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Full Length Research Paper

Detection of bioactive compounds from *Spilanthes acmella* (L.) plants and its various *in vitro* culture products

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In this study, we analyzed the active chemical compounds of *in vitro* plantlets of *Spilanthes acmella.* With 19 samples, GC-MS (Gas Chromatography-Mass Spectrometry) analysis revealed that the naturally occurring insecticide, N-isobutyl-2E, 6Z, 8E-decatrienamide (spilanthol) was present in mother plant, flower heads and *in vitro* plantlets of *S. acmella* with similar retention time (43.18 to 43.21 min). N-isobutyl-2E, 4Z, 8Z, 10E- dodecatetraenamide (an isomer of N-isobutyl-2E, 4E, 8E, 10E-dodecatetraenamide, a potent mosquito larvicide) was only detected in the *in vitro* plantlets of *S. acmella.* Antioxidant, butylated hydroxytoluene (BHT) and fatty acids (n-Hexadecanoic acid and tetradecanoic acid) could be obtained from all the sample extracts of mother plant, flower heads, *in vitro* plantlets, callus, air-dried cells, freeze-dried cells and fresh cells.

Key words: Chemical analysis, spilanthol, micropropagation, *Spilanthes acmella*.

INTRODUCTION

There is growing interest worldwide in the utilisation of plant materials and phytochemicals to support sustainable and environmentally friendly lifestyle. The chemicals extracted from plants are expected to have low toxicity and high degree of biodegradation (Pitasawat et al., 1998; Choochote et al., 1999). Therefore, plant extracted phytochmicals have been used in nutraceuticals, pharmaceuticals, herbal medicines, spices, insect repellents, cosmetics, perfumes and many other beneficial secondary metabolites (Ramlan and Mohamad, 2000).

Spilanthes acmella L. (subang nenek) belongs to the genus *Spilanthes*, family Asteraceae (Compositae), tribe Helianthae and the subtribe Ecliptinae (Nakatani and Nagashima, 1992). It is an herb found all around the world and widely distributed throughout the tropics and subtropics (Ramsewak et al., 1999). *S. acmella* was found to have many medicinal uses. The whole plants can be used in the treatment of dysentery and rheumatism (Baruah and Leclercq, 1993; Nakatani and Nagashima, 1992). The flower heads of *S. acmella* can be chewed to relieve toothache and also as a haemostatic and analgesic (Oliver-Bever, 1986). A decoction of the plant can be taken internally as a diuretic and able to resolve stones in the bladder, while a decoction of the roots can be used as a purgative. It is also used as a preventive medicine for scurvy and stimulates digestion (Burkill, 1966). Besides these medicinal uses, the flower heads have been used as a spice for appetizers by the Japanese and its extract was used as a flavouring material for dentifrices and gum (Lion, 1985).

Phytochemicals analysis is essential to make good use of any medicinal plant. To date, many studies have been done on the chemical analysis and structure determination of pungent alkamides from *S. acmella*. The major pungent compound reported in *S. acmella* is spilanthol, which is an isobutylamide and is well known for its insecticidal properties (Jondiko, 1986; Kadir et al., 1989). However, none of the published paper proposed an *in vitro* mass propagation of this plant for the

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production of active compounds. Currently, the interest in utilisation and conservation of medicinal plants is rapidly increasing, and mass micropropagation of plants via *in vitro* culture techniques has become an alternative approach to produce or extract valuable chemical products rather than from plants grown and harvested in the field (George and Sherrington, 1984). We have established the optimum conditions for micropropagation of this plant (Chan et al., 2005). Hence, the objectives of this research is to detect the presence of spilanthol and other useful compounds from the mother plant, *in vitro* plantlets, callus cultures and cell suspension cultures of *S. acmella* by using GC-MS.

MATERIALS AND METHODS

Preparation of plant materials and extraction

Nineteen different plant materials of *S. acmella* (Table 1) were selected for chemical analysis. The plant materials from the field were washed and air-dried in an air-conditioned room at 25 ± 2°C, while the *in vitro* plant materials were removed from their respective culture medium and washed to remove any traces of culture medium before they were air dried at 25 ± 2°C or freeze dried at -40°C until constant weight was obtained. They were then macerated to powder form with a mortar and pestle. The determination of active compound was carried out according to the method described by Yasuda et al. (1980) with slight modification. One gram (1.0 g) of the macerated plant materials were then soaked with methanol (MeOH) (20 ml) and allowing the homogenate to stand for 3 days. This extraction procedure was repeated three times. The combined supernatant was concentrated to 10 ml below 40°C by using a rotary evaporator (Eyela Rotary Vacuum Evaporator N-N Series and Eyela Digital Waterbath SB-651, Tokyo Rikikai Co. Ltd.) coupled with a water pump (Eyela Aspirator A-3S, Tokyo Rikikai Co. Ltd.) before dissolved in 10 ml of distilled water. The solution was transferred into separatory funnel and extracted three times with 20 ml of n-hexane. After the bottom layer of hexane extract was collected from the funnel, they were concentrated to dryness below 40°C. Just before injection of samples into GC-MS column, the dried extracts were eluted with 1 µL of n-hexane: diethyl ether at a ratio of 1:1. Since, external standard was not employed here, all the chromatograms and mass spectra of the samples were then matched with Mass Spectrometer NIST (National Institute of Standards and Technology) library for authenticity.

Gas chromatography-mass spectrometry (GC-MS) analysis

The active component was identified by Thermo Finnigan Gas Chromatography-Mass Spectrometry (GC-MS) machine. The column used was an Ultra 1 (Crosslinked Methyl Silicone Gum) column (25 m × 0.2 mmØ) coated with 100% dimethyl polysiloxane (0.33 µm phase thickness). The oven was programmed from 50°C (5 minutes hold) at 3.5°C min^{-1} to 250°C. The samples were injected using splitless technique: 2 µL of the sample in n-hexane: diethyl ether (1:1). The column was directly coupled to a Trace MS (Mass Spectrometry). The source temperature and GC interface temperature were 200 and 285°C, respectively. The electron beam energy was 70 eV. Mass spectra and reconstructed chromatograms were obtained by automatic scanning in the mass range m/z 35-250 at 4.4 scan s^{-1}. Chromatographic peaks were checked for homogeneity with the aid of the mass chromatograms for the characteristic fragment ions. NIST (National Institute of Standards and Technology) library was used for automatic identification of GC peaks.

RESULTS

The chemical contents detected from the different plant parts and *in vitro* cultures of *S. acmella* and the percentage of probability or percentage matching of the compound from all detected samples to the NIST database library of the mass spectrometer was shown in Table 1. Two representative total chromatograms of the extract shown in the Figures 1 and 2 were from *in vitro* plantlets of *S. acmella* and leaf callus induced on solid MS medium supplemented with 0.5 mg/L 2,4-D, respectively.

Chemical analysis of mother plant

The active compound N-isobutyl-2E, 6Z, 8E-decatrienamide (spilanthol) in total ion chromatogram (TIC) scan of *S. acmella* mother plant without flower heads was detected at t_R (retention time) 43.30 min. The ion fragments obtained from the mother plant extract were *m/z* (rel. int.): 221$[M]^+$ (5), 206(1), 192(6), 141(71), 126(35), 98(19), 81(100), 79(35), 41(24). These ion fragments of spilanthol were consistent to the ion fragments detected in the NIST (National Institute of Standards and Technology) database library of the mass spectrometer with *m/z* (rel. int.): 221$[M]^+$ $C_{14}H_{23}NO$ (15), 206(2), 192(1), 141(86), 126(24), 98(23), 81(100), 79(24), 41(35) (Figure 3). The comparison of the mass spectrum of spilanthol from the mother plant extract with the NIST database library gave a 92.30% match as well as a confirmatory compound structure match. Since external standard was not employed here, therefore this was then used as the reference spectrum for spilanthol in other sample extracts of plant materials.

Chemical analysis of flower heads

The TIC of the flower head extract of *S. acmella* also showed the presence of spilanthol where the chromatographic peak was produced at t_R 43.18 min. The ion fragments obtained from the extract were *m/z* (rel. int.): 221$[M]^+$ (5), 206(1), 192(3), 141(76), 126(41), 98(28), 81(100), 79(38), 41(33). When compared with the NIST database library, the mass spectrum of spilanthol from the flower head gave 93.19% match as well as a confirmatory compound structure match. Butylated hydroxytoluene was also detected at t_R 31.41 min with 74.52% match as well as a confirmatory compound structure match.

Table 1. List of active compounds present in nineteen different types of *S. acmella* L. plant materials and cultures.

Source	N-isobutyl- 2E, 6Z, 8E-decatrienamide (spilanthol) (%)	N-isobutyl-2E, 4Z, 8Z, 10E-dodecatetraenamide	Butylated Hydroxytoluene (%)	n-Hexadecanoic acid (Palmitic acid) (%)	Tetradecanoic acid (Myristic acid) (%)
Flower	√ (93.19)	–	√ (74.52)	√ (41.55)	√ (54.72)
Mother plant	√ (92.30)	–	√ (56.04)	√ (39.42)	√ (52.75)
In vitro plant	√ (92.37)	√ (96.24 %)	√ (69.07)	√ (23.68)	√ (61.89)
Callus					
Picloram (Petiole)	–	–	√ (78.55)	√ (44.34)	√ (50.23)
2,4-D (Petiole)	–	–	√ (78.45)	√ (33.86)	√ (63.10)
Picloram (Leaf)	–	–	√ (53.22)	√ (33.28)	√ (60.57)
2,4-D (Leaf)	–	–	√ (76.62)	√ (33.04)	√ (54.26)
Cell culture (Air dried)					
9 days	–	–	√ (79.32)	√ (47.06)	√ (30.69)
12 days	–	–	√ (77.85)	√ (47.56)	√ (31.63)
18 days	–	–	√ (78.35)	√ (37.30)	√ (38.12)
27 days	–	–	√ (79.06)	√ (35.02)	√ (34.15)
Cell culture (Freeze dried)					
9 days	–	–	√ (77.64)	√ (42.64)	√ (40.95)
12 days	–	–	√ (78.81)	√ (1.23)	√ (0.16)
18 days	–	–	√ (66.08)	√ (3.39)	√ (1.14)
27 days	–	–	√ (74.17)	√ (18.03)	√ (2.97)
Cell culture (Fresh)					
9 days	–	–	√ (79.65)	√ (39.87)	√ (54.22)
12 days	–	–	√ (80.36)	√ (33.23)	√ (47.33)
18 days	–	–	√ (80.85)	√ (26.55)	√ (45.32)
27 days	–	–	√ (80.59)	√ (42.53)	√ (24.23)

√ = the presence of the compound.
( ) = percentage of probability or percentage matching of the compound to the library database.

Chemical analysis of *in vitro* plantlets

For *in vitro* plantlets of *S. acmella*, the TIC obtained showed the presence of spilanthol labelled with (A) where the chromatographic peak was produced at t_R 43.21 min (Figure 1). The ion fragments from the spilanthol detected in *in vitro* plantlets were *m/z* (rel. int.): 221$[M]^+$ (5), 206(1), 192(3), 141(97), 126(57), 98(32), 81(100), 79(42), 41(56). The comparison of the mass spectrum of spilanthol from *in vitro* plantlets with the NIST database library gave a 92.37% match as well as a confirmatory compound structure match. Besides that, an isomer of mosquito larvicide (N-isobutyl-2E, 4Z, 8Z, 10E-dodecatetraenamide)

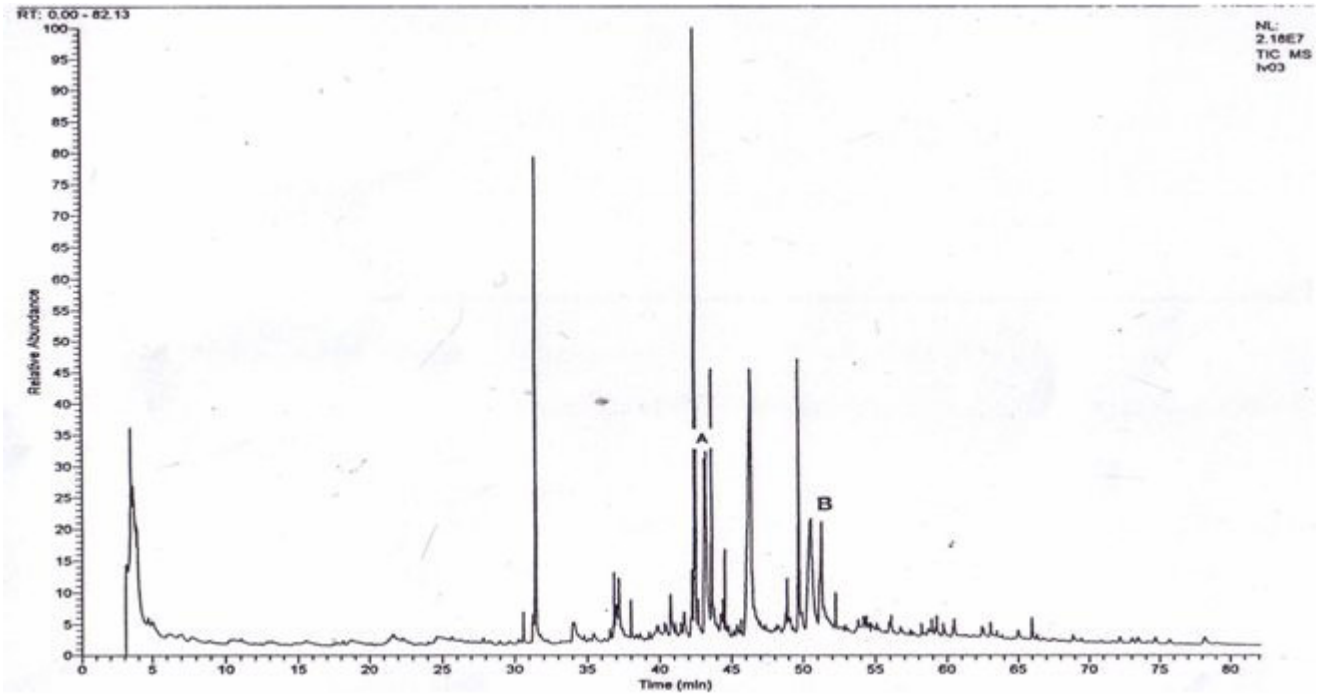

Figure 1. Total ion chromatogram of the extract from *in vitro* plantlets of *S. acmella* (A: the presence of spilanthol; B: the presence of N-isobuty1-2E, 4Z, 8Z, 10E-dodecatetraenamide).

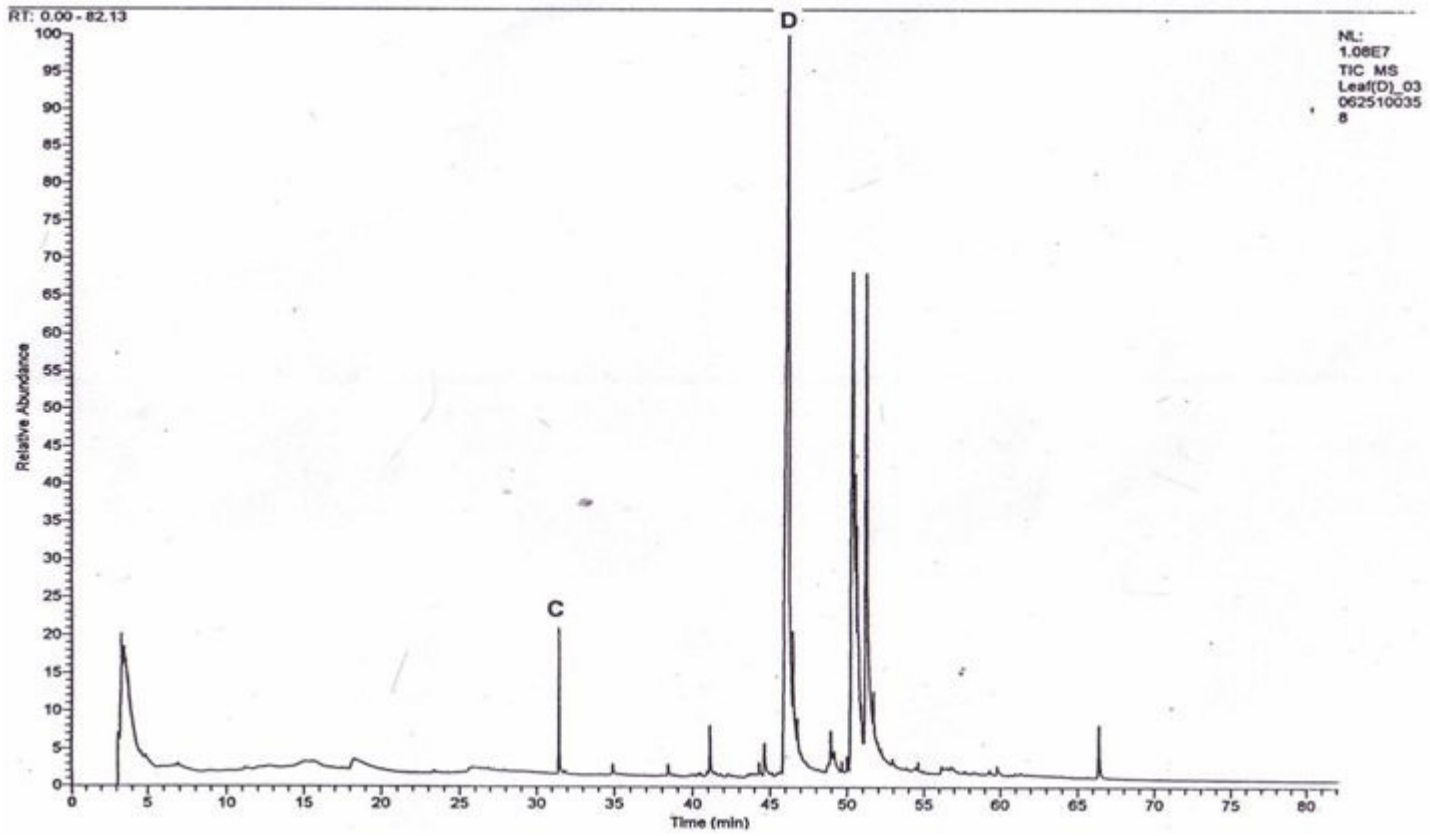

Figure 2. Total ion chromatogram of the extract from leaf callus induced on solid MS medium supplemented with 0.5 mg/L 2, 4-D (C: the presence of butylated hydroxytoluene; D: the presence of n-Hexadecanoic acid and tetradecanoic acid).

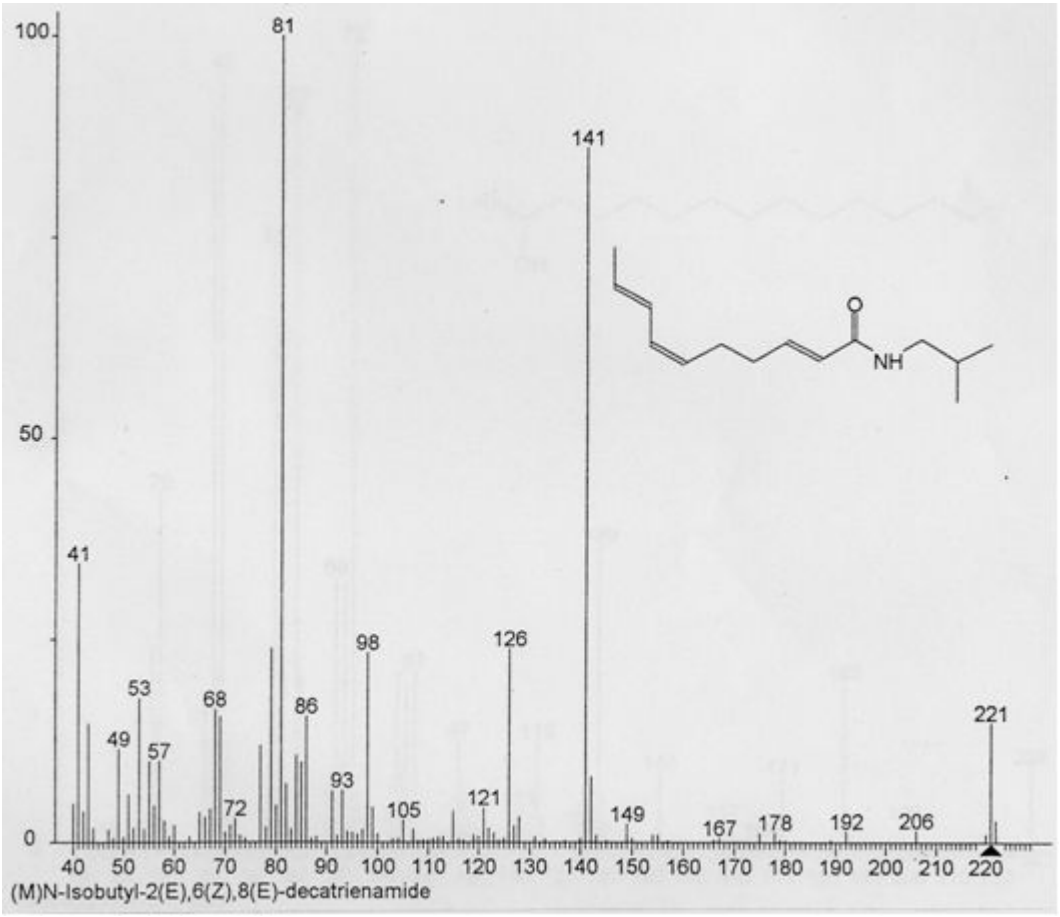

Figure 3. The mass spectrum of spilanthol from NIST database library.

labelled with (B) was detected at t_R 51.20 min in *in vitro* plantlets (Figure 1). The ion fragments detected were *m/z* (rel. int.): 247$[M]^+$ (1), 246(1), 205(0.2), 167(31), 100(10), 81(100), 67(14), 41(35). These ion fragments were consistent to the ion fragments detected in the NIST database library of the mass spectrometer with *m/z* (rel. int.): 247$[M]^+$ $C_{16}H_{25}NO$ (11), 246(3), 205(0.5), 167(96), 100(10), 81(100), 67(27), 41(66) (Figure 4). The mass spectrum of N-isobutyl-2E, 4Z, 8Z, 10E-dodecatetraenamide gave a 96.24% match as well as a confirmatory structure match with NIST database library.

Chemical analysis of callus culture

Spilanthol and N-isobutyl-2E, 4Z, 8Z, 10E-dodecatetraenamide were not detected in four weeks old leaf and petiole derived callus induced on MS medium + 0.5 mg/L 2,4-D and MS medium + 0.5 mg/L picloram. However, the antioxidant (butylated hydroxytoluene) labelled with (C) and fatty acid (n-Hexadecanoic acid and tetradecanoic acid) labelled with (D) were detected in these four different types of calluses (Figure 2). As shown in the TIC, a clear chromatographic peak of butylated hydroxytoluene could be obtained at t_R 31.44 to 31.47 min in these four different types of callus with 53 to 79% of matching referred to the NIST database library (Table 1). Whereas, a clear chromatographic peak of n-Hexadecanoic acid and tetradecanoic acid could be seen at t_R 45.81 to 46.43 min. This indicated that both of the components sharing the same peak**.**

Chemical analysis of plant cell culture

Cell suspension culture of *S. acmella* was derived from friable callus and established in liquid medium MS + 2,4-D 0.5mg/L. Cells cultured for 9 days, 12 days, 18 days and 27 days were harvested and subjected to different drying methods (air-dried, freeze-dried and fresh). Spilanthol and N-isobutyl-2E, 4Z, 8Z, 10E-dodecatetraenamide were not detected in any of the cells regardless drying methods. However, the antioxidant (butylated hydroxytoluene) could be obtained in the TIC with a clear and sharp chromatographic peak at t_R 31.41 to 31.46 min for air-dried cells, t_R 31.41 to

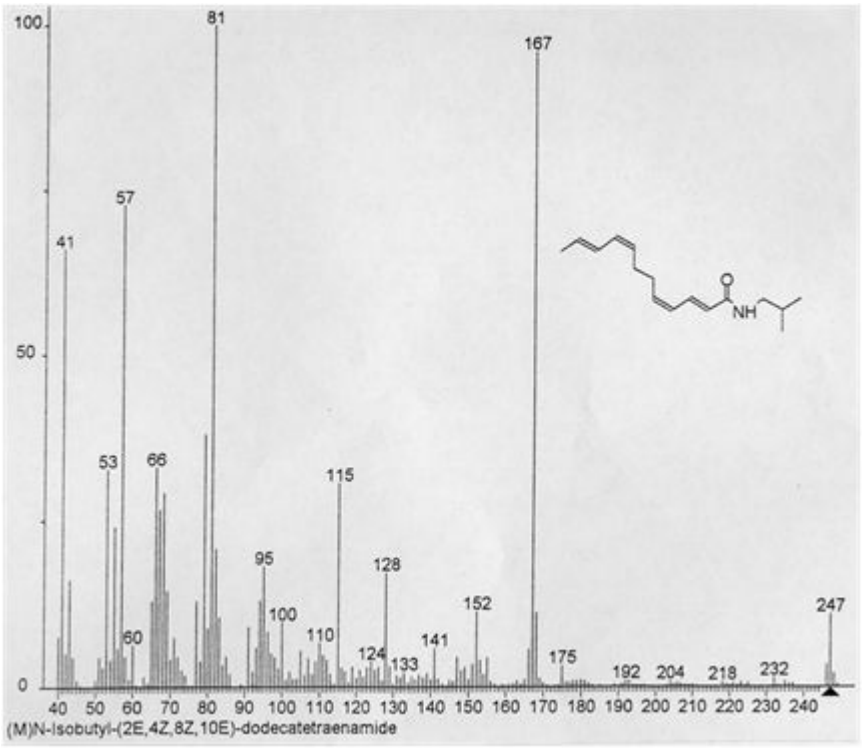

Figure 4. The mass spectrum of N-isobutyl-2E, 4Z, 8Z, 10E-dodecatetraenamide from NIST database library.

31.42 min for freeze-dried cells and t_R 31.41 to 31.45 min for fresh cells, accompanied with more than 75, 65 and 80% of matching to the NIST database library, respectively. Among all the samples examined, fresh cells showed the highest percentage of matching to the NIST database library.

DISCUSSION

Evidence showed that plant cell cultures are able to produce secondary metabolites came quite late in the history of *in vitro* techniques. Many attempts have been made to produce useful secondary metabolites using plant cell cultures, but most have been unsuccessful, because the activity of cells for synthesis of secondary products is lost in most cases when the cells are dedifferentiated and grow rapidly in cultures. Zenk (1991) experimentally demonstrated for the first time that this theory was wrong as they observed dedifferentiated cell cultures of *Morinda citrifolia* yielding 2.5 g of anthraquinones per litre of medium. This finding opened the door to a large community of *in vitro* plant culturists who extensively studied the possible use of plant cultures for the production of secondary compounds of industrial interest (mainly pharmaceuticals and dyes). Our positive results from the present study also confirmed the useful potential of *in vitro* technique in the industry production for active compounds. For *S. acmella*, the production of spilanthol has been detected in mother plants by using ^{1}H and ^{13}C NMR, MS, GC-MS (Ramsewak et al., 1999) and HPLC techniques (Nakatani and Nagashima, 1992). In the present study, GC-MS was employed to detect spilanthol and other useful compounds since the active compound of spilanthol was available in the NIST database library of mass spectrometer. Furthermore, GC-MS is a technique vastly used to qualitatively or quantitatively detect the presence of chemical compounds in volatile prepared substances. Splitless mode was used during the injection of samples in this study because it is suitable for very dilute samples.

In this study, the naturally occurring insecticide, spilanthol was detected in mother plant, flower heads and *in vitro* plantlets of *S. acmella* with similar retention time (43.18 to 43.21 min). The comparison of the mass spectrum with the NIST database library gave more than 90% match as well as a confirmatory compound structure match. Some studies have been carried out on the detection of spilanthol in aerial parts and flower heads of *S. acmella* (Gokhale and Bhide, 1945), but this was the first time reported that spilanthol also present in micropropagated plantlets. N-isobutyl-2E, 4Z, 8Z, 10E-dodecatetraenamide was also firstly detected in *in vitro* plantlets of *S. acmella* with 96.24% match as well as a confirmatory structure match when compared to NIST database library, but this compound was not found in the

mother plant or even flower heads. In fact, it is a potent mosquito larvicide and can provide 100% mortality against third instar larvae of *Aedes aegypti* at 10^{-5} mg/mL (Jondiko, 1986).

Other than spilanthol and N-isobutyl-2E, 4Z, 8Z, 10E-dodecatetraenamide, an antioxidant, butylated hydroxytoluene (BHT), was also obtained in each of the sample extracts (mother plant, flower heads, *in vitro* plantlets, callus, air-dried cells, freeze-dried cells and fresh cells) with a clear and sharp chromatographic peak at t_R 31.30 – 31.50 minutes accompanied with more than 50% match to the NIST database library. BHT, chemically also called 2, 6-di-tert-butyl-p-cresol (DBPC), was obtained by alkylation of p-cresol with isobutene or by monobutylation of m,p-cresol mixtures. The molecular formula is $C_{15}H_{24}O$ and molecular weight is 220. It is an indirect food additive that can be added to food-packaging materials, including adhesives, resinous and polymeric coatings, polyethylene film used for irradiated food packaging, and rubber products. It is used especially for foods that are high in fats and oils because it slows down the development of off-flavours, odours and colour changes caused by oxidation. Besides, BHT can function as an antioxidant, stabilizer and “antiskinning” agent for petroleum products, jet fuels, animal feeds, paints and lacquers, adhesive hardeners, cleaning agents, printing products and thinners (Budavari, 1989; Smolinske, 1992).

It is also used in fibre finishing of resin-bonded filters (Lanigan and Yamarik, 2002). Anderson et al. (1994) reported that BHT is used in sperm extenders to sustain sperm viability and prevent cold shock during freezing and thawing. Topical pharmaceutical products can contain BHT at concentration up to 0.2% (Smolinske, 1992).

Fatty acid, n-Hexadecanoic acid and tetradecanoic acid, could be obtained in all the sample extracts as well. n-Hexadecanoic acid, also known as palmitic, is a 16-carbon saturated fatty acid with $C_{16}H_{32}O_2$ of molecular formula and molecular weight of 256. Generally, it occurs in many natural oils and fats and used in making soap, shampoo, conditioner, emulsifier and emollient. Although the two fatty acids herein did not show a clear chromatographic peak in TIC, their presence was also detected in the samples at t_R 45.82 to 46.43 min. But most of the sample extracts gave only approximately 30% match to the NIST database library as well as a confirmatory compound structure match except 12 days, 18 days and 27 days freeze-dried cells. Moreover, the percentage of matching for fatty acids both in air-dried and fresh cells was rather high, which indicated that different drying methods could influence the secondary metabolites content in cells.

In this study, spilanthol was only detected in mother plants, flower heads and *in vitro* plantlets but was not found in the callus or cell cultures. However, N-isobutyl-2E, 4Z, 8Z, 10E-dodecatetraenamide that was not present in mother plant could be detected in the *in vitro* plantlets. For antioxidant (butylated hydroxytoluene) and fatty acid (n-Hexadecanoic acid and tetradecanoic acid), they were present in all the sample extracts including callus and plant cell cultures of *S. acmella*. In addition, we harvested 9 days, 12 days, 18 days and 27 days old cells to detect the presence of active compounds as secondary metabolites at different growth stages because it has been reported that many secondary metabolites accumulate in specific tissues and cells of plants, or at specific stages during the growth of cultured cells.

The accumulation of most secondary metabolites in cultured cell is maximal during the stationary phase of growth, for example, the accumulation of anthocyanin in suspension cultures of *Daucus carota* (Noé et al., 1980), *Catharanthus roseus* (Hall and Yeoman, 1986) and the accumulation of DOPA in callus cultures of *Stizolobium hassjoo* (Obata-Sasamoto and Komamine, 1983). However, there was no evidence showing the difference in amount of compound at different cell culture stages. From here, it was cleared that secondary metabolites found *in vivo* were not necessary present in the *in vitro* cultures. Vice versa, active compound absent in the mother plant might be obtained in *in vitro* cultures. For *S. acmella*, the amount of fatty acids and other active compounds present in plant cells was not very desirable. Therefore, elicitors could be used to improve the secondary metabolites production. Besides that, biotic elicitors (chitosan, autoclaved mycelium of pathogenic fungi and various protein extracts) or abiotic factors (temperature, UV light, heavy metal salts and pH) can also be used to trigger the production of bioinsecticide that are not produced in plant cells but present in mother plant and *in vitro* plantlets.

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