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Screening and Quantification of Artemisinin and Phytochemicals Content in *Artemisia nilagarica* (C.B. Clarke) Pamp.

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Abstract

The present study reports, for the first time, the quantification of secondary metabolites including artemisinin in field grown plant samples and *in vitro* regenerated plantlets and callus tissues of *Artemisia nilagarica* (C.B. Clarke) Pamp. The influence of different growth regulators on *in vitro* micropropagation and callus induction was investigated. Maximum shoot multiplication from shoot tip explants was observed in explants cultured on Murashige and Skoog medium supplemented with 1 mg/L naphthalene acetic acid in combination with 5 mg/L kinetin followed by medium supplemented with 1 mg/L naphthalene acetic acid in combination with 3 mg/L kinetin and the shoot buds also showed rooting in these media. Rooted plantlets were successfully established in the soil. Friable callus was induced from shoot tip explants of *in vitro* regenerated plantlets on medium supplemented with combination of 1 mg/L naphthalene acetic acid and 5 mg/L 6-benzylaminopurine. Secondary metabolites such as phenol, alkaloid, tannins, saponins, flavonoids, terpenoids, steroids, phlobatannins, chalcones and anthraquinones were found to be present in *A. nilagarica*. The highest amount of alkaloid, saponin, steroid and DPPH free radical scavenging activity was obtained in shoot tips, stems, roots and leaves respectively while the artemisinin content was higher in leaves of field grown plants than in *in vitro* regenerated plantlets and callus tissues.

Abbreviations

BAP: 6-Benzylaminopurine; DPPH: 2,2-Diphenyl-1-Picrylhydrazyl; IAA: Indole-3-Acetic Acid; IBA: Indole-3-Butyric Acid; Kin: Kinetin; MS: Murashige and Skoog medium; NAA: Naphthalene Acetic Acid; TLC: Thin Layer Chromatography; HPLC: High Performance Liquid Chromatography

Introduction

Artemisia is one of the largest genera of the Asteraceae family and about 34 species including *A. nilagarica* (C.B. Clarke) Pamp. are found in India [1]. The genus *Artemisia* is an important medicinal plant and many of the species are economically important as a source of essential oils, secondary metabolites, medicines, food, forage, ornamentals or soil stabilizers in disturbed habitats [2]. Like *A. annua*, the most important source of the anti-malarial drug artemisinin, *A. nilagarica* has also been shown to have high potency against the malaria parasite, *Plasmodium falciparum* [3,4]. *A. nilagarica* is commonly known as 'Laibakngou' in Manipur and its leaf extracts are used by local healers for treatment of wounds on skin, mouth sores and also as a tonic, antiseptic, analgesic, stomachic and insect repellent or as anti-diabetic [5-8]. *A. nilagarica* has been listed in the threatened medicinal plant category [9] and the species is over-exploited for its diverse medicinal properties [10]. Therefore, there is an urgent need to develop an alternative means for increased production of the important secondary metabolites and other important components of the plant. Plants obtained through micro-propagation and *in vitro* cultured cells provide an efficient method for production of useful secondary metabolites irrespective of growing seasons and also minimize the over-exploitation of the plants from nature. There have been few reports on micro-propagation and callus induction in *A.nilagarica* [9] and no study has been conducted to estimate the artemisinin production potential and quantification of secondary metabolites of *in vitro* cultures of the plant. Therefore, the present study was undertaken to comparatively explore the content in field grown plant samples and in *in vitro* regenerated plants and callus tissues of *A. nilagarica*.

Materials and Methods

Micro-propagation

Shoot tips excised from field grown plants of *A. nilagarica* (C. B. Clarke) Pamp. were surface sterilized and cultured on MS medium (Murashige and Skoog, 1962) supplemented with 1 mg/L naphthalene acetic acid (NAA) in combination with 3 mg/L or 5 mg/L kinetin (Kin) for shoot bud multiplication. The multiplied shoot buds were transferred to the same multiplication medium for further multiplication and rhizogenesis. The rooted plantlets obtained after

Table 1: Effects of growth regulators on callus induction from leaf explants of *Artemisia nilagarica* (C.B. Clarke) Pamp.

NAA (mg/L)	BAP (mg/L)	Kin (mg/L)	Nature of callus	Percentage response
-	-	5	Off-white and friable callus	67
-	5	-	Yellowish- green and friable callus	78
1	-	10	Yellowish- green and friable callus	94
1	5	-	Greenish- white and friable callus	100
1	10	-	Greenish- white and friable callus	92

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Table 2: Qualitative analysis of various phytochemical in extracts of different *A. nilagarica* samples prepared by using different solvents.

Sample used	Solvents	PH*	ALK*	TN*	SP*	FLV*	TR*	STR*	PHL*	CHA*	ANT*
Shoot buds	Hexane	+	+	+	+	+	+	-	+	+	-
	Chloroform	+	+	+	+	+	+	+	+	+	+
	EA*	+	+	+	+	+	+	+	+	+	-
	Ethanol	-	+	+	+	+	+	+	+	+	+
	Aqueous	+	+	+	+	-	+	+	+	+	-
Leaves	Hexane	+	+	+	+	+	+	+	+	+	-
	Chloroform	+	+	+	+	+	+	+	+	+	+
	EA	+	+	+	+	+	+	+	+	+	-
	Ethanol	-	+	+	+	+	+	+	+	+	+
	Aqueous	+	+	+	+	-	+	+	+	+	-
***In vitro* leaves**	Hexane	+	+	+	+	+	+	+	+	+	-
	Chloroform	+	+	+	+	+	+	+	+	+	+
	EA	+	+	+	+	+	+	+	+	+	-
	Ethanol	-	+	+	+	+	+	+	+	+	+
	Aqueous	+	+	+	+	-	+	+	+	+	-
Stems	Hexane	+	+	+	+	+	+	+	+	+	-
	Chloroform	+	+	+	+	+	+	+	+	+	-
	EA	+	+	+	+	+	+	+	+	+	-
	Ethanol	-	+	+	+	+	+	+	+	+	-
	Aqueous	+	+	+	+	-	+	+	+	+	-
Roots	Hexane	-	+	+	+	+	+	+	+	+	-
	Chloroform	+	+	+	+	+	+	+	+	+	-
	EA	+	+	+	+	+	+	+	+	+	-
	Ethanol	+	+	+	+	+	+	+	+	+	-
	Aqueous	+	+	+	+	-	+	+	+	+	-
Callus	Hexane	+	+	+	+	+	+	+	+	+	-
	Chloroform	+	+	+	+	+	+	+	+	+	+
	EA	+	+	+	+	+	+	+	+	+	-
	Ethanol	-	+	+	+	+	+	+	+	+	+
	Aqueous	+	+	+	+	-	+	+	+	+	-

*Where, PH: Phenol; ALK: Alkaloids; TN: Tannins; SP: Saponins; FLV: Flavonoids; TR: Terpenoids; STR: Steroid; PHL: Phlobatannins; CHA: Chalcones; ANT: Anthraquinones; EA: Ethyl acetate.

two months were then transferred to plastic cups containing sand:soil mixture (1:1) which were frequently watered and kept covered with perforated clear polythene bags to maintain humidity. The plants were then transferred to field condition after 2 months.

Callus induction

For callus induction, young shoot tips excised from *in vitro* multiplied plantlets were cultured on MS medium supplemented with 5 mg/L 6-benzylaminopurine (BAP) or Kin alone or 1 mg/L NAA in combination with 5 and 10 mg/L BAP or 10 mg/L Kin.

All cultures were incubated in a growth chamber with temperature maintained at 25 ± 1 °C and a 16-h photoperiod. Each treatment for shoot induction, root induction and callus induction had ten replicates and the experiments were repeated thrice.

Phytochemical screening

The coarsely powdered *ex vitro* shoot tips, leaves, stems, roots, *in vitro* leaves and callus of *A. nilagarica* were extracted with different solvents, such as chloroform, ethyl acetate, ethanol, distilled water and hexane. The herb to solvent ratio was kept 1:10 to ensure complete extraction. The plant material was extracted by cold maceration for 72 hours with intermittent agitation. After incubation, the extracts were filtered through Whatman filter paper and the extracts were stored at 4 °C till further use.

Qualitative estimation of secondary metabolites

The extracts obtained were subjected to Mayer's test, Dragendorff's test, Salkowski test, ammonia test, and test for phenols, tannins, saponins, flavonoids, steroids (Glycosides), phlobatannins

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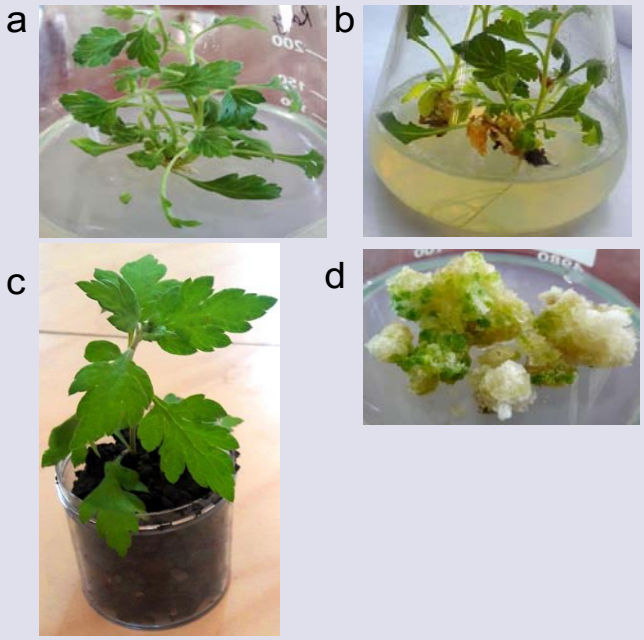

Figure 1: *In vitro* culture of *Artemisia nilagarica* (C.B. Clarke) Pamp., (a) Proliferation of shoot buds from shoot tip explants cultured on medium supplemented with 1 mg/L NAA and 5 mg/L Kin; (b) Rooting from shoot tip explants cultured on medium supplemented with 1 mg/L NAA and 5 mg/L Kin; (c) Transplanted plantlet and (d) Callus induced from shoot tip explants of *A. nilagarica* cultured on MS medium supplemented with 1 mg/L NAA and 5 mg/L BAP.

and anthraquinones as per the methods given by Harborne [11].

Quantitative estimation of secondary metabolites

For quantitative estimation of secondary metabolites, the extracts obtained were subjected to alkaline precipitation gravimetric method for alkaloid determination, double extraction gravimetric method for saponin determination and steroid determination as per the methods given by Harborne [11]. Powdered samples were extracted in methanol with 1:10 sample to solvent ratio and the methanol extracts were used for the quantitative analysis of secondary metabolites and estimation of DPPH free radical scavenging activity. The total phenol, flavonoids, tannin contents and total antioxidant potential in the methanol extracts were determined using Folin-Ciocalteau reagent method [12], aluminum chloride method [12], Folin-Dennis Spectrophotometric method [13] and DPPH analysis method of Shimada et al. respectively [14].

Artemisinin estimation

Stock solution (1 mg/mL) of standard artemisinin (from Sigma Aldrich, New Delhi) was prepared in 95% ethanol. Serial dilution was done for standard curve preparation. Powdered dried samples of young leaves from field grown plants, leaves from six-week old *in vitro* propagated plantlets and callus tissues were used for extraction of artemisinin by using n-hexane. The concentrations of artemisinin present in the samples were determined by UV-Vis Spectrophotometry method specified for artemisinin determination by Tarsisius et al. [15].

Results and Discussion

Multiple shoot buds proliferated from the shoot-tip explants cultured on bud induction medium containing 1 mg/L NAA in combination with 5 mg/L Kin after four weeks of culture and a maximum of 15 shoot buds were obtained per explants (Figure 1a). Earlier, medium supplemented with 1 mg/L BAP in combination with 0.3 or 1 mg/L NAA was found to be effective for micropropagation of *A. nilagarica* through indirect organogenesis from callus [9]. However, in the present study, medium supplemented with combinations of Kin with NAA was more effective than combinations of BAP with NAA and hence BAP was not used for shoot multiplication. The shoot buds cultured on medium containing 1 mg/L NAA in combination with 5 mg/L Kin also showed the induction of rooting and further multiplication was achieved by sub culturing the buds on these media every four weeks (Figure 1b). The *in vitro* regenerated plantlets showed 70-80% survival during transplantation (Figure 1c). The shoot tip explants produced friable callus with the best morphological and growth characteristics suitable for subculture was obtained on medium supplemented with 1 mg/L NAA and 5 mg/L BAP with 100% response (Table 1 and Figure 1d). Similar effectiveness of the combination of NAA and BAP in callus induction in *A. nilagarica* has been reported earlier [9].

Qualitative screening of secondary metabolites revealed the presence of medicinally active constituents like phenol, alkaloid, tannins, saponins, flavonoids, terpenoids, steroids, phlobatannins, chalcones and anthraquinones in extracts of shoots, leaves, stems, roots, *in vitro* leaves and callus of *A. nilagarica* obtained by using different solvents (Table 2). Similar findings have also been reported earlier [4,16-18] except for some minor differences in the presence or absence of the metabolites in the different extracts. The highest alkaloid content was obtained in the methanol extracts of shoot buds (51.97 mg/g) followed by callus (46.64 mg/g), young leaves (37.21 mg/g), *in vitro* leaves (31.38 mg/g), stems (1.57 mg/g) and roots (2.99 mg/g) (Table 3). The saponin content was highest in

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Table 3: Quantification of various secondary metabolites in methanol extracts of different *A. nilagarica* samples.

Sample extract	Alkaloids (mg/g)	Saponins (mg/g)	Steroids (mg/g)	Phenols (mg/g)	Flavonoids (mg/g)	Tannins (mg/g)	DPPH (% inhibition)
Shoot buds	51.97±0.32	7.51±0.02	8.46±0.03	2.3±0.00	0.3±0.00	2.1±0.00	64±0.00
Leaves	37.21±0.01	11.92±0.01	10.22±0.02	2.8±0.00	1.2±0.00	2.4±0.00	65±0.00
stems	1.57±0.00	32.26±0.01	3.86±0.05	1.3±0.00	0.1±0.00	2.5±0.00	57±0.00
roots	2.99±0.41	9.9±0.078	21.96±0.02	1±0.00	0±0.00	1.3±0.00	34±0.00
In vitro leaves	31.38±0.01	9.54±0.23	15.96±0.01	2.7±0.00	1.5±0.00	3.4±0.00	22±0.00
Callus	46.64±0.63	9.47±0.17	7.49±0.008	3.3±0.00	1.8±0.00	3.7±0.00	49±0.1

stems (32.26 mg/g) whereas the steroid content was highest in the roots (21.96 mg/g). The phenol, flavonoids and tannins contents of the methanol extracts were low and it was less than 5 mg/g in all the samples. The highest DPPH free radical scavenging activity in terms of % inhibition of DPPH was shown by leaf extracts (65%) followed by shoot bud extracts (64%).

There is only one earlier report on artemisinin content analysis in *A. nilagarica* and the content was so low that it could not be quantified significantly by HPLC method [19]. However, in the present study, even though the artemisinin concentration was low, it was quantified in leaves of field grown plant (0.14%), *in vitro* leaves (0.07%) and callus tissues (0.05%) of *A. nilagarica*. The artemisinin content in leaves of field grown plants was higher than in *in vitro* regenerated plantlets and callus tissue. Absorption maximum of artemisinin is obtained at 298 nm which was against 290 nm reported by Tarsisius et al. [15]. The regression equation calculated from the standard curve of artemisinin was $Y = 0.128X + 0.358$ (where, 'Y' is the absorbance and 'X' is the concentration) and $R^2 = 1$.

Conclusion

The present study, thus, presents a preliminary report on the quality and quantity of various secondary metabolites including artemisinin and DPPH free radical scavenging activities in various explants of *A. nilagarica*. Further work is being carried out to optimize *in vitro* artemisinin production by cell suspension cultures of the plant.

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