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Comparative Studies on Phytochemical Analysis of Callus and Wild Plants of *Phyllanthus niruri* with Special Reference to Phyllanthin

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Abstract

The main objective of the present study was to induce callus form nodal explants of *Phyllanthus niruri* and, compared the quantification of Phyllanthin in callus and naturally propagated *Phyllanthus niruri*. For callus initiation, different sterile plant parts were transferred on MS medium supplemented with auxins either alone like 0.5-2.0 mg/l 2- 4 D or with varied concentration and combinations of cytokinins like 0.5 mg/l BAP and 0.5 mg/l KN. The petroleum ether, chloroform, ethyl acetate, ethanol and aqueous extracts of *Phyllanthus niruri* plant and callus were prepared and performed its phytochemical analysis. The total flavonoids and polyphenol were investigated to quantify the presence of polyphenol compounds in callus extract and plant extract. The quantification of Phyllanthin in plant extract and callus extract were performed by HPLC. Maximum callus (90.5%) induction from stem/leaf segments on MS medium supplemented with 1.0 mg/l 2,4-D with 0.5 mg/l BAP (6-benzylaminopurine) within 25 days which was fragile in morphology. Preliminary phytochemical revealed the presence of various secondary metabolites in different extract of plant. The concentrations of flavonoids polyphenol in ethanol extract of callus were higher compared to crude plant extract. Further Phyllanthin content in callus was found to be significantly increased in response to field grown plants.

1 Introduction

Today's health care systems rely largely on plant material. Plants occupy an important role in the modern and traditional system in all over the world. Modern medicines are primarily from synthetic or plant origin while synthetic origin may have toxic effects, the plant medicines have less toxicity, and their importance is being realized in both developed and developing countries, plants are an important source of medicines and play a key role in world health[1]. Plant secondary metabolites were found to be sources of various phytochemicals that could be used directly or as intermediates for the production of pharmaceuticals, as additives in cosmetic, food or drink supplements[2].

Phyllanthus family is one of the largest and commonest plant families belong to Euphorbiaceae, containing 550 to 750 species and most of them produce valuable secondary metabolites which have been extracted from whole plants[3]. Phyllanthus are used traditionally to treat different diseases like diabetes, hepatitis B and urolithiasis inflammatory bowel disease. *Phyllanthus niruri* is an important medicinal plant has been used as a medicine for many diseases like diabetes, jaundice, dropsy flu genitourinary infections, skin diseases etc. It is reported that plant shows anti-tumor and anti-carcinogenic activities and has a great potential to the treatment for hepatitis B viral infection by which this plant develop more interest among pharmaceuticals. Several compounds such as alkaloids, tannins, falconoid, lignans, Phenols and terpenes have been isolated and identified in *Phyllanthus niruri* plant and have shown anticipative action in mice and other therapeutic activities[4].

Medicinal plants have been given much attention as a source of curative compound by Pharma industry so the plant is becoming

endangered. Seed dormancy, low germination frequency restricts the propagation of *Phyllanthus niruri.* So callus culture is an alternative for meeting their demand of phytoconstituent of *Phyllanthus niruri.* Callus culture one of the technique of plant tissue culture has its importance in pharma industry since the callus has all the phytoconstituents present in original plant. Which is employed for plant based drug production in pharmaceutical. The objectives of this study was to induce callus form nodal explants of *Phyllanthus niruri* for comparative studies of phytoconstituents profile of callus and wild plants of *Phyllanthus niruri.*

2 Materials and Methods

2.1 Callus induction

For callus initiation, different sterile plant parts like leaves /shoots from 15 to 20 days old *in vitro* raised shoots culture were taken. 3 to 4 millimeter section of leaves /shoots were transferred on Murashige and Skoog (MS)[5] supplemented with auxins either alone like 0.5-2.0 mg/l 2- 4 D (2 4-dichlorophenoxyacetic acid) or with varied concentration and combinations of cytokinins like 0.5 mg/l BAP (6-benzylaminopurine) and 0.5 mg/l KN (kinetin)**.** The cultures were incubated in culture room at 25±2^{o}c under 16 hours photo period provided by cool white fluorescent tubes.For mass production initiated callus of 15 to 20 days, were further transferred either to the same or different medium supplemented with higher concentration of growth hormones like 2.0 mg/l 2, 4.D (2 4-dichlorophenoxyacetic acid) either alone or with combination of cytokinins like 0.2 - 1.0 mg/l BAP (6-benzylaminopurine) and 0.5 -1.0 mg/l KN (Kinetin).

2.2. Preparation of extracts for phytochemical analysis of callus and plant material

2.2.1 Sample preparation

Plant material of *Phyllanthus niruri* were shade dried at room temperature. The shade dried plant material was coarsely powdered and subjected to extraction with petroleum ether, choloroform, ethyl acetate, ethanol and water in a soxhlet apparatus.

Further the callus was extracted with ethanol.

2.2.2 Qualitative phytochemical tests

The extracts of plant material were subjected to various qualitative tests to detect the presence of plant constituents. The method used for the phytochemical screening was reported in various journal[6-10].

2.2.3. Total Polyphenol content

Total polyphenol content was determined using colorimetric method. 1.0 ml of the prepared extract was oxidized using 2.5 ml of Folin- Ciocalteu reagent, and 2.0 ml of sodium carbonate solution (75 g/l) was then added to the reaction mixture. The absorbance readings were taken at 760 nm after incubation at room temperature for 2 hours. The amount was calculated using the Gallic acid calibration curve. The results were expressed as Gallic acid equivalent (GAE) mg per 100 ml of the sample (extract).

A. Calibration curves of Gallic acid

Accurately weighed 100 mg of gallic acid was dissolved in 100 ml of distilled water which gives the concentration of 1000 µg/ml. 10 ml of this solution was taken and made up to 100 ml with gallic acid which contains the concentration of 100µg/ml. Further 10 ml of this solution was taken and made up to 100 ml with gallic acid which contains the concentration of 10 µg/ml. 1 to 10 ml were taken from this solution and made up to 10 ml to get the concentration ranges of 1 to 10 µg/ml. Calibration curve was plotted by mixing 1 ml aliquots of gallic acid solutions with 2.5 ml of Folin-Ciocalteu reagent and 2.0 ml of sodium carbonate solution (75g/l). The absorbance was measured after incubation at room temperature for 2 hr at 760 nm using UV spectrophotometer, against blank solution.

2.2.4 Total flavonoids content

Flavones and flavonoids contents were analyzed by the colorimetric method. 9.8 ml of the prepared extract was mixed with a 10% solution of aluminum chloride (200 µl). After 30 min, absorption was measured at a 425 nm wavelength. The amount was calculated using quercetin calibration curve. The results were expressed as the quercetin equivalent (QE) mg per 100 ml of the sample.

A. Calibration curves of Quercetin

Accurately weighed 100 mg of quercetin was dissolved in 100 ml of distilled water which gives the concentration of 1000 µg/ml. 10 ml of this solution was taken and made up to 100 ml with quercetin which contains the concentration of 100 µg/ml. Further 10 ml of this solution was taken and made up to 100 ml with quercetin which contains the concentration of 10 µg/ml. 1 to 10 ml were taken from this solution and made up to 10 ml to get the concentration ranges of 1 to 10 µg/ml. Calibration curve was plotted by mixing 9.8 ml aliquots of quercetin solutions with a 10% solution of aluminum chloride (200 µl). The absorbance was measured 30 min at 425 nm using UV spectrophotometer, against blank solution.

2.2.5 HPLC analysis of extract

The HPLC analyses were performed on the extracts of plant and extract of callus of *Phyllanthus niruri*.

2.2.5.1 Preparation of standard stock solution

Stock solutions (1000 µg/ ml) of phyllanthin were prepared by dissolving 5 mg of the compound in 5 ml of HPLC grade methanol. The solution was then stored at −20 °C. Quantification was carried out using 5 levels of external

standards obtained by serial dilutions of stock solutions at a concentration range of 100–20 ng/ ml. Each concentration of standard was filtered through a 0.22 µm nylon membrane filter (Millipore, USA) before HPLC analysis.

An accurately weighed quantity of phyllanthin (10mg) was transferred to a 10mL volumetric flask, dissolved and diluted to the mark with Methanol: Water (70:30) to obtain standard stock solution of 1000µg/mL.

2.2.5.2 Sample preparation

100mg extract of plant and callus transferred to 10mL standard flask. Volume is made up to the mark with Methanol: Water (70:30), sonicated for 10 min. It is filtered with 0.22µ filter to obtain sample stock solution. The sample solution further diluted and made upto 100 ng/ml concentration. Then it is filtered with 0.22µ filter. Prepared sample solution was analysed.

2.2.5.3 Instrument and Chromatographic Conditions

Chromatographic separation was achieved on Young Lin ACME 9000 Isocratic HPLC equipped with UV-Visible detector. The separation was carried out on a Promosil C-18 HPLC Column under the following chromatographic conditions: the injection volume was 10 µL. The column temperature was maintained at 35 °C. The wavelength of UV detection was set at 220 nm. The mobile phase was composed of Methanol: Water (70:30) at a flow rate of 1.0 mL/min.

3 Results and Discussions

3.1 Callus induction

Secondary metabolite production has achieved by callus iniduction and production from *in vitro* raised sterile leaves (young and old) and shoot sections excised from micro propagated shoot culture.

Highest percentage of callus induction (90.0%) was observed on medium containing 1.0 mg/l 2, 4-D with 0.5 mg/l BAP within 30 days and the callus formed was light green and soft in morphology followed by 65.0 % of callus production frequency on medium containing 1.5 mg/l 2, 4-D (2 4-dichlorophenoxyacetic acid) within 30 days and the callus was green and soft in morphology (Table 1 and Fig 1).

Table 1: Effect of MS media and growth regulators on callus induction of *Phyllanthes niruri* for secondary metabolite production

MS+Auxin/cytokinin (mg/l)	%age of callus induction	No of days required	Morphology of callus
MS + 0.5 2 4 D	40.3	35	Brown compact
MS + 1.0 2 4 D	75.5	30	Greenish yellow
MS + 1.5 2 4 D	65.0	30	Green and soft
MS + 2.0 2 4 D	50.5	30	Brownish compact
MS + 0.5 2 4 D + 0.5 BAP	85.0	25	Pale yellow and soft
MS + 0.5 2 4 D + 0.5 KN	60.4	30	White friable
MS + 1.0 2 4 D + 0.5 BAP	90.0	30	Light Green and soft
MS + 1.0 2 4 D +0.5 KN	40.8	30	Whitish watery calli
MS + 2.0 2 4 D + 0.5 BAP	60.0	35	Yellow green soft
MS + 2.0 2 4 D + 0.5 KN	40.5	35	Light brown

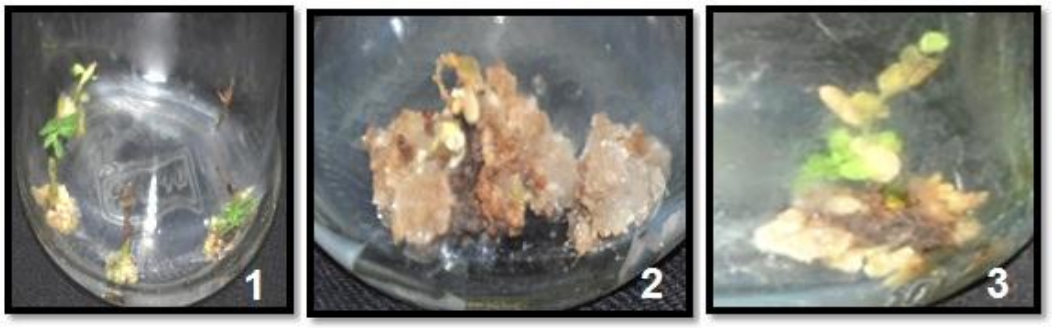

Fig 1: Callus induction and callus production from leaf/shoot of *P. niruri* MS +1.0 mg/l 2,4-D

It was found that in both the media there was maximum growth of callus during second (after 15 days) and third (after 30 days) passage in the same medium. The maximum growth of the callus was observed at the end of 45 days which remained stationary till 60 days suggesting that the life cycle of the cells in culture were completed after 45 days of time**.**

The highest dry weight of callus was observed on medium containing 1.0 mg/l 2, 4-D (2 4-dichlorophenoxyacetic acid), with 0.5 mg/l BAP (6-benzylaminopurine) and the callus was pale yellow in colour. It was observed that only pale yellow and soft callus produced and accumulated phytochemicals. It was confirmed that for the formation of callus of any plant among growth hormones higher concentration of auxins are required[11]. Similar results has reported of callus induction 2, 4-D, with BAP to enhance the formation of secondary metabolites as reported by Thakur *et al.,* (2011)[12].

Few reports have described chemical constituents of *Phyllanthes niruri* growing in the wild, but the field grown plant is not desirable for constant metabolite production at the commercial level because of environmental fluctuation, climatic and geographical variations, diseases and pathogen attack.

In contrast to this, plant cell culture technology shows promise for the large-scale production of high-value secondary metabolites. This technique offers uniform secondary product synthesis by eliminating effect of unforeseen climatic conditions and diseases as observed in field grown.

3.2 Phytochemical analysis

By using different solvent for the extraction it was observed that the highest percentage of yield was noted of Petroleum Ether (8.501%).

Qualitative analysis of the present study of ethanol and aqueous extract of plant and callus of *Phyllanthus niruri* showed the presence of maximum constituent like alkaloids, glycosides, flavonoids, steroids, phenolics, aminoacids, carbohydrates, proteins, deterpines and saponins (Table 2).

Table 2: Phytochemical present in *Phyllanthus niruri*

Constituents	A	B	C	D	E
Alkaloids	-ve	+ve	+ve	+ve	+ve
Glycosides	-ve	-ve	-ve	-ve	-ve
Flavonoids	-ve	+ve	+ve	+ve	+ve
Steroids	-ve	-ve	-ve	-ve	-ve
Phenolics	-ve	-ve	+ve	+ve	+ve
Amino Acids	-ve	-ve	-ve	-ve	-ve
Carbohydrate	-ve	-ve	-ve	-ve	-ve
Proteins	-ve	-ve	-ve	-ve	-ve
Saponins	-ve	-ve	-ve	+ve	+ve
Diterpines	-ve	-ve	+ve	+ve	+ve

A- Pet. Ether, B- Chloroform, C- Ethyl acetate, D- Ethanol, E- water

3.3 Total phenolic content

The ethanol extract of plants and callus of *Phyllanthus niruri* was evaluated for investigation of the total phenolic content concentrations in both extracts. Standard curve of Gallic acid was calculated and plotted in distilled water for determining absorption data (Fig 2).

From this Beer's law range and regression coefficient is determined. The linear equation of Gallic acid was found to be y = 0.0149x - 0.001 (Fig 2). The results of the total phenolic content of the extracts examined, using Folin-Ciocalteu method, are depicted in table 3. The total phenolic content in extracts, expressed as gallic acid equivalents. The total phenolic content of leaves and callus extract of *Phyllanthus niruri* were 55.42 and 83.17 GAE mg/gm, respectively. The callus extracts exhibited highest amount of total polyphenol content compared to plant extracts.

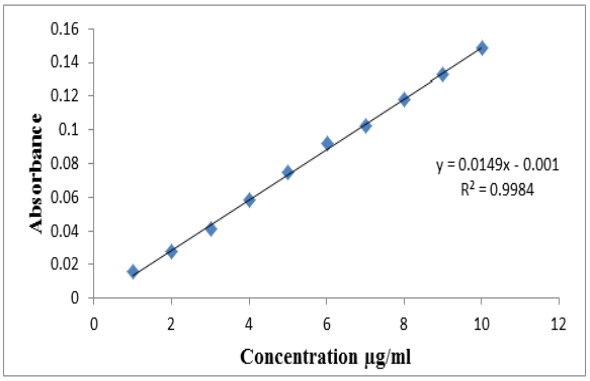

Fig 2: Calibration curve of Gallic acid in distilled water

Table 3: Determination of total Polyphenol content of *Phyllanthus niruri*

Extract	Total polyphenol content (GAE mg/gm)
Leaves	55.42±0.83
Callus	83.17±0.62

Data expressed as gallic acid equivalent (GAE) mg per gm of the extract, Values are mean ± SEM of triplicate determinations

3.4 Total flavonoids Content

The concentration of flavonoids in hydroalcoholic and aqueous extract of *Phyllanthus niruri* were determined by spectrophotometer using aluminum chloride. The content of flavonoids was expressed in terms of quercetin equivalents. Standard curve of quercetin was calculated and plotted in distilled water for determining absorption data (Fig 3). From this Beer's law range and regression coefficient is determined. The linear equation of quercetin was found to be y = 0.0184x + 0.001 (Fig 3). The content of flavonoids identified in the tested extracts is shown in table 4. The concentrations of flavonoids in hydroalcoholic and aqueous extract of *Phyllanthus niruri* were 49.51and 78.29 QE mg/gm, respectively. The callus extracts exhibited highest amount of flavonoids content compared to leaves extracts.

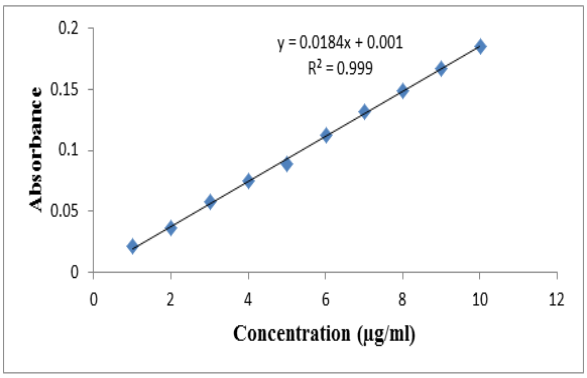

Fig 3: Calibration curve of Quercetin in distilled water

Table 4: Determination of total flavonol content of *Phyllanthus niruri*

Extract	Total flavonol content (QE mg/gm)
Leaves	49.51±0.38
Callus	78.29±0.71

Data expressed as gallic acid equivalent (GAE) mg per gm of the extract, Values are mean ± SEM of triplicate determinations

3.5 HPLC analysis

The HPLC analyses were performed on the extracts of field grown plants/callus of *Phyllanthus niruri* by method for determination of principle compound Phyllanthin the most antiseptic alkaloid in this medicinal plant.

Validation data was collected from three analytical runs. The linear regression equation analyte was y = 47.806x - 55.98. The linear range for phyllanthin was adequate for this method to be used in the determination of quantity of phyllanthin present in extract.

The amount of total Phyllanthin was determined by HPLC method. Phyllanthin was used as a standard compound and the Phyllanthin were expressed as ng/ml using the standard curve equation: y = 47.806x - 55.98 (Fig 4).

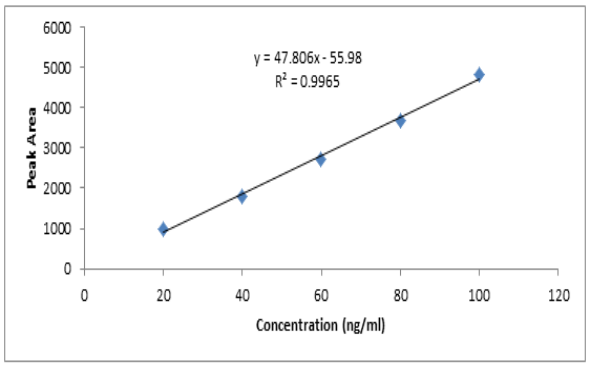

Fig 4: Calibration curve of phyllanthin

The analysis of sample can be completed within 15 min. The chromatogram of HPLC of extract of plant and callus depicted R_t value at 14.5 min, by using solvent system Methanol: Water (70:30). The peaks which could be identified in the chromatogram were Phyllanthin. They were identified by comparison with the compounds chromatogram reported in literature. The HPLC chromatogram exhibited that quantity of Phyllanthin present in callus extract was more compared to extract of plant (Table 5; Fig 5 & 6)

Table 5: HPLC analysis of different samples

Extract	Peak area	Concentration (ng/ml)
Plant extract	1092.438	61.28
Callus extract	1461.745	98.15

Plant cell culture technology shows promise for the mass production of quality and high values. This technique offers uniform secondary product synthesis by eliminating effect of unforeseen climatic conditions and diseases as observed in field grown plants. In *Phyllanthus niruri* very little reports are available on *in vitro Phyllanthin* production. Findings of the present studies have opened up the possibility of producing *Phyllanthus niruri* plants with desired metabolite content, throughout the year, consistently irrespective of the season, which will help the pharmaceutical industries to achieve better yield by using superior quality raw materials. Quantitative Phytochemicals analysis of the Plant of *P. niruri* contains alkaloids, Glycosides, Flavonoids, Steroids, Phenolics,

Aminoacids, Carbohydrates, Proteins, Deterpines and Saponins.

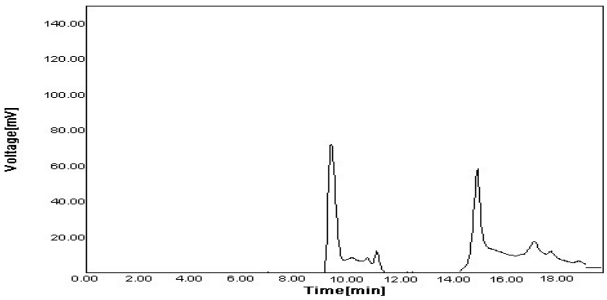

Fig 5: HPLC chromatogram of plant

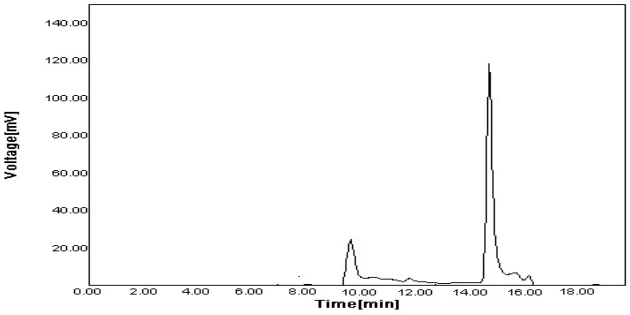

Fig 6: HPLC chromatogram of callus

All the compounds were isolated from methanolic extracts have been also reported by Rajeshkumar *et al.,* (2002)[13]; Manas *et al.,* (2012)[14] and the presence of alkaloids, flavonoids, proteins and carbohydrates in the plant parts of *Phyllanthus niruri.* Similar results has also isolated in other species of Phyllanthus by Filho *et al.,* (1996)[15]; Somanabandhu *et al.,* (1993)[16] Bagalkotkar *et al*., (2006)[17]. Joseph *et al*., (2013)[18] has investigated the qualitative analysis of phytochemicals from five important medicinal plants. In the present experiment same results have reported from the *in vitro* raised callus of *Phyllanthus niruri*.

The results obtained from the present investigation almost related to the results of *P. niruri*, *P. tenellus* and *P. urinaria* the main compounds identified were flavonoids, tannins and phenols[19]. Findings of the present investigation of phytochemical analysis is as similar as the results achieved in *Phillanthus niruri* reported the extracts are rich in by alkaloids, sterols, carbohydrates, flavonoids, tannins and resins[20].

4 Conclusion

The present study demonstrated that the ethanol extract of callus contained higher quantity of polyphenol and flavonoid compared to wild plant extracts. The HPLC study inferred that Phyllanthin in callus was higher compared to wild plant extract. The developed methods herein would prove to be applied to intrinsic quality control of this drug.

5 Conflict of interest

We declared that we have no conflict of interest.

6 Author contributions

AP, PS and SK have carried out the research work in the laboratory. SK compiled and analyzed the data of present work. All authors approved the final manuscript.

7 References

1. Constabel F. Medicinal plant biotechnology. Planta Med. 1990; 5(6): 421–425.
2. Liang OP. Micropropagation and callus culture *of Phyllanthus niruri L, phyllanthus urinaria L and Phyllanthus myrtifolius moon* (euphorbiaceae) with the establishment of cell suspension culture of *Phyllanthus niruri* L. 2007; 45-50.
3. Unander DW. Phyllanthus specious: in vitro culture and production of secondary metabolites. In Biotechnology in agriculture and forestry. (Y.P.S. Bajaj, ed,), V.37 Springer – Verlag, Berlin. 1996; pp. 304 – 318.
4. Cechinel Filho V, Santos, Campos ROP, Miguel OG, Yunes, Ferrari Messana and Calixto JB. Chemical and pharmacological studies of *phyllanthus carolinensis* in mice. Journal of pharmaceutical pharmacology. 1996; 48:1231-1236.
5. Murashige T. Plant Propagation through Tissue Cultures. Annual Review of Plant Physiology. 1974; 25: 135-166.
6. Bama SS, Kingsley JS, Sankaranarayanan S, Bama P. Antibacterial activity of different phytochemical extracts from the leaves of *T. procumbens* Linn: Identification and mode of action of the terpenoid compound as antibacterial. Int J Pharm Pharm Sci. 2012; 4(1): 557-564.
7. Harbone JB. Phytochemical Methods: A guide to modern techniques of plant analysis. London: Chapman and Hall Ltd; 1984.
8. Kenwat R, Prasad P, Sahu RK, Roy A, Saraf S. Preliminary phytochemical screening and in vitro antioxidant efficacy of fruit oil of *Martynia annua*. UK Journal of Pharmaceutical and Biosciences. 2014; 2(1): 16-22.
9. Gupta AK, Ahirwar NK, Shinde N, Choudhary M, Rajput YS, Singh A. Phytochemical Screening and Antimicrobial Assessment of Leaves of *Adhatoda vasica, Azadirachta indica* and *Datura stramonium*. UK Journal of Pharmaceutical and Biosciences. 2013; 1(1): 42-47.
10. Singh B, Ahamad A, Pal V. Evaluation of Antibacterial Activity and Phytochemical Screening of *Azadirachta*

indica Leaves Extracts Against *Staphylococcus aureus*. UK Journal of Pharmaceutical and Biosciences. 2015; 3(4): 43-47.

11. Miller CO, Skoog F, Okumura FS, von Saltza M**H**, Strong FM. Structure and synthesis of kinetin. J Am Chem Soc. 1957; 78**:** 2662–2663.
12. Thakur JS, RK Agarwal, MD Kharya. Enhancing hepatoprotective bioactives of Phyllanthus amarus through immobilization by growth promoters and media changes. the Indian Pharmaceutical sciences. 2011; 73: 271-275.
13. Rajeshkumar NV, Joy KL, Kuttan G, Ramsewak RS, Nair MG, Kuttan R. Antitumour and anticarcinogenic activity of *Phyllanthus amarus* extract. J Ethnopharmacol. 2002; 81(1): 17-22.
14. Manas M, Rashi S, Jaya S, Ruchi P, Raka K. Phytochemical screening and antimicrobial activity of *Phyllanthus niruri.* Linn. Elixir Appl. Botany. 2012; 46: 8487-8489.
15. Filho CV, Santos ARS, Campos ROP, Miguel OG, Yunes RA, Ferrari F, Messana J, Calixto JB. Chemical and pharmacological studies of *Phyllanthus caroliniensis* in mice. *J. Pharmaceu. Pharmacol.* 1996; 48: 1231-1236.
16. Somanabandhu A, Nitayangkura S, Mahidol C, Ruchirawat S, Likhitwitaya wuid K, Shieh HL, Chai H, Pezzuto JM, Cordell GA. 1H-and 14C-NMR assignments of phyllanthin and hypophyllanthin: lignans that enhance cytotoxicity with cultured multidrug-resistant cells. J. Nat. Prod. 1993; 56: 233-239.
17. Bagalkotkar G, Sagineedu S, Saad M, Stanslas J. Phytochemicals from Phyllanthus niruri Linn. and their pharmacological properties: a review. Journal of pharmacy and pharmacology. 2006; 58: 1559-1570.
18. Joseph BS, Kumbhare PH, Kale MC. Preliminary phytochemical screening of selected Medicinal Plants. Int. Res. J. of Science & Engineering. 2013; 1(2): 55-62.
19. Santos AR, Filho VC, Niero R, Vianna AM, Moreno FN, Campos MM, Yunes RA, Calixto JB. Analgesic effects of callus culture extracts from selected species of *Phyllanthus* in Mice. J. Pharmacy and Pharmacol. 1994; 46: 755-759.
20. Samali A, Florence DT, Odeniran OA, Cordelia ON. Evaluation of chemical constituents of *Phyllanthus Niruri* African Journal of Pharmacy and Pharmacology. 2012; 6(3): 125-128.