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Chloroform Extracts of *Ipomoea alba* and *Ipomoea tricolor* Seeds Show Strong *In-vitro* Antibacterial, Antifungal, and Cytotoxic Activity

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Abstract
Extracts from the seeds of two morning glories were studied to determine their anti-microbial activity. *Ipomoea alba* and *Ipomoea tricolor* were analyzed by broth microdilution technique. *I. alba* extract showed very strong antibacterial activity against *Enterococcus faecalis* and *Bacillus cereus*, with minimum inhibitory concentration (MIC) values of 19.5 µg/mL for both. Antifungal activity was also observed against *Cryptococcus neoformans*, with MIC of 78 µg/mL. Strong cytotoxicity against the human breast cancer cell line MDA-MB-231, with IC_{50} value of 35.72 ± 1.86 µg/mL was observed. *I. tricolor* extract showed strong activity against *E. faecalis and B. cereus*, with MIC values of 19.5 and 9.75 µg/mL, respectively. Strong activity was observed against *Cryptococcus neoformans*, with MIC of 9.75 µg/mL. Strong cytotoxicity was also observed against human breast cancer cell lines MDA-MB-231 and Hs 578T, with IC_{50} values of 28.47 ±1.98 µg/mL and <30 µg/mL, respectively.

Keywords: *Ipomoea alba*, *Ipomoea tricolor*, antifungal, antibacterial, cytotoxicity, pharmacognosy

Introduction
Ipomoea alba

Fig 1: *Ipomoea alba* bloom and leaves (left), and *Ipomoea alba* seeds (right).

Class: Magnoliopsida; Superorder: Asteranae; Order: Solanales; Family: Convolvulaceae; Genus: *Ipomoea* L.

Ipomoea alba L., commonly called moonflower, or moonvine, is native to the Caribbean, Mexico, and Central America, and is now grown world-wide as an ornamental. Despite being a "morning-glory", it is a night-blooming flower. It's a tender perennial vine in USDA zones 10 – 12, and is planted as an annual in cooler zones. It is known for its nocturnally blooming and very fragrant large white flowers. It commonly reaches 3-5 m or more in a single season. Its twisted vines have tendrils which it uses to climb. The stems have a milky sap when cut. It has large, rounded, broad-ovate, deep green leaves (10-20 cm long) with cordate bases. Fragrant, white flowers (to 15 cm diameter) bloom at dusk from mid-summer into fall. Flowers unfold in early evening before nightfall from attractive spiraled tubular flower buds. Flowers remain open all night and eventually close before noon the following day. Their rich fragrance attracts moths, particularly the hawk-moth, Sphingidae: *Manduca* spp. and *Agrius*

spp. They also have very large nectaries and produce upwards of 2.5 mg/hr of nectar, which is sucrose dominant [1, 2]. Hummingbirds have been observed drinking their nectar as well. This plant is synonymous with and formerly known as *Ipomoea bona-nox* and *Calonyction aculeatum* [3]. *Ipomoea alba* was used for the processing of latex into rubber by ancient Mesoamericans for thousands of years. Balls, figurines, and tool bindings were made from a mixture of natural latex from Castilla elastic and the juice of the *Ipomoea alba*. They were mixed together so that the latex would remain flexible and not get brittle [4, 5]. The oldest known artifacts are rubber balls from 1600 B.C. from the Olmec burial site, Manati, in Veracruz, Mexico [6]. The rubber balls played a key role in Mayan culture, with ball courts found from Arizona to South America. It even played a part in their origin mythology where two "Hero Twins" played the ball game against the lords of the underworld. Mayans used the rubber for medicine, and European explorers brought this back to the Old World, as well [4, 5].

Ipomoea tricolor

Class: Magnoliopsida; Superorder: Asteranae; Order: Solanales; Family: Convolvulaceae; Genus: *Ipomoea* L.

Fig 2: Ipomoea tricolor (pearly gates variety) in full-bloom, with fresh seed pods (left), and showing leaf-shape (right).

Ipomoea tricolor Cav. is an herbaceous liana, growing 2-5 m tall, with leaves 3-5 cm wide, 3-7 cm long, cordate-ovate. The leaves grow in a spiraling formation around the stem. Flowers are trumpet shaped, 4-8 cm long, blue, with white centers and yellow sex organs. Flowers open in the morning and wilt by late afternoon. Flowers from late spring to fall. Annual above zone 9.

Morning glory (*Ipomoea* spp.) seeds have long been the subject of folklore, myth, and speculation. Some varieties (*I. tricolor* and *I. violacea*) contain lysergic acid derivatives, which are known to be hallucinogenic, and are closely related chemically to the famous LSD molecule [7, 8]. Ethnobotanists have described the ritual use of morning glory seeds by the Mayans and other native tribes of Latin America for religious ceremonies and for divination and ritual psychedelic experiences [9].

Knowledge of this cultural significance led to the hypothesis that members of this genus, in particular the species mentioned in ethnobotanical accounts, likely contain important healing and medicinal properties. Data supporting antibacterial activity of *Ipomoea alba* has been mentioned in previous studies [10].

Methods and Materials

The Extracts

Both species of Ipomoea were grown by the author in Huntsville, Alabama (Lat = N 34.78; Long = W -86.54), in 2016. Seeds were harvested for study in October and November of 2016. Seeds were originally purchased from Burpee seed company. Voucher specimens were pressed and scanned and stored on hard drive of corresponding author.

Ipomoea tricolor seeds (128.4 g) were ground and extracted with 500 mL hexane in an amber jar at room temperature for 8 days. Then the solvent was filtered into a clean, weighed jar and allowed to evaporate in a flow hood. The following day, the seed powder was placed back in the jar and extracted with 1000 mL of $CHCl_3$ at room temperature for 14 days, and then filtered and allowed to evaporate in a clean, weighed jar. The masses of the hexane fraction and the chloroform fraction were obtained. *Ipomoea alba* seeds (233.7 g) were ground and extracted in the same manner as above. When complete, the $CHCl_3$ extract was rinsed with 100 mL hexane to remove residual fats and waxes and this added to the previous hexane extract. These liquid solvent extracts were air dried in a flow hood and the masses of both hexane and chloroform fractions were obtained.

Antimicrobial Assays

The minimum inhibitory concentrations, MICs, were determined using the broth micro-dilution technique with 96 well plates (Falcon brand) [11, 12]. Extracts were screened for antibacterial activity against the Gram-positive bacteria: *Bacillus cereus, Staphylococcus aureus,* and *Enterococcus faecalis*; the Gram-negative bacteria: *Pseudomonas aeruginosa, Escherischia coli, Salmonella typhimurium,* and *Serratia marescens*.

Cation adjusted Müller-Hinton Agar (CAMHA) was used for bacterial culture at 37 °C. After two sequential inoculations, the bacteria were transferred to Cation adjusted Mueller-Hinton Broth (CAMHB). Two generations of the broth

cultures were grown and then diluted to concentrations of approximately 1.5×10^8 colony-forming units (CFU)/mL. The MacFarland turbidity standard of 0.5 was obtained by spectrophotometer, to determine proper concentration of CFU. Dilutions of the $CHCl_3$ extracts were prepared in cation-adjusted Mueller Hinton broth (CAMHB) beginning with 50 µL of 1% w/v solutions of extracts in DMSO, plus 50 µL of CAMHB. The extract solutions and CAMHB were serially diluted (1:1) in 96-well plates with a 12-tipped micro-pipette, and the final 50 µL was discarded. The 96 wells were inoculated with 50 µL each of the bacterial broth cultures. Gentamicin was used as a positive antibiotic control, while DMSO was used as a negative control. Plates were incubated at 37°C for 20 h. The final MIC was determined as the lowest concentration that prevented growth, as compared to the negative control, below 625 µg/mL (DMSO showed some inhibitory effects by itself to approximately 1250 µg/mL). MICs below 156 µg/mL were considered to show activity[13], and MICs of 39 µg/mL or less were considered strong activity. All microorganisms were obtained from the University of Alabama in Huntsville Department of Biology.

Extracts were also screened for antifungal activity against *Candida albicans, Cryptococcus neoformans*, and *Aspergillis niger*, using similar methods to the antibacterial screening (broth micro-dilution technique) [11, 12]. The *C. albicans* and *C. neoformans* were initially grown on Potato Dextrose Agar (PDA), then transferred to potato dextrose broth (PDB) and grown for 48 hours (*C. albicans*) and 72 hours (*C. neoformans*) at 37ºC on a shaker table. The cultures were then diluted with Roswell Park Memorial Institute medium (RPMI 1640) and MOP buffer, to a final concentration of 2000 cells/mL using a hemocytometer to count cells and dilution factor calculations. The *A. niger* was grown on malt extract agar for 7 days, then the spores were harvested by lightly scraping the mature fruiting bodies and filtered through sterile cheesecloth to remove hyphae. These spores were then added to RPMI 1640 buffered with MOPand concentration was calculated with a spectrophotometer set to 625nm. Absorbance of 0.150 was obtained (close to McFarland 0.5 standard). 100 µL of RPMI broth was then added to each well of the 96 well plate. Next, 100 µL of solutions of the $CHCl_3$ extracts at 1% w/v in DMSO was added to the top row of the plate. A positive control of pure DMSO was used and the negative control was un-inoculated RPMI broth. This was serially diluted 1:1 all the way down to the bottom of the plate with a 12-tip micro-pipette, and the last 100 µL was discarded. Finally, each well of the plate was inoculated with 100 µL of fungal broth culture. The plates were incubated at 37°C for 48 and 72 hours for the *C. albicans* and *C. neoformans*, respectively, and 7 days for the *A. niger*. The results were determined by manually viewing the plates through a light table.

Cytotoxicity Assays

The $CHCl_3$ extracts of *I. alba* and *I. tricolor* were screened for cytotoxic activity against Hs578T (human breast tumor cells) and MDA-MB-231 (human breast adenocarcinoma, estrogen receptor negative) cells using MTT-based cytotoxicity assay[14]. Human Hs578T and MDA-MB-231 breast adenocarcinoma cells were grown in a 3% CO2 environment at 37°C in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 100,000 units penicillin, and 10.0 mg streptomycin per liter of medium, 15 mM of HEPES, and buffered with 26.7 mM $NaHCO_3$, pH 7.35. Cells were plated into 96-well cell culture plates at 1.25×10^4 cells per well. The volume in each well was 100 µL. After 48 h, supernatant fluid was removed by suction and replaced with 100 µL growth medium containing 1.0 µL of DMSO solution of the Ipomoea extracts (1% w/w in DMSO), giving a final concentration of 100 µg/mL for each well. Solutions were added to wells in four replicates. Medium controls and DMSO controls (10 µL DMSO/mL) were used. Tingenone was used as a positive control. After the addition of extracts, plates were incubated for 48 h at 37°C in 5% CO_2; medium was then removed by suction, and 100 µL of fresh medium was added to each well. To establish percent kill rates, the MTT assay for cell viability was carried out. After colorimetric readings were recorded (570 nm, using a Spectra MAX Plus microplate reader, Molecular Devices, Sunnyvale, CA, USA), percent kill was calculated.

Results

Table 1: Yields from hexane and chloroform extracts of dried *Ipomoea* seeds.

	Seed mass	Hexane extract (mass/percent)	$CHCl_3$ extract (mass/percent)
Ipomoea alba	233.66 g	11.62 g / 4.97%	11.4 g / 4.87%
Ipomoea tricolor	128.4 g	15.76 g / 12.27 %	2.75 g / 2.14%

Table 2: Antibacterial (MIC, µg/mL), antifungal (MIC, µg/mL), and cytotoxic (IC_{50}, µg/mL) activities of *Ipomoea* $CHCl_3$ seed extracts.

Bioactivity assay	Extract	
Antibacterial	***Ipomoea alba***	***Ipomoea tricolor***
Bacillus cereus	19.5	9.75
Enterococcus faecalis	19.5	19.5
Staphylococcus aureus	625	625
Escherichia coli	1250	1250
Pseudomonas aeruginosa	1250	1250
Salmonella typhimurium	1250	1250
Serratia marescens	1250	625
Antifungal		
Aspergillus niger	312	312
Candida albicans	1250	1250
Cryptococcus neoformans	156	9.75
Cytotoxicity		
Hs578T	N/A	<30
MDA-MB-231	35.72 (±1.86)	28.47 (±1.98)

Discussion

Both of these *Ipomoea* species have been previously studied for their rich chemical content. Morning glory vines are commonly used in areas around Morelos, Mexico, as a ground cover for sugar cane plantations during the fallow season. *Ipomoea tricolor* extracts have shown strong herbicidal properties [15, 16, 17]. Tricolorins A-E are natural tetrasaccharide macrolactones, linear tetraglycosides of jalapinolic acid[18]. Tricolorin-A has been found to be the most potent herbicidal fraction [15, 16]. This same compound has been shown to have in-vitro cytotoxic activity against human breast cancer cells, and all the amphipathic tetrasaccharides from the tricolorin series 1-5, previously exhibited inhibitory activity against *S. aureus* [15, 16]. Structures have been elucidated for at least 10 glycolipids from *Ipomoea tricolor*; they are known as Tricolorin A-J, with Tricolorins F-G being tri-saccharide lactones and H-J appearing as ether dimers of the trisaccharides. Tricoloric acid derivative was also discovered from this seed [18]. These glycolipids have shown inhibition of the ATPase in cell membranes of plant radicles, possibly explaining their herbicidal activity [19].

Ipomoea alba yields indolizine alkaloids [20, 21] and glycolipids as well, in the form of pentasaccharide derivatives of both convolvulinolic and jalapinolic acids[22]. These have been named the albinosides. The structures of approximately 10 of these albinosides have been elucidated[21]. Studies involving these glycolipids as chemosensitizers for cancer chemotherapies as well as possible analgesics have been carried out [23, 24, 25]. Anti-microbial glycosides have also been found in other *Ipomoea* species [26, 27].

Conclusion

The $CHCl_3$ extracts tested here showed strong cytotoxic effects against two lines of breast cancer cells (Hs578T and MD-MBA-231), strong antifungal activity against *Cryptococcus neoformans,* and strong anti-bacterial activity against *E. faecalis* and *B. cereus*. The extracts also showed minimal activity against *Aspergillis niger*, a spore-forming fungus. These studies show evidence that warrants further study into the active compounds and the mechanisms of action of these *Ipomoea* extracts for use as possible antimicrobials and anti-tumor agents.

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There are no known conflicts of interest and no animals were used in these experiments.

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