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Full Length Research Paper

Mutational approach for enhancement of artemisinin in *Artemisia annua*

Fahad Al-Qurainy and Salim Khan*

Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, Kingdom of Saudi Arabia, 11451, Saudi Arabia.

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The potential mutagenic effect of sodium azide (NaN_3) was studied on seeds and callus of *Artemisia annua* for enhancement of artimisinin. The treated and untreated seeds were germinated with half MS liquid medium and the leaves (two-weeks-old) were employed to raise callus on full strength of medium containing α-naphthalene acetic acid (NAA) and kinetin (each 0.5 mg/L). The mutant calluses were obtained from normal callus and foliage leaves of germinated treated seeds with 1 - 5 mM, NaN_3. Artimisinin content was estimated in all mutant calluses with High-performance liquid chromatography (HPLC), and all showed increased level of this compound as compared to those normal calluses. The mutant calluses obtained from foliage leaves of germinated seeds had low content of this compound as compared to those calluses which developed from normal callus with NaN_3 treatment. In all mutant calluses obtained from normal calluses and foliage leaves of treated seeds, the maximum artimisinin was found in T3 and T8, and occurred at 3 mM, NaN_3 as compared to normal ones. Thus, sodium azide is a potent mutagen for enhancement of artemisinin and can be used as an alternative for its *in vitro* production.

Key words: *Artemisia annua*, anti-malarial drug, artimisinin, medicinal plant.

INTRODUCTION

Artemisia annua (L.) is an aromatic and medicinal herb, native to Asia, most probably China and has become naturalized in many countries such as Argentina, Bulgaria, France, Hungary, Romania, Italy, Spain, the United States, and the former Yugoslavia. The most active compound of *A. annua* is 'artemisinin' a sesquiterpene lactone which is used against malaria. Generally, artemisinin and its analogs are relatively safe drugs with no obvious adverse reactions or noticeable side effects (Benakis et al., 1997). At present, these compounds provide the effective remedy for the treatment of schistosomiasis (Xiao, 2005; Mishina et al., 2007), cryptosporidiosis, amoebiasis, giardiasis, clonorchiasis, leishmaniasis (Ma et al., 2004), malaria (Haynes et al., 2006; Li et al., 2006; Mutabingwa, 2005) and cancer (Efferth, 2006). Moreover, artemisinin has been recently indicated as a potential and effective compound against a number of viruses including hepatitis B, C and others (Efferth et al., 2008). Nevertheless, on a global scale, these compounds remain generally unavailable and large-scale isolations of artemisinin from the plant are possible only in a few countries (Chang et al., 2000). The content of artemisinin depends on the level of gene expression such as squalene synthase (SQS), a key enzyme in sterol pathway and has been played a pivotal role in a RNAi (RNA interference) technique (Zhang et al., 2009).

Recently, cytochrome P450 and its associated reductase have been shown to catalyze multiple steps in the biochemical pathway leading to the production of artemisinin with the highest values reaching 31.4 mg/g DW, which is about 3.14-fold the content observed in untransformed control plants (Arsenault et al., 2008). The content of this compound varies in different parts of the plant and has been detected from aerial parts including

*Corresponding author. E-mail: salimkhan17@yahoo.co.in. Tel: +966530020523. Fax: +9664675833.

leaves, inflorescences, seeds and stem (Ferreira et al., 1995). However, it was not detected from pollens and roots. Its content in inflorescence and the bud stage was not higher than in leaves, but in flowers at full bloom, it was 4 to 11 fold higher than in leaves (Ferreira et al., 1995). For organic chemists, it was a challenge to develop a total chemical synthesis pathway for artemisinin due to the complex structure and having 64 theoretical possible stereo-isomers. The low yield and high cost of chemical synthesis suggest its isolation from the plant is the optimum system.

The undifferentiated callus and cell suspension cultures of *A. annua* gave disappointing results with respect to the artemisinin production (Martinez and Staba, 1988) and further their differentiation into shoots, or preferably shoots with roots, is necessary for its significant biosynthesis. However, its content was also improved in the culture fluid from liquid suspension cultures of the callus cells (Nair et al., 1986). Artemisinin content in regenerated plants from stem explants using 0.1 mg/l TDZ (Thidiazuron) was 3.36 ± 0.36 µg/mg DW and two-fold higher than that of *in vitro* grown plants of the same age (Lualon et al., 2008). However, its production by mean of cell, tissue or organ cultures is very low and there are few reports on artimisinin biosynthesis using mutational approach, mainly chemical mutagens. Induced mutations have great potentials and serve as a complimentary approach in genetic improvement of crops for greater yield and quality traits (Mehandjiev et al., 2001; Ahloowalia and Maluszynski, 2001). *In vitro* plantlet variants of *A. annua* were produced with physical mutagen (gamma rays) and evaluated for their artemisinin content and enzymatic activity of amorpha-4, 11-diene synthase (Koobkokkruad et al., 2008). Sodium azide has been investigated for enhancement of yield and quality traits of crops including, medicinal plants such as *Arachis hypogaea* (Menash and Obadoni, 2007), *Vigna radiata* (Samiullah et al., 2004), *Spathoglottis plicata* Blume (Roy and Biswas, 2005), *Halianthus annuus* (Skoric et al., 2008; Venegas-Caleron et al., 2008), *Oryza sativa* (Jeng et al., 2003, 2006; Suzuki et al., 2008), *Hordeum vulgare* (Oliver et al., 2009 and *Phaseolus vulgaris* (Jeng et al., 2010). Its mutagenic effect is mediated through the production of an organic metabolite of azide compound (Owais and Kleinhofs, 1988) which enters into the nucleus and interacts with DNA. Therefore, on the light of limited literature on chemical mutagens on *A. annua*, we employed NaN_3 for enhancement of artimisinin level in the callus cells.

MATERIALS AND METHODS

Seed treatment with NaN_3 and tissue culture

The seeds of *A. annua* were borrowed from Centre for Transgenic Plant Development, Jamia Hamdard, New Delhi, India. They were sterilized in 0.1% $HgCl_2$ solution for 10 min and thereafter washed with autoclaved distilled water three times to remove excess $HgCl_2$ and dried at room temperature. The seeds were treated with various concentrations of NaN_3 solution (diluted in 0.5 M sodium phosphate buffer: pH 3.2) ranged from 1 - 5 mM for 1 h incubation period on rotator shaker at 25 RPM. The treated seeds were washed three times to remove excess NaN_3 with autoclaved distilled water. Subsequently, treated and untreated seeds were germinated on sterilized soil wetted with 1/2 MS liquid basal medium (Murashige and Skoog, 1962) (pH 5.7) in the Petri dish. The foliage leaves of two-weeks-old seedlings were used as explants and sterilized in 0.1% $HgCl_2$ for 10 min and subsequently washed with autoclaved distilled water three times under laminar flow. Subsequently, leaves were transferred to MS medium supplemented with α-naphthalene acetic acid (NAA) and kinetin (each 0.5 mg/L) and kept at 26 ± 1°C in tissue culture chamber with 16 h photoperiod, and 45 µmol/m^{-2}s photosynthetic photon flux density for 30 days. The mutant calluses (T6, T7, T8, T9 and T10) obtained from foliage leaves of germinated treated seeds along with normal callus (Tc) were sub-cultured after three weeks time-interval to obtain large biomass for high content of artimisinin.

Callus treatment with NaN_3

The seven weeks old callus was treated with various concentrations of NaN_3 (1 - 5 mM) diluted in sodium phosphate buffer (0.5 M, pH 3.2). The incubation period for callus was 1 h for all treatments and thereafter washed three times with 1/2 liquid MS medium to remove excess NaN_3. Subsequently, the treated calluses were cultured on fresh callusing medium as used for normal callus development. After two weeks, the callus was sub-cultured on another fresh medium for proper growth development. The mutant calluses (T1, T2, T3, T4 and T5) were harvested after seven-weeks of culture from various treatments. The fresh and dry weight of mutant calluses were recorded and dried callus was employed for artimisinin extraction.

Artimisinin extraction and quantification with high performance liquid chromatography (HPLC)

The dried calluses (1 g) was taken in 10 ml of petroleum ether and kept on shaking water bath at 40°C for 12 h. Extraction was repeated thrice and solvent from each extraction was pooled. Eventually, petroleum ether fractions of each mutant callus were concentrated under reduced pressure and residues defatted with CH_3CN (10 ml × 3). The precipitated fat was filtered out and filtrate was concentrated under reduced pressure. Residues were dissolved in 1 ml of methanol and 100 µl of aliquot of each sample was taken and 4 ml of 0.3% NaOH was added to this extract. The samples were incubated in a water bath at 50°C for 30 min, and thereafter cooled and neutralized with glacial acetic acid (0.1 M in 20% methanol) and pH of the solution was maintained 6.8. The derivatized artemisinin was analyzed and quantified through reverse phase column (C18, 5 µm, 4.6*250 mm) (Waters, USA) using premix methanol: 100 mM K-phosphate buffer (pH, 6.5) in the ratio of 60: 40 as mobile phase at constant flow rate of 1 ml/min, with the detector set at 260 nm. Standard curve was prepared using reference compound of artemisinin (1 mg/ml) and quantified its content in all mutant calluses with HPLC system (Waters, USA).

RESULTS AND DISCUSSION

The content of artimisinin in *A. annua* has been increased with application of traditional and biotechnological tools (Chang et al., 2000; Covello et al., 2007; Wang and

Table 1. Artimisinin content in calluses of *A. annua* produced from the treatment of sodium azide (NaN_3). The mutant calluses T1, T2, T3, T4 and T5 were produced from normal callus with the NaN_3 treatment and T6, T7, T8, T9 and T10 were produced from foliage leaves of germinated treated seeds. Tc is the normal callus and produced from foliage leaves of untreated seeds.

Callus	Artemisinin content (µg/g dw)	Callus	Artemisinin content (µg/g dw)
Tc	520	Tc	520
T1	593	T6	545
T2	619	T7	570
T3	681	T8	630
T4	645	T9	595
T5	614	T10	580

Weathers, 2007; Baldi and Dixit, 2008; Lualon et al., 2008; Zhang et al., 2009). Mutational breeding has important role for improvement of yield and quality traits of the crops including medicinal plants (Khan et al., 2010), for instances gamma irradiation has been used for the enhancement of artimisinin in *A. annua* (Koobkokkruad et al., 2008). Our study showed that percent seed germination of *A. annua* was decreased as the concentration of NaN_3 increased (data not shown). Similar, decrease in seed germination, radicle and coleoptile lengths of *Eruca sativa* was reported as the concentration of this compound increased (Khan and Al-Qurainy, 2009). One hour incubation period was sufficient for better mutant production from callus cells whereas it was insufficient for seed treatment. Therefore, seed treatment needed more incubation period as compared to the callus cells. The growth and biomass of mutants produced from both approaches had wide variations and it may be due to the cell's physiological activities. The fresh and dry weight of mutant calluses were better in T1, T2, T3, T4 and T5 as compared to T6, T7, T8, T9 and T10 (Figures 1 and 2). However, the callus survival (T1-T5) was decreased initially as the concentration of NaN_3 increased but four weeks after treatment, the survived calluses grown better. The fresh and dry weight of mutant calluses (T6-T10) showed different mutagenic effects as compared to those mutant ones which obtained from normal callus treatment (Figures 3 and 4). Thus, it is clear that the mutagenic effect in both approaches depends on the state of the cells, NaN_3 concentration, dilution solution, incubation period and pH of the phosphate buffer solution.

The mutant calluses developed from both approaches at various treatments of NaN_3 on MS medium (Murashige and Skoog, 1962) containing NAA and kinetin hormones (0.5 mg/L each) showed enhanced biosynthesis of artimisinin as compared to normal callus. The mutant calluses (T1, T2, T3, T4 and T5) obtained on treatment with NaN_3 at 1, 2, 3, 4 and 5 mM from normal calluses, had higher artimisinin content than the Tc and it was estimated with standard curve of this compound with HPLC system. The peak of reference compound 'artimisinin' compound is shown in Figure 5. The content of this compound was also increased in T6, T7, T8, T9 and T10 but had lower as compared to those mutant calluses which obtained from normal ones with NaN_3 treatment. The biosynthesis of artimisinin was dose dependent up to 3 mM of NaN_3 and at higher mutagenic concentrations, the content of this compound was found non-significant. Such unpredictable results may be due to random mutations at various loci in the genome. Artimisinin content in mutant calluses T1, T2, T3, T4 and T5 (Figures 7, 8, 9, 10 and 11) obtained with NaN_3 at 1, 2, 3, 4 and 5 mM was 593, 619, 681, 645 and 614 µg/g DW as compared to normal callus which had 520 µg/g DW (Figure 6 and Table 1). Like wisely, the biosynthesis of artimisinin was increased in those calluses which obtained from foliage leaves of germinated seedlings of treated seeds with NaN_3. The content of this compound in these calluses (T6, T7, T8, T9 and T10) was 545, 570, 630, 595 and 580 µg/g DW (Figures 12, 13, 14, 15 and 16) which obtained at 1 - 5 mM, NaN_3 as compared to normal ones which had 520 µg/g DW (Figure 6 and Table 1). In both approaches of the mutant production that is callus and seed treatment, the maximum artimisinin was found in mutant calluses (T3 and T8) which obtained with 3 mM, NaN_3. The physical mutagen gamma rays and its dose LD50 enhanced artimisinin content from 0.03 - 0.70% (w/w) of dry weight, comparing with only 0.18%, present in the original non-irradiated samples (Koobkokkruad et al., 2008). The content of artimisinin varies in various parts of Chinese varieties of *A. annua* and highest content has been reported in the leaves (0.01 - 0.5%) (Nair et al., 1986; Singh et al., 1988; Avery et al., 1992). The variation in the biosynthesis of this compound among various parts of the plant has been investigated at nutritional content level and potential anti-oxidant activity (Brisibe et al., 2009). NaN_3 has mutagenic effect on yield and quality traits of crops and its mutagenicity is still unknown in the literature (Khan et al., 2010) and it is assumed that the mutagenicity of this mutagen is mediated through the production of an organic metabolite of azide compound (Owais and Kleinhofs, 1988). It is known that NaN_3 is highly mutagenic in plants and animals (Grant and Salamone, 1994). However, the mutant callus production from direct treatment of callus

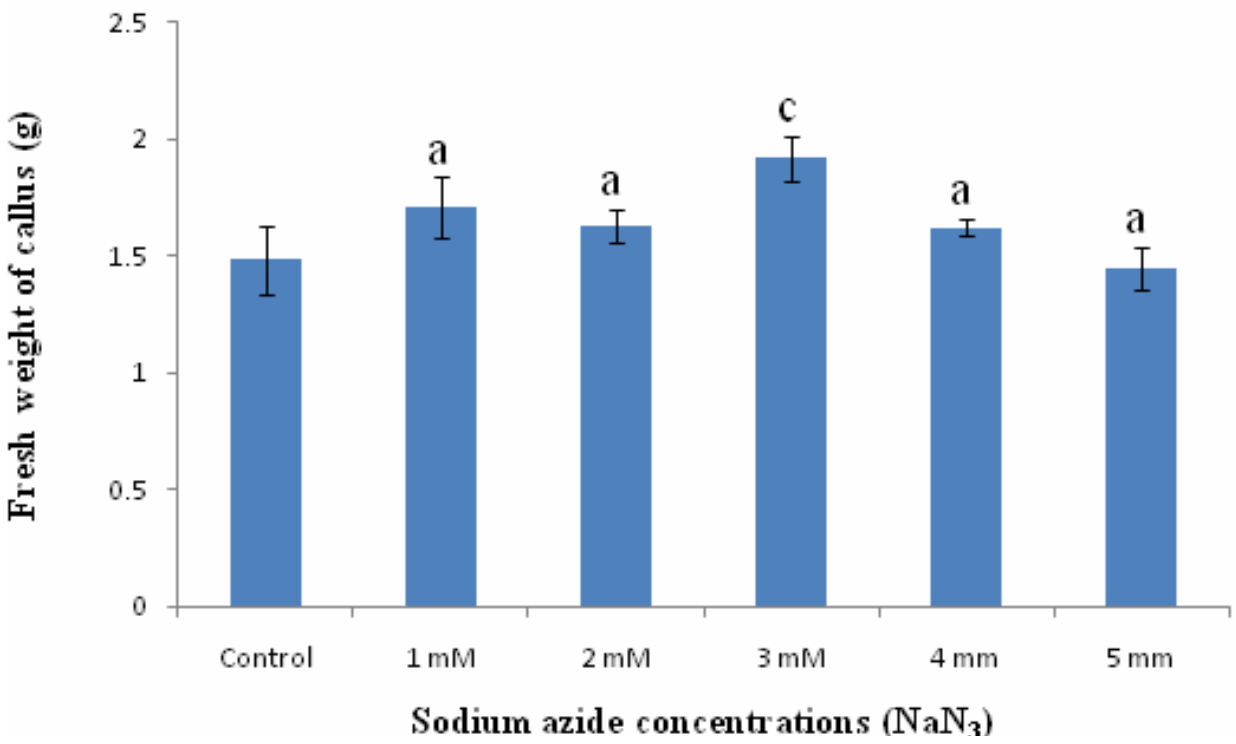

Figure 1. Fresh Data are mean ± SD for three replicates and statistical significance was determined by ANOVA (Dunnett's multiple comparison test). (a) $p > 0.05$ (Non-significant), (c) $p < 0.01$, when compared with control weight of callus after 7-weeks of culture which obtained from normal callus with NaN_3 treatment.

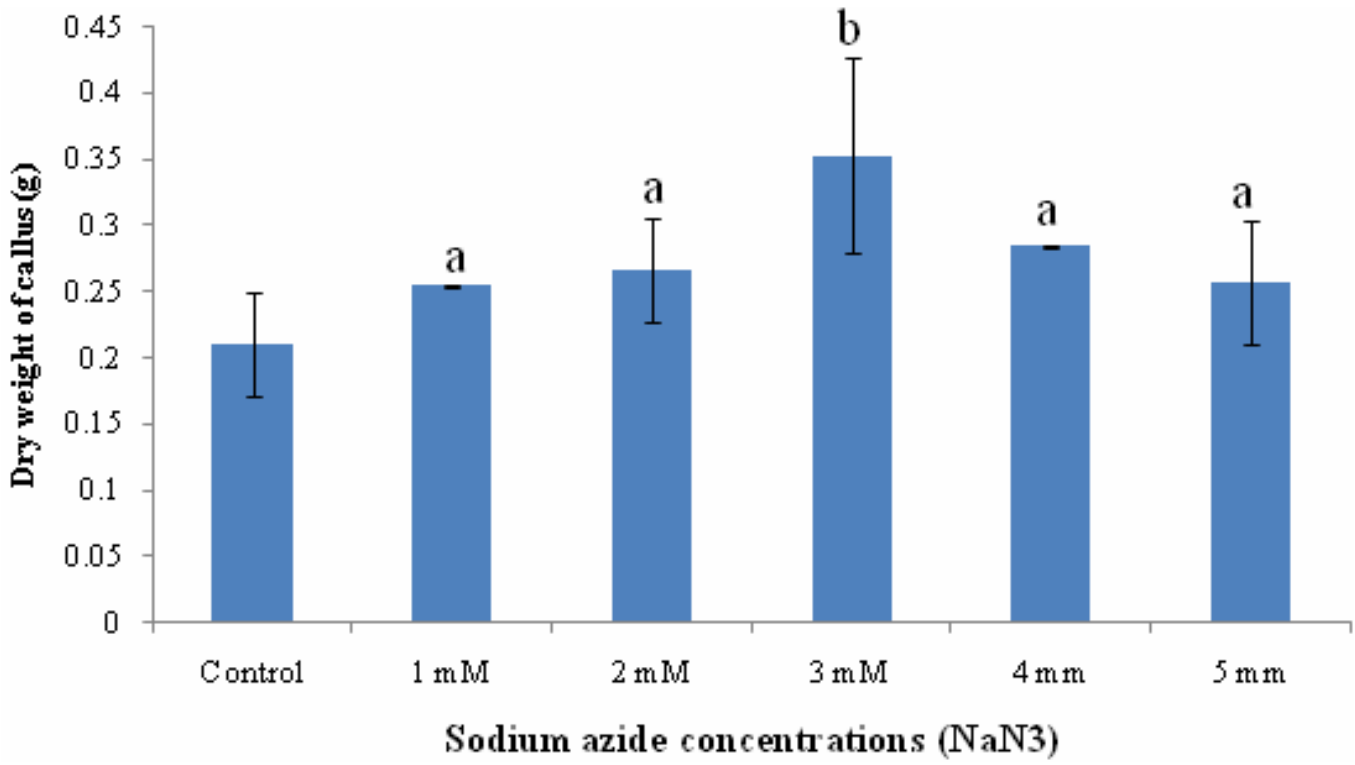

Figure 2. Data are mean ± SD for three replicates and statistical significance was determined by ANOVA (Dunnett's multiple comparison test). (a) $p > 0.05$ (Non-significant), (b) $p<0.05$, when compared with control dry weight of callus after 7-weeks of culture which obtained from normal callus with NaN_3 treatment.

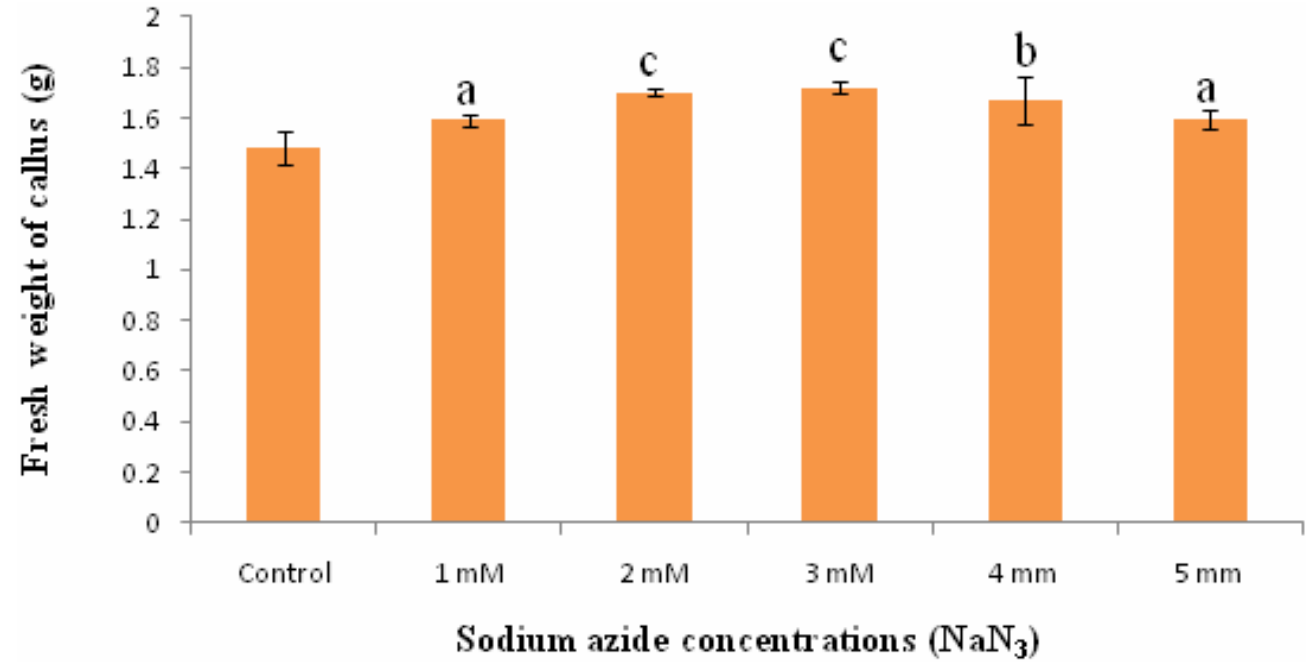

Figure 3. Fresh weight of callus obtained from foliage leaves of germinated treated seeds with NaN_3 after 7-weeks of culture. Note: Data are mean ± SD for three replicates and statistical significance was determined by ANOVA (Dunnett's multiple comparison test). (a) $p > 0.05$ (Non-significant), (b) $p<0.05$, when compared with control, (c) $p< 0.01$, when compared with control.

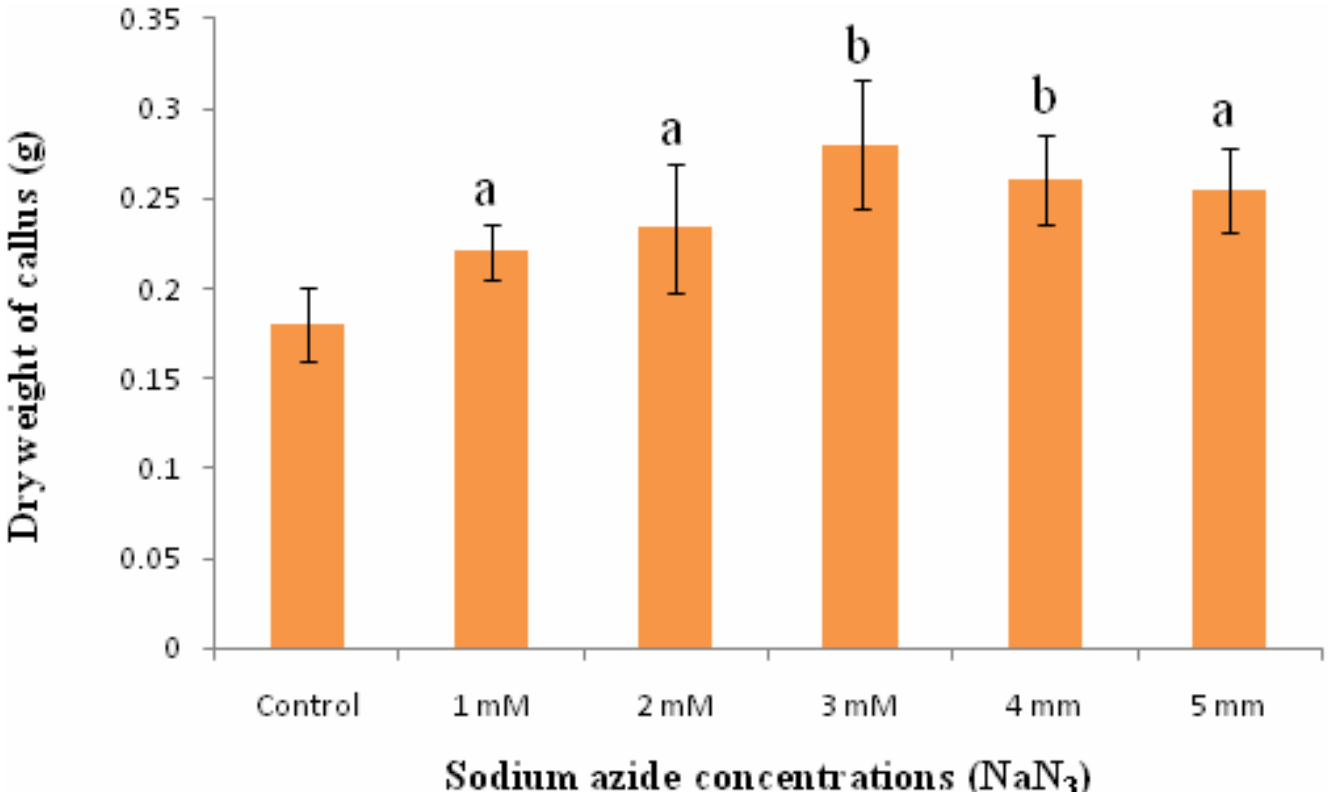

Figure 4. Dry weight of callus obtained from foliage leaves of germinated treated seeds with NaN_3 after 7-weeks of culture. Note: Data are mean ± SD for three replicates and statistical significance was determined by ANOVA (Dunnett's multiple comparison test). (a) $p > 0.05$ (Non-significant), (b) $p<0.05$, when compared with control, (c) $p< 0.01$, when compared with control.

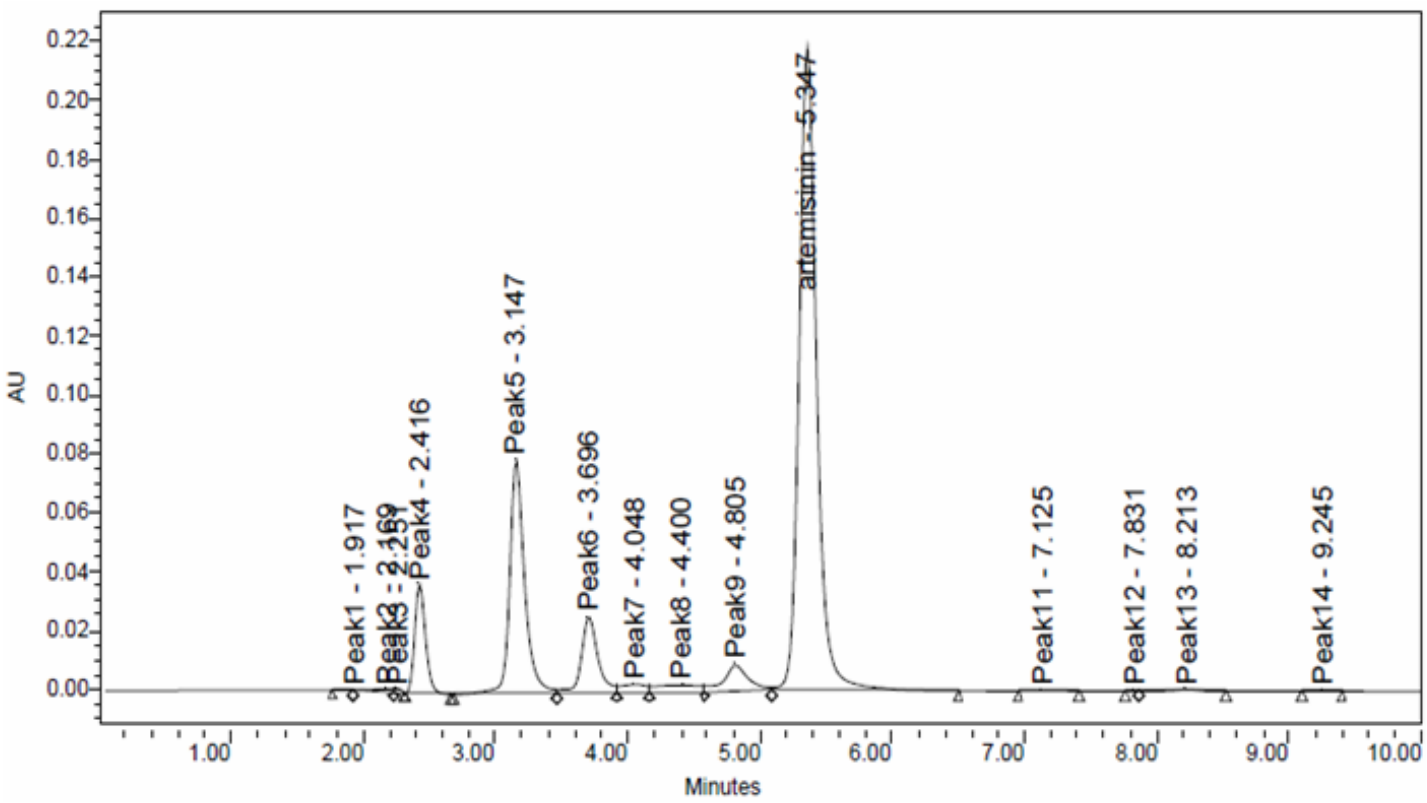

Figure 5. Peak of reference compound (artimisinin).

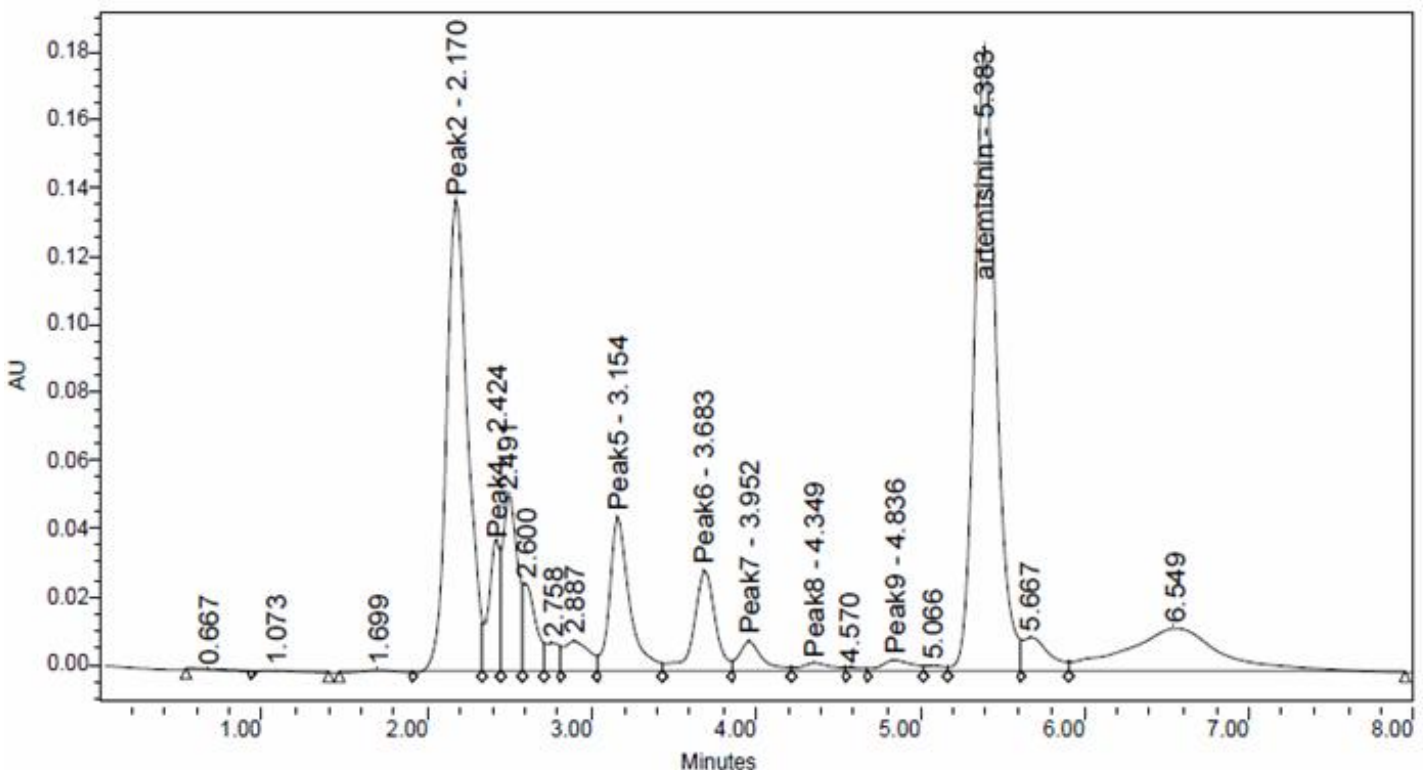

Figure 6. Artimisinin content in normal callus (Tc) estimated with HPLC system.

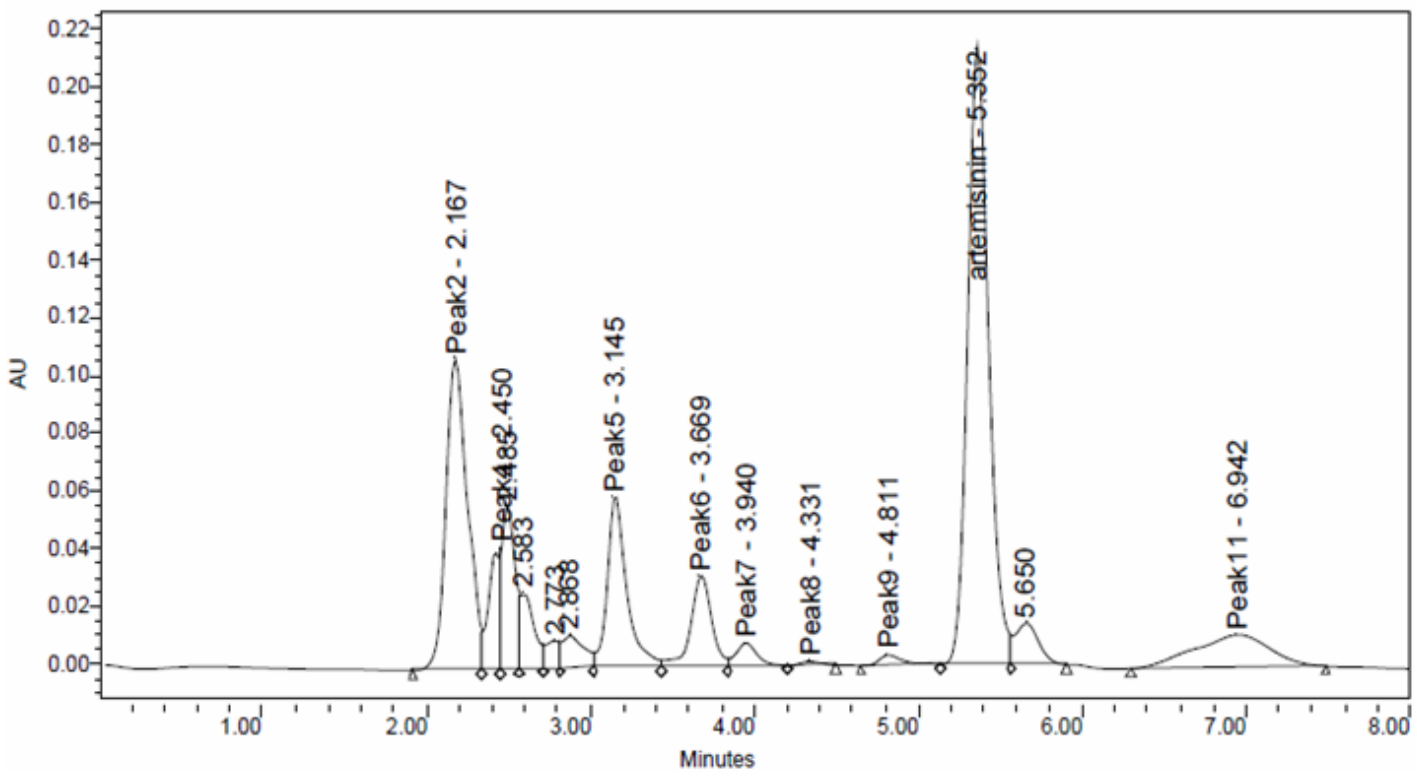

Figure 7. Artimisinin content in mutant Callus (T1) after 7-weeks of culture which obtained from normal callus treatment with NaN_3 at 1 mM concentration.

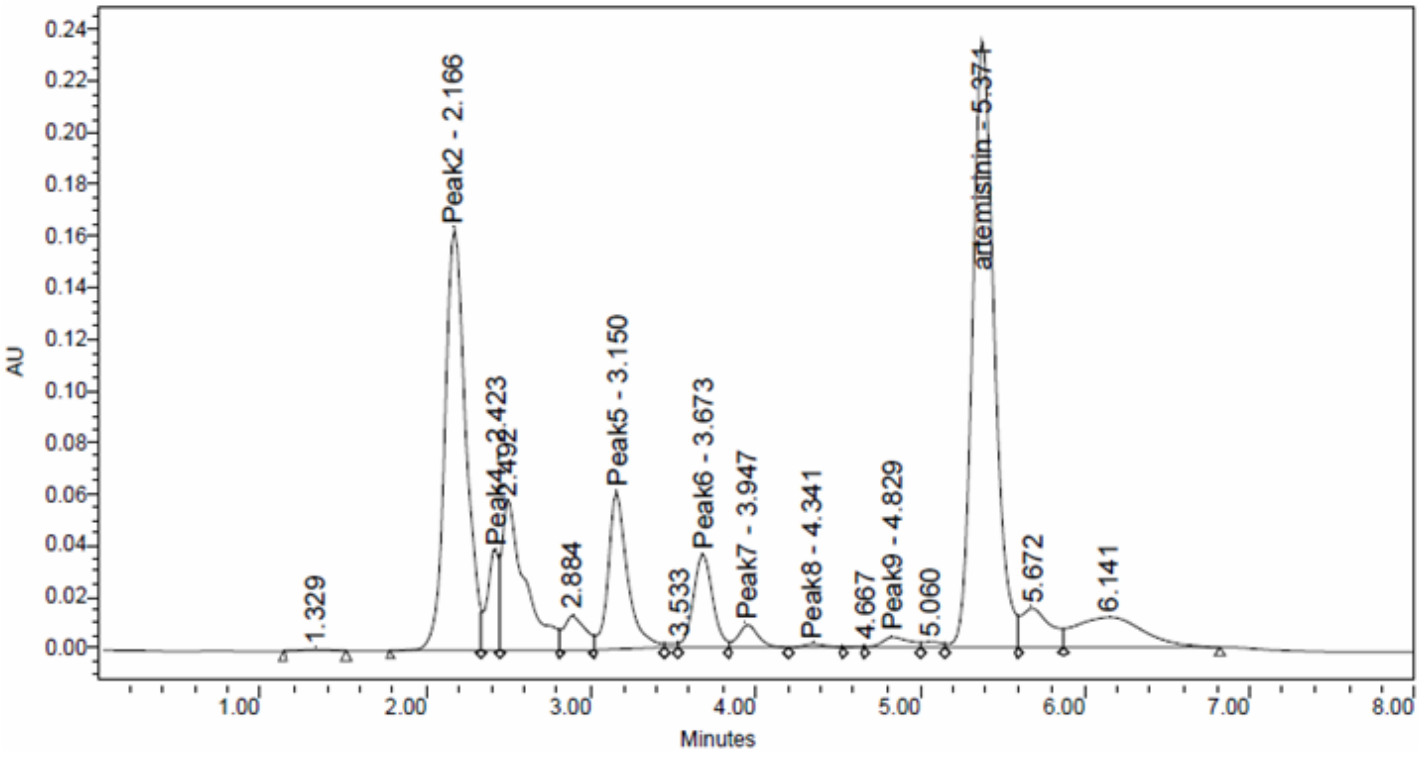

Figure 8. Artimisinin content in mutant Callus (T2) after 7-weeks of culture which obtained from normal callus treatment with NaN_3 at 2 mM concentration.

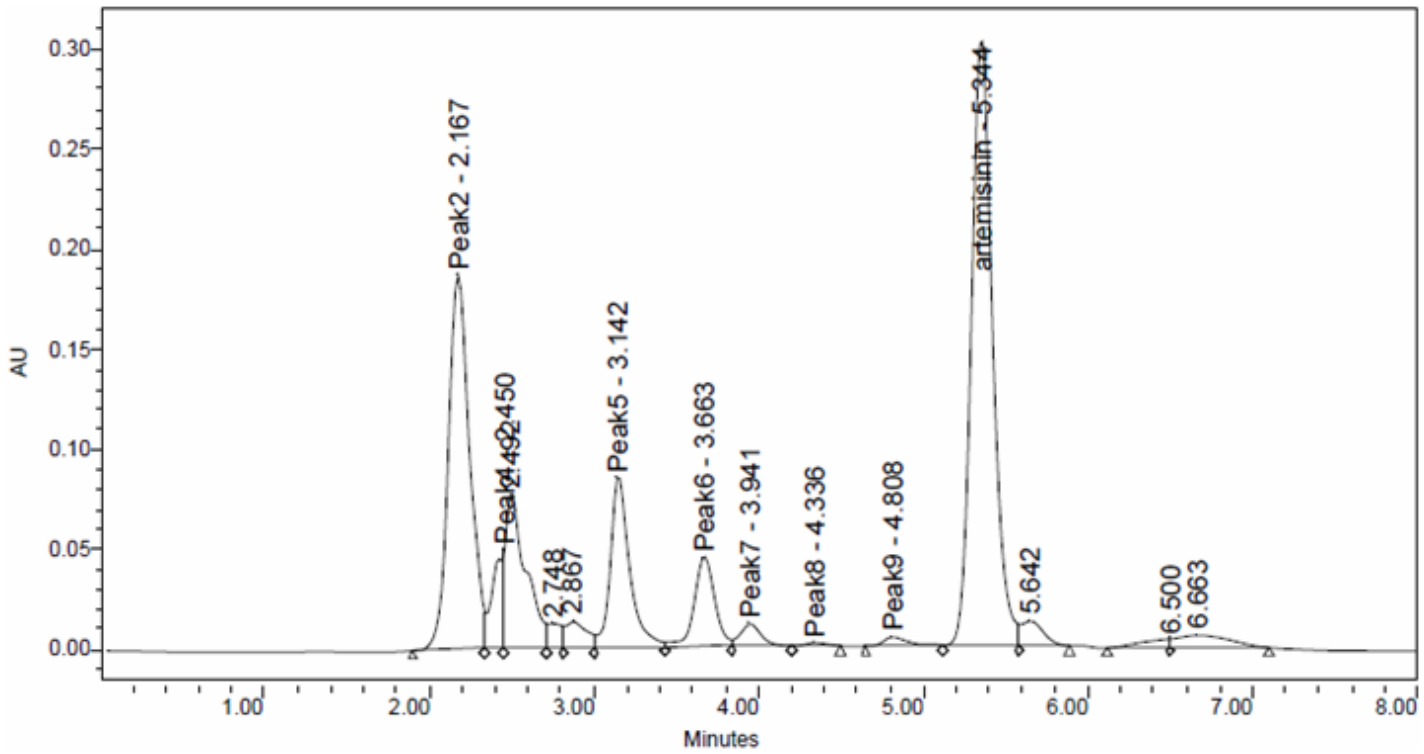

Figure 9. Artimisinin content in mutant Callus (T3) after 7-weeks of culture which obtained from normal callus treatment with NaN_3 at 3 mM concentration.

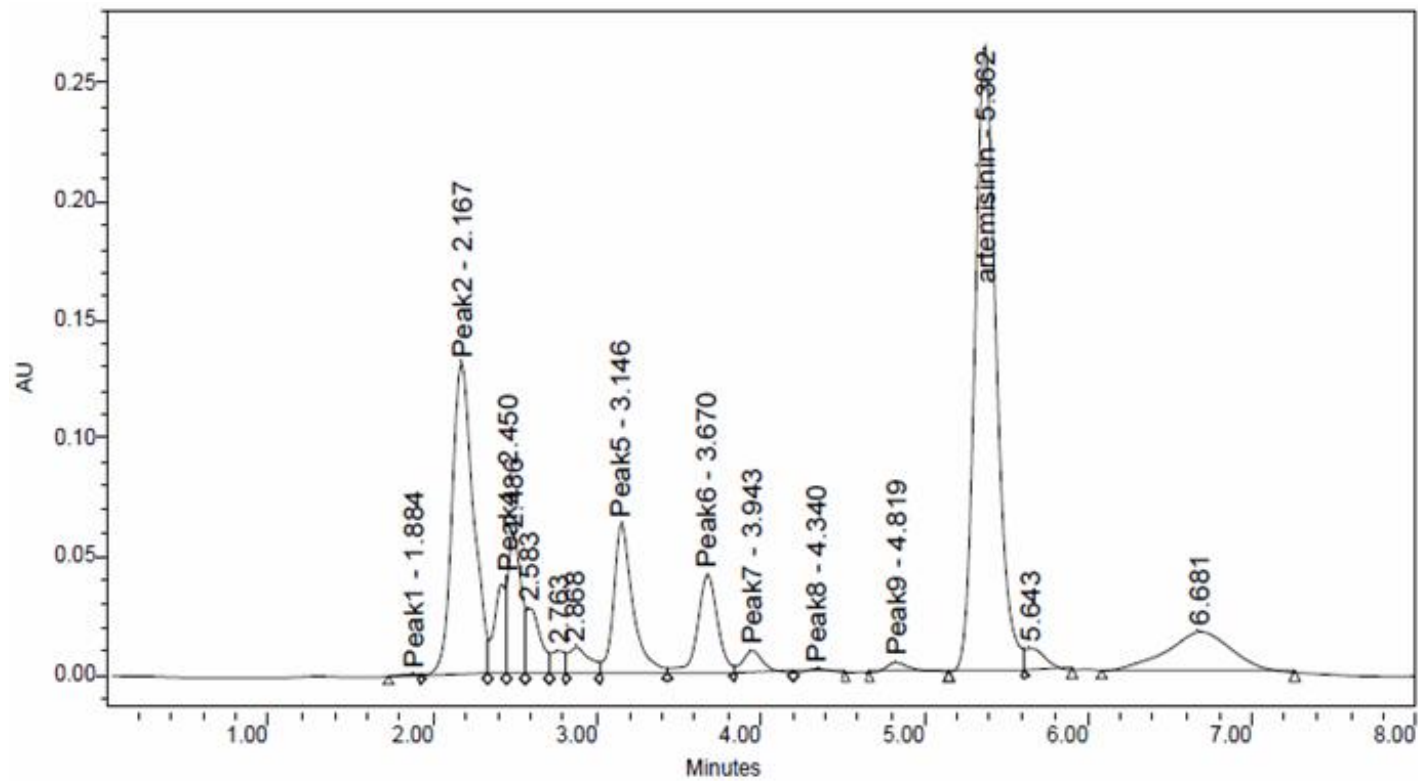

Figure 10. Artimisinin content in mutant Callus (T4) after 7-weeks of culture which obtained from normal callus treatment with NaN_3 at 4 mM concentration.

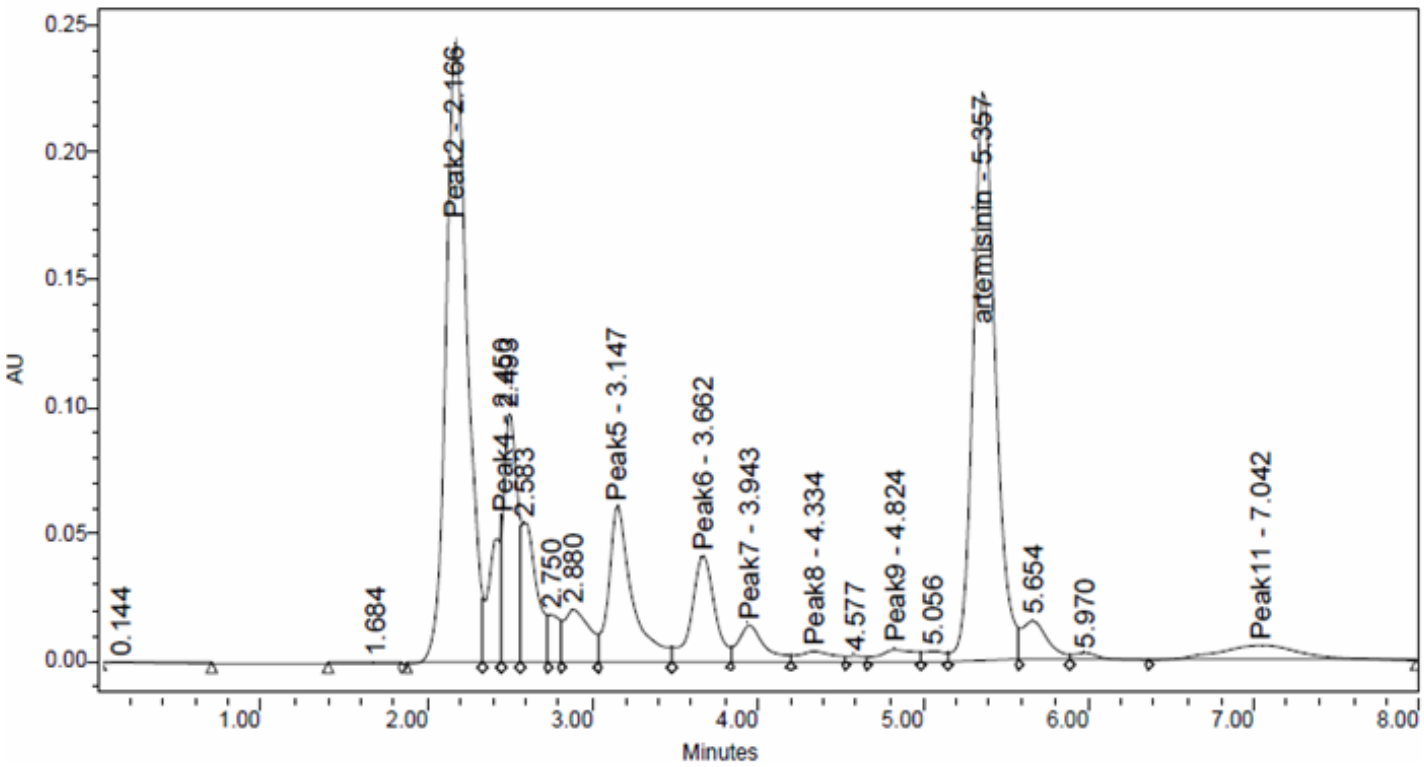

Figure 11. Artimisinin content in mutant Callus (T5) after 7-weeks of culture which obtained from normal callus treatment with NaN_3 at 5 mM concentration.

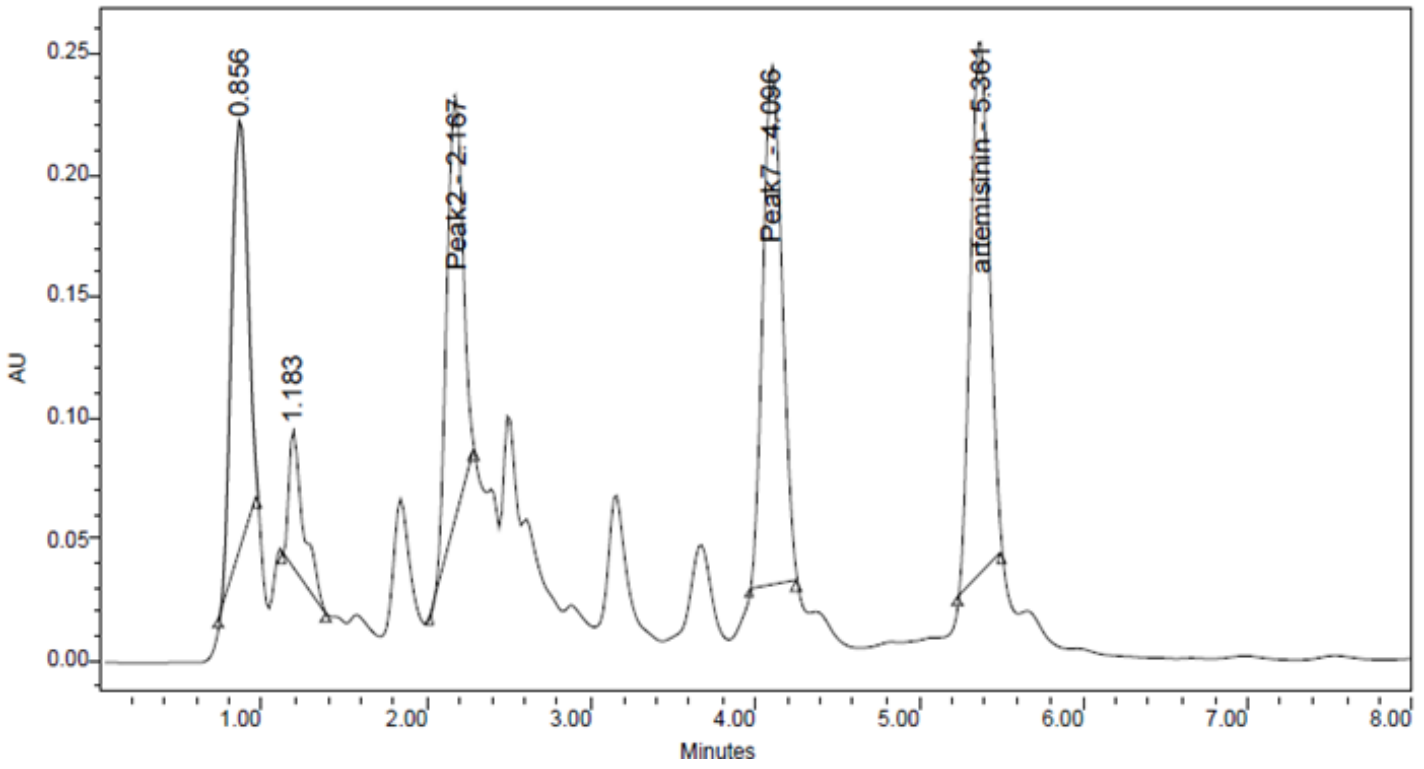

Figure 12. Artimisinin content in mutant Callus (T6) after 7-weeks of culture which obtained from foliage leaves of germinated treated seeds with NaN_3 at 1 mM concentration.

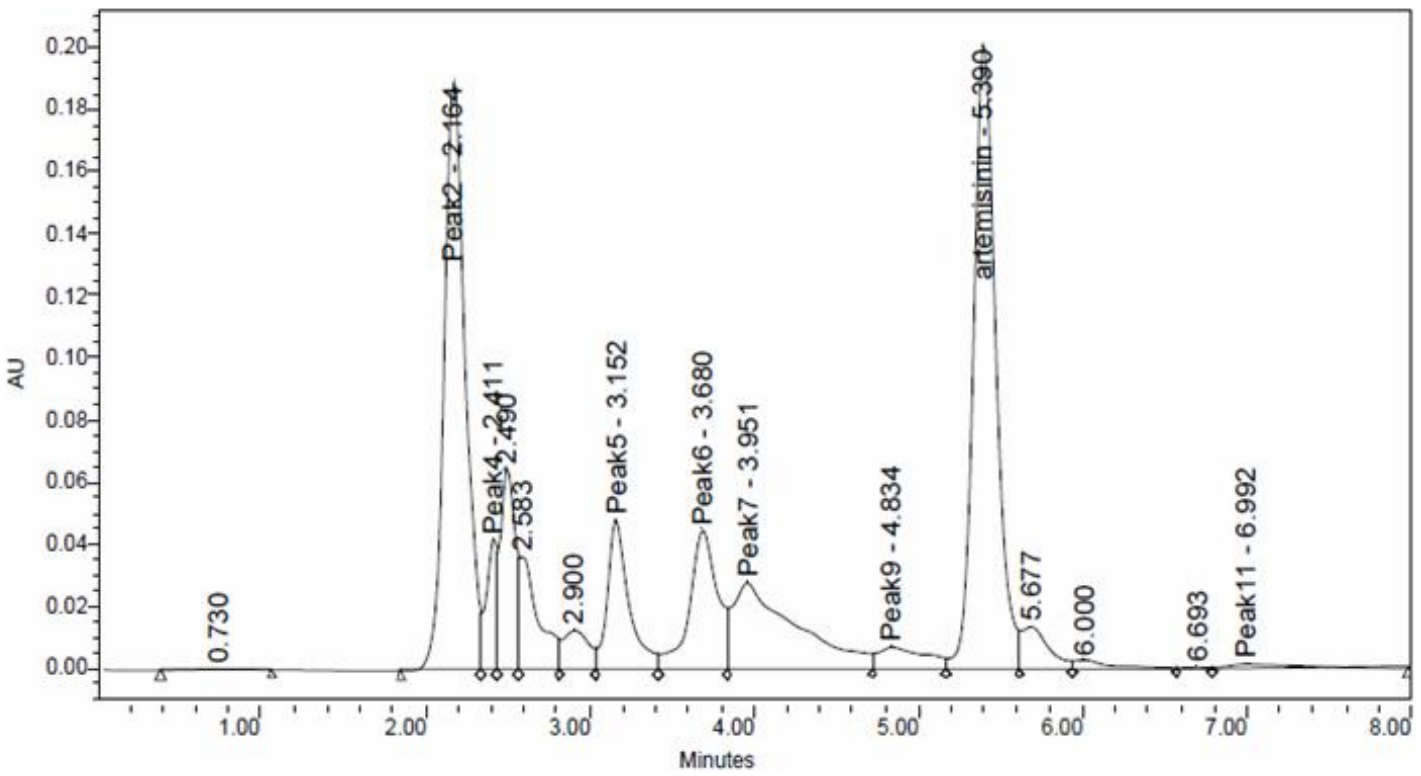

Figure 13. Artimisinin content in mutant Callus (T7) after 7-weeks of culture which obtained from foliage leaves of germinated treated seeds with NaN_3 at 2 mM concentrations.

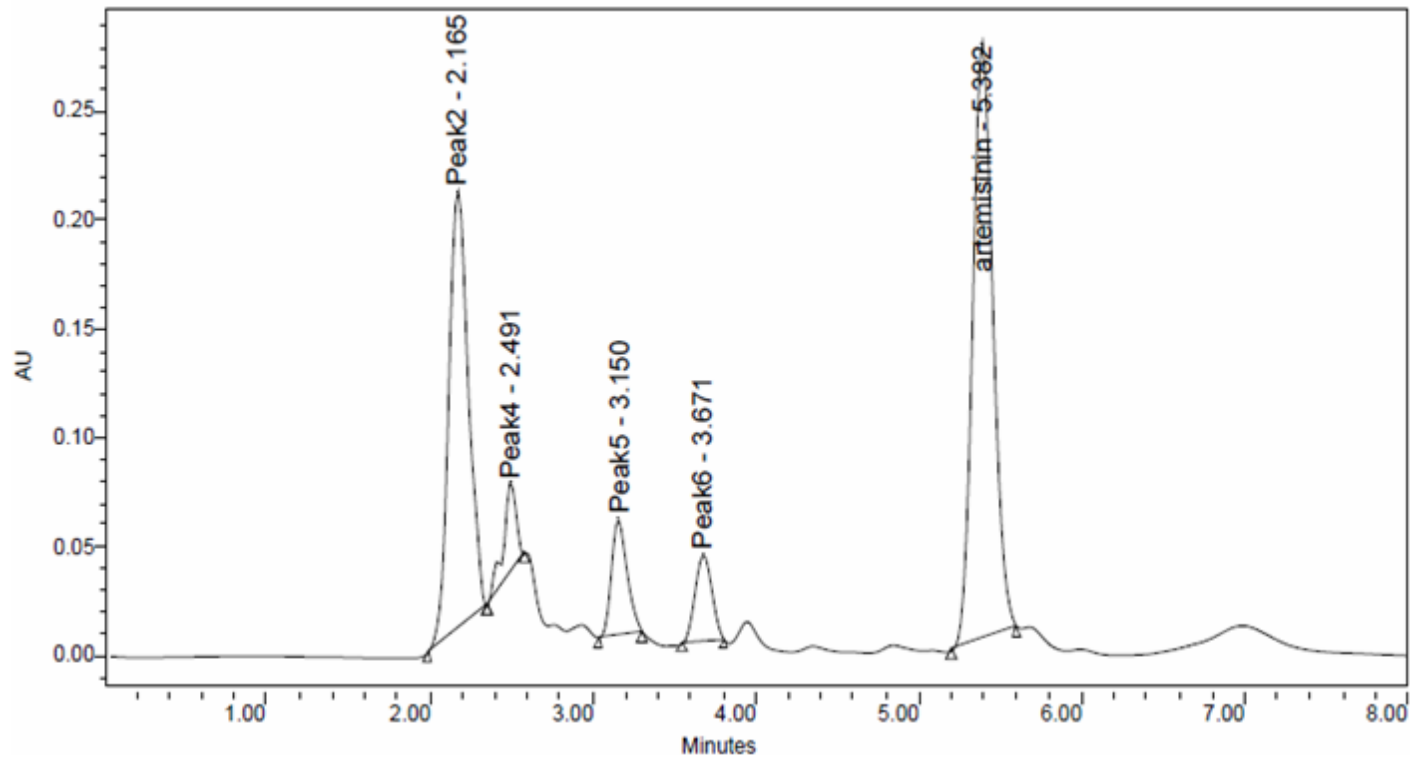

Figure 14. Artimisinin content in mutant Callus (T8) after 7-weeks of culture which obtained from foliage leaves of germinated treated seeds with NaN_3 at 3 mM concentrations.

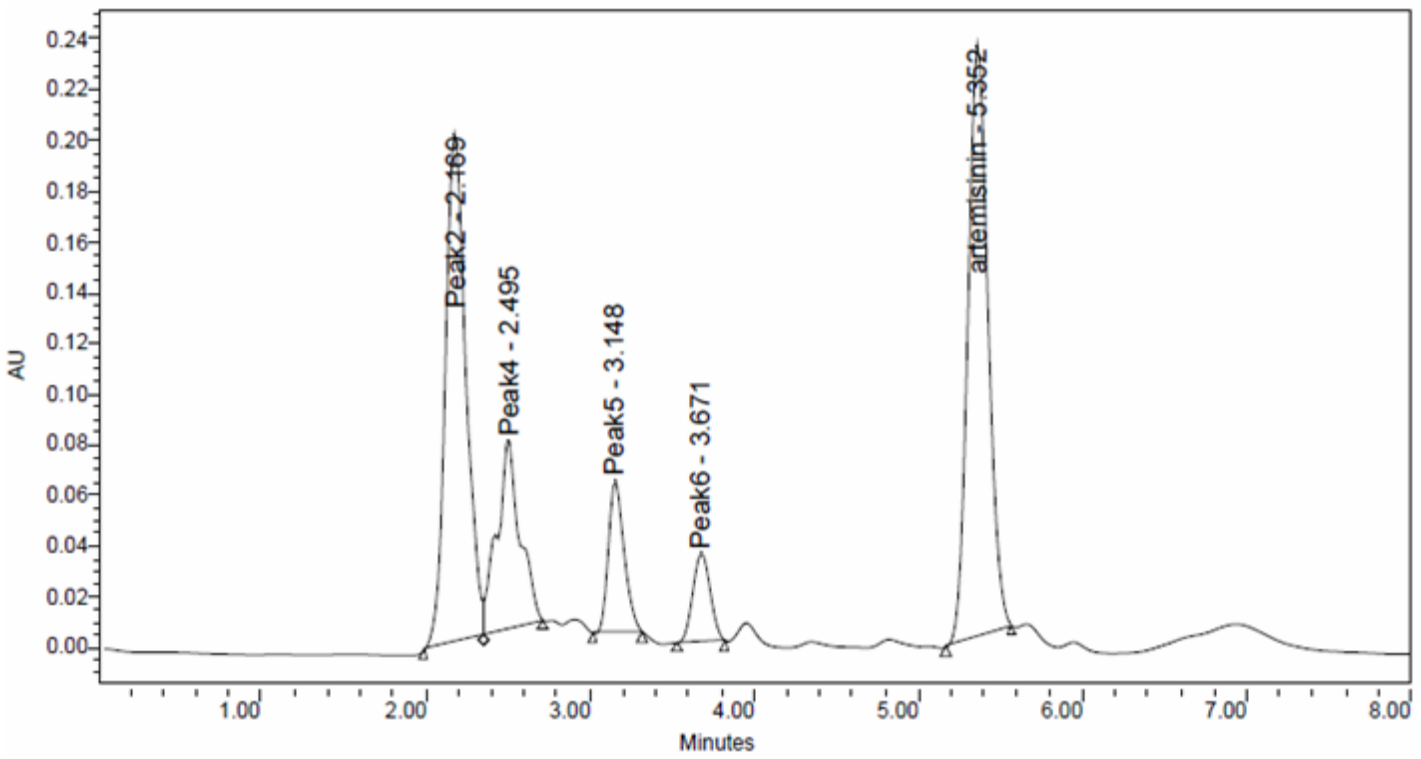

Figure 15. Artimisinin content in mutant Callus (T9) after 7-weeks of culture which obtained from foliage leaves of germinated treated seeds with NaN_3 at 4 mM concentrations.

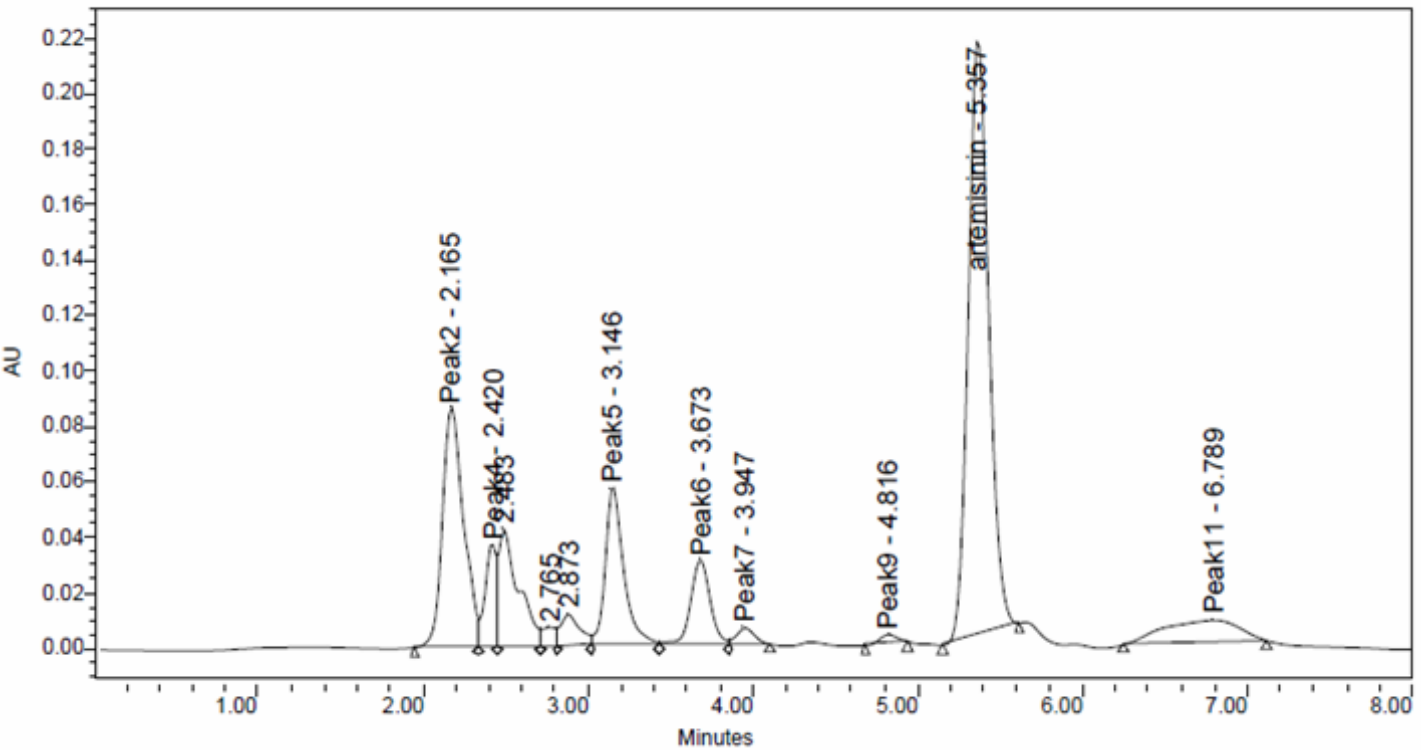

Figure 16. Artimisinin content in mutant Callus (T10) after 7-weeks of culture which obtained from foliage leaves of germinated treated seeds with NaN_3 at 5 mM concentrations.

with NaN_3 is the better approach as compared to seed treatment. The cells in callus stage are extremely sensitive and acquire mutagenic agent as azidoalanine in the cell where it creates mutations. This chemical mutagen is metabolized by plant cells to a extremely mutagenic agent presumably azidoalanine and it is chemically identified as an amino acid analogue, L-azidoalanine (N_3 -CH_2 - $CH(NH)_2$ - COOH) and its production was found to be dependent on the enzyme *O*-acetlserine sulfhydrylase (La Velli and Mangold, 1987). Thus, it is concluded that, the mutagenicity of NaN_3 depends on many physical and physiological parameters. Since, callus and dried seed varies to each other in these parameters; therefore, mutagenicity produced by this compound would be varying.

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