Original Research Article

Phytochemicals analysis in callus, tissue-cultured and conventionally propagated plant roots, and cell culture of *Plumbago indica* L.

ABSTRACT

Aims: *Plumbago indica* L. commonly known as rose-coloured leadwort in English is widely used in the treatment of rheumatism, paralysis, leprosy, headache, leukoderma, enlarged glands, scorpion-sting, cancer, ophthalmia, secondary syphilis, dyspepsia, haemorrhage, piles, flatulence and loss of appetite *etc*. in traditional medicinal systems. Plumbagin is one of the major phytochemicals that is responsible for most of the above biological activities of *P. indica*. This plant is gradually decreasing from ~~their~~ its~~natural~~ natural habitats due to over exploitation for medicinal purposes. The use~~Use~~ of tissue culture plants for mass scale cultivation and phytochemical extraction can protect *P. indica* that grows in the natural environment. The development of cell culture allows extracting plumbagin without growing plants in the field.

Study design: Quantitative and qualitative comparison of phytochemicals in tissue cultured and conventionally propagated *P. indica* plant roots and callus was done using HPLC and GC-MS analysis.

Place and Duration of Study: Department of Botany, Faculty of Applied Sciences, University of Sri Jayewardenepura, Sri Lanka, between July 2018 and April 2022.

Methodology: The amount of plumbagin present in eight months old field-grown tissue cultured and conventionally propagated (shoot cutting) plant roots, callus, and cell culture were quantified by HPLC analysis and phytochemicals were identified qualitatively using GC-MS analysis.

Results: Conventionally propagated plant root extract had the highest plumbagin content 23.53 ± 5.34 µg/mL when compared with tissue cultured plant roots (12.90 ± 2.42 µg/mL), callus (0.009 µg/mL), cell culture pellet (0.015 ± 0.01 µg/mL) and cell culture supernatant extract (4.23 ± 2.07 µg/mL). The GC-MS analysis revealed that conventionally propagated plant root extract contained a higher number of phytochemicals (16) while tissue cultured plant roots contained only four. Callus extract contained only n-Hexadecanoic acid. Nine phytochemicals were detected in cell culture pellet extract while only three phytochemicals were detected in the supernatant.

Conclusion: Tissue cultured *P. indica* roots can be also used as a source to extract plumbagin in mass scale, yet conditions need to be optimized in order to produce high level of plumbagin. The study ~~Study~~ confirmed not only the possibility of obtaining plumbagin through cell cultures in a bioreactor system but also products that are mainly secreted into the medium.

Keywords: Plumbago indica, Plumbaginaceae, Plumbagin, Phytochemicals, ~~cell~~ Cell culture.

1. INTRODUCTION

Plumbago~~-~~ indica (Plumbaginaceae) is a perennial herb or small shrubthat grows well in warm tropical climates. This plant was originated in the Sikkim and Khasi hills of India and migrated to other neighboring countries including Sri Lanka. This plant is cultivated

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worldwide due to its valuable medicinal root system [1]. This plant contains different phytochemicals that belong to alkaloids, flavonoids, saponins, glycosides, and tannins [2]. Different compound natures of phytochemicals have possessed a wide range of biological activities. Tannins have been found to possess wound healing, antibacterial, anti-inflammatory, hypoglycemic and antidiarrheal activity. Flavonoids are free radical scavengers that protect cells from oxidative cell damagers and show antidiarrheal, anti-inflammatory and hypoglycemic, anticancer, anti-allergic, and antimicrobial activities. Alkaloids are heterocyclic indole compounds that possessed anti-inflammatory, hypoglycemic, hypotensive, anticonvulsant, antiprotozoal, antimicrobial, and antimalarial activities [3].

~~*P. indica*~~~~aerial~~ Aerial parts of *P. indica* contain active chemical compounds like 7-0-gencsides, amyrin, palmitic acid, beta-sitosterol [4], stigmasterol, campesterol, Plumbagin, 6-hydroxy plumbagin, plumbaginol. Red colour inflorescence of this plant contains pigments such as pelargonidin, cyanidin, delphinidin that are abundantly found in plants as primary plant pigments and flavanols such as kaempferol. ~~*P. indica*~~Its flowers also contain mono and digalloylglucose. ~~*P. indica*~~*Its* roots contain~~s~~ quinones like plumbagin, 6-hydroxy plumbagin, droserone, elliptinone, plumbagic acid and lactone, flavonoids like flavonyl methyl ethers–ayanin, azaleatin, arachidyl alcohol [1], myricetin-3,3′,5′,7-tetra methyl ether, ampelopsin 3′,4′,5′,7-tetramethylether, carboxylic acids like plumbagic acid, roseanoic acid [5], alkaloids like α-naphthylamine, aliphatic acids like myricyl palmitate, palmitic acid and steroids like β-sitosterol [1]. Plumbagin (5-Hydroxy-2-methyl-1, 4-naphthoquinone) is the naturally occurring active organic compound which is isolated from roots [6]. It is a simple hydroxy naphthoquinone that is commercially important for its broad range of pharmacological activities [7]. Plumbagin is a yellneedle-shaped~~ped~~crystalthat is soluble in alcohol, acetone, chloroform, benzene, and acetic acid and slightly soluble in hot water [1]. Due to the high profile of phytochemicals, this plant has therapeutic properties fora broad range of diseases [8]. Due to the overexploitation, this plant is rare in nature, thus searching for new methods to obtain important phytochemicals without harming natural flora is a timely need.

2. MATERIAL AND METHODS

2.1 Determination of plumbagin content in callus, tissue-cultured and conventionallypropagated plant roots, and cell culture by HPLC analysis

2.1.1 Preparation of standard curve for plumbagin

Different concentrations (10.0 – 200.0 µg/mL) of standard plumbagin solutions were prepared by diluting 1.0 mg/mL stock solution using ethanol and subjected to HPLC analysis.

2.1.2 Extraction of calli, tissue-cultured, and conventionally propagated plant roots

Air-dried powder (10.0 g) of calli, tissue cultured, and conventionally propagated plant roots were extracted with 100.0 mL of absolute ethanol (99.9%) for 4 h in the Soxhlet apparatus at 50 ^{0}C. Excess ethanol was evaporated using rotary evaporator at 40 ^{0}C under reduced pressure. An amount of 3.0 mg from each plant's crude extract was dissolved in 3.0 mL of absolute ethanol.

2.1.3 Extraction of cell culture

To obtain cell culture extracts, three weeks old calli were cultured on liquid MS medium supplemented with 2.5 mg/L 2,4-D, 1.5 mg/L IBA and 1.0 mg/L NAA. Cell cultures were

maintained at 150 rpm on shaker at 25 ± 1 ^{0}C and 16 h photoperiod for three weeks. Phytochemical extraction of cell culture was carried out according to the procedure described by [9]. An aliquot of 50.0 mL was centrifuged at 6,000 rpm for 10 min. The supernatant was extracted thrice with an equal volume (50.0 mL) of ethyl acetate using a separatory funnel at room temperature. Excess ethyl acetate was evaporated using the rotary evaporator at 40 ^{0}C under reduced pressure. Pellet obtained from centrifuged cell culture was crushed with a pinch of acid-washed neutralized sand. Then extracted thrice with 20.0 mL of absolute methanol ((Loba Chemie Pvt. Ltd., Bombay, India)). Excess methanol was evaporated at 40 ^{0}C using the rotary evaporator to obtain crude extract. There were three replicate extractions from each sample.Extracts were weighed and dissolved separately in 2.0 mL of absolute ethanol. All samples were filtered through a 0.45 µm HPLC syringe filter before being subjected to HPLC analysis.

2.1.4 HPLC analysis of samples

A volume of 10.0 µL was injected separately into HPLC for analysis. The HPLC was performed by using the C18 column (length 150.0 mm, inner diameter 4.6 mm, and particle size 5.0 µm) (Ultimate 3000, ThermoFisher Scientific, Germany) with a diode-array detector set to a wavelength 254 nm. Separation was carried out at 33 ^{0}C. The mobile phase consists of 90: 10 mixtures of absolute methanol and deionized water at a constant flow rate of 1.25 mL/min. Resulted chromatograms were analyzed to determine the plumbagin content in each.

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2.2 GC-MS analysis of tissue cultured and conventionally propagated plant roots, callus, and cell culture

2.2.1 Preparation of plant crude extract Samples

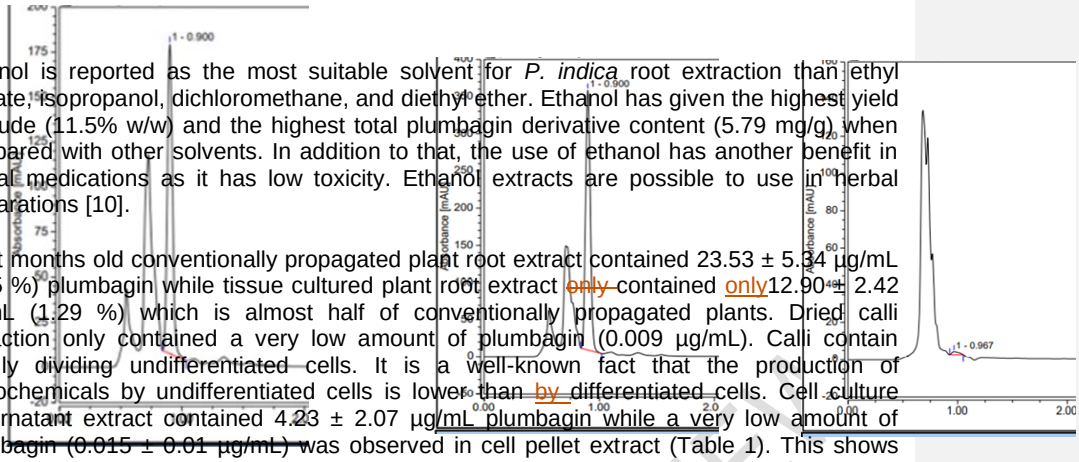
The same crude extracts used for HPLC analysis were used for GC-MS analysis. Samples for analysis were prepared by sonicating 0.2 g of crude extract (obtained by extracting dried calli, roots of tissue cultured, and conventionally propagated plant) with absolute ethanol for 30 min at 30 ^{0}C separately. Then solutions were concentrated up to 2.0 mL before being filtered through a 0.45 µm syringe filter and subjected to GC-MS analysis. Same cell culture extractions prepared for HPLC analysis were used for GC-MS analysis.

2.2.2 GC-MS analysis

An aliquot of 2.0 µL from each sample was injected separately for analysis. Agilent 7890B Gas Chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with MS-5975C inert mass selective detector (MSD) with triple-axis detector was used for GC-MS analysis. The gas chromatograph (GC) was equipped with HP-5MS 5% phenyl methyl siloxane column (Length – 30.0 m, width – 250.0 µm, Film thickness - 0.25 µm). The oven temperature was initially maintained at 50 ^{0}C for 5 min holding time and raised from 50 to 250 ^{0}C at a rate of 2 ^{0}C/min with zero holding time and the total run time was 105 min. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1.0 mL/min.

3. RESULTS AND DISCUSSION

3.1 Determination of plumbagin content in callus, tissue-cultured and conventionallypropagated plant roots, and cell culture by HPLC analysis



Ethanol is reported as the most suitable solvent for *P. indica* root extraction than ethyl acetate, isopropanol, dichloromethane, and diethyl ether. Ethanol has given the highest yield of crude (11.5% w/w) and the highest total plumbagin derivative content (5.79 mg/g) when compared with other solvents. In addition to that, the use of ethanol has another benefit in herbal medications as it has low toxicity. Ethanol extracts are possible to use in herbal preparations [10].

Eight months old conventionally propagated plant root extract contained 23.53 ± 5.34 µg/mL (2.35 %) plumbagin while tissue cultured plant root extract ~~only~~ contained only12.90 ± 2.42 µg/mL (1.29 %) which is almost half of conventionally propagated plants. Dried calli extraction only contained a very low amount of plumbagin (0.009 µg/mL). Calli contain rapidly dividing undifferentiated cells. It is a well-known fact that the production of phytochemicals by undifferentiated cells is lower than by differentiated cells. Cell culture supernatant extract contained 4.23 ± 2.07 µg/mL plumbagin while a very low amount of plumbagin (0.015 ± 0.01 µg/mL) was observed in cell pellet extract (Table 1). This shows that plumbagin produced are secreted into the medium even without any elicitors in cell culture.

Table 1. Plumbagin content of tissue-cultured and conventionally propagated plant roots, callus, cell culture supernatant, and pellet.

Sample	Plumbagin content (µg/mL)
Tissue culture plant roots	12.90 ± 2.42
Conventionally propagated plant roots	23.53 ± 5.34
Callus	0.0085
Cell culture supernatant	4.23 ± 2.07
Cell culture pellet	0.015 ± 0.01

Plumbagin content in twelve months old tissue cultured field-grown, conventionally field-grown, and hydroponically grown *P. indica* plants ~~were~~ was1.80 ± 0.25, 1.33 ± 0.15, and 1.08 ± 0.01 g of plumbagin per 100 g of dry raw material, respectively. Dry callus induced from leaf explants on MS medium supplemented with 2.0 mg/L BA and 3.0 mg/L IBA has contained a considerably low amount of plumbagin (0.26 ± 0.02 g per 100 g of dry ~~row~~ raw material) [11]. The above literature has proven tissue cultured plants as the best source of plumbagin. However, in contrast,the current study showed higher plumbagin content in conventionally propagated plants

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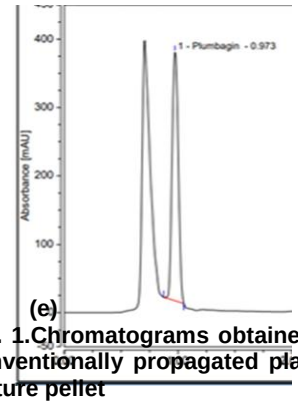
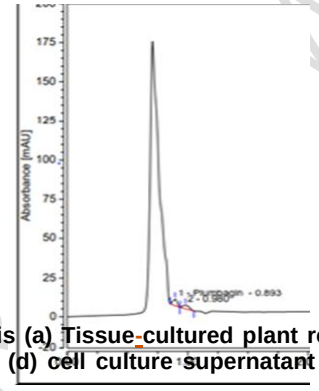
Plumbagin is one of the main phytochemicals present in *P. indica* roots. Production of phytochemicals can be varied according to the nutrient composition of the soil, developmental stage of the plants, climatic season [11] state of mother stock, and other environmental factors. Also, both researchers used two different extraction methods and solvents for phytochemical extractions. In the present study, 10.0 g of dried root sample was extracted with 100.0 mL of absolute ethanol using the Soxhlet apparatus for 4 h. As mentioned in [11], the extraction was carried out by refluxing 2.0 g of dried root sample with methanol for 1 h. The extraction method and solvent used for extraction also can affect the plumbagin content in the tested crude extract.

(a) **(b)** **(c)**





(d) **(e)**

Fig. 1.Chromatograms obtained by HPLC analysis (a) Tissue-cultured plant roots (b) conventionally propagated plant roots (c) callus (d) cell culture supernatant (e) cell culture pellet

Many members of Plumbaginaceae family contain plumbagin as one of the active ingredients in the root system. It is reported that *P. indica* contains a higher amount of plumbagin than *P. capensis* and *P. zeylanica* roots. (0.569 %, 0.429 %, and 0.247 % w/w of plumbagin, respectively) [12]. The quantification technique and extraction procedure used by [12] is different than the one used in the present study. Plumbagin was quantified by the HPTLC method and extraction was done by static cold maceration using 50 % methanol (hydro alcohol) with 100.0 g of dried roots. The difference in results obtained might have been affected by all the above factors.

3.2 GC-MS analysis of tissue-cultured and conventionally propagated plant roots, callus, and cell culture

The GCMS analysis also revealed that conventionally propagated plant root extract contained the highest number of chemical compounds (~~sixteen~~ 16compounds) than tissue culture plant root extract (~~four~~ 4compounds) and callus extract (~~one~~ 1compound). Cell culture pellet extract only contained nine phytochemicals while supernatant extract contained only three phytochemicals having more than 70% similarity. Similarity percentages of phytochemicals present in different samples are shown in Table 2.

None of the observed phytochemicals were common in all tested five samples. Even though a considerably low number of phytochemicals were detected in tissue cultured plant root extract, it contains plumbagin (1,4-Naphthalenedione, 5-hydroxy-2-methyl-). Only tissue culture and conventionally propagated plant root extracts contained a detectable amount of plumbagin. Butyrolactone ethanol, 2,2-diethoxy, 2,4-Dihydroxy2,5-dimethyl-3(2H)-furan-3-one, Succinic anhydride, 4H-Pyran-4-one, 3,5-dihydroxy2-methyl-, 1,4-Naphthalenedione, 3,5-dihydroxy-2-methyl-, 9,12-Octadecadienoic acid (Z, Z), Oleic acid, and Hexadecanoic

acid, ethyl ester was found only in conventionally propagated plant root extracts while Dihydroxyacetone was only found in tissue cultured plant root extracts. Levoglucosenone and 2-Furancarboxaldehyde, 5- (chloromethyl) were only detected in cell culture pellet extract while Benzyl butyl phthalate and Dibenzyl phthalate were only detected in cell culture supernatantextracts.

Table 2. Phytochemicals present in tissue-cultured and conventionally propagated plant roots, callus, cell culture supernatant, and pallet

Chemical compound	Percentage Similarity					Medicinal properties
	CPPRE	TCPRE	CE	CCPE	CCSE	
Furfural	79.3	-_	-_	77.8	-_	Antibacterial, Tyrosinase inhibitor [13]
Butyrolactone ethanol, 2,2-diethoxy	79.5	-_	-_	-_	-_	Unknown
2-Furancarboxaldehyde, 5-methyl-	85.4	-	-	89.5	-	Antimicrobial, Antioxidant [14]
2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	91.0	-	-	-	-	Antioxidant [14]
Succinic anhydride	82.4	-	-	-	-	Unknown
2,5-Furandicarboxaldehyde	88.4	-	-	70.3	-	Unknown
2-Furaldehyde diethyl acetal	76.7	-	-	77.4	-	Anti-microbial [15]
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	97.1	77.8	-	94.9	-	Anti-microbial, Anti-inflammatory [16]
4H-Pyran-4-one, 3,5-dihydroxy-2-methyl-	82.4	-	-	-	-	Antioxidants [17]
5-Hydroxymethylfurfural	92.3	72.5	-	94.9	-	Anti-oxidant, Anti-proliferative [16]
1,4-Naphthalenedione, 5-hydroxy-2-methyl-	88.7	84	-	-	-	Cytotoxicity, Anti-inflammatory, Antimicrobial, Antimalarial, Leishmanicidal, Anti-Oxidant,Wound healing [18]
1,4-Naphthalenedione, 3,5-dihydroxy-2-methyl-	72.6	-	-	-	-	Unknown
n-Hexadecanoic acid	77.7	-	73.2	74.5	75.6	Anti-oxidant, Hypocholesterolemic,Nematicide,Anti-androgenic, Hemolytic, and Antipsychotic [3]
9,12-Octadecadienoic acid (Z,Z,)	92.5	-	-	-	-	Anti-inflammatory, Hypocholesterolemic, Anti-cancer, Hepatoprotective, Anti-arthritic [16], Nematicide, Antihistaminic, Antiacne, 5-Alpha reductase inhibitor, Antiandrogenic [19]
Oleic acid	90.1	-	-	-	-	Antimicrobial [20,21],

						Hypocholesterolemic, Anti-cancer, Anti-inflammatory [22], Antihypertensive, Cutaneous wound healing [23]
Hexadecanoic acid, ethyl ester	75.0	-	-	-	-	Antioxidant, Hemolytic, Hypocholesterolemic, Nematicide, Anti-androgenic [3]
Dihydroxyacetone	-	83.8	-	-	-	Anti-diabetic [24]
Benzyl butyl phthalate	-	-	-	-	82.1	Unknown
Dibenzyl phthalate	-	-	-	-	88.7	Unknown
Levoglucosenone	-	-	-	93.3	-	Chiral agent, Catalyst [25], Anti-viral, Anti-cancer [26]
2-Furancarboxaldehyde, 5-(chloromethyl)	-	-	-	89.6	-	Unknown
9,12-Octadecadienoic acid (Z,Z,)	-	83.8	-	-	-	Anti-diabetic [24]
Oleic acid	-	-	-	-	82.1	Unknown
Hexadecanoic acid, ethyl ester	-	-	-	-	88.7	Unknown
Dihydroxyacetone	-	-	-	93.3	-	Chiral agent, Catalyst [25], Anti-viral, Anti-cancer [26]
Benzyl butyl phthalate	-	-	-	89.6	-	Unknown
Dibenzyl phthalate	-	83.8	-	-	-	Anti-diabetic[24]
Levoglucosenone	-	-	-	-	82.1	Unknown
2-Furancarboxaldehyde, 5-(chloromethyl)	-	-	-	-	88.7	Unknown

**CPPRE – Conventionally Propagated Plant Root Extract, TCPRE – Tissue Culture Plant Root Extract, CE – Callus Extract, CCPE – Cell Culture PelletExtract, CCSE – Cell Culture Supernatant Extractoisture content on oven dry weight basis*

According to [18], three chemical compounds that have more than 70% similarity have been detected in the methanol extract of *P. indica* root bark. Those were methyl 16-methylheptadecanoate, 8-Octadecenoic acid, methyl ester, and Hexadecanoic acid, methyl ester. Behenic alcohol was the only chemical compound detected in the ethyl acetate fraction that has more than 70% similarity. 5-Hydroxy-2-methyl-1,4-naphthalenedione (Plumbagin) was detected in ethyl acetate fraction but the similarity was lower to 3%.

4. CONCLUSION

The conventionally propagated plant root extract has a higher number of phytochemical compounds and higher plumbagin content when compared with tissue cultured plant root extract, calli, cell culture supernatant, and pellet extracts. Even though, conventionally propagated plant roots are the best source to extract plumbagin, tissue cultured plant roots also have a considerably high amount of plumbagin. Thus, results revealed that tissue cultured *P. indica* roots can be also used as a source to extract plumbagin ~~in~~ ona mass scale, yet conditions need to be optimized to produce a high level of plumbagin. The study confirmed not only the possibility of obtaining plumbagin through cell cultures in a bioreactor system but also products are mainly secreted into the medium which is promising results by optimizing conditions for the production. it is not necessary to use mechanical product secretion.

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COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly used products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by the personal efforts of the authors.

REFERENCES

1. Jose B, Dhanya BP, Silja PK, Krishnan PN, Satheeshkumar K. *Plumbago rosea* L. – A review on tissue culture and pharmacological research. International Journal of Pharmaceutical Sciences Review and Research. 2014;25(1):246-256.

2.Eldhose B, Notario V, Latha MS. Evaluation of phytochemical constituents and *in vitro* antioxidant activities of *Plumbago indica* root extract. Journal of Pharmacognosy and Phytochemistry. 2013;2(4):157-161.

3.Tyagi T, Agarwal M. Phytochemical screening and GC-MS analysis of bioactive constituents in the ethanolic extract of *Pistia stratiotes* L. and *Eichhornia crassipes* (Mart.) solms. Journal of Pharmacognosy and Phytochemistry. 2017;6(1):195-206.

4.Kumar SG, Joseph LH, Thangavel K. *in vitro* propagation of *Plumbago rosea* L. Journal of Applied Biology and Biotechnology. 2014;2(2):1-7.

5. Ariyanathan S, Saraswathy A, Rajamanickam GV, Connolly JD. Polyphenol from the roots of *Plumbago rosea*, Indian journal of chemistry, 2010;(49B):386-389.

6. Gangopadhyay M, Chakraborty D, Bhattacharyya S, Bhattacharya S. Regeneration of transformed plants from hairy roots of *Plumbago indica*. Plant Cell Tissue Organ Culture. 2010;102:109-114.https://doi.org/10.1007/s11240-010-9702-z

7. Silja PK, Gisha GP, Satheeshkumar. Enhanced plumbagin accumulation in embryogenic cell suspension cultures of *Plumbago rosea* L. following elicitation. Plant Cell Tissue Organ Culture. 2014;119(3):469-477.https://doi.org/10.1007/s11240-014-0547-8

8. Bhadra SK, Akhter T, Hossain MM. *In vitro* micro-propagation of *Plumbago indica* L. through induction of direct and indirect organogenesis. Journal of Plant Tissue Culture and Biotechnology. 2009;19(2):169-175.http://dx.doi.org/10.3329/ptcb.v19i2.5434

9. Komaraiah, P., Kishor, P.B. K., & Ramakrishna, S.V. (2001). Production of plumbagin from cell cultures of *Plumbago rosea* L. Biotechnology Letters, 23, 1269–1272. https://doi.org/10.1023/A:1010545630018

10. Kaewbumrung, S., & Panichayupakaranant, P. (2014). Antibacterial activity of plumbagin derivative rich *Plumbago indica* root extracts and chemical stability. Natural Product Research, 28(11), 835–837. http://dx.doi.org/10.1080/14786419.2013.879585

11. Lenora RDK, Dharmadasa RM, Abeysinghe DC, Arawwawala LDAM. 2012. Investigation of plumbagin content in *Plumbago indica* Linn. Grown under different growing system. Pharmacologia, 2012;3(2), 57-60.

12.Dorni AIC, Vidyalakshmi KS, Vasanthi HR, Rajamamckam GV. HPTLC method for quantification of plumbagin in three *Plumbago* species. Research journal of phytochemistry. 2007;1(1):46-51.

13. Chai W, Liu X, Hu Y, Feng H, Jia Y, Guo Y, *et al*. Antityrosinase and antimicrobial activities of furfuryl alcohol, furfural and furoic acid. International Journal of Biological Macromolecules. 2013;57:151-155.https://doi.org/10.1016/j.ijbiomac.2013.02.019

14. Sriram V, Vignesh RC, Velavan S, Nethaji S. Identification of phytochemicals in a hydro alcohol extract of *Annona muricata* fruit using GC-MS analysis. Journal of Pharmacognosy and Phytochemistry. 2018;7(2):120-122.

15. Sethupathy S, Nithya C, Pandian SK, 2-Furaldehyde diethyl acetal from tender coconut water (*Cocos nucifera*) attenuates biofilm formation and quorum sensing-mediated virulence of Chromobacterium violaceum and Pseudomonas aeruginosa. The Journal of Bio adhesion and Biofilm Research. 2015;31:721–733.https://doi.org/10.1080/08927014.2015.1102897

16. Kar P, Dey P, Misra AK, Chaudhuri TK, Sen A. Phytometabolomic fingerprinting of selected actinorhizal fruits popularly consumed in North-East India. Symbiosis, 2016;70:159-168.https://doi.org/10.1007/s13199-016-0415-x

17. Ashwathanarayana R, Naika R. Study on aphrodisiac activity of *Olea dioica* roxb. Bark, leaf extracts and its pure compound using wistar albino rats. Asian Journal of Pharmaceutical and Clinical Research, 2017;10(12):85-98.

18. Dissanayake DMIH, Perera DDBD, Keerthirathna LR, Heendeniya S, Anderson RJ, Williams DE, *et al*. Antimicrobial activity of *Plumbago indica* and ligand screening of plumbagin against methicillin-resistant *Staphylococcus aureus*. Journal of Biomolecular structure and dynamics. 2020. https://doi.org/10.1080/07391102.2020.1846622

19. Kartika IGAA, Insanu M, Safitri D, Putri CA, Adnyana IK. New update: Traditional uses, Phytochemical, Pharmacological and Toxicity review of *Peperomia Pellucida* (L.) Kunth. Pharmacology online. 2016;2:30-43.

20. Malarvizhi D, Anusooriya P, Meenakshi P, Sundaram S, Oirere E, Gopalakrishnan VK. Isolation, Structural Characterization of Oleic Acid from *Zaleya decandra* Root Extract, Analytical. 2016. http://dx.doi.org/10.1080/22297928.2016.1238319

21. Abubacker MN, Devi PK. *In vitro* antifungal potentials of bioactive compound oleic acid, 3-(octadecyloxy) propyl ester isolated from *Lepidagathis cristata* Willd. (Acanthaceae) inflorescence. Asian Pacific Journal of Tropical Medicine. 2014;7:190-193.https://doi.org/10.1016/s1995-7645(14)60230-3

22. Ghavam M, Afzali A, Manca ML. Chemotype of damask rose with oleic acid (9 octadecenoic acid) and its antimicrobial effectiveness. Scientific reports. 2021. https://doi.org/10.1038/s41598-021-87604-1

23. Sales-Campos H, Souza PRD, Peghini BC, Silva, JSD, Cardoso CR. An Overview of the Modulatory Effects of Oleic Acid in Health and Disease. Mini-Reviews in Medicinal Chemistry. 2013;13:201-210.https://doi.org/10.2174/138955713804805193

24. Rabinowitch IM. 1925. Observations on the Use of Dihydroxyacetone in the Treatment of Diabetes Mellitus: (Preliminary Report). The Canadian medical association journal, 1925; 15(4):374-381.

25. Zekeya N, Chacha M, Shahada F, Kidukuli A. Analysis of phytochemical composition of *Bersama abyssinica* by gas chromatography – mass spectrometry. Journal of Pharmacognosy and Phytochemistry. 2014;3(4):246-252.

26. Altameme HJ, Hameed IH, Abu-serag NA. Analysis of bioactive phytochemical compounds of two medicinal plants, *Equisetum arvense* and *Alchemila valgaris* seeds using Gas Chromatography – Mass Spectrometry and Fourier – Transform Infrared Spectroscopy. Malaysia. Appl. Biol. 2015;44(4):47–58.