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Preparation and properties of lantadene a complex with DNA

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Abstract
Lantana (*Lantana camara* L.) is a hepatotoxic plant with a number of medicinal properties. Lantadene A (LA), a pentacyclic triterpenoid present in the leaves of lantana plant is one of the most important bioactive constituents of this plant. Pentacyclic triterpenoids exhibit a number of bioactivities like antitumor, anti-HIV, anti-inflammatory, antitubercular, inhibition of nitric oxide production, inhibition of DNA polymerase and DNA topoisomerase. LA was isolated from lantana leaves and purified by batch processes, column chromatography and fractional crystallization. The purity as ascertained by HPLC analysis. The chemical structure was confirmed as 22β-angeloyloxy-3-oxoolean-12-en-28-oic acid by spectroscopic analysis. LA-DNA interaction was ascertained y electrophoresis. The observations on interaction of LA with DNA as evaluated by agrose gel electrophoresis were very interesting. LA masked the binding of ethidium bromide to DNA implying thereby that lantadene. An interacted with DNA by intercalation. Future studies are required to use the LA-BSA for development of immunoassay and for development of possible hepatoprotective immunogen against lantana toxicity. The observation on the intercalation of LA with DNA implies that LA could be potential antitumor compound for future drug development.

Keywords: lantadene A, HPLC, DNA, *Lantana camara*

Introduction
Lantadenes are pentacyclic triterpenoids of lantana *(Lantana camara)* plant (Sharma *et al*., 1981). Most of the triterpenoids isolated from the leaves of L *camara* are pentacyclic and belong to oleane series, a few belong to ursane and lupane series (Sharma *et al*., 1989 and Ghisalberti, 2000) [19, 4]. The content of triterpene acids is lower in young leaves than in mature ones (Sharma *et al*., 2000) Lantana plant grows wild in many parts of our country and provides huge amount of biomass. Currently, there is a lot of interest to exploit its natural products in drug research and preparation of value-added products (Sharma *et al*., 1989, Ghisalberti, 2000) [19, 4]. Different parts of the plant are used in folk medicine for the treatment of cuts, ulcers, fever, toothache etc. (Siddiqui *et al*., 1995) [28].

Phytochemical studies undertaken by different groups of workers, on different parts of the plant, have resulted in the isolation of various natural products (Sharma and Sharma, 1989) [19]. Triterpenoids occur in nature in free ester or their glycosidic form called saponins. LA, LB, LC, RLA and RLB have been shown to be hepatotoxic (Seawright *et al*.1977; Heikel *et al*., 1960, Sharma *et al*., 1991a, 1992) [17, 5, 24, 26]. LA, LB and LC are the major constituents of *L camara* red leaf sample from Kangra valley another important triterpenoid in *L camara* Red leaves is LD (Sharma *et al*., 1990) [22]. The only *L. camara* variety in which icterogenin could be determined is Townsville prickly orange which has oleanolic acid and ursonic acid as major constituents in its leaves and stem. LA and LB are the major constituent of other taxa of L *camara* viz. Townsville Red centered pink, Mackay Red centered pink and large flowered orange as well (Sharma and Sharma, 1989) [19]. The major triterpenoids of common pink taxon are lantanolic acid and lantic acid which have an oxide bridge from C-3 to C-25. Lantanolic acid and lantic acid have also been found in some samples of Indian lantana plant (Baura *et al*., 1975; Sharma and Sharma 1989) [19]. Leaves of L. *camara* have been utilized for inhibition of spore germination in *Alternaria alternate, Aspergillus niger, A. fumigatus and Mucor mucede* (Ross1999; Saxena and Tripathi 1985) and in the inhibition of spore germination and mycelial growth of seed borne mycoflora of Sorghum viz. *Alternaria Tetnuis, Aspergillus flavus, Curvularia innata, Fusarium moniliforme* and *Rhizopus stolonifer* (Meena and Mariappan 1993) [11].

Root bark extract of *L camara* exhibited antimalarial activity against the multidrug resistant K1 strain of *Pseudomonas falciparum* (Weenen *et al*., 1990). Antibacterial activity by *L camara* has also been reported against *Bacillus subtilis, Escherichia coli, Micrococcus pyogenes var. aureus, Pseudomonas aeruginosa and Staphylococcus aureus* (Verpoorte and Dihal 1987). The leaves are used in the treatment of itches, cuts, ulcers, swelling, bilious fever, catarrh and rheumatism (Siddiqui *et al*., 1995) [28]. Leaf oil is useful as an antiseptic for wounds. The roots are used for the treatment of toothache and the flowers for chest complaints in children (Kirtikar *et al*., 1981) [8]. Pharmacological investigations indicated that exhale of the shoots of *Lantana Camara* exhibit antibacterial properties. Lancamarone, a steroid from the leaves possess cardiotonic properties while lantamine, an alkaloid from the bark of stems and roots shows strong antipyretic and antispasmodic properties, comparable with those of quinine (Ghisalberti 2000) [4]. Lantadene A is a secondary plant metabolite which has ability to bind with protein and form protein-LA conjugate. This lantadene-protein conjugate results in mild protection against the hepatotoxic effects of lantana poisoning (Stewart *et al*., 1988; Pass and Stewart, 1992) [13].

As a part of biological activity triterpenoids interact with bio macro molecules like protein and nucleic acid and modulate the activity of key enzymes like DNA topoisomerase and DNA polymerase. DNA topoisomerase catalyses the concerted breaking and rejoining of DNA strands and is involved in producing necessary topological and conformational changes in DNA (Kornberg *et al*., 1992; Wang 1985) [9,]. DNA polymerase catalyses the addition of deoxyribonucleotides to the 3'- hydroxyl terminus of primed double- stranded DNA molecules (Kornberg *et al*., 1992; Mizushina *et al*., 2000) [9, 12]. Ursolic acid has been shown to inhibit the enzymes topoisomerase I and II (Syrovets *et al*., 2000; Mizushina *et al*., 2000) [12]. Spectroscopic evidence for tingenone interacting with DNA (Campanelli *et al*., 1980) [3] and molecular modeling studies support intercalation into DNA with concomitant nucleophilic addition of purine base (Setzer *et al*., 2001; Setzer *et al*., 2003) [16, 15]. Campanelli *et al*., (1980) [3] reported that the size and shape of tingenone (pentacyciic triterpenoid) molecule is favorable for their inclusion in the narrow groove of DNA and, furthermore, hydrogen bonds can be formed between the hydroxyl group of tingenone and the phosphate group of DNA. Keeping this in mind present studies is carried out to evaluate the interaction of LA with DNA.

Materials and Methods

Isolation and Purification of Lantadene A

Lantana (L. *camara* var. *aculeata)* leaves were collected from the vicinity of Palampur and dried at 70 °C. Dried leaves were ground to fine powder of 1 mm particle size using a grinder. The leaf powder (200 g) was extracted with 1 litre methanol by shaking well and keeping overnight. The methanolic extract was filtered through two layers of muslin cloth and treated with 30 g activated charcoal for 1 h with intermittent shaking. The charcoal treated methanolic extract was filtered through coarse filter paper, distilled and the residue was dried *in vacuo* at 70-80 °C. Dissolved the residues in small amount of methanol and made the volume to 180 ml by addition of more methanol. Mixed this solution with water and chloroform (100 ml each), transferred to a separating funnel and left overnight for partitioning and layer formation. The lower layer (chloroform) was decanted and washed with small amount of water. Chloroform layer was subjected to column chromatography on silica gel.

Column Chromatography

The partially purified lantadenes sample from the previous step was loaded on the column of Silica gel (50 g, 60-120 mesh). The elution was done with 200 ml chloroform followed by 600 ml chloroform: methanol (99:1) and then 400 ml chloroform: methanol (95:5) (Sharma *et al*., 1987) [21]. Fractions (25 ml each) were collected during elution with each of the three mobile phases. Fractions obtained during different steps of purification were monitored by thin layer chromatography.

Thin Layer Chromatography

TLC was done on glass plates with coated with silica gel G. TLC of fractions obtained from column chromatography was done. The solvent system was petroleum ether: ethyl acetate: acetic acid (88:10:2). The developed plates were dried at room temperature and detection was done by exposing the plates to iodine vapour (Sharma and Dawra, 1991) [24].

Fractional Crystallization

The pale colored LA enriched fractions containing minor amount of LB, LE and reduced lantadenes eluted with chloroform: methanol (99:1) were pooled up, the solvent was removed by distillation and residue was dried *in vacuo* at 50-60 °C. The residue was dissolved in boiling methanol and kept at 0-4 °C for crystallization. Crystallization through methanol was repeated thrice to get pure lantadene A. Purity of lantadene A was ascertained by HPLC analysis (Sharma *et al*., 1997) [20].

HPLC of pure LA

Standard solution of LA (2 mg/5 ml) was prepared in methanol. Sample was filtered through Whatman stainless steel syringe assembly using 0.22 µm Durapore (Millipore, U.S.A.) membrane filter. An aliquot (20 nl) was used for HPLC injection. LA purified (2 mg) from lantana leaves was also dissolved in 5 ml HPLC grade methanol and was also subjected to HPLC analysis. HPLC analysis was done using Waters HPLC system with Waters HPLC system with 510 and 515 pumps, Rheodyne injector, Novapak C-18 (4u, 4.6 X 250 mm) column, 490E multichannel detector and Millennium 2010 data manager. The mobile phase was: methanol: acetonitrile: water: Acetic Acid (68:20:12:0.01). The elution was in the isocratic mode. The mobile phase was filtered using Durapore (0.22pm) membrane filter.

Agarose Gel Electrophoresis

Reagents

Stock solution

1. 10 X TBE Buffer 1M Tris, 1 M Boric acid, 20 mm EDTA, pH 8.3 - 22.22 g 1 M Tris, 12.366 g I M boric acid and 20 mm EDTA were dissolved in water, pH was adjusted to 8.3 with concentrated HCI and volume was made to 200 ml.
2. Ethidium Bromide -10 mg/ml.
3. 1 X TE Buffer: pH 8.0 - 0.037g EDTA (1mM) and 0.157g Tris -CI (10 mm) was dissolved in water, pH was adjusted to 8.0 and volume made up to 100 ml. Preparation of loading buffer (6X) - 0.25% Bromophenol blue, 50% glycerol.

4. DNA sample was prepared by dissolving in 1X TE buffer and lantadene A was dissolved in dimethyl sulfoxide (DMSO).

Procedure

1. Horizontal gel electrophoresis apparatus was assembled.
2. Dissolved 0.96 g agarose (0.8%) in 120 ml 1 X TBE buffer.
3. Heated it in boiling water until gel completely dissolved. After cooling the solution to about 60°C, added 7.5 ml ethidium bromide (final concentration 0.5 mg/ml) and poured the gel into casting tray containing sample comb. Allowed gel to solidify at room temperature.
4. Removed comb after the solidification of gel.
5. Inserted casting tray into horizontal electrophoresis chamber and covered gel with 1XTBE buffer.
6. Added 10 X gel loading buffer to sample and loaded the sample into wells.
7. The gel was run at 75 V for 1 *V2h* viewed it on UV transilluminator.

Results and Discussion

Leaves of *Lantana camara*, collected from the vicinity of Palampur, were used in this study. The results from the present investigations have been divided into the following sections:

Isolation and Purification of Lantadene A

Lantana leaf powder was dried to remove traces of moisture and extracted with methanol. The green colored pigments were removed by adsorption on activated charcoal. Excess of charcoal on prolonged charcoal treatment can cause adsorption of lantadenes as well in addition to the pigments, resulting in decrease in the yield of lantadenes. Methanol: Chloroform: water partitioning of the decolorized methanolic extract facilitates the separation of lantadenes from the more polar compounds which move to the upper aqueous layer. Lantadenes essentially appeared in the chloroform layer as shown in the TLC chromatogram (Figure1). The chloroform layer was subjected to silica gel (60-120 mesh) column chromatography with the elution scheme given in Table 3.1. Elution of LA enriched fractions commenced on elution with the solvent system chloroform: methanol (99:1). The fractions enriched in LA were pooled and the solvent was removed *in vacuo.*

1: Standard LA sample, **2:** Sample of methanoiic-aqueous layer, **3:** Chloroform layer.

Fig 1: Thin layer chromatography of methanol and chloroform layer Solvent system: Petroleum Ether: Ethyl acetate: Acetic acid: 88:10:2)

Fractional Crystallization

The residue contained substantial amount of LB, RLA, RLB and colored impurities in addition to LA and was subjected to further purification by fractional crystallization. The residue from the preceding step was subjected to fractional crystallization through methanol till no impurity could be detected using TLC analysis (Figure 2).

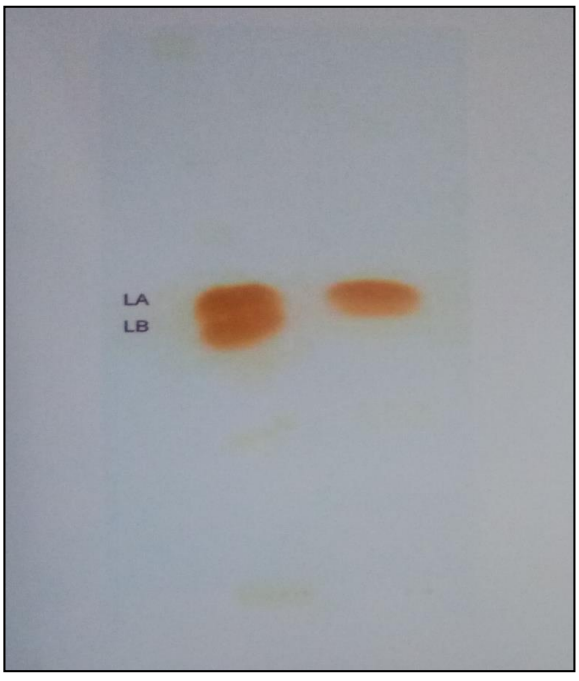

Fig 2: Thin layer chromatography of Pure LA.
Solvent system: Petroleum Ether: Ethyl acetate: Acetic acid: (88:10:2) **1.** Mixture of lantadene A (LA) and lantadene B (LB) **2.** A sample of purified LA

HPLC Analysis

The purity of LA was further ascertained by HPLC analysis. The HPLC chromatograms of standard LA and the sample obtained by purification are shown in Figure 3 and 4. It showed single peak at the same retention time as that of standard LA. The purity of LA was nearly 87.37% as determined by HPLC.

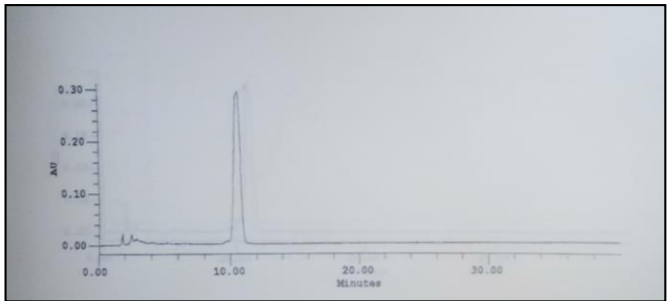

Fig 3: HPLC chromatogram of standard LA (2mg/5ml methanol)

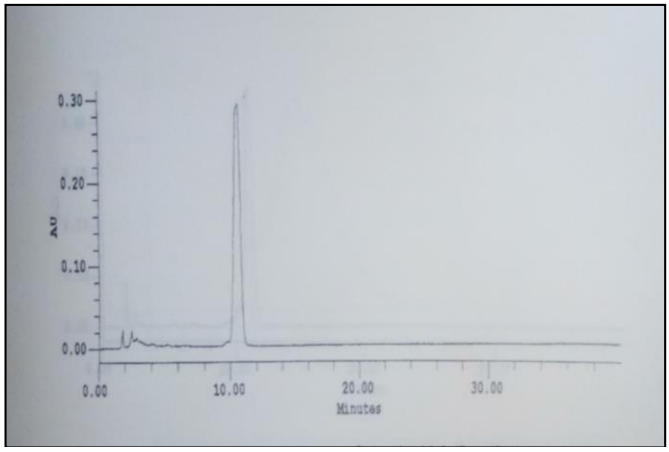

Fig 4: HPLC chromatogram of sample LA (2mg/5ml methanol)

Lantadene A was purified from lantana leaf powder by slight modification of the protocol already reported (Barton *et al*., 1956; Sharma *et al*., 1987) [1, 21]. The purity was determined by HPLC analysis (Sharma *et al*., 1997) [20]. The structure was confirmed as 22 (3- angeloyloxy- 3 - oxoolean-12-en-28-oic acid (Barton *et al*., 1956, Sharma *et al*., 1991) [1]. Till nearly a decade ago lantadenes were known *to* induce only hepatoxicity (Sharma *et al*, 1988, 1991, 1992) [18, 26]. However, some strategic studies revealed that lantadenes inhibited the activity of Epstein Barr virus and had antitumor activity as well (Inada *et al*, 1995, 1997) [7, 6]. These observations triggered a lot more interest in research on lantadenes. A research group at University Institute of Pharmaceutical Sciences Panjab University led by Prof. P.D. Sharma is already preparing a number of derivatives of lantadene A to develop more potent anticancer compounds. No information is available on as to how lantadenes interact with biomolecules, especially macromolecules like DNA.

Interaction between DNA- LA

The interaction of DNA with LA was studied by agrose gel electrophoresis. LA and DNA were mixed in different ratio and then loaded in the wells. DNA is detected by ethidium bromide due to the intercalation of the fluorescent dye amongst the bases. The figure 4.24 shows that prior interaction of LA with DNA inhibited the interaction of DNA with ethidium bromide (lanes 6-11). Similarly, LA and ethidium bromide did not form any fluorescent adduct (lane 4). The studies on interaction of LA with DNA provided more interesting information. Earlier a number of workers have investigated the interaction of triterpenes with DNA (Setzer *et al*., 2001; Campanelli *et al*., 1980; Syrovets *et al*., 2000; Mizushina *et al*., 2000) [16, 3, 12]. Some observations of the workers are: boswellic acid derivatives are inhibitors of DNA topoisomerase I and II (Syrovets *et al*., 2000). Ursolic acid is inhibitor of both DNA polymerase and DNA topoisomerase I and II (Syrovets *et al*., 2000; Mizushina *et al*., 2000) [12] whereas oleanolic acid was inhibitor of DNA polymerase (Ma *et al*., 1999).

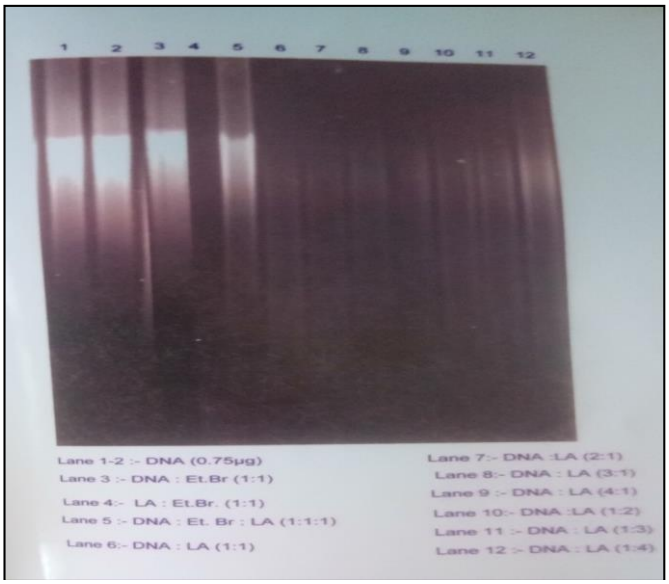

Fig 5: Agrose Gel electrophoresis of LA-DNA

Spectroscopic evidence for tingenone interacting with DNA (Campanelli *et al*., 1980) [3] and molecular modeling studies support intercalation into DNA with concomitant nucleophilic addition of purine base (Setzer *et al*., 2001; Setzer *et al*., 2003) [16, 15]. Campanelli *et al*., (1980) [3] reported that the size and shape of tingenone (pentacyclic triterpenoid) molecule is favorable for their inclusion in the narrow groove of DNA and, furthermore, hydrogen bonds can be formed between the hydroxyl group of tingenone and the phosphate group of DNA. Since tingenone is a bulky molecule, an intercalation mechanism seems to be unlikely, unless only the nearly planar A and B rings are engaged. Mizushina *et al*. (2000) [12] reported that triterpenoids do not bind to ds DNA but they inhibit the enzyme activities by interacting with enzymes directly. In the present study it was observed that LA masked the interaction of DNA with ethidium bromide. Ethidium bromide is known to intercalate with DNA (Scaria *et al*., 1991; Varedevangan *et al*., 2003). So, it is inferred from our data that one of the ways in which LA interacts with DNA is by intercalations. The compounds which intercalate with DNA are expected to be potential inhibitors of DNA polymerase and DNA topoisomerase (Mizushina *et al*. 2000) [12]. Such compounds are potential anticancer compounds as well (Setzer *et al*., 2003, 2001) [15, 16]. Thus, lantadene A and by corollary other lantadenes as well are potential natural products for drug research.

Conclusion

Lantana (*Lantana camara* L) is a hepatotoxic plant with a number of medicinal properties as well. Lantadene A (LA), a pentacyclic triterpenoid present in the leaves of lantana plant is one of the most important bioactive constituents of this

plant. LA was isolated from lantana leaves and purified by batch processes, column chromatography and fractional crystallization. The extent of purification at each step was monitored by TLC. The purity as ascertained by HPLC analysis was 87.4%. LA-DNA interaction was ascertained by electrophoresis on agarose gel. The observations on interaction of LA with DNA as evaluated by agarose gel electrophoresis were very interesting. LA masked the binding of ethidium bromide to DNA implying thereby that lantadene. An interacted with DNA by intercalation. The observation on the intercalation of LA with DNA implies that LA could be a potential antitumor compound for future drug development.

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