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Isolation and structural elucidation of an isothiocyanate compound from *Indigofera tinctoria* Linn. extract

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Indigofera tinctoria **is a well-known medicinal plant that possesses several therapeutic activities. Isothiocyanate derivative from hydroethanolic extract of *Indigofera tinctoria* (HEIT) was isolated by means of chromatographic techniques, i.e. adsorption chromatography, thin layer chromatography and high-pressure liquid chromatography. Structural characterization of isolated compound was done using various spectroscopic techniques (liquid chromatograph–mass spectrometry, 1H nuclear magnetic resonance and Fourier transform-infrared spectroscopy) and the possible structure was identified as 1-[1,2-Diisothiocyanato-2-(3-isothiocyanato-2,2-dimethyl-propylsulphanyl)-ethoxy]-3-isothiocyanato-2,2-dimethyl-propane ($C_{16}H_{22}N_4OS_5$; *m/z* 446.70; ITC-1). Maximum yield of ITC-1 was obtained as 22 mg/5 g HEIT with 97% purity.**

Keywords: Column chromatography, HPLC, *Indigofera tinctoria*, isothiocynate compounds, LC–MS.

ISOTHIOCYANATE compounds (ITCs) belong to the category of organosulphur compounds, i.e. the compounds that contain sulphur moiety, possess R–N=C=S group[1]. Brasicaceae and Fabaceae families are known to contain ITCs[2]. These are weak electrophilic in nature. Some naturally occurring ITCs like PEITC (phenethyl isothiocyanate), BITC (benzyl isothiocyanate) have also been isolated from various cruciferous vegetables. ITCs are known for their various types of therapeutic activities[3], hence have a great significance in medicine.

Indigofera tinctoria belongs to the family Fabaceae. It is found throughout South East Africa, tropical Africa as well as tropical America and is cultivated in Southern India, especially Tamil Nadu[4]. It is a deciduous shrub, reaching 1–2 m in height and may be annual, biennial or perennial. This plant has been reported to possess a wide range of therapeutic activities[5–7].

Keeping the need of naturally derived target specific drug to treat various health problems in medicine, the present study was designed to isolate and characterize the ITC from hydroethanol extract of *I. tinctoria* (Indigo).

Silica gel G and silica gel (60–120) were procured from Himedia, Mumbai, India. Solvents for HPLC were of HPLC grade and procured from RanKem, India. Ethanol, *n*-hexane, chloroform, methanol and petroleum ether

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were obtained from reliable firms, i.e. Central Drug House (CDH) and RanKem, India.

Indigofera tinctoria sample (shaded dried) was purchased from S.M. Heena Industries, Jaipur, Rajasthan, India. They had collected plant sample from Tindivanam (12°15′0″N; 79°39′0″E), Tamil Nadu, India. The plant sample was identified, authenticated and submitted at herbarium section of Department of Bioscience and Biotechnology, Banasthali University, Rajasthan (BURI-13515).

Dried plant material was minced in a grinder (model: UNOVA, VISHVA Enterprises, Mumbai), sieved (Sethi Standard Test Seive, Yamberzal International, Jaipur) and extracted with Soxhlet using 80% ethanol after defatting with petroleum ether. This extract was filtered and dried under reduced pressure in a rotary evaporator (Heidolph InKarp Instruments, Germany) to afford 25.825 g/100 g plant powder and was designated as hydroethanolic extract of *I. tinctoria* (HEIT).

Presence of sulphur containing compound in crude HEIT extract was estimated by 'lead acetate test'. Brown or black colour appearance indicates the presence of sulphur due to PbS formation[8]. HEIT was dissolved in the respective solvent and then adsorbed with silica gel (60–120 mesh size) that formed the coarse uniform powder.

The sintered glass column (5 × 15 cm in height and 4 cm diameter; Borosil, India) was packed by 'wet packing method'. Non-polar solvent, i.e. *n*-hexane was filled in half of the column. Then the dipped silica gel in the same solvent (before 30 min of packing) was poured into the column with continuous tapping to form a uniform bed. The adsorbed HEIT powder was then poured into the column. A cotton plug was also placed above the extract to prevent any damage while extracting the layer.

Silica gel served as stationary phase whereas gradient elution of various solvents was used as mobile phase [*n*-hexane, pure, fraction number 1; *n*-hexane : chloroform (95 : 5, fraction number 2; 90 : 10, fraction number 3–5; 85 : 15, fraction number 6–10; 80 : 20, fraction number 11–15; 70 : 30, fraction number 16–49; 65 : 35, fraction number 50–77; 60 : 40 fraction number 78–110; 50 : 50, fraction number 111–147; 35 : 65, fraction number 148–169; 25 : 75, fraction number 170–186; 10 : 90, fraction number 187–195); chloroform, pure, fraction number 196–326; chloroform : methanol (90 : 10; fraction number 327–450; 80 : 20, fraction number 451–479). To avoid any air bubbles or break, the charged column was left for 3–4 h. Various fractions were eluted with gradual increase in polarity of various solvents.

Different fractions were collected from column and then chromatographed on activated silica gel G plate via thin layer chromatography (TLC) technique to know the extent of mixing of compounds and the presence of desired compound. The sub-fraction '437–448' of large fraction '327–450' was chromatographed using solvent system chloroform : methanol (6 : 4) and then sprayed with NaOH-lead acetate solution to derivatized organo-sulphur compounds. The sub-fraction 437–448 was then subjected to HPLC.

The gradient elution of mobile phase water : acetonitrile was used to separate the components from fraction which was eluted by the column. HPLC (Dionex-ultimate 3000 HPLC) with quaternary gradient pump, variable wavelength UV detector (Ultimate 3000 RS), Genetix G Chrom chromatography, ultra P silica RP C18 HS, standard column, size 5 μm, 250 mm × 4.6 mm was used. Fraction was filtered with 5 μ membrane filter before being loaded in HPLC. Flow rate was 0.800 ml/min; sample injection amount was 200 μl with pressure 113 bars. Total running time was 35 min. Gradient elution system was programmed as 95% water at 0 min; 80% at 3 min; 50% at 10 min; 50% at 20 min; 80% at 25 min; 95% at 30 min and 95% at 35 min. Fraction components were separated and collected with the help of auto-collector and further chromatographed through HPLC to see its purity. Collected sub-fractions were also previously chromatographed via TLC and derivatized using the same solvent system and spray reagent as described above to confirm the organo-sulphur compound. Isolated compound was then lyophilized (Henil Science Industrial Pvt Ltd, Korea; model: Clean Vac 8 vacuum freeze dryer) to get pure compound designated as ITC-1.

Various functional groups in isolated compound (ITC-1) were recorded with the help of FT-IR (Bruker, OPUS, Germany) in the range of 4000–1000 cm^{-1}.

NMR basically deals with the study of interaction of electromagnetic radiation with the compound. 1H NMR spectrum of ITC-1 was recorded with the help of NMR (Bruker Advance III 500 MHz). ITC-1 was dissolved in respective solvent D_2O and about 600 μl was poured in NMR tube and then the peak that arose by the applied magnetic field was observed.

Figure 1. TLC chromatogram of (***a***) active sub-fraction (437–448) and (***b***) isolated compound, derivatized with NaOH-lead acetate solution, showed compound of interest (in circle).

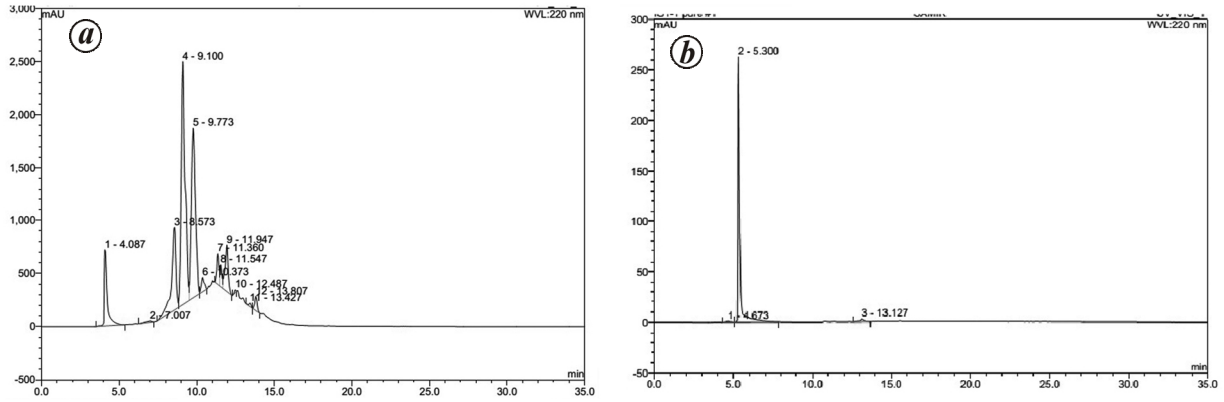

Figure 2. HPLC chromatogram of (***a***) fraction (437–448) shows 10 different compounds with interest of one polar peak on 5.3 min retention time (Rt), (***b***) purified isothiocyanate derivative from *I. tinctoria* hydroethanolic extract.

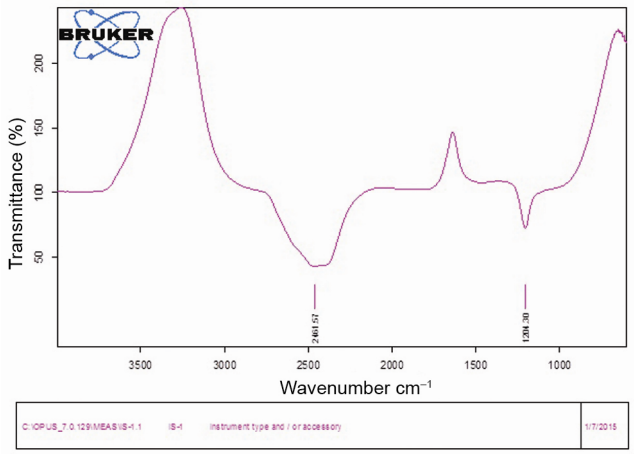

Figure 3. FT-IR spectra of the isolated compound (ITC-1).

LC–MS was used to analyse the mass spectrum of ITC-1. Total ionic chromatogram (TIC) by electrospray ionization method (ESI) of LC–MS (Agilent Technologies, California) with protein digest method, Q-TOF B.05.00 (B5042.0) version, 6200 series TOF/6500 series was used to get the mass spectrum.

HEIT gave a yield of 25.825 g/100 g after soxhlet extraction. It gave a positive result for the presence of organo-sulphur compounds. The collected sub-fraction (437–448) showed a blackish spot when chromatographed on silica plates. This indicates the presence of orgnao-sulphur compound when derivatized with NaOH-lead acetate solution (Figure 1 *a*). The residue yield of this sub-fraction was 192 mg/5 g crude HEIT. The isolated single compound via HPLC from this fraction showed retention time (Rt) 5.3 on HPLC chromatogram with a purity of 96.61% and height 262.807 (Figure 2). This peak approximately resembles with one of the peaks in HPLC chromatogram of sub-fraction 437–448 (4.08 Rt). This drifting difference of 1.22 Rt may be due to involvement of several minor factors like difference in solvent polarity, changes in mobile phase composition, variations in temperature, reservoir capping, minute changes in flow rate, presence of some contaminating species in the sample that can cause irreversible bind to the functional ligand[9].

TLC analysis also confirmed this sub-fraction as organo-sulphur compound (Figure 1 *b*). The isolated compound was then lyophilized (Henil Science Industrial, Korea; model: Clean Vac 8 vacuum freeze dryer) that yielded it in pure form as 22 mg/5 g of crude HEIT. The isolated compound was designated as ITC-1.

The isolated compound obtained after lyophilization was light pale yellow in colour, liquid in nature, stable at room temperature and viscous in nature. This compound was completely soluble in water.

The isolated compound was then further processed to LC–MS for the mass spectra, 1H NMR to get an idea about the number of protons present in the compound and FT-IR to get the spectrum of functional groups. IR spectrum of ITC-1 exhibits two prominent peaks at 2461 and 1204 cm^{-1}. Literature reports that –N=C=S (isothiocyanate) group showed its appearance at around 2400 cm^{-1} whereas –C–O group showed its appearance at around 1200 cm^{-1} (–C–O). So, both spectra gave an idea about the presence of these two groups in ITC-1 (Figure 3).

The 1H NMR spectrum of ITC-1 was taken at 500 MHz operating frequency in D_2O and two doublets at δ 4.47–4.44 ppm (–CH–), and 4.18–4.10 ppm (–CH–S) were obtained. A singlet was observed at 3.63 ppm corresponding to methylene hydrogen. A sharp singlet was observed at 1.30–1.14 ppm corresponding to methyl protons (Figure 4).

The LC–MS spectrum of the ITC-1 was observed by ESI method. The fermented voltage in LC–MS of ITC-1 was 200 V. TOF was used to analyse the mass spectra of ITC-1 in which an ion's *m*/*z* ratio is determined via a time measurement. Time depends on *m*/*z* ratio of the particle. The molecular weight of the isolated compound was observed by TOF-MS *m*/*z* 456 which is the approximate

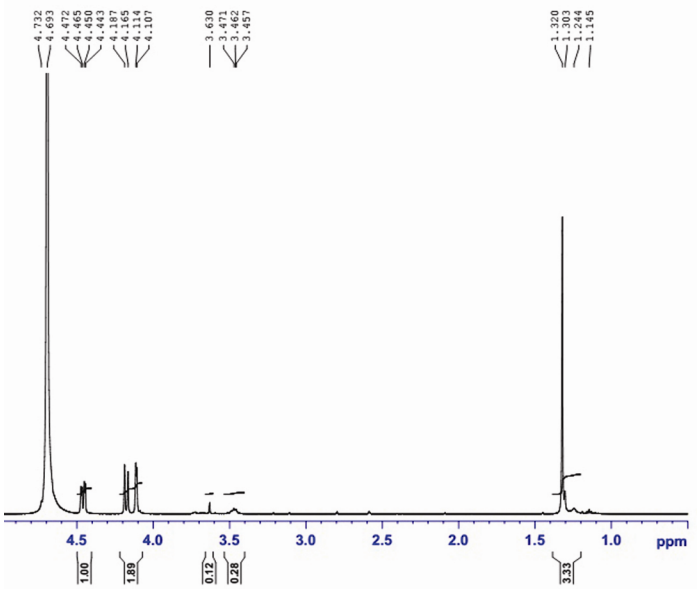

Figure 4. Proton NMR spectra of isolated compound (ITC-1).

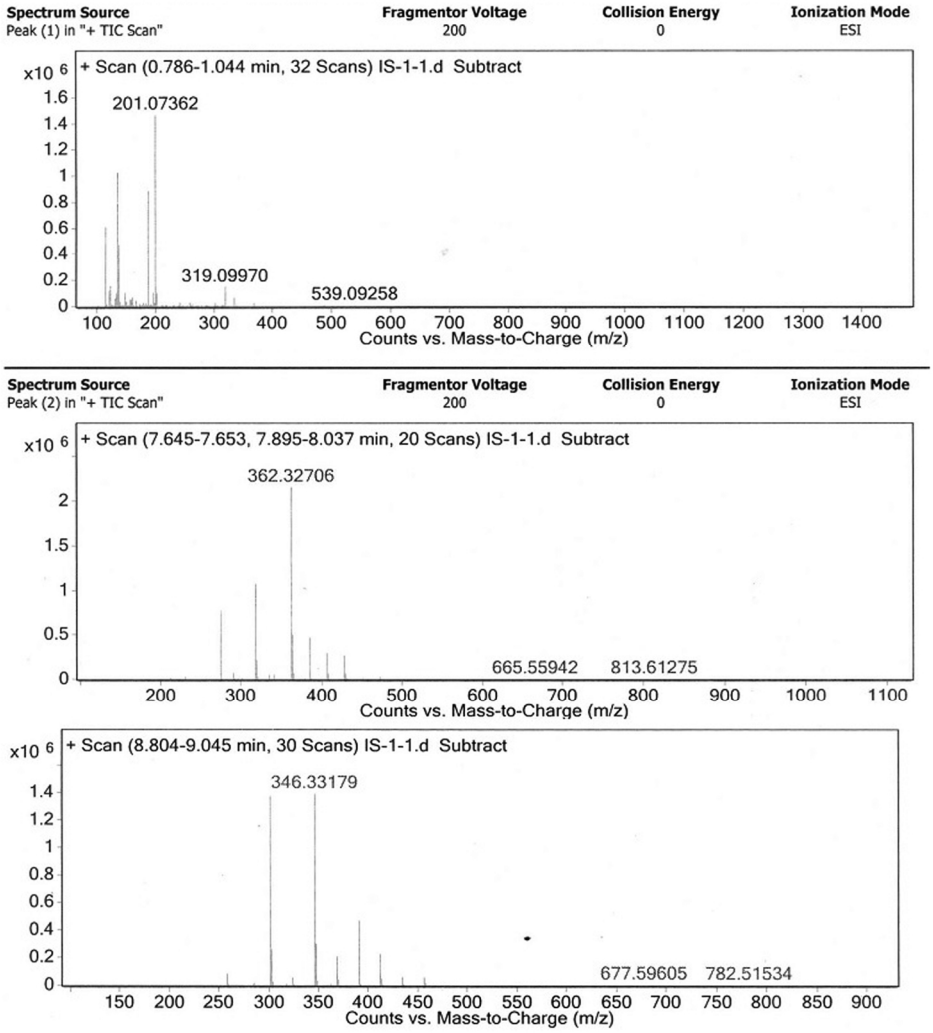

Figure 5. Liquid chromatography–mass spectrometry spectra of ITC-1.

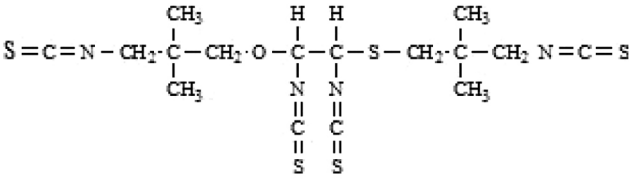

Figure 6. Possible structure of isolated compound (ITC-1), i.e. isothiocyanate derivative having IUPAC name 1-[1,2-diisothiocyanato-2-(3-isothiocyanato-2,2-dimethyl-propylsulphanyl)-ethoxy]-3-isothiocyanato-2,2-dimethyl-propane ($C_{16}H_{22}N_4OS_5$).

equivalent for calculated values of ITC-1 *m/z* 446.70 (Figure 5).

On the basis of these spectral techniques, the possible structure of the isolated compound (ITC-1) was drawn and presented in Figure 6. The IUPAC name for the compound is 1-[1,2-diisothiocyanato-2-(3-isothiocyanato-2,2-dimethyl-propylsulphanyl)-ethoxy]-3-isothiocyanato-2,2-dimethyl-propane and molecular formula is $C_{16}H_{22}N_4OS_5$. Presence of –N=C=S group revealed that this compound is the natural derivative of isothiocyanate that is present in *I. tinctoria* plant. Previously, various compounds have been isolated from *I. tinctoria* plant like indigo, indirubin[10], six different rotenoids (Tephrosin, Rotenone, Deguelin, Rotenol, Dehydrodeguelin and Sumatrol)[11], histamine[12], trans-tetracos-15-enoic acid (TCA)[13], apigenin, kaempferol, tutetion, galactomannan and trigonelline[14] that also have potent therapeutic activities.

Organosulphur compound is an important class of phytochemicals widely distributed in various plants. Sulphur is a very vital element for life. Chemopreventive properties of various organosulphur compounds were previously reported[15,16]. They exert several biological activities through various pathways like inducing carcinogen detoxification, inhibitory effect on proliferation of tumour cells, and free radicals scavenging process. Overproduction of various free radicals is responsible for severe cell damage. Hence, this would be a major pathway to prevent cell damage from various xenobiotic compounds or carcinogen, prevention of various DNA adduct formation in cell and induction of cell apoptosis[17]. Isothiocyanate is one of the important classes of organosulphur compounds that possess R–N=C=S group. Several isothiocynate derived natural compounds were already isolated from various plants like PITC (phenethyl isothiocyanate), BITC (benzyl isothiocyante), sulphoraphane, etc. Isothiocyanate compounds are widely found in nature and have a great interest in medicine. It is reported that various natural isothiocyanate derived phytoconstituents have a remarkable chemopreventive and anticancerous activity[18–21]. Isothiocyantes have been shown to be effective against lung, mammary, liver, pancreatic, colon and esophageal cancers[22–28]. Literature reported that ITCs are formed via myrosinase mediated enzymatic hydrolysis of glucosinolates to their corresponding isothiocyantes derivatives in plants[29].

Nowadays, importance of organosulphur compounds especially isothiocyantes in treatment of various health problems may increase need towards its isolation from medicinal plants which may have quite possibility in future drug invention for cancer or other health related problems.

From the aforementioned studies, it was analysed that the studied plant contained organosulphur compounds especially isothiocyantes. The isolated isothiocyanate derived phytoconstituent named 1-[1,2-diisothiocyanato-2-(3-isothiocyanato-2,2-dimethyl-propylsulphanyl)-ethoxy]-3-isothiocyanato-2,2-dimethyl-propane ($C_{16}H_{22}N_4OS_5$) was isolated from *I. tinctoria* aerial parts. This novel compound may have possible anticancerous property. To explore its therapeutic efficacy, it can be further tested for *in vivo* studies.

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Callus-mediated organogenesis in *Lilium polyphyllum* D. Don ex Royle: a critically endangered Astavarga plant

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***Lilium polyphyllum* D. Don ex Royle (Liliaceae) is a critically endangered herbaceous perennial, commonly known as white lily or Ksheerkakoli. Bulbs of the plant are of immense medicinal use and have astringent and anti-inflammatory properties. Overexploitation of the species from the wild and degradation of habitats are posing threats to its existence. In the present study, a protocol was standardized for micropropagation and mass multiplication of the species from scale leaves. Callusing was induced in basal MS medium containing 2,4-D (6.78 µM) and BAP (4.4 µM), where maximum effect (95.32%) was recorded. Maximum shooting (97.45%) was found in the calluses when shifted to MS medium fortified with BAP (4.4 µM), NAA (0.53 µM) and GA_3 (20 ppm) with an average of 19.2 shoots/per culture. The well-developed *in vitro* regenerated shoots were shifted to the rooting medium and 100% rooting was achieved in half-strength MS basal medium enriched with IBA (9.8 µM). The *in vitro* regenerated plantlets were shifted to a glasshouse for acclimatization and finally transferred to the open environment with 85% success.**

Keywords: Callusing, *Lilium polyphyllum*, micropropagation, organogenesis.

LILIUM POLYPHYLLUM, a bulbous perennial herb of the Liliaceae family, is commonly known as white lily and Ksheerkakoli. IUCN has categorized it as critically endangered medicinal herb and included it under the Red Listed species[1]. It commonly grows in dense, humus-rich forest floors at an altitudinal range 2100–3000 m. The plant grows up to 1.0–1.5 m height, produces white–creamy flowers with purple spots and triangular capsules bearing winged seeds[2,3]. Distribution of the species is restricted to the Himalayan region and it is sparsely distributed from Afghanistan to North-West Himalaya in India. In the Indian Himalaya, the species is surviving with a few populations in Himachal Pradesh (Pulga–Kullu, Dhauladhar and Shimla), Jammu and Kashmir (Chatru and Doda) and Uttarakhand (Chakisain, Gargia-Pithoragarh, Chakrata, Raithal Harsil, Gangotri, Valley of Flowers, Kaddukhal and Dhanaulti)[1–6]. Bulbs are used for

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