Chapter 2
Phytochemical Screening and HPLC Analysis of *Artemisia amygdalina*

Abstract This chapter deals with the qualitative analysis of wild and tissue culture raised regenerants of *Artemisia amygdalina*, for the amount of bioactive principles particularly the antimalarial compound, artemisinin. 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 greenhouse acclimatized plants). Further, HPLC of *A. amygdalina* extracts has revealed the presence of artemisinin in petroleum ether extracts of wild aerial part, tissue culture raised plant, and greenhouse acclimatized plants. Acetonitrile and water in 70:30 ratios at a flow rate of 1 ml/min have been optimized as mobile phase. It has been observed that wild inflorescences and callus do not produce artemisinin.

Keywords Qualitative analysis · HPLC · Artemisinin · Tissue culture · Regenerants · Wild plants

2.1 Qualitative Analysis

Qualitative analysis has been carried out for both wild and tissue culture obtained plants. Five plant samples of *Artemisia amygdalina*, viz., wild aerial (A), wild inflorescence (I), in vitro cultured plants (T), callus (C),and acclimatized greenhouse plants (G) have been sequentially screened for the presence of bioactive compounds. Different quantitative tests performed by the authors' group include:

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2.1.1 Presence of Tannins

Ferric chloride has been used to detect the presence of tannins in the extract solutions of *A. amygdalina*. As a normal procedure, 2 ml of 5 % $FeCl_3$ has been added to 2 ml aqueous extract of each sample. Yellow brown precipitate indicated the presence of tannins (Jigna and Sumitra 2007; Rasool et al. 2010). It has been observed that tannins were present in the methanolic extracts of wild inflorescence (I), in vitro cultured plants (T), callus (C), and acclimatized greenhouse plants (G) (Table 2.1).

2.1.2 Presence of Alkaloids

Dragendroffs test has been used to detect the presence of alkaloids. The methanolic extracts of wild aerial (A) wild inflorescence (I), in vitro cultured plants (T), callus (C) tested positive for the presence of alkaloids while the acclimatized greenhouse plants (G) tested negative for the alkaloid content (Table 2.1).

Table 2.1 Qualitative phytochemical screening of wild and tissue culture raised regenerants of *A. amygdalina*

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

2.1.3 Presence of Saponins

The presence of Saponins was confirmed by the addition of a few drops of sodium bicarbonate solution to 2 ml aqueous extract of all samples (Harborne 1973; Oguyemi 1979; Trease and Evans 1989; Sofowora 1993; Jigna and Sumitra 2007). As per the authors' group all the methanolic extracts of *A. amygdalina* Decne tested negative for the presence of saponins (Table 2.1).

2.1.4 Presence of Cardiac Glycosides

Keller Kiliani test was used to test the presence of cardiac glycosides (Sofowora 1993; Rasool et al. 2010). All the methanolic extracts of *A. amygdalina* tested positive for the presence of cardiac glycosides except for the callus extracts (Table 2.1).

2.1.5 Presence of Terpenes

All the methanolic and aqueous extracts of *A. amygdalina* have been found to be devoid of terpenes. All the low polar petroleum ether extracts have been found to contain terpenes. In case of ethyl acetate extracts, only the wild aerial (A), tissue culture grown plants (T) and greenhouse acclimatized petroleum ether extracts have been found to contain terpenes (Table 2.1).

2.1.6 Test for Steroids

Leiberman Buchard test was successfully applied for the presence of steroids (CCRUM 1987). All the low polar petroleum ether extracts were found to contain steroids. However, the high polar methanolic extracts were found to be devoid of steroids (Table 2.1).

2.1.7 Test for Flavonoids

Shinoda's test involves addition of few drops of conc. HCl followed by 0.5 g of zinc turnings to about 2 ml aqueous or methanolic extracts and then boiling for a few minutes to furnish magenta red or pink color has been used to detect the

presence of flavonoids (Martinez and Valencia 2003; Jigna and Sumitra 2007). Both the methanolic and aqueous extracts of *A. amygdalina* Decne have been found to be negative for the occurrence of flavonoids (Table 2.1).

2.1.8 Presence of Phenolics

Ferric chloride test has been used to detect the presence of phenolics (CCRUM 1987; Martinez and Valencia 2003). Only the methanolic extracts of wild aerial (A) and greenhouse acclimatized plants (G) have been found to contain phenolics (Table 2.1).

2.1.9 Presence of Resins

Methanolic extracts have been treated with 5 ml acetic anhydride. Solutions were heated and subsequently cooled. 0.5 ml of sulfuric acid was added to all sample solutions. Since no color change was found, therefore it was predicted that these extracts do not contain resins (Table 2.1).

2.2 HPLC Analysis

Standardization of artemisinin in *A. amygdalina* by HPLC-UV has been carried out using the optimized conditions. Detection has been done at 210 nm. Acetonitrile and water in 70:30 ratios have been found to be suitable for quantification. 2.5, 5, 10, 15, 20 µl injections of standard and unknown sample have been run. The typical chromatogram of artemisinin with r.t 6.7 is shown in Fig. 2.1. Among various extracts prepared such as methanolic, ethyl acetate, and aqueous extracts only the low polar petroleum ether extracts have shown the presence of

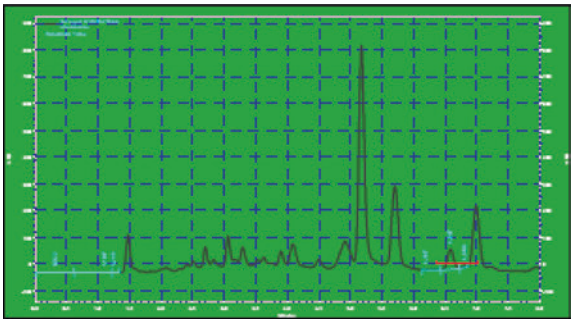

Fig. 2.1 Chromatogram of standard artemisinin with RT 6.7

artemisinin. Petroleum ether extracts of wild aerial (A), in vitro grown (T) and greenhouse acclimatized plants (G) have been found to contain artemisinin while inflorescence (I) and callus (C) have been found to be devoid of this wonder molecule. The peak area ratios of standard and sample solutions have been calculated via external standard method of the Chrom-Quest software. The calibration curve depicted linearity ($y = 6.52951e - 006x + 0.033$). The authors have highlighted their results in Table 2.2 and Figs. 2.1, 2.2, 2.3, 2.4 and 2.5.

The variation in phytochemical profile has been attributed to somaclonal variations that arise during tissue culture cycle and acclimatization process. Appearance of desirable or undesirable variants is a chance event. Genotype, source of explants, duration of culture,and culture conditions all have effect on regenerants.

Table 2.2 Percentage of artemisinin (Mean ± SD % *w/w*) in *A. amygdalina* wild and tissue culture raised regenerants using petroleum ether extracts

Type of extracts	Wild aerial (A)	Wild inflorescence (I)	Tissue culture raised plants (T)	Callus (C)	Greenhouse grown plants (G)
Percentage	0.36 % ± 0.015	0	0.25 % ± 0.07	0	0.28 % ± 0.012
Goodness to fit (r^2)	0.998	0	0.9993	0	0.997

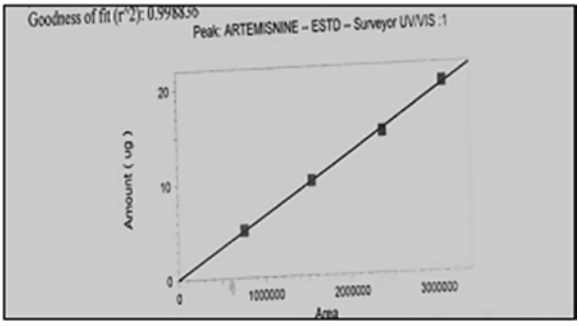

Fig. 2.2 Calibration curve of standard artemisinin

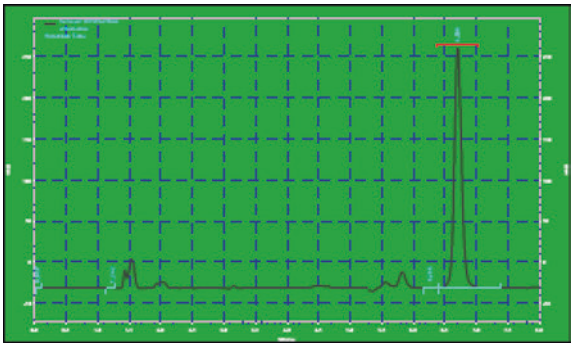

Fig. 2.3 Chromatogram of *A. amygdalina* (wild aerial part)

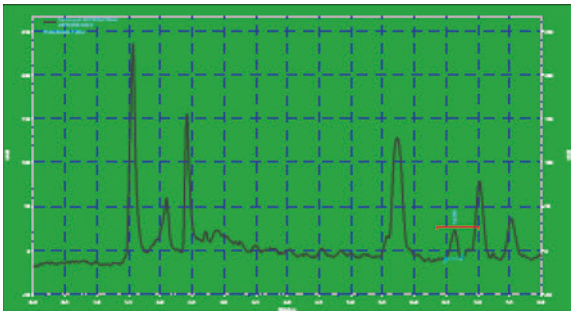

Fig. 2.4 Chromatogram of *A. amygdalina* (in vitro raised)

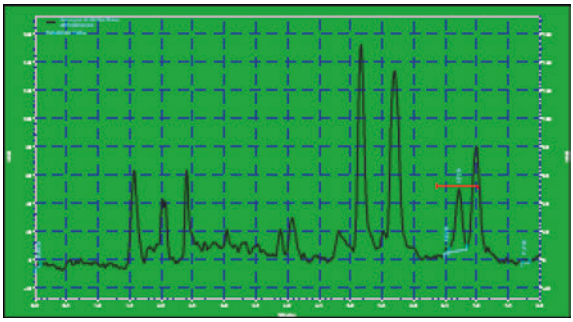

Fig. 2.5 Chromatogram of *A. amygdalina* (Greenhouse raised plants)

Artemisinin, a sesquiterpene lactone has been detected in wild aerial part, invitro grown plant, greenhouse acclimatized plant and was found absent in callus and inflorescences. Artemisinin possesses weak chromophore, so derivatization using NaOH and acetic acid could help in detection (Mannan et al. 2010), but acetic acid used in the procedure here has led to noise production with pressure fluctuations in column. UV absorption of artemisinin in HPLC system, with inbuilt default wavelength range of 190–800 nm, has been found to be 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 the system in equilibrium. So work has been carried without alkaline hydrolysis treatment.

As per (Singh and Sarin 2010) *Artemisia scoparia* callus culture is an alternative to *Artemisia annua* for the production of artemisinin. The yield of artemisinin in *A. annua* has been reported to be higher in aerial plant parts (0.015 %) in comparison to callus culture (0.001 %), which is in accordance with that of *A. amygdalina,* where aerial parts also showed higher concentration of artemisinin (0.36 %) but not in callus (Table 2.2). As per Mannan et al. 2010, the highest artemisinin concentration has been detected in the leaves (0.44 ± 0.03 %) and flowers (0.42 ± 0.03 %) of *A. annua*, followed by the flowers (0.34 ± 0.02 %) of *Artemisia bushriences* and leaves (0.27 ± 0 %) of *Artemisia dracunculus*. Varying concentrations of artemisinin 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 Wallaart et al. 1999), 0.79 % in *A. annua* in leaves in China (Charles et al. 1990), 0.0006 % in *Artemisia cina* in Indonesia (Aryanti et al. 2001), 0.2 % *Artemisia seibri* in Iran (Arab et al. 2006) have been reported worldwide.

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