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In Vitro Screening of the Antibacterial Activity and Identification of Bioactive Compounds From Plants against Selected *Vibrio* spp. Pathogens

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Abstract: In vitro investigation of the vibriocidal activity of acetone extracts of *Saraca indica* Linn (Caesalpinaceae) and *Datura stramonium* Linn (Solanaceae), and aqueous extracts of *Allium sativum* Linn (Liliaceae) showed that they have antibacterial activity against multidrug-resistant human pathogens. Disc diffusion assay showed that there was significant concentration-dependent antibacterial activity against standard strains of *Vibrio cholerae*, *Vibrio parahaemolyticus*, and 12 multidrug-resistant isolates of *Vibrio cholerae* non-O1. The crude plant extracts had zones of inhibition in the range of 7-27 mm against 1 or more test bacteria, with MIC ranging from 2.5 to 15 mg/ml. Acetone extracts of *S. indica* and *D. stramonium*, in most cases, had more potency (low MIC value) than ethanol and aqueous extracts; however, the aqueous extract of *A. sativum* was the most effective. Thin layer chromatography (TLC), bioautography, and high performance liquid chromatography (HPLC) analysis of the crude extracts revealed that saponin, tannin, scopolamine, atropine, and allicin were the active constituents.

Key Words: Vibriocidal activity, MIC, TLC, TLC-bioautography, HPLC

Vibrio spp Patojeni Karşı Bitkilerden Elde Edilen Biyoaktif Bileşiklerin Tanımlanması ve Antibakteriyal Etkilerinin in vitro Görüntülenmesi

Özet: *Saraca indica* Linn (Caesalpinaceae) ve *Datura stramonium* Linn (Solanaceae) bitkisinin aseton eksteresi; ve *Allium sativum* Linn (Liliaceae) bitkisinin sıvı ekstresi in vitro olarak vibrosidal aktiviteleri çalışılmıştır. Bu ekstreler çoklu direnç gösteren insan patojenlerine etkili bulunmuşlardır. Disk difüzyon yöntemi ile *Vibrio cholerae*, *Vibrio parahaemolyticus* ve 12 çoklu direnç gösteren *Vibrio* cholerae non-O1 izolatlarına karşı önemli ölçüde konsantrasyon bağımlı antibakteriyal etkiler gözlenmiştir. Bitki ekstrelerinin denenen bakterilere karşı inhibisyon zon çapları 7-27 mm arasında, MIC değerleri ise 2,5 ile 15 mg/ml arasında bulunmuştur. *S. indica* ve *D. stramonium* bitkisinin aseton ekstresi su ve etanol esktresine gore en yüksek potansiyele (düşük MIC) sahip olmuştur. Fakat, *A. sativum* bitkisinin su ekstresi en etkili bulunmuştur. Ekstrenin ince tabaka kromotografisi (TLC), biyootografi ve Yüksek Performanslı Sıvı Kromotografi (HPLC) analizleri saponin, tannin, skopolamin, atrofin ve allisin bileşiklerinin varlığını ortaya çıkarmıştır.

Anahtar Sözcükler: Vibriosidal aktivite, MIC, TLC, TLC-biootografi, HPLC

Introduction

Among diarrheal diseases, cholera is a serious epidemic disease caused by the gram-negative bacterium *Vibrio cholerae* (1,2). *Vibrio cholerae* serotypes O1 and O139 have the ability to produce an enterotoxin, cholera toxin (CT), which is a major determinant of the virulence of cholera (3). Enterotoxins affect enterocyte functions by stimulating the secretion of transepithelial electrolytes, thus increasing the osmotic flux of water and ions to the intestinal lumen (4). Developing countries face numerous resource constraints, and so it is necessary to focus on particular interventions that are very expensive and likely

to reduce the burden of disease attributable to a specific risk factor. Evaluating the risk of diarrheal diseases requires knowledge of the complex interactions between biological, socioeconomic, behavioral, and environmental factors over time (5).

Antibiotics have revolutionized the treatment of life-threatening infections; however, as bacterial resistance to available antibiotics is increasing, it has now become essential to develop new antibiotics (6).

The therapeutic and prophylactic treatment of cholera with antibiotics, viz. tetracycline, norfloxacin, etc., has probably contributed to the sporadic appearance of drug-resistant strains in different parts of the world (7). Ethnopharmacology and natural product drug discovery remain a significant hope in the current target-rich, lead-poor scenario (8). Ayurveda, the science of life, is a comprehensive medical system that has been the traditional system of healthcare in India for more than 5000 years (9). This comprehensive medical system uses many preparations made with Indian medicinal plants. In the traditional system of healthcare in India the use of medicinal plants to treat diarrheal diseases such as watery diarrhea is widespread (10).

The present study aimed to evaluate the vibriocidal activity of acetone extracts of *Saraca indica* and *Datura stramonium*, and an aqueous extract of *Allium sativum*, which are prescribed by the traditional Indian healthcare system for the treatment of cholera, diarrhea, and gastroenteritis. Bioassay-guided fractionation/ bioautography was performed to identify the bioactive phytoconstituents and assess the vibriocidal activity of the plant extracts against *Vibrio* isolates.

Materials and Methods

Ethnomedical Data and Plant Collection

Based on data provided by the ethnobotanical survey of India and local medicine men, 60 plants were collected for the present study from tribal regions of Mandla and Dindori (M.P.), and were authenticated by SFRI, Jabalpur (M.P.), India (Table 1). Each specimen was labeled, numbered, and annotated with the date of collection; collection locality and medicinal uses were also recorded.

Plant Extraction

For aqueous extraction, 10 g of air-dried powder was placed in distilled water and boiled for 6 h. At 2-h intervals the extracts were filtered through 8 layers of muslin cloth and centrifuged at 5000 × g for 15 min. The supernatants were collected and concentrated to make final volumes that were 25% of the original volume (11).

For solvent extraction, organic (acetone, ethanol, and methanol) extracts of the plant materials were prepared according to the method described by Nair et al. (11), with certain modifications. First, 10 g of air-dried, crushed, and blended powder of each plant was transferred to a conical flask with 50 ml of each solvent and kept on a rotary shaker (220 rpm) for 30 min. Then, they were filtered through 8 layers of muslin cloth and centrifuged at 5000 × g for 15 min. Supernatants were collected and the solvents were evaporated to make final volumes that were 25% of the original.

Microorganisms Tested

The in vitro antibacterial activity of the extracts was tested against *Vibrio* spp. The 12 multidrug-resistant environmental isolates of *V. cholerae* non-01 (BGCC#59, BGCC#60, BGCC#61, BGCC#62, BGCC#63, BGCC#64, BGCC#65, BGCC#66, BGCC#67, BGCC#68, BGCC#69, and BGCC#70) were procured from the Bacterial Germplasm Collection Center, Bacteriology Laboratory, R. D. University, Jabalpur, India. The standard strains of *V. cholerae* (H17004) and *V. parahaemolyticus* (KX-V138) were obtained from the National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India.

MIC of the Plant Extracts

The MIC of each plant extract against *Vibrio* spp. was determined on Mueller-Hinton agar medium, following the method of Rios et al. (12) and Collins et al. (13). Discs (5 mm in diameter) were impregnated with 10 µl of different concentrations of each plant extract, which were placed on the surfaces of the test plates, and incubated at 37 °C for 24 h. Concentrations used ranged from 2.5 to 20 mg/ml (2.5, 5, 10, 15, and 20 mg/ml). To assess the antibiotic resistance/sensitivity pattern, as described by the Clinical Laboratory Standards Institute (14), commercially available HiComb strips (Himedia) containing 4 antibiotics–ceftazidime (Ca), ciprofloxacin (Cf), norfloxacin (Nx), and cephotaxime (Ce)–were used. The HiComb strips were placed on the surface of each test plate with sterilized forceps and gently pressed to ensure contact. Plates were incubated within 30 min at 37 °C for 24 h and inhibition zone diameters were measured to the nearest millimeter.

Table 1. Plants used in the present study.

S. No.	Botanical Name	Family	Vernacular name	Parts used
1	*Aloe vera*	Liliaceae	Aloe	Flower, root
2	*Azadirachta indica*	Meliaceae	Neem	Leaf
3	*Adhatoda vasica*	Acanthceae	Vaska (Adusa)	Leaf
4	*Aegle marmelos*	Rietaceae	Bael	Fruit pulp
5	*Acacia arabica*	Mimosaceae	Babool	Stem bark
6	*Allium sativum*	Liliaceae	Lahsun	Bulb
7	*Acalypha indica*	Euphorbiaceae	Indian nettle	Leaf
8	*Acacia catechu*	Mimosoidae	Khair	Bark
9	*Allium cepa*	Liliaceae	Pyaj	bulb
10	*Andrographis paniculata*	Acanthaceae	Kalmegha	Root
11	*Abutilon indicum*	Malvaceae	Atibala	Bark
12	*Argemone maxicana*	Papaveraceae	Satyanasi	Leaf, seed
13	*Asparagus racemosus*	Liliaceae	Satavari	Tuberous root
14	*Bacopa monnieri*	Scrophulariaceae	Brahmi	Leaf
15	*Bauhinia variegata*	Caesalpinaceae	Kachnar	Stem bark
16	*Beta vulgaris*	Chenopodiaceae	Beet root	Root
17	*Bombax ceiba*	Bombacaceae	Semal	Stem bark
18	*Boerhavia diffusa*	Nyctaginaceae	Punarva	Leaf
19	*Butea monosperma*	Fabaceae	Palash	Bark
20	*Catharanthus roseus*	Apocynaceae	Sadasuhagan	Flower
21	*Cuminum cyminum*	Aplaceae	Jira	Fruit
22	*Calotropis procera*	Asclepiadaceae	Madar	Leaf
23	*Cynodon dactylon*	Poaceae	Dub	Upper part
24	*Coriandrum sativum*	Umbelliferae	Dhania	Fruit
25	*Cinnamomum cassia*	Luraceae	Cinnamon	Bark
26	*Cuscuta reflexa*	Convolvulaceae	Amarbel	Stem
27	*Celastrus paniculata*	Celastraceae	Kujri	Seed
28	*Curcuma longa*	Zingiberaceae	Haldi	Rhizome
29	*Cassia fistula*	Caesalpiniaceae	Amaltas	Flower
30	*Cissus cuadrangula*	Vitaceae	Hadjora	Stem
31	*Commiphora mukul*	Burseraceae	Guggul	Gum-resin
32	*Datura stramonium*	Solanaceae	Datura	Leaf
33	*Drosera burmannll*	Droseraceae	Sundew	Root
34	*Euphorbia hirta*	Euphorbiaceae	Dudhi	Leaf
35	*Emblica officinalis*	Euphorbiaceae	Amla	Fruit
36	*Ficus treligiosa*	Moraceae	Pipal	Leaf
37	*Grewia hirsute*	Tiliaceae	Gulsakari	Root
38	*Hemidesmus indicus*	Asclepiadaceae	Anantamul	Root
39	*Holarrhena antidysenterica*	Apocynaceae	Kurchi	Stem bark
40	*Moringa pterygosperma*	Aplaceae	Munga	Stem bark
41	*Madhuca latifolia*	Sapotaceae	Mahua	Stem bark
42	*Mimusops elengi*	Sapotaceae	Maulsari	Leaf
43	*Ocimum basilicum*	Labiatae	Kali tulsi	Leaf
44	*Ocimum sanctum*	Labiatae	Tulsi	Leaf, seed
45	*Phyllanthus niruri*	Euphorbiaceae	Bhuiamla	Whole plant
46	*Pureria tuberosa*	Fabaceae	Patal kohra	Root
47	*Piper longum*	Piperaceae	Pipali	Dry fruit
48	*Piper nigrum*	Piperaceae	Kalimirch	Dry fruit
49	*Rauwolfia serpentina*	Zingiberaceae	Sarpgandha	Leaf, seed
50	*Ricinus communis*	Euphorbiaceae	Castor (Arandi)	Seed
51	*Salmalia malabarica*	Malvaceae	Semur	Stem bark
52	*Solanum xanthocarpum*	Solanaceae	Bhat Kataiyan	Fruit
53	*Saraca indica*	Caesalpinaceae	Ashoka	Stem bark
54	*Solanum nigrum*	Solanaceae	Makoi	Fruit
55	*Terminalia arjuna*	Combretaceae	Arjuna	Stem bark
56	*Trachyspermum copticum*	Umbelliferae	Ajwain	Seed
57	*Trigonella foenum-graecum*	Leguminasae	Methi	Seed
58	*Withania somnifera*	Malvaceae	Aswagandha	Root, leaf
59	*Zizyphus jujuba*	Rhamnaceae	Ber	Stem bark
60	*Zingiber officinale*	Zingiberaceae	Ginger	Rhizome

Separation of Phytochemicals by Thin Layer Chromatography

Preparation of Plant Extracts

Milled plant powder (1 g) was shaken for 20 min in 10 ml of ethanol. The extract was filtered through glass wool and evaporated to dryness at a temperature not exceeding 40 °C. The dried residue was re-dissolved in 5 ml of solvent and was used as a sample for analysis (15).

TLC Analysis

Plant extract (5, 10, and 15 µl) was applied 2.5 cm from the base on duplicate TLC plates and was developed with ethyl acetate:methanol:water (81:11:8). One set was used as the reference chromatogram and other set was used for bioautography. Spots on reference TLC plates were developed using an anisaldehyde/sulfuric acid spray reagent (465 ml of ethanol, 5 ml of glacial acetic acid, 13 ml of p-anisaldehyde, and 13 ml of sulfuric acid mixed in order), heated at 110 °C for 10 min, and visualized under visible and ultraviolet light (16,17)

TLC-Bioautography

Vibrio cholerae inoculum containing 10^{6f} CFU/ml in molten Mueller Hinton agar was distributed over a prepared TLC plate. After solidification of the suspension, the TLC-bioautography plate was incubated at 37 °C for 24 h. The bioautogram that developed was sprayed with a 1% aqueous solution of 2,3,5-tri phenyl tetrazolium chloride (TTC) and incubated at 37 °C for 4 h. Inhibition zones indicated the presence of active compounds. Growth inhibition areas were compared with the R_f of the related spots on the reference TLC plates. Preparative TLC plates 1 mm thick were prepared using the same stationary and mobile phases as above, with the objective of isolating the plant extract components that inhibited the growth of *V. cholerae* isolates. The substances were eluted from silica gel with methanol and identified via high performance liquid chromatography (HPLC) (18,19).

HPLC Analysis

HPLC fingerprints were prepared using a Chemito LC 6600 equipped with an isocratic pump and UV-VIS detector. Solvents were pre-filtered using a millipore system and analysis was performed on a reverse phase Lichrosphere C_{18} column (250 mm × 4.6 mm i.d., 5 µm). For injection into the HPLC system, the active spots were scraped from the reference TLC plates and dissolved in a solvent containing methanol and water v/v (70:30). The injection volume was 20 µl in all cases. All the extracts were detected at the UV wavelength of 310 nm. The flow rate was 0.7 ml/min in all cases. The mobile phase used for the different extracts was methanol and water (70:30, v/v) (17).

Results and Discussion

Medicinal plants commonly used by tribes of the Mandla and Dindori region were screened for in vitro vibriocidal activity against standard strains of *Vibrio cholerae*, *V. parahaemolyticus*, and 12 multidrug-resistant isolates of *V. cholerae* non-01. Among the 60 plants screened for vibriocidal activity in the present study, 3 plants–*Saraca indica*, *Datura stramonium*, and *Allium sativum*–had high vibriocidal activity and were used for further study. The MIC of the bark extract of *Saraca indica*, the leaf extract of *Datura stramonium,* and the bulb extract of *Allium sativum* was 2.5-10, 2.5-15, and 5-15 mg/ml, respectively, against the tested *Vibrio* spp. (Table 2). In the present study acetone and aqueous extracts had greater activity than the ethanolic extracts. Successive isolation of botanical compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. The acetone extract of *S. indica* exhibited the best antibacterial activity, with an MIC of 2.5 mg/ml against the standard strain of *V. cholera*, and *V. cholerae* non-01 BGCC#60, BGCC#61, BGCC#62, BGCC#64, BGCC#67, and BGCC#70 isolates. The acetone extract of *Datura stramonium* exhibited the best antibacterial activity with an MIC of 2.5 mg/ml against the standard strain of *V. cholerae*, *V. parahaemolyticus*, and *V. cholerae* non-01 BGCC#62 and BGCC#67 isolates. The MIC value of aqueous the extract of *A. sativum* against the standard strain of *V. cholerae* was 10 mg/ml. *Allium sativum* has been used as a medicinal plant and has long been known to have antibacterial, antifungal, and antiviral properties (20). In a recent study garlic extract was reported to be an effective agent for controlling methicillin-resistant *Staphylococcus aureus* (21). Garlic is one of the most extensively researched medicinal plants (22). Its antibacterial action depends on allicin and is thought to be due to multiple inhibitory effects on various thiol-dependent enzymatic systems (23). The MIC of synthetic antibiotics was determined using the disc diffusion method (Table 2).

Table 2. MIC of the plant extracts (mg/ml) and synthetic antibiotics (µg/ml) against *Vibrio* spp.

Plants / Antibiotics	Standard isolates		*Vibrio cholerae* non-O1 isolates (BGCC No. 59-70)											
	V. ch	*V. para*	59	60	61	62	63	64	65	66	67	68	69	70
Saraca indica (Acetone)	2.5	5	10	2.5	2.5	2.5	10	2.5	10	5	2.5	10	5	2.5
Datura stramonium (Acetone)	2.5	2.5	15	15	5	2.5	5	5	5	5	2.5	5	10	15
Alliumsativum (Water)	10	10	5	5	5	10	10	5	10	15	5	5	5	5
Ceftazidime	30	10	60	10	30	10	30	10	60	5	30	60	30	10
Ciprofloxacin	10	10	30	10	10	60	30	10	60	120	30	30	60	10
Norfloxacin	30	120	30	10	10	30	30	60	30	10	30	60	120	30
Cephotaxime	10	30	120	10	10	60	10	10	60	120	60	120	30	5

V. ch: Standard strain of *Vibrio cholerae* (H17004); *V. para*: standard strain of *Vibrio parahaemolyticus* (KX-V138); BGCC: Bacterial Germplasm Collection Center; 59-70: isolates of *V. cholerae* non-O1.

TLC using ethyl acetate:methanol:water (81:11:8) as the developing solvent separated different chemicals present in plant extracts into distinct visible fractions with different R_f values, viz. *Saraca indica* (R_f = 0.530, 0.794, and 0.808), *Datura stramonium* (R_f = 0.463, 0.527, and 0.680), and *Allium sativum* (R_f = 0.310, 0.597, and 0.627) (Figure 1, Panel-A). TLC is a standard technique that separates low molecular weight organic compounds according to their polarity. It is the most common technique used for the separation of natural substances (24).

Bioautography of the thin layer chromatographic plates showed clear zones containing substances that inhibited the growth of *Vibrio cholerae* over the region containing the components with high and medium polarity. These clear zones gave an indication of the vibriocidal activity of the compounds present in those areas. The active compounds were present in the extracts of *Saraca indica* at R_f 0.794, *Datura stramonium* at R_f 0.463, and *Allium sativum* at R_f 0.597 (Figure 1, Panel-B). In conjunction with microorganisms and other biological agents, TLC bioautography can be used to screen bioactivity (25).

HPLC analysis of the plant extracts showed peaks with different retention times. The peaks were identified using

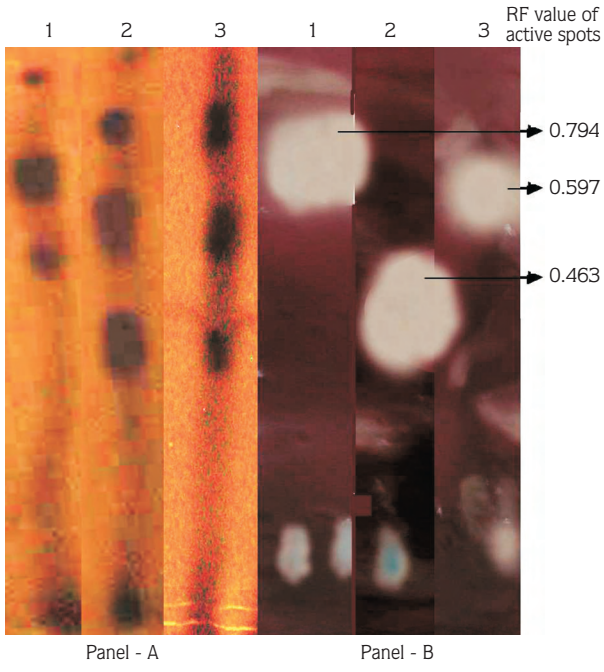

Figure 1. Thin layer chromatography plates of 3 medicinal plants were run in duplicate and 1 set was visualized by anisaldehyde/sulfuric acid spray reagent (Panel-A). The other set was used for bioautography with *Vibrio cholerae* (Panel-B). 1: *Saraca indica*; 2: *Datura stramonium*; 3: *Allium sativum*. Arrows indicate antibacterial activity.

standard solutions under similar conditions. HPLC analysis revealed the following: *Saraca indica* extract contained saponin and tannin with retention times of 3.30 and 8.05 min, respectively; *Datura stramonium* extract contained scopolamine and atropine with retentions times of 20.10 and 21.50 min, respectively; *Allium sativum* contained allicin with a retention time of 11.8 min (Figure 2). HPLC analysis of the bulb extracts of *A. sativum* identified allicin as active compound. The leaves of *D. stramonium* contain the flavonoids, chrysin, liquiritigenin, naringenin, kaempferol, quercetin, withanolide, and withastramonolide (26, 27).

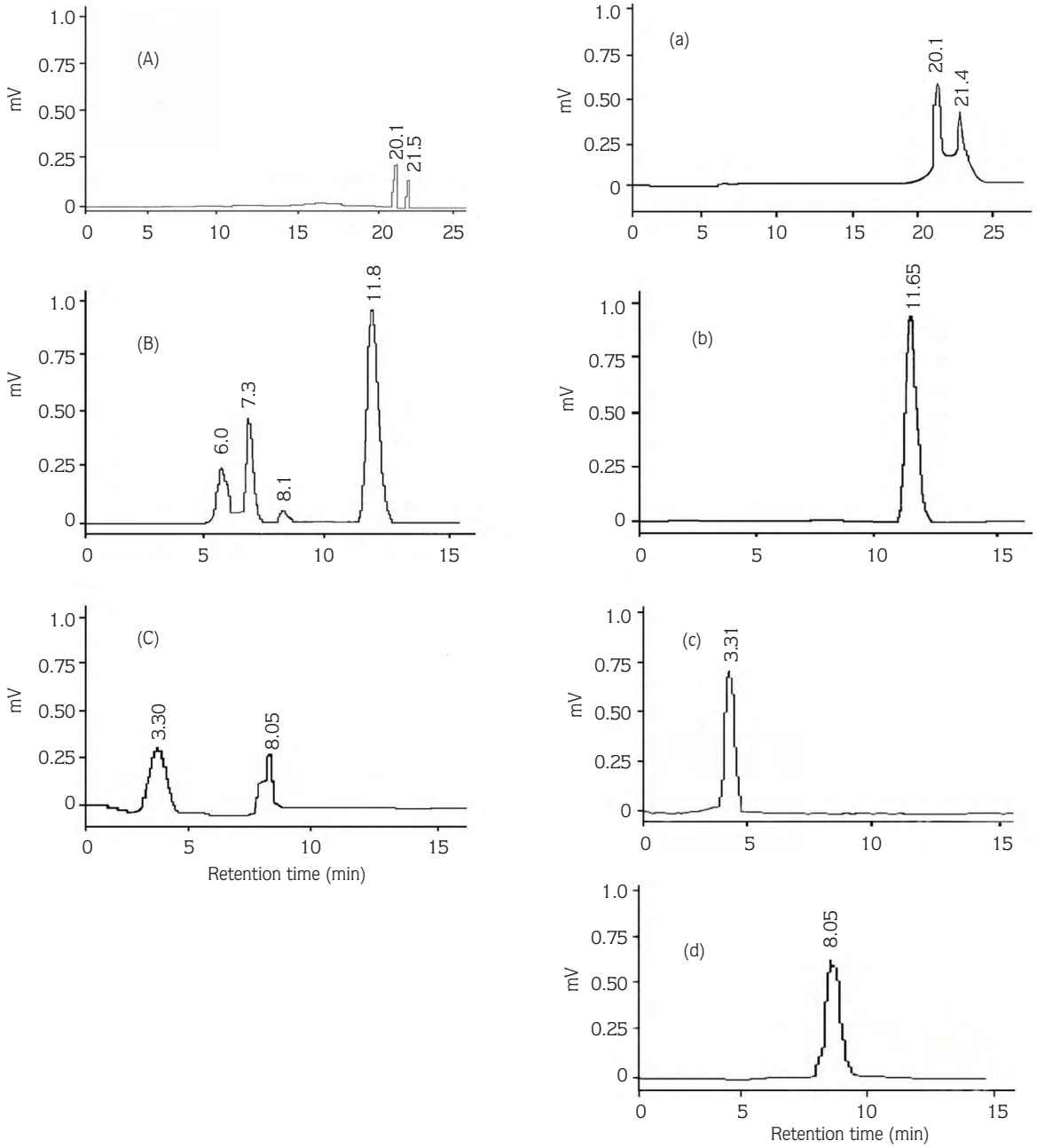

Figure 2. HPLC chromatograms of the methanolic extract of (A) *Datura stramonium*, (B) *Allium sativum*, (C) *Saraca indica*, and a standard solution of (a) scopolamine and atropine, (b) allicin, (c), saponin and (d) tannin, which were obtained using a C18 column, the mobile phase methanol/water (70:30), and a UV detector at 310 nm.

HPLC fingerprinting is the best method for chemical characterization and the present study determined the HPLC fingerprints of the active vibriocidal compounds. The chemical fingerprint profiles of the antibacterial compounds of plants identified in the present study may enable drug manufacturers to adjust the proportion of herbs and prepare a standardized product with consistent biological activity. Thus, the fingerprints developed for the studied plant extracts using HPLC, TLC, and TLC-bioautography represent a detailed chemical profile, which may be useful in the identification and quality evaluation of drugs based on plants. These chromatographic fingerprints may also be useful for differentiating plant material from adulterants. These findings support the traditional knowledge of local users and provide preliminary scientific validation for the use of these plants for their antibacterial activity. To promote proper and sustainable use of such plant resources awareness of local communities should be enhanced and traditional knowledge must be taken into consideration along with scientific findings.

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