Original Article

Pyrroloquinazoline Alkaloids from Tissue Cultures of *Adhatoda vasica* and their Antioxidative Activity

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ABSTRACT

Adhatoda vasica (L.) Nees (Acanthaceae) is a well known plant drug in Ayurvedic medicine, used for the treatment of different types of diseases and disorders. The tissue cultures of this plant species were established on Murashige and Skoog culture medium by using various concentrations of growth hormones (0.5-5.0 mg/L α-napththaleneacetic acid + 0.5-3.5 mg/L 6-benzylaminopurine). The concentration of vasicine acetate was found higher (0.51 ± 0.519%) in six week old callus than *in vivo* (0.47 ± 0.556%) plant material (roots). The antioxidant activity was evaluated by using DPPH-radical scavenging, reducing power and superoxide anion scavenging models. We found that 2-acetyl benzyl amine showed maximum antioxidant activity against various antioxidant models, including DPPH-radical scavenging (40.59 ± 0.774%), reducing power (461.55 ± 0.402%) and superoxide anion scavenging (52.54 ± 0.553%) than other isolated alkaloids at 10-30 µM concentrations. Our observed results suggest that the pyrroloquinazoline alkaloids of *A. vasica* demonstrated strong antioxidant activity, which could be used as potent drugs against different types of oxidative stresses.

Keywords: HPLC; DPPH-radical scavenging; Reducing power; Superoxide anion scavenging models.

INTRODUCTION

Ayurveda, the ancient Indian therapeutic system is renowned as one of the major systems of alternative and complementary medicine. The thorough knowledge about the medicinal plant is mandatory for all who is working in the field of Ayurveda, in order to identify and select the appropriate plant for a cure of specific disease[1]. Vasicine, isolated from *A. vasica* is used in the preparation of Vasaca, a well known drug in the Ayurvedic system of medicine in India. The drug is recommended for a range of ailments viz, bronchitis, asthma, jaundice, diseases of respiratory system, diphtheria, gonorrhea and uterotonic abortifacient[2]. The whole plant has been

used worldwide as an herbal remedy for treating cold, cough, whooping cough, respiratory tract infections, chronic bronchitis, asthma as sedative expectorant, antispasmodic, anthelmintic, rheumatism, and rheumatic painful inflammatory swellings[3,4].

Phytochemical studies of *Adhatoda vasica* revealed that the aerial parts contain viz. vasicine, vasicinone[5,6,7,8], vasicine acetate and 2-acetyl benzyl amine[9,10,11]. The other compounds like as adhatodine, vasicoline, vasicol[12], vasicinolone and deoxyvasicinone have also been reported from *A. zeylanica* Medic.[13]. *Adhatoda* species have antimicrobial and several other important activities[14,15,16,17]. However, there are no published reports available on isolation of pyrroloquinazoline alkaloids from the roots and tissue cultures of *A. vasica,* nor are there any reports of antioxidant activity. Therefore, the study was carried out.

MATERIALS AND METHODS

Plant Materials

The plant materials of *Adhatoda vasica* were collected (Feb, 2012) from the Aravali Hilly areas of Jaipur and botanical authentication was confirmed by Professor R. S. Mishra. The voucher specimens were deposited in the Herbarium, Department of Botany, University of Rajasthan, Jaipur, India (sheet no. RUBL - 200431).

General Experimental Conditions

The melting points of purified compounds were recorded on capillary Toshniwal melting point apparatus and are uncorrected. The spectral data were obtained on the following instruments: ir, NICOLET – AVATAR 330 FTIR; ms, Hewlett Packard HP 5930 A; gc–ms, equipped with a HP 5933 data system, direct inlet at 70 eV; uv, Perkin - Elmer, model–200; nmr, Bruker AM 400 system at 400 and 100 MHz. Adsorbents as silica gel 60 (230-400 mesh, Merck) used for column chromatography and silica gel G (Merck) for preparative thin layer chromatography.

Extraction and Characterization

Shade-dried powdered plant roots (10.0 kg) were soaked in ethanol (10.0 l) for 15 days and then filtered. The extraction was repeated three times. The filtrate was concentrated under reduced pressure at 40 °C to gum. This crude- gum (98.563 g) was treated with 5.0% HCl (250 mL), warmed for 30 min and filtered. The filtrate was basified with ammonia (pH 10.0) and then successively fractionated with hexane (300 mL × four times) and chloroform (300 mL × four times). The chloroform-soluble fraction (yield 20.678 g) was used for isolation of pyrroloquinazoline alkaloids. The five pyrroloquinazoline alkaloids were purified and characterized by column chromatography over silica gel (200 g) using $CHCl_3$: MeOH: EtOAc mixtures with increasing polarity. Total 10 fractions (A-J) were collected from chloroform-soluble fraction.

Vasicine (**1**) *and vasicinone* (**2**): A portion of fraction A–B, were combined (5.432 g) and rechromatographed on silica gel and purified by preparative TLC. Solvent system used as -MeOH: H_2O (60–40, v/v), R_f ~0.70, crystallized with methanol, odorous volatile compound, detection on TLC by Dragendorff's reagent, yielded **1**, vasicine **(**556 mg), mp 210-211 °C, C_{11} $H_{12}N_2O$, UVλ_{max} 270 (0.857 nm), HPLC solvent system–MeOH: H_2O (4: 6), RT (min) 3.12, IR $(KBr)_v$: 3416.2 (O–H stretching), 3065.3 (Ar C–H stretching), 2930.2 (C–H stretching), 1613.4 (C=N stretching), 1584.1 (C=C stretching), 1299.6 (C–N stretching), 1164.7 cm^{-1} (C–O stretching). MS showed $(M)^+$ peak at *m/z* 188, ^1H NMR (DMSO): δ 2.66 (*s*, 2H, CH_2), 3.23 (*s*, 2H, CH_2), 4.23 (*s*, 2H, CH_2), 5.23

(*s*, 1H, OH), 7.42-7.83 ppm (*m*, 4H, Ar –H). Fractions C–D pooled together (3.836 g), rechromatographed on silica gel and purified by preparative TLC with development by $CHCl_3$–MeOH–EtOAc (85–15–10 v/v), R_f ~0.62, crystallized with methanol, positive to Dragendorff's reagent, yielded to compound **2** (vasicinone, 833 mg), mp 198-200 °C, $C_{11}H_{10}N_2O_2$, $([\alpha]^D_{23}$ 97=0.9). UV $(MeOH)_{max}$ log(ε) 273 nm. IR (KBr) V_{max} 3169, 1683, 1463 cm^{-1}, ^1H NMR ($CDCl_3$, 200 MHz): *δ* 2.20-2.45 (*m*, 1H), 2.60-2.80 (*m*, 1H), 3.90-4.15 (*m*, 1H), 4.15-4.30 (*m*, 1H), 5.27 (H), 7.40-7.60 (*m*, 1H), 7.65-7.85 (*m*, 2H), 8.31 (*d, J* = 6H = 1H). ^{13}C NMR ($CDCl_3$ 50 MHz) *δ* 29.4, 43.5, 72.0, 121.1, 126.7, 126.8, 134.4, 148.6, 160.1, 160.6. MS (*m/z*): 202, 185, 174, 146, 130, 119, 102, 90, 76, 63, 55. Anal: C 65.34: H4.99: N13.85 calculated for $C_{11}H_{10}N_2O_2$, found C. 65.18, H5.06, and N13.77.

Vasicine acetate (**3**) *and 2-acetyl benzyl amine* (**4**): Fractions E–H pooled together (2.248 g), rechromatographed on silica gel and purified by means of preparative TLC. Compound **3**, vasicine acetate (749 mg), solvent system: $CHCl_3$: MeOH: EtOAc (60:20:10, v/v), R_f ~0.54, mp 122 °C, $C_{13}H_{14}N_2O_2$, positive to Dragendorff's reagent, white powder, IR data: max cm^{-1} (400-4000) 1720 (acetate), 1629 (> C=N) 1579, 1498, 1406 (aromatic system), 1385, 1345, 1241 (acetate), 1162, 1129, 1105, 1072, 933, 903, 767, 722 (aromatic system). ^1H NMR data for the active molecule were the following: *δ* 7.14 (2H, *m*, H–5 and H–6), 6.96 (1H,*brs*, H–7) 6.84 (1H, *bs*, H–8), 4.72, 4.80 (each 1H, *d, J* = 14.5 HZ, H–9), 2.09 (3H, *s*, $-OCOCH_3$), 3.52, 3.70 (1H each, *m*, H–1), 2.25, 2.55 (1H each, *m*, H–2), 5.19 (1H, *brt*, H–3). ^{13}C NMR: *δ* 50.7 (C–1), 28.0 (C–2), 70.2 (C–3), 163.9 (C–3a), 134.0 (C–4a), 129.5 (C–5), 126.6 (C–6), 126.2 (C–7), 120.3 (C–8), 116.3 (C–8a), 46.8 (C–9), 167.3 (–$OCOCH_3$), 23.3 (–OCOCH). MS: $[M]^+$ *m/z* 230. Compound (**4**) was purified by preparative TLC (solvent system: hexane-ethyl acetate, 8:2 v/v), R_f ~0.65, yielded 2-acetyl benzyl amine (446 mg), white powder, tested positive for primary amine. The IR spectrum gave the following data: Max cm^{-1} = (500-4000) 3400 ($-NH_2$), 2928, 1685 (Ar CO–), 1628, 1576, 1498, 1459 and 1406 (aromatic system), 1188, 775 (aromatic system). ^1H NMR data for the active molecule were the following: *δ* 7.91 (2H, *m*, H–1 and H–3), 7.52 (2H, *m*, H–2 and H–4), 4.42 (1H, *brs*, $-CH_2NH_2$), 2.6 (3H, *s*, COCH3). The ^{13}C NMR spectrum of the active molecule was the following: *δ* 129.1 (C–1), 127.8 (C–2), 132.6 (C–3), 128.2 (C–4), 136.1 (C–5), 137.6 (C–6), 54.3 ($-CH_2NH_2$), 197.6 (–$COCH_3$), 26.6 (–$COCH_3$). MS: $[M]^+$ *m/z* 147, m/f–C_9H_9NO.

Vasicinolone (***5***): The fractions I-J combined together (3.345 g), rechromatographed on silica gel and purified by preparative TLC (solvent system: $CHCl_3$: MeOH, 8–2 v/v), crystallized with methanol, pale yellow crystals, mp 278-280 °C, positive to Dragendorff's reagent, R_f ~0.59, UV λmax 270 (0.832) nm, yielded to the compound V, vasicinolone (198 mg). HPLC solvent system–$CHCl_3$: MeOH (8: 2 v/v), RT (min) 3.12, IR $(KBr)_\upsilon$: 3221.0(O-H), 3021.0 (Ar C–H), 2926.1 (C–H), 1680.8 (C=O), 1590.5 (C=N), 1541.3 (C–C), 1216.2 cm^{-1} (C–N). MS showed $(M)^+$ peak at *m/z* 218.^1H NMR (DMSO): *δ* 2.48 (*s*, 2H, CH_2), 4.31 (*s*, 2H, CH_2), 5.29 (*s*, 1H, OH), 7.21 (*s*, 1H, Ar–OH), 7.22–7.67 ppm (*m*, 3H, Ar–H).

Tissue Culture

The unorganized callus tissue of *A. vasica* was induced by roots. The roots were surface sterilized (size 2-4 mm) with 0.1% (w/v) $HgCl_2$ solution for 1-1.5 min and then rinsed three times with sterilized distilled water. To initiate callus from root explants, the explants were grown on MS culture

medium[18] supplemented with different concentrations of growth regulators: 0.5-5.0 mg/L α-napththaleneacetic acid (NAA) and 0.5-3.5 mg/L 6-benzylaminopurine (BAP). The culture medium was also supplemented with 3.0% sugar. The root parts started differentiated tissue formation after 20-25 days of inoculation. These cultures were incubated at 25 ± 1 °C with 60% relative humidity under room light conditions (300 Lux). The callus tissue sample was transferred onto the fresh MS medium after 4-5 weeks intervals. The callus tissue was harvested at the transfer age of 2, 4, 6, 8 weeks and the growth index was calculated (GI = Final dry weight of callus-Initial dry weight of callus/Initial dry weight of callus; 0.98 in 2 weeks; 3.65 in 4 weeks; 4.89 in 6 weeks; 4.67 in 8 weeks old cell cultures). The compounds were isolated as per the above mentioned methods.

HPLC Analysis

A Shimadzu (Japan) gradient HPLC instrument equipped with two LC–8A pumps, a model 7725 I manual injector valve (Rheodyne). Solvents were filtered through a Milipore system and analysis was performed on a Merck Hibar C_{18} column (250 mm x 4.0 mm I. D. 10 µm). Mobile phase was optimized by varying percentage of acetonitrile in phosphate buffer and peak purity and similarity results of compounds 1-5 (isolated and standard) detected by using photodiode detector, these resulted in the following operating conditions: MeOH–H_2O (4.0: 6.0, v/v) and $CHCl_3$–MeOH (8.0: 2.0, v/v)[10,19].

DPPH-radical Scavenging Activity

The free radical scavenging activity of pyrroloquinazoline alkaloids was determined by DPPH assay[20]. The reaction takes place when a solution of DPPH was prepared in methanol (150 µM). Then 0.5 ml of DPPH solution was added to 0.5 mL of isolated pyrroloquinazoline alkaloids at different concentrations (10, 20, 30 µM). The reaction mixture was then shaken vigorously and incubated at room temperature for 1 h; absorbance values were measured at 517 nm in a spectrophotometer against methanol as blank (negative control) and (±)–α–tocopherol as positive control. The inhibition percentage was calculated of DPPH–radical scavenging activity as follows:

$$\text{Inhibition (\%)} = \frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}})}{\text{Absorbance}_{\text{control}} \times 100}$$

Reducing Power

Reducing power of pyrroloquinazoline alkaloids was measured using the established protocol[21]. Varied concentrations of pyrroloquinazoline alkaloids (10, 20, 30 µM) were mixed with phosphate buffer (pH 6.8) and potassium ferric cyanide. The reaction mixture was then allowed to stand at 52 °C for 15 min. A portion of trichloroacetic acid was added to the reaction mixture and finally centrifuged for 15 min at 1000 *g*. The upper layer of reaction mixture (1.0 mL) was mixed with distilled water (1.0 mL) and $FeCl_3$ (1.0 mL) for 15 min and at last the absorbance was measured at 700 nm in a spectrophotometer. The inhibition percentage was calculated of reducing power activity as follows:

$$\text{Inhibition (\%)} = \frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}})}{\text{Absorbance}_{\text{control}} \times 100}$$

Superoxide Anion Scavenging Activity

The determination of superoxide scavenging activity of pyrroloquinazoline alkaloids was performed by using the method

of Robak and Gryglewski[22]. The superoxide radicals were generated in phenazine methosulfate – nicotinamide adenine dinucleotide systems by nicotinamide adenine dinucleotide oxidation and assayed by nitroblue tetrazolium reduction. The generated superoxide radicals were incubated with different concentrations of pyrroloquinazoline alkaloids and (±)-α-tocopherol (10, 20, 30 µM). The chemical reaction was started with addition of 1.0 mL of phenazine methosulfate (110 µM) solution to the reaction mixture. The reaction mixture was incubated at room temperature for 10 min and the absorbance was measured at 560 nm against blank sample by using spectrophotometer. The inhibition of superoxide anion scavenging activity was calculated as follows:

Inhibition (%) =

$$\frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}})}{\text{Absorbance}_{\text{control}} \times 100}$$

RESULT AND DISCUSSION

The tissue cultures of *A. vasica* were established by using different concentrations of NAA (0.5-5.0 mg/L) and BAP (0.5-3.5 mg/L). The maximum growth of tissue was achieved at 3.5 mg/L NAA and 1.25 mg/L BAP (Fig. 1). Growth of *A. vasica* cell cultures was increased up to 6 weeks and later on started to decrease up to 8 weeks old cell cultures. The growth index was maximum (4.89) in 6 weeks while minimum (0.98) in 2 weeks old cell cultures (0.98 < 3.65< 4.89 > 4.67).

The five pyrroloquinazoline alkaloids were isolated and characterized from the chloroform-soluble fraction of *A. vasica* roots (**1**, 556 mg; **2**, 833 mg; **3**, 749 mg; **4**, 446 mg; **5**, 198 mg; Fig. 2). The pyrroloquinazoline alkaloids were also estimated from the chloroform-soluble fraction of 2, 4, 6, 8 weeks old callus. The vasicine acetate accumulated in higher concentration (0.51 ± 0.519%) in 6 weeks old callus tissue than *in vivo* (roots) plant material (0.47 ± 0.556%). The quantities of isolated alkaloids were compared in both *in vivo* and *in vitro* studies and found that *in vitro* cells produced higher quantities of alkaloids (Table 1).

Present investigation revealed the comparison of antioxidative activity of pyrroloquinazoline alkaloids (10-30 µM) and (±)-α-tocopherol (10-30 µM) using different antioxidative models such as DPPH-radical scavenging, reducing power and superoxide anion scavenging. The DPPH-radical has been used widely to test the potential of phytocompounds as free radical scavengers of hydrogen donors. 2-acetyl benzyl amine demonstrated maximum DPPH-radical scavenging activity (40.59 ± 0.774%) than other pyrroloquinazoline alkaloids and positive control showed 65.34 ± 0.509% of inhibition respectively (Fig. 3). The transformation of Fe^{3+}–Fe^{2+} was monitored in presence of pyrroloquinazoline alkaloids. The reducing power activity 2-acetyl benzyl amine was observed as 461.55 ± 0.430% much higher than other isolated compounds (Fig. 3). The maximum superoxide anion scavenging activity was showed (52.54 ± 0.553%) by the 2-acetyl benzyl amine in comparison to other pyrroloquinazoline alkaloids (Fig. 3).

The accumulation of pyrroloquinazoline alkaloids was estimated in callus tissue and also compared with accumulation of phytochemicals in roots of *A. vasica*. Manipulation of physical parameters and nutritional elements in a culture is perhaps the most fundamental approach for optimization of culture productivity[23,24]. The total protein and carbohydrate values increased slowly up to day 12-14 but later reached a sharp peak at day 30–35 day and then gradually diminished to the initial inoculum value. The microscopical

observations suggest that much increase in cell dry weight (6 weeks old) is due to accumulation of starch grains, which subsequently disappear during the growth cycle[25].

The DPPH scavenging activity is totally based on the decolorization of DPPH by the antioxidant compounds[26]. The 2-acetyl benzyl amine is a primary amine and the primary amines are extremely potent radical scavengers capable of reacting directly with carbon centered radicals and interacting the auto-oxidation cycle earlier than primary and secondary antioxidants. The primary antioxidants consist mainly of hindered aromatic amines. They scavenge and destroy the chain propagating peroxy and alkoxy radicals before they can react with the polymer[27,28]. Therefore, DPPH-free radical scavenging effect of chloroform-soluble fraction and isolated pyrroloquinazoline alkaloids might be attributed to a direct role in the trapping of free radicals by donating hydrogen atom. Similarly, superoxide anion radical is also considered as a one of the strongest reactive oxygen species among the free radicals[21].

CONCLUSION

From the observation it may be concluded that the roots of *A. vasica* are the good source of natural antioxidants and might be useful in treating the diseases associated with oxidative stress. The data obtained from literature as well as results reveal the great potential of *A. vasica* for therapeutic treatment, in spite of the fact that they have not been completely investigated. Therefore, more studies need to be conducted to search for new compounds.

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DECLARATION OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this research paper.

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Table 1. Quantity of isolated pyrroloquinazoline alkaloids from A. vasica Nees *in vivo* and *in vitro*

Isolated Compounds	Percentage of isolated compounds (w/w) ± SD				
	In vivo	*In vitro* (Age of callus in weeks)			
		2	4	6	8
I	0.35 ± 0.212	0.071 ± 0.721	0.099 ± 0.469	0.32 ± 0.111	0.31 ± 0.808
II	0.44 ± 0.413	0.064 ± 0.437	0.084 ± 0.555	0.24 ± 0.813	0.25± 0.718
III	0.47 ± 0.556	0.081 ± 0.444	0.11 ± 0.616	0.51 ± 0.519	0.36 ± 0.777
IV	0.24 ± 0.661	0.076 ± 0.228	0.091 ± 0.319	0.18 ± 0.448	0.16 ± 0.331
V	0.31 ± 0.784	0.0232 ± 0.187	0.071 ± 0.228	0.096 ± 0.559	0.089 ± 0.353

Isolated compounds: I, vasicine; II, vasicinone; III, vasicine acetate; IV, 2- acetyl benzyl amine; V, vasicinolone. Values are mean of triplicate readings

Figure 1. *A. vasica* (a) view of whole plant; (b) callus cultured on MS culture medium supplemented with 3.5 mg/L α-napththaleneacetic acid + 1.25 mg/L 6-benzylaminopurine

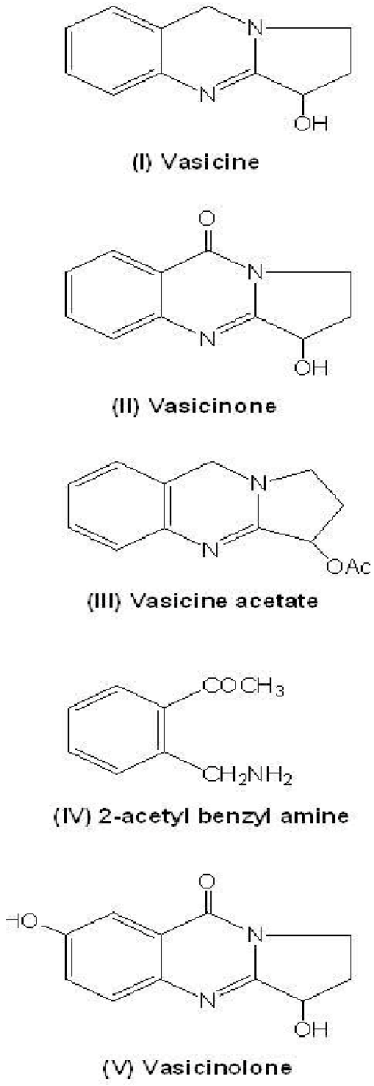

Figure 2. Structures of isolated pyrroloquinazoline alkaloids from *A. vasica* Nees

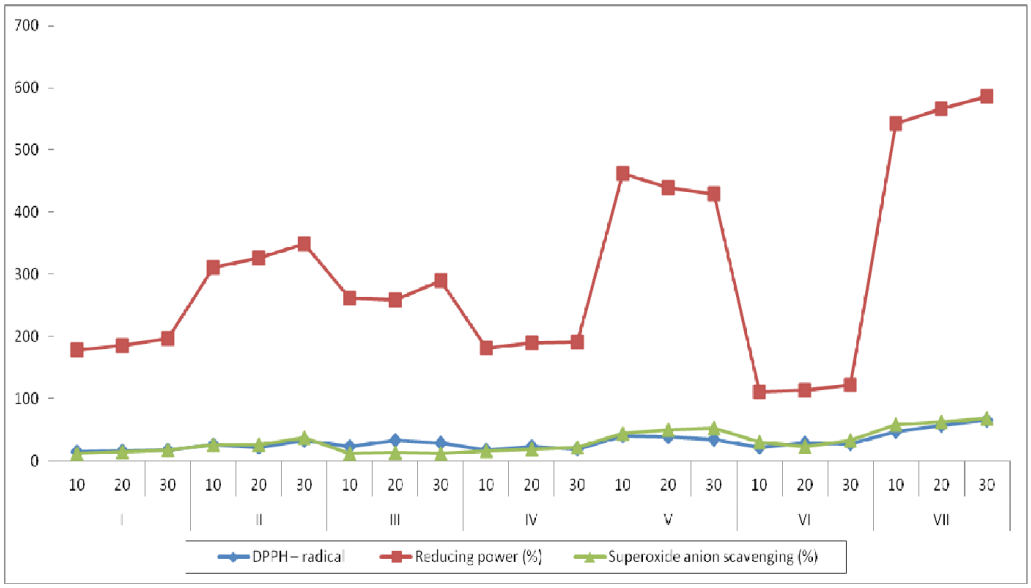

Figure 3. Antioxidant activity of isolated pyrroloquinazoline alkaloids from *A. vasica* Nees