American Journal of Life Sciences
2014; 2(6-1): 1-6
Published online August 19, 2014 (http://www.sciencepublishinggroup.com/j/ajls)
doi: 10.11648/j.ajls.s.2014020601.11
ISSN: 2328-5702 (Print); ISSN: 2328-5737 (Online)

Isolation and characterization of antimicrobial alkaloids from *Plumeria alba* flowers against food borne pathogens

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To cite this article:
Sibi G., Apsara Venkategowda, Lepakshi Gowda. Isolation and Characterization of Antimicrobial Alkaloids from *Plumeria alba* Flowers against Food Borne Pathogens. *American Journal of Life Sciences.* Special Issue: Recent Developments in Health Care through Plants and Microbes. Vol. 2, No. 6-1, 2014, pp. 1-6. doi: 10.11648/j.ajls.s.2014020601.11

Abstract: Many plants have been identified for their applications in preventing food pathogens but identification of active compounds are yet to be defined in most cases. The objective of this study was to explore the potential alkaloids from *Plumeria alba* and their activity against food pathogenic and spoilage microorganisms. A total of six food borne pathogens namely *Bacillus cereus* ATCC 10876, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Salmonella typhimurium* MTCC 3224, and *Shigella flexneri* ATCC 12022 were tested against the alkaloid extract from *P. alba* flowers under in vitro conditions. Antibacterial assay was evaluated using well diffusion assay and minimum inhibitory concentration (MIC) values were tested by broth microdilution method. Alkaloid profile of the extracts was determined by gas chromatography-mass spectroscopy (GC-MS). Minimum inhibitory concentration (MIC) values revealed that *Shigella flexneri* ATCC 12022 was found to be the most sensitive organism (7.5 µg ml-[1]) followed by *Staphylococcus aureus* ATCC 6538 (15 µg ml-[1]). *Escherichia coli* ATCC 8739 was more resistant to the extract with an MIC value of > 60 µg ml-[1]. Examination of the alkaloid profile of *Plumeria alba* flowers using gas chromatography-mass spectrometry resulted in the presence of 11 alkaloids of which 3 isoquinoline alkaloids, 2 pyridine alkaloids, 1 indole alkaloid, 1 vinca alkaloid and 1 resperine alkaloid were identified. According to the results, *S. flexneri* was found to be the most susceptible organism and it can be concluded that formulation of antimicrobial drugs containing *P. alba* flower extract to control food borne pathogens is feasible.

Keywords: *Plumeria Alba*, Alkaloids, Antimicrobial, Food Borne Pathogens

1. Introduction

Alkaloids are defined as cyclic compounds containing nitrogen in a negative oxidative state [1]. They are part of chemical defense in plants [2] and have antibiotic activities [3]. Approximately 60% of the drugs from plant origin are from alkaloids [4]. Many alkaloids have been used in medicine over the years and some are still prominent drugs which have physiological effect on animals [5].

Plants have been exploited for their secondary metabolites as sources of medicinal agents since time memorial and many plants were discovered with pharmacological properties [6]. The increasing demand and research on herbal medicines due to their potential phytochemicals are proving plants as the major pharmacological sources. Food borne pathogens pose a major health threat around the world. Using natural antimicrobials in food where plant derived compounds in particular are reported to play a major role in preventing food-borne diseases caused by pathogenic and spoilage microorganisms [7]. The significant global development of medicinal plant research has encouraged assessment of the activity of a number of plant extracts. Although many plants have been identified for their applications in preventing food pathogens, identification of active compounds are yet to be defined in most cases. Exploring the phytoconstituents of a plant material is necessary to establish a relation between pharmacology and chemistry of the plant. *Plumeria* belongs to the Apocyanaceae family have been reported to possess antimicrobial activities [8- 12]. This study was aimed to explore the potential alkaloids from *Plumeria alba* and their activity against food pathogenic and spoilage microorganisms.

2. Materials and Methods

2.1. Plant Material Collection

Fresh flowers of *Plumeria alba*, a species of the family Apocyanaceae known as Frangipani were collected, identified, shade dried and pulverized.

2.2. Alkaloid Extraction

Dried flowers of *Plumeria alba* were collected, and extracted with 95% ethanol in Soxhlet apparatus for 24 hrs. The extract was filtered and dried in rotary vacuum evaporator under reduced conditions followed by the addition of 1 N hydrochloric acid. Ethanol from the extract was removed by rotary evaporator and 10 % ammonium hydroxide was added until the pH reached 9. The basic solution was extracted with petroleum ether (60° - 90°C) by separating funnel followed by drying the solvent with sodium sulphate and used for further studies.

2.3. Test Organisms

Bacillus cereus ATCC 10876, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Salmonella typhimurium* MTCC 3224, and *Shigella flexneri* ATCC 12022 were obtained from American Type Culture Collection (ATCC) and Microbial Type Culture Collection (MTCC), Chandigarh. The bacteria were then standardized by adjusting the bacterial suspension to absorbance reading within the range of 0.08 to 0.10 at OD 625 nm which was equivalent to 1.2 x 10^8 CFU/ml.

2.4. Antibacterial Assay

Antibacterial activities of petroleum ether extract of *Plumeria alba* flowers were evaluated using well diffusion assay. 100µl of the appropriate bacterial suspension was inoculated on Mueller Hinton agar using sterile swabs. 20 µl of the extract was added into the 5 mm wells and the plates were allowed for pre-diffusion of the extract before incubation. The diameter of zone of inhibition mean of two replicates ± SD as indicated by clear area which was devoid of growth of microbes was measured to determine antibacterial activity. The experiment was replicated thrice for reproducible results.

2.5. Minimum Inhibitory Concentration

Minimum inhibitory concentrations (MIC) of *Plumeria* flower extracts were tested by broth microdilution method with 96 well plates. The extract was dissolved in 5% DMSO to obtain a stock concentration of 120 mg ml^{-1}. Various concentrations of extract were prepared to achieve 600, 300, 150, 75, 37, 18 µg ml^{-1} and the wells were inoculated with 1 x 10^6 CFU of bacteria. The incubation period was 24 h at 37°C. The MIC testing was performed according to standard methods [13] along with positive control. The lowest concentration which inhibits the visible growth of tested organism was determined as MIC. All determinations were run in triplicate.

2.6. Statistical Analysis

The data are expressed as mean±SE. statistical analysis was done by using paired and unpaired student's t-test.

2.7. GC-MS Analysis

GC-MS analysis of alkaloid extract was performed using a Thermo TRACE GC Ultra comprising an Thermo DSQ II auto-sampler equipped with TR-5 (5% phenyl methyl poly siloxane) fused a capillary column (30 m x 0.25 mm ID x 0.25 um). For GC-MS detection, an electron ionization system was operated in electron impact mode with ionization energy of 70 eV. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1ml/min and an injection volume of 2 µl was employed with a split ratio of 10:1. The injector temperature was maintained at 275°C and the ion source temperature was 220°C. Mass spectra were taken at 70 eV with a scan speed of 500 amu/s at the interval of 0.755s. The solvent delay was 0 to 2 min and the total GC/MS running time was 41 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The mass analyzer used in the analysis was Quadrupole and the software adopted to handle mass spectra and chromatograms was Xcalibur.

2.8. Identification of Phytocomponents

Interpretation on GC-MS spectrum was conducted using the database of National Institute of Standards and Technology (NIST). The name, molecular weight and structure of the components of the test materials were ascertained from NIST library.

3. Results

3.1. Antibacterial Activity

In this study, the crude plant extract was shown to be more active against the pathogens tested (Fig-1). *S. flexneri* ATCC 12022 was found as the most susceptible organism (23.7± 0.1 mm) followed by *S. aureus* ATCC 6538 (19.0 ± 0.3 mm). *B. cereus* ATCC 10876, *B. subtilis* ATCC 6633 and *S. typhi* MTCC 3224 were sensitive with a zone inhibition range of 15.2 - 18.6 mm. *E. coli* ATCC 8739 was inhibited with a zone diameter of 9.6 ± 0.8 mm.

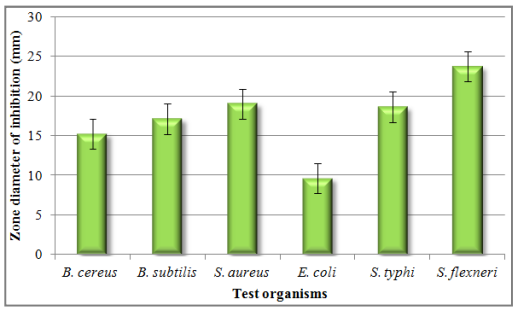


Fig 1. *Antibacterial activity of P. alba crude alkaloid extract*

Table 1. *MIC values (μg ml^{-1}) of alkaloid extract from P. alba flowers*

Bacterial strains	Alkaloid extract	Chloramphenicol
Bacillus cereus ATCC 10876	30	<2.0
Bacillus subtilis ATCC 6633	30	1.0
Staphylococcus aureus ATCC 6538	15	0.50
Escherichia coli ATCC 8739	>60	4.0
Salmonella typhimurium MTCC 3224	<30	>1.0
Shigella flexneri ATCC 12022	7.5	<0.50

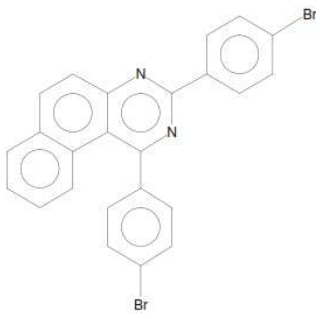
Regarding the MIC activities (Table-1) against the pathogens presented by the dilutions obtained from crude extract, it is remarkable that the low MIC value against *S. flexneri* (7.5 μg ml^{-1}). Noteworthy is also the MICs against *S. aureus* (15 μg ml^{-1}). The extract was also active against the species of *Bacillus* (30 μg ml^{-1}). On the other hand, neither crude extract nor its dilutions displayed any significant activity against *E. coli* (> 60 μg ml^{-1}).

3.2. Gas Chromatography-Mass Spectroscopy Data

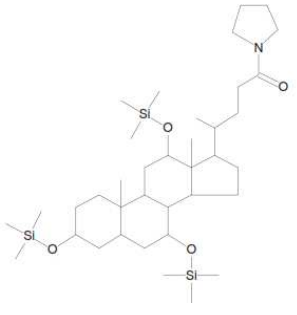
The results pertaining to GC-MS analysis of the petroleum ether extract of *Plumeria alba* flowers lead to the identification of a number of compounds. The various alkaloids present in the extract were detected are shown in (Table-2). The GC-MS spectrum confirmed the presence of various components with different retention times as illustrated in Fig-2. The mass spectrometer analyzes the compounds eluted at different times to identify the nature and structure of the compounds. The large compound fragments into small compounds giving rise to appearance of peaks at different m/z ratios. These mass spectra are fingerprint of that compound which can be identified from the data library.

Table 2. *GC-MS analysis of alkaloid extract from P. alba flowers*

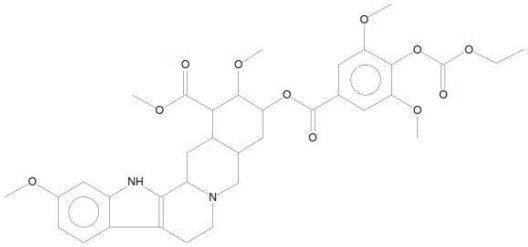
RT (min)	Compound	m/z (M+)	Mol. wt	Mol. formula	Category
5.2	1,3-Di(4-bromophenyl) benzo[f]quinazoline	99	488	$C_{24}H_{14}Br_2N_2$	Alkaloid
23.7	Pyrrolidine 1-[3.alpha., 7.alpha., 12.alpha.-tris(trimethylsiloxy)-5.beta.-cholan-24-oyl]	132	677	$C_{37}H_{71}NO_4Si_3$	Alkaloid
23.74	Syrosingopine	291	666	$C_{35}H_{42}N_2O_{11}$	Reserpine alkaloid
35.5	Voacamine	95	704	$C_{43}H_{52}N_4O_5$	Indole alkaloid
39.64	Vobtusine, 2',3'-didehydro-2'-deoxy	150	700	$C_{43}H_{48}N_4O_5$	Vinca alkaloid
39.7	1,4-Naphthalenedione, 2-(3,7,11,15,19,23,27,31-octamethyl-2,6,10,14,18,22,26,30-dotriacontaoctaenyl), (all-E)-	172	702	$C_{50}H_{70}O_2$	Alkaloid
39.72	Evonine	138	761	$C_{36}H_{43}NO_{17}$	Pyridine alkaloid
48.2	Evonimine, 8-(acetyloxy)-O2-benzoyl-O2-deacetyl-8-deoxo-26-hydroxy-, (8.alpha.)	340	883	$C_{43}H_{49}NO_{19}$	Pyridine alkaloid
53.01	Curine	55	594	$C_{36}H_{38}N_2O_6$	Isoquinoline alkaloid
53.07	Tubocurarine chloride	241	680	$C_{37}H_{42}Cl_2N_2O_6$	Isoquinoline alkaloid
53.1	Cycleanine, O7,O7'-didemethyl-,(1.alpha.,1'.alpha.)	29	594	$C_{36}H_{38}N_2O_6$	Isoquinoline alkaloid



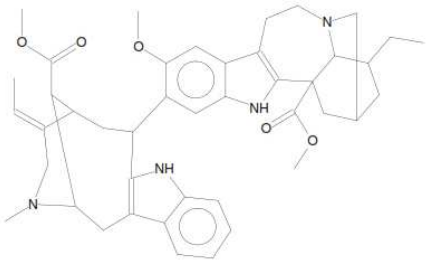
1,3-Di(4-bromophenyl) benzo[f]quinazoline



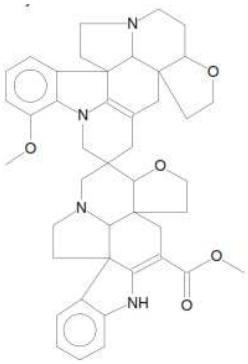
Pyrrolidine 1-[3.alpha., 7.alpha., 12.alpha.-tris(trimethylsiloxy)-5.beta.-cholan-24-oyl]



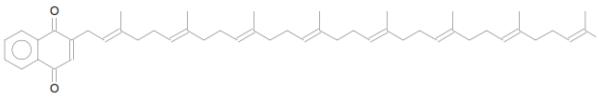
Syrosingopine



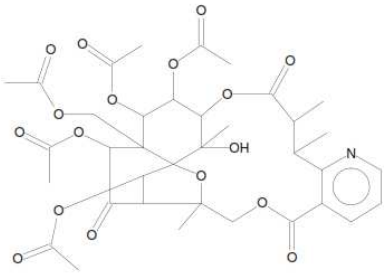
Voacamine



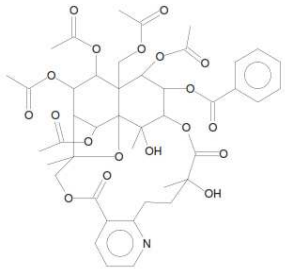
Vobtusine, 2',3'-didehydro-2'-deoxy



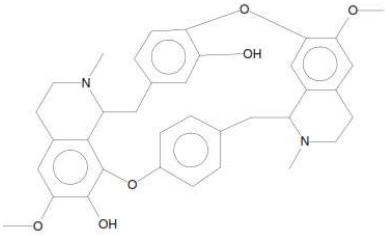
1,4-Naphthalenedione, 2-(3,7,11,15,19,23,27,31-octamethyl-2,6,10,14,18,22,26,30-dotriacontaoctaenyl)-, (all-E)-



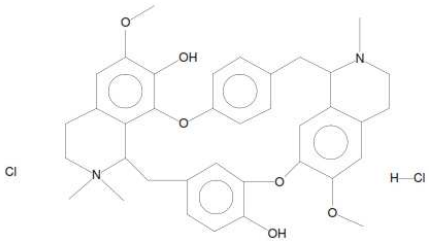
Evonine



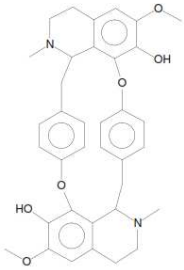
Evonimine, 8-(acetyloxy)-O2-benzoyl-O2-deacetyl-8-deoxo-26-hydroxy-, (8.alpha.)



Curine



Tubocurarine chloride



Cycleanine, O7, O7'-didemethyl-, (1.alpha.,1'.alpha.)

Fig 2. *Alkaloid profile of Pluemria alba flowers*

4. Discussion

Among all the bacterial strains tested, *S. flexneri* was found to be the most susceptible organism. All antibacterial activity occurred in a concentration dependent manner as suggested by MIC values. However, the efficacy of the extract was lesser than the standard antibiotic, chloramphenicol. Alkaloids are group of secondary metabolites have an array of pharmacological activities. The results of GC-MS analysis of *Plumeria alba* flower extract revealed the presence of 11 alkaloids based on their retention time and retention indexes. The mass spectra of these alkaloids were matched with those found in the NIST databases. Of the 11 alkaloids present in the extract, 3 isoquinoline alkaloids, 2 pyridine alkaloids, 1 indole alkaloid, 1 vinca alkaloid and 1 resperine alkaloid were identified.

Antimicrobial activity of pyrrolidine was reported in earlier studies [14]. Voacamine demonstrated antimicrobial activity against Gram positive and Gram negative bacteria [15, 16]. In addition to that, it was reported as a competitive antagonist of cytotoxic agents, inducer of autophagy [17, 18] and antiplasmodial compound [19]. Antibacterial effect of 1,4-napthalene dione was reported along with beta lactamse inhibitor activity [20-22]. Quinazoline alkaloids were reported to possess antibacterial activities [23]. Curine is reported as vasodilator [24]. Cycleanine has been reported to contain antimicrobial and cytotoxic activities [25].

5. Conclusion

In the process of controlling food borne pathogens, antimicrobials from plant origin have more potential and effective. On the basis of this study, it can be concluded that the antimicrobial activity of *P. alba* flower extracts might be correlated to the presence of alkaloids which justify this plant for its various ailments. All these data along with urgent need of new effective drugs invite further work on *Plumeria*.

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