Solamargine production by a fungal endophyte of Solanum nigrum

El-Hawary, S.S.; Mohammed, R.; AbouZid, S.F.; Bakeer, W.; Ebel, Rainer; Sayed, A.M.; Rateb, Mostafa

Published in:
Journal of Applied Microbiology

DOI:
10.1111/jam.13077

Published: 01/04/2016

Document Version
Peer reviewed version

Solamargine production by a fungal endophyte of *Solanum nigrum*

Seham S. El-Hawary [a], Rabab Mohammed [b], Sameh F. AbouZid [b],Walid Bakeer [c], Rainer Ebel [e], Ahmed M. Sayed [b,d], and Mostafa E. Rateb [b,e,*]

[a]*Pharmacognosy Dept., Faculty of Pharmacy, Cairo University, Cairo, Egypt 11787*,

[b]*Pharmacognosy Dept., Faculty of Pharmacy, Beni-Suef University, Beni-Suef, Egypt 62514,*

[c]*Microbiology Dept., Faculty of Pharmacy, Beni-Suef University, Beni-Suef, Egypt 62514,*

[d]*Pharmacognosy Dept., Faculty of pharmacy, Nahda University, Beni-Suef, Egypt 62513*,

[e]*Marine Biodiscovery Centre, University of Aberdeen, Scotland, UK, AB24 3UE.*

***Correspondence:** Mostafa E. Rateb, m.rateb@abdn.ac.uk , +441224274536, Fax +44 1224 272 921

Abstract

Aims: The aim was to isolate, identify and characterize endophytes from *Solanum nigrum* L, as a new source of the anticancer solamargine and other bioactive metabolites for pharmaceutical and agricultural applications.

Methods and Results: Three endophytic fungi; SNFSt, SNFL, and SNFF were isolated from stems, leaves and fruits of *Solanum nigrum*, respectively and identified by molecular methods. Screening by TLC showed the production of the plant steroidal alkaloid solamargine **1** by one of these fungi, SNFSt, which was confirmed by LC-HRESIMS analysis together with its characteristic MS^2 fragmentation pattern. Solamargine production could be followed for a total of 10 generations of this fungus which was found to be related to *Aspergillus flavus* based on phylogenetic analysis of ITS sequences. Large scale fermentation of all three fungal strains led to the identification of known fungal metabolites (**2-10**) using HRESIMS and NMR analyses. Moreover, triacylglyceride composition of three fungal endophytes was determined using a simple and fast ^{1}HNMR approach.

Conclusions: The production of solamargine (found in the host plant) by a cultivable fungal endophyte is a novel observation. It would be interesting to further study the solamargine sustainable production by this fungal endophyte.

Significance and Impact of the Study: These fungal endophytes are a potential handle for scientific and probably commercial utilization. Although the current titer of solamargine doesn't satisfy the mass production, media optimization could provide adequate quantity of the anticancer drug. Furthermore, those fungal endophytes effectively produced diverse bioactive secondary metabolites which could have potential role in survival of their host plant in the harsh desert environment.

Keywords: *Solanum nigrum*, Endophytes, *Aspergillus flavus*, Solamargine, triacylglyceride.

Introduction

Solanum nigrum L., family Solanaceae is a well-known medicinal plant which possesses several biological activities such as antioxidant, hepatoprotective, anti-inflammatory, antipyretic, diuretic, antimicrobial and anticancer activity due to its flavonoid and steroidal alkaloid content (Jain et al., 2011). Solamargine, one of the major steroidal alkaloides in *S.nigrum* (Mohy-Ud-Din et al., 2010) has been demonstrated to exhibit potent anticancer activity against colon, prostate, breast, hepatic (Hu et al., 1999) and lung cancer cell lines (Liu et al., 2004). Its aglycone, solasodine is an inhibitor of the growth of the prostate gland adenocarcinoma cell line PC-3 (Zha et al., 2010).

Endophytes are fungi or bacteria that can survive inside plant tissues and establish a symbiotic relationship with their host plant (Rodriguez et al., 2009). Fungal endophytes are a valuable source for bioactive compounds with diverse biological activities like anticancer, antibiotic and immunosuppressive effects. Some of them have the ability to produce secondary metabolites specific to their host plant. The paclitaxel producing endophytic fungus *Taxomyces andreanae* isolated from Pacific yew tree (Stierle et al., 1993) opened up the door for the exploration of the ability of plant-derived fungal endophytes to produce potent bioactive secondary metabolites previously exclusively isolated from their host plants. Thereafter, paclitaxel production by fungal endophytes was reported from various other *Taxus* species, including *Pestalotiopsis microspora* from *Taxus wallachiana* (Strobel et al., 1996), *Tubercularia* sp. strain TF5 from *Taxus mairei* (Wang et al., 2000),*Pestalotiopsis* sp. from *Taxus cuspidate* (Kumaran et al., 2010), *Nigrospora* sp. from *Taxus globosa* (Sanchez et al., 2010),and *Metarhiziuma nisopliae* and other speciesfrom *Taxus chinensi* (Liu et al., 2009). Endophyte production of paclitaxel had been considered restricted to the genus *Taxus* until it was discovered in the endophytic fungus *Bartalinia robillardoides* isolated from *Aegle marmelos* (Gangadevi and Muthumary, 2008) and recently in the endophytic fungus of hazel *Penicillium aurantiogriseum* NRRL 62431 (Yang et al., 2014). In the latter case, it was genetically established that this endophytic fungus can independently produce paclitaxel, and that horizontal gene transfer between this fungus and its host plant is unlikely (Yang et al., 2014).However, it should be mentioned that in all previous reports, paclitaxel yield was very low, and that its titer decreased upon sub-culturing, in most cases below the detection limit. It is interesting to note that in the study aimed at enhancing its yield in the endopytic

fungus *Nigrospora* sp., paclitaxel production was found to be higher in solid-state fermentation than in liquid fermentation (Sanchez et al., 2010).

Further examples of fungal endophytes that produce bioactive plant metabolites are as follows: camptothecin by *Fusarium solani* (isolated from *Camptotheca acuminata*) (Kusari et al 2009a), vinblastine and vincristine by *Fusarium oxysporum* (from *Catharanthus roseus*) (Kumar et al., 2013),podophyllotoxin by *Fusarium oxysporum* (from *Juniperus recurva*) (Kouret al., 2008), gentiopicrin by an unidentified endophyte (from *Gentianam acrophylla*) (Yin et al., 2009),piperine by *Colletotrichum gloeosporioides* (from *Piper nigrum*) (Chithra et al., 2014), capsaicin by *Alternaria alternate* (from *Capsicum annuum*) (Devari et al., 2014), the steroidal alkaloid speimisine and peiminine by *Fusarium sp*. (from *Fritillaria unibracteata*) (Pan et al., 2014) as well as sipeimine by an unidentified endophyte (from *Fritillaria ussuriensis*) (Yin and Chen, 2008),and the flavonolignans, silybin A, silybin B, and isosilybin A by *Aspergillus iizukae* (from *Silybum marianum*) (El-Elimat et al., 2014).These examples clearly illustrate that endophytic fungi quite frequently have been implicated to be involved in the biosynthesis of a series of known plant bioactive metabolites comprising different structural classes and with important medicinal, pharmaceutical or agricultural applications. However, in all cases, the titers of these compounds were very low, and often disappeared with increasing rounds of cultivation which has led to the suggestion that media optimization or genetic approaches may be required to maintain the production (El-Elimat et al., 2014; Pu et al., 2013).

In this study, three fungal endophytes, SNFSt, SNFF, and SNFL were isolated from fresh material of *Solanum nigrum* collected from the East Desert in Beni-Suef, Egypt. Using DNA amplification and sequencing of the fungal internal transcribed spacer (ITS) region, strains SNFSt and SNFL were found to be closely related to *Aspergillus flavus*, whereas strain SNFF was related to *Lasiodiplodia theobromae*. Their extracts were screened for the potential production of steroidal alkaloids. Preliminary analysis was done by TLC and spraying with Dragendorff's reagent, and in comparison to an extract of *S. nigrum*. Only one out of these three tested fungal endophytes, strain SNFSt, tested positive and was subsequently subjected to chemical profiling using LC-HRESIMS to determine the exact nature of steroidal alkaloids. Additionally, larger scale fermentation of these three strains led to the identification of a series of known fungal metabolites. Finally, the triacylglyceride composition of

the three fungal endophytes was determined using a simple and fast ^{1}H-NMR-based technique, indicating that fungal endophytes could be a potential source for polyunsaturated fatty acids (PUFA).

Materials and methods

Plant material

Solanum nigrum L. selected for the present study was collected from Nahda University campus, East Desert, Beni-Suef, Egypt in January 2012. It was authenticated by Prof. Abd El-Halim Mohamed, Department of Flora and Phytotaxonomy Research, Horticulture Research Institute, Agriculture Research Center, Dokki, Cairo, Egypt. The samples of the plant material were collected and brought to laboratory in plastic bags and kept at 4 °C till immediate processing.

Isolation and identification of endophytic fungi

Plant material used for the isolation was first washed with tap water to remove the attached soil, then surface sterilized to remove epiphytes using serial washing in 70% ethanol for 1 min, 3% sodium hypochlorite solution for 3 min, followed by three rinses in sterilized distilled water (Long et al., 2003). Fruits, leaves and stem were cut aseptically into smaller segments using a sterile blade; each segment was placed onto a Petri dish with malt extract agar medium supplemented with ampicillin (0.5µg/mL) to suppress bacterial growth. All the plates were incubated at 28 °C and were regularly monitored for any mycelial growth. Pure fungal isolates were obtained upon repeated sub-culturing and were kept at 4 °C.

Molecular identification

Taxonomic identification of the fungal strains was achieved by genomic DNA extraction, amplification and sequencing of the fungal internal transcribed spacer (ITS) region using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4(5′-TCCTCCGCTTATTGATATGC-3′) according to Kjer's protocol (Kjer et al., 2010).Quality and integrity ofthe extracted DNA were screened by electrophoresis on 1% (w/v) agarose gel. Sequences thus obtained were submitted to GenBank, National Centre for Biotechnology Information (NCBI) with accession numbers KM504167, KM504168, and KM504169, respectively. Alignment with published sequences in GenBank showed that the fungal strain SNFSt had 99% identity with *Aspergillus flavus* (Genbank accession No.

KC131548), while strain SNFF had 98% identity with *Lasiodiplodia theobromae* (Genbank accession No.HM346880) and strain SNFL had 97% identity with *Aspergillus flavus* (Genbank accession No.JQ675308) or *Aspergillus oryzae* (Genbank accession No.KJ650333). All the isolated strains were deposited in the Microbiology Department, School of Pharmacy, Beni-Suef University, Egypt.

Fermentation, extraction and isolation of secondary metabolites

The isolated sub-cultured fungal endophytes (3rd generation) were fermented in 1.5 L liquid malt extract medium using 5 L Erlenmeyer flasks following inoculation by placing pieces of fresh growing agar cultures into the liquid medium. The flasks were incubated at 20 °C for four weeks. Subsequently, the fungal mycelia were separated from the culture broth by filtration, cut into small fragments and subjected to ultrasonic-assisted extraction with MeOH, while the culture broth was extracted with EtOAc (3 × 300 mL). The MeOH and the EtOAc extracts were evaporated separately using a rotary evaporator. Leaves, stems and fruits of the plant were dried separately, powdered, extracted with MeOH, and the resulting MeOH extracts were likewise concentrated by rotary evaporation. The concentrated MeOH extracts were suspended in distilled water and fractionated with hexane (4 × 200 mL), CH_2Cl_2 (3 × 200 mL), EtOAc (4 × 200 mL), and *n*-butanol (3 × 200 mL). Hexane fractions of SNFSt (2.5 g), SNFF (2 g) and SNFL (3 g) were subjected to column chromatography on silica gel (200 g, 70–230 mesh) using hexane:EtOAc (100:0 – 50:50) to obtain seven fractions. Crude fungal oils (850 mg, 450 mg and 700 mg, respectively) were obtained from Fr.I. Fraction V from strain SNFSt (200 mg) was further chromatographed on silica gel (20 g, 70–230 mesh) using CH_2Cl_2:MeOH(99:1), followed by column chromatography over Sephadex LH-20 and MeOH as mobile phase to obtain **3** (20 mg) and **4** (10 mg). The EtOAc extract of fermentation broth of strain SNFF (2.5 g) was chromatographed twice on silica gel (100 g and 200 g, 70–230 mesh) using CH_2Cl_2:MeOH (100:0–50:50) to obtain **5** (30 mg) and **6** (55 mg). The CH_2Cl_2 fraction of strain SNFF was subjected to semipreparative HPLC to give **2**(2 mg). HPLC separation was carried out using a reversed-phase column (C18, 250 × 10 mm, L×i.d., Phenomenex Luna) connected to an Agilent 1200 series binary pump and was monitored using an Agilent photodiode array detector. Detection was carried out at 215, 230, 254, 280, 320, and 380 nm. Elution was achieved using a gradient of CH_2CN and H_2O (10% - 100% over 30 min) at a flow rate of 2 mL/min. The EtOAc extract of the fermentation broth of strain

SNFL(3 g) was chromatographed on silica gel (300 g, 70–230 mesh) using CH_2Cl_2:MeOH(100:0–50:50) to obtain **7** (60 mg).

TLC screening for steroidal alkaloid production

Primary qualitative analysis of all extracts was performed using TLC. Silica gel plates were developed in solvent systems of CH_2Cl_2:MeOH (9:1 and 9.5:0.5, v/v). The TLC plates were sprayed with Dragendorff's reagent. The R_f values of the spots were determined and compared with an extract of the host plant.

Time course of solamargine production in SNFSt.

Strain SNFSt was fermented on malt extract agar and subcultured on fresh medium every 3 days. From each generation, a liquid culture was obtained in malt extract broth and fermented for 14 days. Liquid media obtained from the 1st, 3rd, and 5th until 11th generation were screened for steroidal alkaloid production by TLC as described above. Additionally, the1st and 3rd generation liquid cultures were analyzed by LC-HRESIMS.

LC.MS/MS analysis and identification

High resolution mass spectral data was obtained from a Thermo Instruments MS system (LTQ XL/ LTQ Orbitrap Discovery) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler and Accela Pump). The following conditions were used: capillary voltage 45 V, capillary temperature 260 °C, auxiliary gas flow rate 10-20 arbitrary units, sheath gas flow rate 40-50 arbitrary units, spray voltage 4.5 kV, mass range 100–2000 amu (maximum resolution 30000). Gradient separation was achieved using a SunFire C_{18} RP analytical HPLC column (5µm, 4.6 × 150 mm, Waters) with a mobile phase of 0-100%MeOH over 30 min at a flow rate of 1 mL/min.

Results

LC-HRESIMS/MS characterization of steroidal alkaloids

After visualization with Dragendorff's spraying reagent, extracts from the stem-derived fungal strain SNFSt and the stem of *Solanum nigrum* showed matching orange spots with similar R_f values (data not shown). Subsequent LCMS analysis of the fungal and the stem extracts (Fig.1) revealed a common

peak with retention time of 12.1min. HRESIMS data including fragmentation patterns of this peak in the two chromatograms were virtually identical, and were consistent with the presence of solamargine **1**. This assignment is based on the quasimolecular ion observed at *m/z* 868.5053 $[M+H]^+$which indicated the molecular formula $C_{45}H_{74}NO_{15}$, while its in-source fragmentation pattern revealed theconsecutive loss of a rhamnosyl group (146) to give *m/z* 722.4476(β_1-solamargine), another rhamnosyl group to give *m/z* 576.3894(γ-solamargine), and a glucosyl group (162) yielding the aglycone solasodine, *m/z* 414.3366 $[M+H]^+$. The MS^2 spectrum of *m/z* 414.3366 revealed a loss of H_2O (*m/z* 396.3261) and further ions due to fragmentation of the E-ring at *m/z* 271.2056, 253.1947 and 126.1277 (Fig. 2-3). In addition, fragmentation of the B-ring of *m/z* 253.1947 gave *m/z* 157.1010. This fragmentation pattern is characteristic ofsolamargine **1** according to the Metlin MS^2 database (Scripps Center For Metabolomics and Mass Spectrometry) and literature data (Cahill et al., 2010; Wu et al., 2013). These data prove that solamargine **1** which is a common metabolite in *S. nigrum*, was also present in the extract of the mycelium of the stem-derived endophytic fungus SNFSt. However, the EtOAc extract of the culture broth of the fungus did not show such a peak upon similar analysis (Fig.1), indicating that solamargine is retained in its mycelia, but is not excreted. Likewise, using the extracted ion chromatograms for *m/z*868.5, solamargine **1** was not detected in any of the extracts obtained from strains SNFF and SNFL (data not shown).

Phylogenetic analysis of solamargine producing strain SNFSt

The amplified ITS region of the strain SNFSt (genBank accession number: KC131548) was sequenced and compared with the ITS sequences of microorganisms represented in the NCBI database gene bank using BLAST search and MEGA 5.1 to generate a phylogenetic tree (Fig. 4) with the method of Neighbor-joining. Sequence analysis of the ITS regions of rDNA showed 99% identity with *Aspergillus flavus* isolate CZF-3 (Genbank accession No. KC131548) and 98% identity with *Aspergillus flavus* isolate A2S4_12 (Genbank accession No. JX501383). Therefore, Strain SNFSt was identified as *Aspergillus flavus*.

Time course of solamargine production in strain SNFSt.

The time course of solamargine production was investigated by analyzing 11 consecutive generations of strain SNFSt on agar plates which were sub-cultured every three days. Liquid cultures corresponding to each generation were screened by TLC, once the identity of the single Dragendorrf positive spot had

been established by LC-HRESIMS. The intensity of the spot due to solamargine remained more or less consistent until the 7th generation, started to gradually fade away through the 8th-10th generations, whereas the compound was no longer detected by TLC starting from the 11th generation (data not shown).

Structural characterization of the isolated compounds

Applying different chromatographic techniques to the fungal strain SNFSt total extract led to the isolation of (*S,S*)-sapinofuranone B **2** (Fig. 5) which was identified based on the HRESIMS, NMR spectra and optical rotation that were consistent with the previously reported data (Clough et al., 2000). Similarly, ergosterol **3** (Wang, 2012), ergosterol-5α,8α-peroxide **4** (Wang et al., 2014;Yue et al., 2001) were characterized from the same fungus. Indole-3-carboxylic acid **5** (The Human Metabolome Database (HMDB)) and indole-3-acetic acid **6** (Yue et al., 2000) were characterized by NMR spectral comparison and accurate mass analysis after their isolation from the fungal strain SNFF. Finally, penicillic acid **7** isolated from the fungus SNFL was identified based on the comparison of its NMR and HRESIMS data with previously reported data (Bladt et al., 2013; Ciegler et al., 1971).

Additionally, the characteristic MS^2 fragmentation patterns were used for the identification of cyclo(Leu-Pro) **8** and cyclo(Phe-Pro) **9** in the CH_2Cl_2 fraction of the fungus SNFF (Furtado et al., 2007), while the presence of 3-indolaldehyde **10** in the EtOAc fraction of the same fungus was established by comparison with an authentic standard.

Determination of fungal endophytes triacylglyceride composition using ^1H NMR spectroscopy

Fatty acid composition of fungal triacylglycerides was determined using an established protocol based on^{1}H-NMR spectroscopy (Barison et al., 2010). This method uses the integral ratios of signals characteristic for monounsaturated and diunsaturated fatty acids, and glycerol, respectively to give an estimate of the relative percentages of saturated, monounsaturated and diunsaturated fatty acids in triglyceride mixtures.

The ^{1}H-NMR spectra (Fig. 6) of triacylglycerides contain 9 signals of variable intensity depending on the proportions of the different acyl groups, indicating that these triglycerides are mixtures containing *bis*-allylic fatty acids such as linoleic acid (L), allylic fatty acids such as oleic acid (O) and saturated

fatty acids, and the proportion of each of these can be determined using the following equations (Guillen and Ruiz, 2003).

L (%) = 100 (E/D), O (%) = 100 [(C/2D) – (E/D)], S (%) = 100 [1 – (C/2D)].

A, C, D and E are the integrals pertaining to peaks 1, 4, 5 and 6 (Fig. 6).

The composition of triglycerides in each of the three endophytic fungi is shown in Table 1.

Discussion

Herein, we report the production of the potent anticancer steroidal alkaloid solamargine from the mycelia of the fungal endophyte SNFSt isolated from the stem of *S. nigrum*. To date, there are very few reports on production of steroidal alkaloids by microbes, and none so far from solanaceous plants, so this endophytic fungus is considered a new potential source of this valuable anticancer metabolite. Our findings indicate that solamargine was genuinely produced by the endophytic fungus SNFSt as the LC-HRESIMS analysis was carried out using its 3rd generation culture, so it is highly unlikely that residual steroidal alkaloids were still adsorbed onto the fungal cell wall as has been suggested for other cases (Heiniget al., 2013). Furthermore, we were still able to detect the presence of solamargine using Dragendorff's reagent till the 10th generation culture. Although solamargine production was unambiguously confirmed by HRESIMS and its MS^2 characteristic fragmentation pattern, it was produced in trace amount which did not allow us for its isolation even when the fungus was cultivated at larger scale. Therefore, further experiments such as media optimization or the study of epigenetic modifiers appear rewarding in order to establish whether higher titers of solamargine can be achieved for strain SNFSt.

Endophytic fungi have recently received a great deal of attention based on numerous cases where they produce the same metabolites as their host plants. Currently, there are two hypotheses to explain this striking phenomenon. Firstly, biosynthesis of the same compounds by the host and an endophyte could be due to horizontal gene transfer where entire gene clusters required for the biosynthesis of secondary metabolites in the host plant are transferred to the endophytic fungus or *vice versa* (Sachinet al., 2013). Alternatively, the endophytic fungus could independently synthesize its host plant secondary metabolites through a different biosynthetic pathway. For example, *Thielavia subthermophila* from *Hypericum perforatum* that is able to produce hypericin was shown to lack the *hyp*-1gene responsible

for hypericin biosynthesis in its host plant (Kusari et al., 2009b), and gene sequencing of the putative terpene cyclases in the paclitaxel-producing endophyte *Penicillium aurantiogriseum* from hazel and comparison with 13 known paclitaxel biosynthetic genes from *Taxus* spp. revealed little homology, making horizontal gene transfer an unlikely explanation (Yang et al., 2014).

In the course of our chemical analysis of the three fungal endophytes SNFSt, SNFF and SNFL, we have identified different classes of bioactive metabolites such as sterols, a γ-lactone, the auxin indole-3-acetic acid and diketopiperazines. Recently, DKPs such as **8** and **9** have been implicated in playing a role as quorum sensing signaling molecules in *Shewanella baltica* (Gu et al., 2013) while **9** additionally has been demonstrated to act as a plant-like auxin (Castroet al., 2010).These findings raise interesting questions regarding the role and control of these endophytes within *S. nigrum* and their potential role in survival of their host plant in the harsh desert environment from where the plant was collected.

Moreover, fungal endophytes have recently been reported as an alternative source of polyunsaturated fatty acids (PUFA) (Zhao et al., 2013). In our study, triacylglyceride mixtures containing a high percentage of unsaturated fatty acids were detected using a simple ^{1}H NMR technique which did not need sample pre-treatment such as derivatization.

Fungal endophytes could have the potential to produce their host plant specific secondary metabolites in addition they are considered a powerful and versatile production tool for nutraceuticals, agriculture and pharmaceutical industry.

Acknowledgement

We thank the College of Physical Sciences, University of Aberdeen, for provision of infrastructure and facilities in the Marine Biodiscovery Centre, and A. Raab, Marine Biodiscovery Centre for LCMS analysis.

References

Barison, A., Da Silva, C.W.P., Campos, F.R., Simonelli, F., Lenz, C.A. and Ferreira, A.G. (2010) A simple methodology for the determination of fatty acid composition in edible oils through ^{1}H-NMR spectroscopy. *Magn Reson Chem* 48, 642–650.

Bladt, T.T., Dürr, C., Knudsen, P.B., Kildgaard, S., Frisvad, J.C., Gotfredsen, C.H., Seiffert, M., and Larsen, T.O. (2013) Bio-Activity and dereplication-based discovery of ophiobolins and other fungal secondary metabolites targeting leukemia cells. *Molecules* 18, 14629–14650.

Cahill, M.G., Caprioli, G., Vittori, S. and James, K.J. (2010) Elucidation of the mass fragmentation pathways of potato glycoalkaloids and aglycones using Orbitrap mass spectrometry. *J Mass Spectrom* 45, 1019–1025.

Castro, R.O., Pérez, C.D., Trujillo, M.M., Río, R.E., García, J.C. and Bucio, J.L. (2010) Transkingdom signaling based on bacterial cyclodipeptides with auxin activity in plants. *PNAS* 108, 7253–7258.

Chithra, S., Jasim, B., Sachidanandan, P., Jyothis, M. and Radhakrishnan, E.K. (2014) Piperine production by endophytic fungus *Colletotrichum gloeosporioides* isolated from *Piper nigrum*. *Phytomed* 21, 534–540.

Ciegler, A., Detroy, R.W. and Lillehoj, E.B. (1971) *Microbial toxins* (chapter 6): patulin, penicillic acid, and other carcinogenic lactones. Academic Press, New York, p 417

Clough, S., Raggatt, M.E., Simpson, T.J., Willis, C.L., Whiting, A. and Wrigley, S.K. (2000) Structure elucidation and synthesis of (4S,5S,6Z,8E)-5-hydroxydeca-6,8-dien-4-olide[(S,S)-sapinofuranone B]–a novel γ-lactone metabolite of *Acremonium strictum*. *J. Chem. Soc. Perkin Trans. I* 15, 2475–2481.

Devari, S., Jaglan, S., Kumar, M., Deshidi, R., Guru, S., Bhushan, S., Kushwaha, M., Gupta, A.P., Gandhi, S.G., Sharma, J.P., Taneja, S.C., Vishwakarma, R.A. and Shah, B.A. (2014) Capsaicin production by *Alternaria alternata*, an endophytic fungus from *Capsicum annum*; LC–ESI–MS/MS analysis. *Phytochem* 98, 183–189.

El-Elimat, T., Raja, H.A., Graf, T.N., Faeth, S.H., Cech, N.B. and Oberlies, N.H. (2014) Flavonolignans from *Aspergillus iizukae,* a fungal endophyte of Milk Thistle (*Silybum marianum*). *J Nat Prod* 77, 193–199.

Furtado, N.A.J.C., Vessecchi, R., Tomaz, J.C., Galembeck, S.E., Bastos, J.K., Lopes, N.P. and Crotti, A.E.M. (2007) Fragmentation of diketopiperazines from *Aspergillus fumigatus* by electrospray ionization tandem mass spectrometry (ESI-MS/MS). *J Mass Spectrom* 42, 1279–1286.

Gangadevi, V. and Muthumary, J. (2008) Taxol, an anticancer drug produced by an endophytic fungus *Bartalinia robillardoides* Tassi, isolated from a medicinal plant, *Aegle marmelos* Correa ex Roxb. *World J Microbiol Biotechnol* 24, 717–724.

Gu, Q., Fu, L., Wang, Y. and Lin, J. (2013) Identification and characterization of extracellular cyclic dipeptides as quorum-sensing signal molecules from *Shewanella baltica*, the specific spoilage organism of *Pseudosciaena crocea* during 4 °C Storage. *J Agric Food Chem* 61, 11645−11652.

Guillen, M.D. and Ruiz, A. (2003) ^{1}H nuclear magnetic resonance as a fast tool for determining the composition of acyl chains in acylglycerol mixtures. *Eur J Lipid Sci Technol* 105, 502–507.

Heinig, U., Scholz, S. and Jennewein, S. (2013) Getting to the bottom of Taxol biosynthesis by fungi. *Fungal Diversity* 60, 161–170.

Hu, K., Kobayashi, H., Dong, A.J., Jing, Y.K., Iwasaki, S. and Yao, X.S. (1999) Antineoplastic agents III: steroidal glycosides from *Solanum nigrum*. *Planta Med* 65, 35–38.

Jain, R., Sharma, A., Gupta, S., Sarethy, I.P. and Gabrani, R. (2011) *Solanum nigrum*: current perspectives on therapeutic properties. *Altern Med Rev* 16, 78–85.

Kjer, J., Debbab, A., Aly, A.H. and Proksch, P. (2010) Methods for isolation of marine-derived endophytic fungi and their bioactive secondary products. *Nature protocols* 5, 479–490.

Kour, A., Shawl, A.S., Rehman, S., Sultan, P., Qazi, P.H., Suden, P., Khajuria, R.K. and Verma, V. (2008) Isolation and identification of an endophytic strain of *Fusarium oxysporum* producing podophyllotoxin from *Juniper usrecurva*. *World J Microbiol Biotechnol* 24, 1115–1121.

Kumar, A., Patil, D., Rajamohanan, P.R. and Ahmad, A. (2013) Isolation, purification and characterization of vinblastine and vincristine from endophytic fungus *Fusarium oxysporum* isolated from *Catharanthus roseus*. *PLoS One* 8(9):e71805.

Kumaran, R.S., Kim, H.J. and Hur, B.K. (2010) Taxol-producing fungal endophyte, *Pestalotiopsis* species isolated from *Taxus cuspidata*. *J Biosci Bioeng* 110, 541–6.

Kusari, S., Zühlke, S. and Spiteller, M. (2009a) An endophytic fungus from *Camptotheca acuminate* that produces camptothecin and analogues. *J Nat Prod* 72, 2–7.

Kusari, S., Zühlke, S., Košuth, J.,Čellàrovà, E. and Spiteller, M. (2009b) Light-independent metabolomics of endophytic *Thielavia subthermophila* provides insight into microbial hypericin biosynthesis. *J Nat Prod* 72, 1825–1835.

Liu, K., Ding, X., Deng, B. and Chen, W. (2009) Isolation and characterization of endophytic taxol-producing fungi from *Taxus chinensis*. *J Ind Microbiol Biotechnol* 36, 1171–1177.

Liu, L.F., Liang, C.H., Shiu, L.Y., Lin, W.L., Lin, C.C. and Kuo, K.W. (2004) Action of solamargine on human lung cancer cells-enhancement of the susceptibility of cancer cells to TNFs. *FEBS Lett* 577, 67–74.

Long, H.H., Furuya, N., Kurose, D., Takeshita, M. and Takanami, Y. (2003) Isolation of endophytic bacteria from *Solanum* sp. and their antibacterial activity against plant pathogenic bacteria. *J Fac Agr Kyushu Univ* 48, 21–28.

Mohy-Ud-Din, A., Khan, Z., Ahmad, M. and Kashmiri, M.A. (2010) Chemotaxonomic value of alkaloids in *Solanum nigrum* complex. *Pak J Bot* 42, 653–660.

Pan, F., Hou, K., Gao, F., Hu, B., Chen, Q. and Wu, W. (2014) Peimisine and peiminine production by endophytic fungus *Fusarium sp*. isolated from *Fritillaria unibracteata* var. *wabensis*. *Phytomed* 21, 1104–1109.

Pu, X., Qu, X., Chen, F., Bao, J., Zhang, G. and Luo, Y. (2013) Camptothecin-producing endophytic fungus *Trichoderma atroviride* LY357: isolation, identification, and fermentation conditions optimization for camptothecin production. *Appl Microbiol Biotechnol* 97, 9365–9375.

Rodriguez, R.J., White, J.F.,Arnold, A.E. and Redman, R.S. (2009) Fungal endophytes: diversity and functional roles. *New Phytol* 182, 314–330.

Sachin, N., Manjunatha, B.L., Kumara, P.M., Ravikanth, G., Shweta, S., Suryanarayanan, T.S., Ganeshaiah, K.N. and Shaanker, R.U. (2013) Do endophytic fungi possess pathway genes for plant secondary metabolites. *Curr Sci* 104, 178–182.

Scripps Center For Metabolomics and Mass Spectrometry - METLIN last accessed in Dec 2014.

Stierle, A., Strobel, G. and Stierle, D. (1993) Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. *Scince* 260, 214–216.

Strobel, G., Yang, X., Sears, J., Kramer, R., Sidhu, R.S. and Hess, W.M. (1996) Taxol from *Pestalotiopsis microspora*, an endophytic fungus of *Taxus wallachiana*. *Microbiol* 142, 435–440.

Sanchez, J.R., Bustamante, Z.R., Dendooven, L., Torres, E.F., Soca-Chafre, G., Galindez-Mayer, J. and Flores-Cotera, L.B. (2010) A comparative study of taxol production in liquid and solid-state fermentation with *Nigrospora* sp. a fungus isolated from *Taxus globosa*. *J Appl Microbiol* 109, 2144–2150.

The Human Metabolome Database (HMDB)last accessed in Dec 2014.

Wang, F.W. (2012) Bioactive metabolites from *Guignardia* sp., an endophytic fungus residing in *Undaria pinnatifida*. *Chin J Nat Med* 10, 72–76.

Wang, H., Liu, T. and Xin, Z. (2014) A new glucitol from an endophytic fungus *Fusarium equiseti* Salicorn. *Eur Food Res Technol* 239, 365–376.

Wang, J.F., Li, G.L., Lu, H.Y., Zheng, Z.H., Huang, X.J. and Su, W.J. (2000) Taxol from *Tubercularia* sp. strain TF5, an endophytic fungus of *Taxus mairei*. *FEMS Microbiol Lett* 193, 249–253.

Wu, S.B., Meyer, R.S., Whitaker, B.D., Litt, A. and Kennelly, E.J. (2013) A new liquid chromatography–mass spectrometry-based strategy to integrate chemistry, morphology, and evolution of eggplant (*Solanum*) species. *J Chromatogr A* 1314, 154–172.

Yang, Y., Zhao, H., Barrero, R.A., Zhang, B., Sun, G., Wilson, I.W., Xie, F., Walker, K.D., Parks, J.W., Bruce, R., Guo, G., Chen, L., Zhang, Y., Huang, X., Tang, Q., Liu, H., Bellgard, M.I., Qiu, D., Lai, J. and Hoffman, A. (2014) Genome sequencing and analysis of the paclitaxel-producing endophytic fungus *Penicillium aurantiogriseum* NRRL 62431. *BMC Genomics* 15, 69.

Yin, H. and Chen, J.L. (2008) Sipeimine-producing endophytic fungus isolated from *Fritillaria ussuriensis*. *Z. Naturforsch* 63c:789–793.

Yin, H., Zhao, Q., Sun, F.M. and An, T. (2009) Gentiopicrin-producing endophytic fungus isolated from *Gentiana macrophylla*. *Phytomed* 16, 793–797.

Yue, J.M., Chen, S.N., Lin, Z.W. and Sun, H.D. (2001) Sterol from the fungus *Lactarium volemus. Phytochem* 56, 801–806.

Yue, Q., Miller, C.J., White, J.F., Jr and Richardson, M.D. (2000) Isolation and characterization of fungal inhibitors from *Epichloe festucae*. *J Agric Food Chem* 48, 4687–4692.

Zha, X.M., Zhang, F.R., Shan, J.Q., Zhang, Y.H., Liu, J.O. and Sun, H.B. (2010) Synthesis and evaluation of in vitro anticancer activity of novel solasodine derivatives. *Chin Chem Lett* 21, 1087–1090.

Zhao, Y., Wang, H., Liu, T. and Xin, Z. (2013) The individual lipid compositions produced by *Cunninghamella* sp. Salicorn5, an endophytic oleaginous fungus from *Salicornia bigelovii* Torr. *Eur Food Res Technol* 238, 621–633.

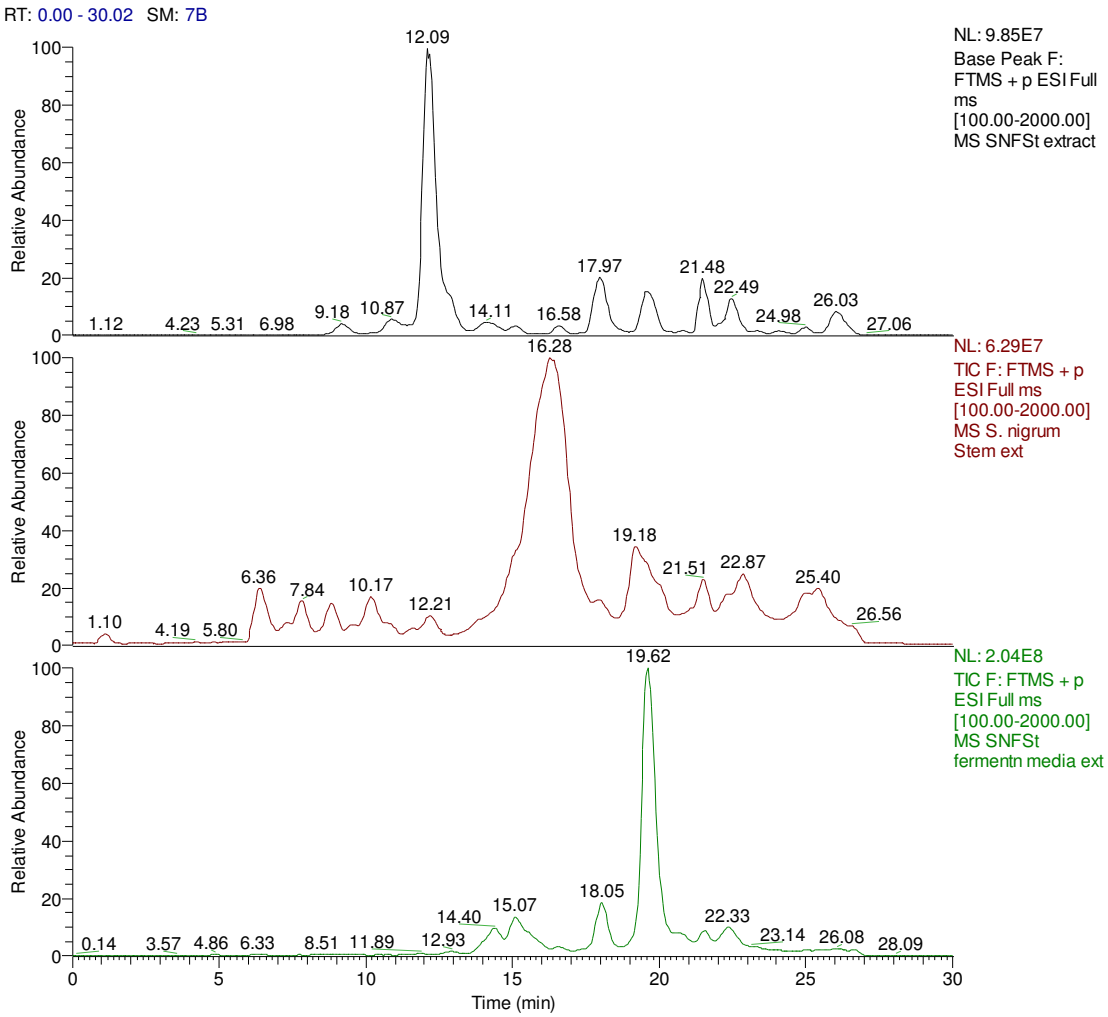

Fig.1. MS traces of extracts from (a) mycelium of SNFSt, (b) stem of *S. nigrum*, (c) culture broth of SNFSt.

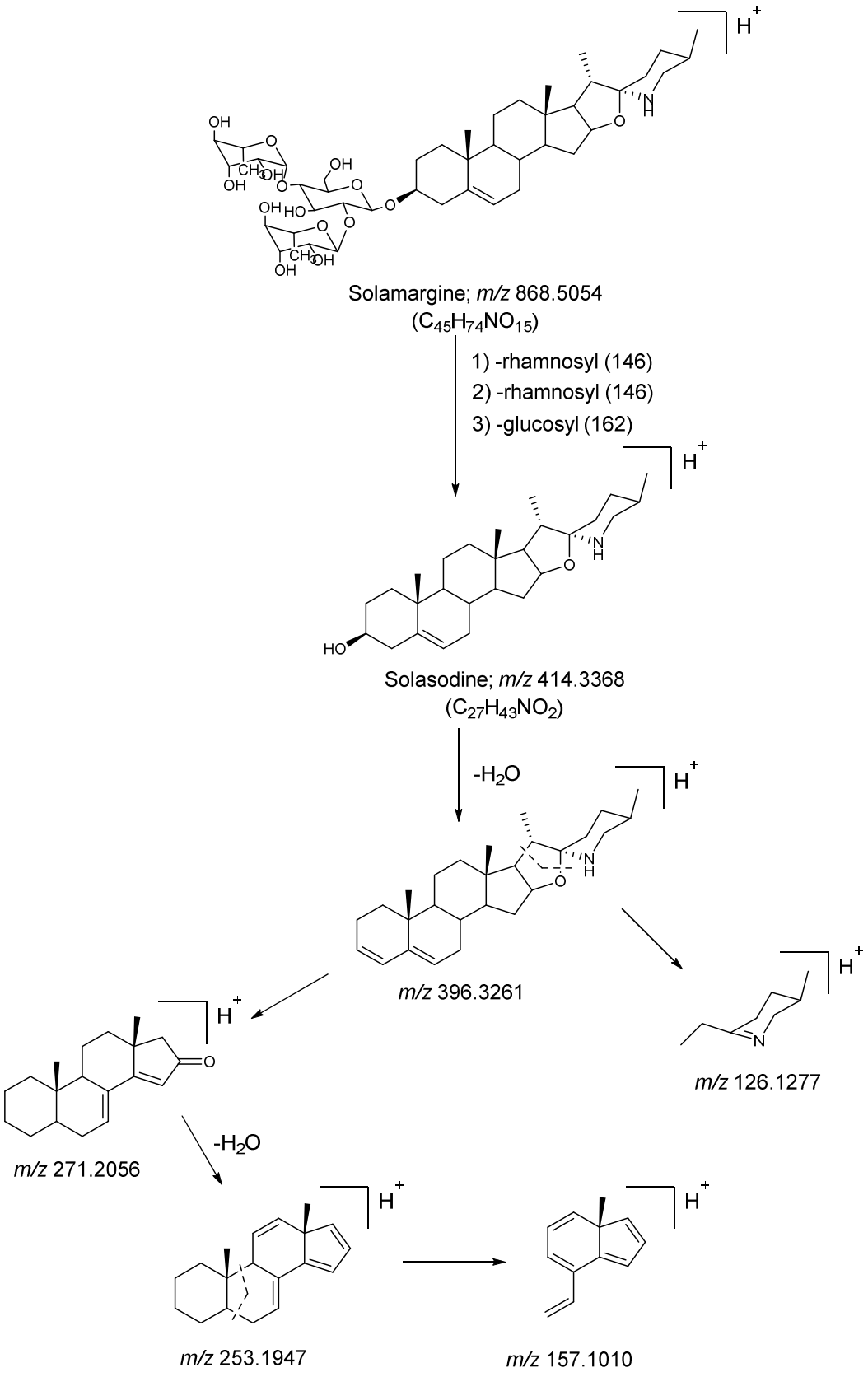

Fig.2. Fragmentation patterns of solamargine **1** and its aglycone.

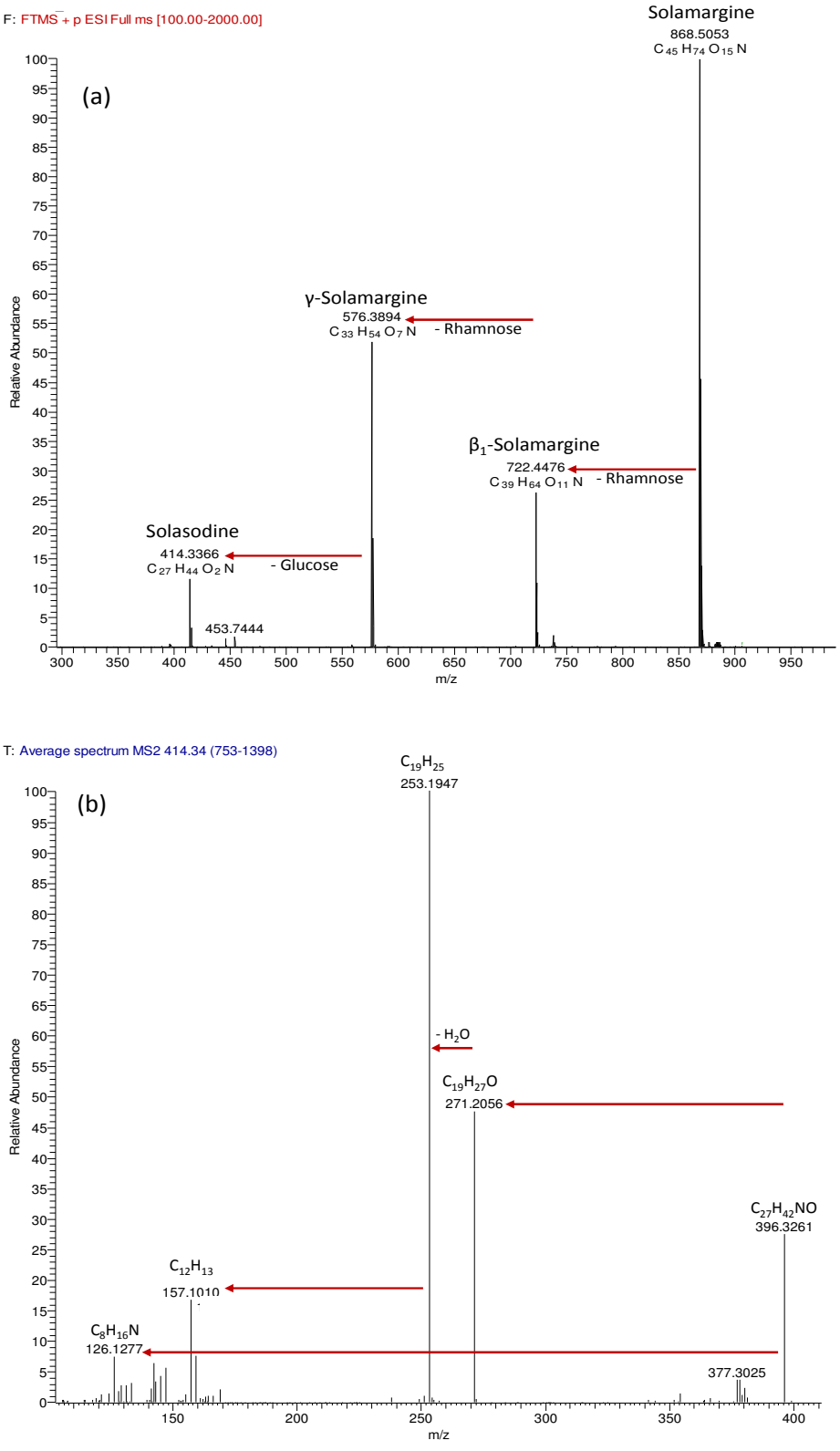

Fig.3.HRESIMS analysis of (a)solamargine **1** and (b) MS^2 of its aglycone from the mycelium of SNFSt.

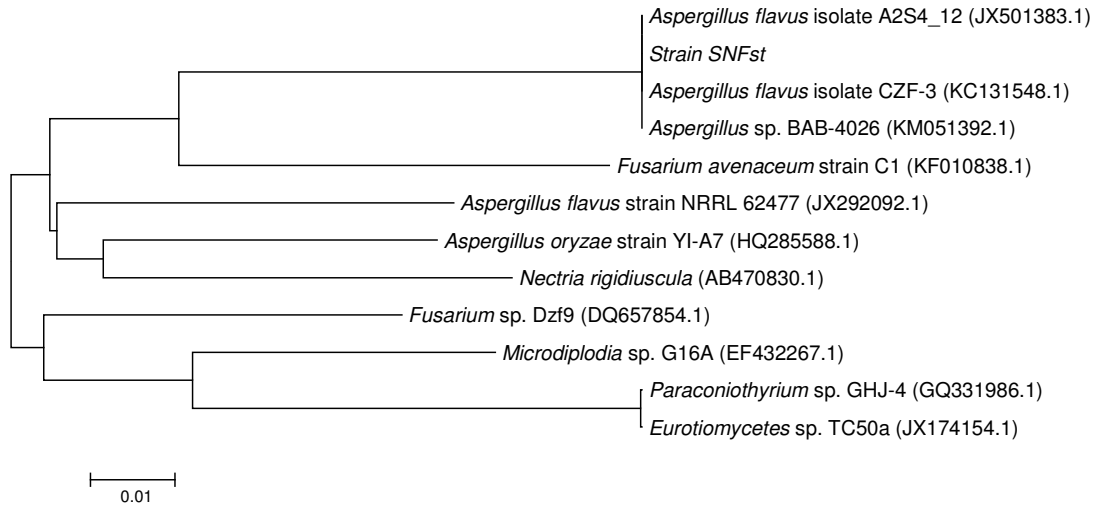

Fig. 4. Neighbor-joining phylogenetic tree based on ITS-rDNA sequences of fungal isolate SNFSt and related sequences from GenBank.

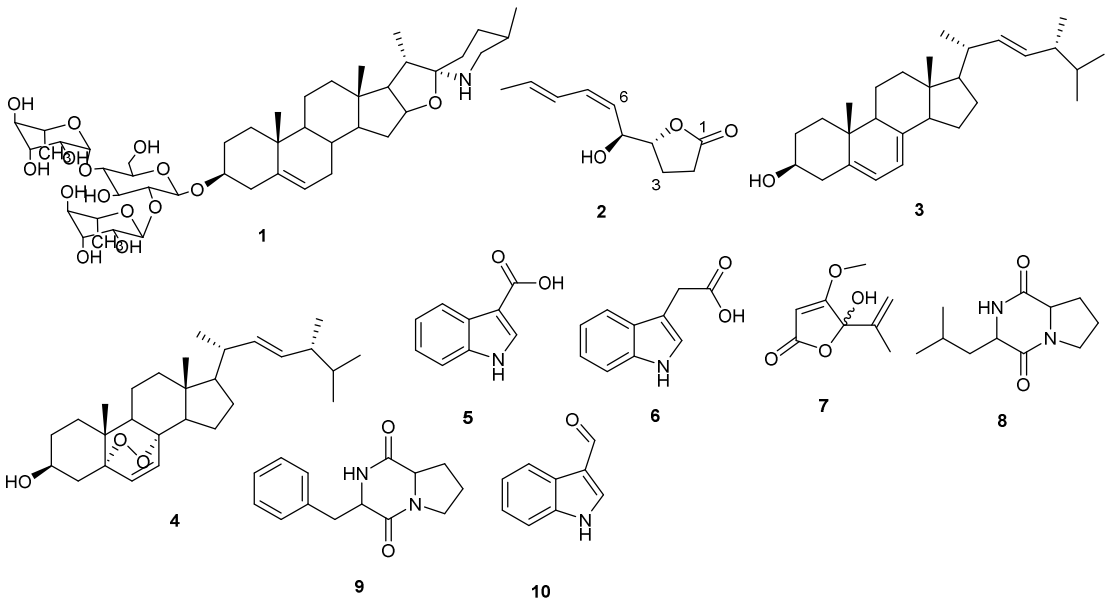

Fig.5. Compounds isolated from the plant-derived endophytic fungi.

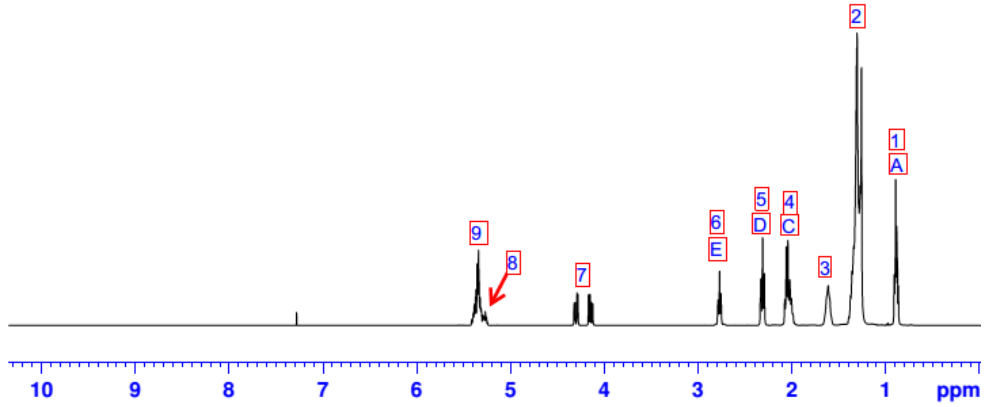

Fig. 6. ^{1}H-NMR spectrum of triacylglyceride mixtures obtained from strain SNFL.

Table 1.Triacyl glycerol composition of fungal endophytes established by^{1}H NMR analysis.

fungal strain	***bis*-allylic (linoleic)**	**allylic(oleic)**	**saturated**
SNFSt	41.4%	27.3%	31.3%
SNFL	61.8%	23.6%	14.6%
SNFF	17.0%	16.9%	66.1%