

Oleanolic acid and Ursolic acid in cell cultures of *Lantana camara* L. and their activity against *Streptococcus mutans*

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Abstract

Lantana camara L. commonly known as red or wild sage, belonging to family Verbenaceae, holds a distinct place in the list of medicinally important plants as it is a reservoir of several important bioactive molecules. Hitherto, this plant has been overlooked as far as its exploitation by biotechnological means is concerned. Recently, natural products from Lantana, have been implicated in the prevention and cure of many serious diseases including cancers. These molecules of natural origin promote human health without recognizable side effects.

In the present work, we report establishment of aseptic in vitro cultures from the leaves of L. camara plant, bearing pink-yellow variety of flowers, on MS medium supplemented with 6- benzylaminopurine (5 μ M) + 2,4-dichlorophenoxyacetic acid (1 μ M) + α -naphthalene acetic acid (1 μ M). Thin layer chromatography of cell derived extract revealed the presence of pharmacologically active anti-cancerous pentacyclic triterpenoids - Oleanolic acid and Ursolic acid. The extract also exhibited antimicrobial activity against Streptococcus mutans, a facultative anaerobe responsible for dental cavities in humans. Apart from this, batch kinetics studies have been carried out that disseminate the pH profile, dissolved oxygen profile, substrate consumption rate of the suspension cultures. This may help to determine experimental parameters for the future research related to scale-up studies, for the harvest of significant anti-cancerous compounds present in Lantana.

Keywords: *Batch Kinetics, Lantana camara, Leaf culture, Organic extract, Streptococcus*

Introduction

Streptococcus mutans, a facultative anaerobic gram-positive bacterium commonly found in the human oral cavity is a significant contributor to tooth decay^{9,20}. The growth and metabolism of pioneer species such as these change local environmental conditions like pH, coaggregation and substrate availability, thereby, enabling more fastidious organisms to further colonize after them, forming dental plaque²². It plays a major role in tooth decay by metabolizing sucrose to lactic acid. The acidic environment created in the mouth by this process is what causes the highly mineralized tooth enamel to be vulnerable to decay. *S. mutans* is also one of a few specialized organisms equipped with receptors that help for better adhesion to the surface of teeth. Sucrose is utilized by *S. mutans* to produce a sticky, extracellular, dextran-based polysaccharide that allows them to cohere to each other forming plaque. It is the combination of plaque and acid that leads to dental decay¹¹. Due to the role the *S. mutans* plays in tooth decay, there have been many attempts to make a vaccine for the organism but none have been successful in humans⁸. Many plant derivatives have been put to use since ancient times for problems related to teeth.

One such plant, *Lantana camara* is one of the wild plants of tropical and sub-tropical areas of the world that contains innumerable compounds belonging to different classes of secondary metabolites including triterpenoids. The genus is a difficult one to classify taxonomically since plant is an aggressive, obligate outbreeder and hybridisation is widespread; shape of inflorescence changes with age, and flower colors vary with age and maturity⁶. Given that there are large areas infested with this plant, it is reasonable to consider if large-scale use could be made of its biomass and, therefore, currently, there is a lot of interest to exploit its natural products for drug research⁶. This plant had been mentioned in ayurveda for treatment of various vitiated body conditions⁷. Ursolate acetate from its leaves was found to be active against *Staphylococcus aureus* and *S. typhi*¹. Ngouela et al.¹⁶ have discussed antimicrobial activity of Ursolic and Oleanolic acid, two pentacyclic triterpenoids, obtained from the seeds of *Symphonia globulifera*. Antimutagenic activity of these triterpenoids have also been observed on mice by Resende et al.¹⁸ Other classes like phenylethanoid glycosides in *Lantana*, particularly, Verbascoside has also been shown to possess anti-bacterial activity.

Till date there are no investigations on anti-bacterial activity of this plant on *Streptococcus*, although there have been reports on the use of its leaves and shoots as a cure for dental tribulations in certain parts of Assam. Therefore, our aim is to validate this asset of the plant apart from raising its in vitro cultures and studying its batch kinetics.

Materials and Methods

Plant Material and Cell Cultures

Leaves were collected from healthy *Lantana* plants bearing pink-yellow variety of flowers. They were disinfected using 1% Tween-20 for 15 min and 0.1% mercuric chloride for 10 min followed by three rinses in sterile distilled water after each step. The leaf disc explants were prepared using a cork-borer of 5 mm diameter. The basal media used in all the experiments related to callus induction and proliferation consisted of MS¹⁴ macro and microsalts, vitamins, 100 mg l⁻¹ myoinositol, enriched with 30 g l⁻¹ sucrose and solidified with 0.8% agar (HiMedia Laboratories, India) along with the following growth regulator combinations: 6-benzylaminopurine (BAP) (1-5 μ M); 3-indole acetic acid (IAA) (5-10 μ M); BAP (5 μ M) + IAA (10 μ M); BAP (10 μ M) + IAA (5 μ M); BAP (5 μ M) + α -naphthalene acetic acid (NAA) (10 μ M); BAP (10 μ M) + NAA (5 μ M); BAP (5 μ M) + 2,4-D (1 μ M) + NAA (1 μ M).

Media was adjusted to a pH of 5.8 before addition of agar and autoclaved at 121°C at 15 psi for 15 min. The heat unstable growth regulators like IAA were filter-sterilized and added to the medium after autoclaving. Twenty milliliters media was dispensed in each culture tube (Borosil, India) and cultures were incubated at 25°C under 16/8 h light/dark regime. They were subcultured at every 4-week interval.

Establishment of Suspension cultures

After 7-8 subcultures of calli obtained on semi solid media, some were chemically analysed and some were used to obtain cell suspension cultures. Suspension cultures were initiated in 250 ml Erlenmeyer flasks containing 50 ml MS medium supplemented with BAP (5 μ M) + 2,4-D (1 μ M) + NAA (1 μ M). The cells were subcultured every 3 weeks and maintained in an orbital shaker at 25 °C at 120 rpm.

Extraction of Oleanolic acid and Ursolic acid

The dried cell mass (50 g) was drenched in methanol (200 ml) for 48 h and was sonicated thereafter at 30% amplitude (pulser 5 sec on/off) for 40 min, then again for 20 min at pulser 3 sec on/off cycle. The methanolic mixture, thus, obtained was filtered and the filtrate was centrifuged at 10,000 rpm for 10 min. Supernatant was pooled, filtered and dried in a rotary evaporator at 40 °C. This methanolic extract was further fractionated into an organic (ethyl acetate) and aqueous fractions. The ethyl acetate extract was further dried under reduced pressure in a rotary evaporator at 40°C (Buchi Rotavapor R-200, Japan) and used for further study.

Thin Layer Chromatography

The organic extract was qualitatively analysed by thin layer chromatography on the glass plates coated with silica gel (Merck, India). The extract was dissolved in chloroform and was eluted with 4% methanol in chloroform mixture. Spot visualization was accomplished in an iodine chamber. The Retention factor or the R_f value of the spots obtained from the extracts were compared with those of the standard compounds and was calculated as: Distance travelled by the sample/ Distance travelled by the solvent.

Microorganism and Culture Media

The cultures of *Streptococcus mutans* were kindly provided by Dr Chandana Kalita of Guwahati Medical College. The bacterial cultures were maintained in blood agar medium at 37°C.

Determination of dry cell weight, pH profile, nitrate and phosphate

For determination of dry weight, 0.2 g calli were inoculated into 250 ml Erlenmeyer flasks each containing 50 ml of liquid medium of same composition. At the end of the passage, the cells were harvested from the flasks, washed with distilled water and filtered under vacuum. Thereafter, the cells were dried in oven at 30°C ± 2°C until a constant weight was achieved. The drying temperature was kept low to avert decomposition of thermolabile compounds. The supernatant from the suspension cultures was used for the analysis of pH profile, residual nitrate and phosphate according to standard methods available in literature.

Results and Discussions

Establishment of Cell Lines

Callus differentiated from the axenic explants on MS medium supplemented with BAP (5µM), 2,4-D (1µM) and NAA (1µM). 100% of the explants callused on this medium and the callus growth was profuse after the first subculture. In all the other combinations tried, explants turned brown soon after inoculation and were unable to revive thereafter. In the responding medium, the leaf disc explants first turned brown but after a week, bright-green, hard, compact calli started developing from the margins of the leaf-disc. These compact calli were dissected out and subcultured on the fresh medium of the same growth regulator composition. Until 10th subculture, the cells in the callus were a mixture of green and brown cells. With an aim to screen elite cell lines, the green and brown calli were subcultured separately to see whether they give rise to similar kind of cells, but

to our surprise both of them gave rise to a mixture of green and brown cells. Also, the chemical analysis of both green and brown calli was performed separately but no difference was observed in the chemical profile with respect to the compounds under investigation. This is understandable, because triterpenoids are colorless solids with high melting point². It took about 26 weeks of regular subculturing at 4-week interval to improve the nature of callus and enhance the rate of callus proliferation (Fig.1).

Browning of the explants and the culture media is usually due to phenolic exudation^{13,15}. It has been suggested that the addition of anti-oxidants like ascorbic acid, polyvinylpyrrolidone (PVP), polyvinylpolypyrrolidone (PVPP) can be used to overcome browning and death of explants. Apart from this, repeated sub-culturing at regular intervals is also an efficient alternative way to overcome browning of the cultures^{12,19}. In the present study, repeated subculturing was adapted as a route to eliminate phenolic exudation from the explant into the medium. This may omit any alteration in secondary metabolite profile of the cultured cells due to anti-oxidants.

Chemical analysis of the extract

TLC of the standard samples and organic extract revealed OA and UA spots with an R_f value of 0.28 and 0.17, respectively (Fig.2). Natural products, until recently, have been the prime source of commercial medicines and drugs. A recent survey revealed that the origin of 61% of the 877 drugs introduced worldwide can be traced back to the natural products. However, at the start of 1990's natural product drug discovery was eliminated in most of the big pharmaceutical companies, primarily due to the promise of the then emerging field of combinatorial chemistry³, whereby huge libraries of man-made small molecules could be rapidly synthesized and evaluated as drug candidates; but nothing substantial came out of it. Of roughly 3,50,000 species of plants believed to exist, 1/3rd of those have yet to be discovered. Of the quarter million that have been reported, only a fraction of them have been chemically investigated.

Antibacterial activity assay

Anti bacterial evaluations were performed by agar streak method. Working solutions of 1:1, 1:2, 1:4, 1:8 and 1:16 were obtained by serial dilution 10 mg/ml stock solution. Maximum inhibition was observed at a dilution of 1:4 (Fig. 3).

Quite a few reports are available that mention the antimicrobial activity of *Lantana* on different bacterial species. White and orange varieties were tested for antibacterial activity against *Staphylococcus aureus* ATCC 25923,

Escherichia coli ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853⁵. Alcoholic and aqueous extracts of both varieties showed following zones of inhibition against *S. aureus*: 14.6 and 14.8 mm, respectively, for the white variety; 14.2 and 13.1 mm, respectively, for the orange variety. Antibacterial activity against human pathogen has also been observed in the Petroleum ether extract from the leaves of *Lantana*²¹.

Growth of cell cultures, pH profile, residual nitrate and phosphate

The growth curve of *L. camara* cells cultured on MS + BAP (5 μ M), 2,4-D (1 μ M) and NAA (1 μ M) is represented in figure 4a. Cultures reached stationary phase of growth by the 9th day with a specific growth rate (μ) of 0.1072 d⁻¹. The pH of the shake flask suspension culture medium was monitored after every two days. It was observed that after showing a slight decrease in the value, the pH dropped sharply between 4-6 days, which dropped further after 10 days. This may be concomitant to the synthesis of acid triterpenes in the medium. However, it remains to be investigated whether the production of OA and UA is growth linked or both processes are uncoupled. Studies regarding this are in progress. The kinetic profile of consumption of phosphate and nitrate in the culture medium is shown in figure 4b. It was invariably observed that phosphate was almost completely consumed by the 10th day of culture. Its utilization was very fast in the initial days (lag phase) of culture than in the later stages of growth. Uptake of nitrate was at slower rate in comparison to phosphate. It was present in the culture medium till the last days of cultivation. Hence, it may be concluded that the complete utilization of phosphate from the culture medium resulted in the onset of stationary phase and it was a major limiting nutrient for growth. Similar kind of kinetic profiles, where phosphate is assimilated faster than the nitrate has been observed and reported by many workers in different plant species^{4, 17}.

Oleanolic acid and ursolic acid are ubiquitous triterpenoids in plant kingdom, medicinal herbs, and are integral part of the human diet. They are well known for their hepatoprotective effects for both acute chemically induced liver injury and chronic liver fibrosis and cirrhosis¹⁰. They are still used alone or in combination with other hepatoprotective ingredients as oral medications. Several articles have been published on their research, reflecting incredible interest and progress in our understanding of these triterpenoids. However this report is the first attempt to detect the presence of these terpenoids in in vitro cultures of *L. camara* plant. Furthermore, the growth kinetics study will aid in deciding scale up parameters for large scale production and isolation of these compounds.

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Figure Legends

Figure 1: Leaf-derived axenic cultures of *Lantana camara*

- (a) 26-week-old callus cultures
- (b) 2-week-old suspension cultures

Figure 2: Thin Layer Chromatography of the organic extract (1st lane is the crude sample, 2nd and 3rd are a mixture of crude and standard while 4th lane represents the standard compound)

- (a) Oleanolic acid
- (b) Ursolic acid

Figure 3: Anti bacterial assay on *Streptococcus mutans*

- (a) Bacterial culture treated at 1:16 dilution of stock showing full colony strength
- (b) Bacterial culture treated at 1:8 dilution of stock; colony strength reduced
- (c) Bacterial culture treated at 1:4 dilution of stock; no colonies observed

Figure 4: Batch Kinetics of *L. camara* cell suspension cultures

- (a) Graphs representing dry cell weight and pH variation in the cultures
- (b) Phosphate and Nitrate consumption profile of the liquid cultures

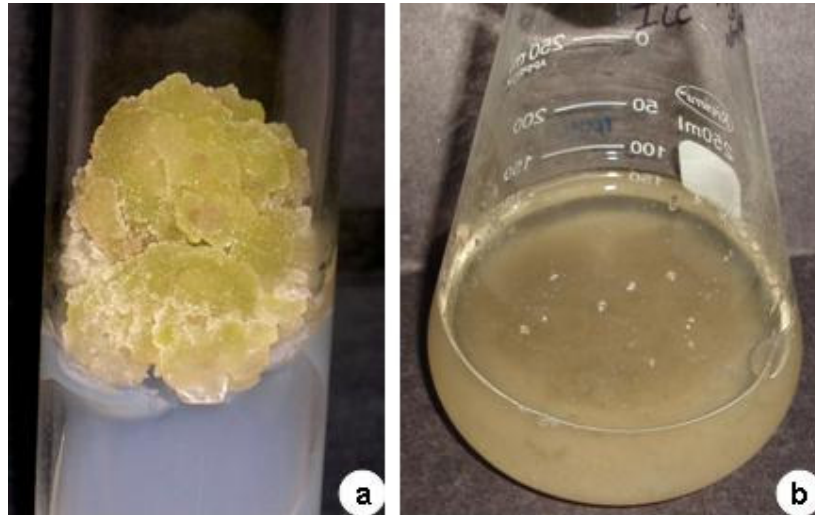


FIG. 1

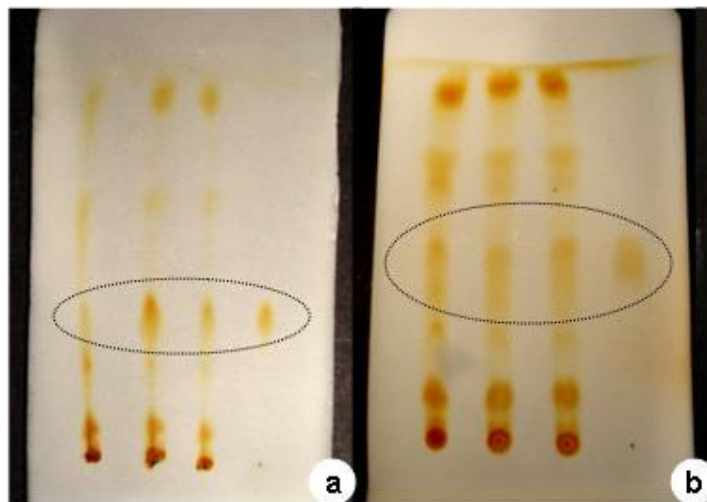


FIG. 2

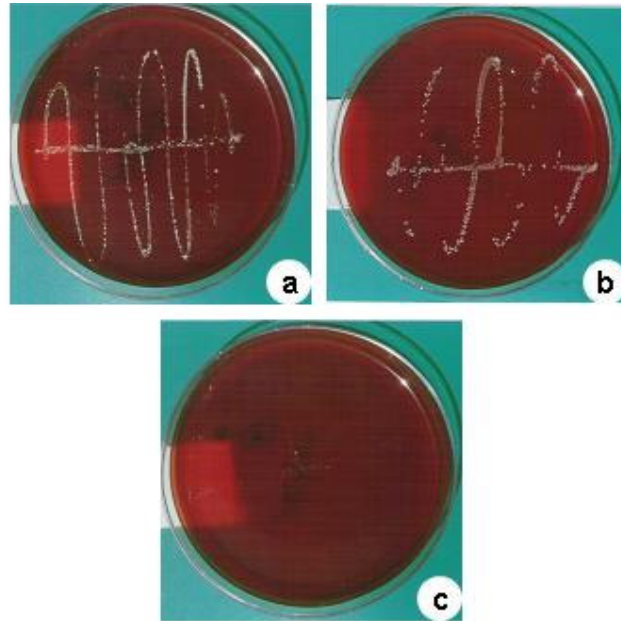


FIG. 3

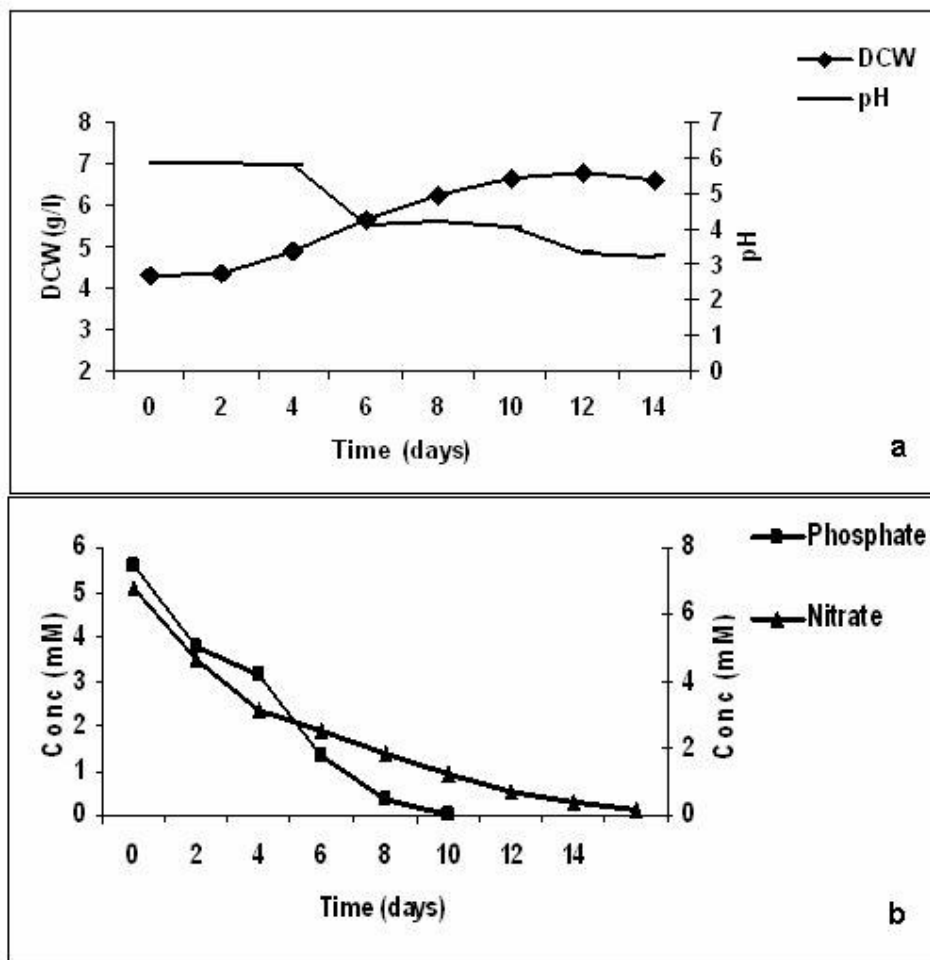


FIG. 4