CLONAL PROPAGATION OF VIETNAMESE *ORTHOSIPHON STAMINEUS* BENTH. ACCESSION CONTAINING HIGH CONTENT OF SINENSETIN USING PLANT TISSUE CULTURE TECHNIQUE

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Introduction

Around the world, plant tissue culture techniques have been successfully applied to conservation and clonal propagation of elite genotypes of numerous medicinal plants. Regenerated plants from *Mentha spp.* [1], *Digitalis purpurea* [2], *Gingko biloba* [3], *Petasites hybridus* [4], *Andrographis paniculata* [5] by using plant tissue culture techniques were verified to be phytochemically and genetically identical to the donor plants.

Orthosiphon stamineus Benth. (known as "Cat's whiskers" in English and "Rau meo" in Vietnamese), a member of the family Laminaceae, is a popular medicinal herb in Vietnam. According to the traditional medicine, *Orthosiphon* tea is widely used for removing uric acid stones from the kidney. It is also used in treatment of diabetes and hypertension [6]. Recently, the diuretic activity of this plant's extracts and components have been demonstrated by various pharmacological and clinical studies [7, 8]. As a result, several pharmaceutical products derived from this medicinal plant, such as Uriphron® (from PhytoPharmica®, USA), Rau Meo – Chuoi Hot™ (from Domesco, Vietnam) and Kim Tien Thao – Rau Meo™ (from Fidofarm, Vietnam) has been commercialized and commonly used in treatment of kidney stones, urinary tract and renal diseases.

In a previous publication, we reported our attempts in establishment of an in-vitro propagation protocol for Vietnamese *Orthosiphon stamineus* Benth. genotypes [9]. This paper describes some recent data involving the RP-HPLC screening for Vietnamese *O. stamineus* Benth. clones containing high content of sinensetin, one of the major active principles present in this medicinal plant. The highest sinensetin-containing genotype was subjected to the mass propagation using shoot-bud culture techniques. The homogeneity of regenerated plantlets were examined by DNA (RAPD-PCR) analyses.

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2. Materials and methods

Plant materials. Ten accessions of *Orthosiphon stamineus* Benth. were collected from different locations in Northern Vietnam (Table 1), and authenticated by Dr. Nguyen Van Tap at the Vietnam Institute of Medicinal Materials Herbarium, where vouchers specimens are held for reference (reference no. 85, 1735, 2918 A/B/C). Samples obtained from 1-year old plants of each accession were air-dried at temperature below 40°C by a Memmert® drying oven, and used for extraction and HPLC analyses of sinensetin. Whereas, the shoot-buds of the same plants were subjected to tissue culture experiments.

Chemicals. Standard sinensetin (HPLC grade) were supplied by Extrasynthese (Lyon, France). Plant growth regulators were obtained from the Sigma (St. Louis, USA). All other chemicals were of analytical and HPLC grade and purchased from the Merck & Co.Ltd. (Darmstadt, Germany).

Table 1. Names and origins of Orthosiphon stamineus Benth. accessions collected in the study

N_o.	Name of accessions	Collection site		Ref. No. at IMM Herbarium(*)
		Commune, District	City / Province	
1	HN_1	Ngu Hiep, Thanh Tri		1735 A
2	HN_2	Ngu Hiep, Thanh Tri	Hanoi	1735 B
3	HN_3	Ngu Hiep, Thanh Tri		1735 C
4	TH_0	Ha Trung		2918
5	TH_1	Thanh Hoa Town	Thanh Hoa	
6	TH_2	Than Hoa Town		
7	PT_1	Viet Tri		
8	PT_2	Viet Tri	Phu Tho	
9	PT_3	Viet Tri		
10	QN_1	Tra My	Quang Nam	85

(*) Herbarium at the Vietnam Institute of Medicial Materials (IMM).

Sinensetin analyses of Orthosiphon stamineus Benth. extracts by RP-HPLC. HPLC analyses of sinensetin were carried out according to [10] with some minor modification on an analytical Shimpac MRC (250mm × 4,6mm ID, 5μm) column using a Shimadzu LC 10ATvp system coupled to a diode array detector (SPD-M10Avp). The samples were eluted with 40% acetonitrile and 60% acetic acid (0.05 M) at a flow rate of 0.5 ml/minute within 30 minutes. The identification of sinensetin in plant extracts was based on comparing the retention time and peak areas between the samples and the standard sinensetin. Each sample was separately extracted at least twice and analyzed by HPLC. Analytical data are given as mean ± standard deviation (SD).

Plant tissue culture. Shoot buds and axillary buds obtained from the highest sinensetin containing accession determined by HPLC analyses were employed for plant tissue culture experiments according to the protocol we optimized previously [9]. Each treatment was repeated at least with 24 replicates. Data were analyzed by one-way ANOVA calculated using Excel® 2003 (Microsoft Inc., USA), and represent as mean ± standard error (SE).

RAPD-PCR analyses. Leaves of regenerated plants of different ages ranging from 3 to 12 months-old were used for DNA extraction and analyses. RAPD primers obtained from Operon Technologies (USA) were employed to amplify genomic DNA of regenerated plants according to an optimized protocol (data not shown) in 25µl PCR reaction containing 30ηg sample ADN, 200µM dNTPs, 2.5U *Taq* polymerase, 2.5µl *Taq* polymerase buffer (10x) added with 0.3 µM $MgCl_2$.

3. Results and discussion

HPLC analyses of sinensetin

Based on the standard sinensetin (Extrasynthese, France), we established and verified the protocol for extraction and RP – HPLC analyses of sinensetin in Vietnamese *Orthosiphon stamineus* Benth. accessions. Figure 1 represents the linearity of the protocol determined by the standard sinensetin at 5 different concentrations, i.e. 0.0, 2.5, 5.0, 7.5 and 10.0 mg/ml with the regression coefficient (R) of 0,996, providing an evidence for the accuracy and precision of the analytical method. The details of HPLC conditions are described in the section of materials and methods. By this RP-HPLC protocol, sinensetin eluted at ca. 16.15 min. after sample injection (see Figure 2 for the comparison of the UV-VIS spectra between the sample's eluent at ca. 16.15 min. and the standard sinensetin).

Table 2 represents the sinensetin contents present in the leaf extract of the Vietnamese *Orthosiphon stamineus* Benth. accessions which were collected in this study. These data revealed that the senensetin content in the leaf extracts derived from different Vietnamese accessions fluctuated remarkably, ranging from 0,002% (QN_1) to 0,188 % (TH_0) of dry weight. The concentration of this compound in the leaf extracts of the other accessions, i.e. HN_1, HN_2, HN_3, TH_1, TH_2, PT_1, PT_2, and PT_3, were 0.091%, 0.121%, 0.087%, 0.128%, 0.087%, 0.033%, 0.049%, and 0.012%, respectively. As all samples were collected from plants of the same age (1 year old), the variation of sinensetin content among different accessions indicated that apart from genetic factor (genotype), the growing conditions and post-harvesting technique might have played an important role in the accumulation of sinensetin in Cat's whiskers herbal products.

Remarkably, in this study we also found sinensetin existing in stem extracts of the accession TH_0 (Table 2). However, it appeared in stems only in trace amount, i.e. 0.006%, as compared to leaves (0.188%). As reported, in Vietnam traditional medicine practitioners often employed both leaves and stems of Cat's whiskers to prepare herbal remedies, whereas in some European countries, only leaf extracts are of concern. Our data proved that if higher amount of sinensetin is expected in *Orthosiphon stamineus* Benth. herbal remedies, only leaf should be the part of use.

As an outcome of HPLC analyses, we found plant extracts from the accession TH_0 contained the highest concentration of sinensetin. Hence, the plants of this accession was then selected for in-vitro propagation studies.

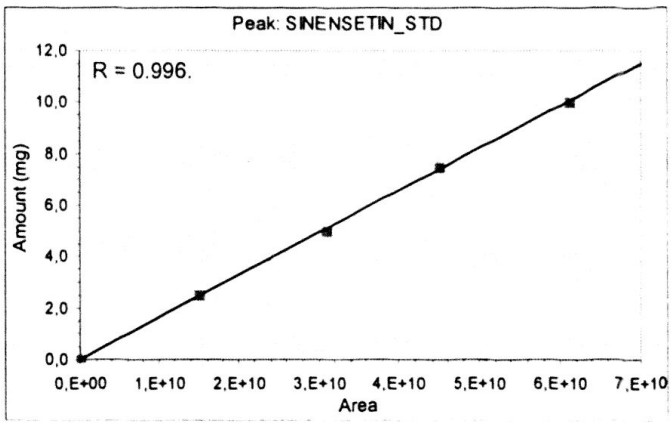

Figure 1. Linearity of HPLC protocol revealing the precision and accuracy of the analytical method. Y axis shows the amount of standard sinensetin in mg/ml. X axis is the peak area determined.

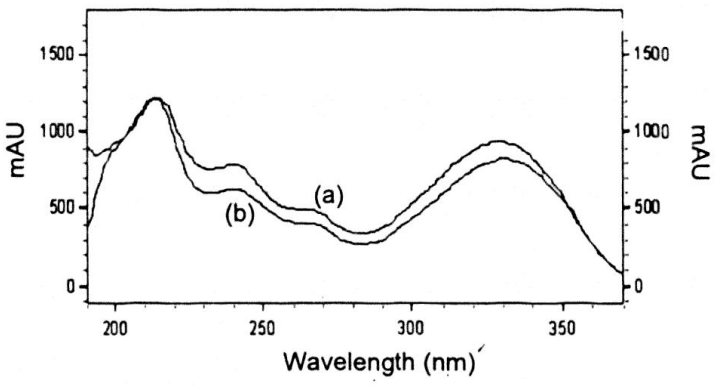

Figure 2. UV-VIS absorbance spectra of standard sinensetin (a), and of the plant sample's eluent at ca. 16.15 min. (b).

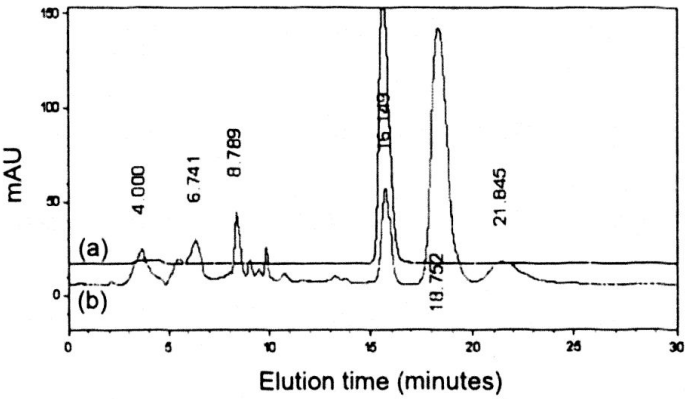

Figure 3. RP-HPLC chromatograms of standard sinensetin (a), and of the leaf extract derived from the accession TH_0 (b).

In-vitro propagation

Results presented in Figures 4 and 5 indicate that the formation and growth of *Orthosiphon stamineus* Benth. shoots in vitro were obviously regulated by the presence of NAA and BAP in the culture medium. The best rate of shoot proliferation and elongation were observed for the MS medium [11] containing 0.10 mg/l BAP and 0.05 mg/l NAA that permitted a multiplication rate of 4 – 5 folds after every 4 weeks with vigorous shoots.

An increase of the BAP concentration from 0 mg/l to 0.10 and 0.25 mg/l correspondingly enhanced the average number of regenerated shoots per explant. However, further increase of BAP concentration to 0.50 and 0.70 mg/l inhibited the growth rate of regenerated shoots, produced less axillary buds, and consequently lower multiplication rate.

Table 2. Sinensetin content in the Vietnamese Orthosiphon stamineus Benth. accessions(*)

No.	Analytical specimens			*Sinensetin content*
	Name of accession	Collection site	*Type of extracts*	*(% dry weigth)*
1	HN_1	Thanh tri, Ha nội	Leaf	0,091 ± 0,012
2	HN_2	Thanh tri, Ha nội	Leaf	0,121 ± 0,010
3	HN_3	Thanh tri, Ha nội	Leaf	0,087 ± 0,005
4	TH_0	Ha Trung, Thanh Hoa	Leaf Stem + Leaf Stem	0,188 ± 0,002 0,059 ± 0,015 0,006 ± 0,003
5	TH_1	Thanh Hoa City	Leaf	0,128 ± 0,005
6	TH_2	Thanh Hoa City	Leaf	0,087 ± 0,013
7	PT_1	Viet Tri, Phu Tho	Leaf	0,033 ± 0,021
8	PT_2	Viet Tri, Phu Tho	Leaf	0,049 ± 0,018
9	PT_3	Viet Tri, Phu Tho	Leaf	0,012 ± 0,005
10	QN_1	Tra My, Quang Nam	Leaf	0,002 ± 0,002

(*) Values represent means of at least twiçe replicated experiments ± SD.

Supplementing NAA at a low concentration (0.05 mg/l) to the BAP-containing media improved the shoot elongation and vigor. However, a higher concentration of NAA (0.10, 0.15 and 0.20 mg/l) induced callusing in some explants and reduced the shoot proliferation.

Root formation of regenerated shoots in-vitro was found in the highest efficiency with the MS/2 medium supplemented with 0.1 mg/l NAA (data not shown). 100% shoots produced roots within 3 weeks in this culture medium.

After rooting stage, almost all regenerated plants were successfully transferred to soil. For *O. stamineus* Benth., we encountered no major problem in regard to the adaptation of in-vitro regenerated plants to ex-vitro conditions, although we have observed slight effects of humidity and water-logging on the survival rate of the plants. For this reason, a soil condition that provides a good drainages is recommended during the initial period after transfer. We obtained satisfactory results when the micropropagated

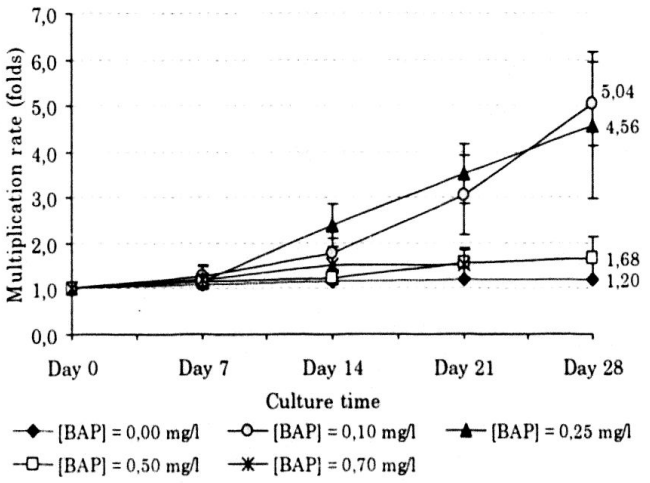

Figure 4. Effects of BAP on the regeneration rate of Orthosiphon stamineus Benth. shoots in-vitro (medium supplemented with 0.05 mg/l NAA).

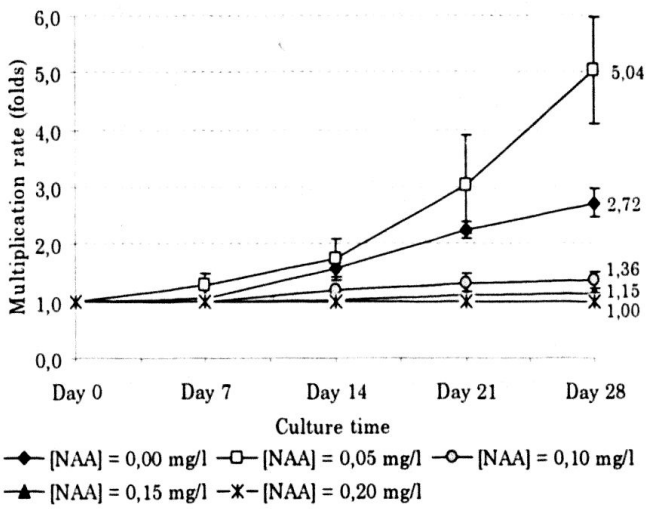

Figure 5. Effects of NAA on the regeneration rate of Orthosiphon stamineus Benth. shoots in-vitro (medium supplemented with 0.1 mg/l BAP).

plants were first grown in sand within first 3 weeks before being transferred to soil. More than 90% of the plants were successfully adapted to ex-vitro under these conditions. No extraordinary feature was observed for the micropropagated plants as compared to the donor and wildly growing plants (see Figure 6).

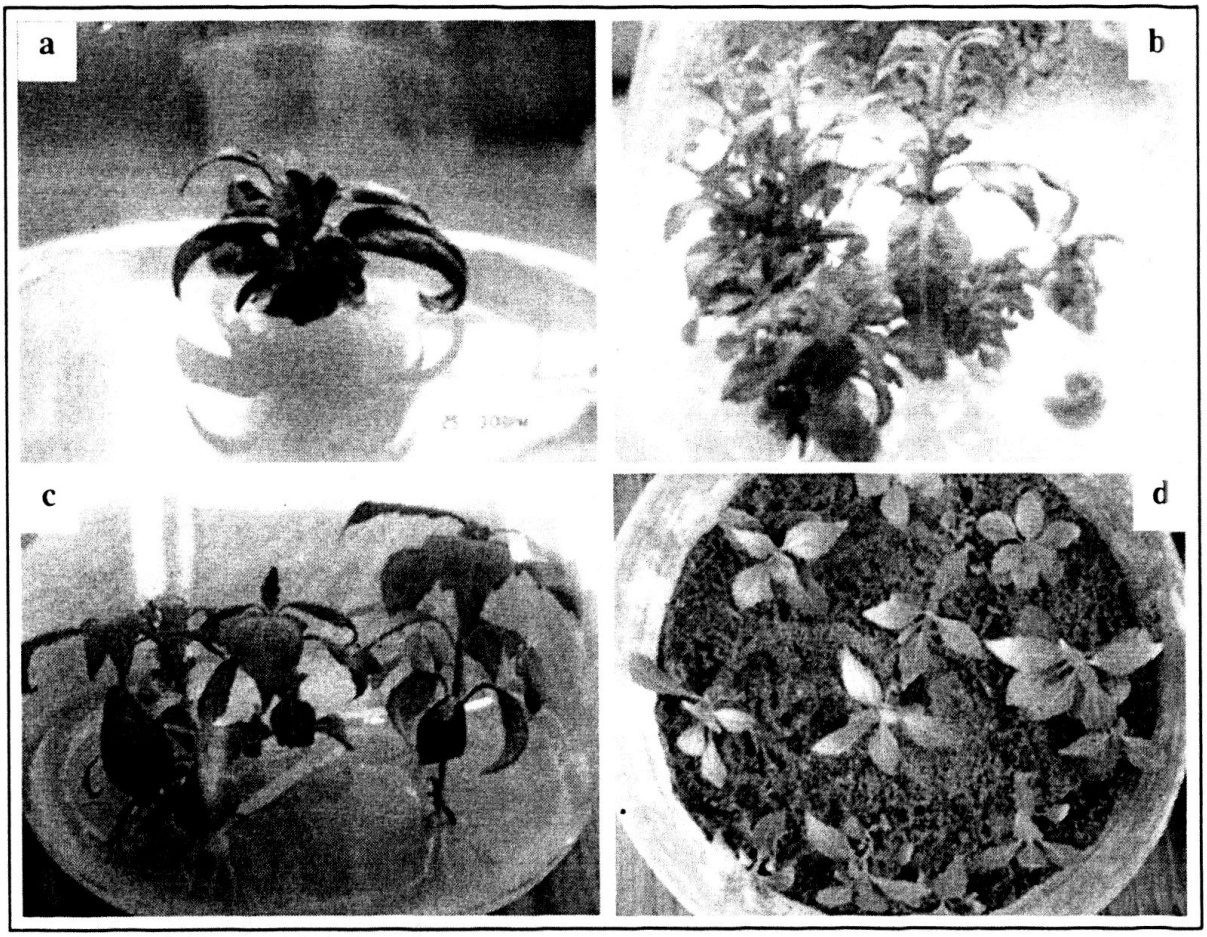

Figure 6. In-vitro propagation of Cat's whisker (Orthosiphon stamineus Benth.) (a) Regeneration of shoots from aseptic cultures, (b) Rapid propagation of shoots in the multiplication medium, (c) In-vitro shoots produced roots in the rooting medium, (d) In-vitro regenerated plants after transfer to ex-vitro conditions.

RAPD-PCR analyses of regenerated plants

Regenerated plants were examined for the genetic homogeneity and uniformity as compared the donor plant by analyses of DNA fingerprinting using RAPD-PCR technique. Different RAPD-PCR primers purchased from Operon Technologies (USA), including OPA-3, 4, 5, 8, 9, 10, 14, 15, 17, 18, 19, and 20 (see http://www.operon.com), were employed for the assays. No variation in DNA fingerprintings was found in regenerated plants of different ages as compared to each

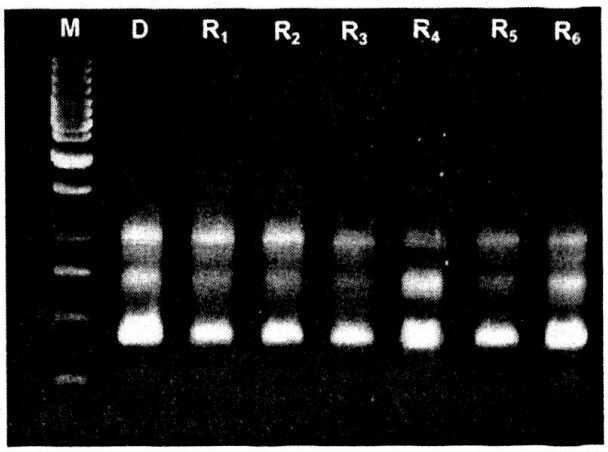

Figure 7. DNA fingerprintings using RAPD-PCR technique reveals the genetic homogeniety and uniformity among regenerated plants (R1 - 6) as compared to the donor plant (D). M: molecular weight marker.

other as well as to the donor plant (see an example using RAPD-PCR primer OPA-18 shown in Figure 7).

These results revealed that the *Orthosiphon stamineus* Benth. plants produced by shoot-bud tissue culture technique showed a high level of genetic homogeneity and uniformity. Such results have been previously reported in micropropagation of various medicinal plant species (Banthorpe, 1996; Camper et al., 1997; Wildi et al., 1998; Shoyama et al., 1997).

4. Conclusions

Our experiments on HPLC analyses of sinensetin in Vietnamese *Orthosiphon stamineus* Benth. accessions and in-vitro propagation of the clone containing high content of sinensetin led to the following main conclusions:

- Variation of sinensetin content were observed between leaf and stem extracts and among ten *Orthosiphon stamineus* Benth. accessions collected in Northern Vietnam. Apart from genetic factor, the growing conditions and post-harvesting technique might have played important role in the accumulation of sinensetin.

- MS basal medium supplemented with 0.1 mg/l BAP and 0.05 mg/l NAA was the most effective shoot induction medium, permitting a multiplication rate of about 4 – 5 every 4 weeks for the highest sinensetin containing accession, i.e. TH_0. Whereas, MS/2 medium supplemented with NAA (0.1 mg/l) was the most efficient rooting medium, inducing 100% shoots to form roots in vitro within 3 weeks of culture initiation. No major problem was encountered for *Orthosiphon stamineus* Benth. regenerated plants after the transfer to ex vitro conditions.

- Regenerated plants showed a high level of genetic homogeneity and uniformity revealed by DNA fingerprintings using RAPD-PCR technique.

Hence, our study demonstrates that plant tissue culture technique could be used as an efficient tool for rapid propagation of Cat's whisker clones and as an adjunct method for standardized production of medicinal materials derived from Vietnamese *Orthosiphon stamineus* Benth. genotypes in the future.

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XÂY DỰNG QUY TRÌNH NHÂN NHANH IN-VITRO DÒNG CÂY THUỐC RÂU MÈO (*ORTHOSIPHON STAMINEUS* BENTH.) CỦA VIỆT NAM CÓ HÀM LƯỢNG DƯỢC CHẤT SINENSETIN CAO

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Cây Râu mèo (Orthosiphon stamineus Benth) là loại cây thuốc quý đã được sử dụng từ lâu trong y học cổ truyền Việt nam. Gần đây cây thuốc này được nhiều phòng

thí nghiệm tại một số nước tiên tiến quan tâm nghiên cứu và dược điển một số nước Châu Âu ghi nhận là một loài cây thuốc có khả năng điều trị các bệnh liên quan đến đường tiết niệu như viêm thận, sỏi thận... Trong đó, hợp chất thuộc nhóm flavonoid là sinensetin được quan tâm hơn cả.

Kết quả phân tích HPLC trong nghiên cứu này cho thấy hợp chất sinensetin có mặt trong dịch chiết lá của tất cả các dòng Râu mèo thu thập ở Việt nam. Tuy vậy, hàm lượng hợp chất này rất dao động, từ 0,002% đến 0,188% (hàm lượng chất khô). Senensetin cũng có trong thân, nhưng chỉ ở lượng vết. Kỹ thuật nuôi cấy mô thực vật là một phương pháp hiệu quả để nhân giống các dòng cây Râu mèo. Trong đó, môi trường nhân giống có thành phần khoáng cơ bản MS (Murashige và Skoog, 1962) bổ sung 0,1 mg/l BAP và 0,05 mg/l NAA cho hệ số nhân chồi đạt 4 – 5 lần sau cứ 4 tuần nuôi cấy. Các cây con nuôi cấy mô sau khi được chuyển ra ngoài đất trồng có các đặc điểm sinh trưởng và phát triển đồng đều, giống nhau và giống cây mẹ ban đầu. Tính ổn định di truyền của chúng còn được khẳng định bằng chỉ thị ADN (RAPD-PCR).

Kết quả nghiên cứu của chúng tôi cho thấy kỹ thuật nuôi cấy mô thực vật in vitro kết hợp với các kỹ thuật phân tích chỉ thị hóa học và chỉ thị ADN có thể được áp dụng phục vụ mục đích bảo tồn, phát triển nguồn gen cây dược liệu quý và tiêu chuẩn hóa nguồn nguyên liệu làm thuốc từ các loài thảo mộc ở nước ta trong tương lai.