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Rapid mass propagation of endangered valuable medicinal plant *Salacia chinensis* L. and GC-MS/LC-MS analysis of active compounds produced in callus and leaf extracts

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Abstract

A protocol for indirect organogenesis of *Salacia chinensis* was established. Normally *S. chinensis* is propagated through seeds. However, due to the difficulty to obtain uniform plants in a short time period by seed germination, micro-propagation is a possible alternative method. For the micro-propagation, media with different concentration combinations of cytokinins and auxins were used to induce callus formation in three explants types: leaf segment, nodal segment and seeds. The rate of recurrence of callus formation from leaf on Murashige and Skoog (MS) basal medium supplemented with thidiazuron (TDZ) 0.5mg and TDZ 1mg was 100% and the maximum percentage of shoot let development from nodal segment on MS basal medium supplemented with BAP 6-(benzylamino purine) 3.5+ indole3-butyric acid (IBA) 1 mg/l was 78.3% when compared to other plant growth regulators (PGR) combinations used. The highest shoot regeneration response (85%) and the determined shoots (12.33 ± 0.33%) per callus were attained from leaf explants on MS medium containing 1-napthaleneacetic acid (NAA) 1mg/l + BAP 0.5 mg/l. The seeds showed highest percentage of shoot formation on MS medium supplemented with BAP 2mg + indole 3-acetic acid (IAA) 2mg/l and BAP 2mg + isopentenyl adenine (2iP) 2mg/l. Highest root formation (70±1.3%) was found in shoot regenerated using leaf segment on MS medium supplemented with IBA 0.5mg. The gas chromatography mass spectrometry (GC-MS) analysis of methanolic extract of callus showed more compounds at higher percentage. HPLC-MS analysis of methanolic extract of callus showed higher concentration of *Mangifera* than in leaf extracts are reported for the first time.

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1. Introduction

Salacia chinensis L. is a medicinally important plant used as herbal medicine in Ayurveda (Ghanam *et al*., 2016) and different parts of this plant have been extensively used to treat variety of ailments (Sikarwar *et al*., 2012). Biologically active compounds such as mangiferin, salacinol, kotanalol, phenolic compounds, glycosides and triterpenes have been isolated from the plant (Matsuda *et al*., 2005). For the treatment of diabetes, the compound Salacinol along with other compounds, present in *S. chinensis* is increasingly being used (Patwardhan *et*

al., 2014). Extracts of *S. chinensis* have preventive effect on various metabolic disorders (Waltenberger *et al*., 2016).

Due to the lack of proper cultivation practices, devastation of plant habitats, excessive and unselective collection to fulfil global demands, especially for its anti-diabetic property, this medicinal plant is severely threatened and categorized as endangered species (Bhagya *et al.*, 2011, Maheswari *et al*., 2016, Majid *et al*., 2016, florakarnataka.ces.iisc.ac.in., www.iucnredlist.org 2015). There is a need for its conservation and large-scale production. Very little efforts have been reported on propagation (Dhanasri *et al*., 2015; Majid *et al*., 2016). In this study, we report a reproducible and reliable micro-propagation protocol developed for its effective propagation.

2. Materials and Methods

2.1 Explants source and surface sterilization

Young and healthy leaves and nodal segments were collected from four- year old *S. chinensis* plants maintained in Botanical garden of College of Forestry, Sirsi and seeds were collected from Amruta herbals, Bargal Karwar. The undamaged collected explants were washed thrice under running tap water and were pre-washed with concentrated dish washer gel (Sodium LAS, Disodium EDTA, SLES, Concentrated Lime Juice, CI 19140, CI 42051, Water) (4-5drops/100ml double distilled water (ddH_2O) for 5minutes followed by rinsing five times in ddH_2O. Subsequently, the explants were submerged in 70% (v/v) ethanol for 2 minutes. Nodal segments were surface sterilized with 0.1% of mercuric chloride ($HgCl_2$) while leaf explants were sterilized with 1.0% (w/v) of Sodium hypochlorite (NaOCl) plus Tween 20 (2-3 drops/100ml ddH_2O) and seeds were sterilized in 2% NaOCl solution for 15minutes. The solutions were discarded and surface sterilized explants were washed thoroughly with ddH_2O for four times under aseptic conditions and placed on a sterilized petridish covered with autoclaved filter paper to remove excess moisture.

2.2 Medium composition and culture conditions

Basal medium used for inoculation was Murashige and Skoog's medium (MS) (Murashige and Skoog 1962) supplemented with 3% (w/v) sucrose and 0.8% agar. The pH of the medium was adjusted to 5.7 before autoclaving at 121°C at 15 psi pressure for 20 min for medium sterilization. The explants were cut into suitable sized pieces (1.0 cm for leaf and 1.5cm for nodal segment). Each excised explant was incubated in pre-sterilized test tubes, petridishes or suitable containers containing 20ml medium supplemented with various combinations of auxins and cytokinins.

2.3 Preparation of stock solutions of plant growth hormones (PGRs)

The desired PGRs stock solutions (1.0mg/l) were prepared by weighing 13mg of desired PGR dissolving in 13ml of ddH_2O with the help of respective solvents (Table 1). The stock solutions were stored in micro centrifuge tubes at suitable temperature (at 4°C). IAA was stored in amber coloured bottle in order to prevent photodecomposition.

2.4 Incubation conditions

Cultures were incubated at temperature of 25 ± 2°C, air humidity of 55% and a 16-h photoperiod supplied by cool-white florescent tubes.

Table 1: Preparation growth hormone

Sl. No	PGRs	Solvents	Storage conditions
1	indole-3-acetic acid (IAA)	95% ethanol or KOH	At -20° C indefinitely
2	indole-3-butyric acid (IBA)	1N NaOH or KOH	At -20° C indefinitely
3	2,4-dichlorophenoxy acetic acid (2,4-D)	1 N NaOH or KOH	At 4° C for several months or At -20° C indefinitely
4	1-napthaleneacetic acid (NAA)	95% ethanol, 1 N NaOH or KOH	At 4° C for several months or At -20° C indefinitely
5	6-benzylamino purine (BAP)	0.1 N HCl or 95% ethanol	At 4° C for several months or At -20° C indefinitely

2.5 Tissue culture studies:

2.5.1 Direct regeneration:

Shoot differentiation and shoot proliferation and rooting of regenerated shoots from nodal segment explant and seed:

Surface sterilized nodal explants were inoculated on MS medium supplemented with different growth regulators viz., gibberellic acid (GA_3) (1-3mg/l), TDZ (1-3mg/l) + kinetin (Kn) (0.5-1mg/l), Bap (1-4mg/l) + IBA (1mg/l), BAP (1-4mg/l) + IAA (1mg/l), Kn (2.5-4mg/l) + IBA (0.5-1mg/l), Kn (2.5-

4mg/l) + IAA (0.5-1mg/l), polyvinylpyrolidine (0.1%), L-Proline (0.1%) and activated charcoal (0.1%) were added to MS media to inhibit formation and release of phenolic into media. The shoots longer than 3 cm were excised from the axils and transferred into half strength MS rooting medium, supplemented with or without IAA (0.5-2mg/l), IBA (5-15mg/l), NAA (5-15mg/l). After root initiation, micro shoots were transferred to half strength MS medium supplemented with IAA (2.0mg/l) for normal growth of roots.

Seed explants were inoculated on MS medium supplemented with different combinations of PGR's (Table 1). Treatments were arranged in a completely randomized design 20 seeds per treatment one seed/tube and the experiment was repeated thrice.

2.5.2 Indirect organogenesis:

Callus induction, shoot differentiation, shoot proliferation and rooting of regenerated shoots from leaf explant:

For callus induction, the excised healthy mature leaves were inoculated on MS medium supplemented with different growth regulators viz., 0.5 or 2.0mg/l Indole-3-butyric acid (IBA) or α-naphthalene acetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D) or 6-benzylaminopurine (BAP) or thidiazuron (TDZ) or 6-(γ-γ, dimethylallyamino purine) (2iP). Leaf explants were cultured on the media in a growth chamber at 25°C under 16 h light/8 h dark photoperiod provided by white fluorescent tubes. Effect of various auxin/cytokinins on callus induction was evaluated based on the appearance and consistence of callus formation in each treatment. After 4- weeks of callus induction, only well-developed regenerating calli (2-3 mm diameter pieces) were transferred to half strength MS media supplemented with 0.5-1.0mg/l NAA in combination with 0.5-1.0mg/l 2iP or 0.5-1.0mg/l TDZ or 0.5-1.0mg/l BAP. The regenerating callus cultures were maintained in growth chambers on the same medium with controlled conditions for 8 weeks for shoot induction. There were four replicate culture flasks with 5 explants each per treatment and the experiment was repeated thrice. The mean percentage of shooting response, mean number of shoot buds formed, mean shootlet length and mean number of leaves per callus were recorded after 90 days of subculture. Two-three cm long shoots were isolated from shoot clusters from elongation medium and transferred individually to half strength MS medium supplemented with IBA, NAA or IAA (0.5-2mg/l) and incubated for 4 weeks for root development. After root initiation micro shoots were transferred to half strength MS medium supplemented with IAA (2.0mg/l) for normal growth of roots.

2.6 Preparation of leaf extract for GC-MS and HPLC-MS analysis:

The fine powdered granules from shade dried leaves of *S. chinensis* were subjected to extraction. In 400-500ml methanol about 30g of dried powdered granules were soaked and extracted at temperature between 60-65 °C by continuous hot percolation method using Soxhlet apparatus. The extraction was continued for 72 hours. The extract was then filtered and concentrated using rotary evaporator to the volume of 25ml. The dark yellowish brown concentrate was obtained (Jayaraj *et al*., 2016). This concentrate was used for phytochemical analysis using Gas chromatography mass spectrometry (GC-MS). The dark yellowish brown concentration was further dried to obtain powder which was used for High performance liquid chromatography mass spectrometry (HPLC-MS) technique analysis of mangiferin concentration in root and stem samples (Dhanasri *et al*., 2015). The experiments were repeated three times.

2.6.1 Preparation of leaf extract for GC-MS and HPLC-MS analysis:

Callus was dried at 40˚C for 24hrs, ground into powder using mortar and pestle, then subjected to extraction. About 30g of callus powder was extracted with 400-500ml methanol using Soxhlet apparatus for 72 hours at 60°C. The solution was then evaporated to dryness as mentioned in 2.6, and then the extract was stored at 4°C for future use.

2.6.2 *Mangiferin concentration analysis of leaf and callus developed from leaf explant:*

In this study we have analysed the phytoconstituents using GC-MS MS (Shimadzu model number QP2010S) equipped with Rxi- 5Sil MS fused silica capillary column (30 m X 0.25 mm ID, film thickness 0.25µm). As carrier gas pure helium gas was used at constant flow rate of ±1 mL/min. Injector temperature was set at 260 °C. The oven temperature was programmed initially at 80°C and then programmed to increase at 280°C at a rate of 10°C ending with a 20min. The spectrums of the components were compared with database of spectrum of known components stored in GC MS libraries NIST 11 & WILEY 8. Measurement of peak areas and data processing were carried out by GC MS solutions software (Sivakumar & Dhivya 2015).

The concentration of mangiferin was determined using HPLC-MS (Shimadzu LC-10 AT vp) equipped with luna 5u C18 analytical column (250 x

4.6 mm) and SPD-M10A vp photo diode array detector (PDA). Mobile phase used for mangiferin quantification was A: Acetonitrile (15%) and B: 0.1% Ortho Phosphoric acid (85%). The 20µl (1mg sample dissolved in 1ml methanol) of sample was injected for flow of 1ml/min with total 12min run time at wavelength of 257nm. Calibration was carried out using standard Mangiferin (Jayaraj *et al*., 2016) in callus part derived from leaf explant for comparative analysis of mangiferin between plants collected naturally and callus culture of *S. chinensis*.

2.7 Statistical analysis:

All the experiments described above were conducted in a completely randomized design and repeated thrice. All the tissue culture experiments such as callus induction, shoot regeneration, shoot number and shoot length under different plant growth regulator concentrations were computed and analysis of variance (ANOVA) was used for data analysing using the IBM SPSS statistical package (Version 20.0. Armonk, NY, USA:IBM Corp.) The significant differences between means were scored using Duncan's Multiple Range Test ($P = 0.05$).

3. Results

3.1 Leaf explants:

Leaf, nodal and seed explants cultured on MS medium without PGRs failed to produce shoots even after 4 weeks of inoculation. Whereas swelling and expansion was observed in MS medium supplemented with PGR's and callus induction was started at the cut ends after 6-7 days of incubation of leaf segment explants of *S. chinensis*. Two types of calli were formed at the cut ends of leaf explants. One comprises of soft, white calli and the other has nodular, greenish white calli (Plate 1a). The maximum frequency (100%) of callus formation was observed with explants supplemented with TDZ (0.5 and 1mg/l) compared to other PGRs. Callus induction was decreased when higher concentration of PGRs were used. The callus obtained was regularly sub cultured on fresh medium with the same PGR concentration. The callus thus formed was transferred to regeneration medium (plate 1b-d). Maximum rooting (8.5±0.28%) was observed on half strength MS medium supplemented with IAA 0.5mg/l. The different PGRs treatments used and mean percentage of callus induction was represented in table 2.

Table 2: In vitro response of leaf explants to various concentration of plant growth hormones (PGR)

Concentration of plant growth regulators (PGRs) mg/l						No. of explants inoculated	Mean percentage of Callus induction (%)
BAP	TDZ	2,4 D	IBA	2iP	NAA		
0	0	0	0	0	0	20	0.0
0.5	-	-	-	-	-	20	65
1	-	-	-	-	-	20	70
2	-	-	-	-	-	20	55
-	0.5	-	-	-	-	20	100
-	1	-	-	-	-	20	100
-	2	-	-	-	-	20	90
-	-	0.5	-	-	-	20	35
-	-	1	-	-	-	20	40
-	-	2	-	-	-	20	30
-	-	-	0.5	-	-	20	0.0
-	-	-	1	-	-	20	25
-	-	-	2	-	-	20	15
-	-	-	-	0.5	-	20	50
-	-	-	-	1	-	20	55
-	-	-	-	2	-	20	45
-	-	-	-	-	0.5	20	40
-	-	-	-	-	1	20	42.5
-	-	-	-	-	2	20	35

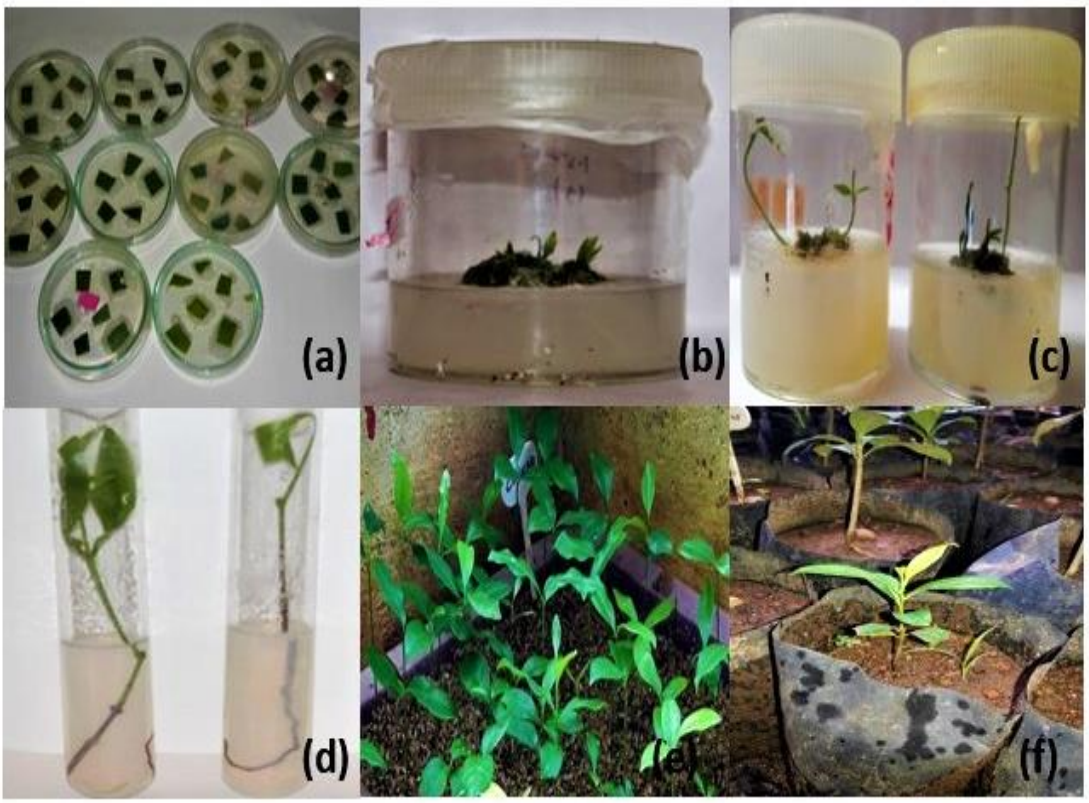

Plate 1: Callus morphogenesis and shoot bud regeneration of *Salacia chinensis*: (a) The induced leaf callus tissue on MS medium supplemented with TDZ (0.5mg/l) (b) Adventitious shoot bud regeneration with primary callus (c) Emerging of multiple shoots (d) rooting of the regenerated shootlet (e) Hardening of explant bio compost nutrients f) Hardened plantlets with thick roots were transferred into polythene bags containing bio compost nutrients

3.1.1 Regenerating callus and shoot:

Soft, green nodular organogenic callus sub-cultured on MS medium with different combinations of BAP, TDZ and IBA in combination with NAA (0.5-1mg/l) produced embryogenic callus and shoots were induced after few days. Maximum shoot regeneration was observed when callus cultured on MS medium supplemented with BAP (1.0mg/l) along with NAA (0.5mg/l). Responses of different concentration of PGRs on shoot bud regeneration from callus cultures, mean of shooting response, mean number of shoot buds observed and mean height of shootlet of *S. chinensis* were listed in Table 3. Elongated shoots were then transferred to half strength MS medium containing different concentration of PGR's i.e., IBA, IAA or NAA (0.5-1mg/l) (Table 4).

3.2 Nodal explants:

Nodal explants cultured for regeneration, on full strength MS medium supplemented with different combinations of PGRs i.e. high concentration of GA_3 (2-3mg/l), TDZ + Kn (1-3+0.5mg/l), Kn + IBA (2.5-3+0.5-1mg/l), Kn + IAA (2-3+0.5-1mg/l) showed significant changes in the first week of inoculation *i.e.*, small protuberance. Plate 2 shows different stages of micro propagation of *S. chinensis* L nodal explants. The percentage of explants showing the same response at the axils is more or less similar in all the PGRs combinations used. The sprouts in the axils increased in size to form shootlets in 2 weeks. The maximum percentage of shootlets development was observed on the phytohormones combinations BAP 3.5mg/l with combination of IBA 1mg/l (78.3%) and BAP 3mg/l with combination of IAA 1mg/l (43%) when compared to other PGRs combinations used. Full strength medium supplemented with GA_3 (0.5-1.5mg/l) induced shoots but the shoots were dwarf.

Full strength medium supplemented with different concentration of BAP in combination with IAA or IBA induced shoot formation but BAP 3.5 mg + IBA 1 mg gave better results in regeneration of number of shoots 15±0.6 and enhancement of shoot elongation (table 5, Plate 2b). The shootlets increased in length during the 3rd week and the maximum shoot length of 2.83 ± 0.44 was observed in BAP 3.5mg/l + IBA 1mg/l.

Table 3: Responses of different concentration of plant growth regulators (PGR) on shoot bud regeneration from callus cultures of S. *chinensis*

Mean of shooting response %				Mean of Shooting response %	Mean number of shoot buds/explant (cm) ± SE	Mean height of shootlet (cm) ± SE	Mean number of Leaves per explant
BAP	**TDZ**	**2iP**	**NAA**				
0	0	0	0	0.0	0	0	0
		0.5	**0.5**	35	2 ± 0.57	1.2 ± 0.2	3.5 ± 0.1
		1	**1**	30	1.6 ± 0.33	1.8 ± 0.4	3 ± 0.23
		1	**0.5**	40	2.3 ± 0.33	2 ± 0.11	6 ± 0.11
	0.5		**0.5**	75	8 ± 1	2.8 ± 0.11	7.5 ± 0.28
	1		**1**	62	5.3 ± 0.33	2.4 ± 0.1	5.8 ± 0.23
	1		**0.5**	80	10 ± 0.57	3.8 ± 0.11	11.7 ± 0.78
0.5			**0.5**	70	7 ± 0.57	3.2 ± 0.11	5.4 ± 0.2
1			**1**	60	4 ± 0.57	2.6 ± 0.11	5 ± 0.11
1			**0.5**	85	12.3 ± 0.33	3.4 ± 0.11	7 ± 0.28

Table 4: Response of different PGRs (Auxins) on the induction of rooting on half strength MS medium

Concentration of plant growth regulators (mg/l)		Percentage of explants rooted (%)	Mean number of rootlets per explants ± SE	Mean root length ± SE
Control	0	0.0	0	0
IBA	0.5	70	7 ± 0.23	2.8 ± 0.11
IBA	1	30	4 ± 0.11	2 ± 0.11
IBA	2	25	2 ± 0.23	1.8 ± 0.11
IAA	0.5	75	8.5 ± 0.28	3 ± 0.11
IAA	1	38	6 ± 0.41	2.4 ± 0.23
IAA	2	25	3± 0.41	2.2 ± 0.23
NAA	0.5	28	3.4 ± 0.30	1.9 ± 0.28
NAA	1	22	2.4 ± 0.30	1.6 ± 0.23
NAA	2	20	1.9 ± 0.2	1.4 ± 0.23

The number of explants inoculated per each treatment, mean number of explants showing bud emergence, mean number of explants showing shoot elongation, number of explants rooted and number of explants hardened were listed in Table 5. Opening of small leaves from the shootlets was observed during 4th week (Plate 2b). In few explants, the explant started yellowing from the bottom and the yellowing reached to top, causing death of that explant in 8-9 weeks. Shoots longer than 3cm, with leaves opened were transferred to half MS rooting media, supplemented with different concentrations of PGRs *i.e.* IBA (0.5-1mg/l) or NAA (0.5-1mg/l) or IAA (0.5-1mg/l). Maximum number of rooting observed in half MS media supplemented with IAA 0.5mg/l. small roots were initiated in explants that were grown on BAP (3.5mg/l) + IBA (1mg/l) (Plate 2c) where the explants from all other PGRs combinations failed to root and they showed yellowing of leaf and browning of tissue.

3.3 Seed explant:

Seed explants cultured on full strength MS medium supplemented with BAP (1-3mg/l) with different combinations of PGRs *i.e.* IAA (1-3mg/l) or 2iP (2mg/l) or NAA (1-3mg/l). Even at the lowest concentration BAP alone induced adventitious shoot bud from seed explants. However, The seeds showed highest percentage of shoot on MS medium supplemented with BAP 2mg + IAA 2mg/l and BAP 2mg + 2iP 2mg/l whereas the former medium induced shoot regeneration after the callusing of the

explant, the latter stimulated direct shoot formation. (Table 6, Plate 3a). The combination of BAP with IAA or NAA exhibited formation of shoot buds. However, the differentiation of shoots was preceded by callusing of the explants. At lower concentration, IAA (68%) and NAA (1mg/l) (64%) direct shoot buds regeneration was observed with an occurrence comparable to BAP (3mg/l) + 2iP (2mg/l). Nevertheless, the intensity of shoot bud formation was high with the latter treatment (Table 6). Shoots were then transferred to MS medium containing only BAP 1mg/l for well and healthy development. On this medium shoots were well developed and attained height of 2.63 ± 0.29 after 20 days (Plate 3b). For the initiation of roots shoots were individually transferred to MS medium supplemented with NAA 0.5mg/l. In this treatment 90% of shoots produced roots directly at the bottom within 7 days, with an average of five roots per explants (Plate 3c).

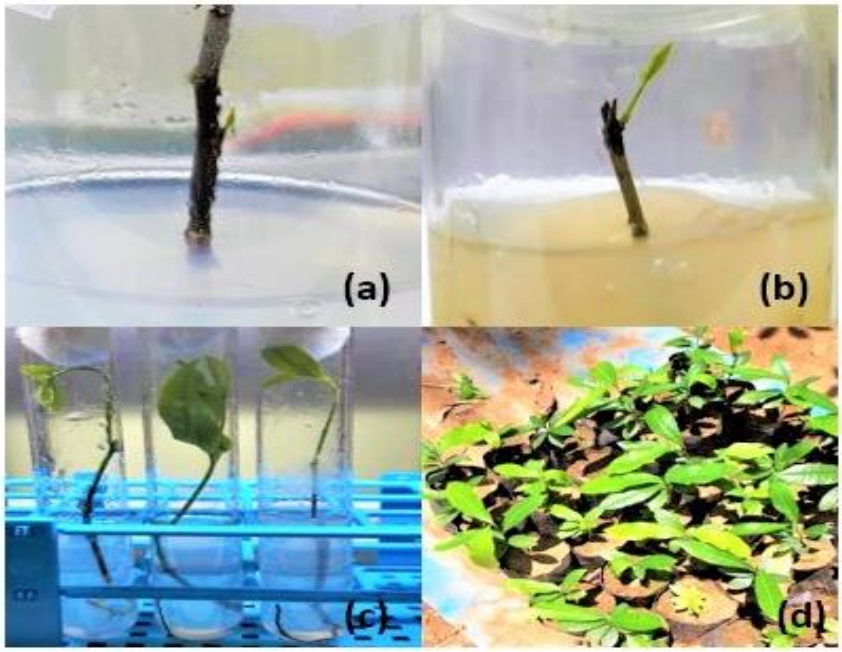

Plate 2: Different stages of micro-propagation of S. chinensis L. using nodal segments:

(a) Bud emergence from axils of nodal explants supplemented with BAP (3.5mg/l) + IBA (1mg/l)

(b) initiation of shoot elongation from emerged axillary buds

(c) rooting observed on MS medium supplemented with IAA (0.5mg/l)

(d) Acclimatized plantlets with thick roots were transferred to polythene bags

Plate 3: Different stages of micro-propagation of S. chinensis L. using seed explants:

(a) shoot bud emergence from seed explant supplemented with BAP (3mg/l) + 2iP (2mg/l),

(b) (c) rooting observed on MS medium supplemented with NAA (0.5mg/l)

(d) Hardened plants

Table 5: In-vitro response of nodal segment explants to various concentration of PGR

Phytohormone used (mg/l)	No. of explants inoculated	Mean No. of explants showing bud emergence ± SE	Mean No. of explants showing shoot elongation ± SE	No. of explants rooted	No. of explants hardened
GA_3 (0.5)	20	1.6 ± 0.8	0.6 ± 0.3	0	0
GA_3 (1)	20	4.6 ± 0.6	1.63 ± 0.27	0	0
GA3 (1.5)	20	1.3 ± 0.8	0.3 ± 0.3	0	0
GA3 (2)	20	0	0	0	0
GA3 (2.5)	20	0	0	0	0
GA3 (3)	20	0	0	0	0
TDZ (1) + Kn (0.5)	20	0	0	0	0
TDZ (2) + Kn (0.5)	20	0	0	0	0
TDZ (3) + Kn (0.5)	20	0	0	0	0
TDZ (1) + Kn (1)	20	1.6 ± 0.3	0.3 ± 0.3	0	0
TDZ (2) + Kn (1)	20	4.3 ± 0.3	0.76 ± 0.14	0	0
TDZ (3) + Kn (1)	20	3.3 ± 0.8	1.6 ± 0.6	0	0
BAP (1) + IBA (1)	20	0	0	0	0
BAP (2) + IBA (1)	20	0	0	0	0
BAP (2.5) + IBA (1)	20	2.3 ± 0.3	1	0.3 ± 0.3	0.3 ± 0.3
BAP (3) + IBA (1)	20	8.6 ± 0.6	2.6 ± 0.2	4.6 ± 0.6	2.6 ± 0.3
BAP (3.5) + IBA (1)	20	15.6 ± 0.6	2.8 ± 0.4	10 ± 1	8 ± 1
BAP (4) + IBA (1)	20	0	0	0	0
BAP (1) + IAA (1)	20	0	0	0	0
BAP (2) + IAA (1)	20	2.3 ± 0.3	1	0	0
BAP (2.5) + IAA (1)	20	5 ± 1.1	1.73 ± 0.3	0	0
BAP (3) + IAA (1)	20	8.6 ± 0.6	1.9 ± 0.8	0	0
BAP (3.5) + IAA (1)	20	0	0	0	0
BAP (4) + IAA (1)	20	0	0	0	0
Kn (2.5) + IBA (0.5)	20	0	0	0	0
Kn (2.5) + IBA (1)	20	0	0	0	0
Kn (3) + IBA (0.5)	20	0	0	0	0
Kn (3) + IBA (1)	20	2.6 ± 0.3	1.46 ± 0.26	0	0
Kn (4) + IBA (0.5)	20	7.6 ± 0.8	1.4 ± 0.23	0	0
Kn (4) + IBA (1)	20	4.3 ± 0.3	0.9 ± 0.1	0	0
Kn (2.5) + IAA (0.5)	20	0	0	0	0
Kn (2.5) + IAA (1)	20	0	0	0	0
Kn (3) + IAA (0.5)	20	0	0	0	0
Kn (3) + IAA (1)	20	3.3 ± 0.8	1.33 ± 0.57	0	0
Kn (4) + IAA (0.5)	20	9.3 ± 0.3	1.0 ± 0.11	0	0
Kn (4) + IBA (1)	20	5 ± 1	1.23 ± 0.14	0	0

*Significant at $p \leq 0.05$ level of probability

Table 6: In -vitro response of seed explants to various concentrations of PGR

No. of explants inoculated				No. of explants used	Percentage of Seed germinated (%)	Percentage of Culture with adventitious shootlet		Mean no. shootlets per explants ± SE	Mean height of shootlet ± SE
BAP	2iP	NAA	IAA			direct	Through callus		
1				25	0	20.92	0	2.43 ± 0.29	1.46 ± 0.29
2				25	0	25.32	0	3.33 ± 0.66	1.7 ± 0.20
3				25	0	33.04	0	5.33 ± 0.29	2.0 ± 0.23
1	2			25	0	41	0	6.1 ± 15	2.1 ± 0.17
2	2			25	0	54.12	0	6.6 ± 0.30	2.4 ± 0.23
3	2			25	0	73	0	12.3 ± 0.40	2.63 ± 0.29
1			1	25	0	68	0	8.5 ± 0.28	2.5 ± 0.28
2			2	25	99.32	0	64	7.33 ± 0.35	2.0 ± 0.1
3			3	25	94.64	0	60	6.1 ± 0.20	1.9 ± 0.1
1		1		25	0	32.24	0	5.13 ± 0.29	1.83 ± 0.12
2		2		25	82.5	0	32	4.8 ± 0.24	1.6 ± 0.32
3		3		25	75	0	28.2	4.6 ± 0.23	1.5 ± 0.36

After 5 weeks of incubation in light, the rooted shoots were carefully removed from culture vessels and washed in running tap water to free agar. Healthy rooted shoots were transferred to plastic cups containing sand: soil: vermiculite in the proportion of (1:1:1) and covered with polythene covers to maintain humidity under aseptic conditions inside the culture room. After 2 weeks the bag was carefully removed and transferred to earthen pots with garden soil (plate 1f, 2d and 3d).

3.4 GC-MS and HPLC-MS analysis of leaf and callus extract of S. chinensis

S. chinensis leaf methanolic extract were analysed using GC-MS technique, their chemical composition showed 20 components. Table 7 and Figure 1a shows that most copious component is phytol.

Table 7: Comparison between phyto-components identified in methanol extract of leaf parts and callus obtained from *S. chinensis* using GC-MS peak report TIC

IUPAC name; molecular formula	Area %	
	Leaf	Callus
Phenol, 2,4-bis(1,1-dimethylethyl)-; $C_{14}H_{22}O$	7.42	6.11
Octadecane; $C_{18}H_{38}$	--	0.39
3-Hexadecanol; $C_{16}H_{34}O$	--	0.59
Neophytadiene; $C_{20}H_{38}$	--	2.35
3,7,11,15-Tetramethyl-2-hexadecen-1-ol; $C_{20}H_{40}O$	--	0.83
3-Heptadecanol; $C_{17}H_{36}O$	1.09	--
Hexadecanoic acid, methyl ester; Methyl hexadecanoate	1.64	1.73
Hexadecanoic acid; $CH_3(CH_2)_{14}COOH$;	16.37	5.04
Cyclohexanol, 1-butyl-; $C_{10}H_{20}O$	--	0.39
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-; $C_{19}H_{32}O_2$;	--	1.28
Phytol; $C_{20}H_{40}O$	29.69	14.12
Tetratriacontane; $C_{34}H_{70}$	--	1.26
Tetracosanoic acid, methyl ester; $C_{25}H_{50}O_2$	1.37	--
cis,cis,cis-7,10,13-Hexadecatrienal; $C_{16}H_{26}O$	3.45	--
Octadecanoic acid; $C_{17}H_{35}CO_2H$	2.33	0.53
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester; $C_{19}H_{38}O_4$	27.26	8.95
Squalene; $C_{30}H_{50}$	2.34	40.32
dl-.alpha.-Tocopherol; $C_{29}H_{50}O_2$	--	15.60
Octadecanoic acid, 2,3-dihydroxypropyl ester	7.04	--
(Z,Z)-6,9-CIS-3,4-Epoxy-nonadecadiene	--	0.52

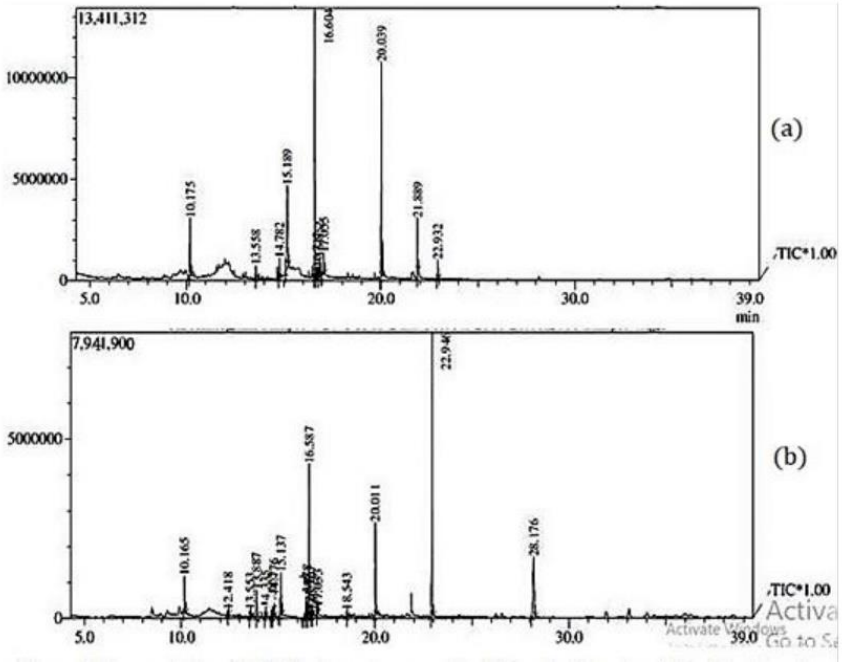

Figure 1: Representative of GC-MS chromatograms of leaf (a) and callus extract (b) of *S. chinensis*

HPLC analysis has shown the presence of mangiferin in both leaf and callus extracts of *S. chinensis* (Figure 2). The study showed that *S. chinensis* callus extract (26mg/ml) contain higher quantity of mangiferin as compared to leaf extract (4mg/ml) (Figure 3).

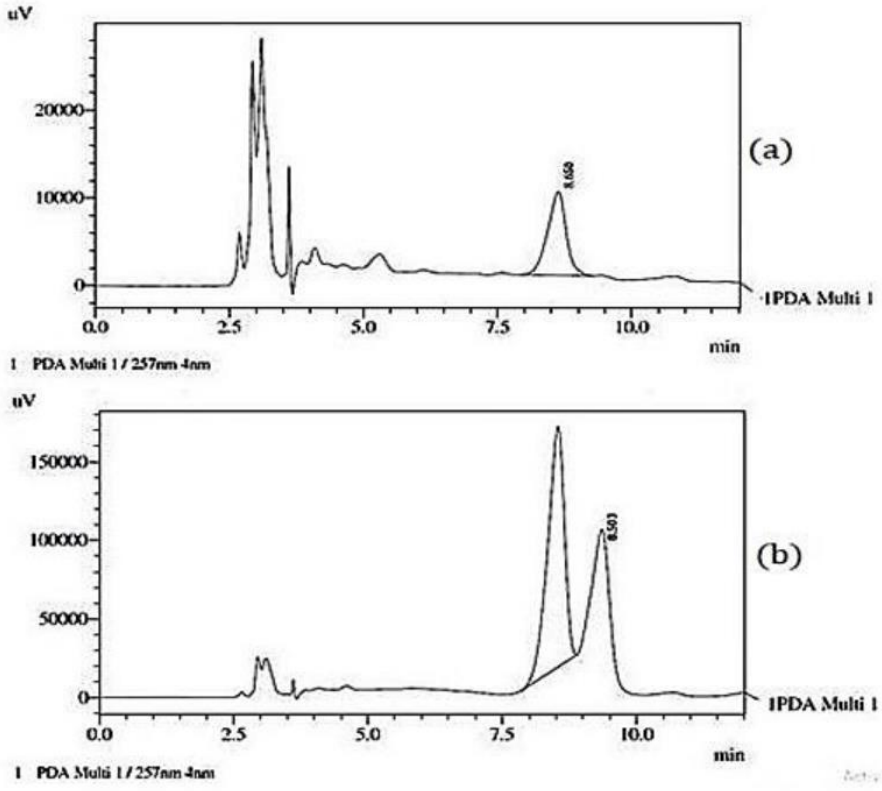

Figure 2: Representative of HPLC-MS chromatograms of leaf (a) and callus extract (b) *S. Chinensis*

Table 7 and Figure 1b shows that the most abundant compound is squalene. The extract also contains hexadecanoic acid (5.04%), Phenol, 2, 4-bis(1,1-dimethylethyl) (6.11%), squalene (40.32%). Phytochemical dl-.alpha.-Tocopherol (15.60%) was found only in callus and has great therapeutic value. The extract also contains hexadecanoic acid (16.35%), Phenol, 2, 4-bis(1,1-dimethylethyl) (7.45%), squalene (2.38%) as major components. The chemical composition of callus extract of *S. chinensis* showed 16 components.

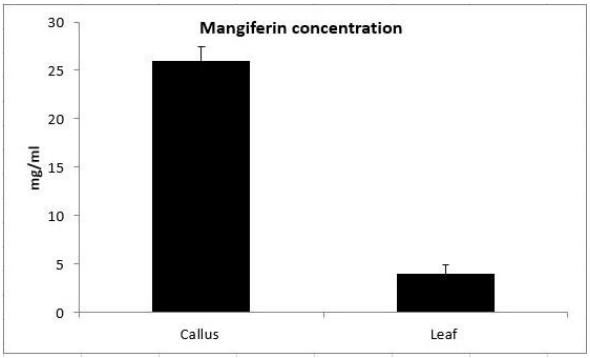

Figure 3: Comparison of Mangiferin concentrations (mg/ml) in the methanol extracts of the stem and root parts of *Salacia* species.

4. Discussion

The present study comprehensively investigated the effects of various kinds and concentrations of PGRs on 3 different explants of *S. chinensis* micro-propagation. The ability to regenerate complete plants from mature leaf derived callus, nodal segment and seeds of *S. chinensis* has been found.

Leaf explants and nodal explants are the most suitable plant source for regeneration. BAP among the different cytokinins that have the higher potential for both shoot and root induction and IAA for root initiation among the auxins (Elangomathavan *et al*., 2017). An increase in the auxin concentration with the higher concentration of cytokinins resulted in the production of callus at cut ends of leaf segments, proliferation of shoot and root for the leaf derived callus, nodal segment and seed explants which may be due to the cell proliferation stimulated by the accumulation of auxins in the presence of cytokinins (Marks and Simpson 1994; Dhanshri *et al*., 2015). In the present study, cytokinins promoted intensive shoot multiplication. Cytokinins are reported to overcome apical dominance, induce high number of shoot buds and also release lateral buds from dormancy (Rout *et al*., 2000; Rout, 2005).

The MS medium supplemented with BAP 3.5 mg/l + IBA 1 mg/l and NAA 0.5mg/l +TDZ 0.5mg/l resulted highest number of shoot elongation and root formation. The negative effect on shoot regeneration was observed when the MS medium supplemented with BAP 4 mg/l and continued exposure of explants to high concentration of BAP during shoot induction inhibited the growth of shoots. The results of the present study are in accordance with Deepak *et al*., (2015) with the use of BAP for shoot multiplication. The presence of auxins at lower concentration in medium facilitated better root formation. MS medium supplemented with 15 mg/l IAA was found superior to IBA and NAA in inducing roots.

In our previous study we have analysed the phytoconstituents and mangiferin concentration in different species of *Salacia* (Kamat *et al*. 2019). We have noticed that the leaf, stem and root extract of *S. chinensis* showed the higher concentration of mangiferin compared to other species. Although many compounds were reported from different species of *Salacia*, it is an eminent fact that mangiferin is important for its antidiabetic property and is a well explored molecule in *in vitro* and preclinical studies. Mangiferin projected to be the most active principle from *Salacia* spp.

The anti-inflammatory activity and anti-arthritic activity of 9,12-octadecadienoic acid were reported (Murray 2012). The anti-inflammatory, antimicrobial, and antioxidant activity of phenol, neophytadiene, 9,12-octadecadienoic acid and dl-.alpha.-Tocopherol were reported (Frank 1962; Owen *et al*., 2000; Maruthupandian and Mohan 2011). Based on the literature, above compounds could effectively contribute to the antibacterial activities of selected plants.

The phenolic compounds are known to be synthesized by plants in response to microbial infection. It is therefore possible that they can act as effective antimicrobial substances against a wide array of microorganisms. However, the antimicrobial activity of plant extracts depends not only on phenolic compounds but also by the presence of different secondary metabolite (Gordana *et al*., 2007) like hydroxyl groups on the active constituents, because these substances are capable of binding to bacterial adhesions and interacting with the supply of receptors on the surface. The phenolic compound observed in this study is phenol, 2,4-bis (1,1-dimethylethyl) in natural as well as callus extract.

5. Conclusions

In the present study, the successful protocol for callus induction and shoot regeneration from leaf derived callus of *S. chinensis* has been established. About 64% seeds exhibited regeneration. Leaf

explants and nodal segments are the most suitable plant materials for regeneration. The protocol presented in this study may provide a high efficiency regeneration system for successful regeneration of adventitious shoots for *ex situ* preservation of *S. chinensis* as well as genetic improvement studies for pharmaceutical uses and future research investigations. This optimized protocol helps in providing a promising method for the large scale propagation of this species. Like mangiferin, other compounds identified in leaf and callus extract have their own biological importance and further study of this plant's phytochemicals by *in silico* and *in vitro* methods can prove its medicinal importance in future and can be effective and efficient drug source.

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References

Bhagya, N., Sheik, S., Sharma, M.S., Chandrashekar, K.R. (2011) Isolation of endophytic colletotrichum gloeosporioides penz. From *Salacia chinensis* and its antifungal sensitivity. *Journal of Phytology* 3(6):20-2.

Deepak, K.G.K., Suneetha, G., Surekha (2015) In vitro clonal propagation of *Salacia oblonga* WALL an endangered medicinal plant. *Annals of phytomedicine* 4(2): 67-7

Dhanasri, G., Srikanth, R.M., Naresh, B., Pratibha, Devi. (2015) Molecular genetic analysis of *Salacia reticulata*, a threatened medicinal plant for the study of genetic diversity. *International Journal of Pure and Applied Bioscience* 3(1): 92-99.

Elangomathavan, R., Beaulah, N., S, Hariharan., P, Kalaivanan. (2017) Indirect shoot organogenesis from leaf explants of threatened medicinal plant *Cleistanthus collinus. International Journal of Advanced Research in Biological Sciences* 4(1):58-68.

Frank, D. Popp. (1962) Synthesis of Potential Anticancer Agents. VIII. Benzaldehyde Mustard Derivatives and Related Compounds *Journal of Medicinal Chemistry* 5 (3):627-629.

Ghanam, K., Srivastava, V., Deshpande, J., Juturu, V. (2016) *Salacia chinensis* extract (SCE) modulates carbohydrates and lipid metabolism: in vitro and in vivo models. *International Journal of Endocrinology and Metabolism*: 3(6): 1-8.

Herbarium JCB. Flora of Karnataka. Indian Institute of Science. *florakarnataka.ces.iisc.ac.in.*

IUCN red list of threatened species (2015). *www.iucnredlist.org*

Jayaraj, R., Sasidharan, N., Beenu, T., Muhammad, A. 2016: Comparative phytochemical profiling and quantification of mangiferin content in species of *Salacia* from Southern Western Ghats of India. *Journal of Biologically Active Products from Nature*., 6(3): 209-222.

Kamat, S.G., Vasudeva, R., Patil, C.G. (2019) GC-MS and LC-MS based phytochemical profiling and quantification of mangiferin in six species of *Salacia* from the Western Ghats of India. *South Asian Journal of Experimental Biology* 8(4):132-148.

Maheswari, J. (2011) Patenting Indian medicinal plants and products. Indian *Journal of Science and Technology* 4(3):298-301.

Majid, B.N., Sampath, K.K.K., Prakash, H.S., Geetha, N. (2016) Rapid mass propagation of *Salacia chinensis* L., an endangered valuable medicinal plant through direct organogenesis. *Indian Journal of Science and Technology* 9(4).

Marks, T.R., and Simpson, S.E. (1994) Factors affecting shoot development in apically dominant Acer cultivars in vitro. *Journal of Horticultural Science* 69: 543-551.

Maruthupandian, and V, R. Mohan. (2011) GC-MS analysis of some bioactive constituents of *Pterocarpus marsupium* Roxb. *International ournal of. Chemtech. Research.* 3 (3), 1652-1657.

Matsuda, H., Yoshikawa, M., Morikawa, T., Tanabe, G., Muraoka, O. (2005) Antidiabetogenic constituents from *Salacia* species. *Journal of Traditional Medicine*: 22 (1):145-53.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bio assays with tobacco tissue culture. *Physiologia plantarum* 15:473-497.

Murray, J.A.H., Jones, A., Godin, C., Traas, J. (2012) Systems analysis of shoot apical meristem growth and development: integrating hormonal and mechanical signalling. *Plant Cell* 24:3907–3919

Patwardhan, A., Pimputkar, M., Joshi, R. (2014) Evaluation of anti-diabetic property of extracts of different plant parts of *Salacia chinensis* Linn. *Journal of Biodiversity, Bioprospecting and Development* 1:107.

R.W. Owen, A. Giacosa, W.E. Hull, R, Haubner., B, Spiegelhalder., and H, Bartsch. (2000) the antioxidant/anticancer potential of phenolic compounds isolated from olive oil. *European Journal of Cancer* 36(10):1235-47.

Rout, G.R. (2005) Micropropagation of *Clitoria ternatea* Linn. (Fabaceae) -An important medicinal plant. *In vitro Cellular and Developmental Biology. Plant* 41:516-519.

Rout, G.R., Samantaray, S., Das, P. (2000) *In vitro* manipulation and propagation of medicinal plants. *Biotechnology Advances* 18:91-120.

S, C. Gordana., M, C. Jasna., M, D. Sonja. (2007) Antioxidant potential, lipid peroxidation inhibition and antimicrobial activities of Satureja montana L. Subsp. kitaibelii extracts.

International Journal of Molecular Science 8:1013-1027.

Sikarwar, M.S., and Patil, M.B. (2012) Antihyperlipidemic activity of *Salacia chinensis* root extracts in triton-induced and atherogenic diet-induced hyperlipidemic rats. *Indian Journal of Pharmacology*: 44(1): 88-92.

Sivakumar R, Dhivya A (2015) GC-MS analysis of bioactive compounds on ethyl acetate extract of *Cordia Monoica* Roxb. Leaves. IJRDPL 4(1): 1328-1333.

Waltenberger, B., Mocan, A., Smejkal, K., Heiss, E.H. (2016) Natural products to counteract the epidemic of cardiovascular and metabolic disorders. *Molecules* 21:807.