Analytical Techniques used for Isolation and Identification of Phytoconstituents in Phyllanthus Urinaria: A Review

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Abstract—*Phyllanthus urinaria (P. urinaria) is a herb (Family: Phyllanthaceae) which is endowed with multiple medicinal properties. The decoction made from whole plant has been used as folk medicine for treating urinary diseases, diabetes, stomach problems, fever and malaria. Pharmacognostic and pharmacological studies of plant extract have revealed antiviral, antioxidant, anticancer, analgesic and anti-inflammatory activity. Further, many phytochemicals that can be grouped as lignans, flavonoids, sterols etc. have been isolated from P. urinaria, using several analytical techniques (eg.TLC, HPLC, HPLC-SPE-NMR and HPLC-PDA-MS). Many of the phytoconstituents isolated from the herb have been experimentally shown to have implication in antiviral, anti-inflammatory, analgesic and antiangiogenic activity. The plant holds much promise for future utilization as a herbal drug and further investigations demonstrating pharmacological activity of its phytoconstituents should be done. Therefore, chemical and structural identification and characterization studies of the bioactive phytochemicals found in P. urinaria are essential for effective utilization in therapeutics.*

Keywords*: Phyllanthus urinaria, Phytoconstituent, Analytical techniques, Anti-inflammatory, Anticancer, Antiviral, Antiangiogenic.*

1. INTRODUCTION

Phyllanthus urinaria (*P. urinaria*) is a herb (Family: Phyllanthaceae) which is of immense ethnobotanical value. It has several medicinal properties which makes it important to study its pharmacognosy and phytochemistry. The plant extracts have been used as folk medicine for treating urinary diseases, diabetes, stomach problems, fever, malaria and many other common ailments. Pharmacognostic studies in this herb, have revealed antiviral, antioxidant, anticancer, analgesic and anti-inflammatory properties. These medicinal properties can be attributed to several phytochemicals that have been isolated from *P. urinaria*. Nine major classes of compounds have been isolated from this herb that is lignans, ellagitannin, flavonoid, acid, alkanol, phyallate, sterol, triterpenes and coumarin. Lignans, phyllanthin, hypophyllanthin, niranthin, nirtetralin, virgatusin and heliobupthalmin lactone are commonly present in this herb and also in *P. amarus* and *P. maderaspatensis* [1]. One of the lignin phyltetralin is common to *P. amarus, P. fraternus, P. tenellus, P. virgatus and P. urinaria* [2]. Some of flavonoids like rutin, quercitrin, quercetin, kaempferol and astragalin have been identified in *P. urinaria*. Ellagitannins such as geraniin, corilagin and phyllanthusiins are found in *P. amarus and P. urinaria*. Several analytical techniques have been used to isolate and characterize these phytoconstituents (see Table 1). With advancement of analytical technology, the structures of several phytoconstituents in *P. urinaria* have also been assessed. For understanding the activity of the phytoconstituents for therapeutic uses, it is important to identify, analyze and characterize each of the phytoconstituent. Keeping this in view, a short review of the analytical techniques used for analyzing some of the phytoconstituent of *P. urinaria* has been presented.

Table 1: List of phytoconstituents identified, isolated and analyzed through analytical techniques.

Species	Analytical Techniques	Phytoconstituents	References
P.urinaria	HPLC-PDA-MS	Phyllanthin Hypophyllanthin Nirtetralin Niranthin Virgatusin Heliobuphthalmin	[Shanker et al., 2011]
	HPLC-SPE-NMR	Phyllanthin Hypophyllanthin Virgatusin Nirtetralin Lintetralin Niranthin 5-demethoxyniranthin Urinatetralin Phyltetralin	[Wang and Lee, 2005] [Chang et al., 2003]

	HSCCC	Corilagin Ellagic acid	[Jikai et al., 2002]
	HPLC	Geraniin Gallic acid	[Okuda et al., 1980] [Huang et al., 2010]
	TLC	Quercitrin Rutin Rhamnocitrin β-sitosterol Methylgallate Trimethyl 3,4 dehydrochebulate Methylbrevifolin carboxylate	[Fang et al., 2008]

2. ANALYTICAL TECHNIQUES

Some of the analytical techniques used for isolation and identification of phytoconstituents in *P. urinaria* are:

- Hyphenated High performance liquid chromatography photo diode array mass spectrometry (HPLC-PDA-MS)
- High performance liquid chromatography Solid phase extraction Nuclear magnetic resonance (HPLC-SPE-NMR)
- High speed countercurrent chromatography (HSCCC)
- High performance liquid chromatography (HPLC)
- Thin layer chromatography (TLC)

2.1. HPLC-PDA-MS

Hyphenated techniques are combination of chromatographic and spectral methods to use the optimum advantages of both. The quantitative and qualitative analysis of unknown compounds in complex natural product extracts and fractions have been carried out by these hyphenated techniques. The coupling of separation and detection techniques can involve more than one separation or detection techniques like LC-PDA-MS (Liquid chromatography photo diode array mass spectrometry), LC-MS-MS (Liquid chromatography mass spectrometry), LC-NMR-MS (Liquid chromatography nuclear magnetic resonance mass spectrometry) and LCPDA-NMR-MS (Liquid chromatography photo diode array nuclear magnetic resonance mass spectrometry) etc.

The basic principle of HPLC-PDA-MS is separation of components of a mixture by liquid chromatography and their identification and quantification by mass spectrometry. HPLC-PDA-MS is a powerful technique due to its very high sensitivity and selectivity. This technique has been used in the separation and potential identification of chemicals of specific masses. Phytoconstituents of *P. urinaria* namely phyllanthin, hypophyllanthin, nirtetralin, niranthin, virgatusin and heliobuphthalmin have been isolated and characterized using HPLC-PDA-MS technique. For this experiment, an online hyphenated high performance liquid chromatography photo diode array mass spectrometry (HPLC-PDA-MS) method was developed and validated for the simultaneous identification and quantification of six phytoconstituents. These six phytoconstituents are active lignans of *P. urinaria* species. The standard stock solutions 1mg/ml of standard or reference lignans (namely phyllanthin, hypophyllanthin, nirtetralin, niranthin, virgatusin and heliobuphthalmin) purity >99% isolated from *P. amarus* were used. They were characterized by spectral analysis infrared spectroscopy IR, UV, ^{1}HNMR,^{13}CNMR and mass [3-7]. Standard stock solutions were prepared in methanol. The calibration curves for each standard lignans were constructed by plotting the peak area vs. working lignan standard concentration (0.042-0.166mg/ml).

Aerial part of *P. urinaria* was air dried under shade and powdered with mechanical grinder. 100mg of powdered plant material was extracted with different extraction procedures. The extract was filtered through whatman no.1 filter paper and concentrated under vaccum and also dried using N2 gas flux. The concentrated residue was reconstituted in 1ml of methanol and filtered through 0.45µm filter prior to LC-PDA-MS (ESI-APCI) analysis.

LC-PDA-MS optimized condition for analysis

For this technique, the monolithic reverse phase silica chromolith column (Merck, 4.6mm*100mm) was used. The mobile phase was water with 0.5% dioxane and methanol (40:60, v/v). The column temperature, injection volume and flow rate was optimized for 28ºC, 20 µl and 1ml/min respectively. The column effluent was delivered into PDA and dual ESI-APCI ion source of MS. The quantification of targeted six lignans was performed at 230nm and characteristic selected ion mode (SIM) using PDA and MS detector. Mass spectrometric conditions were also optimized for curved desolvation line (CDL) temperature and heat block temperature which was at 250 and 480ºC respectively. Other conditions were probe voltage of +4.5kv; detector voltage of 1.5kv; CDL voltage of -20V; and Q-array Bios was 50V. Nebulizing gas was nitrogen at a flow rate of 1.5L/min. All six compounds showed a good linearity ($R^2 \geq 0.99$).

Quantitative and qualitative fingerprint analysis using LC-PDA-MS of targeted lignans viz. hypophyllanthin, phyllanthin, nirtetralin and niranthin were confirmed in *P. urinaria* by their abundant molecular adduct ions, retention time, UV and mass spectra as compared to reference or standard 6 compounds [1]. The method was validated according to international guidelines. This method can be used for assays of *P. urinaria* extracts [1].

2.2 HPLC-SPE-NMR

This is also a hyphenated technique where high performance liquid chromatography is coupled with solid phase extraction and nuclear magnetic resonance spectroscopy. The common HPLC-MS method does not characterize chemical structure completely. The technique of HPLC-NMR helps in detailed

study of plant extracts at the microgram level [8,9]. The interference of the eluted solvent and sensitivity problem has been solved by application of solid phase extraction (SPE) cartridges which have revolutionized the analytical procedures.

Nine lignin phytocompounds namely phyllanthin (1), hypophyllanthin (2), virgatusin (3), nirtetralin (4), lintetralin (5), niranthin (6), 5-demethoxyniranthin (7) and urinatetralin (8) and phyltetralin (9) were analysed and identified in *P. urinaria* whole plant by HPLC-SPE-NMR.

A reversed phase HPLC system with tetrahydrofuran-water (30:70%) and 40% methanol was used as mobile phase. Elution of sample using C8 column, seven lignans from P. urinaria with good resolution was detected at 225nm. Coupling of HPLC with SPE-NMR elucidated clean ^{1}H-NMR spectra for nine lignans present in 4mg of a lignin rich fraction [5]. Moreover, the investigation of the aerial and root parts of P. urinaria. resulted in 13 isolated lignans. Out of 13 lignans, four lignans namely 5-demethoxyniranthin, urinatetralin, dextrobursecernin and urinaligran were new and nine were known lignans. Their stereochemistry were elucidated by spectral analysis. Ethanolic extract of the aerial part of P.urinaria that consists water insoluble part was divided into n-hexane and chloroform soluble fractions and a residue by trituration with the corresponding solvent. Repeated chromatography over Sephadex LH-20, aluminium oxide and silica gel of two soluble fractions yielded 13 lignans. These compounds were found to be phyllanthin [3], niranthin [10], phyltetralin [11], hypophyllanthin [3], nirtetralin [10], lintetralin [12], isolintetralin [13], heliobuphthalmin lactone [14] and virgatusin [15]. These were confirmed by comparing their physical data (specific rotation $[\alpha]_D$, NMR, MS) with that reported in literature. Lignan compounds namely 5-demethoxyniranthin, urinatetralin, dextroburseherin, urinaligran structures and molecular formula were established through spectral data and HR-EI-MS data. The ethanolic extract of *P. urinaria* root part was partitioned by the solvent system $CHCl_3$-MeOH-H_2O (2:2:1) to give two fractions aqueous and organic layers. The organic layer yielded 12 lignans whose structures were identified and characterized by ^{1}HNMR signals. Intensity analysis of aromatic and methoxy signals distinguished various lignans and determined their abundance in the aerial and root parts. It was observed that phyllanthin (6.63%) phyltetralin (3.06%) and hypophyllanthin (0.76%) are abundant in the aerial parts whereas phyllanthin (5.36%), niranthin (2.27%) and hypophyllanthin (1.41%) are most abundant in the roots [6].

2.3 HSCCC

High speed countercurrent chromatography is a liquid chromatography technique. It is based on liquid-liquid partition chromatographic separation technique. The separation process occurs in three stages mixing, settling and separation of two phases. The phytoconstituent corilagin and ellagic acid in *P. urinaria* were isolated and characterized using one step high speed countercurrent chromatography. Jikai *et al.*,compared two isolation techniques HPLC and HSCCC for purification of corilagin and ellagic acid from *P. urinaria* [16].

The aerial parts of *P. urinaria* (1g) was dissolved in 10ml methanol and centrifuged to prepare a crude methanolic extract. The supernatant was subjected to gel filtration over sephadex LH-20 (100*5 cm i.d column) which was eluted with a gradient of methanol (20-100%) in water. Purification of 100% methanol fractions was performed by preparative HPLC to yield 25mg of corilagin. Preparative HPLC was performed with C18 column eluted with a gradient of 0.05% trifluroacetic acid in water: acetonitrile at a flow rate of 12 ml/min. This whole process requires 2-3 days of labor. HSCCC experiments were also performed. High speed countercurrent chromatography is demonstrated to be powerful tool that limits time, labor and enhances efficient isolation of bioactive natural products due to its ability to handle crude material directly with no binding to a solid sorbent.

2.4 HPLC

HPLC is a separation technique that is based on mechanisms of adsorption, partition and ion exchange, depending on the type of stationary phase used. HPLC involves a solid stationary phase, normally packed inside a stainless-steel column, and a liquid mobile phase. Separation takes place due to difference in the relative distribution ratios of the solutes between the two phases.

Geraniin was isolated from *P. urinaria* by HPLC. Leaves were air dried, homogenized and centrifuged. Supernatant was evaporated in vacuum at room temperature. The dried extracts were dissolved in 50% methanol with concentration of 5mg/ml and 1-6 µl of the solutions were injected in the column of HPLC. The mobile phase mixture of 0.5 MKh2PO4, EtOH (10 ml) and EtOAc (1ml) and 100cm*7.9mm I.D stainless steel column packed with Zipax hydrocarbon polymer was used. The compound was identified with 254nm UV detector equipped with liquid chromatography. Geraniin 1.1% was quantified in leaves of *P. urinaria* [17]. Also, water extracts of *P. urinaria* was subjected to high performance liquid chromatography-mass-spectrometry (HPLC/MS) for identification of gallic acid and other compounds. The mass structure was identified based on mass data mining. The identification of gallic acid was further confirmed by chemical markers like mass and HPLC retention time [18].

2.5 TLC

TLC is a chromatography technique used to separate non-volatile mixtures, for identifying substances and testing the purity of compounds. TLC has advantage over other techniques as it requires small quantities of material.

Phytoconstituents of other classes of P. urinaria namely quercitrin, rutin, rhamnocitrin, β-sitosterol, methylgallate, trimethyl 3, 4 dehydrochebulate and methylbrevifolin carboxylate were extracted from dried plant material of P. urinaria. The 80% ethanolic extract of P. urinaria was concentrated under vacuum at 35ºC to give dark brown syrup (28g) and suspended in distilled water. The chloroform extract was purified by silica gel chromatography eluted with mobile phase of n-hexane/EtOAc. Twenty five column fractions were collected and analyzed by TLC and again rechromatographed on a silica gel column. This yielded trimethyl 3, 4 dehydrochebulate (0.0008%), methylgallate (0.0094%) and rhamnocitrin (0.0018%). The concentrated n-butanol extract (1.375%) was subjected to repeated column chromatography on a silica gel and eluted with CH_2Cl_2/MeOH with gradual increase in ratio of methanol. Methyl brevifolincarboxylate (0.0033%, β-sitosterol-3-O-β-D-glucopyranoside (0.0031%), Quercitrin (0.001%) and Rutin (0.004%) were obtained. The structures of compounds were established through NMR and mass spectral studies [19].

3. FUTURE PROSPECT

Phytoconstituents of *P. urinaria* have shown antitumour, antiviral, antioxidant, antithrombosis and antinociceptive activities, and further analytical and pharmacological research can lead to development of better herbal drugs for many diseases. Research for optimal production of active compounds may also help pharmaceutical industries. The identification and characterization of new phytoconstituents in this herb and study of their biological activity could be beneficial and can boost pharmaceutical industries.

4. ACKNOWLEDGEMENT

The work is supported by Bioinformatics Infrastructure Facility, Department of Biotechnology (Ministry of Science and Technology), Government of India.

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