Analysis of variation in petiole extracts from regenerant of *Jatropha curcas* L.

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ABSTRACT

Jatropha curcas L. is a perennial shrub with medicinal properties. Ethanol, methanol and aqueous extracts from petioles of the mother plant and plants regenerated from callus induced from leaf disc explants of *J. curcas* were prepared and variation in the phytochemical content was analyzed. The extracts were tested for the presence of alkaloids, tannins, glycosides, flavonoids, anthraquinones and saponins. Antibacterial activity and MIC (minimum inhibitory concentration) of petiole extracts from both the motherplant and regenerant were tested against six test microorganisms: *Staphylococcus aureus, Escherichia coli, Salmonella typhi, Salmonella typhimurium, Klebsiella pneumoniae* and *Bacillus cereus*. Extracts from both mother plant and regenerant showed activity against all the tested bacteria, ethanol extracts showed higher activity than methanol and aqueous extracts. The inhibitory action of ethanol extract from petiole of regenerant against *K. pneumoniae, S. typhimurium, S. typhi, E. coli* and *B. cereus* was higher than that of the mother plant extract. Methanol extract of the regenerant showed higher activity than that of mother plant against *S. aureus, S. typhimurium* and *B. cereus*. Thin layer chromatography profile showed variation in the number of constituents and Rf value, when visualized under UV at 365nm. Rf value of the antibacterial component in the extracts could be detected using bioautographic overlay analysis. The study shows the possibility for the induction of variation in tissue culture regenerants.

Keywords: *Jatropha curcas*, petiole extracts, antibacterial activity, minimum inhibitory concentration, tissue culture regenerants, thin layer chromatography, phytochemicals

INTRODUCTION

Jatropha curcas L. (purge nut, physic nut) belonging to the family Euphorbiaceae, is a multipurpose perennial shrub with many medicinal attributes apart from its high seed oil content. The genus name Jatropha derives from the Greek *jatros* (physician), *trophe* (food), which implies its medicinal uses [1]. The extracts of many *Jatropha* species including *Jatropha curcas* displayed potent cytotoxic, antitumour and antimicrobial activities in different assays [2]. The active component in its latex jatrophone has shown anti-cancer properties [3]. Curcacycline B [4], pohlianin A has antifungal and antimalarial activity [5] and jatrophidin I has antifungal activity [6]. Fagberno-Beyioku et al. [7] investigated and reported the anti-parasitic activity of the sap and crushed leaves of *J. curcas*. Phytochemical screening of petiole methanol extracts showed the presence of alkaloids, glycosides, tannins and phenols, flavonoids and phlobatannins [8]. Methanolic extract of *J. curcas* plant has shown antiulcer activity on aspirin-induced gastric lesions in Wistar rats [9]. The juice of the leaves has both procoagulant and anticoagulant activities [10]. The antimicrobial and larvicidal activities of the plant leaves have been reported by Kalimuthu et al. [11,12]. Zeng et al. [13] studied the *in vitro*

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antibiotic effect of alcohol extract from Jatropha leaf on *E. coli* and *S. aureus* and found that extract inhibited both the organisms. The activity against *E. coli* was found to be better than that against *S. aureus*. Methanolic extracts of stem and petiole of the plant showed, antibacterial, antifungal activities against gram-positive and gram negative bacteria with varying magnitudes [14].

Commercial exploration by means of plant tissue culture for antimicrobial activity, biopharmaceuticals and bio-energy production are some of the prospective future potential of this plant [8]. Plants are subjected to phenotypic or genotypic variation concomitant with changes in the environment. Plants regenerated from calli often display qualitative and quantitative phenotypic alterations, cytological abnormalities, sequence changes and gene activation and silencing. These cell culture induced changes, collectively called somaclonal variation, may be stable or unstable, reversible or irreversible, meiotically reset or transgenerationally transmitted [15-17]. Somaclonal variation has been reported at different levels in micropropagated plants. It can be morphological, cytological, cytochemical, biochemical, and molecular variations [18]. Kalimuthu et al. [12] reported that methanol extract from leaf callus of *Jatropha curcas* at high concentrations (1.0 and 1.2%) inhibited the growth of *Staphylococcus aureus* and *Pseudomonas* sp. with diameter of inhibition 20 and 23 mm respectively. Though, the antifungal activities of leaf extract were noteworthy, the methanol extract of leaf derived callus of *J. curcas* showed higher antifungal activity with concomitant increase in concentrations.

In the current study ethanol, methanol and aqueous extracts from petioles of the mother plant and plants regenerated from callus induced from leaf disc explants of *J. curcas* were prepared and variation in the phytochemical content was analyzed.

MATERIALS AND METHODS

Collection of plant material

The variation in bioactive compounds of extracts from mother plant, *Jatropha curcas* L. (TBGT 70000), and plants regenerated from callus induced from leaf disc on media containing 0.2 mg/L BAP and 0.4 mg/L IBA was analysed. In the study, extracts were taken from each plant part of four regenerants and compared with the mother plant. Petioles were harvested from regenerants and mother plants and washed with distilled water and tween 80 solution so as to remove dust and other foreign particles. The samples were then left on a clean surface and were air-dried in the shade for 10-12 days. The dried samples were pulverized to fine powder using an electric grinder and stored in air tight bottles. The powdered material was used, for preparation of extracts for studying the variation in *in vivo* and *in vitro* generated plants.

Preparation of extracts

0.25 g of the powdered plant material was cold extracted in analytical grade ethanol, methanol and distilled water. The extracts were then centrifuged for 5 minutes at 10000 rpm (Sigma laboratory centrifuge 3K30; rotor: 12154). The supernatant was collected and observed for its physical properties. It was then concentrated to dryness by evaporation. The dried samples were re dissolved (10 mg/mL) in corresponding solvents.

Phytochemical analysis of plant extracts

Various phytochemical tests were done to detect the presence of active chemical constituents like alkaloids, tannins, glycosides, flavonoids, anthraquinones, oils and fats and saponins in callus extracts and in petiole extracts (Table 1).

Table 1. Tests to detect the presence of phytochemicals in the extracts.

Phytochemicals	Test
Flavonoids Sulphuric acid test	A few drops of 10% concentrated sulphuric acid were added to solvent free extract, followed by 1mL of ammonia [19]
Shinoda test	To the test Solution, few fragments of Magnesium ribbon was added followed by drop wise addition of concentrated Hydrochloric acid.
Glycosides	0.5g of each extract was dissolved in 2 mL of chloroform. H_2SO_4 was carefully added to form a lower layer.
Phenolics	About 2mL of the extract was measured into a test tube and three drops of ferric chloride solution added.
Saponins	About 2mL of the extract was taken into a test tube and shaken vigorously
Tannins	1 mL of 5% ferric chloride was added to solvent free extract and observed for the formation of bluish black or greenish black precipitate [19].
Sterols and Triterpenoids Salkowski test	Treated extract in Chloroform with few drops of conc. sulphuric acid, shaken well and allowed to stand for some time. Observed for red color appears at the lower layer which indicates the presence of steroids and formation of yellow colored lower layer indicates the presence of triterpenoids.
Quinines	To the test solution added sodium hydroxide solution which gives blue, green and red precipitate within short time if quinine present.
Alkaloids Mayer's test	To the 1 mL of extract, added 1 mL of Mayer's reagent (potassium mercuric iodide solution).
Wagner's test	To the 1 mL of extract added 2 mL of Wagner's reagent (iodine in potassium iodide) and observed for the formation of reddish brown precipitate which indicates the presence of alkaloids.
Anthraquinones	To 150mg of solvent free extract, 2 mL of water followed by few drops of dilute sulphuric acid was added and allowed to heat on a water bath for 10mins followed by that 1 mL of dilute ammonia was added [19].

Antibacterial activity

The test microorganisms used in this study were *Staphylococcus aureus* (NCIM: 2127), *Escherichia coli* (NCIM: 2343), *Salmonella typhi* (NCIM: 2263), *Salmonella typhimurium* (NCIM: 2501), *Klebsiella pneumoniae* (NCIM: 2957) and *Bacillus cereus* (NCIM: 2155). The bacterial cultures of these microbes were revived in Nutrient broth medium and incubated at 37°C for 48 hours. Each bacterial culture was further maintained at 37°C on nutrient agar slants at 2-8°C and nutrient broth after every 48 hours of transferring. Nutrient agar medium was used as bacterial culture medium in the antibacterial assays. The antibacterial activity of the crude extracts was determined in accordance with the agar-well diffusion method described by Irobi et al. [20]. The bacterial isolates were first grown in a nutrient broth for 18 h before use. The petri plates were autoclaved at 121°C for 15 minutes and were cooled under laminar air flow. 20 mL of media was added into each sterile petri dish and solidified. 100µl of the cell suspensions were spread on nutrient agar media plates. Wells were then made in the agar using a sterile 3 mm diameter cork borer. Approximately 30µl of the crude extract was introduced into each wells, kept at room temperature for about 2 h and then incubated at 37°C for 24 h. Control without plant extracts and reference control using standard antibiotic (ampicillin 20mg/mL) were included in the assay. The plates were observed for zones of inhibition after 24 h and antibacterial activity was determined by measuring the diameters of zone of growth inhibition. The growth inhibition caused by the extract was corrected based on control and compared with those of reference control. The experiments were conducted in triplicate. The data was expressed as mean and standard error (M±SE).

Minimum inhibitory concentration (MIC)

The MIC estimation of the extract was determined using the methods of Gunasekaran [21]. An aliquot (100µl) of the extract solution at concentration of 10 mg/ mL was added to 900µl of presterilized nutrient broth. Subsequently, 100µl from the first test tube was transferred to the second test tube and this continued up to the sixth test tube. Thereafter, 100µl of each 24 h test bacterium (1.0×10^6 cells/mL) was inoculated into each test tube and mixed thoroughly. The test tubes were then incubated at 37°C for 24 h.

Thin Layer Chromatography (TLC)

In order to analyse variations in chemical constituents, extracts from regenerated plants which showed high bioactivity and from the mother plant were subjected to thin layer chromatographic analysis. Thin layer chromatography was performed on readymade plates (Merck plates silicagel $60F_{254}$, 20 × 20 dimension) by applying 10µl of the extract, with toluene: acetone: aceticacid (9:1:0.5) [22], as mobile phase. The separated components were visualized under visible and ultraviolet light (365 and 254nm) using UVi tech of Genei and the Retention values (Rf value) were calculated.

Bioautographic overlay assay

Bioautographic agar-overlay assay was done with TLC plate of plant extracts showing significant antimicrobial activity. For bioautographic analysis, developed TLC plates were dried to remove the solvent and placed over nutrient agar plates. It was overlayed with soft agar seeded with a concentrated suspension of actively growing *B. cereus* cells and incubated at 37°C for 24 hours. The plates were then sprayed with aqueous solution (1mg/mL) of 2, 3, 5-triphenyltetrazolium chloride and incubated for 24 h. The inhibition zones appears colorless against a purple background. Spots showing any inhibition were noted and compared with the Retention factor (Rf) of the related spots on the reference TLC plate. All samples were tested in triplicate and the tests were repeated thrice.

RESULTS AND DISCUSSION

The physical properties such as colour and viscosity of the extracts were found to vary with different solvents and sources used for extraction. Ethanol, methanol and aqueous extracts of petioles of regenerants and mother plant were odorless and slightly viscous. The color of the extracts varied from pale brown to dark green. Flavonoids, phenolic compounds, saponins, sterols, steroids, terpenoids, tannins, quinines, anthraquinones and alkaloids were found to be present in ethanol and methanol extracts of petiole of mother plant and regenerant where as phenol, terpenoids and sterols were not detected in aqueous extract (Table 2). Glycosides were absent in all the extracts. There was no variation between phytochemical profile of mother plant and *in vitro* generated plant for the tested secondary metabolites.

In the agar well diffusion assay, petiole extract of *J. curcas* mother plant and regenerant showed activity against all the tested bacteria. It is evident from figure 1 that the ethanol extract showed the maximum activity compared to the other two extracts. The inhibitory action of ethanol extract from petiole of regenerant against *K. pneumoniae*, *S. typhimurium*, *S. typhi*, *E. coli* and *B. cereus* was higher than that of the mother plant extract. Methanol extract of the regenerant showed activity against *S. aureus*, *S. typhimurium* and *B. cereus* than the same of mother plant petiole. Study by Adebayo and Kofi [23] revealed that the ethanol extract of the plant has greater antibacterial potential than the aqueous extract. The higher activity of the ethanolic extracts as compared to the

aqueous extract can be attributed to the presence of higher amounts of polyphenols as compared to aqueous extracts. It may possibly because ethanol penetrates the cellular membrane easily to extract the intracellular ingredients from the plant material [24]. Methanol is more polar than ethanol but due to its cytotoxic nature, it is unsuitable for extraction in certain kind of studies [25].

Table 2. Phytochemical screening of extracts from *J. curcas* mother plant and regenerant.

Extract	Flavanoids	Glycosides	Phenols	Saponins	Sterols	Tannins	Terpenoids	Quinines	Anthroquinones	Sterols	Alkaloids
Mother plant ethanol extract	+	-	+	+	+	+	+	+	+	+	+
Regenerant ethanol extract	+	-	+	+	+	+	+	+	+	+	+
Mother plant methanol extract	+	-	+	+	+	+	+	+	+	+	+
Regenerant methanol extract	+	-	+	+	+	+	+	+	+	+	+
Motherplant aqueous extract	+	-	-	+	-	+	-	+	+	-	-
Regenerant aqueous extract	+	-	-	+	-	+	-	+	+	-	-

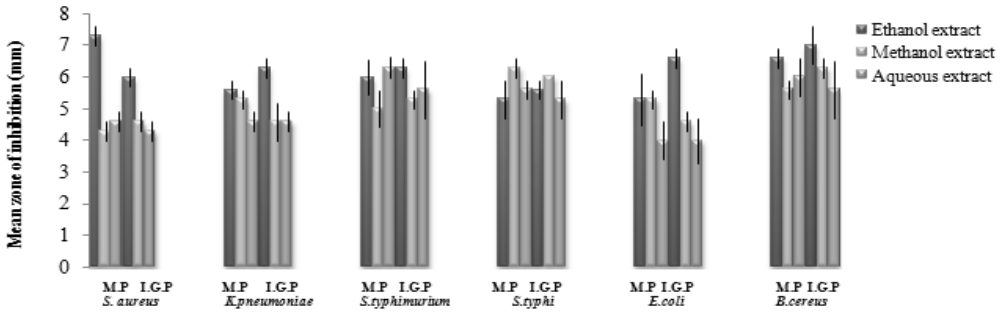

Figure 1. Antibacterial activity of extracts from petiole of *J. curcas* mother plant and *in vitro* regenerated plantlets. Values represent mean ± SE, MP- Mother Plant, IGP- *In vitro* Generated Plant.

The decrease in activity of aqueous extract can be ascribed to the enzyme polyphenol oxidase, which degrades polyphenols in water extracts, whereas in methanol and ethanol they are inactive [26]. The compounds found to be present in phytochemical screening in this study are known to be biologically active and therefore may aid in the antimicrobial activities of *J. curcas*. The larger zones of inhibition exhibited by the ethanolic extract of *J. curcas* against the test organism may be due to the presence of variety of active compounds induced to produce secondary metabolites such as tannins, alkaloids, flavonoids and saponins [27].

In the comparative study by Gawri and and Upadhyay, [8] on antimicrobial activity and phytochemicals, secondary metabolites like alkaloids, glycosides, flovonoids, tannins and phlobatannis were found to be present in the petiole samples as well as the cultured callus raised from petiole but the concentration of alkaloids and glycosides were higher in the callus. Both the samples showed high antimicrobial activity also. The extent of enhancement in the activity depends on many factors like type and composition of media, plant growth regulators and culture conditions; among which plant growth regulators play vital role in the production of the active compounds [28].

Ethanolic extract from petiole of *J. curcas* exhibited considerable antimicrobial activity, than methanol and aqueous extracts, hence minimum inhibitory concentration (MIC) of extracts from mother plant and *in vitro* regenerated plantlets analyzed with 6 bacterial strains. MIC of Ampicillin control tested against the same organisms depicted in the table 3 for comparison with the test extracts. In the MIC analysis, *S. aureus* and *B. cereus* required the extract at MIC, 1.0 mg/L, where as bacterial growth was observed with 1.0 mg/mL ethanolic extract and found that 10 mg/mL of the extract is necessary for completely inhibiting the bacterial growth.

TLC profiling showed the variation in number of components in the ethanolic extract of mother plant and regenerant. The number of fractions were eight in the ethanolic extract of *Jatropha curcas* petiole from motherplant under thin layer chromatography analysis, whereas it was nine in the extract from regenerant as depicted in table 4. The chromatogram was visible under uv at 365nm (Figure 2). Overlay bioautography proved their antibacterial activity against bacterial strain. Rf value of the antibacterial component in the extracts could be detected using bioautographic overlay analysis. Table 3 shows the TLC bioautographic results of *J. curcas* petiole ethanol extract.

Table 3. MIC of ethanol extracts from petiole of *J. curcas* mother plant and *in vitro* regenerants.

Test bacteria	Ampicillin (mg/mL)	Mother plant petiole extract (mg/mL)	Regenerant petiole extract (mg/mL)
Staphylococcus aureus	2×10^{-1}	1.0	1.0
Klebsiella pneumoniae	2×10^{-2}	10.0	10.0
Salmonella typhimurium	2×10^{-2}	10.0	10.0
Salmonella typhi	2×10^{-4}	10.0	10.0
Escherichia coli	2×10^{-1}	10.0	10.0
Bacillus cereus	2×10^{-4}	1.0	1.0

Table 4. Retention factor of thin layer chromatography analysis of ethanol extracts from petiole of *J. curcas* mother plant and *in vitro* regenerants with solvents, toluene: acetone: acetic acid (9:1:0.5).

Ethanol extract	Number of components	Color of the band	Rf value	Inhibitory effect on *B. cereus*
Mother plant	8	Blue	0.19	+
		Red	0.20	+
		Red	0.48	+
		Blue	0.52	-
		Red	0.55	-
		Blue	0.65	-
		Red	0.74	-
		Red	0.87	-
Regenerated plant	9	Blue	0.19	+
		Red	0.23	+
		Blue	0.30	+
		Red	0.47	+
		Blue	0.53	-
		Red	0.57	-
		Blue	0.60	-
		Red	0.73	-
		Red	0.85	-

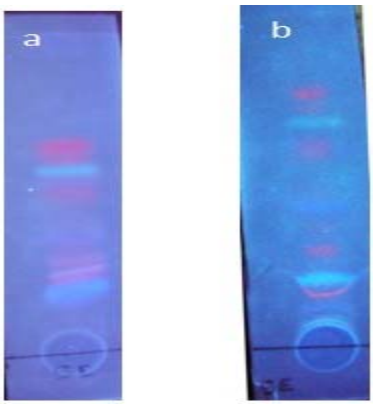

Figure 2. Thin layer chromatogram of ethanol extracts from petiole of *J. curcas* (a) mother plant and (b) *in vitro* regenerants under uv at 365nm.

The bioautography revealed zones of growth inhibition for *B. cereus*, where as in other bacteria the zones of inhibition were less visible. Clear zone observed in the Rf zone lies between 0.19-0.48 in both the sample. *In vitro* regenerated plantlets have been reported to produce higher yields of active compounds [29-31]. The results of the present study suggest that *in vitro* culture may affect the phytochemical content of the regenerant. Further identification of minor constituents and molecular dissection of the stress induced pathway can bring forth a significant enhancement in the pharmaceutically important secondary metabolite production.

REFERENCES

[1] Agarwal D, Agarwal AK. Appl. Therm. Eng. 2007, 27:2314-2323.
[2] Singh R, Agarwal T, Rastogi R, et al. J. Pharmaceutic. Biomed. Sci. 2012, 15(15):1-4.
[3] Kosasi S, Van Der Sluis WG, Labadie RP. J. Ethanopharmacol. 1989, 27(1-2):81-90.
[4] Auvin C, Baraguey C, Blond A, et al. Tet. Lett. 1997, 38:2845-2848.
[5] Auvin C, Baraguey C, Blond A, et al. Tet. Lett. 1999, 55:11495-11510.
[6] Devappa RK, Makkar HPS, Becker K. J. Agric. Food Chem. 2010, 58:6543-6555.
[7] Fagberno-Beyioku AF, Oyibo WA, Anuforom BC. East Afr. Med. J. 1998, 75:508-511.
[8] Gawri S, Upadhyay A. J. Phytol. 2012, 4(3):18-20.
[9] Kannappan N, Jaikumar S, Manavalan R, Muthu AK. Pharmacol. Online 2008, 1:279-293.
[10] Osoniyi O, Onajobi F. J. Ethnopharmacol. 2003, 89:101-105.
[11] Kalimuthu K, Kadarkaral M, Savariar V, Siva K. Parasitol. Res. 2011, 109 (5):1251-1257.
[12] Kalimuthu K, Vijayakumar S, Senthilkumar R. Int. J. Pharm. Bio. Sci. 2010, 1(3):1-5.
[13] Zeng LH, Yan F, Chen F. Chin. Poult. Sci. 2004, 8(1):35-37.
[14] Kumar S, Chaube A, Jain SK. Energy Policy 2012, 41:775-781.
[15] Karp A. Mol. Cell Biol. 1991, 7:1-58.
[16] Phillips RL, Kaeppler SM, Olhoft P. Proc. Natl. Acad. Sci. USA 1994, 91:5222-5226.
[17] Kaeppler SM, Kaeppler HF, Rhee Y. Plant Mol Biol. 2010, 43:179-188.
[18] Rani V, Raina S. In Vitro Cell. Dev. Biol. Plant 2000, 36:319-330.
[19] Siddiqui AA, Ali M. Practical Pharmaceutical Chemistry, 1st ed., CBS Publisher and distributors, New Delhi 1997, 126-131.
[20] Irobi ON, Moo-Young M, Anderson WA, Daramola SO. Int. J. Pharmacog. 1994, 34:87-90.
[21] Gunasekaran P. Laboratory Manual in Microbiology, 1st ed., New Age International Publishers, New Delhi, 2005, 40-41.
[22] Vandana AS. Int. J. Pharm. Tech. 2010, 2(4):2438-2440.
[23] Adebayo OL, Alphonse, Kofi P. Int. J. Pharm. Phytopharmacol. Res. 2011, 1(3):117-123.

[24] Wang GX. Veterinary Parasitol. 2010, 171:305-313.
[25] Tiwari P, Kumar B, Kaur M, et al. Int. Pharmaceut. Sci. 2011, 1(1):98-106.
[26] Lapornik B, Prosek M, Wondra AG. J. Food Eng. 2005, 71:214-222.
[27] Abo KA, Adeyemi AA, Jegede IA. Niger. Sci. Forum 2000, 3(2):57-63.
[28] Morris P, Scragg AH, Smart NJ, Stafford A. In: Plant cell culture - a practical approach, Dixon RA (ed.), IRL Press, London, 1985, 230.
[29] Fowke LC, Attree SM, Pomeroy MK. Plant Cell Rep. 1994, 13:601-606.
[30] Mahagamasekera MGP, Doran PM. Phytochem. 1998, 47: 17-25.
[31] Han KH, Gordon MP, Floss H. In: Biotechnology in Agriculture and Forestry, No. 44, Transgenic Trees, Bajaj YPS (ed.), Springer-Verlag, Berlin 1999, 291-306.