ORIGINAL ARTICLE

Antibacterial and phytochemical studies on *Calotropis gigantia* (L.) R. Br. latex against selected cariogenic bacteria

Kalpesh B. Ishnava *, Jenabhai B. Chauhan †, Akanksha A. Garg †, Arpit M. Thakkar †

Ashok and Rita Patel Institute of Integrated Studies and Research in Biotechnology and Allied Sciences (ARIBAS), New Vallabh Vidyanagar 388 121, Gujarat, India

Received 8 June 2011; revised 2 October 2011; accepted 8 October 2011
Available online 21 October 2011

**KEYWORDS**
Calotropis gigantia;
Latex;
Cariogenic bacteria;
Phytochemical investigation

**Abstract** In *vitro* antibacterial potential of the chloroform, ethyl acetate, hexane, methanol and aqueous extracts of *Calotropis gigantia* (L.) R. Br. was evaluated by using five cariogenic bacteria, *Actinomyces viscosus*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Streptococcus mitis* and *Streptococcus mutans*. Agar well diffusion method and minimum inhibitory concentration (MIC) were used for this purpose. The chloroform extracted fraction of latex showed inhibitory effect against *S. mutans* and *L. acidophilus* with MIC value of 0.032 and 0.52 mg/mL, respectively. Qualitative investigation on structure elucidation of bioactive compound using IR, NMR and GC–MS techniques revealed the presence of methyl nonanoate, a saturated fatty acid.

© 2011 King Saud University. Production and hosting by Elsevier B.V. All rights reserved.

1. Introduction

Dental caries is the most common chronic oral disease which affects from 60% to 90% of young population (Petersen, 2003). Despite the advances concerning its prevention and control, dental caries is still considered a public health problem that affects many countries in the world (Bowen, 2002). During the past few decades, changes have been observed in prevalence, distribution and pattern of dental caries in the population. Observed, especially in the western part of the world, in nations like Germany, England, USA, Scandinavia, Scotland, Norway and Australia (Birkeland et al., 2002; Davies et al., 1997; Petersson and Bratthall, 1996; Schulte et al., 2006). Indians have less dental caries when compared with the western population (Gupta and Ray, 2004).
Oral disease, including dental caries, gingival inflammation, periodontal disease, and tooth loss, may significantly affect overall health. Among these, dental caries is a multi-factor infectious disease in which diet, nutrition, microbial infection and host response all play important roles. Their key role of the aetiology of periodontitis and dental caries, the most prevalent diseases in the world, is well established (Meyer and Fives-Taylor, 1998). Formation of dental caries is caused by the colonization and accumulation of oral microorganisms, and adherence is the first step in the colonization process. Streptococci especially *Streptococcus mutans* have been implicated as a primary causative organism, but other major group of bacteria like *Actinomyces* sp., *Lactobacillus* sp. and *Streptococcus* sp. are also present in dental caries (Hamada and Slade, 1980). Medicinal plants have been used in traditional folk medicines for thousands of years and have shown promise as a source of components for the development of new antibacterial, antifungal, antiviral, anticancer and anti-hypertensive drugs (Newman et al., 2003). Natural products have been recently investigated more thoroughly as promising agents for the prevention of oral diseases, especially plaque-related diseases such as dental caries (Bakri and Douglas, 2005; Fernandes-Filho et al., 1998; Katsura et al., 2001; Marsh, 1992; Matsumoto et al., 1999; Pai et al., 2004; Shouji et al., 2000; Wennstrom and Lindhe, 1985). The increasing resistance to available antimicrobials has attracted the attention of the scientific community regarding a search for new cost-effective drugs of natural or synthetic origin (Fine et al., 2000). There are more than 2500 species of plants that produce natural latex. The Euphorbiaceae, Apocynaceae and Asclepiadaceae families include many laticiferous species (Trease and Evans, 1977). Most of these latexes remain to be scrutinized for their biological usefulness. Among those latexes of biological importance, the medicinal usefulness of opium is amply recognized. Bioactive potential of latex bearing plants is reported in the scientific literature against various pathogenic organisms (Kareem et al., 2008; Ricardo, 2004).

*Calotropis gigantia* (L.) R. Br. (Asclepiadaceae) is a widely growing and native plant of India (Lindley, 1985). It is a 3–4 m tall shrub with milky latex. Bark ash coloured, leaves opposite, decussate, sessile or subsessile; flower 2–4 cm across, purplish white, complete, fruit follicles, seed numerous, broadly ovate, plano-convex. Traditionally the milky juice of *C. gigantia* has been used as a violent purgative, gastrointestinal irritant and abortion inducer (Chopra et al., 1956; Maurya et al., 2004; Nadkarni and Nadkarni, 1976). It has also been used in the treatment of toothache, earache, headache, sprain and stiff joints (Manandhar and Manandhar, 1990).

In the present study, *C. gigantia* latex extracts were evaluated for their efficacy against different cariogenic bacteria under *in vitro* conditions. Further phytochemical analysis was carried out to identify the bioactive constituent present in the latex of this plant.

2. Materials and methods

2.1. Collection of plant latex

*C. gigantia* plants latex was collected between January and March 2009 from the surrounding area of Vallabh Vidyanagar.

2.2. Extraction of latex

First of all the *C. gigantia* plants latex were aseptically collected and subjected to oven drying at 60 °C for 12 h. The dried matter (100 mg) of latex was extracted by using 1.0 mL of organic solvents (each of chloroform, distilled water, dimethyl sulfoxide, ethyl acetate, hexane and methanol). The resultant mixture was vortexed and centrifuged at 3000 rpm for 10 min. The supernatant was used for antibacterial activity.

2.3. Cariogenic bacteria

A group of bacteria known to cause dental caries were selected and purchased from Microbial Type Culture Collection (MTCC) bank, Chandigarh as a freeze dried pure culture. The bacterial cultures were revived by using MTCC specified selective growth medium (*Actinomyces viscosus* (AV)-Pikovskaya agar medium, *Lactobacillus acidophilus*(LA)-Nutrient agar medium, *Lactobacillus casei* (LC)-MRS medium, *Streptococcus mutis*(SMI)-Nutrient agar medium, *Streptococcus mutans*(SMU)-Brain Heart infusion agar medium) and preserved as glycerol stocks.

2.4. Inoculum preparation

Fresh microbial cultures were prepared by streaking loopful of bacterial suspension into organism specific selective media (Hi-media) and incubated at optimal temperature in order to maintain approximately uniform growth rate of each organism. The bacterial cultures from fresh media were compared with 0.5 McFarland turbidity standard, which is equivalent to approximately $1 \times 10^8$ bacterial cell count per ml (Kachhiya, 2008; Perilla, 2003) was maintained throughout the experimentation.

2.5. Bioassay for antibacterial activity of *C. gigantia* latex extracts

2.5.1. Agar well diffusion method

In the present study, to test antibacterial activity, *C. gigantia* plant latex extracts were used. The antibacterial activity was studied by agar well diffusion method (Peres et al., 1990). From the stock, 100 mg of latex extracts was suspended in 1 mL of each of chloroform, distilled water, dimethyl sulfoxide, ethyl acetate, hexane and methanol. Selective medium agar plates were marked and divided into four equal parts, labelled for specific organism and extract name. A fresh bacterial culture of 100 μl having $10^8$ CFU/ml was spread on agar plates with glass spreader. A well of 10 mm diameter punched off at previously marked petriplates into agar medium with sterile cup borer and then it was filled with 100 μl of *C. gigantia* latex supernatant and fresh latex. Plates were placed for 30 min in refrigerator for diffusion of extracts and then incubated at 37 °C (or specified temperature) for 24 h or more depending upon the bacterial species, until appearances of inhibition zone. The zone of inhibition (including well diameter) was measured as a property of antibacterial activity. Antibiotic, ampicillin was used as a standard at a concentration of 10 μg/ml and all the organic solvents were used as positive control and negative control, respectively. Bioassay was performed in duplicate and repeated twice.
2.6. Phytochemical characterization

2.6.1. Preliminary phytochemical analysis
Qualitative phytochemical analysis of *C. gigantia* crude latex extracts selected based on MIC analysis was performed as per the methodology of Parekh and Chanda, 2008 to determine the presence of tannin, alkaloids, saponis, cardiac glycosides, steroids, terpenoids and phenolic compound.

2.6.2. Analytical thin layer chromatography
Analytical TLC was performed to find out suitable solvent system for the development of chromatogram. Different solvent system was tried on precoated TLC plates (Merck, silica gel 60 F254 plate, 0.25 mm) for the development of chromatogram. Among all, chloroform:methanol (8:2) solvent system was found best and used for subsequent analysis.

2.6.3. Bioautography
By using capillaries 10 μl of chloroform extract of *C. gigantia* latex (100 mg/ml stock solution) was spotted onto 0.25 mm thick precoated silica gel 60 F254 plate (Merck, Germany). The band length was 2 mm thick. After air drying the TLC plate was run using pre-standardized solvent system, chloroform:methanol (8:2). The chromatogram was observed under UV illumination and used for bioautography. Brain heart infusion agar medium, seeded with *Streptococcus mutans*, was overlaid onto the silica gel plate loaded with sample and incubated at 37 °C for 24 h. On the next day, the plate was flooded with 2, 3, 5-triphenyl tetrazolium chloride (0.1%) to visualize growth inhibition. The area of inhibition zone was appeared as transparent against red black background (lawn of living bacteria).

2.6.4. Preparative thin layer chromatography (PTLC)
The preparative thin layer chromatography was performed at the final step of the purification of pure compound prior to the structure elucidation. Band that showed antibacterial activity was pulled together and further purified by preparative thin layer chromatography (PTLC). For the PTLC, sample aliquots were loaded on TLC plates and developed in chloroform:methanol (8:2) solvent system. Bioautography of the TLC plate was used to confirm the position of compound showing antibacterial activity. The compound was eluted from the developed plate by scraping off silica gel and mixed well with D/W and centrifuged at 10,000 rpm for 10 min. The supernatant was collected and used for further analysis.

2.6.5. Fourier transformer infra red (FTIR) spectroscopy
A thin film of *C. gigantia* plant latex active eluted fraction in D/W was applied on the glass and IR spectra were recorded by using Perkin Elmer spectrophotometer, Spectrum Instrument.
2.6.5. Gas chromatography–mass spectroscopy (GC–MS)

The GC–MS analysis was done by electron impact ionization (EI) method on Auto system XL gas chromatography (Perkin Elmer Instrument, Germany) coupled to a Turbo Mass Spectrophotometer (Perkin Elmer Instrument, Germany) at Sophisticated Instrumentation Centre for Applied Research and Training (SICART), Vallabh Vidyana-gar, Gujarat. The column was fused with silica capillary column, 30×0.25 mm ID; coated with D-I, 0.25 μm film thickness. The temperature of column was programmed at 70–250°C at the rate of 10°C/min increase, injection port temperature at 250°C. Helium was used as carrier gas at constant pressure of 100 kPa and flow rate of 20 ml/min. Samples which dissolved in chloroform were run fully at a range of 60–550 amu and the results were compared by using NIST 107 Spectral library search programme.

2.6.6. NMR spectroscopy

1H NMR spectra were recorded in CDCl3 using a BRUKER and 400 MHz for proton NMR spectrometer at the Department of Chemistry, Sardar Patel University, Vallabh Vidyana-gar, Gujarat, India.

3. Results and discussion

In the present study, the antibacterial sensitivity assay of *C. gigantia* latex extracts against cariogenic bacteria was carried out. The latex of *C. gigantia* was extracted using chloroform, DMSO, distilled water, ethyl acetate, hexane and methanol and used for antibacterial assay. The result of antibacterial sensitivity of cariogenic bacteria was assessed by visualizing the presence or absence of inhibition zone and measuring the zone diameter (Table 1). Chloroform extract of this plant showed very high activity against LA and SMU (19 mm) and low activity against AV (12 mm) and no activity against LC and SMI. There was no activity of DMSO extract of this plant against AV, LA, LC and SMU compared to SMI (13 mm). Methanol extract of this plant showed moderate activity against SMI (13 mm) and low activity against SMU (11 mm) and no activity against AV, LA and LC. Further, MIC determination was done for Chloroform extract of this plant (Table 1). Distilled water, ethyl acetate and hexane extracts of this plant showed no activity against any of the selected bacteria. MIC of chloroform extract of this plant against LA and SMU was 0.52 and 0.032 mg/ml, respectively. Latex: DMSO (1:1) extract of this combination showed low activity against LA and LC (12 mm) and very low activity against SMU (11 mm) and no activity against AV and SMI. Latex: D/W(1:1) combination showed activity against LA (13 mm) and LC (14 mm) and low activity against SMU (12 mm) and no activity against AV and SMI (Table 1). The latex of *Calotropis procera* has been reported to possess in vitro larvicidal, antifungal and anthelmintic activities (Sehgal et al., 2005). Kareem et al. (2008) reported promising antibacterial activity of *C. procera* latex on selected pathogenic bacteria and fungi but not on cariogenic bacteria. To the best of our knowledge there is meagre information on study of plant latex cariogenic bacteria, it is quite difficult to compare our finding. Phytochemical screening of *C. gigantia* latex showed the presence of alkaloids, steroids, cardiac glycosides and terpenes (Table 2). The TLC was also done from chloroform extract of latex of this plant. The bioactive compound was separated from the crude chloroform extract (RF value: 0.95) by using TLC technique and pre-standardize solvent system, chloroform:methanol (8:2). The chromatogram was used for bioautography against *S. mutans*.

The analysis of TLC plate run from eluted sample showed no fluorescence at 254 nm and blue florescence at 366 nm, respectively, the single band was confirmed by using iodine vapour. The study of infrared spectra revealed the presence of H-bonded, C–H stretching –alkene and C–N stretch aliphatic as a major functional group. The peak showing maximum percentage area at RT 22.36 in GC–MS analysis and scan 2.726 through mass spectrophotometer (Fig. 1), revealed the presence of methyl nonanoate (C10H20O2) and have molecular weight of 172.15, pK is 22.36 (Fig. 2). The compound identified as methyl nonanoate, a saturated fatty acid. It has been reported that the latex of *Calotropis procera* contains...
seven types of saturated fatty acid and 11 types of unsaturated fatty acid (Samina et al., 2008). For the taxonomic point of view, Calotropis procera and C. gigantia are very close. Therefore, there is a chance for the presence of similar chemical constituents (Kalita and Saikia, 2004). Methyl nonanoate identified in the present study is also reported by Samina et al. (2008) from Calotropis procera, a closely related plant species.

4. Conclusion

The results obtained from the current study suggest that latex of C. gigantia (L.) R. Br. (Asclepiadaceae) possesses significant anticariogenic activity against two of the selected cariogenic bacteria. The chloroform extracted fraction of latex showed inhibitory effect against S. mutans and L. acidophilus with MIC value of 0.032 and 0.52 mg/ml, respectively. The RF value of cariogenic compound was 0.95. Spectroscopic analysis using FTIR, GC–MS and NMR showed the presence of methyl nonanoate (C9H20O2), a saturated fatty acid may be responsible for anticariogenic activity. The latex of C. gigantia may be useful as anticariogenic agent.

Acknowledgements

Authors are thankful to Drs. A.K. Ray, Department of Chemistry, Sardar Patel University, Vallabh Vidyanagar and Jogesh Raval, Ashok and Rita Patel Institute of Integrated Study and Research in Biotechnology and Allied Sciences (ARIBAS), New Vallabh vidyanagar, Gujarat, India, for the interpretation of IR and NMR data. Authors are also thankful to Charutar Vidya Mandal (CVM), Vallabh Vidyanagar, Gujarat, India and Director of Ashok and Rita Patel Institute of Integrated Studies and Research in Biotechnology and Allied Sciences (ARIBAS), New Vallabh Vidyanagar 388121, Gujarat, India, for providing necessary support for research and laboratory facility.

References