International Journal of Medicinal Plants Research ISSN: 2169-303X Vol. 5 (4), pp. 262-267, April, 2016.
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Full Length Research Paper

Identification of phytochemical constituents of *L. nodiflora* using TLC and chemical derivatization method

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Accepted 30 March, 2016

There are a numbers of bioactive compounds in plants, such as alkaloids, tannins, flavonoids, sterols, triterpenes, etc., noted to have the major role in nutrition, physiology and control of diseases. Triterpenoids constitute one of the most characteristic classes of compounds in higher plants. The foremost important task in this paradigm is the screening of these compounds in the plants. The chromatographic study of the compounds serves to be a very useful and reliable source in the process of bioactive compounds screening in plants. According to the ethnobotanical information, it has been reported that the plant *Lippia nodiflora* possesses the antihypertensive potential. Hence in the present study, an attempt has been made to identify the phytochemical constituents of *L. nodiflora* using TLC and chemical derivatization method. Further, the isolation of the same compound is carried out by preparative HPTLC using the standardized solvent system viz., hexane: toluene: ethyl acetate (2:15:0.5). The confirmation of the isolation was done by GC-MS.

Key words: Preparative HPTLC, isolation of triterpenoids, *Lippia nodiflora*, solvent system for triterpenoids separation.

INTRODUCTION

The chemicals which are responsible for colours and smell into the plant are known as secondary metabolites or phytochemicals. The term, phytochemicals, is generally used to refer to those chemicals that may have biological significance but have not been established as essential nutrients. Phytochemicals have been used as drugs since long in the past. Many of the secondary metabolites have been screened form medicinal plants and have been used in herbal therapy. Recent studies are involved in the identification and isolation of new therapeutic compounds of medicinal importance from the plants for specific diseases (Khan et al., 2009; Khan et al., 2010a; Khan et al., 2010b; Sahreen et al., 2010).

The secondary metabolites present in the plants, such as alkaloids, tannins, flavonoids, sterols, triterpenes, etc. noted to have the major role in nutrition, physiology and control of diseases (Sahreen et al., 2011; Khan et al., 2011a; Khan et al., 2011b). Triterpenoids are a large family of compounds synthesized by plants that have a common chemical structure. Triterpenoids are metabolites of isopentenyl pyrophosphate oligomers and represent the largest group of phytochemicals. It has been estimated that more than 20,000 triterpenoids exist in nature (Liby et al., 2007). They predominantly are found in various plants including sea-weeds as well as in wax-like coatings of various fruits and medicinal herbs, including apples, cranberries, figs, olives, mistletoe, lavender, oregano, rosemary and thyme (Rabi and Bishayee, 2009; Laszczvk, 2009; Ovensna et al., 2004; Neto, 2007; Gerhauser, 2008). Triterpenoids are biosynthesized in plants by the cyclization of squalene, a triterpene hydrocarbon and precursor of all steroids (Phillips et al., 2006). They can further be subclassified into diverse groups including cucurbitanes, cycloartanes, dammaranes, euphanes, friedelanes, holostanes,

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hopanes, isomalabaricanes, lanostanes, limonoids, lupanes, oleananes, protostanes, sqalenes, tirucallanes, ursanes and miscellaneous compounds (Setzer and Setzer, 2003; Petronelli et al., 2009; Mullsuer et al., 2010). Although triterpenoids were considered to be biologically inactive for a long period of time, accumulating evidence on their broad spectrum pharmacological activities coupled with a low toxicity profile has sparked renewed interest with regard to human health and disease. Triterpenoids are used for medicinal purposes in many Asian countries for antiinflammatory, analgesic, antipyretic, hepatoprotective, cardiotonic, sedative and tonic effects (Ovensna et al., 2004; The Wealth of India: Raw Materials; Huang, 1993). Recent studies have not only confirmed some of the aforementioned pharmacological properties of several triterpenoids, but also identified a variety of additional biological activities including antioxidant, antimicrobial, antiviral, antiallergic, antipruritic, antiangiogenic and spasmolytic activity (Sultana and Ata, 2008; Shah et al., 2009). An increasing number of triterpenoids have been reported to exhibit cytotoxicity against a variety of cancer cells without manifesting any toxicity in normal cells (Laszczyk, 2009; Petronelli et al., 2009; Setzer and Setzer, 2003). They also demonstrate antitumor efficacy in preclinical animal models of cancer (Laszczyk, 2009; Petronelli et al., 2009). A large number of triterpenoids have been synthesized by structural modification of natural compounds for optimization of bioactivity, and some of these semi-synthetic analogs are considered to be the most potent antiinflammatory and anticarcinogenic triterpenoids known to mankind (Liby et al., 2007). The antitumor efficacy of several triterpenoids are currently being evaluated in phase I clinical trials (Petronelli et al., 2009).

A major contradicting task in the field of the phytochemical is the screening of these compounds in the plants and there isolation in the pure forms. The chromatographic methods are very useful and reliable source in the process of phytochemicals screening. This made the authors to use highly reliable, basic chromatographic methods for screening of plant for the triterpenoid constituents, referring to as the Thin Layer Chromatography and High Performance Thin Layer Chromatography on the plant *Lippia nodiflora*. The further isolation of the separated compound has been carried out by the preparative HPTLC method and the final separation was confirmed by the GC-MS followed by the Ultraviolet - Visible Spectroscopy.

MATERIALS AND METHODS

Collection of samples

Fresh plants was collected from, Shree Bapalal Vaidhya Botanical Graden, located in the Veer Narmad South Gujarat University Campus, Udhna Magdalla Road, Surat, Gujarat, India. The plant was authenticated and voucher specimen of the plant was deposited in the herbarium of the university. The whole plant was washed under the running tap water followed by distilled water and dried at 40°C in the oven for 3 days. The dried plant was pulverized into the fine powder and passed through a 30 mesh sieve and stored for the future use.

Preparation of extracts

The ground plant material was subsequently extracted with methanol using a soxlet apparatus. The resulting crude methanolic extract was filtered by passing through a Wattman no 3 filter paper followed by concentration in vaccume at 40°C using a rotary evaporator and freeze drying. This freeze dried sample is used for further analysis (Reddy and Mishra, 2012).

Chemicals and reagents

All the chemicals and reagents including Methanol, Ethyl Acetate, hexane and toluene were of Analytical Grade and purchased from Merck. The TLC silica plates were purchased from Merck of HPTLC Grade.

Screening of triterpenoids

The initial screening of the triterpenoids in the methanolic extract was carried out with the basic qualitative test for triterpenoids, where 0.5 ml of the extract was mixed with acetic anhydride, heated and cooled. 1 ml of Concentrated H_2SO_4 was added along the side of the tube and formation of purple colour indicates the presence of triterpens. Further, Thin Layer Chromatography of the extract was carried out with the modification in the method given by Wagner and Bladt (1996). The solvent system was selected as, Hexane**:** Toluene: Ethyl acetate (2:15:0.5). In the TLC Screening procedure, a thin strip of 3 × 10 cm of TLC Silica Plate (TLC Silica gel 60 F254, Merck), was taken and impregnated with the fine drop of extract. The plate was then air dried and kept for the development in chromatographic chamber containing 10 ml of the prepared solvent system. After the successful development, the plate was examined under the UV Chamber at 366 nm. The presence of triterpens constituent was confirmed by the chemical derivatization, where the developed plate was sprayed with anisaldihyde sulphuric acid (Wagner and Bladt, 1996).

Preparative HPTLC of extract

From the successful development of TLC plate with the prepared solvent system, the Preparative High Performance Thin Layer Chromatography of the extract was carried out on the CAMAG HPTLC System. Prior to sample application, 20 × 10 cm HPTLC plate (HPTLC

Table 1. Rational classification of the terpenes has been established based upon the number of isoprene (or isopentane) units incorporated in the basic molecular skeleton:

S/No.	Terpenes	Isoprene units	Example	Carbon atoms	Biological activity
1.	Monoterpenes	2	Geraniol, citral, menthol, myrcene, limonene, citronellal	10	Perfumery and flavouring industries, local anaesthetic and refreshing effects
2.	Sesquiterpenes	3	Caryophylle, humulene, cedrene, longifolane	15	Antibiotic property
3.	Diterpenes	4	Abiotic acid, podocarpic acid, neutral resin, manoyl oxide	20	Insect and plant hormone, medicinal uses
4.	Sesterpenes	5	Manoalide	25	Antibacterial activity
5.	Triterpenes	6	Squalene, cortisone, lanosterol	30	Female steroid hormones, antioxidant activity, immunosuppressive activity, wood resins
6.	Carotenoids	8	Lycopene, monocyclic γ-carotene and bicyclic α and β carotenes	40	Antioxidant activity, precursor of vitamin A
7.	Rubber	>100		>500	

Table 2. Screening of triterpens by chemical method with other phytochemicals

S/No	alkaloids	sterols	phenols	Cardiac glycosides	Tanin	flavonoids	triterpenoids
1.	+	+	+	+	+	+	++

+ = present, ++ = present in appreciable quantity.

Silica gel 60 F254, Merck) was activated at 110°C for 30 min. 2000 µL of the extract was then applied as a single band of 180 mm length on the activated HPTLC plate using a CAMAG automatic TLC sampler III (CAMAG, Switzerland). The plate was then developed with the 10 ml of standardized solvent system, Hexane: Toluene**:** Ethyl acetate (2:15:0.5) in the twin trough chromatographic chamber. After the successful development, the plate was examined under the UV Chamber at 366 nm.

Isolation of triterpenoids constituent

After the development of plate it was subjected for the isolation of triterpenoids. The band was then selected with the graphite tip using the scale markings of 1 to 10 cm. and then was scratched out along with the silica with a sharp scalpel and eluted with the methanol in eppendorf tubes. The contents of the tubes were then pooled to form a single sample which was then given the code “LN- 1”. The excess concentration of the methanol was evaporated by placing the tube open at the room temperature until the final volume was retained to 1/3rd of the original volume (Markham, 1975; Hostettman et al., 1998). The LN- 1 was then co-chromatographed with the crude extract for the confirmation of the band at the same Rf.

Confirmation of isolation of terpenoids

The developed plates after HPTLC were then scanned for the available possible bands using the CAMAG automatic TLC scanner 3. Further confirmation of the isolation of triterpenoid was done by analyzing the LN- 1 in the UV–Visible Spectrophotometer for a single peak and GC-MS for further analysis.

RESULTS AND DISCUSSION

Screening of terpenoids

The initial screening of the terpenoids in the plant extract with basic preliminary procedures was the first step in the process. The screening of the terpenoids with primary phytochemical screening process revealed the presence of appreciable quantity of the terpenoids (Tables 1 and 2). The thin layer chromatography (TLC) process confirms the possible presence of terpenoids by revealing

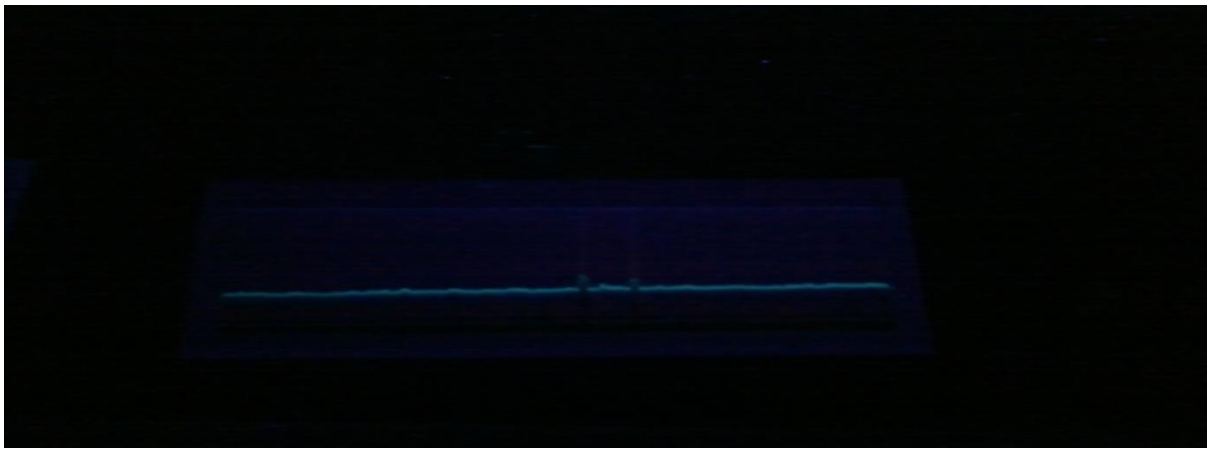

Figure 1. Preparative HPTLC at 366 nm (Methanolic extract of *Lippia nodiflora*).

Figure 2. HPTLC of LN 1 at 366 nm.

Figure 3. Co-chromatographic screening of LN1 with methanolic extract of *Lippia nodiflora*. LN1 = Fraction collected by preparative HPTLC. MCE = Methanolic crude extract of *Lippia nodiflora.*

the fluorescent bands which on further derivatization gave blue fluorescence on long wavelength (360 nm) and forms purple colour zone when heated at 100°C for 5 to 10 min (Hostettman et al., 1998).

Preparative HPTLC of extract

The method of extraction of phytochemicals is the most important procedure in the development of pharmaceutical use of any plant species which is known or reported to have medicinal importance. Preparative HPTLC is one of the cheapest and yet highly reliable procedure in the fantasy of collecting pure compound from the crude plant extract. The crude extract revealed the several bands at different Rf under the TLC scanner (Figures 1 and 4). The clear bright fluorescent band was considered to be of the possible compound of interest and was selected for isolation (Figure 1).

Isolation of the terpenoids constituent

The co-chromatographic screening of LN- 1 by HPTLC with the methanolic crude extract revealed the sharp, single, blue fluorescent band which was considered of triterpenoid only at Rf = 0.41 cm under 366 nm (Figures 2 and 3).

Confirmation of isolation of terpenoids

The spectra comparison of the crude extract in TLC scanner revealed the maximum absorption of bands at 365 to 370 nm wavelength (Figure 4); hence the spectrum analysis of LN- 1 was carried out with UV-Vis Spectrophotometer in the range of 200 to 800 nm. The fraction, LN 1 showed the presence of a single peak in the complete spectrum at approximately 370 to 380 nm (Figure 4). This resolves the approximate true

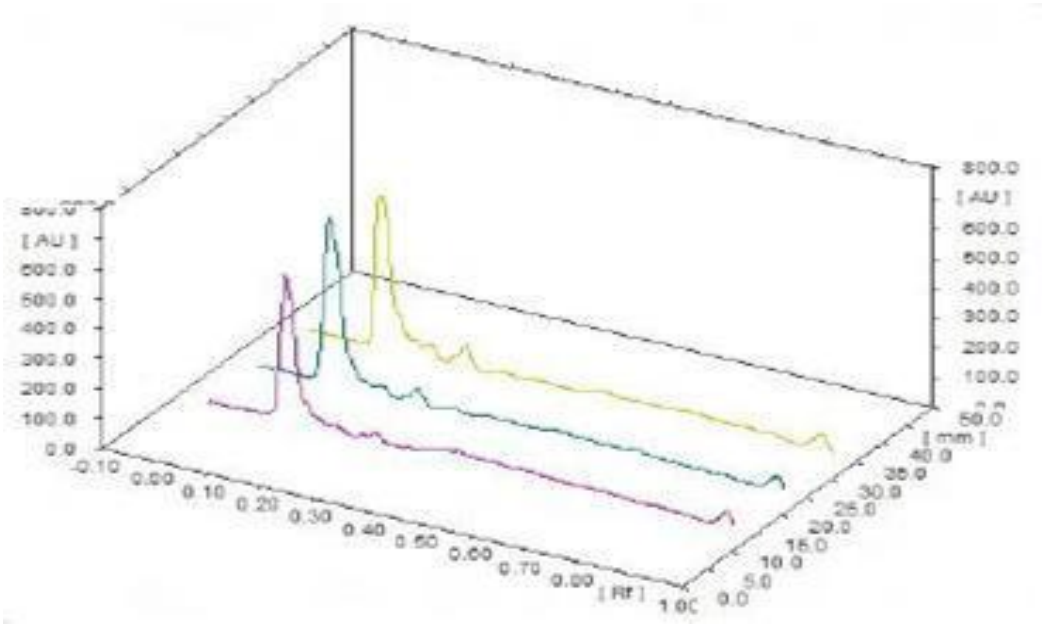

Figure 4. HPTLC Chromatogram of LN1.

confirmation of isolation of compound.

CONCLUSION

From the above procedural workout, it can be clearly concluded that the plant *L. nodiflora* does contain the triterpenoids. The above stated procedure is therefore results to be very simple in the procedural workout isolation of compounds which seems to be very difficult in the case of plant extracts. This procedure, hence therefore, though being a very simple process, is remarkably very efficient for the purification of the compounds from crude extracts of the plants. Also the solvent system, standardized for the separation of triterpenoids found to be suitable for the separation of terpenoids, where the clear separation of bands is one of the great hurdle to be faced during the isolation of pure compounds from plant extracts.

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