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Evaluation of Phytochemical, Antimicrobial and GC-MS analysis of Extracts of *Indigofera trita* L.F. SPP. *subulata* (vahl ex poir)

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ABSTRACT

Indigofera trita L.F. SPP. *subulata* (vahl ex poir) distributed in the southern part of India particularly in Tamilnadu and it has potential medicinal properties and used in the treatment of tumours and liver disorders. The work carried out in the plant is much less, the present work was designed to investigate the preliminary phytochemical, antibacterial and GCMS analysis of various parts of the plant. The medicinally compounds from crude extracts of leaf, stem and root portions were fractionated in different solvents (aqueous, chloroform, petroleum ether and ethanol) subjected to preliminary phytochemical and antibacterial activities. The potential extracts were analysed through FTIR and GCMS. Phytochemical screening of leaves, stem and root extracts of *Indigofera subulata* revealed the presence of alkaloids, Quinones, reducing sugars, saponins, terpenoids and tannins. The ethanol extract of leaves and stem was found to exhibit activity against *Pseudomonas aeruginosa* Fourteen compounds were identified by GC-MS analysis. Phytomedicines avenues for the identifications of medicinally significant compounds with potential activity and it will lead to the isolation of potential antibiotics. Thus *Indigofera subulata* can be used as multi resistant drug in future.

Key words: Phyto-compounds, plant extracts, *Pseudomonas aeruginosa*, antibacterial activity, GC-MS

INTRODUCTION

India is the birth place of renewed system of indigenous medicine such as Siddha, Ayurvedha and Unani. Traditional systems of medicines are prepared from a single plant or combinations of more than one plant. These efficacy depends on the current taxonomic identity of plant species, use of proper plant part and its biological potency which in turn depends upon the presence of required quantity and nature of secondary metabolite in a raw drug. Traditional system of medicine continued to be widely practiced. Global estimate indicates that 80% of about 5 billion population cannot afford the products of the western pharmaceutical industry but they offered the uses of traditional medicines which are mainly derived from plant materials. In this modern world, nowadays plant based drugs are widely used and many countries contributes 40-50% of their total, health budget in the production of novel drugs (Karthishwaran *et al*., 2010; Sati *et al*., 2010).

Indigofera is a large genus of about 700 species of flowering plants belonging to the family Fabaceae. They occur throughout the tropical and subtropical regions of the world, with a few

species reaching the temperature zone in eastern Asia. The species are shrubs, though some are herbaceous and a few can become small trees up to 5-6 m (16-20 ft) tall (Bhalla and Dakwale, 1978; Augustine, 1993).

Of the various *Indigofera* species and *Indigofera tinctoria* and *Indigofera suffruticosa* are especially used to produce the dye indigo (Leite *et al*., 2003) . Several species of this group are used in anticancer therapy (Vieira *et al*., 2006). The herbs are generally regarded as an analgesic with anti-inflammatory activity. *Indigofera articulate* is used for toothache and *Indigofera oblongifolia* was used as an anti-inflammatory for insect stings, snakebites and swellings. *Indigofera aspalthoides* have also been used as anti-inflammatories (Rajkapoor *et al*., 2005). A patent was grant for use of *Indigofera arrecta* extract to relieve ulcer pain. The aim of the study was to carry out the preliminary phytochemical, antimicrobial and compound identification from the extracts of *Indigofera trita* L.F. SPP. *subulata* (vahl ex poir).

It was reported that the distribution and description of *Indigofera trita* L.F. SPP. *subulata* (vahl ex poir)-a rare plant in the kolli hill of Tamil nadu. (Ramachandran *et al*., 2006). The colour of the flower is reddish yellow. *Indigofera trita* Linn. (Family: Fabaceae) is an under shrub with wide distribution, mostly found in India, Ceylon, South Africa and North Australia (Sanjappa, 1984). The plant is known as Kattuavuri and Punal Murungai in Tamil. The entire plant is traditionally used for various ailments including liver disorders and tumors (Nadkarni, 1996; Kirtikar and Basu, 1993). The antitumour activity of ethanol Extract of Indigofera Trita L.f (EIT) was evaluated against Ehrlich Ascites Carcinoma (EAC) tumour model in Swiss albino mice on dose dependent manner (Kumar *et al*., 2007). Free radicals or oxidative injury now appears the fundamental mechanism underlying a number of human neurologic and other disorders. So it is very essential to determine the speciality of the plant with respect to the medical application.

MATERIALS AND METHODS

Plant material: The plant was collected in perumal hills Turaiyur (Trichirappalli District) Tamilnadu, South India. The plant was located at Latitude 10.2667, Longitude 77.5500, Altitude (feet) 5032 m. Plants were confirmed in the Rabinat Herbarium, St. Joseph's College, Tiruchirappalli, South India. The collected plant materials (stem, root and leaf) were air dried and grounded well to obtain a homogeneous fine-grade powder. The powder was dewaxed by treating it with petroleum ether. The treated powders were extracted with water, ethanol, petroleum ether and chloroform successively at room temperature for 3 days. Thirty gram of each powdered material was soaked in 300 mL of water, ethanol, petroleum ether and chloroform. The solvents were filtered and evaporated at 37°C under reduced pressure. The percentage yield of extracts from different solvents ranged from 5-16% w/w.

Preliminary phytochemical screening: All the extracts such as aqueous, ethanol, petroleum ether and chloroform of *Indigofera subulata* were subjected to routine qualitative chemical analysis to identify the nature of phytochemical constituents present in them.

Alkaloids: The 0.25 g of each extracts was diluted to 5 mL of 2 N HCl. Aqueous layer formed was decanted and then it was added with one or a few drops of Mayer's reagent. Formation of precipitate or turbidity formed indicates the presence of alkaloids.

Aminoacids: The 1% ninhydrin was added to the 2 mL of the test solution. Formation of blue or violet color indicates the presence of aminoacids.

Coumarins: A 10% NaOH was added with the each of the test solution. Formation of yellow color indicates the presence of coumarins.

Glycosides: To the 2 mL of test solution, 2 mL of glacial acetic acid added with one drop of ferric chloride and 1 mL of concentrated H_2SO_4. Mix well and the formation of brown greenish ring indicates the presence of glycosides.

Phenols: Few drop of 5% ferric chloride solution was added to the test solutions. Formation of intense blue color indicates the presence of phenols.

Quinones: A few drops of concentrated H_2SO_4 were added with the 0.5 g of test solution. Formation of red color indicates the presence of quinones.

Reducing sugars: To the 2 mL of the test solution, 2 mL of fehling's solution and 3 mL of distilled water was added and boiled for 2 min. The formation of reddish orange color indicates the presence of reducing sugars.

Sugars: The 0.5 g of each extract was added with very small quantity of anthrone and a few drops of concentrated H_2SO_4 and heated. Formation of green or purple color indicates the presence of sugars.

Saponins: The 2 mL of each test solution was added with H_2O and shook. Formation of foamy lather indicates the presence of saponins.

Terpenoids: The 2 mL of test solution was added with 2 mL of chloroform and a 3 mL of conc. H_2SO_4 mixed well and the formation of reddish brown indicates the presence of terpenoids.

Tannins: A 2 mL of each test solution was added with distilled H_2O and a pinch of lead acetate, formation of white precipitate indicates the presence of tannins.

Test microorganisms and growth media: Preliminary antimicrobial activities of the crude extracts of water, chloroform, petroleum ether, ethanol fractions were carried out using 50, 100 mg mL^{-1} of stock concentrations. The anti bacterial activity was carried out using 7 pathogenic micro-organisms. Gram-positive bacteria; *Bacillus subtilis, Staphylococcus aureus.* Gram-negative bacteria; *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhi.* These bacterial strains procured from the Department of Microbiology, Jamal Mohammed College, Trichirappalli, Tamilnadu, India were used for screening of antibacterial activity of *Indigofera subulata.*

Disk diffusion assay: The antimicrobial activity of the water, ethanol, petroleum ether and chloroform extracts was evaluated by the paper disk-agar diffusion method (Mutai *et al.*, 2009; Satnami and Yadava, 2011). Test plates were prepared with nutrient agar (Himedia) and then the

organisms were inoculated on the surface of the plates. In all cases, the concentration of the inoculum was adjusted to 1.5×108 cfu mL^{-1}. Sterile paper discs (10.0 mm) were aseptically impregnated with 10 µL of the resulting solutions (extract and solvents alone) and then deposited on the surface of inoculated plates. After 24 h of incubation at 37°C, the activity was determined by the presence of clear zones of inhibition around the test extracts.

FTIR analysis: Fourier transform infrared spectroscopy was used to analyse the crude leaf and stem extracts of *Indigofera subulata*. The spectrum was focused in the IR ranges between 600-4000 cm by the Kbr pellet technique. The spectrum of both the leaf and stem samples were recorded.

GC/MS analysis:

Preparation of Extracts: Crude leaf ethanol fraction of *Indigofera subulata* was subjected to routine qualitative chemical analysis to identify the nature of phytochemical constituents present in them.

GCMS conditions: The analysis of the leaf sample was carried out using a GC (GC Clarus 500 Perkin Elmer) interfaced with a Mass detector Turbo mass-Perkin Elmer equipped with an Elite-1 Dimethyl poly siloxane capillary column (30×0.25 mm×1 µmdf). The carrier gas was helium with a constant flow rate of 1 mL min^{-1} with split ratio of 10:1. The oven temperature was operated according to the following oven temperature: 110°C held for 2 min, raising at the rate of 10°C min^{-1} up to 200°C with no held, raising at the rate of 5°C min^{-1} up to 200°C with 9 min held, injector temperature and volume 250°C and 1 µL, respectively. The total GC running time was about 36 min. The MS operating conditions were ionization voltage 70 eV, source temperature of 200°C, inlet line temperature of 200°C, mass scan (m/z)-45-450, solvent delay: 0-2 min, total MS running time-36 min.

Compound identification: Compounds were identified by comparison of their mass spectra with those in the NIST 2005 library

RESULTS

Priliminary phytochemical analysis: *Indigofera subulata* was screened for the preliminary phytochemical analysis and it was summarized in the Table 1. Terpenoids were present in all the 4 different solvent fractions. But it is absent in leaf and stem extracts of chloroform fractions. Aminoacids, coumarins and phenolic compounds were completely negligible in all fractions. Glycosides were absent in ethanol and chloroform fractions of leaves. Tanins were present in leasf, stem and root extracts of water, petroleum ether and chloroform. Water and chloroform fractions of stem and root indicated the presence of saponins.

Anti-microbial activity: The antimicrobial activity of the leaf, stem and root extracts were examined against the 6 different micro-organisms in 2 different concentrations namely 50 µg mL^{-1} and 100 µg mL^{-1}. In present study, *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae* and *Salmonella typhi* showed the negligible activity against the water, ethanol, chloroform and petroleum ether extracts. Ethanol extracts of *Indigofera subulata* was found to be significantly active exhibiting the highest potency with MIC of 100 µg mL^{-1} of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The ethanol extract of leaves and stem of *I. subulata* was found to

Table 1: Priliminary phytochemical analysis of screened plant in different solvents

Solvent	Plant parts	Aminoacids	Alkaloids	Glycosides	Phenol	Quinones	Reducing sugar	Sugar	Saponin	Terpenoids	Tannin	Coumarins
Water	Leaf	---	++	+++	---	---	+++	---	---	++	++	---
	Stem	---	+++	++	---	---	+++	---	++	++	++	---
	Root	---	---	+++	---	+++	+++	---	++	+++	++	---
Ethanol	Leaf	---	---	---	---	---	+++	---	---	+++	-	---
	Stem	---	---	++	---	++	+++	---	---	++	---	---
	Root	---	---	++	---	---	+++	---	---	++	++	---
Chloroform	Leaf	---	---	---	---	---	+++	---	---	---	---	---
	Stem	---	---	++	---	---	+++	---	++	---	++	---
	Root	---	++	++	---	---	+++	++	++	++	+++	---
Petroleum ether	Leaf	---	---	++	---	---	+++	---	---	++	---	---
	Stem	---	---	++	---	---	+++	---	---	++	++	---
	Root	---	---	++	---	++	+++	---	---	++	++	---

+++: Present, ++: Present in least amount ---: Not present

Table 2: Screening for antibacterial activity of various plant extracts

		E.coli		*P.aeroginosa*		*B.subtilis*		*K.pneumoniae*		*S.typhi*		*S. aureus*	
	Plants	----------		----------		----------		----------		----------		----------	
	parts	50	100	50	100	50	100	50	100	50	100	50	100
Solvent	used	--(µg mL^{-1})--		--(µg mL^{-1})--		--(µg mL^{-1})--		--(µg mL^{-1})--		--(µg mL^{-1})--		--(µg mL^{-1})--	
Water	Leaf	---	---	---	---	---	---	---	---	---	---	---	---
	Stem	---	---	---	---	---	---	---	---	---	---	---	---
	Root	---	---	---	---	---	---	---	---	---	---	---	---
Ethanol	Leaf	---	---	---	13 mm	---	---	---	---	---	---	---	12 mm
	Stem	---	---	---	15 mm	---	---	---	---	---	---	---	---
	Root	---	---	---	---	---	---	---	---	---	---	---	---
Chloroform	Leaf	---	---	---	---	---	---	---	---	---	---	---	---
	Stem	---	---	---	---	---	---	---	---	---	---	---	---
	Root	---	---	---	---	---	---	---	---	---	---	---	---
Petroleum ether	Leaf	---	---	---	---	---	---	---	---	---	---	---	---
	Stem	---	---	---	---	---	---	---	---	---	---	---	---
	root	---	---	---	---	---	---	---	---	---	---	---	---

exhibit activity against *Pseudomonas aeruginosa* with 13-15 mm zone of inhibition and also the ethanol leaves fraction has high potential to form a zone of inhibition of about 12 mm against *Staphylococcus aureus* (Table 2).

FTIR: The compounds present *in vivo* leaf included alkanes (-CH_2-), amino acids, alkynes (RC = CH) and Halides. The following compounds were present in *in vivo* leaf explant namely secondary amides, free (NH) trans, alkenes (CHR_1 = CHR_2-Cis), sulfites, aliphatic esters (CH_3-COOR) and sulphonic acids (Table 3). The following compounds are present *in vivo* stem explant namely alkenes (CHR_1 = CHR_2), alkanes, alkynes (RC = CH), alkenes (RCH = CH_2 or $R1R_2$= CH_2), Sulphonyl chlorides, amino acids and bromides. The compounds like secondary amides, free NH (trans), amino acids, carbamates, aliphatic esters (CH_3COOR), Sulfoxides were present in *in vivo* leaf explant (Table 4).

Table 3: Infrared absorption and structure of compounds present in *in vivo* stem explant of *Indigofera subulata*

Frequency range	Intensity	Type and group	Bond
3433.30	s	Sec. amides, free NH (trans)	N-H Str.
2925.15	m	Alkanes (-CH_2-)	C-H Str.
2372.40	w	Amino acids	-
2123.25	w	Alkynes (RC=CH)	C=C Str.
1637.84	s	Carbamates, amide I band	C=O Str.
1383.61	s	Sulphonyl chlorides	S=O Str.
1255.16	v	Aliphatic esters CH_3COOR	-
1056.74	s	Sulphoides	S=O Str.
609.90	s	Bromides	C-Br Str.

s: Strong, m: Medium, v: Variable, w: Weak

Table 4: Infrared absorption and structure of compounds present in *in vivo* leaf explant of *Indigofera subulata*

Frequency range	Intensity	Type and group	Bond
3429.41	s	Sec. amides, free NH (trans)	N-H Str.
2923.20	m	Alkanes (-CH_2-)	C-H Str.
2359.59	w	Amino acids	-
2110.92	w	Alkynes (RC=CH)	C=C Str.
1638.34	s	Alkenes (CHR_1=CHR_2)	C=O Str.
1385.12	s	Sulphites	S=O Str.
1265.61	v	Aliphatic esters CH_3COOR	-
1070.16	s	Sulphoic acids	S=O Str.
610.00	s	Halides	C-Cl Str.

s: Strong, m: Medium, v: Variable, w: Weak

Table 5: Phyto-components identified by GC-MS study

RT	Name of the compound	Molecular formula	Molecular weight	Peak area (%)
11.26	l-[-]-4-hydroxy-1-methylproline	$C_6H_{11}NO_3$	145	4.77
13.30	3-O-Methyl-d-glucose	$C_7H_{14}O_6$	194	59.87
14.40	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296	2.38
16.15	N-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	2.99
16.31	Ethyl 9-hexadecenoic acid	$C_{18}H_{34}O_2$	282	0.34
16.44	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284	1.08
17.83	13-Hexyloxacyclotridec-10-en-2-one	$C_{18}H_{32}O_2$	280	1.86
18.40	Phytol	$C_{20}H_{40}O$	296	4.44
18.83	Oleic acid	$C_{18}H_{34}O_2$	282	2.60
18.97	9,12-Octadecadienoic acid, ethyl ester	$C_{20}H_{36}O_2$	308	0.53
19.06	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	$C_{20}H_{34}O_2$	306	1.45
19.43	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	312	0.27
21.67	Ricinoleic acid	$C_{18}H_{34}O_3$	298	8.33
28.87	Squalene	$C_{30}H_{50}$	410	9.08

GC MS analysis: By GCMS analysis 14 compounds were identified in *Indigofera trita* L.F. SPP. *subulata* (vahl ex poir) by GC-MS analysis. .The active principles with their Retention Time (RT), molecular formula, molecular weight and peak area (%) were presented in Table 5 and Fig. 1. The prevailing compound was 3-O-Methyl-d-glucose (59.87%), Squalene (9.08%), Ricinoleic acid (8.33%), l-[-]-4-hydroxy-1-methylproline (4.77%), N-Hexadecanoic acid (2.99%), Oleic acid (2.60%) and 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (2.38%).

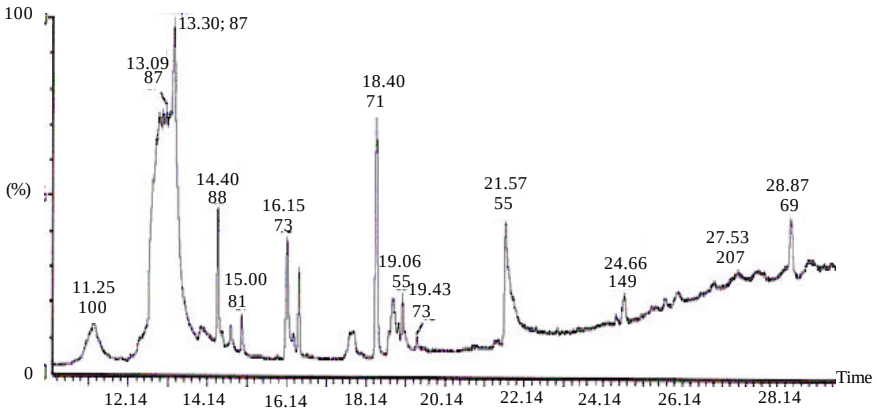

Fig. 1: Gas chromatography and mass spectroscopy (GC-MS) analysis of ethanolic leaf sample of *Indigofera subulata*

DISCUSSION

The medicinal properties of plant can be determined by the presence of one or more plant natural products. Phytochemical screening of leaves, stem and root extracts of *Indigofera subulata* revealed the presence of alkaloids, Quinones, reducing sugars, saponins, terpenoids and tannins. The preliminary phytochemical studies have revealed the presence of alkaloids, tannins, phytosterols and flavonoids in *Indigofera aspalathoides* (Rajkapoor *et al*., 2005). Plant extracts is the best source of phytochemicals and it is the natural medicine for many diseases. Among the variety of secondary metabolites in *Indigofera subulata*; tannins and terpenoids are predominant antioxidant compounds present in it Phytochemicals such as saponins, terpenoids and flavonoids have anti inflammatory and hyperglycemic activities (Cherian and Augusti, 1995). In clinical studies it has been proved that the terpenoids strengthen the skin, cures the wounds and it is used in other skin diseases. It is reported that the terpenoids have the ability to deduce the blood glucose levels in animal studies (Luo *et al*., 1999).

A number of species of *Indigofera* contain an amino acid indospicine (Christie *et al*., 1969; Aylward *et al*., 1087) and nitropropanoyl esters of glucose (Benn *et al*., 1992; Garcez *et al*., 2002) as some natural toxic product. Phytochemical analysis suggests that the presence of biologically activity compounds (alkaloids, steroids, flavanoids, proteins-lectin, carbohydrates, indigo, etc.,) in the aqueous extracts of leaves of *I. suffruticosa* could be correlated to anti-inflammatory and antimicrobial activities (Leite *et al*., 2003; Jose *et al*., 2006).

Various reports imply that the terpenoids and tannins possess the potent antimicrobial activity. The anti microbial activity of terpenoids is due to their ability to complex with extra cellular and soluble protein and to complex with bacterial cell wall. While, tannins have the potent to inactivate the enzymes responsible for microbial adhesions (Cowan, 1999). Antimicrobial resistance is a major cause of significant morbidity and mortality. Phytomedicines avenues for the identifications of compounds with antimicrobial activity and it will lead to the isolation of potential antibiotics. A novel abietane diterpenoid was identified from *Indigofera longeracemosa* with potential antituberculous and antibacterial activity (Thangadurai *et al*., 2002). This activity may be attributed to the rich tannins potent of leaves and stem of the plant.

In present study *Staphylococcus aureus* and *Pseudomonas aeruginosa* shows response against leaf and stem parts of *Indigofera subulata*. This implies that these extracts are potent antibacterial

against the multi resistant pathogens. The *Pseudomonas aeruginosa* and *Salmonella typhi* showen the zone of inhibiotion against *Schinus terebinthifolius* extracts (Moustafa *et al.*, 2007). Thus present findings support that antibacterial activity of *Indigofera subulata* different extracts were used in higher concentration (Ramachandran *et al.*, 2006). The inhibitory activity found in this plant study was more pronounced than other *Indigofera* species that reported by Nisar *et al.* (2009) where he evaluated certain plant extracts used in South-Western Nigerian unorthodox medicine against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. This may probably be due to solvents and or method of extraction used. In addition, the greater potency against *Staphylococcus aureus* and *Pseudomonas aeruginosa* was predicted. It was observed in ethanol extracts due to its ability to solubilize more antimicrobials from plants than water (Eloff *et al.*, 1998). The essential oils from *Phyllanthus amarus* exhibited strong activity against the *B. subtilis, S. aureus* and *C. albicans* (Ogunlesi *et al.*, 2009).

The over all test pathogens are sensitive to polar solvents namely ethanol and water while the extracts dissolved in non polar solvents has less antimicrobial activity. It is reported that the alcohols to be reliable and consistant solvents for the extractions of anti microbial compounds from medicinal compounds (Ahmad *et al.*, 1998).

Thermo Nicolet Corporation 2001 (http://www. scribd. com/doc/52432067/FTIRintro) publishes the FT-IR stands for Fourier infrared, the preferred method of infrared spectroscopy. In infrared spectroscopy, IR radiation is passed through a sample. Some of the infrared radiation is absorbed by the sample and some of the sample passed through (transmitted). Result of spectrum represents the molecular adsorption and transmission, creating a molecular fingerprint of the sample. The smaller retention peaks are found to be ambiqous. Identification of specific phyto constituents that are medicinally important has to be carried out for the future studies that leads to drug discovery. FTIR analysis gave results that suggest the Processing, Croom Helm, London. presence of different functional groups ranging from O-H stretching, hydroxyl (3472.7-3362.6 cmG1), C-H stretching, alkyl (2933.7-1460.6 cmG1), C = C stretching aromatic ring (1667.3-1414.4 cmG1), C-O bending and alcohols, ethers, esters, carboxylic acid and anhydrides.

Through GCMS analysis 7 bioactive compounds were identified in the methanol extracts of *Acanthus ilicifolius* leaves (Ganesh and Jannet Vennila, 2011). In present study 14 compounds were identified in which, 3-O-methyl-D-glucose (3-OMG), a nonmetabolizable glucose derivative, as a new means of providing protection for keratinocytes undergoing desiccation. We show that with decreasing water contents, viability of the cells decreases; however, at the same water content the immediate post-rehydration viability and long-term survival of the cells exposed to 3-OMG are much higher than those of controls (Norris *et al.*, 2006).

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