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In vitro Callus Induction and Antioxidant Activity of *Rauwolfia vomitoria* Afzel. (Apocynaceae)

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: *Rauwolfia vomitoria* Afzel. (Apocynaceae) is a medicinal plant valued for its antipsychotic effect and used in various herbal preparations.

Objectives: This study was designed to develop a protocol for callus initiation in *Rauvolfia vomitoria* and to investigate the antioxidant activity of the wild plant and the leaf-derived callus of the plant.

Materials and methods: Callus initiation of the leaf explants was achieved on Murashige and Skoog's medium fortified with α-naphthaleneacetic acid (NAA), 2, 4-dichlorophenoxyacetic acid (2,4- D) and 6-benzylamino purine (BAP). Methanol extracts of leaf and root of the wild plant and callus were analyzed for their antioxidant activity using 2, 2, diphenyl-1-picryl hydrazyl (DPPH) for the radical scavenging activity (RSA) and Folin Ciocalteau spectrophotometric method for the total phenolic content (TPC).

Results: Of the various plant growth hormones employed in this study for callus establishment, 1.0 mg/L NAA + 4.0 mg/L BAP induced maximum callus formation of 83.0%, while 1.0 mg/L 2,4-D gave 20.0% formation. Leaf explants placed in the dark phase produced whitish, friable calli. The highest antioxidant activity was obtained from the root extract of the wild plant with IC_{50} values of 3.56±1.67 and 429.72±19.83 µgGAE/g for RSA and TPC, respectively. Leaf-derived callus had the least antioxidant effect. Ascorbic and Gallic acid included in the study as standards had IC_{50} values of 6.9±0.18 and 8.6±0.65 µg/mL, respectively.

Conclusion: An efficient protocol has been established for the induction and proliferation of callus of *Rauwolfia vomitoria*, justifying the use of tissue culture technique for the conservation of this important medicinal plant.

Keywords: *Rauwolfia vomitoria*, *In vitro* callus induction, Antioxidant, Plant tissue culture, Conservation.

INTRODUCTION

Traditional medicine is still the predominant means of health care in developing countries where about 80% of the total population depends on it for their well-being (WHO, 2003). Public interest in alternative medicine is growing exponentially due to increased incidence of the adverse drug reactions and economic burden of the modern synthetic drugs. *Rauwolfia vomitoria* Afzel., a tropical shrub in the family of Apocynaceae, is a medicinal plant commonly used in various ethnomedicines in the West African sub-region. The plant occurs in bush vegetation, gallery forest, secondary vegetation where fallow periods are prolonged, and along roadsides. This medicinal plant is cultivated in many parts of the tropics and subtropics. It is commonly known as Poison devil's pepper, African snakeroot, African serpent wood or swizzle stick (English). It is a medicinal plant valued for its antipsychotic property and used in various herbal preparations for the treatment of hypertension, insomnia, nervous disorder, jaundice, diarrhoea, scabies and malaria (Schmelzer, 2007; Orwa *et al.*, 2009). The parts mostly used are the root and leaves (Burkill, 1994; Orwa *et al.*, 2009). Also, it is reported to possess multiple therapeutic properties *viz.* hepatoprotective activity (Ezejindu *et al.* (2013); hypoglycemic activity (Opajobi *et al.* (2011).

Rauwolfia vomitoria contained more than 50 active indole alkaloids, each possessing remarkable pharmacological activities (Derick, 2002). Reserpine, isolated from the root of the plant, is commonly used as a marker to identify *Rauwolfia*. Despite the decline in the sales of reserpine-based pharmaceuticals in Europe owing to side effects, *Rauwolfia* preparations are still used in the management of hypertension and in certain neuropsychiatric disorders. Moreover, the costs of reserpine-based drugs are relatively exorbitant compared to other drugs employed in the management of hypertension. For example, a tablet of Brinedrine and Regroton go for about $7 and $4, respectively.

Rauwolfia vomitoria is usually propagated by seed, although stem and root cuttings can also be employed. Seed propagation is considered the best method for raising commercial plantation. The major constraints to successful establishment of *in vitro* culture of many plants in the Apocynaceace family to which *R. vomitoria* belongs are prolonged seed dormancy and the recalcitrant nature of the seed. Seed germination in *R. vomitoria* is usually very poor (about 60%) and viability is lost after six months, while the growth rate is slow to medium (Ofori *et al*., 2012). Also, due to the reduction in fallow periods as a result of forest degradation, rapid agricultural encroachment, urbanization, to mention a few, *R. vomitoria* populations are at a higher risk of massive extinction than one would expect (Orwa *et al*., 2009). In addition, studies on distribution, ecology and conservation of *R. vomitoria* are rarely investigated in Nigeria. The management of traditional medicinal plant resources has become the matter of urgency. The need to provide reliable information on the trend of natural regeneration is of utmost importance to ensure sustainable management and prevent species extinction. Hence, there is need to employ biotechnological tool for the propagation and conservation of this plant of great medicinal significance. The present study was therefore designed to develop a protocol for the *in vitro* callus regeneration of *Rauwolfia vomitoria* and to evaluate the antioxidant and total phenolic content of the *in vitro* callus and the wild plant.

MATERIALS AND METHODS

Plant Collection, Authenication and Extraction

Leaves and roots of *R. vomitoria* were collected in July, 2015 from the field at Premier Hill, Premier Hotel, Mokola, and National Centre for Genetic Resources and Biotechnology (NACGRAB), Moor plantation, Apata, Ibadan, Southwest Nigeria, respectively. The specimens were identified and authenticated by Mr. Patrick Agwu at the Department of Pharmacognosy Herbarium, University of Ibadan (DPHUI) where the voucher specimen (DPHUI 1698) was deposited. The samples were air dried for four weeks and pulverized into coarse powder. The pulverized materials were macerated separately by exhaustive extraction in three litres of methanol. The solvent from each plant material was filtered with Whatman filter paper No.1 and concentrated *in vacuo* (Searchtech Instruments RE52-2) at 45°C to recover the methanol.

Source and preparation of explants for callus Induction

Juvenile, meristematic leaves of *Rauwolfia vomitoria* (about 60 weeks old) were collected at NACGRAB, Moor Plantation, Apata, Ibadan, Nigeria between the months of April and September, 2015. To produce sterile leaf explants for the experiment, the healthy leaves were selected and washed in a sterilized bottle with 3 drops of Tween 20 (liquid detergent) (3 drops per 150 mL solution) and with frequent agitation for 20 min. They were rinsed in distilled water until explants were completely free of Tween 20 bubbles and allowed under running tap water for another 30 min. The explants were treated with 70% ethanol for 5 min and rinsed in sterilized distilled water. Finally, the explants were disinfected with 5% Clorox (v/v; domestic household bleach containing 8.25% Sodium hypochlorite (NaOCL) for another 10 min and rinsed four times with sterile distilled water before inoculation abaxially in Murashige and Skoog‘s (MS) (1962) medium supplemented with various plant growth regulators. Explants were excised and pieces of about 0.5 cm were inoculated (Figure 1) in glass test tubes (150 mm x 25 mm) and in disposable petri dishes (90 mm) containing 15 mL and 20 mL, respectively, of MS medium supplemented with MS basal, 3% sucrose (30 g/L), myoinositol 100 mg/L (0.1 g/L), 0.7% (7 g/L) agar and different concentrations of α-Naphthalene acetic acid (NAA), 2,4-Dichlorophenoxy acetic acid (2, 4-D), 6-benzyl amino purine (BAP) were used either singly or in combination (Table 1). All disinfection and inoculation processes were carried out under the laminar flow hood.

The medium was adjusted to pH 5.7 with 1 N NaOH/HCL and sterilized at 121 °C at 15 psi for 15 min. The cultures were maintained in the growth room at 27 ± 1°C at an intensity of 10-20 µmol $m^{-2}s^{-1}$(Philips ThD 36W/84) and photoperiod of 16 h/8h dark. All surface sterilization processes were carried out in the laminar airflow cabinet. Each treatment of 10 samples was replicated three times. The cultures were subcultured on a fresh medium after 6 weeks and were maintained for another 6 weeks before harvesting for further analysis.

Table 1: Percentage Yield of crude extracts of wild leaf, Root and *in vitro* generated callus of *Rauwolfia vomitoria*

Plant parts	Treatments(Callus)	Weight of powder(g)	Weight of Extract(g)	Colour	Yield (%)
Leaves (wild)		800	33.64	Dark green	4.21
Roots (wild)		200	15.44	Brownish	7.72
Callus	1.0 mg/L NAA+ 2.0 mg/L BAP	1.06	0.28	Brownish (Oily)	26.41
	1.0 mg/L NAA+ 4.0 mg/L BAP	1.07	0.21	Brownish (Oily)	19.63

Aseptic inoculation of explant

Sterilized explants were inoculated in cultures in a laminar flow cabinet/hood. The explants were implanted horizontally (abaxially) on the sterile media in sigma glass tubes (150 mm x 25 mm) using sterilized forceps. The sigma glass tubes were sealed with paraffin film to prevent contamination and labeled appropriately.

Evaluation of growth of *Rauvolfia vomitoria* in cultures

Growth of plant was assessed weekly after culturing. Calli obtained after 6 weeks were subcultured in MS media supplemented with same growth hormones to further maintain the callus. Observations were recorded 2 weeks following inoculation and subculturing. The growth indicators used were: colour, size, and nature of calli formed.

Preparation of callus extract

Fresh mass of 6 weeks old calli with pale green colouration *viz*. 1.0 mg/L NAA + 2.0 mg/L BAP and 1.0 mg/L NAA + 4.0 mg/L BAP were harvested, air-dried, pulverized and extracted exhaustively with distilled methanol until constant weight were attained. Wet weight of 8.0 g and 9.05 g were obtained for 1.0 mg/L NAA + 2.0 mg/L BAP and 1.0 mg/L NAA + 4.0 mg/L BAP, respectively. Air-dried samples (1.06 g and 1.07 g, respectively) were agitated with 100 mL of methanol at room temperature for 24 h. The methanol extract was filtered using Whatman filter paper 90 mm.

Residues were further extracted using 80 mL methanol for 24 h and filtrates were combined and evaporated. The residues obtained were then weighed and stored in a refrigerator at 4 °C until further use (Sonibare and Adeniran, 2014).

Phytochemical analysis of crude extracts of wild *Rauwolfia vomitoria*

Standard phytochemical screening was performed on the crude extracts of the wild plant to test for the presence of secondary metabolites following the methods of Sofowora (2008), Trease and Evans (2006) and Harborne (1973). Colour intensity was used to categorize the presence of each phytochemical into copious, moderate or slight (trace).

Estimation of Total Phenolic Content

The total phenolic content (TPC) was determined using the Folin-ciocalteu method described by Singleton *et al*. (1999) which was based on a colorimetric oxidation and reduction reaction. Five milliliters of Folin-ciocalteu reagent was added to 100 µg/mL aliquots of the extracts. After 3 min, 4 mL of 7.5 % Na_2CO3 solution in distilled water was added to the mixture; the content was thoroughly mixed. The mixture was incubated for 30 min after which the absorbance was determined spectrophotometrically at absorbance of 765 nm. A linear dose – response regression curve was generated using absorbance reading of gallic acid at 765 nm wavelength. Results were expressed as µgGAE/g of the dry weight of the extracts. Absorbance values were measured at 765 nm using Model 752 UltraViolet Grating Spectrophotometer and the standard curve was drawn. All determinations were carried out in triplicate. The total phenolic content in the extract in gallic acid equivalents (GAE) was calculated using:

$$TPC = \frac{C \times V}{M}$$

Where TPC – Total Phenolic Content (mg/g plant content) in GAE.

C – the concentration of gallic acid established from the calibration curve (mg/mL)

V – the volume of plant extract (gram)

DPPH free radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl(DPPH) assay employed in the determination of free radical scavenging activity of crude extracts was performed as described by previous workers (Karioti *et al*., 2004, MacDonald-Wicks *et al*., 2006), with slight modifications. The DPPH

(0.0035 mg) in 100 mL was prepared to make 0.01 mM of DPPH. Extracts (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL and 1.562 µg/mL) were added to 0.5 mL of 0.01 mM DPPH–methanol solution. The concentrations of the extracts stock solution were obtained through multifold serial dilutions.

Various concentrations of the crude extracts and standard antioxidants (ascorbic acid and gallic used as a positive control) in methanol were prepared under dim light, shaken vigorously and incubated at room temperature for 30 min in the dark after which absorbance was read at 517 nm using a Model 752 Ultraviolet Grating Spectrophotometer. Absolute methanol was used as a blank. Background correction of the sample absorbance (without DPPH) was done by subtracting the absorbance readings of the sample extracts from the corresponding readings obtained in the presence of DPPH (Karioti *et al*., 2004). Each sample extract and standard antioxidant was replicated three times. The reaction was carried out in test tubes that were tightly wrapped with aluminum foil. The DPPH radical stock solution was freshly prepared for the reaction, and precautionary measures were taken to reduce the loss of free radical activity during the experiment. The inhibition percentage of DPPH radicals as determined by the decolouration of the deep purple DPPH solution was calculated according to the formula:

% Free Radical Scavenging Activity = Abs blank – Abs sample⁄ Abs blank x 100

Where; Abs blank = absorbance of the control reaction (all reagents except plant extract)

Abs sample = absorbance of the sample (plant extract)

Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted (inhibition percentage against extract concentration). Tests were carried out in triplicate and data were recorded as means plus standard error.

Statistical Analysis

All data were presented as mean ± S.D and mean ± S.E.M using Microsoft Office (Microsoft Excel, 2007 program). Similarly, correlation analysis of the percentage free radical scavenging activity and total phenolic content of the methanol extracts of wild leaf, root and callus were determined using the correlation and regression by Microsoft Excel 2007 package.

RESULTS AND DISCUSSION

The percentage yields of the leaf and root methanol extracts of *R. vomitoria* after concentration were approximately 4.21% and 7.72%, respectively. *In vitro* generated calli had higher yield (Table 1). The success of callus induction and micropropagation as a whole largely depends on the selection of suitable plant part as explants (Shah *et al*., 2003). Equally important is the specific concentration of plant regulators utilized for the propagation. In the present study, tender leaf explants were selected as source of primary explants (Figure 1).

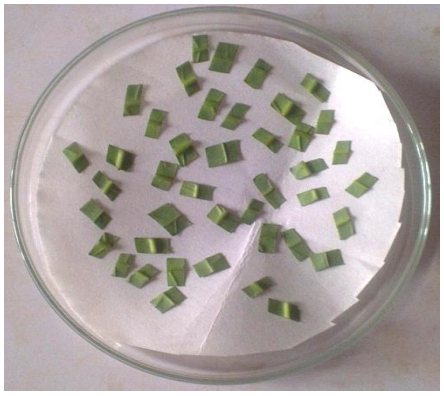

Figure 1: Trimmed juvenile leaf explants of *R. vomitoria* prior to inoculation

The results of callus induction with various plant hormones are presented in Table 2. Callus emerged at the cut edges between 10-14 days (Figure 2). The whole explants surface produced callus, which grew continuously up to six weeks of culture before subculturing was done in the same media for another 6 weeks. The explants enlarged and were pale green in colour on MS medium supplemented with different concentrations of BAP, NAA or 2, 4-D either alone or in combination (Figure 3). The explant placed in the dark for 6 weeks produced whitish, friable calli when supplemented with 0.1 mg/L NAA and 0.5 mg/L BAP. The explants grown on hormone-free medium failed to initiate calli. No callus initiation was recorded for MS medium supplemented with 0.5 mg/L NAA + 0.5 mg/L 2, 4-D, (0.1-0.5 mg/ L) + NAA (0.5 mg/L), NAA (0.1-1.0 mg/ L), (0.1 mg/L) NAA + 0.1 mg/ L BAP, (0.1 mg/ L) NAA + (1.0-2.0 mg/L) BAP, (0.1-0.5 mg/L), (0.1-0.5 mg/L) BAP and (0.1- 0.5 mg/L) 2,4-D (Table 2).

Table 2: Response of different hormones at different concentrations to the explants after 6 weeks of culture

Hormone Concentration (mg/L)	% of Response	Colour	Size/ Intensity of callus induction	Nature of callus
NAA				
NAA 0.1	0	—	—	—
NAA 0.5	0	—	—	—
NAA 1.0	0	—	—	—
NAA 2.0	13.3	Pale green	++	Hard
BAP				
BAP 0.1-2.0	0	—	—	—
NAA + BAP				
NAA 0.1+ BAP 0.1	0	—	—	—
NAA 0.1+ BAP 0.5	80	Whitish	+++	Hard
NAA 0.1+ BAP 1.0	0	—	—	—
NAA 0.1+ BAP 2.0	0	—	—	—
NAA 0.5+ BAP 0.1	0	—	—	—
NAA 0.5+ BAP 0.5	0	—	—	—
NAA 0.5+ BAP 1.0	13.3	Pale green	++	Hard
NAA 0.5+ BAP 2.0	33.3	Pale green	++	Hard
NAA 1.0+ BAP 1.0	20	Pale green	+++	Hard
NAA1.0+BAP 2.0	73	Pale green	**++++**	Hard
NAA 1.0+ BAP 3.0	0	—	—	—
NAA 1.0+ BAP 4.0	83	Pale green	++++	Hard
NAA+BAP 0.75	27	Pale green	++	Compact
NAA+ BAP 1.5	23	Pale green	++	Compact
2,4 –D				
2,4 –D 0.1	0	—	—	—
2,4 –D 0.5	0	—	—	—
2,4 –D 1.0	20	Pale	+++	Hard
2,4 –D 2.0	37	Pale	+++	Hard
NAA + 2,4 –D				
NAA 0.5+ 2,4-D 0.1	0	—	—	—
NAA 0.5 + 2,4-D 0.5	0	—	—	—
NAA 0.5 + 2,4-D 1.0	36.7	Pale green	++	Hard
NAA 0.5 + 2,4-D 2.0	20	Pale green	++	Hard
2,4 –D + BAP				
2,4-D 1.0 + BAP 1.0	0	—	—	—
2,4-D 1.0 + BAP 2.0	70	Pale green	++++	Hard
2,4-D 1.0 + BAP 3.0	0	—	—	—
2,4-D 1.0 + BAP 4.0	70	Pale green	++++	Hard

Number of explants inoculated (30). Each treatment consisted of 10 replicates and the experiment was repeated three times. + < 1 cm; ++ = 1-2 cm; +++ = 2-3 cm; ++++ = 3-4 cm.

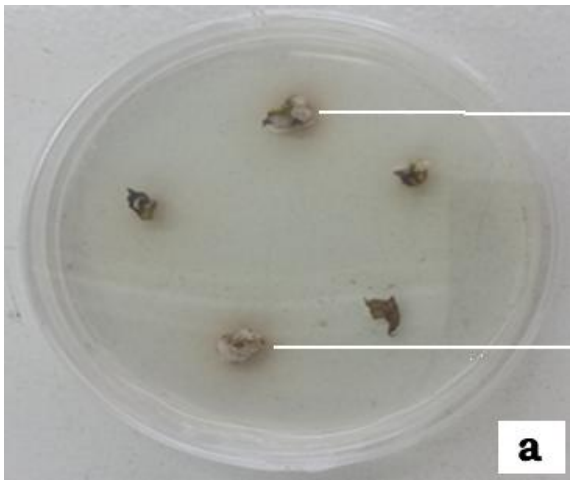

Figure 2: (a) Callus formation of *R. vomitoria* on MS media + NAA (0.1 mg/mL) + BAP (0.5 mg/mL) using juvenile leaf explant after 2 weeks of culturing in dark regime.

Explants grown on 1.0 mg/L NAA + 2.0 mg/L BAP (Figure 3a) and 1.0 mg/L 2,4-D + 2.0 mg/L BAP (Figure 3c-d) resulted in 73% and 70% callus induction, respectively. They had a larger size of approximately 4 cm, hard texture and pale green calli, while explants cultured in the dark on 0.1 mg /L NAA + 0.5 mg/L (Figure 2) produced 80% whitish, friable calli of medium size (2-3 cm). The highest percentage of callus induction (83%) was achieved using MS medium containing 1.0 mg/L NAA and 4.0 mg/L BAP (Figure 3g-i), whereas the medium containing 1.0 mg/L 2,4-D, and 0.75 mg/L NAA + 0.75 mg/L BAP showed a low rate of callus induction ranging between 20-30% (Table 2). Subsequent subculturing of induced calli in the same medium for another 6 weeks resulted in the transformation of initial hard texture calli into more friable ones. The calli, which did not show any sign of organogenesis after 6 weeks, were harvested separately per treatment, air-dried at 37°C, pulverized, exhaustively extracted with methanol and stored for other analyses. The presence of auxin alone showed poor callusing as seen in the media composed of 1.0 mg/L NAA (showing no callus) and 2,4-D at 1.0 mg/L and 2.0 mg/L producing 20% and 37% calli, respectively. No callus was observed for 2, 4-D + 0.1 mg /L and 0.5 mg/L. Also, the presence of cytokinins alone failed to initiate callus growth at all concentrations. This is in line with previous work done by Bhatt *et al.* (2008). Of the two auxins tested in this study, callus induction was better on the medium containing NAA than 2,4-D, especially when combined with various concentrations of BAP. Alpha-naphthaleneacetic acid and 2, 4-D are frequently used with BAP for callus initiation in various systems (Khateeb *et al.*, 2012; Mohammad *et al.*, 2014).

Optimum callusing has been initiated with NAA with BAP as reported by Ahmad *et al*. (2002), Pant and Joshi (2008), Cheruvathur *et al*. (2010), Khateeb *et al*. (2012). Other scientists have also found that the combination of 2,4 –D and BAP was suitable for callus proliferation (Chang *et al*., 2000; Wani *et al.*, 2010). The synergistic effect of auxin and cytokinins on callus initiation has been well documented in several systems, for instance in *Lycium barbarum* L. (Goji) (Osman *et al.*, 2013) and *Celosia argentea* var. *cristata* (Bakar *et al.*, 2014). The effects of NAA or 2,4-D and BAP for callus initiation observed in this study corroborates previous reports on callus induction in *Rauwolfia serpentina* (L.) Benth. ex Kurz (Ahmad *et al*., 2002; Pant and Joshi, 2008), *Artemisia annua* L. (Mohammad *et al*., 2014), *Cichorium pumilum* Jacq. (Khateeb *et al.*, 2012) and *Withania somnifera* (L.) Dunal. (Adhikari and Pant, 2013).

The phytochemical analysis of methanol root extract of *Rauwolfia vomitoria* as presented in Table 3 revealed the presence of abundant alkaloids and triterpenoids with moderate steroids and saponins. Tannins, phlobatannins, flavonoids, anthraquinones, cardiac glycosides were not detected in the root. The methanol leaf extract showed trace quantities of steroids and saponins, while tannins and cardiac glycosides were found in moderate amount compared to the root. The presence of alkaloids in the wild root justifies its usage in traditional medicine in treating malaria, mental illness, measles and other skin related infections (Gaoue *et al.*, 2008). Alkaloids have pharmacological applications as analgesic, anti-spasmodic, anesthetic, CNS stimulants and antibacterial properties and are the most significant plants substances medicinally (Madziga *et al.*, 2010). The presence of steroids in the root of *R. vomitoria* is of importance because steroidal compounds are potent precursors for the synthesis of sex hormones (Edeoga *et al*., 2005). Terpenoids are active against bacteria, fungi, viruses, and protozoa. Terpenes act as antimicrobial agent by membrane disruption while their antidiarrhoeal activity is attained by inhibiting the release of autocoids and prostaglandins (Ciocan and Băra, 2007).

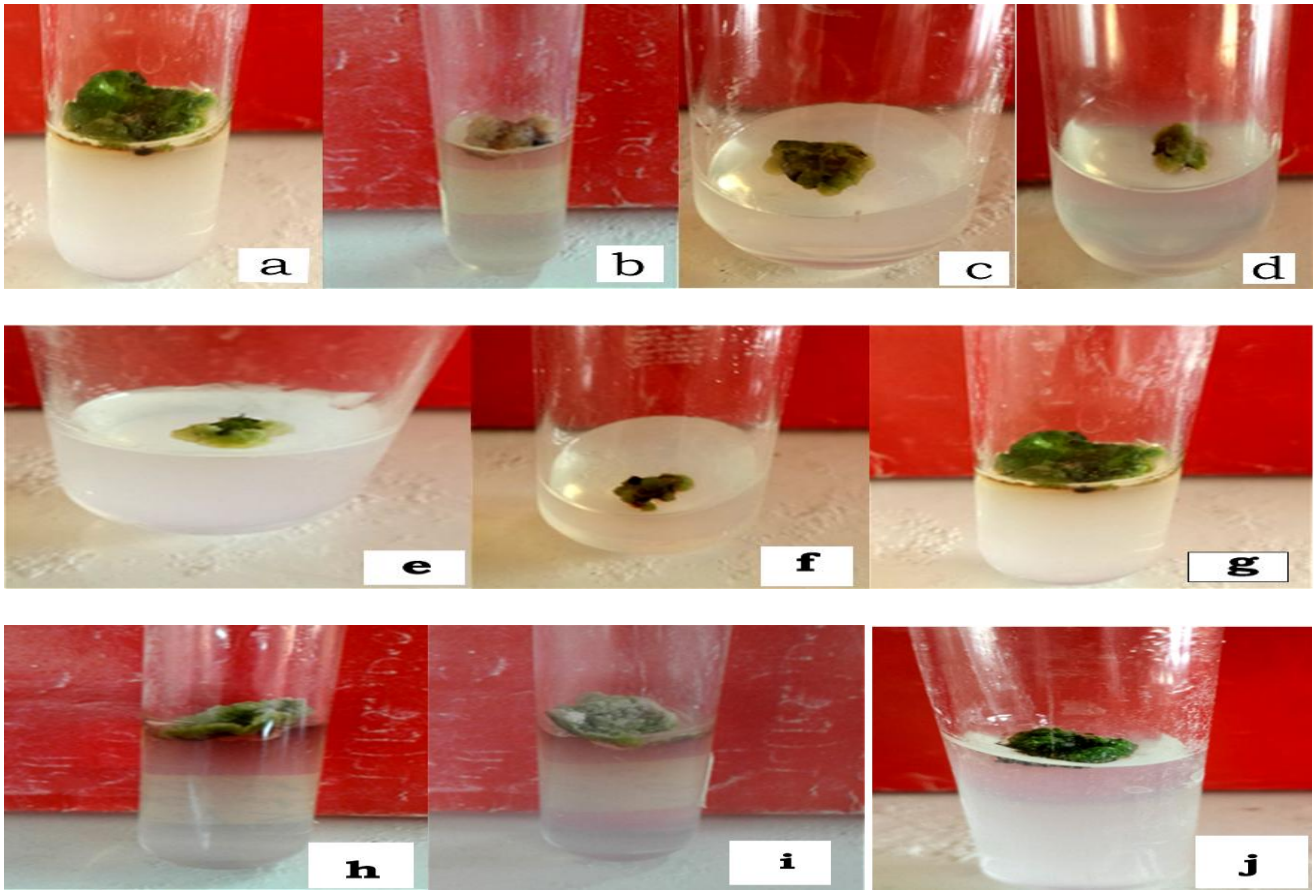

Figure 3: Callus initiation and development in *Rauvolfia vomitoria*

a. Callus formation of *Rauwolfia vomitoria* on MS media + NAA (1.0 mg/mL) + BAP (4.O mg/mL) using juvenile leaf explant after 4 weeks of culturing.
b. Callus formation of *R. vomitoria* on MS media + 2, 4-D (1 mg/mL) + BAP (2.0 mg/mL) using juvenile leaf explant after 6 weeks of subculturing.
c. Callus formation of *R. vomitoria* on MS media + 2, 4-D (1.0 mg/mL) + BAP (2.O mg/mL) using juvenile leaf explant after 3 weeks of culturing.
d. Callus formation of *R. vomitoria* on MS media + 2, 4-D (1.0 mg/mL) + BAP (2.O mg/mL) using juvenile leaf explant after 4 weeks of culturing.
e. Callus formation in *R. vomitoria* on MS media + 2, 4-D (2.0 mg/mL) using juvenile leaf explant after 4 weeks of culturing
f. Callus formation of *R. vomitoria* on MS media + 2, 4-D (1.0 mg/mL) + BAP (2.O mg/mL) using juvenile leaf explant after 3 weeks of culturing.
g. Callus formation of *R. vomitoria* on MS media + NAA (1.0 mg/mL) + BAP (4.O mg/mL) using juvenile leaf explant after 4 weeks of culturing.

(h-i) Callus formation of *Rauvolfia vomitoria* on MS media NAA (1.0 mg/mL) BAP (4.O mg/mL) using juvenile leaf explant after 6 weeks of first subculturing.

j. Callus formation (compact) of *R. vomitoria* on MS media + NAA (0.75 mg/mL) + BAP (0.75 mg/mL) using juvenile leaf explant after 4 weeks of culturing

Saponins are bitter phenolic compounds produced by plants as deterrence mechanisms to stop attacks by foreign pathogens, thus saponins are natural antimicrobials (Okwu and Emenike, 2006). They enhance the penetration of proteins through cell membranes (Sule *et al.*, 2011). They are also important therapeutically as they are shown to possess antitussive, hypolipidemic and anticancer activity (Skene and Sutton, 2006; Sarker and Nahar, 2007). Tannins are useful in the treatment of intestinal disorders such as diarrhea and dysentery, as well as urinary tract infections (Fahey, 2005; Akinpelu and Onakoya, 2006).

Table 3: Results of phytochemical screening for crude extracts of field grown *Rauwolfia vomitoria's* leaf and root

Secondary metabolites	Leave crude extract	Root crude extract
Alkaloids	—	+++
Steroids	+	++
Triterpenoids	-	+++
Tannins	++	—
Phlotannin	—	—
Flavonoids	—	—
Anthraquinones	—	—
Saponins	+	++
Cardiac glycosides	++	—

Key +++ Copious; ++ Moderate; + Trace; – Absent

Table 4: Total phenolic content of methanol extracts of field-grown leaf, root and callus of *Rauwolfia vomitoria*

Extract/ Standard Drug	Total phenolic content Assay IC_{50} (µg/GAE/g)
Wild Root	429.72 ± 19.83
Field-grown leaf	78.01 ± 2.6.46
Callus raised from (1.0mg/L) NAA+ (2.0 mg/L)BAP	6.908 ± 3.99
Callus raised from (1.0 mg/L) NAA + (4.0 mg/L)BAP	14.858 ± 8.58

Table 5: DPPH free radical Scavenging activity of methanol extracts of field-grown leaf, root and callus of *Rauwolfia vomitoria*

Extract/ Standard Drug	DPPH Assay IC_{50} (µg/ mL)	% DPPH Inhibition
Wild Root	3.56 ± 1.67	70.36
Field-grown leaf	46.75 ± 3.24	S47.95
Callus raised from (1.0 mg/L)NAA+(2.0 mg/L) BAP	62.71 ± 2.6	45.75
Callus raised from (1.0 mg/L) NAA + (4.0 mg/L) BAP	105 ± 0.25	29.04
Ascorbic acid	6.9 ± 0.18	66.15
Gallic acid	8.6±0.65	62.35

The occurrence of saponins, steroids, and glycosides have been shown to possess antidiarrhoeal effect, while saponins, alkaloids, terpenoids, essential oils, polyphenols, tannins and flavonoids, have been proven to possess both antimicrobial and antidiarrhoeal effects (Tiwari *et al*., 2011). The presence of saponins, triterpenoids, steroids and alkaloids in *Rauwolfia vomitoria's* root confirm its use in folklore medicine as antidiarrhoeal and antimicrobial agents. The results of these findings reveal that the abundance of phytochemicals in the methanol extracts of *R. vomitoria* plant contribute significantly to its antimicrobial property and justify its various roles in folk medicine.

Quantitative estimation of total phenolics content showed that the wild root had the highest phenolics (429.72 ± 19.83 µg/GAE/g), while 1.0 mg/mL NAA ± 2.0 mg/mL leaf-derived callus had the least (6.908 ± 3.99 µg/GAE/g) (Table 4). The free radical scavenging activity of methanolic extracts of root, leaf and calli of *Rauwolfia vomitoria* was determined from a reduction of absorbances of DPPH radical at 517 nm. *Rauwolfia* species extracts have been reported to possess excellent antioxidant activity, as evaluated by using DPPH Radical Scavenging and Reducing Capacity Assay (Njau *et al*., 2014). In the present study, root extracts exhibited higher antioxidant activity with IC_{50} of 3.56 ± 1.67 than gallic acid (with IC_{50} of 8.6 ± 0.65) and ascorbic acid (with IC_{50} of 6.9 ± 0.18) standard antioxidant compounds used (as shown in Table 6). The order of activity was wild root > Ascorbic acid (with IC_{50} of 6.9 ± 0.18) > Gallic acid (with IC_{50} of 8.6 ± 0.65) > wild leaf (IC_{50} of 46.75 ± 3.24) > callus with 1.0 mg/L NAA + 2.0 mg/L (IC_{50} of 62 ± 2.6) > callus with 1.0 mg/L NAA + 2.0 mg/L (IC_{50} of 105 ± 0.25). All the extracts showed concentration-dependent activity with increase in activity as the concentration increases (Table 5). The high antioxidant activity may be attributed to the abundance of alkaloids in wild root. This result is in line with previous report by Njau *et al*. (2014) indicating that alkaloid extracts obtained from the root bark of *R. caffra* exhibited higher antioxidant effect than ascorbic and gallic acid. Also, the present result corroborates previous studies which reported that alcohol (methanol) is a better solvent for more consistent extraction of bioactive compounds from medicinal plants against microbes compared to other solvents (Ellof, 1998; Cowan, 1999; Das *et al.,* 2010). This could be due to the fact that the root of *R. vomitoria* might contain more secondary metabolites, responsible for the antimicrobial activity and inhibition of the growth of microbes.

CONCLUSION

The *in vitro* propagation methods utilized in this study was efficient and could be employed for germplasm conservation and mass multiplication of *Rauwolfia vomitoria*. *In vitro* culture protocol for callus induction in this study is in tandem with literature report and may be developed for mass propagation of the plant for possible extraction, isolation and characterisation of compounds of interests that could be of pharmaceutical use in the future.

Profuse callus induction was observed within 4 weeks with 1.0 mg/L NAA + 4.0 mg/L BAP with 83% callus response. The results of this study justify the use of tissue culture technique as an alternative propagation technique for the conservation of *Rauwolfia vomitoria* for material supply in support of its several medicinal applications.

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