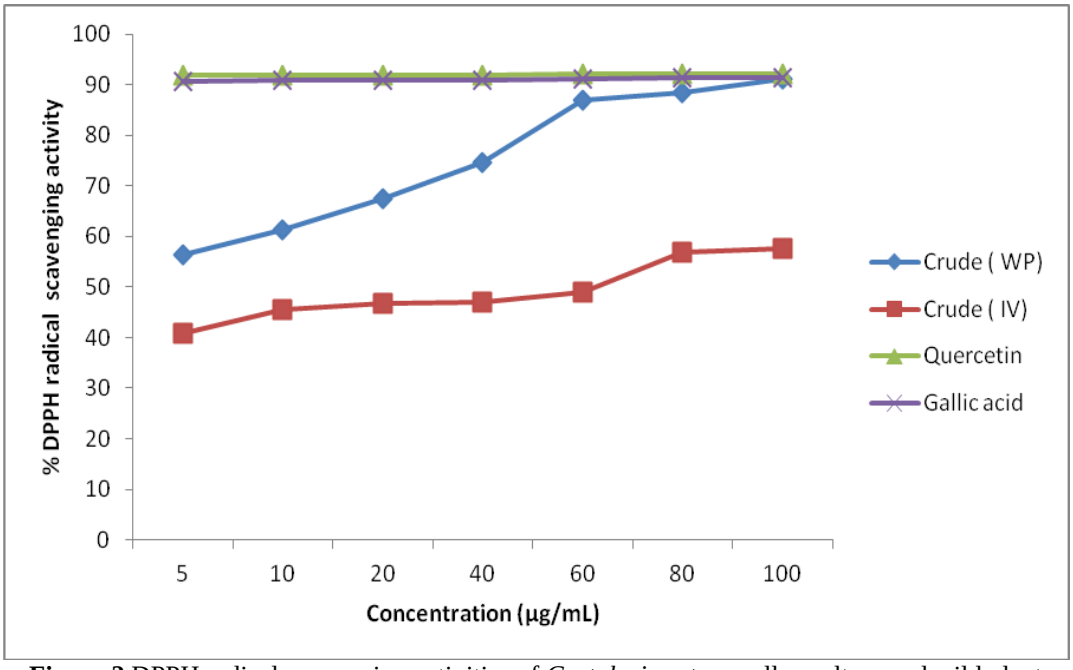

Figure 2 DPPH radical-**s**cavenging activities of *Crotalaria retusa* callus culture and wild plant

The antimicrobial activity observed in the CCE over WPE could be due to higher phenolic contents in the callus. This trend in which extracts with high phenolic contents exhibited higher antimicrobial activity had been reported in several literatures (Papadopoulou *et al.*, 2005; Alberto *et al.*, 2006; Thitilertdecha *et al.*, 2008). In part, the variation observed in the phenolic contents of the WPE and CCE could be due to the aseptic micro-propagation factors which may modify the active constituents of the plant positively.

Further, on the basis of DPPH radical scavenging activity, higher antioxidant potential was observed in the WPE compared to the CCE probably due to a higher proportion of flavonoids content in the wild plant. The presence of higher flavonoids contents in the WPE could suggest that most of the phenolic contents in the wild plant were flavonoids, whereas the micropropagation technique might have favoured the accumulation of other phenolic constituents different from flavonoids in the callus culture. Flavonoids had been explained as a group of polyphenols with known properties such as free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action (Frankel, 1995). The biological activity of flavonoids has been attributed to their ability to exert antioxidant action either through their ability to scavenge reactive oxygen species or through their possible influences on intracellular redox status (Lard *et al.*, 2006; Rice-Evans *et al.*, 1996).

There was no correlation between total phenolic contents and antioxidant activity of the plant extracts but the WPE with higher total flavonoids content also exhibited higher antioxidant potential. Although some studies have demonstrated a correlation between phenolic content and antioxidant activity (Yang *et al.*, 2006), our result is in agreement with several other findings in which the antioxidant activity had correlation only with the total flavonoids content. In previous findings, no correlation had been reported between total phenolic content and antioxidant capacities of a number of medicinal plant extracts (Bajpai *et al.*, 2005). In this study, since no correlation was observed between the total phenolic content and antioxidant activity, the antioxidant potential demonstrated by the crude extract of *C. retusa* through the DPPH radical scavenging activity could be said to be possibly due to the presence of flavonoids. The mechanisms of action of flavonoids are through the scavenging or chelating process (Kessler et al., 2003). Compounds such as flavonoids, which contain hydroxyl functional groups have been reported to be responsible for the antioxidant effect in plants (Das and Pereira, 1990). Therefore, *C. retusa* extract could be considered as a potential source of natural antioxidants which might be helpful in preventing the progress of various oxidative stresses. Further investigations should be carried out, to isolate and identify the antioxidant compounds present in the plant extracts. Furthermore, the *in vivo* antioxidant activity of this extract should be assessed prior to clinical use.

Thus, in the comparative study of the antimicrobial and antioxidant activities of *Crotalaria retusa* wild plant and its callus culture, results clearly showed that the callus culture extract had the potential to produce the desired antibacterial, antifungal, antioxidant and phenol metabolites. This depicts the ability to adopt the tissue culture technique as a biotechnological tool towards the enhancement of desired bioactive metabolites in callus culture of *Crotalaria retusa* instead of the continuous use of wild plants, employed in pharmaceutical purposes.

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