Variation in bioactive principles of *Artemisia amygdalina* Decne. in wild and tissue culture regenerants

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Abstract: Wild and tissue culture raised regenerants of *Artemisia amygdalina*, a critically endangered and endemic plant of Kashmir and North West Frontier Provinces of Pakistan were screened for the amount of bioactive principles and in particular antimalarial compound artemesinin. Phytochemical screening of extracts revealed the presence of terpenes, alkaloids, phenolics, tannins (polyphenolics), cardiac glycosides and steroids in wild (aerial, inflorescence) and tissue culture regenerants (*in vitro* grown plant, callus and green house acclimatized plants). HPLC of *Artemisia amygdalina* revealed the presence of artemesinin in petroleum ether extracts of wild aerial part, tissue culture raised plant and green house acclimatized plants. Acetonitrile and water in 70:30 ratios at flow rate of 1ml/min was standardised as mobile phase. Retention time for standard chromatogram was 6.7. Wild inflorescences and callus does not produce artemesinin. This is the first report of phytochemical screening and artemesinin estimation of wild and tissue culture raised regenerants of *Artemisia amygdalina.*

Keywords: Bioactive principles, Kashmir, Artemesinin, *Artemisia amygdalina.*

INTRODUCTION

The great interest in the use of plants as medicine is attributed to the presence of active principles whose pharmacological activities had been investigated. They are used in different pharmacopoeia drugs. Moreover plants are available on a continuous basis. However no concerted effort has been made against constant threats posed by increased demand and extensive destruction of plant habitats. Drugs obtained from the wild plants are sold in markets all over the region. They provide ingredients of medicines in medical traditions (Suri, 2010).

Multinational pharmaceutical companies are active in the field of herbal medicinal products (HMP), which subjects many plants to degradation and extinction within few years (Suri, 2010). Production system, *in situ* conservation, *ex situ* conservation and propagation all together in a proper manner can bring a positive change. Present study is an attempt to analyze effect of conservation strategy on the amount of bioactive principles in *Artemisia amygdalina* Decne. a critically endangered and endemic plant species of Kashmir valley and North West Frontier Provinces of Pakistan (NWFP). The plant has been collected after 40 years (Singh *et al*., 1971; Dar *et al*., 2006). It belongs to asteracea family and is tall aromatic perennial herb with simple serrate leaves commonly known as "Veer Thetven" in Kashmir.

MATERIALS AND METHODS

Plant collection

Plants were collected from Gurez Nallah of forest division in Bandipora in May-June, 2009. Specimens of plants were deposited in Kashmir University herbarium, vide voucher no: KASH (1013) and KASH (1014). The wild plant parts of *A. amygdalina* i.e. aerial (A) and inflorescence (I) were cleaned, washed and dried under shade for few days and then crushed in grinder to a fine texture. *In vitro* raised plants (T) and callus (C) using tissue culture technique were simultaneously collected after 1 year of culture period, washed to remove any medium and were kept for shade drying (Rasool *et al.*, 2011a; 2011b). The samples were then grinded to a fine powder in electric grinder and then stored in separate airtight bottles. Similar procedure was done to the green house grown plants (G).

Qualitative screening

Five plant samples of *A. amygdalina* i.e. wild aerial (A), wild inflorescence (I), *in vitro* cultured plants (T), callus (C) and acclimatized green house plants (G) were sequentially screened for the presence of bioactive compounds.

Tannins 2 ml of 5% $FeCl_3$ was added to 2 ml aqueous extract of each sample. Yellow brown precipitate indicated presence of tannins (Jigna and Sumitra, 2007, Rasool *et al*., 2010).

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Alkaloids 1.5 ml of 1% HCl was added to 2 ml methanolic filtrate of samples. The solution was heated and 6 drops of dragendroff reagent was added. Orange precipitate confirmed alkaloids (Jigna and Sumitra, 2007, Rasool *et al*., 2010).

Saponins 2 ml aqueous extract of all samples were mixed with few drops of sodium bicarbonate solution. Persistence of froth indicated saponins (Harborne, 1973; Oguyemi, 1979; Trease and Evans, 1989; Sofowora, 1993 and Jigna and Sumitra, 2007).

Cardiac glycosides (Keller Kiliani test) 1 ml glacial acetic acid, 2 drops of $FeCl_3$ and 1 ml of conc. H_2SO_4 was added to 2 ml of methanolic extract of wild and regenerant extracts of *A. amygdalina*. Brown ring at the interface shows presence of cardiac glycosides (Sofowora, 1993 and Rasool *et al*., 2010).

Terpenes To 1 ml of petroleum ether or ethyl acetate extract, 2 ml chloroform, 2 ml conc. H_2SO_4 was added along sides. Formation of reddish brown color of interface indicated terpenes (Harborne, 1973 and Rasool *et al*., 2010).

Steroids (Leiberman Buchard test) 2 ml of acetic anhydride solution was added to 1 ml of petroleum ether extract of each sample in chloroform followed by 1 ml of conc. H_2SO_4. A greenish color developed if turned to blue indicated steroids(CCRUM, 1987).

Flavonoids (Shimoda's test) In 2 ml aqueous or methanolic extracts, few drops of conc. HCl followed by 0.5 g of Zinc turnings were added. Tubes were boiled for few minutes. Formation of magenta red or pink color indicated flavonoids (Martinez, 2003 and Jigna and Sumitra, 2007).

Phenolics To 2 ml of aqueous or methanolic extracts, 1 ml of 1% ferric chloride solution were added. Blue or green color indicates phenols (CCRUM, 1987; Martinez, 2003).

Resins Methanolic extracts were treated with 5 ml acetic anhydride. Solutions were heated and subsequently cooled. 0.5 ml of sulfuric acid was added to all sample solutions. If violet color appears and changes to red that indicates resins (CCRUM, 1987).

Quantitative screening

Alkaloid 2.5 g of the plant powder of each sample was extracted using 100 ml of 20 % acetic acid in ethanol. The resulting solutions were covered for 4 hours. Filtrate obtained was concentrated to 25 ml. Concentrated ammonium hydroxide was added to filtrate carefully to attain precipitation. Precipitate was allowed to settle. Precipitate was collected and washed with dilute ammonium hydroxide and then filtered. Filtrate was dried and weighed (Harborne, 1973; Edeoga *et al*., 2005 and Rasool *et al.,* 2010).

Phenolics 1g of sample powders was extracted with 80 % ethanol. Filtrate obtained was evaporated to dryness and again redissolved in water. Different aliquots (0.1-1 ml) were pipetted out and volume was made to 3 ml by distilled water. 0.5 ml of Folin's reagent followed by 2 ml 20% Na_2CO_3 solution was added. Tubes were vortexed, heated in boiling water for 1 min and finally cooled. Absorbance was measured at 650 nm against blank. A standard curve using different conc. of 2 mg % catechol was prepared (Malick and Singh, 1980).

Tannins 2 g plant powder from each sample was extracted thrice in 70% acetone. Samples were centrifuged and supernatant was collected. Different aliquots were prepared and final volume was made to 3 ml by distilled water and vortexed. 1 ml of 0.016 M K_3 [Fe $(CN)_6$], 1 ml of 0.02M $FeCl_3$ in 0.1M HCl were added. Tubes were shaken and then kept as such for 15 min. 5 ml of stabilizer (3:1:1 ratio of water, H_3PO_4 and 1% gum arabic) was added and tubes were again revortexed. Absorbance was taken at 700 nm. Standard curve was plotted using different conc. of 1.9 mg % gallic acid (Graham, H.D, 1992).

Total Lipids 1g of each plant sample was dissolved in ether and stirred for 1 hour. Mixture was centrifuged and supernatant was dried, redissolved in ethanol. 0.1 ml of alcohol was used as blank, olive oil as standard (500 mg % in alcohol) and test sample as unknown respectively. 2 ml of conc. H_2SO_4 followed by 5 ml of phosphovanillin reagent was added and vortexed well. Tubes were incubated for 30 min and finally absorbance of each tube was recorded at 540 nm. Lipids (mg) were quantified using formulae

$$\left[\frac{\text{At-Ab}}{\text{As-Ab}}\right]\times 500$$

where At is absorbance of test sample, As is absorbance of standard and Ab is absorbance of blank (Ganai *et al*., 2005).

High performance liquid chromatography (HPLC)

Acetonitrile, water, petroleum ether, methanol and glacial acetic acid were HPLC-grade, purchased from Merck (Darm stadt, Germany) and artemisinin was purchased from Sigma-aldrich (St Louis, MO, USA) and stored at -20ºC. Standard artemesinin (361593, 98%, CAS 63968-64-9) was prepared by weighing 1 mg of crystals on microbalance (Citizen CM21) and solubilizing in 1ml of HPLC-grade acetonitrile carefully. Samples were kept in HPLC autosampler vials 12mm × 32mm, 1.8ml (Thermo Fisher, A4954-010) on ice tray until used.

Sample preparation

1 mg of petroleum ether (P) extracts of wild (A, I) and tissue culture regenerants (T, C, G) were dissolved in 1 ml of HPLC grade acetonitrile in 1 ml eppendorf tube and samples were sonicated for 30 mins (PCi 3.5L(H), Mumbai, India) before use. Each sample (PA, PI, PT, PC and PG) was filtered using nylon 25 mm, 0.45 µm micropore qualisil syringe filters (LCGC, Chandigarh, India) before injecting into HPLC for analysis.

Method development

HPLC (Thermofisher Scientific, Pittsburgh, PA, USA) composed of a Finnigan Surveyer UV plus detector (80018SRYYR/UV5P; firmware 50 mm light5/3.12) set to scan from 190 to 800 nm, Finnigan Surveyer autosampler (80249/SRVYR-AST; firmware 2.15), Finnigan Surveyer LC Pump plus 80149/SRVYR-LCMPP; firmware 2.00) was used for analysis. It also contains six port injection valve (Valco C2). The whole setup works via ChromQuest 4.2.34 software. 5 to 25 µl of each sample can be injected in column. Method was standardized with Kromasil Gold E27918 C-8 column (250 × 4.6 mm i.d.; 5 µm). The absorbance was measured at 210 nm. After number of standardization trials, an isocratic binary mobile phase solution was chosen using acetonitrile and water in (70:30 ratio) with a flow rate of 1 ml/min. Premixing was also done whenever necessary followed by 30 min sonication. Acetonitrile was given as blank before injecting sample.

RESULTS

Phytochemical screening

Screening of the plant extracts of wild aerial (A), wild inflorescence (I), *in vitro* grown (T), callus (C) and green house grown plant (G) revealed the presence of the terpenes, steroids, alkaloid, phenols, tannins (polyphenolics) and cardiac glycosides. Results of screening and quantitative estimations of bioactive constituents are summarized in tables 1 and 2.

Table 1: Qualitative phytochemical screening of wild and tissue culture raised regenerants of *Artemisia amygdalina*

Bioactive agents	Type of extract	Presence(+)/Absence(-)				
		Wild aerial [A]	Wild Inflorescence [I]	Callus [C]	Tissue culture grown plants [T]	Green house raised plants [G]
Alkaloid	Methanol	+	+	+	+	-
Phenolics	Methanol	+	-	-	-	+
Tannins (polyphenolics)	Methanol	-	+	+	+	-
Cardiac glycosides	Methanol	+	+	-	+	+
Flavonoids	Methanol	-	-	-	-	-
	Aqueous	-	-	-	-	-
Saponins	Methanol	-	-	-	-	-
Terpenes	Methanol	-	-	-	-	-
	Aqueous	-	-	-	-	-
	Pet. ether	+	+	+	+	+
	Ethyl acetate	+	-	-	+	+
Steroids	Methanolic	-	-	-	-	-
	Pet. ether	+	+	+	+	+
Resins	Methanolic	-	-	-	-	-

Table 2: Quantitative estimation of wild and tissue culture raised regenerants of *Artemisia amygdalina*

Bioactive agents	Type of extract	Quantity (g %)*				
		Wild aerial [A]	Wild Inflorescence [I]	Callus [C]	Tissue culture grown plants [T]	Green house raised plants [G]
Alkaloid	Methanolic	3.77 ± 0.01	4.49 ± 0.05	2.98± 0.002	2.505 ± 0.1	-
Phenols	Methanolic	0.011± 0.001	-	-	-	0.020 ± 0
Tannins	Methanolic	-	0.0477± 0.0019	0.0342± 0.001	0.022 ± 0.0027	-
Total Lipids (incl. steroids & terpenes)	Pet ether	10.97 ± 0.05	10.69 ± 0.7	9.39 ± 1	8 ± 2	8.8± 1.5

*Results are mean ± SD of triplicate determination on the basis of dry weight.

Table 3: Percentage of artemesinin (Mean ± SD % w/w) in *A. amygdalina* wild and tissue culture raised regenerants using petroleum ether extracts

Type of extracts	Wild aerial (PA)	Wild Inflorescence (PI)	Tissue culture raised plant (PT)	Callus (PC)	Green house grown plant (PG)
Percentage	0.36 % ± 0.015	0	0.25 % ± 0.07	0	0.28 %± 0.012
Goodness of fit (r^2)	0.9988	0	0.9993	0	0.997

High performance liquid chromatography (HPLC)

Standardization of artemesinin in *A. amygdalina* by HPLC-UV was carried out using the optimized conditions. Standard was found to be partially soluble in methanol but completely soluble in acetonitrile. Detection was done at 210 nm. Acetonitrile and water in 70:30 ratios was found to be suitable for quantification. 2.5, 5, 10, 15, 20 µl injections of standard and unknown sample series were run. The typical chromatogram of artemesinin with RT 6.7 is shown in fig.1a. Among methanolic, ethyl acetate, aqueous extracts only petroleum ether extracts showed presence of artemesinin. Petroleum ether extracts of wild aerial, *in vitro* grown and green house acclimatized plants (PA, PT, and PG) showed presence of artemesinin while as inflorescence and callus (PI, PC) does not. The peak area ratios of standard and sample solutions were calculated via external standard method of ChromQuest software. The calibration curve was linear (y=6.52951e–006x + 0.033). The procedure was repeated three times and amount of marker in sample was calculated. Results are shown in table 3 and figs. 1 a-e.

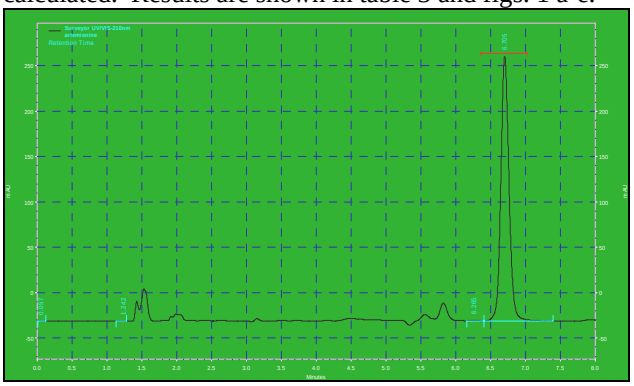

Fig. 1 (a): Chromatogram of standard artemesinin with RT 6.7.

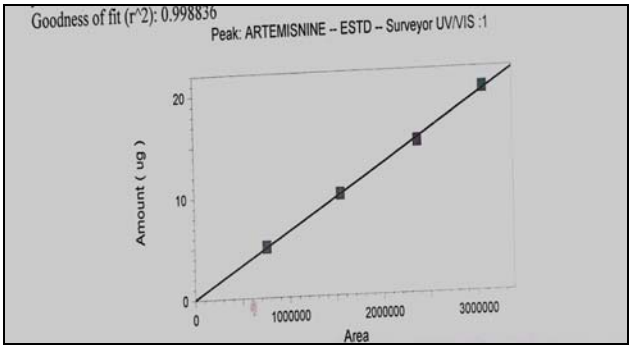

Fig 1 (b): Calibration curve of standard artemesinin.

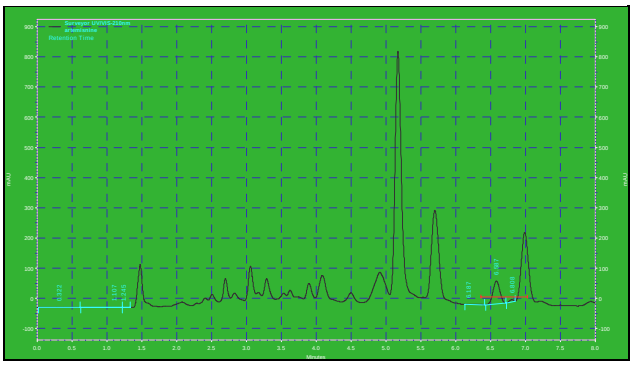

Fig 1(c): Chromatogram of *Artemisia amygdalina* (wild aerial part)

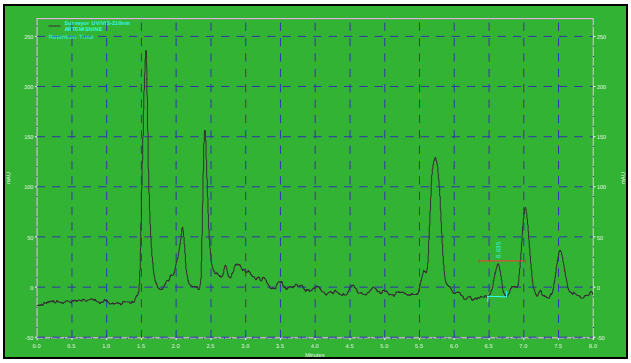

Fig. 1(d): Chromatogram of *Artemisia amygdalina* (*in vitro* raised plants)

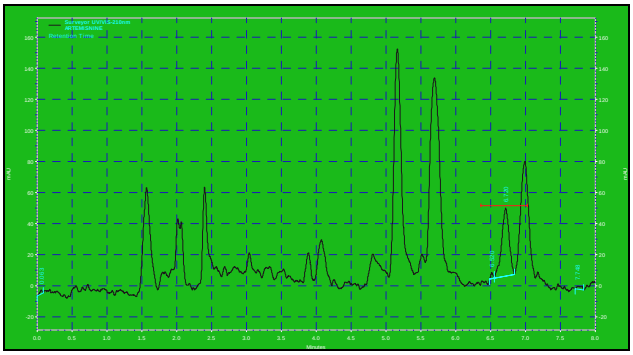

Fig. 1(e): Chromatogram of *Artemisia amygdalina* (Green house raised plants)

DISCUSSION

Quantitative estimation revealed variation in type and amount of phytochemicals from samples of both wild and

regenerant plants. Phenolics considered as effective free radical scavengers were more in green house grown plants than wild grown plant. Alkaloids and tannins were not detected in greenhouse grown plants. This variation in phytochemical profile can be accounted due to somaclonal variations arised during tissue culture cycle and acclimatization process. As appearance of desirable or undesirable variants is a chance event. Genotype, source of explants, duration of culture and culture conditions all lay effect on regenerants. Artemesinin, a sesquiterpenes lactone was detected in wild aerial part, *in vitro* grown plant, green house acclimatized plant and was absent in callus and inflorescences. Artemesinin possesses weak chromophore so derivatization via NaOH and acetic acid could help in detection (Mannan *et al*., 2010) but acetic acid used in the procedure produced noise and causes pressure fluctuations in our column. UV absorption of artemesinin in our HPLC system with inbuilt default wavelength range of 190-800 nm was high enough that allowed the quantification without alkaline hydrolysis treatment which is in conformity with the findings of Ferreira and Gonzalez, 2009. It also maintained system in equilibrium. So work was carried without alkaline hydrolysis treatment.

Singh and Sarin, 2010 find *A. scoparia* callus culture as an alternative of *A. annua* for the production of artemisinin. The yield of artemisinin in *A. annua* was higher in aerial plant parts (0.015%) in comparison to callus culture (0.001%) which in accordance with our results where aerial parts also showed higher concentration of artemesinin (0.36%) but not in callus (table 3). Our results are also in accordance with Mannan *et al*., 2010 where the highest artemisinin concentration was detected in the leaves (0.44 ± 0.03%) and flowers (0.42 ± 0.03%) of *A. annua*, followed by the flowers (0.34 ± .02%) of *A. bushriences* and leaves (0.27 ± 0%) of *A. dracunculus.* Varying concentrations of artemesinin in various species ranging from 1.38 % in *A. annua* leaves in Switzerland (Delabays *et al*., 1993), 0.86% in *A. annua* leaves in Vietnam (Wallart *et al*., 1999), 0.79% in *A. annua* in leaves in China (Charles *et al*., 1990), 0.0006% in *A. cina* in Indonesia (Aryanti *et al*., 2001), 0.2% *A. seibri* in Iran (Arab *et al*., 2006), 0.022 % in *A. absinthium* in Pakistan (Zia *et al*., 2007) have been reported worldwide.

CONCLUSION

The present study showed the potential of *in vitro* cultured plants that can be used as an substitute against endangered wild germplasm but to maximize the yield of metabolites proper stage of formation in tissue culture cycle, their distribution in organized plant formed inside lab or plant acclimatized outside and unorganized callus, collection period need to be sought out and that needs further studies.

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