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Screening for antifungal activity of decoction preparation of yellow seahorse (*Hippocampus kuda)* from Kauswagan, Lanao del Norte, Philippines as used in Traditional Chinese Medicine (TCM) and its implication to conservation

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Key words: Seahorse, *Hippocampus kuda,* decoction, antifungal, conservation**.**

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Abstract

Seahorses are charismatic creatures that are highly vulnerable to changing environment, overfishing and habitat destruction. They have been widely used in Traditional Chinese Medicine (TCM). Nowadays, these are good sources in the treatment for various ailments. However, various studies are also done to investigate its potential in order to alleviate pressure on wild populations. One of the seahorse species found in the Philippines particularly in Mindanao is the yellow seahorse (*Hippocampus kuda).* In TCM, seahorses are usually taken orally using decoction preparation however, the antifungal activity of seahorses (*H. kuda)* using decoction preparation is not yet known, hence this study. Antifungal activity of various concentrations of the decoction preparation of *H. kuda* was investigated against two (2) potentially pathogenic fungal strains: *Aspergillus niger* and *Candida tropicalis* using the Kirby-Bauer (disc diffusion) method. Results show that there was no formation of any inhibition zone as observed after 24-48 hours based on various decoction concentrations examined. This indicates that the decoction preparation was insufficient to unleash the antifungal property of seahorses (*H. kuda*). Herewith, the negative results for antifungal activity help alleviate pressure on wild seahorses to conserve its population.

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Introduction

Since time immemorial, wild marine organisms have been used in the discovery and development of traditional and pharmaceutical drugs used in allopathic medicine. Noteworthy, is that the chemical diversity of isolated compounds reflects the biodiversity of the source organisms which have evolved and adapted to their environment. Seahorses, a known marine teleost fish, believed to have managed to survive a milieu of pathogenic organisms in their natural environment and become one of the important organisms which have been used in Traditional Chinese medicine (TCM). In fact, every year, about 20 million seahorses were collected from the wild to be used for medical purposes (Binh *et al.*, 2016). Traditional medication came into existence lengthy before Western medicine advanced in Europe. Recently, the capability of natural products developed from organisms, and mainly from marine medicinal organism used in traditional medicine has been recognized in the Western world (Qian *et al.,* 2012). Intensive research since 1970s has proved that marine organisms are magnificent sources of bioactive compounds. So far there are 10 species of seahorses in the Philippines (Lourie *et al.*, 1999). In TCM, seahorses are believed to have the capability to cure infertility, baldness, asthma and arthritis. A reliable research work on the biomedical validation of seahorses proved that they have the ability to cure arthritis and its associated inflammation (Kumaravel *et al.,* 2012). Scientific researches further revealed that these organisms exhibit biomedical properties like anti-aging, anti-fatigue, anti-cancer, anti-oxidant and anti-microbial. For this, dried seahorses had been exploited for trading and aquarium around 33 countries worldwide. Alongside, finding sources with antibacterial and antifungal properties, seahorses become important candidates however, the antifungal activity of seahorses are not fully investigated hence, this study.

In this respect, *H. kuda,* become a valuable species in trade for traditional medicine, curios and aquaria (Perry *et al.*, 2010). In Southeast Asia, they are among the most heavily traded seahorse species particularly in the Philippines (Garcia and Garcia, 2009). The call for this species is excessive because of its large length, smooth texture, and pale complexion when dried, which characterize all acceptable traits for traditional medicine purposes. This species is likewise incidentally caught as bycatch in different fisheries and is greatly affected by habitat loss (Giles *et al.*, 2006; Perry *et al.*, 2010; Vincent *et al.*, 2011).

Accordingly, TCM preparations include decoction administered to patients, submerging the whole organism in alcohol as seahorse wine, grinding and mixing them with other ingredients or in powdered form (Lourie, 2016). In most cases, dried whole specimens were allowed to boil in water or pulverized and taken in the form of tea. For decades, TCM preparation has been extensively used.

At present, the natural populations of seahorses had been dramatically reduced as a result of the increasing demand of TCM (Qian *et al.,* 2012), which greatly affects the species' mortality rate. Moreover, if proven that antifungal activity is relatively absent in seahorse preparations based on TCM, it will alleviate pressure on rare seahorse species thus, aide in conservation of the species.

In this study, decoction preparation of *H. kuda* in water, was screened for antifungal activity using Kirby Bauer (disk diffusion) method against two potentially pathogenic fungi: *Aspergillus niger* and *Candida tropicalis*. *A. niger,* is a parasitic fungi that penetrate citrus fruit tissue through micro-wounds and bruises (Ladaniya, 2008) and *C. tropicalis,* is a fungi that mainly causes candidiasis and candidaemia (Ann Chai *et al.,* 2010). These two are common pathogens which posed problems in terms of health. Important findings serve as baseline data for future researches and for tailor-fit conservation strategies.

Materials and methods

Sampling area and sample specimens

Opportunistic sampling was done such as seahorse samples were donated as dead bycatch adult samples from fishermen from the municipality of Kauswagan

in the province of Lanao del Norte (Fig.1). The municipality was bounded by coastal areas thus communities mainly rely on fishing as their mode of living. Identification of samples was done through illustrated keys, Guide to the identification of Seahorses (Lourie *et al*., 2004) and consultation of experts. A total of three (3) *H. kuda* were obtained (1 female and 2 males). The samples collected were placed in zip lock containers and were stored in an icebox for laboratory processing.

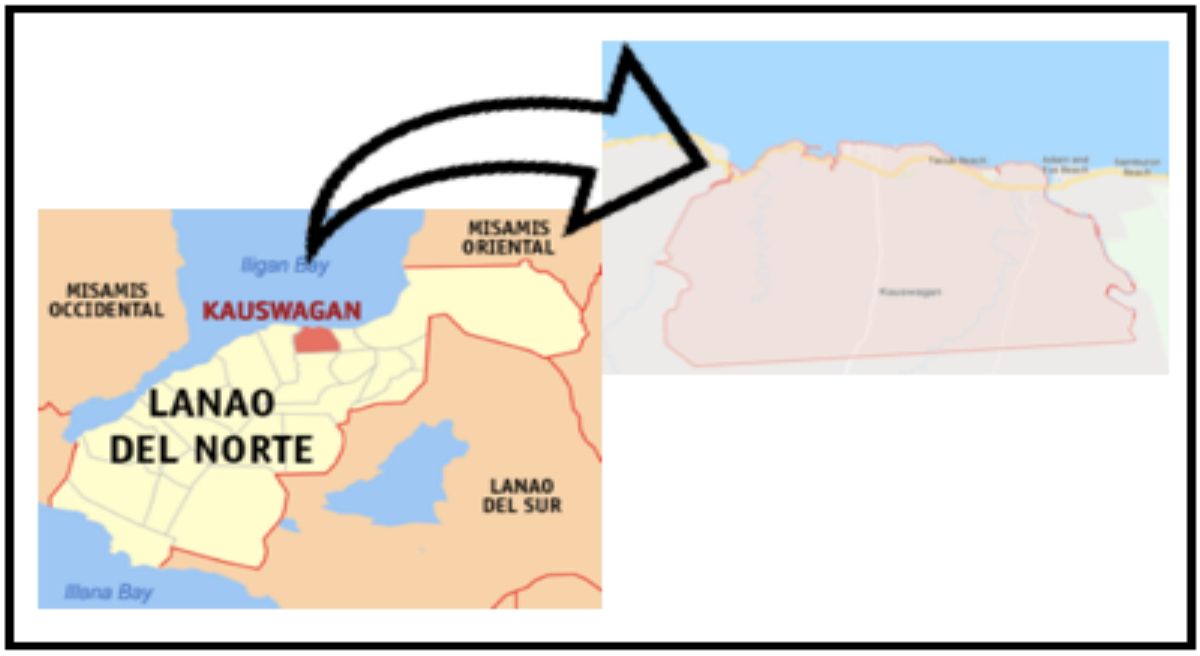

Fig. 1. Map of the sampling area: Kauswagan, Lanao del Norte, Philippines.

Decoction preparation

A total of only three (3) individuals (2 males and 1 female) of *H. kuda* (Fig.2) were used. Sample specimens were washed and air-dried for approximately 20 days. Following a consistent folkloric way in decoction preparation, dried seahorses (*H. kuda)* were pulverized using mortar and pestle. Powdered form was weighed in the analytical balance to come up with the following concentrations: 30%, 50%, 70% and 90% w/v for both male and female *H. kuda*. Desired amount of powder per concentration were placed in desired volume of sterilized distilled water and allowed to boil for 2-5 minutes just like preparing tea as practiced in traditional Chinese medicine. Volumes of respective prepared concentrations were used for the disk diffusion method.

Test microorganisms

Two strains of test organisms were used and obtained from the University of the Philippines-Los Baños: *Aspergillus niger* BIOTECH 3080 and *Candida tropicalis* BIOTECH 2085. These were sub-cultured on appropriate media as prescribed by the BIOTECH, and were incubated overnight at room temperature. A bacterial suspension for each test microorganisms were conducted using 0.5 McFarland Turbidity Standard, roughly $1.5x10^8$ cells/ml. These test organisms were used for the preliminary screening of antifungal activity.

Aseptic technique was done throughout the experiment to avoid contaminations. A sterile cotton swab was dipped into the tubes containing the fungal suspension and seeded unto the surface of the properly labeled Mueller Hinton Agar (MHA) plates. Standard discs for the decoction concentrations and antifungal discs were aseptically placed at standard distance from each other.

Kirby-Bauer disk diffusion test

Fungal test organisms were cultured separately in

Mueller Hinton agar (MHA) media and incubated for 24 hours. After incubation, plates were divided into four quadrants. One quadrant for the positive control, another for the negative control and the remaining two quadrants were used for the different concentrations of decoction preparation.

Kirby-Bauer disk diffusion test was done where Fluconazole, a synthetic triazole antifungal drug, used for the treatment of serious infections caused by fungi was used as the positive control while, sterilized distilled water served as the negative control. A volume of 10µl of various concentrations of decoction preparation: 30%, 50%, 70% and 90% w/v were dispensed in sterilized blank discs of around 6mm in diameter and placed in the agar surface aseptically along with the negative (10 µl) and positive control (10 µl). The assay was performed in triplicates. The resulting plates were incubated at 27-30°C for 24hrs and maximum of 72 hrs. Observations from 24 to 72 hrs were recorded. Photographs were then taken for all the plates examined with the ruler that served as a scale (Bauer *et al.*, 1966; Hudzicki, 2009).

Results and discussion

Antifungal screening of different concentrations of decoction preparation of Hippocampus kuda

Decoction preparation has been used in Traditional Chinese Medicine (TCM) to treat various ailments. In TCM, seahorses are believed to have the capability to cure infertility, baldness, asthma and arthritis.

They are thought to exhibit antimicrobial properties as well. This study screened the antifungal activity of different concentrations of the decoction preparation of *Hippocampus kuda* through Kirby-Bauer method. Table 1 shows the negative results obtained from the antifungal screening of different concentrations of the decoction preparation of *H kuda* against two (2) potentially pathogenic microorganisms *(Aspergillus niger* and *Candida tropicalis)* observed after 24-48 hours.

Table 1. Antifungal screening of different concentrations of decoction preparation of male and female *Hippocampus kuda*.

Sex of the Seahorse	Concentration	Zones of inhibition (mm)	
(*Hippocampus kuda)*	(g/ml H_2O)	*A. niger*	*C. tropicalis*
Female	30%	NI	NI
	50%	NI	NI
	70%	NI	NI
	90%	NI	NI
Male	30%	NI	NI
	50%	NI	NI
	70%	NI	NI
	90%	NI	NI

Using varied concentrations of decoction preparation, the antifungal activity of the male and female *Hippocampus kuda* were tested. The antifungal activity was tested on *Aspergillus niger* and *Candida tropicalis* respectively. Increasing concentrations did not have any effect on the two (2) fungal strains (*A. niger* and *C. tropicalis)* since, the results show no zone of inhibition (Fig. 3 and 4). The sex of the seahorses did not have any influence on the antifungal potential of seahorse (*H. kuda)*. Hence, the result was negative for any antifungal potential.

Apparently, the results obtained were contrary to the study performed by Kumaravel *et. al,* 2010. Antimicrobial activity of five (5) species of seahorse which included *Hippocampus kuda* and was investigated with various extractions using Methanol, chloroform, and n-butanol.

Fig. 2. Dried yellow seahorse (*Hippocampus kuda*), male (left) and female (right).

In the antifungal screening, the results show that extracts are possible to bring antifungal effect against the fungal strains used. However, out of the five species, only the *H. kuda* has given antifungal effect on two (2) out of twenty (20) strains. This shows that the seahorses are extremely strange to the fungal contact and its mediated infection.

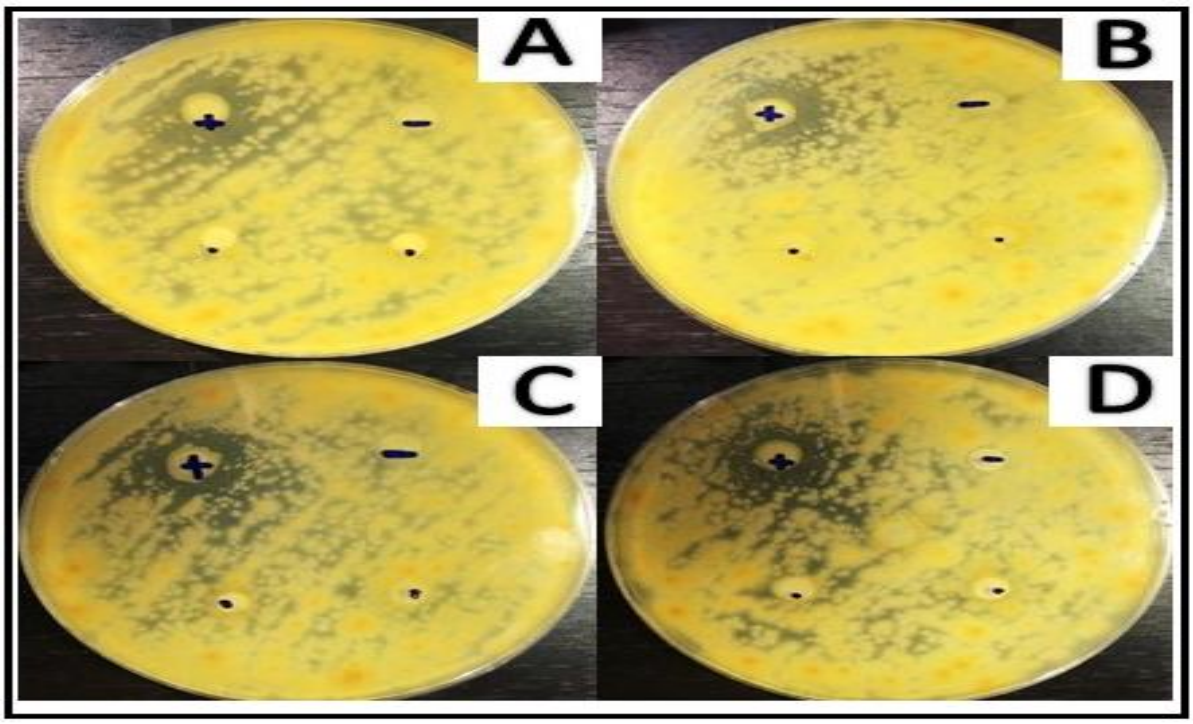

Fig. 3. Test for antifungal activity of different concentrations of decoction preparation of (A-B) male and (C-D) female *H. kuda* on *Aspergillus niger* using disc diffusion method. Varied concentrations of seahorse decoction: (A&C) 30% (bottom left), 50% (bottom right); (B) 70% (bottom left), 90% (bottom right) and (D) 70% (bottom left), 90% (bottom right); No zone of inhibition produced compared to positive control (Fluconazole: positioned on the top leftmost part of the plate); negative control is sterilized distilled water (positioned on the top rightmost part of the plate).

There is growing proof that pathogens have vital impacts on marine systems (Harvell *et al.*, 1993). Fungal pathogens are present in reef communities and emerge once conditions favor them, and when hosts are stressed and their defenses compromised (Kim *et al.,* 2000). Herewith, *H. kuda* species activity was characterized through the methanolic and butanol fraction and these fractions was selected to cover a broad range of polarities and consequently a wide range of the active substances present in the various extracts.

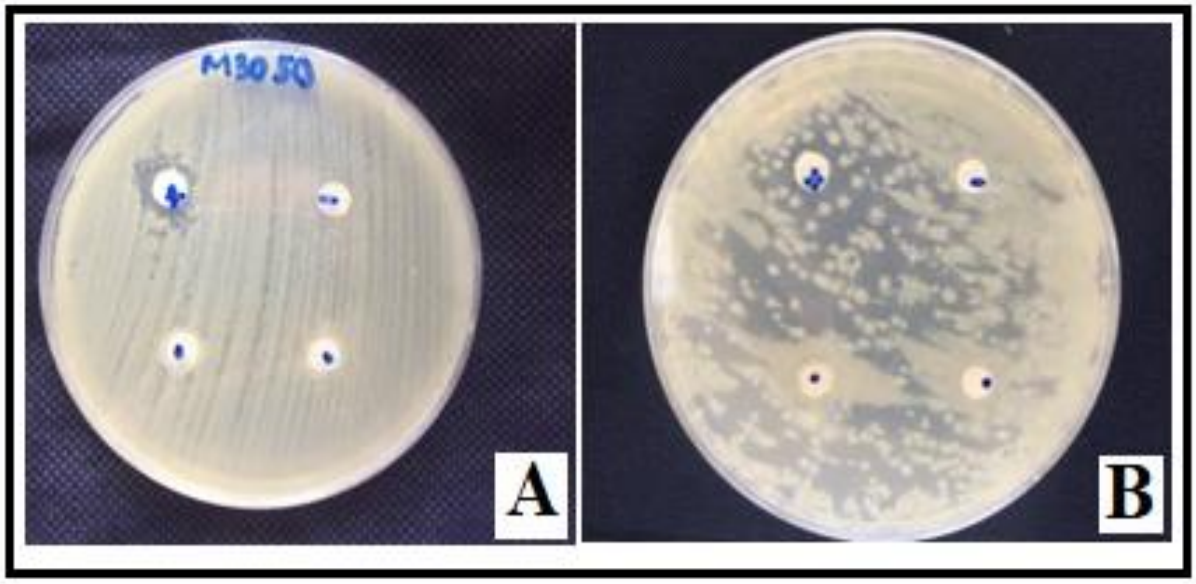

Fig. 4. Test for antifungal activity of different concentrations of decoction preparation of (A-B) male *H. kuda* on *Candida tropicales* using disc diffusion method. Varied concentrations of seahorse decoction: (A) 30% (bottom left), 50% (bottom right); (B) 70% (bottom left), 90% (bottom right); No zone of inhibition produced compared to positive control (Fluconazole: positioned on the top leftmost part of the plate); negative control is sterilized distilled water (positioned on the top rightmost part of the plate).

The study of Kumaravel *et.al,* 2010 were contrary to the results obtained from decoction preparation for the screening of antifungal property of *H. kuda*. It is apparent that the decoction procedure as practiced in Traditional Chinese Medicine (TCM) was insufficient to release the antifungal properties of *H. kuda*.

Moreover, a study by Khattab *et al.*, 2008 on screening for antibacterial and antifungal activities in some selected marine organisms that included seahorses, out of the eight methanolic extracts of the studied marine organisms assayed which included a pathogenic fungal species (*Candida albicans*), only two organisms: soft coral (*Sarcophyton glaucum)* and sea cucumber (*Bohadscia graeffae*) showed clear effect on isolates of *C. albicans* using the well diffusion assay thus possessing antifungal property and the seahorse (*Hippocampus fuscus*) did not exhibit any activity. Hence, this study may also support the negative antifungal activity of seahorses. Furthermore, seahorses are called the jewels of the ocean however, their population is in danger. For a long time, seahorses have been used in the discovery and development of traditional and pharmaceutical drugs used in allopathic medicine (Qian *et. al.,* 2012). In fact, seahorses (*H. kuda*) have been used as one of the most famous and expensive materials of TCM and have been believed to be a treatment to some ailments. Populations are depleting because of growing demand on the market. In this study, the entire organism was used and different concentrations were made for the decoction process. Light was shed regarding the antifungal potential yielding a negative result since various concentrations of decoction preparation was insufficient in unleashing its antifungal activity thus, results obtain

may help in conservation purposes of seahorse (*H. kuda*) populations.

Conclusion

The study was done to screen the antifungal activity of decoction preparation of seahorse (*Hippocampus kuda)* as used in traditional Chinese medicine. Various concentrations of decoction preparation (30%, 50%, 70%, 90% w/v) were tested against two (2) potentially pathogenic strains: *Aspergillus niger* and *Candida tropicalis* using the Kirby-Bauer method. The results obtained were negative. These indicate that the decoction procedure was insufficient to unleash the active compounds and substances present in the seahorse (*H. kuda)* that are responsible for its antifungal properties. Hence, results obtain may help in conservation purposes of seahorse (*H. kuda*) populations in the wild.

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