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ANTIMICROBIAL ACTIVITY OF *DRACAENA CINNABARI* RESIN FROM SOQOTRA ISLAND ON MULTI DRUG RESISTANT HUMAN PATHOGENS

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Abstract

Background: Few studies showed that *Dracaena cinnabari* resin, collected from Soqotra Island, Yemen, has antimicrobial activity. This study is the first to investigate antimicrobial activity of the resin on both antibiotic multi-resistant human pathogens and on poly-microbial culture.
Material and Methods: Antimicrobial activity of ethanolic extract of *Dracaena cinnabari* resin from Soqotra Island on multidrug resistant Gram-positive and Gram-negative human ATCC standard pathogens and *Ascosphaera apis*, the causal organism of chalkbrood disease of honeybee was studied using the agar disc diffusion method. The minimal inhibitory concentration of extracts was carried out by the broth micro dilution method.
Results: Ethanolic extract of *Dracaena cinnabari* resin showed a considerable antimicrobial activity against all the pathogens tested. The zone of inhibition were between 4.9-11.5 mm. The most sensitive microbe was *Staphylococcus aureus* and least sensitive was *Aspergillus nidulans*. The minimal inhibitory concentration of the extract against *Escherichia. coli* ATCC 10402, *Klebsiella pneumonia* ATCC 10031, and *Staphylococcus aureus* ATCC 29212 was 1.25 µg/mL (w/v) and for the other pathogens (*Candida albicans* ATCC 10231, *Salmonella typhimurum* ATCC 3311 and *Pseudomonos aeruginosa* ATCC 2785) was 2.5 µg/mL (w/v).
Conclusion: Ethanolic extract of *Dracaena cinnabari* resin has a considerable antimicrobial activity against Gram-positive and Gram-negative human pathogens and fungi. This extract might possess a role in the management of microbial infections in human and honeybee disease.

Key words: Antimicrobial Activity, *Dracaena cinnabari*, Human pathogens, Minimum Inhibitory Concentration

Introduction

Socotra Island is characterized by a high level of endemism (37%) comparable with other oceanic islands such as Mauritius, Galapagos and the Canary islands (Miller and Morris, 2004). *Dracaena cinnabari* (Agavaceae) (*D. cinnabari*) is arguably the main endemic flagship species of Socotra, commonly known as *Damm Al-akhwain* in Yemen. It is one of the six arboreal species (Dragon blood tree) of the genus (Marrero et al., 1998). *D. cinnabari* is threatened for the collection of resins (Dragon blood). Dragon's blood is a deep red resin, which has been used as a famous traditional medicine since ancient times by many cultures (Gupta et al., 2008). Dragon's blood was used by the early Greeks, Romans, and Arabs for its medicinal properties. Locals of Moomy city on Socotra island used the Dragon's blood (*Dracaena*) as a sort of cure-all, using it for many diseases such as wounds, diarrhea, fevers, dysentery diseases, ulcers of mouth, throat, intestines and stomach, respiratory disease and eczema. In Yemeni folk medicine, dragon's blood (*Dracaena*) is used for treating dysentery, diarrhoea, hemorrhage and external ulcers (Milburn, 1984).

Infectious diseases are still the number one cause of death world-wide, and in tropical countries they account for approximately 50% of deaths (Iwu et al., 1999). *Staphylococcus aureus* causes conjunctivitis, blepharitis, keratitis, suppuration, abscess formation, a variety of pyrogenic infections and even fatal septicemia (Callegan et al., 2007). *Candida albicans* has become another main cause of morbidity and mortality worldwide among immunocompromised individuals (Morgan, 2005). Importantly, *Candida* has been shown to be the third most commonly isolated blood pathogen from patients in US hospitals (Ramage et al., 2001).

Antibiotics provide the main basis for the therapy of microbial (bacterial and fungal) infections. However, overuse of antibiotics has become the major factor for the emergence and dissemination of multi-drug resistant strains of several groups of microorganisms (Harbottle et al., 2006). Apart from resistance, some antibiotics have serious undesirable side effects which limit their application. Thus, there is a need to develop new antimicrobial agents that are effective with minimal side-effects (Maureer-Grimes et al., 1996).
The extract of *D. cinnabari* showed a high antiplasmodial and antileishmanial activity (Mothana et al., 2012). The antimicrobial activities of *D. cinnabari* resin toward various pathogens have been investigated by some researchers (Mothana and Lindequist, 2005; Gupta & Gupta, 2011; Yehia et al., 2013). However, very little information is available regarding its effects against multi drug-resistant human pathogens. Regarding polymicrobial culture, no study investigating the effect of *D. cinnabari* resin on the growth of multiple pathogens cultured on the same media has been published. Therefore, the present study is the first to investigate the effect of the resin on multidrug resistant poly-microbial culture.

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Materials & Methods

Microorganisms

Cultures of various ATCC human standard isolates were obtained from College of Pharmacy, King Saud University Riyadh. The isolates were identified by the standard bacteriological techniques. The *Ascosphaera apis*, causal organism of chalkbrood disease was isolated from infected chalkbrood of hybrid carniolan honeybee at bee research chair, King Saud University Riyadh. Standard isolates included *Candida albicans* ATCC 10231, *Escherichia coli* ATCC 10402, *Salmonella typhimurum* ATCC 3311, *Staphylococcus aureus* ATCC 29212, *Pseudomonos aeruginosa* ATCC 2785, *Klebsiella pneumonia* ATCC 10031 and *Aspergillus nidulans*. The Kirby-Bauer method was used to test antibiotic sensitivity. Using a 10-ml standard loop, a colony of each isolate was picked from a plate, grown in 10 ml nutrient broth, and used after 24 h culture in 37°C for bacteria and at 30°C for fungi. The cultural media and materials were supplied by King Saud University. Cefoxitin antibiotic (Sigma-Aldrich,India) was used as a standard control.

Plant Materials

D. cinnabari resin was collected from Soqotra Island and identified as *Dracaena cinnabari* Balf f. resin. The air-dried and powdered plant materials (5 g) were extracted under shaking with 70% ethyl alcohol using a soxhlet assembly for 12 h at room temperature. The extract was filtered and concentrated under vacuum in a rotary evaporator until completely dry and then stored at 4°C. The extracts were mixed with sterile nutrient broth to obtain different concentrations.

Agar Disc diffusion assay

Antimicrobial activity of the resin extract was evaluated using the agar disc diffusion method against different human pathogens and *Ascosphaera apis* (Bauer et al., 1966). Fresh culture suspension (100 µl) of each microorganism was spread on respective media agar plates. The concentration of cultures was 1×10^8 CFU/ ml. For screening, 6 mm diameter filter paper sterile discs were impregnated with 10 µl of extract equivalent to 0.1 mg of extract after being placed on the surface of the inoculated media agar plates. The plates were incubated at 37° for 24 h. Clear zone of inhibition around the discs indicated the presence of antimicrobial activity. The diameters of the zone of inhibition were measured in millimetres, including the diameter of the disc. The controls were set up with equivalent quantities of Dimethyl Sulfoxide and nutrient broth. A zone of inhibition at10 mm or more was considered as a high antibacterial activity.

Antimicrobial effects of resin on single pathogen culture and determination of minimal inhibitory concentration

In order to study the antimicrobial activity of the resin extract on the pathogens and to measure the minimal inhibitory concentration (MIC) of the extracts against the microbes, micro-broth dilution method was used (Valgas et al., 2007). Alcohol free extract was dissolved in Dimethyl Sulfoxide solvent (300µg/mL, final concentration) and were diluted with nutrient culture broth to obtain different concentrations (0.25, 0.5. 1.25. 2.5. 5.0, 25, 50, 75, 100, 125, 150, and 175 µg/mL). A sample from each dilution (180 µl) was distributed in 96-well plates. Controls were set up as a sterility control (containing culture medium only) and a growth control (containing culture medium plus Dimethyl Sulfoxide). Each test well as well as the growth control well were inoculated with 5 µl of a microbes (Bacteria or Fungi) suspension (10^8 CFU/ well). All the experiments were performed in triplicate. The microdilution plates were incubated at 37°C for 24 h. For detecting any growth, 10 µl loop were taken from each well and inoculated on nutrient agar. After 24h, MIC values were defined as the lowest concentration of the resin extract in nutrient broth, which completely inhibite microbial growth on solid culture media after 24 h of incubation.

Antimicrobial Effects of resin on Polymicrobial cultures

To study the effect of the resin extract on polymicrobial culture, four types of mixed microbial cultures of ATCC standard isolates were prepared: mixture 1 contained *Staphylococcus aureus* and *E.coli*; mixture 2 contained *Staphylococcus aureus* and *Candada albicans*; mixture 3 contained *E.coli* and *Candada albicans*; and mixture 4 contained all three isolates. Sample from each dilution (180 µl) were distributed in 96-well plates. Controls were set up as a sterile control (containing culture broth medium only) and a growth control (containing culture medium plus Dimethyl Sulfoxide). Five µl of each microbe (Bacteria and Fungi) suspension (10^8 CFU) were mixed into the well and then were incubated at 37°C for 24 h. Then a loopful of the cultures of each of the specimens of the mixture was streaked onto appropriate selective solid media agar plates to assess the viability of the isolates. Selective solid media included a mannitol salt agar for *Staphylococcus aureus*, a MacConkey agar medial for *E. coli*, and Sabouraud media for *Candada albicans*. The streaked plates were incubated aerobically at 37°C and inspected after 24 h.

Statistical Analysis

Mean and standard deviation of MIC of the resin against Gram-positive and Gram-negative bacteria and fungi were used. ANOVA and *t* test were used to compare means of MIC of the resin samples to the control; $p < 0.05$ was statistically significant.

Results

Antibiotic sensitivity

Antimicrobial resistance testing showed that all the bacteria were multidrug resistant (**Table 1**).

Antimicrobial activity and Minimum Inhibitory Concentration against single culture

The results showed that *Staphylococcus aureus* ATCC 29212 was the most sensitive microbe to the resin (zone of inhibition= 11.5±0.9), whereas *Aspergillus nidulans* was the least sensitive pathogen (zone of inhibition=4.9±0.7). Other sensitive microbes were *E. coli*

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ATCC 10402 and *Klebsiella pneumonia* ATCC 10031 with zone of inhibition, 10.2±1.9 mm and 9.8±1.2 mm respectively. Cefoxitin antibiotic was used as a control, which has the approximately similar zone of inhibition obtained with the use of resin except for *Staphylococcus aureus* ATCC 29212 (zone of inhibition= 6.8±0.7) and *Salmonella typhimurum* ATCC 3311 (no zone of inhibition). MIC of the extract against *E. coli* ATCC 10402, *Klebsiella pneumonia* ATCC 10031, and *Staphylococcus aureus* ATCC 29212 was 1.25 (µg/mL; w/v) and for the other pathogens was 2.5 (µg/mL; w/v) (Table 2).

Table1: Antibiotics (Drugs) to which the pathogens are resistant

Name of the microbes	Name of the Drugs
Escherichia coli ATCC 10402	LZG, VA 30, E 15, CXM, AMC 30
Klebsiella pneumonia ATCC 10031	VA 30, CXM, AMP 25
Staphylococcus aureus ATCC 29212,	CXM, AMC 30, AMP 25
Pseudomonas aeruginosa ATCC 2785,	F 300, LZG, C 30, VA 30, E 15, CXM
Salmonella typhimurum ATCC 3311	LZG, VA 30, E 15, CXM, AMC 30, FOX 30
Candida albicans ATCC 10231	-
Aspergillus nidulans	-
Ascosphaera apis	-

F 300, nitrofurantoin; LZD, linezolid; C 30, chloramphenicol, VA 30, vancomycin; E 15, erythromycin; CIP, ciprofloxacin; CXM, cefuroxime; AMC30, amoxicillin; FOX30, cefoxitin; AMP25, ampicillin.

Table2: Antimicrobial activity of *D. cinnabari* resin against selected pathogens (Single Culture)

Microorganism	Zone of Inhibition (mm)		MIC (µg/mL)
	Ethanolic Extract (Resin)	Cefoxitin	
E. coli ATCC 10402	10.2±1.9	9.5±0.4 (p>0.05)	1.25
Klebsiella pneumonia ATCC 10031	9.8±1.2	8.8±0.4 (p>0.05)	1.25
Staphylococcus aureus ATCC 29212,	11.5±0.9	6.8±0.7 (p<0.05)	1.25
Pseudomonas aeruginosa ATCC 2785,	7.4±0.6	8.0±0.6 (p>0.05)	2.5
Salmonella typhimurum ATCC 3311	7.8±0.9	-	2.5
Canadada albicans ATCC 10231	6.7±0.5	-	2.5
Aspergillus nidulans	4.9±0.7	-	2.5
Ascosphaera apis	5.8±0.2	-	2.5

Table 3: MIC of resin dissolved in broth toward single and polymicrobial cultures. HG: heavy growth, *** not tested.

Pathogens	Control culture growth	MIC when resin dissolved in broth used in single culture (µg/mL)	MIC when resin dissolved in broth used in polymicrobial cultures (µg/mL)			
			E.coli* + *C. albicans	***E.coli* + *S. aureus***	***C. albicans* + *S. aureus***	***E.coli* + *C. albicans* + *S. aureus***
E. coli	HG	1.25	2.50	2.50	***	2.50
Staphylococcus aureus	HG	1.25	***	2.50	1.25	1.25
Candada albicans	HG	2.50	2.50	***	2.50	2.50

Effect of resin on Polymicrobial Culture

Various concentrations of resin inhibited the four kinds of mixed microbial growth (Table 3). The grade of the growth of each microorganism cultured together did not differ considerably from the grade of growth of each microorganism cultured alone in the broth. However, minor increase was noticed in the growth of *E. coli* when cultured with *Candada albicans*; *Staphylococcus aureus* when cultured with *E. coli*; and *E. coli* when cultured along with *Staphylococcus aureus* and *Candada albicans* (Table 3). MIC of the resin increased for *E. coli* when cultured with *Staphylococcus aureus* and MIC of the resin for *Staphylococcus aureus* increased when *Staphylococcus aureus* was cultured with *E. coli*. No changes were observed in MIC of the resin in case of *Candada albicans* when it was cultured with other microorganisms.

Discussion

The present work was designed to evaluate the potential antimicrobial activity of *D. cinnabari* resin in the core of searching a novel

therapeutic agent. The main findings are: 1) *D. cinnabari* resin prevents growth of a single pathogenic microbe inoculated separately in broth containing resin, 2) resin prevents growth of mixed isolates inoculated together in broth containing resin, 3) polymicrobial culture with resin affects growth of each isolate except for *Candada albicans*, and 4) the most sensitive microorganism was *Staphylococcus aureus* when the isolates were cultured separately in broth media and with others when the isolates were cultured together. This is the first study to report the effect of *D. cinnabari* resin on polymicrobial culture of human multi-drug resistant pathogens. Resin inhibits growth of *Candada albicans*, which is supported by an earlier study showing that *D. cinnabari* resin could inhibit the growth of *Candada albicans* (Gupta & Gupta, 2011).

In one study by Yehia et al., the zone of inhibition of ethanolic extract of *D. cinnabari* against *Staphylococcus aureus* was 16±1.5 mm and against *E. coli* was 19±0.6 respectively, while *Pseudomonas auriginosa* was the most resistant bacterium (Yehia et al., 2013). In another study conducted in India, it was found that *Candada albicans* was the most resistant pathogen to the methanolic extract of the resin whereas *Staphylococcus aureus* (zone of inhibition= 10.33 ± 0.58), and *E. coli* (zone of inhibition= 9.0 ± 0.0) were the most sensitive (Gupta & Gupta, 2011). In the present study, *Staphylococcus aureus* ATCC 29212 was the most sensitive microbe (zone of inhibition= 11.5±0.9), whereas *E. coli* ATCC 10402 (zone of inhibition, 10.2±1.9 mm) and *Candada albicans* ATCC 10231(ZOI= 6.7±0.5) were less sensitive. Conclusively, *D. cinnabari* resin prevented growth of both gram positive and gram negative bacteria and *Candada albicans*. This property makes resin a good intervention that required clinical application and testing. Furthermore, our results suggest that the *resin* depresses the *A. apis* growth. It is possible that this resin can be used in an integrated pest management strategies in bee colonies.

The phytochemical constituents of plants such as flavonoids, alkaloids, phenolics and several other aromatic compounds that are secondary metabolites of plants serve as a defence mechanism against pathogens, insects and herbivores (Doughari, 2006). Several biflavonoids, homoisoflavanoids and flavonoids were isolated from *D. cinnabari* (Masaoud et al.1995, Vachálková et al., 1995). The antimicrobial activity of *D. cinnabari* resin might be due to the presence of these compounds (Yehia et al., 2013). However, more studies are needed to explore mechanism of action.

The spread of antibiotic resistance is a global public health problem and a challenging issue (Davies & Davies, 2010). The U.S. Centers for Disease Control and Prevention (CDC, 2000) has described antibiotic resistance as one of the world's most devastating health problem in the 21st century (Arias & Murray; 2009). It is well established that the number of bacteria resistant to antibiotics has increased, and many bacterial infections become resistant to antibiotic treatments. WHO has identified antibiotic resistance as one of the three greatest threats to human health. A recent database revealed the existence of more than 20,000 potential resistance genes (r genes) of nearly 400 different types (Liu & Pop, 2009). The European Commission decided on an unprecedented approach to drive the search for novel antibiotics by integrating pharmaceutical industry, research capacities of universities and small companies supported by public funding along with pricing/reimbursement and regulatory bodies (Theuretzbacher, 2012).

Multi-growth cultures are common finding when cultured specimens were collected from various human specimens including wounds and ulcers. Therefore, attempt to find an agent which can work in such clinical entity is warranted. The study showed that the resin has property to work against cultures with multi-microbial growth including fungi. If this finding is proved in clinical setting, the resin will represent an additional natural intervention for the treatment of single and multiple microbial infection.

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