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Comparison of the antifungal effects of isolates B*atis maritima* extracts against animal pathogens

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Abstract

Batis maritima is a halophytic shrub, one which has historically been used in the making of traditional medicine to treat ailments of microbial origins. Both of these facts suggest that the plant possesses compounds with antimicrobial properties, which may prove helpful against diseases of microbial origins. In the current research, the isolates of the alcoholic extracts of the leaves of *B. maritima* were compared to determine their efficacy as antifungal agents against two animal pathogenic fungi viz., *Aspergillus flavus* and a *Malassezia sp*.

Normal phase gradient column chromatography was utilised to isolate the components of the alcoholic extracts. These were then prepared in 1% solutions and tested for their antifungal properties. The Well-Diffusion method was employed in the antifungal assay.

The separation of the extracts yielded 6 components, of which 4 were tested, labelled C_1, C_2, C_3 and C_0. Of these, C_1, the least polar isolate, was seen to be effective against the two fungi, displaying partial inhibition against both. Partial inhibition was observed in C_2 and C_0 against the *Malassezia sp.* only. However, no inhibition was noted from C_3 against any of the fungi, showing no activity against either.

From the results of the study it may be seen that certain isolates of *B. maritima* display antifungal activity against the pathogens tested, especially the *Malassezia sp*. Thus, it is recommended that further research be carried out on these isolates, particularly C_1, and the possibility of their incorporation into treatments against the fungi and their associated ailments.

Keywords: *Batis maritima*; Saltwort; Crabgrass; *Aspergillus flavus; Malassezia sp*

1. Introduction

Batis maritima is a tropical, succulent shrub [1] that grows in many coastal regions of the world, including southeastern United States, the Caribbean, northern South America and Hawaii [2]. It is a halophyte, withstanding high levels of salinity, and, as a shrub, usually grows to a height of 1 to 4 ft. Its active growth period is during the spring season and flowers by the end of the season. The flowers are green and the leaves are also green and deciduous [3]. *B. maritima* usually grows in salt marshes, and, in Guyana, is commonly found within the salt marshes of the mangrove spread along the Coastal Plain [4]. It is also capable of growing in direct seawater and is able to endure short periods of flooding as well as prolonged water-logged environments [1].

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Historically, *B. maritima* has been used in traditional medicine in the treatment of various ailments. It was used in Puerto Rico to treat ailments such as gout, eczema, psoriasis, rheumatism, blood disorders, and thyroid disorders. It was also noted to have been used in Mexico for the treatment of other cutaneous infections [5] and blood disorders [1].

With respect to its phytochemistry, *B. maritima* has been found to contain a wide range of active metabolites. Alcoholic extracts of the leaves were found to contain glycosides, alkaloids, flavonoids, saponins, sterols, tannins and terpenoids [6]. The presence of these active metabolites, strongly suggests antimicrobial properties, since in many cases these properties are derived from the compounds themselves [7]. Additionally, the seeds of the plant were found to contain carbohydrates, proteins and fats in relatively high percentages (46.5%, 17.3% and 25.0% by mass, respectively) [8].

In terms of its antimicrobial properties, alcoholic extracts of the leaves were tested against two human pathogenic bacteria, *Pseudomonas aeruginosa* and *Streptococcus mutans*, and two animal pathogens, *Vibrio harveyi* and *Vibrio parahaemolyticus* and it was found to be effective against all four bacteria tested with Zones of Inhibition of 12 ± 2 mm for *V. parahaemolyticus*; 10 ± 2 mm for *V. harveyi*; 8.33 ± 1.52 mm for *P. aeruginosa* and 6.66 ± 2.51 against *S. mutans* [9]. Furthermore, alcoholic extracts of the leaves were tested at various concentrations against two animal pathogenic fungi, *Aspergillus flavus* and *Malassezia sp*., and were found to be effective against both. The Zones of Inhibition against *A. flavus* at 10%, 5% and 1% were found to be 55.9 ± 3.40 mm, 52.7 ± 1.72 mm and 47.0 ± 3.68 mm, respectively, while the Zone of Inhibition against *Malassezia sp*. was found to be 25.3 ± 5.44 at 50% [10].

Malassezia sp. is a genus of animal pathogenic fungi which are associated with tinea. There are 14 species within the genus, the more common ones being *globosa* and *cuniculi* species. These fungi cause cutaneous infections (yellowing of nail) and other forms of tinea that may lead into skin diseases and inflammation of the skin [11].

Aspergillus flavus is an opportunistic pathogen to both animals and humans, [12] [13] [14] and is the leading cause of aspergillus's in humans and other animals [12]. *A. flavus* is the main *Aspergillus* species that produces aflatoxins which causes this condition, attacking the skin, sinuses, kidneys and heart of humans and animals [14]. Aflatoxins may be found in contaminated goat, cow and human milk, and all domestic animals are susceptible to this fungus [13].

Since, in the previously mentioned study, the alcoholic extracts were proven to be effective, to a certain degree, against the fungi tested [10], it was therefore surmised that there may be compounds present in the crude extract which may display higher levels of antifungal activities than the crude extract itself. This was likely since the extract was made up of a mixture of multiple compounds found in the leaves of the plant. Some of these compounds could theoretically mask the effects of the active compounds and thus lower their activities. It was therefore, the aim of this study to isolate the compounds within the crude alcoholic extract of the *B. maritima* leaves and, thus, determine the antifungal activities of each of these isolates against *A. flavus* and *Malassezia sp*.

2. Material and method

2.1. Plant Materials and Test Fungi

The plant material utilized in the study was the leaves of the *B. maritima* shrub.

The test fungi used were *A. flavus* and *Malassezia sp*. These were obtained from samples around the University of Guyana Berbice Campus Johns Science Centre. These were cultured and isolated on Potato Dextrose Agar (PDA) plates, and stored for later use.

2.2. Collection of Samples

Batis maritima leaves were collected from various locations along the Corentyne Coast, close by the university campus. These were then taken to the University to be prepared for the study.

2.3. Preparation of Samples and Extraction

Extraction of the sample was done following the method outlined by Sivanandham [15], with necessary adjustments.

The leaves of the plant were separated from the stems and washed in distilled water. These were the left to oven dry for 5 days at a temperature range of 40 – 50℃. The dried leaves were then ground by a mill, reducing them to a fine powder. The dried, ground leaves was then allowed to soak in 90℃ ethanol at room temperature for 7 days. Re-

extraction was done twice more for an additional 7 days each. After the extraction process, the crude extracts were pooled and concentrated, using a rotary evaporator.

2.4. Separation of Components

Separation of the components (isolates) of the crude extract was done, using a chromatography column loaded with 100-200 mesh silica gel, via normal phase elution [16]. This was achieved using solvent media of hexane, ethyl acetate, acetic acid and methanol, respectively. The fractions obtained were compared using Thin Layer Chromatography (TLC) and pooled based on their retardation factors. The solvents were then removed from each pooled fraction, isolating the individual components of the crude extract.

2.5. Antifungal Assay

For the antifungal assay, each of the isolates were made up to 1% solutions with a 7:3 acetone-water mixture as the solvent.

Each of the test fungi were inoculated in PDA onto 90 mm petri plates. These were then subjected to the Well-Diffusion Assay [17] against each of the 1% isolate solutions as separate treatments. Each test was carried out in triplicates.

3. Results

As a result of the breakdown of the crude alcoholic extract of *B. maritima* leaves, 6 components in total were isolated. These were labelled C_0 to C_5, respectively. C_0 was obtained during the removal of the solvent from the crude extract, while C_1 to C_5 were isolated as a result of the column elution, with C_1 being the least polar and C_5 being the most, due to the separation being carried out via normal phase elution.

Of these isolates, four, viz. C_0 to C_3 were tested for their antifungal efficacy against the test fungi, *A flavus* and a *Malassezia sp*. The results of the assay may be seen on Table 1 and Figure 1.

Table 1 Results of Antifungal Assay against Isolates tested

Test Fungi	Zones of Inhibition of Isolates (mm)				
	C_1	C_2	C_3	C_0	Control
A. flavus	49.7 ±1.06	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Malassezia sp.	20.3 ± 0.91	22.3 ± 2.84	0.0 ± 0.00	23.7 ± 3.02	0.0 ± 0.00

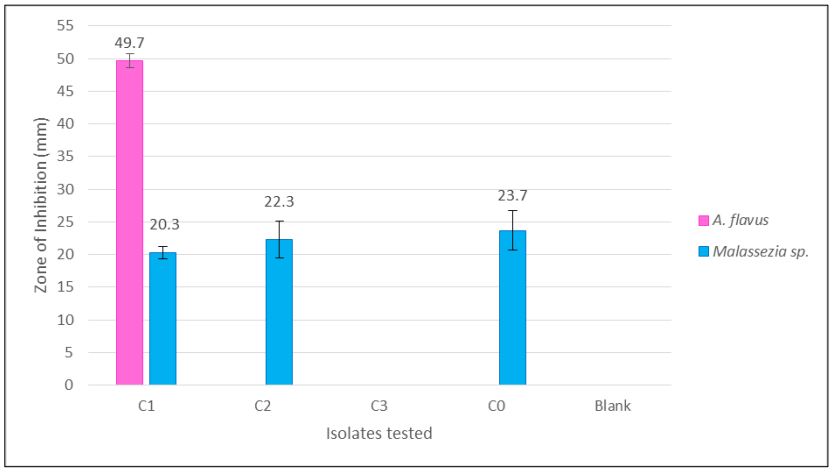

Figure 1 Comparison of Zones of Inhibition of Isolates

From the results of the assay, it may be seen that, with the exception of C_3, all of the isolates tested demonstrated some amount of antifungal activity against one or both of the fungi. For C_1, a ZOI of 49.7 ±1.06 mm was seen against *A. flavus*

and 20.3 ± 0.91 mm against *Malassezia sp*. Furthermore, a ZOI of 22.3 ± 2.84 mm was seen in the treatment of C_2 against *Malassezia sp.*, while 23.7 ± 3.02 mm was noted for C_0 against *Malassezia sp*. C_2 and C_0 proved ineffective against *A. flavus*, while C_3 showed activity against neither of the test fungi. No ZOI was observed from the control treatment.

From the comparison of the antifungal activities of each of the isolates, as seen on Figure 1, it may be noted that there was an obvious difference between the antifungal activity of C_1 and the rest of the isolates with respect to *A. flavus*, as C_1 was the only isolate that was effective against this fungal species. However, with respect to the *Malassezia sp.*, statistically similar activities were seen among the isolates C_1, C_2 and C_0, with probability values of $p = 0.326$ between C_1 and C_2, $p = 0.143$ between C_1 and C_0, and $p = 0.560$ between C_2 and C_0.

4. Discussion

Batis maritima, as mentioned, is a halophyte, growing along the coast of several sub-tropical countries and regions [1], thus, as a halophyte, it was expected to contain several biologically active compounds, a common property among this type of plant [18]. This was corroborated in literature, as *B. maritima* was recorded to have been put to use in several traditional medicines in different regions [1] [5]; plants used in traditional medicines have also been found to contain active compounds demonstrating antibacterial and antifungal properties [19]. Evidence of the antifungal properties of the plant was confirmed in a previous study, where it was seen that the crude alcoholic extract of leaves of *B. maritima* displayed significant activity against *A. flavus* and *Malassezia sp.* [10]. Thus, in this study the alcoholic extract of the leaves of this plant was separated into the individual components and the antifungal activity of these isolates, against the identical fungal species as in the previous study, were compared.

In the current study, a 90% alcoholic crude extract of the leaves of the *B. maritima* was prepared and separated into various fractions, based on polarity, to obtain the individual compounds. This was done via Thin Layer Chromatography (TLC) and Column Chromatography. It should be noted that, upon the removal of most of the extraction solvent from the crude extract, prior to the implementation of the Liquid Chromatography, one of the compounds (denoted C_0) was readily precipitated out of the solution and separated via sedimentation and decantation of the extract. After the separation of C_0, TLC was used to confirm that it was a pure compound.

For the rest of the extract, gradient elution was deemed appropriate for the column chromatography. This method of elution is extremely useful in extracts that contain compounds of varying polarities, where the attraction of each compound to the stationary phase differs greatly [20]. Due to this, it was proven to be ideal for use in the current study since it allowed for better separation of compounds of similar polarity while speeding up the elution of the others [21]. In this way, much solvent was conserved since the solvent was repeatedly altered to facilitate the efficient elution of the next compound.

The gradient elution of the crude extract of the *B. maritima* was done in normal phase, allowing the least polar components to be obtained first. The solvents used were hexane, ethyl acetate, acetic acid and methanol, respectively. From this elution, 5 components were obtained, designated C_1 to C_5, respectively, with C_1 being the least polar compound. Elution was initiated with 19:1 hexane-ethyl acetate. The low polarity of this solvent allowed for a greatly discrete separation of the two compounds. After this, the moderately polar compound, C_3, was eluted with a solvent of 2:1 ethyl acetate-acetic acid. Finally, compounds C_4 and C_5 were eluted with 1:2 acetic acid-methanol, and pure methanol, respectively, attesting to the extremely high polarity of these compounds, corresponding with that of the solvents.

For the antifungal assay, the individual components designated C_0, C_1, C_2 and C_3 were each made up to 1% solutions (m/V), using acetone as the solvent. Isolates C_4 and C_5, the components of highest polarity, were obtained in quantities too small to be quantifiable with the instruments available and therefore could not be accurately made up to specific concentrations.

As may be noted from the results, 2 fungal species were used in the analyses of the antifungal properties of the components: *A. flavus* and *Malassezia sp*. *Malassezia sp.* is noted to be the fungus commonly associated with rash and other skin related disorders [11], similar to the conditions which were historically treated with concoctions of *B. maritima* [5]. It was therefore predicted that, since ailments related to *Malassezia sp.* were successfully treated with the plant extracts, then *B. maritima* would be shown to contain compounds that are active against the fungus. This was also noted in a previous study, as it was seen that crude alcoholic extracts of *B. maritima* leaves significantly hindered the growth of *Malassezia sp.* (in addition to *A. flavus*) [10].

In terms of assessing the activity of the isolates, it could be seen that C_1 was the only one to show any activity against *A. flavus*, with a zone of inhibition of 49.7 ± 1.06 mm, and was therefore the only active compound confirmed in this regard. Upon comparison with previous research it was seen that positive results were also noted in that study, with the 1% crude alcoholic extract displaying a similar ZOI of 47.0 ± 3.68 mm against the same fungi [10], it may be surmised that C_1 is the main (if not the only) active isolate against *A. flavus*, in the extract. However, as C_4 and C_5 were not tested, this may not be definitely confirmed.

With respect to the *Malassezia sp.*, Compounds C_0, C_1 and C_2 also yielded positive results with zones of inhibition of 23.7 ± 3.02 mm, 20.3 ± 0.91 mm and 22.3 ± 2.81 mm respectively. With three of the isolates testing favorably against the fungus, the effectiveness of the crude extract against *Malassezia sp.* may be appreciated.

However, it must be noted that in a previous study, an effect against the *Malassezia sp.* was only seen with a 50% crude extract solution, suggesting the minimum inhibition of the fungus by this solution to be between concentration levels of 10% to 50% [10]. Since isolates show activity at the 1% level while the crude extract only shows activity at 50%, it may be surmised that this may be an instance of antagonism between the active compounds and the others within the crude extracts, where the masking components are more effective at lower concentrations, with the masking compounds being any of C_3, C_4 or C_5.

Nonetheless, this positive activity against the *Malassezia sp.* may explain why *B. maritima* has been documented to have been used in herbal medicine to treat certain skin disorders and rashes [1], as this fungus is responsible for a wide range of rashes [11]. If the herb were to be ground, made into a paste and applied to the rash, as was usually the method of application of various traditional herbal treatments, employed by different cultures throughout the ages, against dermatologic conditions [22], then this may have resulted in a high enough concentration that would have yielded results analogous to the 50% concentration of the extract in the previous study, and which would, therefore, have led to an appropriate concentration for an effective herbal treatment.

In comparing the antifungal properties among the isolates tested, it may be seen that, against the fungi utilised in this study, the isolate which seemed to possess the least antifungal activity was C_3, showing no effectiveness, while C_1 (the least polar of the isolates) appeared to possess the most, providing inhibition against both fungi. Furthermore, in the case where multiple isolates demonstrated activity against the same fungus, it may be seen that there was no significant difference between the ZOI of the three, implying similar efficacy against the fungus in question.

5. Conclusion

From the results of the study it may be seen that the least polar isolate, C_1, displayed activity against both fungi tested, While C_3, of intermediate polarity displayed no activity. Further, in terms of the degree of inhibition, the 3 active isolates, C_1, C_2 and C_0, displayed similar strengths against the susceptible fungus.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest.

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