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Total Phenolic Content and Antioxidant Activity of Standardized Extracts from Leaves and Cell Cultures of Three *Callistemon* Species

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ABSTRACT

A comparative study was carried out with ethanolic (80%) *extracts from leaves and cell cultures of three Callistemon species, namely C. lanceolatus* (*CL*), *C. viridiflorous* (*CV*), *and C. comboynensis* (*CC*). *Cell suspensions of the three species were grown in liquid Murashige and Skoog* (*MS*) *medium* (100 *ml*) *supplemented with* 0.9 $mg{\cdot}g^{-1}$ *kinetin in combination with* 1.1 $mg{\cdot}g^{-1}$ *NAA. The CL leaf extract was standardized to contain the highest amount of phenolics* (104 ± 2.0 $mg{\cdot}g^{-1}$), *followed by CC* (95.8 ± 1.2 $mg{\cdot}g^{-1}$) *and CV* (79.8 ± 4.6 $mg{\cdot}g^{-1}$). *On the other hand, cell cultures of CV contained more phenolics* (14.9 ± 0.6 $mg{\cdot}g^{-1}$) *than those of the other two species, CL and CC, which contained* 12.2 ± 0.16 *and* 9.12 ± 0.16 $mg{\cdot}g^{-1}$, *respectively. Nevertheless, CV leaf extract exhibited the highest antioxidant activity* (91.4% ± 0.4%) *at a concentration of* 1000 $\mu g{\cdot}ml^{-1}$, *comparable to* 100 $\mu g{\cdot}ml^{-1}$ *gallic acid* (90.8% ± 1.5%).

Keywords*: *Callistemon*, *Phenolic Content*, *Antioxidant Activity*, *Callus*, *Cell Cultures

1. Introduction

The genus *Callistemon* (Myrtaceae) contains 34 species of beautiful evergreen shrubs and small trees. The majority of the *Callistemon* species is endemic to the more temperate regions of Australia, four species are found in New Caledonia and seven species have been introduced to India as ornamental trees [1]. They are commonly known as bottle brushes because of their cylindrical brush-like flowers resembling the traditional bottle brush.

C. lanceolatus, also named *C. citrinus*, is a well-known shrub. Leaves of this plant are used as a tea substitute and have a refreshing flavor. Many phenolic compounds of this plant have been identified [2]. Due to the over-exploitation for its volatile oil and secondary metabolites, there is a great need to develop alternative strategies of conservation and industrial production of the bioactive compounds from this plant [3]. No reports of works were found concerning the other two species, *C. viridiflorous* and *C. comboynensis*.

In vitro cultures have the potential to form secondary metabolites and to exhibit bioactivity comparable to the original plant [4,5]. Cultured cells may serve industrial purposes, e.g. by immobilization of cells in a matrix for use in bioreactors. Besides the genetic potential of the donor plant for callus induction and growth of this callus in *in vitro* cultures, a medium containing sufficient nutrients, such as the preferred MS medium, is required [4].

Antioxidants play an important role in the prevention of human diseases. Antioxidant compounds may function as free radical scavengers, complexing agents for pro-oxidant metals, as well as reducing agents and quenchers of singlet oxygen formation [6-8]. Antioxidants are often used in oils and fatty foods to retard their autoxidation. Therefore, the importance of the search for natural antioxidants has greatly increased in recent years [9]. A focus is on plant-derived polyphenols because of their potential antioxidant and antimicrobial properties. Phenolic compounds exhibit considerable free-radical scavenging activity, which is determined by their reactivity as hydrogen- or electron- donating agents, their reactivity with other antioxidants and their metal chelating properties, as well as the stability of the resulting antioxidant-derived radicals [10,11].

Our present work is a comparative study of leaves and

cell cultures of three *Callistemon* species with respect to their potential as antioxidant agents in relation to their total content of phenolic compounds.

2. Materials and Methods

2.1. Plant Material

Plants of three *Callistemon* species, *C. lanceolatus* (CL), *C. viridiflorus* (CV), and *C. comboynensis* (CC), were collected from a cultivated area in Cairo, Alexandria Road, Egypt. They were kindly authenticated by Prof. Dr. M. Gebali (Plant Taxonomy and Egyptian Flora Department, National Research Center, Giza, Egypt). A voucher specimen of each was deposited at the herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Cairo, Egypt.

2.2. Calli and Cell Cultures

Callus of CL was induced by a combination of 0.9 $mg{\cdot}L^{-1}$ kinetin and 1.1 $mg{\cdot}L^{-1}$ NAA [12]. Calli of CV and CC were similarly induced (the detailed methodology will be published later on). Calli material (0.3 g each) were collected in the active growth phase (after the 15th day of subculture) and placed in 250 ml flasks containing 100 ml liquid MS medium supplemented with 0.9 $mg{\cdot}L^{-1}$ kinetin in combination with 1.1 $mg{\cdot}L^{-1}$ NAA. The resulting cell cultures of the three *Callistemon* species were incubated in a horizontal shaker at 100 rpm and 25˚C for 21 days.

2.3. Preparation of the Extracts

The three cell suspension cultures were aseptically filtered and the cells dried in a vacuum oven at 40˚C, together with the leaves of the three species. They were then macerated in 80% ethanol for two days, filtered and macerated for another two days. After filtration, they were concentrated under vacuum at 50˚C.

2.4. Evaluation of the Antioxidant Activity

Determination of the free radical scavenging activity of the different extracts was carried out using a modified quantitative DPPH (1,1-diphenyl-2-picrylhydrazyl; Sigma-Aldrich, St. Louis, MO, USA) assay [13]. Various concentrations of sample extracts in methanol were prepared (1000, 500, 250, and 100 $\mu g{\cdot}ml^{-1}$). Gallic acid was used as a positive control at concentrations of 100, 50, 25, and 10 $\mu g{\cdot}ml^{-1}$. Blank samples were run using 1 ml methanol in place of the test extract. One ml of 0.2 mM DPPH in methanol was added to 1 ml of the test solution, or standard, plus 1 ml of methanol for dilution and allowed to stand at room temperature in a dark chamber for 30 min. The change in colour from deep violet to light yellow was then measured at 517 nm. Inhibition of free radical in percent (I%) was calculated according to the following equation: $I\% = \left[\left(A0 - A1\right)/A0\right] \times 100$, with A0 being the absorbance of the control reaction (containing all reagents except for the extract) and A1 the absorbance of the extract. Measurements were carried out in triplicates.

2.5. Determination of Total Phenolic Content

A spectrophotometric method after MacDonald [14] was adopted for the determination of total polyphenols in the prepared extracts. Folin-Ciocalteu reagent from Merck (Darmstadt, Germany) was used and a standard calibration curve was prepared using different concentrations of gallic acid in methanol (0.025 - 0.400 $mg{\cdot}ml^{-1}$). Cell culture and leaf extracts were prepared in methanol at a concentration of 0.06 g/3 ml and 0.06 g/20 ml, respectively. Absorbance was measured at 765 nm. For each sample, three replicate assays were performed. The total phenolic content was calculated as gallic acid equivalent (GAE) by the following equation: $T = C \times V/M$. T is the total phenolic content in $mg{\cdot}g^{-1}$ of the extracts as GAE, C is the concentration of gallic acid established from the calibration curve in $mg{\cdot}ml^{-1}$, V is the volume of the extract solution in ml and M is the weight of the extract in g.

3. Results and Discussion

3.1. Phenolic Content of the Extracts

Ethanolic (80%) extracts from the leaves and cell cultures of the three *Callistemon* species were standardized for their contents of phenolic compounds. The calibration curve showed linearity for gallic acid in the range of 25 - 400 $\mu g{\cdot}ml^{-1}$, with a correlation coefficient (R2) of 0.999 (**Figure 1**). Leaves of CL contained the highest content of phenolics (104 ± 2.0 $mg{\cdot}g^{-1}$), followed by CC (95.8 ± 1.2 $mg{\cdot}g^{-1}$) and CV (79.8 ± 4.6 $mg{\cdot}g^{-1}$). On the other hand, cell cultures of CV were standardized to contain more phenolics (14.9 ± 0.6) than the cell suspensions of the other two species, CL and CC, which contained 12.2 ± 0.16 and 9.12 ± 0.16 $mg{\cdot}g^{-1}$, respectively (**Figure 2**).

3.2. Antioxidant Activity of the Extracts

It is well known that there is a strong relationship between total phenol content and antioxidant activity, as phenols possess strong scavenging ability for free radicals due to their hydroxyl groups. Therefore, the phenolic content of plants may directly contribute to their antioxidant action [11,15,16].

The standardized *Callistemon* extracts were assessed for their capacity to scavenge DDPH free radical along with gallic acid as a positive control. The antioxidant activity data are presented as percent of free radical inhi-

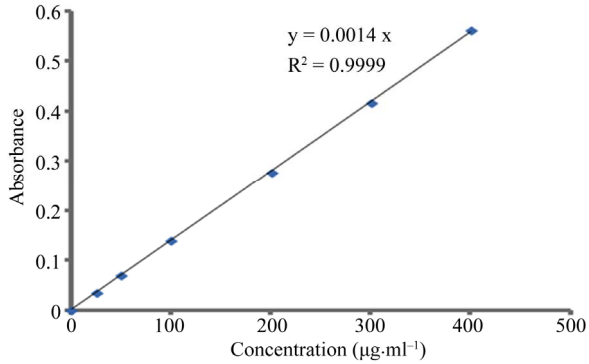

Figure 1. Standard calibration curve of gallic acid at concentrations of 25, 50, 100, 200, 300 and 400 µg·ml^{-1}. Spectrophotometric detection was at 765 nm.

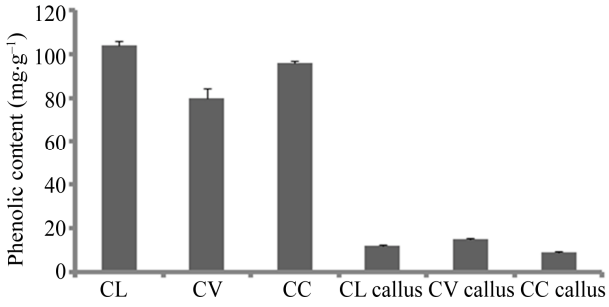

Figure 2. Total phenolic content of leaf and cell culture extracts from three *Callistemon* species determined by the Folin-Ciocalteu assay and calculated as GAE in mg·g^{-1} extract based on dry weight. Results are the average of triplicates ± SD.

Table 1. Antioxidant activity of *Callistemon* leaf and cell culture extracts assayed by the DPPH assay.

Conc. of extract µg/ml	*CL leaves*	*CV leaves*	*CC leaves*	*CL cultures*	*CV cultures*	*CC cultures*	*Conc. of standard µg/ml*	*Gallic acid*
1000	73.5 ± 3.2	91.4 ± 0.4	74.4 ± 0.3	50.7 ± 0.2	71.1 ± 0.4	47.3 ± 1.9	100	90.8 ± 1.5
500	67.3 ± 0.2	78.4 ± 0.2	66.9 ± 0.9	41.7 ± 1.4	68.4 ± 0.2	44.4 ± 0.3	50	83.7 ± 0.6
250	60.3 ± 2.0	75.4 ± 0.4	57.8 ± 0.7	38.3 ± 0.4	53.5 ± 0.2	35.3 ± 0.2	25	76.3 ± 0.2
100	48.9 ± 3.7	57.4 ± 0.4	56.3 ± 0.3	35.9 ± 0.8	46.7 ± 0.2	34.1 ± 0.1	10	65.4 ± 0.1

Activity is expressed as inhibition of free radical in percent, I% ± SD (n = 3). Leaf and cell culture extracts were tested at 1000, 500, 250 and 100 µg·ml^{-1} and the positive control (gallic acid) at 100, 50, 25 and 10 µg·ml^{-1}.

bition in **Table 1**. The ethanolic (80%) extracts of the leaves of CV exhibited pronounced antioxidant activity (91.4% ± 0.4%) at a concentration of 1000 µg·ml^{-1}, comparable to 100 µg·ml^{-1} gallic acid (90.8% ± 1.5%), although its phenolic content was less than that of CL and CC (**Figure 2**). Furthermore, extracts of CV cell cultures showed antioxidant activity (71.1% ± 0.4%) comparable to that of leaf extracts of CL and CC at 1000 µg·ml^{-1}, even though their phenolic contents were approximately 7-fold that of CV cell cultures (**Figure 2**). It was previously reported that non-phenolic antioxidants might also contribute to the antioxidant activity of plant extracts [17,18]. Thus, compounds other than phenolics might be responsible for the pronounced antioxidant activity observed with CV extracts, which requires further investigation. Polyphenolic compounds are also believed to have chemopreventive and suppressive activities against cancer cells by inhibition of metabolic enzymes involved in the activation of potential carcinogens or arresting the cell cycle [19]. Nevertheless, a compound with strong antioxidant potential can also contribute to DNA protection and prevent apoptosis [20]. Further studies are therefore required to detect potential anticancer activities of the extracts reported here.

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