OXFORD

BIOLOGY Methods & Protocols

Biology Methods and Protocols, 2023, bpad019

https://doi.org/10.1093/biomethods/bpad019

Advance Access Publication Date: 19 September 2023

Pre-registered study protocols

In vitro callus induction and evaluation of antioxidant activity of *Rhinacanthus nasutus* (L.) Kurz

Wipa Yaowachai[1], Prathan Luecha[2] Worasitikulya Taratima [1,*]

[1]Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand

[2]Department of Pharmacognosy and Toxicology, Faculty of Pharmaceutical Science, Khon Kaen University, Khon Kaen 40002, Thailand

*Correspondence address. Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand. Tel: +66 994599622, E-mail: worasitikulya@gmail.com

Abstract

Rhinacanthus nasutus (L.) Kurz is used in Thai traditional medicine for the treatment of skin diseases, ringworm, and eczema. This research studied the effects of cytokinin and auxins on callus induction and evaluated antioxidant activity of *R. nasutus*. Nodes, young, and mature leaf explants were cultured on MS medium containing 0, 1, 2, 3, and 4 mg/l kinetin (6-furfurylaminopurine) and 0, 1 mg/l 1-naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), and 2,4-dichlorophenoxyacetic acid (2,4-D) for 6 weeks to induce callus. Calli derived from nodes, young and mature leaves, and other plant parts were ultrasonically extracted with methanol to determine total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity by ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhtdrazyl (DPPH), and 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assays. Nodal explants on MS medium containing 1 mg/l kinetin combined with 1 mg/l 2,4-D were most efficient in callus production with the average fresh and dry weight per explant of 2.29 ± 0.14 and 0.18 ± 0.01 g, respectively. Addition of kinetin combined with NAA or 2,4-D had a positive effect on callus induction from young and mature leaf explants. The leaf extract showed the highest TPC, TFC, FRAP, and IC_{50} of DPPH and ABTS assays (ca 113 mg GAE/g extract, 45 mg QE/g extract, 121 mg TE/g extract, 53 μg/ml and 14 μg/ml, respectively), followed by callus derived from nodes. Overall, phenolic content was higher than flavonoid content. A strong positive correlation was found between FRAP assay, TPC ($r = 0.973$), and TFC ($r = 0.798$), indicating that phenolic and flavonoid compounds are responsible for antioxidant activity of *R. nasutus*.

Keywords: bioactive compound; chemical constituents; medicinal plant; snake jasmine

Introduction

Plants belonging to the *Rhinacanthus* genus in the Acanthaceae family are distributed throughout Southeast Asia, including Thailand [1]. *Rhinacanthus nasutus* (L.) Kurz (known as Thong Pan Chang in Thai) is a slender-stemmed, erect, branching, and sometimes hairy shrub, reaching a height of 1–2 m. The leaves are opposite, simple, and can grow up to 8 cm in length, while the flowers are white with a slight brown spot at the base [2]. Plant parts of *R. nasutus* are used in traditional medicine to treat various ailments such as skin diseases, ringworm, eczema, inflammation and atopic dermatitis, and infectious disease caused by *Mycobacterium tuberculosis* [3]. Root extracts showed hepatoprotective effects in rats [4], while the leaves are used to treat allergy-related diseases [5]. Stem extracts are used for the treatment of ringworms which causes a ring-shaped infection on the skin [6]. A phytochemical analysis of *R. nasutus* showed that naphthoquinones were a major component including rhinacanthin-A to -D and rhinacanthin-G to -Q, rhinacanthone, lignan groups and polyphenolic compounds [7]. Wu *et al.* [8] also reported on the isolation and identification of anthraquinone compounds from the leaf and stem. *Rhinacanthus nasutus* is a valuable medicinal herb in terms of quantitative and qualitative bioactive compounds.

The general method of propagation of *R. nasutus* is through stem cuttings and seeds which fall easily but are sensitive to physical factors, especially the weather [9]. Unregulated collecting of this medicinal plant in the wild has resulted in an alarming reduction in the natural population, with efforts urgently required to conserve and propagate its natural fertility. Plant tissue culture is now widely used to produce plantlets from various types of explants such as cell, tissue, organ, or protoplast using aseptic techniques, with the morphogenetic potential of the explants greatly impacted by medium composition. A suitable medium contains macronutrients and micronutrients, amino acids, organic compounds, vitamins, carbon sources, solidifying agents, and plant growth regulators (PGRs) [10]. The culture medium is modified to promote development and create the required morphogenesis for individual species [11].

Callogenesis is an important aspect of indirect organogenesis for research into bioactive molecules in medicinal plants [12]. Callus is defined as a group of parenchyma cells derived from somatic tissues that are cultured in the medium. Callus can be white or cream-colored, green, or purple in some parts or all of the plant [13]. The types and concentrations of PGRs and explants affect successful callus production. Cytokinins and auxins are commonly used as PGRs in plant tissue culture media. Callus induction can be promoted by suitable exogenous hormones such as auxin and cytokinin, either alone or in combination [14]. Callus cultures can be used to stimulate the bioactive production of cells or tissue cultures

Received: July 17, 2023. **Revised:** August 27, 2023. **Editorial decision:** August 28, 2023. **Accepted:** September 15, 2023

[15], while PGRs play a critical role in the growth, development, and spread of plant system nutrients. Changing environmental conditions drive plants to use a variety of hormonal alterations to adapt and survive [16]. In the preliminary study, it was found that the addition of cytokinin higher than auxin resulted in the formation of callus and new shoots in some explants. Therefore, this study chooses to use a high concentration of cytokinin and a low concentration of auxin. High concentrations of auxin resulted in the death of plant parts, so a concentration of 1 mg/l was selected. Cytokinin concentrations of 1–4 mg/l were at a level that could well induce callus and shoot formation.

Previous research mainly reported on the phytochemical constituents in *R. nasutus* extracts. Phenolic compounds account for most of the antioxidant activity in plants [17] but the phytochemical constituents and antioxidant activities of callus derived from different types of *R. nasutus* explants have not been investigated. Therefore, this study assessed the combined effects of auxins, cytokinins, and explant types on callus induction of *R. nasutus*. Total phenolic and flavonoid contents and antioxidant activities by ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhtdrazyl (DPPH), and ABTS assays were also evaluated for calli from other parts of wild *R. nasutus* plant extracts.

Materials and methods

Plant material

Node explants were collected from *R. nasutus* plants growing in Khon Kaen Province, Thailand (16°28′53.1″N 102°49′05.9″E). The specimens were deposited at the Department of Biology, Faculty of Science, Naresuan University, Phitsanulok, Thailand and identified by Dr. Pranee Nangngam (Researcher at the Department of Biology, Faculty of Science, Naresuan University, Phitsanulok, Thailand). Explants used in this research were permissible by the Plant Varieties Protection Office, Department of Agriculture, Thailand. The nodes were washed for 15 min under running tap water and soaked for 1 min in 70% (v/v) ethanol, followed by disinfection with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 15 min under aseptic conditions. The nodes were then rinsed in sterilized distilled water for 5 min, three times, and cultured on MS medium [18] with 30 g/l sucrose for shoot induction. The MS medium was solidified using 8 g/l of agar with pH adjusted to 5.8. The medium was autoclaved at 121°C for 20 min for sterilization.

Callus induction

Nodes, young leaves, and mature leaves from 8-week-old *in vitro* grown plants were selected for callus induction. The nodal explants were cut into 0.5 cm lengths, with the leaves cut into 0.5 × 0.5 cm squares. The explants were cultured on solid MS medium supplemented with kinetin at 0, 1, 2, 3, and 4 mg/l in combination with NAA, IAA, or 2,4-D at 0 and 1 mg/l. All media were solidified using 8 g/l of agar, with pH adjusted to 5.8 before autoclaving. The IAA used in this study was Sigma (Product No. 12886), which is classified in the CA/F type sterilization category. Therefore, IAA can be autoclaved [19]. Callus induction cultures were incubated at 25 ± 2°C with 16/8 h (light/dark) photoperiod under white cool fluorescent light at 40 μmol/m^2/s for 6 weeks. Response, survival, and callus induction percentages were recorded after 6 weeks of culture. Survival percentages in different media were estimated from the characteristics of explants that survived. Explants that produced callus were used to calculate callus induction percentage, fresh weight, and dry weight after drying for 3 days at 45°C.

Preparation of *R. nasutus* extracts

Calli derived from the node, young leaf, and mature leaf of *in vitro* cultured (1 mg/l kinetin + 1 mg/l 2,4-D) explants and leaf, stem, stem with bark, and lateral taproot of wild *R. nasutus* plants (Khon Kaen Province, Thailand) were dried in a hot air oven at 50°C for 7 days and then ground into powder. All dried sample powders (1 g) were extracted with methanol (10 ml) by ultrasound-assisted extraction (UAE) for 30 min using 40 Hz ultrasonic power at 30°C. The extractions were repeated nine times and the extract solutions were combined. The supernatant was collected and evaporated to dryness in SpeedVac equipment at room temperature for 24 h and stored at −20°C for further analysis. Extract percentage yield was calculated using the following formula:

$$\text{Percentage yield} = (\text{actual yield}/\text{theoretical yield}) \times 100. \quad (1)$$

Determination of total phenolic content

Total phenolic content (TPC) was determined using the Folin–Ciocalteu method described by Hmamou *et al.* [20]. Aliquots of 20 μl of sample extracts were dissolved in methanol (1 mg/ml) and added to 100 μl of 10% (v/v) Folin–Ciocalteu solution and 80 μl of 7.5% (v/v) sodium carbonate (Na_2CO_3) in a 96-well microplate. The microplates were incubated in the dark at room temperature for 30 min followed by measurement of absorbance at 765 nm using a microplate reader spectrophotometer. A standard curve was prepared using 2-fold dilutions of gallic acid (5-320 μg/ml) and all values were expressed as mg gallic acid equivalent per g dry weight extract (mg GAE/g extract).

Determination of total flavonoid content

Total flavonoid content (TFC) was determined using the aluminum chloride method described by Oh *et al.* [21]. Aliquots of 100 μl of sample extracts were dissolved in methanol (1 mg/ml) and added to 20 μl of 0.5% (w/v) sodium nitrate ($NaNO_3$) and 35 μl of 1% (w/v) aluminum chloride ($AlCl_3$) in a 96-well microplate. The reaction mixture was incubated in the dark at room temperature for 10 min. Absorbance values were measured at 430 nm with a microplate reader and a standard curve was prepared using 2-fold dilutions of quercetin (5–320 μg/ml). All values were expressed as mg quercetin equivalent per g dry weight extract (mg QE/g extract).

Evaluation of antioxidant activities

FRAP assay

The FRAP assay was conducted as described by Niroula *et al.* [22] with slight modifications. The FRAP reagent was freshly prepared every day by mixing acetate buffer (300 mM, pH 3.6), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ; 10 mM in 40 mM of HCl), and $FeCl_3$ (20 mM in distilled water) in a ratio of 10:1:1 (v/v/v). The FRAP reagent was incubated at 37°C before use. Aliquots of 190 μl of FRAP reagent were mixed with 10 μl of sample extracts and dissolved in methanol (2 mg/ml) before incubating in the dark at 37°C for 15 min. The reaction was measured by the absorbance at 593 nm using a microplate reader and a standard curve was prepared using 2-fold dilutions of Trolox (1.25-320 μg/ml). All values were expressed as mg Trolox equivalent per g dry weight extract (mg TE/g extract).

DPPH radical scavenging assay

The DPPH radical scavenging assay was performed according to the modified method described by Li *et al.* [23]. Briefly, DPPH was

prepared at 0.2 mM in methanol. Aliquots of 100 µl of diluted samples were mixed with 100 µl of DPPH solution in a 96-well microplate and incubated in the dark at room temperature for 30 min. After incubation, the absorbance was measured at 517 nm using a microplate reader. Free radical scavenging activity (FRSA) was calculated using the following formula:

$$\%\ \text{FRSA} = [1-(A_{\text{sample}}/A_{\text{control}})] \times 100, \quad (2)$$

where A_{sample} is the absorbance of the sample solution and A_{control} is the absorbance of the negative control. The antioxidant activity was expressed as the half-maximal inhibitory concentration (IC_{50}) by plotting graphs of scavenging activity against sample concentration and comparing with ascorbic acid.

ABTS radical scavenging assay

The ABTS radical scavenging assay was determined as described by Ehiobu *et al.* [24] with slight modifications. Briefly, ABTS radical cation solution was prepared by mixing 7.0 mM ABTS solution and 2.45 mM potassium persulfate 1:1 (v/v) followed by 12–16 h incubation in the dark at room temperature. Before use, the $ABTS^{+\cdot}$ solution was diluted with distilled water to obtain absorbance at 734 nm between 0.7 ± 0.03. Then, 50 µl of diluted samples was added to 150 µl of $ABTS^{+\cdot}$ solution and incubated in the dark at room temperature for 10 min. After incubation, the absorbance at 734 nm was measured using a microplate reader. The scavenging activity percentage was calculated using formula (2), with antioxidant activity expressed as IC_{50} using ascorbic acid as a standard.

Statistical analysis

Ten replicates of all samples were carried out for callus induction, with five replicates for TPC, TFC, and antioxidant activities for each treatment. All data were presented as mean ± standard error (SE) and analyzed by one-way analysis of variance (ANOVA), with mean comparisons tested by Duncan's multiple range test at $P < .05$ level (IBM SPSS® Statistics version 22.0). Correlations between TPC, TFC, and antioxidant activities (FRAP, DPPH, and ABTS assays) were presented as values of Pearson's correlation coefficient, with significant differences in correlations determined at the $P < .01$ level. Multivariate analysis was conducted by a principal component analysis (PCA) biplot using Pearson-type matrices. A hierarchical cluster analysis (HCA) and heatmap were performed on explant extract types based on TPC, TFC, and antioxidant activities, with Pearson's correlation coefficient, PCA, and HCA implemented by OriginPro, Version 2022 software.

Results

In vitro callus induction

For nodal explants, results showed that MS media containing 1–2 mg/l kinetin, 2–4 mg/l kinetin + 1 mg/l NAA, 1–2 mg/l kinetin + 1 mg/l IAA, and without PGRs obtained 100% survival (Table 1). Survival percentages declined for other PGR types and concentrations. Callus induction was effective in all treatments, except for media containing 1 mg/l 2,4-D. Highest callus induction percentage (100%) was obtained for combinations of 3–4 mg/l kinetin + 1 mg/l NAA. Interestingly, media containing kinetin alone and without PGRs produced new shoot formations (Fig. 1A). Stereomicroscopic observation showed that the callus tissues were compact. Calli were induced from the cut end of the node, mostly with established pigments of light green to dark green (Fig. 1B). Significantly highest fresh and dry weights were obtained from media containing 1 mg/l kinetin + 1 mg/l 2,4-D at 2.29 and 0.18 g, respectively ($P < .05$, Table 1). Fresh and dry weights of calli induced from nodal explants were higher than calli from young and mature leaf explants.

For young leaf explants, 100% survival and callus induction were observed on MS media containing 1 and 4 mg/l kinetin combined with 1 mg/l 2,4-D (Table 2). After incubation, several young leaf explants were enlarged one to two times in size after 2 weeks. Callus induction from young leaf explants was obtained only in the media supplemented with kinetin + NAA or kinetin + 2,4-D. Callus tissue was compact with light green, dark green, and slightly brown pigments, and many calli were induced from

Table 1. Effect of different cytokinin and auxin combinations on callus induction from nodal explants.

PGRs (mg/l)				Survival (%)	Callus induction (%)	Fresh weight (g)	Dry weight (g)
Kinetin	NAA	IAA	2,4-D				
0	0	0	0	100.00 ± 0.00^{a}	10.00 ± 10.00^{ef}	0.01 ± 0.00^{hi}	0.00 ± 0.00^{g}
1	0	0	0	100.00 ± 0.00^{a}	$40.00 \pm 16.33^{c-f}$	$0.16 \pm 0.00^{f-i}$	$0.02 \pm 0.00^{g-i}$
2	0	0	0	100.00 ± 0.00^{a}	$50.00 \pm 16.67^{b-e}$	0.26 ± 0.01^{f}	0.03 ± 0.00^{g}
3	0	0	0	$70.00 \pm 15.28^{a-c}$	$70.00 \pm 15.28^{a-d}$	0.23 ± 0.02^{fg}	0.03 ± 0.00^{g}
4	0	0	0	80.00 ± 13.33^{ab}	$40.00 \pm 16.33^{c-f}$	0.55 ± 0.11^{e}	0.06 ± 0.01^{f}
0	1	0	0	80.00 ± 13.33^{ab}	$70.00 \pm 15.28^{a-d}$	$0.17 \pm 0.01^{f-i}$	0.03 ± 0.00^{gh}
1	1	0	0	80.00 ± 13.33^{ab}	$60.00 \pm 16.33^{a-d}$	0.63 ± 0.05^{e}	0.08 ± 0.00^{e}
2	1	0	0	100.00 ± 0.00^{a}	$60.00 \pm 16.33^{a-d}$	0.04 ± 0.00^{hi}	0.01 ± 0.00^{ig}
3	1	0	0	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	1.16 ± 0.03^{c}	0.14 ± 0.00^{b}
4	1	0	0	100.00 ± 0.00^{ab}	100.00 ± 0.00^{a}	0.96 ± 0.02^{d}	0.11 ± 0.01^{c}
0	0	1	0	$70.00 \pm 15.28^{a-c}$	$30.00 \pm 15.28^{d-f}$	$0.07 \pm 0.01^{g-i}$	$0.01 \pm 0.00^{h-g}$
1	0	1	0	100.00 ± 0.00^{a}	90.00 ± 10.00^{ab}	$0.18 \pm 0.00^{f-i}$	$0.02 \pm 0.00^{g-i}$
2	0	1	0	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	1.92 ± 0.10^{b}	0.19 ± 0.01^{a}
3	0	1	0	90.00 ± 10.00^{a}	$90.00 \pm 10.00^{a-d}$	1.80 ± 0.02^{b}	0.15 ± 0.00^{b}
4	0	1	0	$70.00 \pm 15.28^{a-c}$	$70.00 \pm 15.28^{a-d}$	$0.19 \pm 0.02^{f-h}$	0.03 ± 0.00^{g}
0	0	0	1	0.00 ± 0.00^{d}	0.00 ± 0.00^{f}	0.00 ± 0.00^{i}	0.00 ± 0.01^{g}
1	0	0	1	40.00 ± 16.33^{b}	$60.00 \pm 16.33^{a-d}$	2.29 ± 0.14^{a}	0.18 ± 0.01^{a}
2	0	0	1	80.00 ± 13.33^{ab}	$80.00 \pm 13.33^{a-c}$	0.99 ± 0.04^{d}	0.09 ± 0.00^{d}
3	0	0	1	$70.00 \pm 15.28^{a-c}$	$70.00 \pm 15.28^{a-d}$	1.29 ± 0.09^{c}	0.11 ± 0.01^{c}
4	0	0	1	50.00 ± 16.67^{bc}	$60.00 \pm 16.33^{a-d}$	$0.18 \pm 0.01^{f-i}$	0.03 ± 0.00^{g}

Notes: Mean ± SE values followed by different superscripts in the same column are significantly different according to ANOVA and Duncan's multiple range test ($P < .05$).

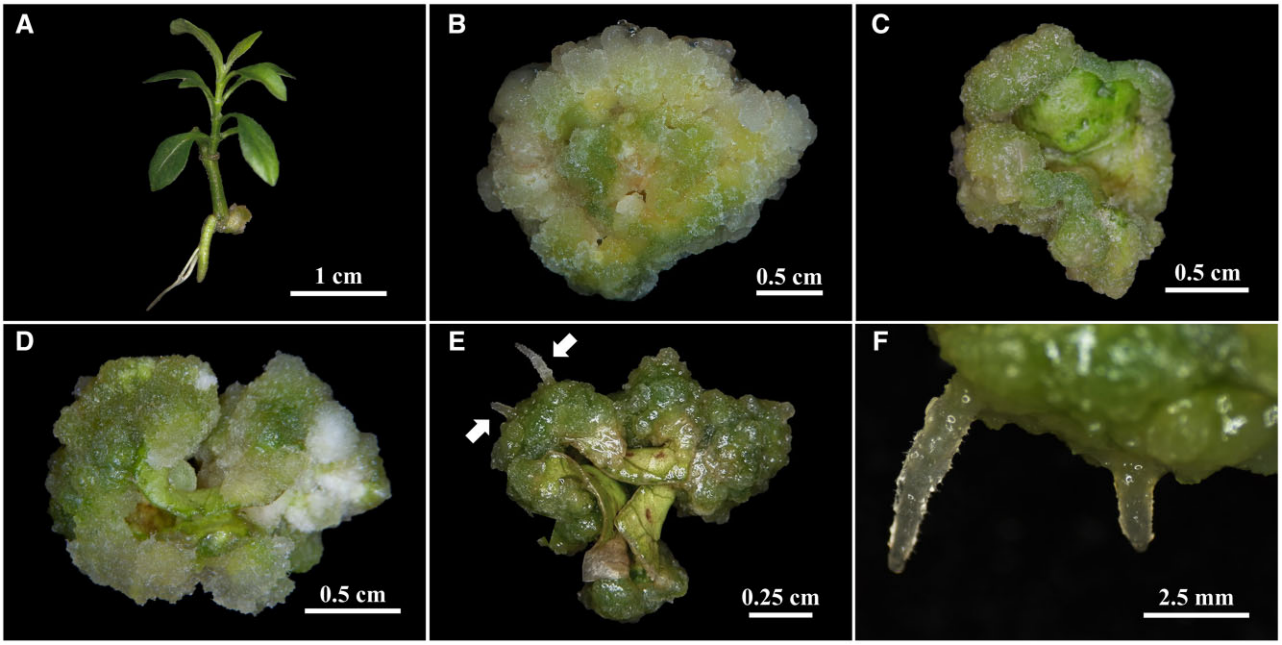

Figure 1 Morphological characteristics of callus established from *R. nusutus*. (**A**) Nodal explants cultured on MS medium without PGRs. (**B**) Nodal explants cultured on MS medium with 1 mg/l kinetin + 1 mg/l 2,4-D. (**C**) Young leaf explants cultured on MS medium with 2 mg/l kinetin + 1 mg/l 2,4-D. (**D**) Mature leaf explants cultured on MS medium with 1 mg/l kinetin + 1 mg/l 2,4-D. (**E**) Mature leaf explants cultured on MS medium with 1 mg/l kinetin + 1 mg/l NAA (arrow). (**F**) Callus derived from node with taproot.

Table 2. Effect of different cytokinin and auxin combinations on callus induction from young leaf explants.

PGRs (mg/l)				Survival (%)	Callus induction (%)	Fresh weight (g)	Dry weight (g)
Kinetin	NAA	IAA	2,4-D				
0	0	0	0	$50.00 \pm 16.67^{b–e}$	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}
1	0	0	0	$30.00 \pm 15.28^{d–f}$	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}
2	0	0	0	20.00 ± 13.33^{ef}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}
3	0	0	0	0.00 ± 0.00^{f}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}
4	0	0	0	0.00 ± 0.00^{f}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}
0	1	0	0	$30.00 \pm 15.28^{d–f}$	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}
1	1	0	0	$60.00 \pm 16.33^{a–e}$	60.00 ± 16.33^{bc}	0.35 ± 0.02^{c}	0.07 ± 0.00^{d}
2	1	0	0	$40.00 \pm 16.33^{c–f}$	40.00 ± 16.33^{c}	0.46 ± 0.06^{b}	0.09 ± 0.01^{c}
3	1	0	0	$80.00 \pm 13.33^{a–c}$	80.00 ± 13.33^{ab}	0.53 ± 0.02^{b}	0.09 ± 0.00^{bc}
4	1	0	0	$60.00 \pm 16.33^{a–e}$	60.00 ± 16.33^{bc}	0.63 ± 0.11^{a}	0.11 ± 0.01^{a}
0	0	1	0	$50.00 \pm 16.67^{b–e}$	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}
1	0	1	0	$60.00 \pm 16.33^{a–e}$	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}
2	0	1	0	$50.00 \pm 16.67^{b–e}$	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}
3	0	1	0	$70.00 \pm 15.28^{a–d}$	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}
4	0	1	0	$70.00 \pm 15.28^{a–d}$	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}
0	0	0	1	0.00 ± 0.00^{f}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}
1	0	0	1	100.00 ± 0.00^{ab}	100.00 ± 0.00^{a}	0.52 ± 0.03^{b}	0.09 ± 0.01^{bc}
2	0	0	1	90.00 ± 10.00^{ab}	90.00 ± 10.00^{a}	0.71 ± 0.06^{a}	0.10 ± 0.01^{ab}
3	0	0	1	90.00 ± 10.00^{ab}	90.00 ± 10.00^{a}	0.63 ± 0.05^{a}	0.09 ± 0.01^{bc}
4	0	0	1	100.00 ± 0.00^{ab}	100.00 ± 0.00^{a}	0.68 ± 0.04^{a}	0.09 ± 0.00^{bc}

Notes: Mean ± SE values followed by different superscripts in the same column are significantly different according to ANOVA and Duncan's multiple range test ($P < .05$).

the leaf edge (Fig. 1C). Dead explants turned a dark brown color. Fresh and dry weights of young leaf explants were significantly highest in media with 2 mg/l kinetin + 1 mg/l 2,4-D and 1 mg/l NAA at 0.71 and 0.10 g, respectively ($P < .05$, Table 2).

For mature leaf explants, the highest survival percentage (100%) was observed on MS media with 1–2 mg/l kinetin + 1 mg/l 2,4-D (Table 3). Several mature leaf explants were one to two times larger after 2 weeks of incubation. Mature leaf explants induced calli at 100% when cultured on MS media containing 1–3 mg/l kinetin + 1 mg/l 2,4-D (Fig. 1D). Callus tissue was induced from the leaf edge, and the tissue and pigment of the calli were similar to calli from young leaf explants. Media containing kinetin alone and combinations of kinetin + IAA did not induce calli. Interestingly, media containing 1 mg/l kinetin + 1 mg/l NAA

Table 3. Effect of different cytokinin and auxin combinations on callus induction from mature leaf explants.

PGRs (mg/l)				Survival (%)	Callus induction (%)	Fresh weight (g)	Dry weight (g)
Kinetin	NAA	IAA	2,4-D				
0	0	0	0	20.00 ± 13.33^{de}	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}	0.00 ± 0.00^{e}
1	0	0	0	30.00 ± 15.28^{cde}	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}	0.00 ± 0.00^{e}
2	0	0	0	$60.00 \pm 16.33^{a–d}$	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}	0.00 ± 0.00^{e}
3	0	0	0	80.00 ± 13.33^{ab}	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}	0.00 ± 0.00^{e}
4	0	0	0	$70.00 \pm 15.28^{a–c}$	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}	0.00 ± 0.00^{e}
0	1	0	0	$30.00 \pm 15.28^{c-e}$	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}	0.00 ± 0.00^{e}
1	1	0	0	$70.00 \pm 15.28^{a–c}$	70.00 ± 15.28^{bc}	0.31 ± 0.01^{e}	0.06 ± 0.00^{d}
2	1	0	0	70.00 ± 15.28^{abc}	60.00 ± 16.33^{cd}	0.26 ± 0.01^{e}	0.05 ± 0.00^{d}
3	1	0	0	80.00 ± 13.33^{ab}	60.00 ± 16.33^{cd}	0.25 ± 0.02^{e}	0.05 ± 0.00^{d}
4	1	0	0	80.00 ± 13.33^{ab}	40.00 ± 16.33^{d}	0.29 ± 0.05^{e}	0.05 ± 0.01^{d}
0	0	1	0	30.00 ± 15.28^{cde}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}
1	0	1	0	40.00 ± 16.33^{bcde}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}
2	0	1	0	70.00 ± 15.28^{abc}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}
3	0	1	0	70.00 ± 15.28^{abc}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}
4	0	1	0	80.00 ± 13.33^{ab}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}
0	0	0	1	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.01^{e}
1	0	0	1	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	0.88 ± 0.06^{a}	0.11 ± 0.01^{a}
2	0	0	1	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	0.77 ± 0.06^{b}	0.10 ± 0.01^{b}
3	0	0	1	90.00 ± 10.00^{a}	100.00 ± 0.00^{a}	0.48 ± 0.03^{d}	0.07 ± 0.00^{c}
4	0	0	1	90.00 ± 10.00^{a}	90.00 ± 10.00^{ab}	0.62 ± 0.02^{c}	0.08 ± 0.00^{c}

Notes: Mean ± SE values followed by different superscripts in the same column are significantly different according to ANOVA and Duncan's multiple range test ($P < .05$).

produced root formation at about 0.5 cm in some explants (Fig. 1E and F). Significantly highest fresh and dry weights of callus were observed on MS media with 1 mg/l kinetin + 1 mg/l 2,4-D at 0.88 and 0.11 g, respectively ($P < .05$, Table 3).

Quantification of phenolic compounds

Methanolic extracts of different types of explants were extracted by UAE, with percentage yields presented in Table 4. Node callus gave the highest yield of 38.22%, which was not significantly different from mature leaf callus (36.92%). Stem with bark extract had the lowest yield of 7.24%. Interestingly, crude extracts from node, young leaf, and mature leaf calli had significantly higher percentage yields than crude extracts from wild plant parts.

TPC values obtained from the extracts were analyzed using the Folin–Ciocalteu method, with results shown in Table 5. The TPC value of the methanolic leaf extract was significantly highest at 113.11 mg GAE/g extract ($P < .05$), followed by node callus extract at 53.21 mg GAE/g extract which was significantly higher than the other extracts. Extracts from node, young leaf, and mature leaf callus had TPC values significantly higher than extracts from wild plants (stem, stem with bark, and lateral root), with methanolic lateral root extract giving the lowest TPC value of 14.01 mg GAE/g extract.

TFC of *R. nasutus* extracts was analyzed using the aluminum chloride method, with results shown in Table 5. The TFC value of leaf extract was significantly higher than other explants at 45.15 mg QE/g extract ($P < .05$), followed by *in vitro* extracts including young leaf callus extract (25.31 mg QE/g extract), mature leaf callus extract (17.56 mg QE/g extract), and node callus extract (10.15 mg QE/g extract). The TFC value of the node extract was not significantly different ($P < .05$) from the taproot extract (10.28 mg QE/g extract), with lowest TFC value observed for the stem extract (6.81 mg QE/g extract) that was not significantly different ($P < .05$) from the stem with bark extract (7.17 mg QE/g extract).

Table 4. Extract percentage yield for different explant types.

Explant type	Yield (%)
Node callus	38.22 ± 0.19^{a}
Young leaf callus	26.42 ± 0.11^{b}
Mature leaf callus	36.92 ± 0.59^{a}
Leaf	24.67 ± 0.58^{c}
Stem	10.60 ± 0.33^{e}
Stem with bark	7.24 ± 0.14^{f}
Lateral root	27.69 ± 0.18^{b}
Taproot	14.31 ± 1.24^{d}

Notes: Mean ± SE values followed by different superscripts in the same column are significantly different according to ANOVA and Duncan's multiple range test ($P < .05$).

Antioxidant activities

The FRAP reducing power scavenging activities are shown in Table 5. A significantly ($P < .05$) highest value of FRAP was obtained for the methanolic leaf extract at 121.10 mg TE/g extract, followed by the node extract (46.27 mg TE/g extract) and stem with bark extract (11.88 mg TE/g extract). The FRAP value of methanolic extracts of young leaf callus (7.20 mg TE/g extract), mature leaf callus (7.13 mg TE/g extract), stem (7.18 mg TE/g extract), lateral root (7.75 mg TE/g extract), and taproot (8.32 mg TE/g extract) was not significantly different ($P < .05$).

DPPH FRSA was significantly influenced by explant type. Scavenging activity on DPPH radical was expressed as IC_{50} value (Table 5). The lower the IC_{50} value, the more effective scavenging of DPPH with higher antioxidant activity. The IC_{50} values of *R. nasutus* extracts and standard are shown in Table 5. The IC_{50} value of the standard ascorbic acid was 5.52 µg/ml. For IC_{50} valuations, methanolic leaf extract had significantly ($P < .05$) highest DPPH scavenging activity (52.95 µg/ml) followed by the node callus extract (118.33 µg/ml), with lowest DPPH scavenging activity observed for the lateral root extract (1,247.62 µg/ml).

ABTS-free radical scavenging abilities and standard expressed as IC_{50} values are shown in Table 5. A statistically significant difference was found between ABTS scavenging activity across the

Table 5. TPC, TFC, FRAP, and IC_{50} values by DPPH and ABTS assay of different explant types.

Explant type	TPC (mg GAE/g extract)	TFC (mg QE/g extract)	Antioxidant activities		
			FRAP assay (mg TE/g extract)	IC_{50} by DPPH assay (μg/ml)	IC_{50} by ABTS assay (μg/ml)
Node callus	53.21 ± 0.12^{b}	10.15 ± 0.07^{d}	46.27 ± 0.97^{b}	118.33 ± 0.23^{c}	55.91 ± 0.11^{c}
Young leaf callus	27.40 ± 0.06^{e}	25.31 ± 0.26^{b}	7.20 ± 0.20^{d}	303.19 ± 0.23^{e}	212.43 ± 0.53^{h}
Mature leaf callus	30.72 ± 0.14^{d}	17.56 ± 0.37^{c}	7.13 ± 0.56^{d}	523.82 ± 0.68^{f}	211.75 ± 0.31^{h}
Leaf	113.11 ± 0.98^{a}	45.15 ± 0.40^{a}	121.10 ± 2.46^{a}	52.95 ± 0.16^{b}	14.44 ± 0.06^{b}
Stem	25.99 ± 0.46^{f}	6.81 ± 0.11^{f}	7.18 ± 0.75^{d}	624.52 ± 0.58^{h}	100.55 ± 0.48^{e}
Stem with bark	20.40 ± 0.36^{g}	7.17 ± 0.06^{f}	11.88 ± 0.25^{c}	620.69 ± 1.56^{g}	138.90 ± 0.50^{f}
Lateral root	14.01 ± 0.19^{h}	9.47 ± 0.14^{e}	7.75 ± 0.52^{d}	$1{,}247.62 \pm 1.80^{i}$	209.47 ± 0.85^{g}
Taproot	35.99 ± 0.05^{c}	10.28 ± 0.06^{d}	8.32 ± 1.38^{d}	275.42 ± 0.82^{d}	71.35 ± 0.24^{d}
Ascorbic acid	–	–	–	5.52 ± 0.02^{a}	3.28 ± 0.01^{a}

Notes: Mean ± SE values followed by different superscripts in the same column are significantly different according to ANOVA and Duncan's multiple range test ($P < .05$).

different types of explants, with IC_{50} value of the standard ascorbic acid 3.28 μg/ml. The IC_{50} value of methanolic leaf extract showed significantly ($P < .05$) highest activity (14.44 μg/ml) followed by the node callus extract (55.91 μg/ml). Lowest ABTS radical scavenging (higher IC_{50} value) was observed for young leaf callus (212.43 μg/ml) and mature leaf callus (211.75 μg/ml).

Correlations and multivariate statistics

The relationship between TPC and TFC with antioxidant FRAP, DPPH, and ABTS scavenging activities was studied using Pearson's correlation analysis (Fig. 2). No significant correlations ($P < .01$) were found between TPC and TFC with antioxidant activities, except for correlations between TFC with ABTS. TPC showed significantly strong positive correlations with FRAP ($r = 0.973$) and TFC ($r = 0.828$) and significantly strong negative correlations with ABTS ($r = -0.737$) and DPPH ($r = -0.686$). It also exhibited a significantly positive correlation with FRAP ($r = 0.798$) and a significantly negative correlation with DPPH ($r = -0.521$) but was not significantly correlated with ABTS. FRAP exhibited a significant negative correlation with ABTS ($r = -0.702$) and a less pronounced negative correlation with DPPH ($r = -0.562$). In contrast, DPPH and ABTS demonstrated a strong and significant positive correlation ($r = 0.646$).

A PCA biplot was produced to evaluate the correlations between TPC and TFC with antioxidant FRAP, DPPH, and ABTS scavenging activities of *R. nasutus* extracts. The biplot showed that the two principal components accounted for 90.12% of the total variance. The components PC1 and PC2 explained 83.7% and 14.7% of the total variation, respectively (Fig. 3). The PCA factor loading showed that TPC, FRAP, and DPPH scavenging activities were strongly associated with PC1, while TFC and ABTS were highly correlated with PC2. The PCA biplot separated the leaf extract from the other *R. nasutus* extracts in the positive quadrant of PC1. Conversely, lateral root, mature leaf, and young leaf extracts were separated in the positive quadrant of PC2. The biplot was used to differentiate between several extracts, with the direction and length indicating the contribution of each variable. In PC1, leaf extract had positive coefficients for TPC and FRAP scavenging activity. The direction of the eigenvectors indicated leaf extract characteristics through contained phenolic compounds and antioxidant activity by FRAP assay. By contrast, lateral root, mature leaf callus, and young leaf callus extracts were considered negative coefficients for DPPH scavenging activity. In PC2, the negative coefficient for TFC indicated characteristics of leaf extract. The ABTS scavenging activity had positive coefficients in PC2, demonstrating characteristics for lateral root, mature leaf, and young leaf callus extracts.

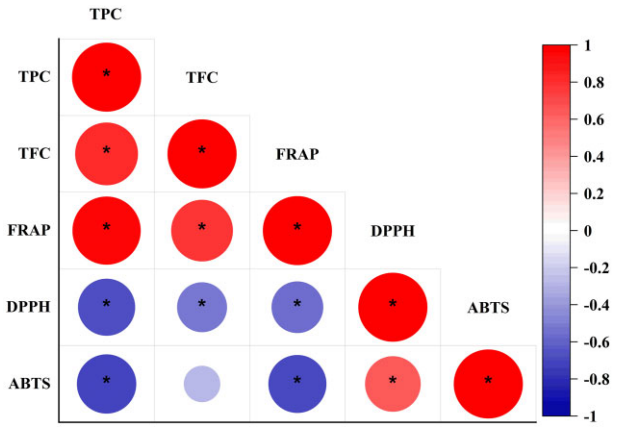

Figure 2 Pearson's correlation coefficient matrix between phenolic compounds and antioxidant activities of *R. nasutus* extracts. FRAP radical scavenging activity; DPPH radical scavenging activity (IC_{50}); ABTS radical scavenging activity (IC_{50}). (* represents significance at $P < .01$).

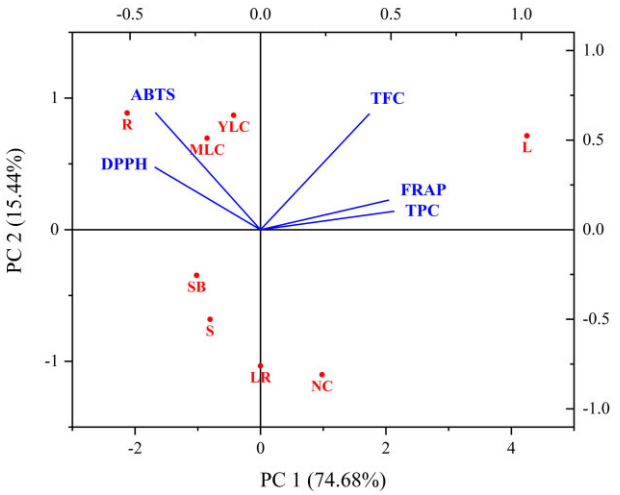

Figure 3 PCA biplot from PC1 and PC2 for *R. nasutus* extracts (red dots) based on their phenolic compounds and antioxidant activities (blue letters). LR, lateral root; MLC, mature leaf callus; YLC, young leaf callus; L, leaf callus; SB, stem with bark; S, stem; TR, taproot, NC, node callus; FRAP radical scavenging activity; DPPH radical scavenging activity (IC_{50}); ABTS radical scavenging activity (IC_{50}).

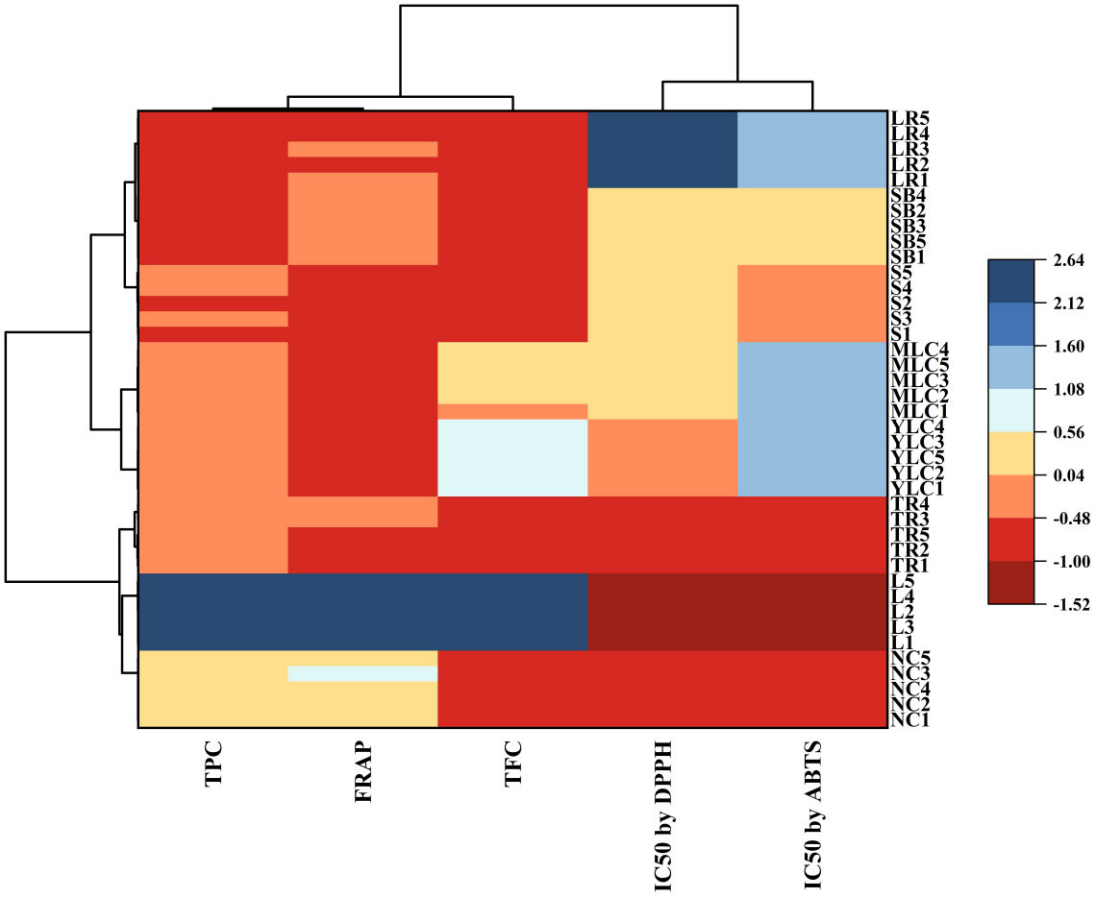

Figure 4 HCA and heatmap of phenolic compounds and antioxidant activities of *R. nasutus* extracts. LR, lateral root; MLC, mature leaf callus; YLC, young leaf callus; L, leaf callus; SB, stem with bark; S, stem; TR, taproot, NC, node callus; FRAP radical scavenging activity; DPPH radical scavenging activity (IC_{50}); ABTS radical scavenging activity (IC_{50}); the numbers 1–5 refer to the number of replications.

The HCA heatmap results quantitatively identified the phenolic compounds, while the antioxidant activity indicated two main clusters in the dendrogram (Fig. 4). Cluster I included TPC, FRAP scavenging activities, and TFC, while IC_{50} values by DPPH and ABTS assays were in Cluster II. The *R. nasutus* extracts were separated into six major clusters, with the variables following the same pattern. Cluster I consisted of the methanolic node callus extract, while young leaf and mature leaf callus were grouped in Cluster IV. The methanolic leaf extract and taproot extract were grouped in Cluster II and Cluster III, respectively. Cluster V included the methanolic stem extract and stem with bark extract, while lateral root extract was grouped in Cluster VI (Supplementary Table S1).

Discussion

In vitro callus induction

Successful, micropropagation of *R. nasutus* has recently been reported for shoot and root multiplication and secondary metabolite production. In this study, the main objective was to develop an efficient *in vitro* protocol for large-scale production of callus from various types of explants. Results indicated that the responses of callus differed at varying concentrations and combinations of kinetin and auxins (NAA, IAA, and 2,4-D). Nodal explants showed higher percentages of callus induction and fresh weight compared with young leaf and mature leaf explants. Sulaiman and Toma [25] achieved successful callus induction in *Arbutus andrachne* using nodal explants. Similar results have also been documented in other plants, including *Pseudarthria viscida* [26] and *Lycopersicon esculentum* [27]. Plant nodes contain meristematic tissues that are easy to reactivate, with high concentrations of plant hormones (cytokinins and auxins) in the plant body providing high callus productivity [28]. The young and mature leaf explants enlarged due to callus growth, showing the sign of dedifferentiation. Results showed that fresh and dry weights of callus derived from young leaves were similar to callus derived from mature leaves. Reshi [29] showed that *R. nasutus* leaf explant had maximum callusing percentage. The success of callogenesis depends on the source and developmental stage of the explant, plant species, PGRs, and culture conditions [30, 31].

Addition of PGRs, such as natural or synthetic phytohormones, at specific phases of growth or maturation, can be used to influence plant development [32]. Cytokinin and auxin hormone groups are essential in tissue culture for plant regeneration. This research successfully developed calli on MS media supplemented with 1 mg/l kinetin + 1 mg/l 2,4-D, with highest fresh and dry weights of callus derived from the nodes, while media added with kinetin alone produced shoots with roots on nodal explants. Therefore, the ratio of auxins to cytokinins plays an important role in explant induction. A high auxin-to-cytokinin ratio induces root formation, while a low ratio induces shoot formation, and a suitable ratio stimulates callus induction [33]. Similar results were also observed by Handayani *et al.* [34] who reported that various concentrations of 2,4-D and kinetin stimulated

callus formation in cinnamon. 2,4-D is widely used to induce callus formation from various types of explants because it converts cells to a dedifferentiated state where they start dividing [35]. Kinetin added to the media stimulates cell division and encourages organ development and the proliferation of shoots [36, 37]. Cheruvathur *et al.* [38] also confirmed that media containing an auxin–cytokinin combination was necessary for callus induction in *R. nasutus*. The pigments of callus are implicated in fundamental developments for plant life such as photosynthesis, plant protection, and reproduction [39]. Our results indicated that nodes of *R. nasutus* could be used as explants for callus induction, while MS media containing 1 mg/l kinetin + 1 mg/l 2,4-D was optimal for mass production of callus.

Quantification of phenolic compounds and antioxidant activities

In vitro calli derived from the nodes, young leaves, and mature leaves extracted with methanol were subjected to quantification of phenolic compounds and antioxidant activities and compared with other *R. nasutus* wild plant parts. Results showed that leaf extracts from wild plants gave higher TPC values than the other extracts, while callus derived from node extracts provided higher values of TPC compared with *in vitro* callus extract. Similar results were observed by Bukke *et al.* [1] who reported that the methanolic leaf extract of *R. nasutus* produced the highest TPC. Recently, research findings on the phytochemicals contained in *R. nasutus* have identified a class of phenolic compounds, plant steroids, isoprenoids, anthracenediones, and naphthoquinones [40]. From our results, the TFC value of leaf extract was higher than the other explants followed by young leaf callus extract. Interestingly, all the methanolic extracts contained higher phenolics than flavonoids, except for the callus derived from the node. Polyphenols are secondary plant metabolites that are involved in growth, reproduction, pigmentation, and defense of pathogens [41]. They are generally classified into phenolic acids, flavonoids, stilbenes, and lignans [42, 43]. Nirmaladevi *et al.* [7] confirmed that the leaves of *R. nasutus* contained compounds such as alkaloids and polyphenols. Therefore, callus culture techniques could be used to enhance the production of phenolic and flavonoid compounds.

Antioxidant activities of *R. nasutus* extracts were evaluated and compared with FRAP, DPPH, and ABTS assays. Results showed that the leaf extract had higher scavenging activity than the other extracts, while the node callus showed higher *in vitro* scavenging activity. Both these results depended on the amounts of phenolic and flavonoid compounds. In this study, node callus showed pigments of light green to dark green, while callus derived from nodal explants showed green and slightly brown pigments. Ahmad Fauzi *et al.* [44] reported that callus formation of *Azadirachta indica* that contained green pigment had higher bioactive pigment content and antioxidant potential activities compared with brown and cream callus, with TPC and TFC more highly correlated with FRAP than DPPH and ABTS radical scavenging activity. Thus, phenolic and flavonoid compounds of *R. nasutus* could be major factors in the FRAP assay. Differences in antioxidant activity depend on the antioxidant reactions; FRAP (reduces metal ions), DPPH (scavenges free radicals), and ABTS (reduces synthetic radical cations) [45, 46]. The activity of phenolics and flavonoids in reducing Fe^{3+} to Fe^{2+} also plays a significant role. Primary active components in *R. nasutus* extracts were classified as naphthoquinones including rhinacanthin-C, -D, -N and -Q, rhinacanthone, and polyphenolic compounds [6]. Results demonstrated that node callus extracts contained higher phenolic and flavonoid content and antioxidant activity than other explants from *R. nasutus* wild plants.

Conclusions

Rhinacanthus nasutus is a medicinal plant that contains bioactive compounds with a variety of pharmacological applications. This study investigated the effects of cytokinins, auxins, and explant types on *R. nasutus* callus induction. Results showed that all explant types could induce callus but nodal explants were more suitable. MS media containing 1 mg/l kinetin + 1 mg/l 2,4-D was appropriate for node callus induction, while leaf extract of *R. nasutus* showed highest phenolic and flavonoid contents and antioxidant activity. Callus derived from nodes showed higher phenolic than flavonoid content and higher antioxidant activity than young leaf and mature leaf explants. Pearson's correlation and PCA results showed that TPC and TFC had strongly positive correlations with the FRAP assay, while HCA and heatmap results showed that the leaf extract gave more effective TPC, TFC, FRAP, DPPH, and ABTS assays. Our findings provide credence to the notion of callus culture to optimize bioactive compounds.

Acknowledgements

The authors acknowledge the Department of Biology, Faculty of Science and Faculty of Pharmaceutical Science, Khon Kaen University, Thailand for facility support.

Author contributions

Wipa Yaowachai (Conceptualization [equal], Investigation [equal], Methodology [equal]), Prathan Luecha (Conceptualization [equal], Visualization [equal]), and Worasitikulya Taratima (Conceptualization [equal], Project administration [equal], Supervision [equal])

Supplementary data

Supplementary data are available at *Biology Methods and Protocols* online.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Funding

This study was financially supported by the Science Achievement Scholarship of Thailand (SAST).

References

1. Bukke S, Raghu PS, Sailaja G *et al.* The study on morphological, phytochemical and pharmacological aspects of *Rhinacanthus nusutus* (L.) Kurz (a review). *J Appl Pharm Sci* 2011;**1**:26–32.
2. Subramaniam A. Promising plants in the development of new life saving drugs for viral and fungal diseases. *Curr Sci* 2006; **90**:480–6.
3. Siripong P, Kanokmedhakul K, Piyaviruyakul S *et al.* Antiproliferative naphthoquinone esters from *Rhinacanthus nasutus* Kurz. roots on various cancer cells. *J Tradit Complement Med* 2006;**23**:166–72. doi:10.11339/jtm.23.166.

4. Shyamal S, Latha PG, Suja SR *et al.* Hepatoprotective effect of three herbal extracts on aflatoxin B1-intoxicated rat liver. *Singapore Med J* 2010;**51**:326–31.
5. Tewtrakul S, Tansakul P, Panichayupakaranant P. Anti-allergic principles of *Rhinacanthus nasutus* leaves. *Phytomedicine* 2009;**16**: 929–34. doi:10.1016/j.phymed.2009.03.010.
6. Kodama O, Ichikawa H, Akatsuka T *et al.* Isolation and identification of an antifungal naphthopyran derivative from *Rhinacanthus nasutus*. *J Nat Prod* 1993;**56**:292–4. doi: 10.1021/np50092a018.
7. Nirmaladevi R, Padma PR, Kavitha D. Analyses of the methanolic extract of the leaves of *Rhinacanthus nasutus*. *J Med Plants Stud Res* 2010;**4**:1554–60.
8. Wu TS, Yang CC, Wu P *et al.* A quinol and steroids from the leaves and stems of *Rhinacanthus nasutus*. *Phytochemistry* 1995; **40**:1247–9. doi:10.1016/0031-9422(95)00427-9.
9. Gouthaman T, Senthil Kumar T, Rao AS *et al. In vitro* plant regeneration in dainty spur [*Rhinacanthus nasutus* (L.) Kurz.] by organogenesis. In: Anis M, Ahmad N (eds), *Plant tissue culture: Propagation, conservation and crop improvement*. Singapore: Springer, 2016, 119–33. doi:10.1007/978-981-10-1917-3_7.
10. Kim DH, Gopal J, Sivanesan I. Nanomaterials in plant tissue culture: The disclosed and undisclosed. *RSC Adv* 2017;**7**:36492–505. doi:10.1039/C7RA07025J.
11. Reed BM, Wada S, DeNoma J *et al.* Mineral nutrition influences physiological responses of pear *in vitro*. *In Vitro Cell Dev Biol Plant* 2013;**49**:699–709. doi:10.1007/s11627-013-9556-2.
12. Abbasi BH, Khan MA, Mahmood T *et al.* Shoot regeneration and free-radical scavenging activity in *Silybum marianum* L. *Plant Cell Tiss Organ Cult* 2010;**101**:371–6. doi:10.1007/s11240-010-9692-x.
13. Bhatia S, Sharma K, Dahiya R *et al.* Chapter 2—Plant tissue culture. In: Bhatia S (eds), *Modern Applications of Plant Biotechnology in Pharmaceutical Sciences*. UK: Academic Press, 2015, 31–107. doi: 10.1016/C2014-0-02123-5.
14. Ikeuchi M, Sugimoto K, Iwase A. Plant callus: mechanisms of induction and repression. *Plant Cell* 2013;**25**:3159–73. doi: 10.1105/tpc.113.116053.
15. Uchendu MR, Shukla BM, Reed DCW *et al.* Improvement of ginseng by *in vitro* culture challenges and opportunities. In: Murray MY (eds), *Comprehensive Biotechnology*. Pergamon, 2017, 329–42. doi:10.1016/B978-0-12-809633-8.09210-4.
16. Patel A, Tiwari S, Parihar P *et al.* Chapter 2—Carbon nanotubes as plant growth regulators: Impacts on growth, reproductive system, and soil microbial community. In: Tripathi DK, Ahmad P, Sharma S *et al.* (eds), *Nanomaterials in Plants, Algae and Microorganisms*. UK: Academic Press, 2019, 23–42. doi: 10.1016/B978-0-12-811488-9.00002-0.
17. Sulaiman CT, Balachandran I. Total phenolics and total flavonoids in selected Indian medicinal plants. *Indian J Pharm Sci* 2012;**74**:258–60. doi:10.4103/0250-474X.106069.
18. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 1962;**15**: 473–97. doi:10.1111/j.1399-3054.1962.tb08052.x.
19. *Growth Regulators—Plant Tissue Culture Protocol* [Internet]. Darmstadt: Merck KGaA [cited 26 August 2023]. https://www.sigmaaldrich.com/technical-documents/technical-article/cell-culture-and-cell-culture-analysis/plant-tissue-culture/growth-regulators.
20. Hmamou A, Eloutassi N, Alshawwa SZ *et al.* Total phenolic content and antioxidant and antimicrobial activities of *Papaver rhoeas* L. organ extracts growing in Taounate region, Morocco. *Molecules* 2022;**27**:854.doi:10.3390/molecules27030854.
21. Oh S, Zheng S, Fang M *et al.* Anti-photoaging effect of *Phaseolus angularis* L. extract on UVB-exposed HaCaT keratinocytes and possibilities as cosmetic materials. *Molecules* 2023;**28**:1407.doi: 10.3390/molecules28031407.
22. Niroula A, Amgain N, Kc R *et al.* Pigments, ascorbic acid, total polyphenols and antioxidant capacities in deetiolated barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) microgreens. *Food Chem* 2021;**354**:129491.doi:10.1016/j.foodchem.2021.129491.
23. Li C, Yu M, Li S *et al.* Valorization of fig (*Ficus carica* L.) waste leaves: HPLC-QTOF-MS/MS-DPPH system for online screening and identification of antioxidant compounds. *Plants* 2021;**10**: 2532.doi:10.3390/plants10112532.
24. Ehiobu JM, Idamokoro ME, Afolayan AJ. Phytochemical content and antioxidant potential of leaf extracts of *Citrus limon* (L.) Osbeck collected in the Eastern Cape Province, South Africa. *S Afr J Bot* 2021;**141**:480–6. doi:10.1016/j.sajb.2021.06.001.
25. Sulaiman LS, Toma RS. Callus initiation and plant organ regeneration of *Arbutus andrachne* L. *from leaves and nodes grown from in vitro seed culture*. *JDU* 2023;**26**:70–8. doi:10.26682/ajuod.2023.26.1.8.
26. Cheruvathur MK, Thomas TD. An efficient plant regeneration system through callus for *Pseudarthria viscida* (L.) Wright and Arn., a rare ethnomedicinal herb. *Physiol Mol Biol Plants* 2011;**17**: 395–401. doi:10.1007/s12298-011-0089-z.
27. Halmagyi A, Coste A, Deliu C *et al.* High frequency direct organogenesis in five Romanian tomato (*Lycopersicon esculentum* Mill.) cultivars. *Horticulturae* 2023;**9**:411.doi:10.3390/horticulturae9030411.
28. Al Ghasheem N, Stănică F, Peticilă AG *et al. In vitro* effect of culture media and growth hormones on three peach (*Prunus persica* L. Batsch) cultivars. *Sci Bull* 2022;**26**:71–6.
29. Reshi N. *In vitro* micropropagation of *Rhinacanthus nasutus* (L) Kurz. *Int J Biodivers* 2018;**10**:357–64. doi:10.5897/IJBC2016.1053.
30. Hoque ME, Mansfield JW. Effect of genotype and explant age on callus induction and subsequent plant regeneration from root-derived callus of indica rice genotypes. *Plant Cell Tissue Organ Cult* 2004;**78**:217–23. doi:10.1023/B:TICU.0000025640.75168.2d.
31. Chirva OV, Ignatenko RV, Ershova MA *et al. Pinus sylvestris* L. mature seeds megagamethophytes cultured *in vitro*. Influence of the genotype and the growth regulators content in nutrient medium on the initiation of callus formation. *Plant Cell Tiss Organ Cult* 2023;**152**:299–308. doi:10.1007/s11240-022-02404-3.
32. Phillips GC, Garda M. Plant tissue culture media and practices: an overview. *In Vitro Cell Dev Biol Plant* 2019;**55**:242–57. doi: 10.1007/s11627-019-09983-5.
33. Rahman NNA, Rosli R, Kadzimin S *et al.* Effects of auxin and cytokinin on callus induction in *Catharanthus roseus* (L.) G. Don. *Fundam Appl Agric* 2019;**4**:1–32. doi:10.5455/faa.54779.
34. Handayani RS, Yunus I, Sayuti M *et al. In-vitro* callus induction of durian (*Durio zibethinus* Murr.) leaves using kinetin and 2, 4-D (dichlorophenoxyacetic acid). *J Trop Hort* 2019;**2**:59–64. doi: 10.33089/jthort.v2i2.23.
35. Dalila ZD, Jaafar H, Manaf AA. Effects of 2,4-D and kinetin on callus induction of *Barringtonia racemosa* leaf and endosperm explants in different types of basal media. *Asian J Plant Sci* 2012; **12**:21–7. doi:10.3923/ajps.2013.21.27.
36. Khalil R, Haroun S, Bassyoini F *et al.* Salicylic acid in combination with kinetin or calcium ameliorates heavy metal stress in *Phaseolus vulgaris* plant. *J Agric Food Res* 2021;**5**:100182.doi: 10.1016/j.jafr.2021.100182.
37. Farjaminezhad R, Garoosi GA. New biological trends on cell and callus growth and azadirachtin production in *Azadirachta indica*. *3 Biotech* 2019;**9**:309.doi:10.1007/s13205-019-1836-z.
38. Cheruvathur MK, Sivu AR, Pradeep NS *et al.* Shoot organogenesis from leaf callus and ISSR assessment for their identification of

clonal fidelity in *Rhinacanthus nasutus* (L.) Kurz., a potent anticancerous ethnomedicinal plant. *Ind Crops Prod* 2012;**40**:122–8. doi:10.1016/j.indcrop.2012.02.043

39. Benítez-García I, Vanegas-Espinoza PE, Meléndez-Martínez AJ *et al.* Callus culture development of two varieties of *Tagetes erecta* and carotenoid production. *Electron J Biotechnol* 2014;**17**:107–13. doi:10.1016/j.ejbt.2014.01.004.
40. Wu TS, Hsu HC, Wu PL *et al.* Naphthoquinone esters from the root of *Rhinacanthus nasutus*. *Chem Pharm Bull (Tokyo)* 1998;**46**: 413–8. doi:10.1248/cpb.46.413.
41. Lattanzio V, Lattanzio VM, Cardinali A. Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. *Phytochem Adv Res* 2006;**661**:23–67.
42. Manach C, Scalbert A, Morand C *et al.* Polyphenols: food sources and bioavailability. *Am J Clin Nutr* 2004;**79**:727–47. doi: 10.1093/ajcn/79.5.727.
43. Plamada D, Vodnar DC. Polyphenols—gut microbiota interrelationship: a transition to a new generation of prebiotics. *Nutrients* 2021;**14**:137.doi:10.3390/nu14010137.
44. Ahmad Fauzi NS, Abd Rahim MH, Abdul Majid N *et al.* Evaluation of the effect of jasmonic acid elicitation on composition of pigments and biological activities in green callus of neem (*Azadirachta indica*). *Front Sustain Food Syst* 2022;**6**:1017398. doi:10.3389/fsufs.2022.1017398.
45. Müller L, Fröhlich K, Böhm V. Comparative antioxidant activities of carotenoids measured by ferric reducing antioxidant power (FRAP), ABTS bleaching assay (αTEAC), DPPH assay and peroxyl radical scavenging assay. *Food Chem* 2011;**129**:139–48. doi:10.1016/j.foodchem.2011.04.045.
46. Santos-Sánchez NF, Salas-Coronado R, Villanueva-Cañongo C *et al.* Antioxidant compounds and their antioxidant mechanism. *Antioxidants* 2019;**10**:1–29. doi:10.5772/intechopen.85270.