Isolation and Analysis of Methanol Extract of Leaf of *Senna siamea*

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ABSTRACT

This study aimed at isolation and characterization of biochemical constituents of the leaf extract of *Senna siamea*. The fresh leaves of *Senna siamea* were air-dried, pulverized and extracted using methanol by maceration method. The extract was screened for phytochemicals using standard methods. Fifty grammas (50 g) of the crude extract was defatted using n-hexane; 20 g was subjected to column chromatographic (CC) analysis using ethyl acetate and n-butanol fractions with similar retardation factor (R_f) were pooled and coded. Subsequently purification of fraction SSM₅ was carried out using CC with solvent [Toluene and n-butanol (4:1) as mobile phase]. The collection was based on colour bands) and TLC was carried out where a sub-fraction SSM₅₄ amongst other fractions gave a single spot on TLC and the R_f value was 0.71. It was then subjected to UV, IR and GC-MS analysis. The extract by maceration yielded 12.60% w/w and the defatted extract yielded 10.0% w/w . The result of the UV-visible gave a wavelength of 289 nm and an absorbance of 4.268. The result of infrared spectroscopy revealed functional groups thus O-H, N-H, C-H stretching group at 3385.8 cm^{-1} , -CHO at 2955.8 cm^{-1} , -CHO stretching at 2926.0 cm^{-1} , -NH₃⁺ at 1640.0 cm^{-1} , carboxylate ion-CO₂⁻ for amino acid at 1449.9 cm^{-1} , -N=N- at 1401.5 cm^{-1} , -N⁺O⁻=N- at 1315.8 cm^{-1} , C-O-C at 1189 cm^{-1} , Si-O at 1084 cm^{-1} and S=O at 1013.8. The GC-MS analysis of SSM₅₄ revealed the presence of Diisooctyl phthalate, Purine-2,6-dione, 8-(3-Ethoxypropylamino)-1,3-dimethyl-3, 9-dihydro-, α -Pinene, 10-(dimethylaminomethyl), 1H-indene-2-ethanamine, N, N- dimethyl-3-[1-(2-pyridinyl) ethyl]-, Benzene, 1, 2- bis(2,5 dimethylphenylaminomethyl)-3,6- dimethyl- and Cinnamic acid, p-(trimethylsiloxy)-, methyl ester were among the probable bioactive compounds.

Key words: *Senna siamea*, Chromatographic, Gas Chromatography-Mass Spectroscopy, Ultraviolet Visible Spectroscopy and Infrared Spectroscopy

Introduction

The use of plants as medicines pre-dates written history. The earliest records of herbs are found from the Sumerian civilization, where hundreds of medicinal plants, including opium, were listed on clay tablets. Over the years they have assumed a central stage in modern civilization as natural source of chemotherapy as well as amongst scientists in search for alternative sources of drugs. About 3.4 billion people in the developing world depend on plant-based traditional medicines. This represents about 88% of the world's inhabitants, who rely mainly on traditional medicine for their primary health care. [1]

In 2001, researchers identified 122 compounds used in modern medicine derived from traditional plant sources; 80% of these have had a traditional use identical or related to the

current use of the active elements of the plant [2]. These include the common drugs aspirin, digoxin, quinine and opium [1]. The compounds found in plants are of many kinds, but most are in four major biochemical classes; the alkaloids, polyphenols, glycosides and terpenes. These classical biocompound found in plants are called phytochemical. Without specific knowledge of their cellular actions or mechanisms, phytochemicals have been considered possible drugs for millennia [1]. For example, salicin, having anti-inflammatory and pain-relieving properties, was originally extracted from the bark of the white willow tree and later synthetically produced to become the common, over-the-counter drug, aspirin [3,4]. The carotenoids, beta-carotene, alpha-carotene and beta-cryptoxanthin, however, are well-

established as provitamin, a compounds essential for normal growth and development, immune system function and vision [5]. Other carotenoid phytochemicals, such as lycopene, lutein and zeaxanthin, are not yet fully understood for their possible nutritional or biological effects [5].

Chromatography is the collective term for a set of laboratory techniques for the separation of mixtures. The technique has been vastly employed by organic chemist for the isolation of natural products, The involved in the mixture is being dissolved in a fluid called the mobile phase, which carries it through a structure holding another material called the stationary phase. The various constituents of the mixture

Fig. 1: Picture of the leave

Materials and Methods

Sample Collection, Identification and Extraction

Fresh sample of the leaf of *Senna siamea* was collected from Faculty of Veterinary Medicine, University of Maiduguri, Maiduguri, Borno State, Nigeria. The Plant material was identified and authenticated by a Plant Taxonomist Prof S.S. Sanusi, Department of Biological Sciences, University of Maiduguri, Borno State, Nigeria to be from *Senna siamea*, deposited at the of the Postgraduate Research Laboratory, Chemistry Department, University of Maiduguri, Maiduguri with voucher number of 549Awas given. The fresh plant sample was cleaned and

travel at different speeds, causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus changing the separation [6].

Senna siamea is a common plant in the Northeast of Nigeria, but in spite of its uses in traditional medicine by the local people in the northeast of Nigeria, only a few scientific studies have been carried out with little documentation on its isolation and characterization to identify the possible bioactive compounds it has. This study reports its isolation and identifying possible bioactive compound(s) in the plant.

air-dried under shade at room temperature for two weeks [7].

Isolation of biochemical constituents of *Senna siamea*

Column Chromatography (CC)

The defatted methanol crude extract was subjected to Column Chromatographic separation to further purify a bioactive compound. Twenty 20 g of the defatted crude extract was used for the Column Chromatographic study solvents system of ethylacetate and n-butanol in various combining ratio as 100:0, 80:20, 60:40, 50:50, 40:60, 20:80 and 0:100 respectively.

A glass tube with a diameter 2.8 cm and a height of 90 cm with a tap at the bottom was used for the column chromatography. A plug of cotton wool was well placed at the bottom of the column very close to the tap so as to prevent the stationary phase from blocking the column. Eighty 80 g of silica gel 60-120 mesh (Quiklem, India) was used to prepare slurry by wet method. The silica gel was mixed with n-hexane and stirred with a clean glass rod until a uniform mixture was obtained, then it was packed cautiously and manually to two third the size of the column tube using a glass funnel. The gel was then allowed to settle and pack for 24 hrs. The air bubbles were avoided and care was taken not to dry the column by maintaining the level of the chloroform to that of the silica gel. 20 g of the mixture of the defatted ethanol crude extract and 5 g of silica gel in methanol was mounted on the already equilibrated silica-fixed column on top of the stationary phase. This was topped with a small layer of cotton, then sand to protect the shape of the organic layer from the velocity of newly added eluent (stationary phase). The eluting solvent initially was 100 % ethyl acetate and the polarity was gradually increased at 90:10, ethyl acetate: n-butanol ratio until 0:100 ethyl acetate: n-butanol ratio was used. Twenty two (22) sub-fractions were collected. The fractions collected were monitored using TLC plate. Those with similar R_f were pooled together, at the end five (5) portions were obtain from the pooling process coded as SSM₁ to SSM₅ respectively, the column chromatographic procedure stated above

RESULTS

Extraction and Defatting Profile of *Senna siamea* Leaf

The extraction of the leaf of *Senna siamea* using methanol produced extract with dark green colour. The methanol extract had the mass of 63.0 g and the percentage yield of 12.6 %. The result of the extraction profile is shown on Table

was applied to portion SSM₄ and SSM₅. This method was used to further purify the column fractions using Toluene and n-butanol in increasing polarity as mentioned above until a compound of single spot on thin layer chromatography was achieved.

Thin Layer Chromatography (TLC)

Commercially prepared aluminium back TLC plates of 20 x 20 cm x 0.25 mm activated silica gel of 60 F₂₅₄ (Merck, Germany) was cut to size of 5 x 5 cm. Methanol leaf extract of *Sennasiamea* which contained the most phytochemicals and had the largest quantity was preferred for the chromatographic analysis. Methanol was used to dissolve the extract and allowed to stand for 30 minutes and then it was spotted at the bottom of the TLC plate (1.5 cm from the base).

The extract which was dissolved in a few drops of methanol was dried in air for 30 minutes and was used for the spotting by the aid of a capillary tube. The spotted plate was placed in a previously saturated developing chamber containing toluene and ethyl acetate in ratio 4:1 the chosen solvent system as mobile phase and was covered with a watch glass and allowed to run 3/4th of the height of the prepared plates (8). Fractions which gave a single spot when developed, using the chromatographic tank was allowed to dry. The Retention Factor (R_f) values of the compounds (pure) measured and then crystallize, was recorded prior to elucidation of possible structures by Gas Column-Mass Spectroscopy (GC-MS).

1. The methanol extract (50 g) which was defatted using n-hexane produced extracts with dark green and brown colour of gummy masses respectively. The crude methanol leaf extract with initial weight of 50 g yielded of 10.0 %. The result of the extraction profile is shown on Table 2

Table 1: Extraction profile of air-dried powdered leaves of *Senna siamea*

S/N	Fraction	Mass (g)	%Yield (%/w)	Colour	Texture
1.	Methanol	63.00	12.60	green	gummy mass

Table 2: The profile of *Senna siamea* methanol crude extract defatted portion

S/N	Fraction	Mass (g)	%Yield (w/w)	Colour	Texture
1.	n- Hexane	50.00	10.00	green	gummy mass

Chromatographic Separation of Methanol Leaf Extract of *Senna siamea* and of the Fractions

Column Chromatographic Analysis of Methanol Leaf Extract of *Senna siamea*

Components present in the methanol leaf extract of *Senna siamea* were separated through column chromatographic technique. Similar fractions were combined together and were coded: SSM (1a-1b) as 1, SSM (2a-2b) as 2, SSM (3a-5a) as 3, SSM (5b-6a) as 4 and SSM (6b-11b) as 5. Percentage yields of the combined fractions determined ranged from 18.5 % with fraction SSM₅ as the highest, to 0.5 % of SSM₁ as the

combined fraction with the lowest weight. The colours of the column fractions ranged from dark green of SSM₁ to brown of SSM₅

Thin Layer Chromatographic Analysis of the Column Fractions

The fractions collected from column chromatographic analysis were monitored by thin layer chromatographic technique. The combined column fractions encoded SSM₄ and SSM₅ did resolve while SSM₁, SSM₂ and SSM₃ fractions didn't resolved when solvent system of toluene and n-butanol (4:1) was used. The recombined fractions of the spots observed were calculated using the equation below while values obtained are shown on Table 3.

$$\text{Retardation factor} = \frac{\text{distance travelled by solute (cm) from the origin}}{\text{distance moved by solvent (cm) from the origin}}$$

Table 3: Fractions, weight of dried fractions, percentage yield of the recombined and colour of the fractions from column chromatographic analysis

Recombined fraction	weight of dried recombined fraction (g)	% w/w of recombined fraction	Component	Colour of fraction
SSM ₁	0.5	2.5	SSM (1a-1b)	dark green
SSM ₂	0.6	3.0	SSM (2a-2b)	green
SSM ₃	1.5	7.5	SSM (3a-5a)	dark brown
SSM ₄	0.7	3.5	SSM (5b-6a)	light brown
SSM ₅	3.7	18.5	SSM (6b-11b)	brown

Initial weight of crude aqueous extract = 20 g, Amount recovered = 7.0 g, Percentage recovered = 35% (w/w), SSM = *Senna siamea* methanol extract, Mobile phase = toluene and butanol (4:1)

Re-Chromatographic Separation of Methanol Leaf Extract of *Senna siamea* of the portion 5 fractions

Components present in the methanol leaf extract of *Senna siamea* were fractionated using micro column with silica gel 30 g and the silica gel as stationary phase and 3.7 g of the extracts, while toluene and n-butanol at different increasing polarity ratio were used as the mobile phase. Fractions were coded: asSSM₅₁ SSM₅₂, SSM₅₃, SSM₅₄ and SSM₅₅. Weights of the combined

fractions determined ranged from 24.86 % with fraction SSM₅₄ as the highest, to 5.68 % of SSM₅₂ as the combined fraction with the lowest weight.

Thin Layer Chromatographic Analysis of the Column Fractions

The fraction from column chromatographic analysis was monitored by thin layer chromatographic technique. The column fractions encoded did not resolve while SSM₅ fractions resolved when solvent system of

toluene and butanol was used. The retardation factors of the spots observed were calculated

using the equation below while values obtained are shown on Table 4.

$$\text{Retardation factor} = \frac{\text{Distance travelled by solute (cm) from the origin}}{\text{Distance moved by solvent (cm) from the origin}}$$

Table 4: Recombined fractions, weight of dried recombined fraction, percentage yield from portion SSM₅ column chromatographic analysis

Recombined fraction	Weight of dried recombined fraction (g)	% (w/w) of recombined fraction	Component
SSM ₅₁	0.35	9.45	1
SSM ₅₂	0.21	5.68	2
SSM ₅₃	0.12	3.24	3
SSM ₅₄	0.80	21.62	4
SSM ₅₅	0.28	7.57	5

Initial weight of crude aqueous extract = 3.7 g, Amount recovered = 1.76 g, Percentage recovered = 47.56 (%/w), SSM= *Sennasiemea* methanol extract, Mobile phase = Toluene and n-butanol

Table 5: Thin layer chromatographic (TLC) profile of the column fractions of methanol leaf extract of *Senna siamea*

Fraction Factor (R _f)	Colour of fractions	No. of spot	Distance (cm)	Retardation
1	pale yellow	NR		
2	yellow	NR		
3	yellow	NR	12.5	0.71
4	yellow	1	11.0	0.63
5	yellow	2	8.6	0.51

NR = Not Resolve, SSM = methanol leaf extract of *Senna siamea*, Solvent front =17 cm, Solvent system = Toluene: n-butanol; 4:1

Ultraviolet-Visible spectroscopy of Fraction SSM₅₄ of the Leaf Extract of *Senna siamea*

The result of the ultraviolet-visible spectroscopy of the isolated compound of the methanol leaf extract of *Senna siamea* is shown in Figures 1. The result of the UV-visible with a wavelength of 289 nm and the absorbance of 4.268.

IR Spectroscopy of the methanol leaf Extract of *Senna siamea* of SSM₅₄

Infrared spectroscopy is used to gather information about compound's structure, assess

its purity, and sometimes to identify it. The infrared spectroscopy has a range of 4000-650 cm⁻¹; the functional groups indentify are O-H, N-H, C-H stretching group at 3385.8 cm⁻¹, -CHO at 2955.8 cm⁻¹, -CHO stretching at 2926.0 cm⁻¹, -NH₃⁺ at 1640.0 cm⁻¹, carboxylate ion -CO₂⁻ for amino acid at 1449.9cm⁻¹, -N=N- at 1401.5 cm⁻¹, -N⁺O⁻=N- at 1315.8 cm⁻¹, C-O-C at 1189 cm⁻¹, Si-O at 1084 cm⁻¹ and S=O at 1013.8 table 4.8 show the function assign to the values obtain from the IR. Figure 2 the IR chart

Figure 2: The UV spectra of fraction SSM₅₄ of the leaf extract of *Senna siamea* showing the Absorbance

Table 6: Infra- red values of functional groups indentified it various frequencies of *Senna siamea* fraction SSM₅₄

S/N	Wavelengths	Functions
1	3385.8	O-H
2	2955.8	-CHO
3	2926.0	-CHO
4	1640.0	-NH ₃ ⁺
5	1449.9	-CO ₂ ⁻ for amino acid
6	1401.5	-N=N-
7	1315.8	-N ⁺ O ⁻ =N-
8	1189.0	C-O-C
9	1084.7	Si-O
10	1013.8	Si=O

Figure 3: Infra red spectrum of methanol leaf extract of *Senna siamea* SSM₅₄

Gas Chromatography-Mass Spectroscopy (GC-MS) of Fraction SSM₅₄ of the Leaf Extract of *Senna siamea*

The gas chromatography-mass spectroscopy of the isolated compound of the SSM₅₄methanol leaf extract of *Senna siamea* is shown six (6) peaks with the major peaks, retention time, area and library ID. Peak number 6, the retention time 32.727, the area 18333446.22 and the library ID Diisooctyl phthalate figure 4 shows the compound, peak number 5, retention time 22.765, the area 458490.25 and the library ID Purine-2,6-dione, 8-(3-Ethoxypropylamino)-1,3-dimethyl-3, 9-dihydro- figure 5, peak number 4, retention time 9.302, the area 402059.29 and the library ID a-Pinene, 10-(dimethylaminomethyl) figure 6, peak number 3, retention time 8.735,

the area 250535.49 and the library ID 1H-indene-2-ethanamine, N, N- dimethyl-3-[1-(2-pyrindinyl)ethyl]- figure 7, peak number 2, retention time 8.449, the area 51617.52 and the library ID Benzene, 1, 2- bis(2,5-dimethylphenylaminomethyl)-3,6- dimethyl- figure 8 and peak number 1, retention time 9.302, the area 402059.29 and the library ID Cinnamic acid, p-(trimethylsiloxy)-, methyl ester figure 9

Figure 4 shows the chromatogram of the sample SSM₅₄. Clear peaks (levels) were marked out for analysis by the mass spectrometer.

Table 8 shows the % area, retention time, peak number, and quality of peaks of the gas chromatogram of fraction SSM₅₄. Peak 22 has the highest % area of 32.90.

Figure 4: Gas chromatogram of leaf extract of fraction SSM₅₄

Table 8: Gas Chromatographic analysis of fraction SSM₅₄ methanol leaf extract of *Senna siamea*

Peak No.	Retention Time	%Area	height	width
1	5.29	2986873.63	24062.06	8.638
2	8.449	241574.50	51617.52	0.175
3	8.735	250535.50	38455.00	0.209
4	9.305	402059.29	56016.94	0.307
5	22.765	458490.25	52317.93	0.452
6	32.727	18333446.22	2620877.88	0.418

Table 9: Gas chromatography-Mass spectroscopy analysis of isolated compound from SSM₅₄

Peak no	Retention Time (min)	Name of Compound	Molecular Formular	Mol. Wt	% Area
1	5.29	Cinnamic acid, p-(trimethylsiloxy)-methyl ester Oxide	C ₉ H ₈ O	148	2986873.63
2	8.45	Benzene, 1, 2- bis (2,5-dimethylphenyl aminomethyl)-3,6-dimethyl- undecene	C ₂₆ H ₃₂ N ₂	372	241574.50
3	8.74	1H-indene-2-ethanamine, C ₂₀ H ₂₄ N ₂		292	250535.49
4	9.30	N, N- dimethyl-3-[1-(2-pyrindinyl)ethyl]-a-Pinene, C ₁₃ H ₂₃ N		193	402059.29
5	22.77	Purine-2,6-dione, 8-(3-Ethoxypropylamino)-1,3-dimethyl-3, 9-dihydro-	C ₁₂ H ₁₉ N ₅ O ₃	281	458490.25
6	32.73	Diisooctyl phthalate	C ₂₄ H ₃₈ O ₄	390 183	33446.22

Fig 5: Cinnamic acid, p-(trimethylsiloxy)-, methyl ester

Fig 6: Benzene, 1,2-bis(2,5-dimethylphenylaminoethyl)-3,6-dimethyl

Fig 7: 1H-indene-2-ethanamine, N, N-dimethyl-3-[1-(2-pyrindinyl)ethyl]

Fig 8: alpha-pinene, 10-(dimethylaminomethyl)

Fig 9: Purine-2, 6-dione, 8-(3-ethoxypropylamino)-1,3-dimethyl-3,9-dihydro-

Fig 10: diisooctyl phthalate

The structure of the compounds isolated from GC-MS analysis of SSM₅₄

DISCUSSION

The value obtained for the sub fraction of SSM₅₄ methanol leaf extract of *Senna siamea* UV the result is in support of Ultraviolet Detection of Monosaccharides: Multiple Wavelength Strategy to Evaluate Results after Capillary Zone Electrophoretic Separation [9].

Results of the IR spectra of fraction SSM₅₄ had values of the functional groups indentified as follows: O-H, N-H, C-H stretching group at 3385.8 cm⁻¹, -CHO at 2955.8 cm⁻¹, -CHO stretching at 2926.0 cm⁻¹, -NH₃⁺ at 1640.0 cm⁻¹, carboxylate ion -CO₂⁻ for amino acid at 1449.9 cm⁻¹, -N=N- at 1401.5 cm⁻¹, -N⁺O=N- at 1315.8 cm⁻¹, C-O-C at 1189 cm⁻¹, Si-O at 1084 cm⁻¹ and S=O at 1013.8.

The successful separation of biomolecules by the chromatographic technique depended upon suitable solvent system which needs an ideal range of partition coefficient (K) for each target compound [10].

Result of GC/MS analysis of sub-fraction SSM₅₄ of the methanol leaf extract of *Senna siamea* indicate the compound Diisooctyl phthalate in the extraction and bioactivity from *Jatropha curcas* L. and also in Diisooctyl phthalate in *plantago major* L. reported the presence of this compound [11, 12]. Research indicates that diisooctyl phthalate has antimicrobial and algicidal activities as reported by [13], Purine-2, 6-dione, 8-(3-Ethoxypropylamino)-1, 3-dimethyl-3, 9-dihydro- was report by [14] in GC-MS analysis.

Reference

1. M. Tsivou, N. Kioukia-Fougia, E. Lyris, Y. Aggelis, A. Fragkaki, X. Kiouisi, P. Simitsek, H. Dimopoulou, I. P. Leontiou, M. Stamou, M. H. Spyridaki, and C. Georgakopoulos, (2006), An Overview of the Doping Control Analysis During the Olympic Games of 2004 in Athens, Greece. *Analytica Chimica Acta*, **555**, 1–13.
2. D. S. Fabricant, and N. R. Farnsworth, (2001). "The value of plants used in traditional medicine for drug discovery". *Environmental. Health Perspectives*, **109**, 69–75.
3. A. Bruneau, M. Mercure, G. P. Lewis, and P. S. Herendeen, (2008), Phylogenetic patterns and diversification in the caesalpinoid legumes, *Botany*, **86**, 697–718.
4. D. Cardoso, R. T. Pennington, L. P. De Queiroz, J. S. Boatwright, B. E. Van Wykd, M. F. Wojciechowskie, and M. Lavin, (2013), Reconstructing the deep-branching relationships of the papilionoid legumes, *South African Journal of Botany*, **89**: 58–75.
5. B. Marazzi, P. K. Endress, L. P. Queiroz, and E. Conti, (2006), Phylogenetic relationships within *Senna* (Leguminosae, Cassiinae) based on three chloroplast DNA regions: patterns in the evolution of floral symmetry and extrafloral nectaries, *American Journal of Botany*, **93**, 288–303.
6. M. H. Laurence, and J. M. Christopher, (1989), *Experimental Organic Chemistry: Principles and Practice* (Illustrated Edition), Wiley Blackwell, pp. 180–185.
7. S. M. K. Rates, (2001), Review, Plants as source of drugs, *Toxicology*, **39**, 204 – 207.
8. E. Stahl, (2005), *Thin Layer Chromatography, A Laboratory Handbook*. 2nd ed., Springer (India); Pvt., Ltd., pp, 53-56.
9. L. Kaijanen, M. Paakkunainen, S. Pietarinen, E. Jernström, S. Reinikainen, (2015) Ultraviolet Detection of Monosaccharides, Multiple Wavelength Strategy to Evaluate Results after Capillary Zone Electrophoretic Separation, *International Journal of Electrochemical Science*, **6**, 2950-2961.
10. Y. Ito, (2005), Golden rules and pitfalls in selecting optimum conditions for high-speed counter current chromatography, *Journal of Chromatography A*, **1065**, 145-168.
11. D. A. Romeh, (2013), Diethyl Phthalate and DioctylPhthate in *Plantago major* L. *African Journal of Agricultural Research*, **8**, 4360-4364pp.
12. L. Soto-Armenta, J. Sacramento-Rivero, A. Gonzalez-Coloma, G. Martinez-Sebastians, and A. J. Rocha-Urbe, (2019), Extraction and Bioactivity from

- Jatropha curcas* L. leaves by Steam Distillation. *Pakistan Journal of Botany*, **51**, 1-6pp
13. C. C. Bie, F. M. Li, Y. F Wang, H. Y Wang, Y. H Zhao, W. Zhao, and Z. Y. Wang, (2012), Mechanism of the inhibitory action of melochemicalDibutyl phthalate on *AlgaeGymnodiniumbreve*, *Environmental Science*, **33**, 228-232.
 14. L. Razeghi, M. Azizi, S. M. Ziaratnia, A. Z. Bagheri, and S. H. Nemati, (2015) Evaluation *in vitro* culture of *Kelussiaodoratissima*Mozaff and secondary metabolites production through suspension cultures, *The Pharma Innovation Journal*, **5**, 74-80.