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Isolation and identification of flavonoid from *in vitro* and natural grown *Nyctanthes arbor-tristis* L. (Harsingar) - a medicinally important plant

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A rapid propagation protocol was established and optimized *in vitro* for *Nyctanthes arbor-tristis* L. a small ornamental tree with medicinal and aromatic properties. Nodal explants were cultured on Murashige and Skoog (MS) medium supplemented with various plant growth regulators: BAP and Kn in combination. Maximum frequency of shoot initiation (FSI) (1-2 wks) and mean shoot number (MSN) 3.02±0.437 were obtained on BAP + Kn (1.0 + 0.1), whereas high mean shoot length (MSL) 2.9±0.356 was observed on BAP + Kn (2.0 + 0.5) Elongated microshoots (3-4 wks) were successfully rooted and regenerated plants were simultaneously hardened and acclimatized. An approximately 91% survival rate was recorded. Natural leaf, stem, *in vitro* leaf and stem were subsequently extracted in 80% methanol and were subjected to thin layer chromatography along with the standard.

Key words: Axillary bud, Micropropagation, Multiple shoots, Nodal explants, *Nyctanthes arbor-tristis*, Thin layer chromatography.

In recent years, tissue culture techniques are intensively used for the propagation of medicinally important plant species, which are important source of compounds for the pharmaceutical industries. *Nyctanthes arbor-tristis* Linn. (Harsingar) belongs to family Oleaceae is a valuable medicinal and ornamental tree with scented white flowers. It is widely cultivated throughout India as a garden plant (Anonymous, 1988). It tolerates moderate shade and is often found as undergrowth in dry deciduous forests (Rout *et al.,* 2007). Its leaves, flowers and seeds are used for various diseases such as chronic fever, bronchitis, asthma, constipation, grayness of hair, baldness and skin diseases (Kirtikar and Basu, 1981). The bitter leaves are used in Ayurvedic system of medicine for treatment of rheumatism, sciatica, diuretic and intestinal worms. The powdered seeds are recommended for the treatment of scurvy (Kirtikar and Basu, 1988). The flower contains a coloring substance nyctanthin used for coloring silk and useful for printing purposes (Das and Mondol, 2012). Seed germination is low on account of the presence of phenolic compounds in the seed coat (Bhattacharya *et al.,* 1999) which results in death of seedlings (Anonymous, 1988). The above mentioned causes prompted us to find an alternate method for micropropagation of this species. Clonal mass propagation can be widely applied to shorten the long sexual cycle and

other problems like limited seed availability and problems of seed physiology (Chandrasekhar *et al.,* 2006). Plant tissue culture is a well known biotechnological tool for the rapid propagation of medicinal plants for the purpose of commercialization (Kitto, 1997), conservation (Anis *et al*., 2007) and cryopreservation (Decruse *et al*., 1999).

Flavonoids are a group of polyphenolic compounds widely distributed throughout the plant kingdom (Anonymous, 1996). A variety of pharmacological effects were confirmed for flavonoids including antioxidative, superoxide scavenging, variety of enzymes inhibitory activity and antiinflamatory, antiviral, anticarcinogenic activities (Bylka *et al*., 2001).

The objective of the present study is to establish a simple protocol for *in vitro* propagation of Harsingar through nodal explants and detection of flavonoids by chromatography methods.

Material and Method:

***In vitro* propagation:**

Nyctanthes arbor-tristris was procured from Jawaharlal Nehru Krishi Vishwavidyalaya, Jabalpur. Plants were identified by Institutional Botanist and voucher specimen (No. 22420). Axillary buds (0.5-0.7 cm) were used as explants. They were washed thoroughly under running tap water then treated with Teepol solution (a liquid detergent) for 10 min followed by washing in tap water. The explants were then surface sterilized with 70% ethanol (v/v) for 2 min followed by 0.1% $HgCl_2$ for 3 min. Then rinsed with sterilized distilled water 5 to 6 times to minimize the contamination caused by microorganisms like fungus, endogenous and exogenous bacteria. The explants then dried on sterile filter paper, sectioned and aseptically inoculated to the Murashige and Skoog's medium supplemented with different concentration of cytokinins *viz.* 6-benzylaminopurine (BAP) and 6-furfurylaminopurine (Kn) in combination. The media was supplemented with 3% sucrose and 0.8% agar as a gelling agent. The pH was adjusted to 5.6±0.3 and autoclaved at 121^{o}C, 15 lb pressure for 45 min. Cultures were maintained under 16 hrs photoperiod of cool white fluorescent light at 25±0.5^{o}C (light intensity 50 µ mol m^{-2} s^{-1}) and 55 to 60% relative humidity. Each experiment was repeated thrice with 12 replicates per treatment. The 8 weeks old *in vitro* grown micro-shoots were rooted according to Bansal *et al.* 2012, transferring to half strength MS medium with 2% sucrose devoid of agar, supplemented with different concentrations of auxins. Plantlets with well developed roots were removed from the culture tubes; treated with 1% Bavistin solution, washed under tap water and then transferred to poly cups containing sterilized soil: sand: farmyard manure (1:1:1). The regenerates were hardened up to 4 weeks, by covering with a thin perforated transparent polythene bags to maintain humidity and watered regularly with distilled water for the first week and later with tap water.

In all the experiments each single treatment consisted of 12 replications and all experiments were repeated thrice, the data were statically analyzed using SPSS version 20 and comparison of means was performed at the $p< 0.05$ level of significance using Tukey's HSD test.

Extraction procedure:

Natural and *in vitro* grown plant material was shade dried and 5gm of sample was weighed and soxhlet extraction was done in 80% methanol for 24 hours (Subramanian and Nagarajan, 1969). The extracts was then filtered and concentrated and subjected to TLC.

Thin layer chromatography (TLC):

The glass plate coated with silica gel F254 was activated at 100^{o}C for 30 minutes in oven. About 5 µl of methanolic fraction of each sample was loaded with the help of a

micropipette 1 cm above the edge of the plate along with the reference standard compound (quercetin 0.1 mg/ml). The plate was then developed in a chromatographic chamber containing about 200 ml of solvent mixture of Toluene: Ethyl acetate: acetic acid (30: 40: 5). After development, the plate was withdrawn from the TLC chamber, dried at room temperature and visualized under UV light.

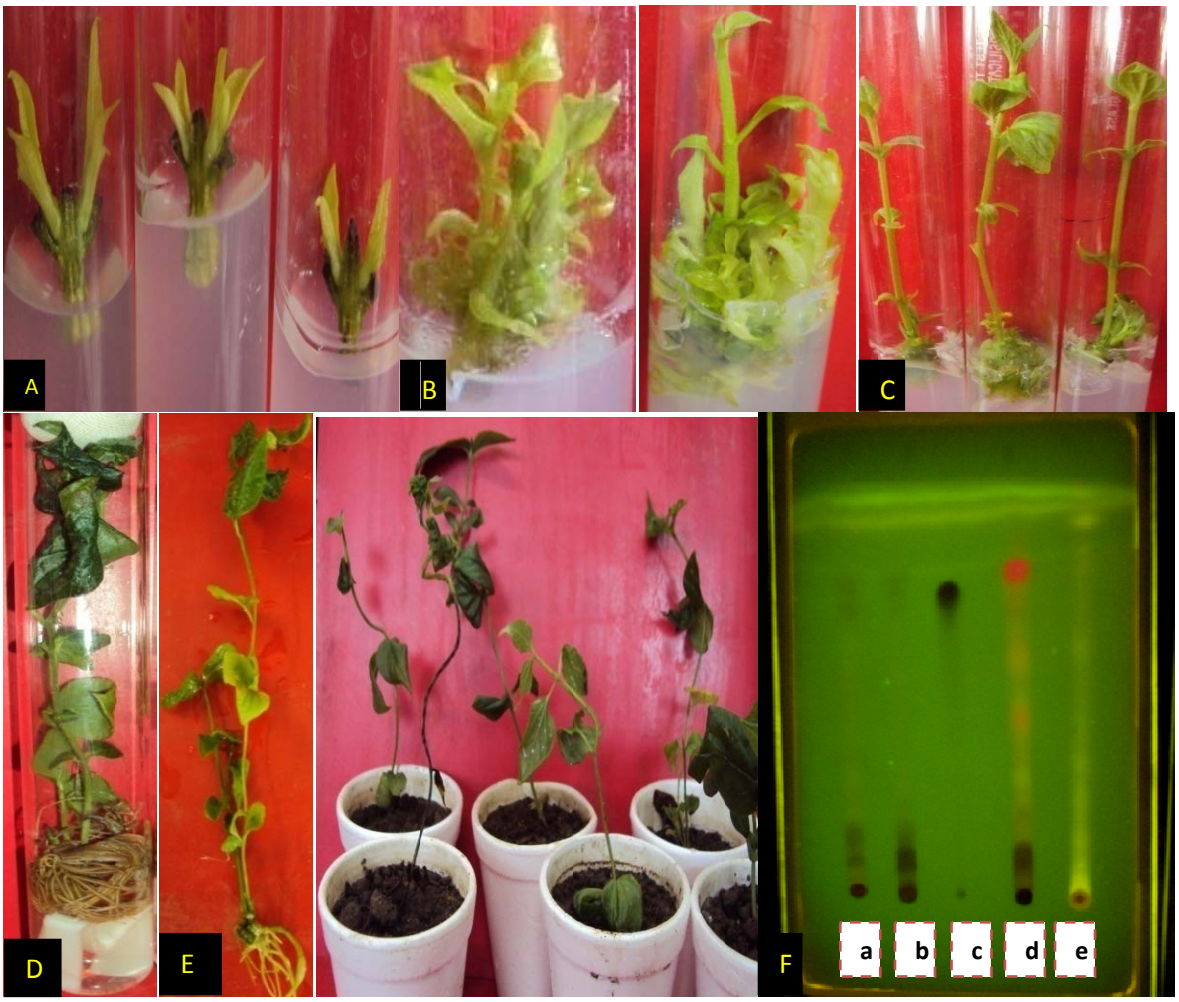
Results and Discussions:

In order to establish an efficient *in vitro* micropropagation system for *Nyctanthes arbor-tristris* the nodal explants were incubated on MS medium (solid) supplemented with BAP and Kn in various combinations and are presented in tables 1. Nodal explants are the best source of multiple shoot induction and have also been suggested in case of other medicinal plants, such as *Vernonia amygdalina* (Khalafalla *et al.*, 2007), *Lagerstroemia indica* (Niranjan *et al.*, 2010), *Pterocarpus marsupium* (Chand and Singh 2004), *Clitoria ternatea* (Singh and Tiwari 2010) and *Zehneria scabra* (Anand, Jeyachandran, 2004). MS supplemented with BAP + Kn (1.0 + 0.1 mg/l) gives maximum frequency of shoot induction (FRI) (91.66), maximum mean shoot number (MSN) (3.02) and maximum Mean node number (MNN) (2.63) whereas maximum mean shoot length (MSL) (2.9) was obtained on BAP + Kn (2 + 0.5).

Table 1. Effect of cytokinins on shoot differentiation from axillary bud explants of *Nyctanthes arbor-tristis*

S. No.	Cytokinin Conc (mgl^{-1})	Frequency of shoot initiation	Mean shoot length (cm)	Mean shoot number	Mean Node number
1.	Control	60.00	0.91±0.1163	0.88±0.196	0.94±0.183
2.	0.1B+0.1K	58.30	1.27±0.162^{ab}	1.72±0.263^{ab}	1.41±0.182^{a}
2.	0.1B+0.5K	56.33	1.32±0.239^{ab}	1.5±0.234^{a}	1.33±0.211^{a}
3.	0.1B+1 K	91.66	1.65±0.293^{ab}	1.33±0.202^{a}	2.02±0.312^{a}
4.	0.1B+2K	66.66	2.56±0.449^{ab}	2.66±0.605^{ab}	2.63±0.438^{a}
5.	0.1B+5K	75.00	1.57±0.214^{ab}	1.77±0.275^{ab}	1.55±0.228^{a}
6.	0.5B+0.1K	50.00	1.31±0.163^{ab}	1.97±0.294^{ab}	1.52±0.248^{a}
7.	0.5B+0.5K	33.33	1.15±0.157^{a}	1.52±0.191^{a}	1.25±0.162^{a}
8.	0.5B+1K	41.66	1.46±0.202^{ab}	1.47±0.249^{a}	1.55±0.260^{a}
9.	0.5B+2K	66.66	1.36±0.176^{ab}	1.66±0.275^{ab}	1.77±0.234^{a}
10.	0.5B+5K	91.66	1.61±0.229^{ab}	1.97±0.288^{ab}	1.61±0.220^{a}
11.	1B+0.1K	66.66	1.95±0.311^{ab}	3.02±0.437^{b}	2.05±0.243^{a}
12.	1B+0.5K	75.00	1.58± 0.373^{ab}	2.00±0.286^{ab}	1.83±0.318^{a}
13.	1B+1K	58.33	1.38±0.154^{ab}	2.30±0.328^{ab}	1.58±0.241^{a}
14.	1B+2K	91.66	1.63±0.267^{ab}	2.25±0.298^{ab}	1.80±0.201^{a}
15.	1B+5K	75.00	1.52±0.314^{ab}	1.88±0.315^{ab}	1.90±0.225^{a}
16.	2B+0.1K	83.33	1.59±0.266^{ab}	1.5±0.205^{a}	1.41±0.206^{a}
17.	2B+0.5K	66.66	**2.9±0.356^{b}**	2.52±0.387^{ab}	2.44±0.446^{a}
18.	2B+1K	75.00	1.35±0.213^{ab}	1.97±0.269^{ab}	1.63±0.234^{a}
19.	2B+2K	77.00	2.55±0.545^{ab}	2.22±0.315^{ab}	2.58±0.374^{a}
20.	2B+5K	58.30	1.39±0.341^{ab}	2.05±0.359^{ab}	1.66±0.300^{a}
21.	5B+0.1K	83.33	1.92±0.396^{ab}	1.75±0.271^{ab}	2.02±0.336^{a}
22.	5B+0.5K	75.00	2.53±0.379^{ab}	2.11±0.372^{ab}	2.16±0.299^{a}
23.	5B+1K	58.33	2.40±0.422^{ab}	1.91±0.256^{ab}	2.19±0.359^{a}
24.	5B+2K	41.66	2.03±0.381^{ab}	1.66±0.197^{ab}	2.11±0.335^{a}
25.	5B+5K	33.33	1.70±0.328^{ab}	2.25±0.363^{ab}	1.94±0.279^{a}

Values represent means ± standard error of 12 replicates per treatment in 3 repeated experiments. Means followed by the same letter are not significant by Tukey's HSD test at 0.05% probability level.



***In vitro* propagation of *Nyctanthes arbor-tristis*. A: Initiation (1-2 wks); B: Multiple shooting (3 wks); C: Shoot elongation (4 wks); D: *In vitro* Rooting in (3-4 wks); E: Hardnening, F: TLC plates showing the presence of flavonoids a. natural leaf, b. *in vitro* leaf, c. standard, d. *in vitro* stem, e. natural stem.**

Extraction is an important step involved in the isolation of bioactive compounds from the plants with medicinal value. Plant secondary metabolites are being studied in various parts of the world to find new molecules, to decipher new cues for drug discovery. The aim of every extraction process is rapid and effective isolation of compounds by using minimum amount of solvent. Traditional methods, for example percolation, exhaustive soxhlet extraction, or direct extraction with boiling solvent under reflux are most often used (Waksmundzka-Hajnos *et al.*, 2007). Flavonoids are usually extracted using water, or more often a mixture of water and miscible polar solvents such as methanol, ethanol, and acetone. Methanol is more frequently used than ethanol and acetone because of its higher extraction efficiency (Marston and Hostettmann, 2006). Extraction is removal of desired substances from undesired ones. Successful extraction involves selection of right solvent which can extract out maximum quantity of targeted chemicals, while minimizing the interference of unwanted components. The solvent system used for TLC of flavonoid in the present study is Toluene: Ethyl acetate: Acetic acid (30: 40: 5),

similar solvent system was used by Ganatra *et al.* (2012) and Marica *et al.* (2004).

Natural leaf extract of *N. arbor-tristis* showed 6 spots with Rf values 0.139, 0.316, 0.481, 0.588, 0.740 and 0.803, whereas *in vitro* leaf extract gives 4 spots with Rf values 0.145, 0.329, 0.746 and 0.810. Natural stem extract of *N. arbor-tristis* gives three spots having Rf 0.064, 0.147 and 0.737, whereas *in vitro* stem extract showed only two spots (0.113 and 0.753). Results obtained indicate that all the samples shows the presence of flavonoid which coincides with the standard (Quercetin) having Rf (0.75) in similar conditions. Similar results were also obtained by Ganatra *et al*. (2012).

The present study shows that the plant *Nyctanthes arbor-tristis* possess flavonoid compounds which have proven medicinal activity, so in this context this plant might serve as a plant based remedy for many ailments such as cancer, ulcer, depression, antioxidant, etc.

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