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Reseach Article
Antioxidant Properties of Leaf, Twig and Calli Extracts of *Neolamarckia cadamba* (Roxb.) Bosser in Sri Lanka

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Abstract

Neolamarckia cadamba (Roxb.) Bosser poses a threat of extinction due to felling for timber, large-scale sample collections for herbal formulations in traditional medicine and research purposes. Use of calli as the source of secondary metabolites instead of destructive sampling conserves *N. cadamba* for future generations. Objectives of the present study were to evaluate antioxidant activity of natural extracts of leaf and twigs of *N. cadamba*, through optimization of calli induction of leaf and internode explants and a comparison of calli and plant extracts for their antioxidant activity. Calli were induced from tender leaves and internodes using two out of ten NAA and BAP hormone combinations; NAA 5.0 mg L^{-1}: BAP 0.5 mg L^{-1} and NAA 2.5 mg L^{-1}: BAP 3.0 mg L^{-1}. Methanolic crude extracts of mature leaves, twigs and calli were obtained and the extracts were screened for antioxidant activity using *in vitro* assays; Total Phenol Content (TPC) assay, DPPH assays and plasmid DNA nicking assay. The TPC was highest in twigs than leaves of natural plant and call extracts which in turn indicated increase antioxidant activity. Calli obtained from leaves and internodes are of important as a potential source of bioactive compounds showing the capability of scavenge the diverse free radicals in different systems and as potential therapeutic agents for treating radical-related pathogenic cell damages. Further studies focusing the enhancement of production of secondary metabolites in calli of *N. cadamba* exposing to different stress conditions is a prerequisite to produce important bioactive compounds in higher concentrations.

Key words: Antioxidants, callus, *Neolamarckia cadamba* (Roxb.) Bosser, natural extracts

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The focus on plant study has been increased during the past years all over the world and a large number of evidence has collected to show the immense potential of using medicinal plants in numerous traditional therapeutic systems. Natural plant products are frequently reported as efficient chemo-preventive agents. Antigenotoxic and antioxidative mechanisms are considered essential for the prevention of some degenerative diseases such as cancer (Leow *et al*., 2011; Thapa and Ghosh, 2012).

Study on antioxidant is an important topic in medicinal study as well as in food industry. The importance of Reactive Oxygen Species (ROS) and free radicals in cellular damage and the aging process has attracted increasing attention over the past 20 years (Valko *et al*., 2007). The ROS, such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^•$) and other free radicals are identified as by-products of biological metabolism. In such biological systems, there are enzymatic systems and chemical scavengers; dietary antioxidants (α-tocophenol, β-carotene, ascorbic acid, glutathione and uric acid), some hormones (estrogen, angiotentensin) and endogenous enzymes (superoxide dismutase, glutathione peroxidase and catalase). All these are able to remove free radicals formed in cells and therefore protect against oxidative damage (Uttara *et al*., 2009; Rizzo *et al*., 2010). The oxidation induced by ROS can result in cell membrane breakdown, membrane protein damage and DNA mutation. Such reactions can further initiate or proliferate the development of many diseases, such as cancer, liver injury and cardiovascular diseases (Bancirova, 2010; Gulcin *et al*., 2009).

The antioxidant phytochemicals obtained from plants, especially flavonoids and other polyphenols, are reported to inhibit the propagation of free radical reactions, to protect the human body from disease and longer life expectancy (Ozcan *et al*., 2011; Khoobi *et al*., 2011). Therefore, interest has been developed studies on naturally occurring antioxidants for the use in foods or medicinal material as compared to the synthetic antioxidants. Because of the presence of their non-side effects nature (Ito *et al*., 1983).

Neolamarckia cadamba (Roxb.) Bosser is known as Bakmee in Sinhala and Wild cinchona in English and popular in India as Kadamb. The fruits and inflorescences (Fig. 1) are reportedly edible to humans. The fresh leaves are fed to cattle. A yellow dye is obtained from the root bark (Mhaske *et al*., 2013). It is also used as a low-grade timber and fuel. The plant is very significant in Indian mythology and religion due to its connection with Lord Krishna (Mishra, 2011). Most importantly, *N. cadamba* is an important medicinal plant in the family Rubiaceae used in traditional ayurvedic medicine for the treatment of fever, uterine diseases, skin diseases, dysentery and diabetes (Patel and Kumar, 2008; Dubey *et al*., 2011). In ayurvedic medicine the plant is used for uterine complaints, blood diseases, leprosy. Therefore, large-scale destructive collection of plant parts for herbal formulations and study purposes possibly pose a potential threat of extinction on this plant. The production of secondary metabolites is influenced by various environmental conditions (Kaimoyo *et al*., 2008; Okudera and Ito, 2009) and therefore, calli can be subjected to varying stress conditions for desired secondary metabolites.

Studies have been carried out on phytochemical analysis (Dubey *et al*., 2011), antioxidant activity of leaf and bark (Alekhya *et al*., 2013; Ganjewala *et al*., 2013), antifungal activity of leaf and bark (Patel *et al*., 2011), antibacterial activity of fruit (Mishra, 2011), antidiabetic property of leaf, hypoglycemic activity of leaf (Ahmed *et al*., 2011) of *N. cadamba.* However,

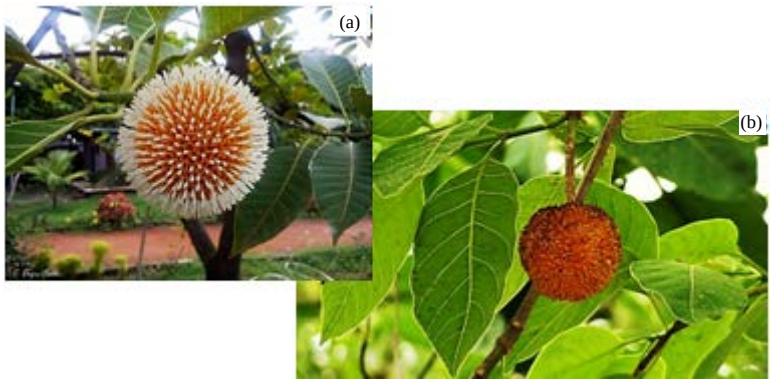

Fig. 1(a-b): (a) Inflorescence and (b) Fruit of *Neolamarckia cadamba* (Roxb.) Bosser

on attempts to raise callus of *N. cadamba* explants for secondary metabolites is not extensively studied.

The objective of the present study is to explore the possibility of deducing whether the calli raised from *N. cadamba* can be used as a potential source for extraction of secondary metabolites instead of destructive sampling. The present study reports a comparison of antioxidative potential of natural plant extracts of leaves and twigs of *N. cadamba* and calli derived from leaf and internode explants of *N. cadamba*.

MATERIALS AND METHODS

Leaf and twig samples of *N. cadamba* were collected from Thalgahawila, Horana in the District of Kalutara, Sri Lanka between February to May, 2014 and samples were identified by comparing herbarium specimens deposited in Botanical Garden, Peradeniya, Sri Lanka and referring related literature.

Calli induction: Tender leaves emerging from axillary buds and internodes used for calli induction were washed and the surface sterilized using 0.05% $HgCl_2$ solution followed by using 70% alcohol and in warm sterile distilled water before placing on the culture medium (Indu *et al*., 2013). A series of ten MS culture media (20.0 mL) each with varying NAA and BAP hormone concentrations (NAA: BAP 0.5:5.0/1.0:4.5/1.5:4.0/2.0: 3.5/2.5:3.0/3.0:2.5/3.5:2.0/4.0:1.5/4.5:1.0 and 5.0:0.5) along with 3 mg mL^{-1} of activated charcoal was used for calli generation. Ten explants were tested with each hormonal treatment.

Crude extraction: Air-dried plant tissues (leaves and twigs) (10 g) and air-dried calli tissue (five leaf-calli and five internode-calli) (5.0 g) were separately macerated and extracted into cold methanol and dried in a vacuum to a constant dry weight.

Antioxidant assays: Antioxidant assay of leaves and twigs of *N. cadamba* and calli extracts were tested by measuring the total phenolic content using Folin-Ciocaltau phenol reagent assay, DPPH radical scavenging assay and plasmid DNA protection assy.

Total Phenol Content (TPC) assay: The total phenolic content of the plant extract was determined by using Folin-Ciocaltau method (Yu *et al*., 2002). Crude extracts (1.0 g) of leaf, twig, leaf-calli and internode-calli were separately added to distilled water (0.9 mL) followed by adding Folin-Ciocaltau Reagent (0.5 mL) and 20% sodium carbonate (1.5 mL). The final volume of the mixture was made up to 10 mL with distilled water. Absorbance of mixture was measured at 750 nm. Phenolic content of extracts was calculated as Gallic Acid Equivalents (GAE) in mg g^{-1} on the basis of standard curve of gallic acid (Chandel *et al*., 2012).

DPPH radical scavenging activity assay: The DPPH radical scavenging activity assay method evaluates the ability of antioxidants to scavenge free radicals. The effect of antioxidants on DPPH is assumed to be due to their hydrogen donating ability. Different concentrations of extracts (20, 40, 60 and 80 μg mL^{-1}) of leaf, twig, leaf-calli and internode-calli were dissolved in methanol and taken in test tubes in triplicates. Then 2.0 mL of 0.1 mM methanol solution of DPPH was added to each test tube and shaken vigorously. After keeping in dark for 30 min, absorption was measured at 517 nm. Results were compared with standard compound rutin (IC_{50} = 54.05 μg mL^{-1}). Radical Scavenging Activity (RSA)% was calculated as follows (Chandel *et al*., 2012):

$$RSA\ (\%) = \frac{Ab_{control} - Ab_{sample}}{Ab_{control}} \times 100$$

where, $Ab_{control}$ is absorbance of DPPH in methanol, Ab_{sample} is absorbance of a DPPH solution with the test sample.

Plasmid DNA protection assay: To measure the hydroxyl radical scavenging effect of leaf, twig and calli extracts of *N. cadamba*, DNA nicking equipment was performed according to the protocol of Lee *et al*. (2002). Plasmid DNA was incubated with Fenton's reagent containing the different extracts and then the final volume of the mixture was raised up to 20 μL. Then, the mixture was incubated for 30 min at 37°C and added the loading dye. Electrophoresis was carried out in Tris Acetic acid EDTA (TAE) buffer and DNA was analyzed after ethidium bromide staining.

Statistical analysis: Statistical analysis of data were performed on the statistical software package SPSS PC Version 20. The significance of results was checked at $p \leq 0.05$.

RESULTS

Calli induction: The following hormone combinations used in MS medium were able to induce and develop calli from leaf and internode explants of *N. cadamba* (Fig. 2).

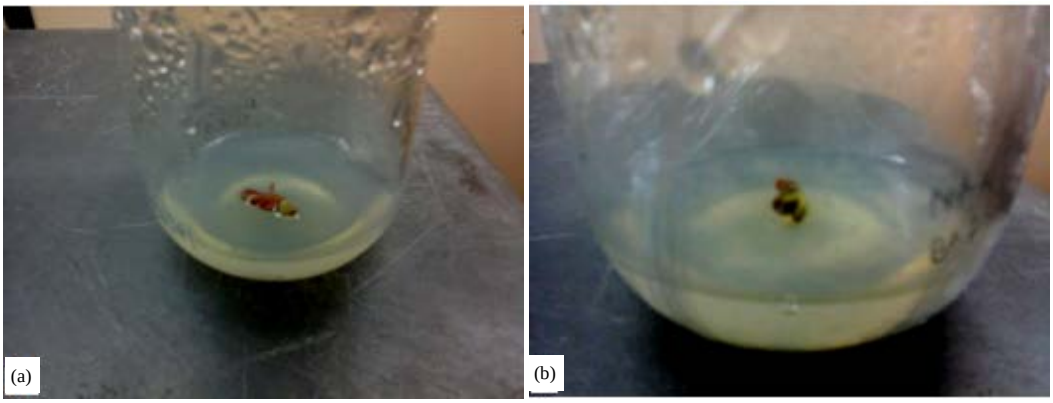

Fig. 2(a-b): Development of calli from leaf and internode of *N. cadamba* (a) Developed callus of leaf and (b) Developed callus of internode

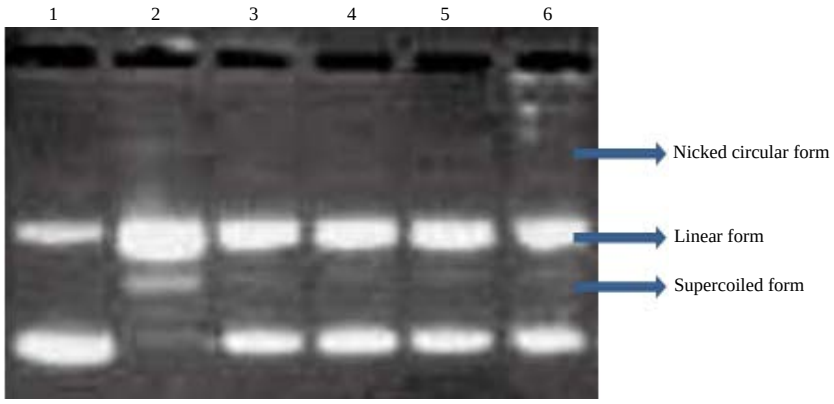

Fig. 3: Effect of *N. cadamba* extracts of leaves, twigs, leaf-calli and internode-calli in DNA protection assay. Lane 1: Negative control, Lane 2: Fenton's Reagent (FR)+DNA, Lane 3: pBR322 plasmid DNA+FR+Leaf extract, Lane 4: pBR322 plasmid DNA+FR+twig extract, Lane 5: pBR322 plasmid DNA+FR+leaf-calli extract and Lane 6: pBR322 plasmid DNA+FR+internode-calli extract

- Leaf: NAA 5.0 mg L^{-1}: BAP 0.5 mg L^{-1} and NAA 2.5 mg L^{-1}: BAP 3.0 mg L^{-1} (60% calli development)
- Internode: NAA 5.0 mg L^{-1}: BAP 0.5 mg L^{-1} and NAA 2.5 mg L^{-1}: BAP 3.0 mg L^{-1} (70% calli development)

Total Phenol Content (TPC) and DPPH radical scavenging activity assays: Total Phenolic Content (TPC) (mg g^{-1} GAE) was highest in crude (1.0 g) extracts of twigs (17.0) and leaf (4.0) compared to that of internode-calli (3.5) and leaf-calli (2.0) (Table 1). Radical Scavenging Activity (RSA) % increased with concentration of extracts. In RSA of extracts of *N. cadamba*, activity decreased in the order of twig, leaf, internode-calli and leaf-calli, confirming the findings of Ganjewala *et al.* (2013). The TPC and RSA values showed a similar trend in twig, leaf, internode-calli and leaf-calli.

Plasmid DNA protection assay: The DNA protective activity was assessed by measuring the degree of protection on DNA scission that was induced by the attack of hydroxyl radicals generated by Fenton's reagent. Hydroxyl radical is one of the ROS formed in biological systems, causing DNA strand breakage, which brings about carcinogenesis, mutagenesis and cytotoxicity. The protection against the damage caused by hydroxyl radicals is shown by the agarose gel electrophoresis pattern (Fig. 3). It is clear from the results that the extracts protected the pBR322 plasmid DNA against the DNA damaging effect of hydroxyl radicals.

DISCUSSION

The DPPH assay has been widely used to determine the free radical-scavenging activity of various plant extracts as

Table 1: Total Phenolic content (TPC) and Radical Scavenging Activity (RSA) for different concentrations of leaf, twig and calli crude extracts of *N. cadamba*

Plant part	TPC* (mg g^{-1} GAE) (SD)	RSA (%)* (SD) based on IC_{50} values for different concentrations (µg mL^{-1}) of extracts
Leaf-calli	2.0 (0.00)	20-11.00 (0.58)
		40-20.67 (1.00)
		60-30.33 (1.16)
		80-81.33 (0.58)
Internode-calli	3.5 (0.00)	20-19.00 (1.00)
		40-59.67 (0.58)
		60-85.67 (2.08)
		80-94.33 (0.58)
Leaf	4.0 (0.00)	20-72.67 (6.43)
		40-69.67 (0.58)
		60-86.33 (2.31)
		80-95.00 (1.73)
Twig	17.0 (4.58)	20-61.67 (0.58)
		40-81.33 (1.16)
		60-90.67(0.58)
		80-98.00 (0.00)

*Mean and standard deviation in parenthesis of three replicates

well as pure compounds (Li and Liu, 2009; Dong *et al*., 2012). The DPPH is a stable free radical, which dissolves in methanol. Its purple colour shows a characteristic absorption at 517 nm. An antioxidant when scavenges the free radical by hydrogen donation, the color changes from purple to light yellow. Ganjewala *et al*. (2013) have employed the DPPH to access the antioxidant properties of methanol extracts of leaves and fruits of *N. cadamba* (Roxb.). Similar observations were made by Sun *et al*. (2011) in evaluating the antioxidant activity of flavonoids extract from *Diospyros kaki* L. leaves and attributed the potent antioxidant activity on the DPPH radical to a direct role in tapping free radicals by donating a hydrogen atom.

Although, the extracts of leaf-calli and internode-calli demonstrated positive antioxidant activities in Total Phenol Content (TPC) assay and DPPH radical scavenging activity assay, the activity is less and revealed that secondary metabolites responsible for such properties are produced in calli but to a lesser extent. However, natural environment imposes various conditions of stress on naturally growing plants stimulating to produce more secondary metabolites in higher concentrations (Akula and Ravishankar, 2011). This could have been the reason, why leaf and twig extracts showed higher antioxidant activity compared to that of calli extracts. The leaf, twig and calli extracts of *N. cadamba* showed the potential to protect the pBR322 plasmid DNA from the damage caused by hydroxyl radicals. In all the *in vitro* antioxidant assays, a significant correlation was observed between total phenolic content and antioxidant activity. The antioxidant assays carried out for *Anthocephalus cadamba* (= *Neolamarkia cadamba*), Chandel *et al*. (2012) also showed potential antioxidant activity through Total Phenol Content (TPC) assay and DPPH radical scavenging activity assay. The leaf, twig and calli extracts of *N. cadamba* also showed the potential to protect the plasmid DNA (pBR322) against the attack of hydroxyl radicals generated by Fenton's reagent (Falcioni *et al*., 2002).

As suggested by the literature (Indu *et al*., 2013) if calli are subjected to optimization with stresses such as salinity, water stress, electric stimulation, etc. There may be a possibility of production of secondary metabolites in higher in concentrations (Ye *et al*., 2004).

CONCLUSION

The present study reveals that development of calli from leaves and internodes of *N. cadamba* could be considered as a potential source of bio-active compounds with antioxidant properties to act against free radicals.

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