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Research Article

EFFICACY OF ANTI-MICROFOULING AND TOXICITY FROM RED SEAWEED - *Portieria hornemannii* (Lyngbye) P.C.Silva 1987

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ABSTRACT. Biofouling on drenched structures causes foremost monetary losses in the aquatic system. The point of this work was to screen the phytochemicals and antifouling capability of the different solvent extracts from seaweed *Portieria hornemannii* against fouling bacteria. Our methodology joins *in-vitro* toxicity bioassay, GC-MS and FT-IR analysis were carried out. The main target was to explore the biological activities of this species and to investigate the presence of chemical constituents. Primarily phytochemical analysis deduced the presence of the alkaloids, terpenoids, steroids, tannins, saponins, flavonoids, phenols, coumarins carbohydrates and glycosides. The antibacterial activities were tested against ten biofilm-forming bacteria using hexane, ethyl acetate, acetone and methanol extract of *P. hornemannii*. The methanol extract revealed the highest inhibition zone against *Bacillus flexus* (15.4 mm) and lower inhibition recorded in the acetone extract showed the zone of inhibition against the *Bacillus aryabhattai* (8.5 mm). The toxicity assay was analyzed against *Artemia* nauplii, the 50% inhibitory concentration (LC-50) value of methanol extract was recorded as low toxic (500 µg/ml). The Gas Chromatography-Mass Spectrometry (GC-MS) analysis of methanol fraction had shown the presence of bioactive compounds such as Oxirane, n-Hexadecanoic acid, 25-methyl-methyl ester and Lauroyl peroxide. Fourier Transform-Infrared Spectroscopy (FT-IR) reveals the presence of functional groups in the methanol extract of *P. hornemannii*. The present work recommended that the methanol extract of *P.hornemannii* might be further explored for testing biological activities after the isolation of individual components. The mixture and various combinations of these chemicals may hint at actual potent agents which may be novel against vast varieties of biofilm creatures.

Keywords: *FT-IR, GC-MS, phytochemicals, seaweed, toxicity analysis*

INTRODUCTION

In an aquatic environment, bio-fouling is a major problem on submerged man-made structures such as fishing nets, engineering structure underwater pipelines, ship's vessels and hulls [1-3]. Biofouling accumulation in marine environs is made up of thousands of marine creatures including bacteria, fungi, phytoplankton, polychaetes, algae, barnacles, mollusks and ascidians [4, 5]. These fouling communities are very complex and socially organized processes [6]. These are natural processes that can have a significant environmental and economic impact on maritime. For example, corrosion, distortion and alteration of the immersed surface, increased drag leading to the reduction in watercrafts speed and increases the fuel consumption more than 45% [7-9]. The incremental fuel consumption leads to arouses the emission of carbon dioxide, sulfur oxide and nitrogen

oxide [10]. In the aquaculture and coastal industries, hundreds of millions to billions of dollars are spent annually on the anti-fouling process [6].

Tributyltin (TBT) containing antifouling paint was widely used in commercial vessels to control biofouling [11]. However, the use of TBT caused an environmental problem as it is more toxic to non-target marine organisms at low concentrations [12-14]. Because of the environ concern over the utilization of TBT, the International Maritime Organization (IMO) and Marine Environment Protection Committee (MEPC) prohibited the use of TBT for maritime solicitations from January 2008 [15, 16]. In addition, many other compounds are also commonly used as antifouling biocides like irgarol-1051 [17], sea-nine211, chlorothaloril [18], copper pyrithione, diuron, chlorothalonil [19], zinc pyrithione [20], tralopyil, triphenylborane, pyridine, capsaicin [21], maneb, thiram and zineb [22].

Natural products are suggested as an alternative to toxic biocides in antifouling paints for controlling biofouling [23]. Current research to control biofouling is focused on producing non-toxic, effective antifouling natural agents to replace toxic synthetic molecules [24]. In biofouling research, many natural extracts of terrestrial and marine sources have been tested as replacements for toxic antifoulants [25, 26]. Among these investigations, marine natural products have been highlighted as promising environmental friendly antifoulants [27]. Many organisms including marine seaweeds, sponges, corals and ascidians are believed to produce rich natural antifouling substances that could get rid of fouling organisms [28-33].

Among all marine organisms, the marine macroalgae are the most important living resources of the ocean with containing more bioactive compounds such as amino acids, terpenoids, phlorotannins, steroids, phenolic compounds, halogenated ketones and alkanes [34-41]. In this context, the present study was aimed to perform the anti-fouling activity of solvents extracts of *Porteria hornemannii* (Lyngbye) P.C. Silva to find out potential active antifouling agents.

MATERIALS AND METHODS

Microorganisms

Biofouling bacteria such as *Bacillus cohnii*-MT071882 (MBF1), *Bhargavaea cecembensis*-MT071889 (MBF2), *Virgibacillus dokdonensis*-MT071890 (MBF3), *Bacillus idriensis*-MT071893 (MBF4), *Fictibacillus phosphorivorans*-MT071896 (MBF5), *Bacillus sp*.-MT075845 (MBF6), *Bacillus aryabhattai*-MT075846 (MBF7), *Bacillus flexus*-MT075851 (MBF8), *Bacillus megaterium*-MT075850 (MBF9) and *Alcaligenes faecalis*-MT071952 (MBF10) were utilised for this study.

Seaweed sample collection

The seaweed of *Portieria hornemannii,* was collected from the Gulf of Mannar, region (9.284 N, 79.178 E). The collected seaweed sample was cleaned using the running tap water followed by double distilled water to remove the unwanted biota. This sample was dried at room temperature. Finally, this seaweed sample was ground as fine particles.

Preparation of seaweed extraction

Seaweed powder was subjected to sequential extraction with different solvents such as hexane, ethyl acetate, acetone and methanol at the ratio of 1:5 by the maceration method.

The seaweed extract was filtered by the Whatman No.1 filter paper and stored at -20°C for further examination [42].

Phytochemical screening

The crude extracts phytochemicals analysis by a standard method to determine the presence of alkaloids, terpenoids, steroids, tannins, saponins, flavonoids, phenols, coumarins carbohydrates and glycosides [43-45]. The formation of precipitate or any changes of colors is a positive reaction to these investigates.

Test for Alkaloids: For alkaloid detection, 2 mL of concentrated HCl was added to 2 mL algal extract. After that, a few drops of Mayer's reagent were added. The presence of alkaloids is indicated by the presence of green or white precipitate.

Test for Terpenoids: Chloroform (2 mL) and concentrated Sulphuric acid were added to 0.5 mL of algal extract for terpenoids identification. The presence of terpenoids is shown by the formation of a reddish-brown hue at the contact.

Test for steroids: For identify the steroids, 0.5 mL of the algal extract was mixed with 2 mL of chloroform and 1 ml sulphuric acid (H_2SO_4). Steroids are detected by the appearance of a reddish-brown ring at the interface.

Test for tannins: 1 mL of algal extract mixed with 1 mL ferric chloride (5 %). The presence of tannins is indicated by the formation of a dark blue or greenish-black color.

Test for saponins: 2 mL distilled water was added to 2 mL algal extract and agitated for 15 minutes lengthwise in a graduated cylinder. The presence of saponins is indicated by the formation of a 1 cm layer of foam.

Test for flavonoids: 2 mL algal extract was mixed with 1 mL 2N sodium hydroxide (NaOH). The presence of flavonoids was indicated by the formation of a yellow color.

Test for phenol: 1 mL of algal extract was mixed with 2 mL of distilled water and a few drops of 10% ferric chloride. The presence of phenols is indicated by the formation of a blue/green color.

Test for coumarins: 1 mL of 10% NaOH was added to 1 mL of algal extract for coumarin identification. The presence of coumarins is indicated by the formation of a yellow color.

Test for carbohydrate: In a test tube, add 5 mL Fehling's solution with 2 mL seaweed extract and bring to a boil. The presence of carbohydrates in a sample is indicated by the formation of a yellow or brownish-red cuprous oxide precipitate.

Test for glycosides: 3 mL chloroform and 10% ammonium solution were added to 2 mL algal extract for glycosides detection. The presence of glycosides is indicated by the formation of pink color.

In-vitro antibacterial activity

The Antifouling activity was evaluated by the agar well technique in petri dishes by using Zobell Marine Agar (ZMA). Bacterial isolates were spread on ZMA plates with sterile effusion and extracts (100 µl) were loaded. These plates were placed on an incubator at 37°C for 24 h. After incubation clear zone around a well was proof of antimicrobial activity. Diameters of the zones of inhibition were evaluated in millimeter. Each test was repeated in triplicate [46].

Toxicity assay

The toxic activity assay method was followed by Ravichandran et al., 2018, different concentration of *P.hornemannii* seaweed extract was treated with *Artemia* nauplii in the 24 well plates that contain the filtered marine water and matured *Artemia* nauplii (50 numbers). In this lethality assay, triplicate was maintained. After 24 hours the survival rate of *Artemia* nauplii was recorded [47].

Percentage of *Artemia* survival =No. of live *Artemia*÷ Initial number of live *Artemia*×100

Gas Chromatography-Mass spectrometry [GC-MS]

Gas Chromatography-Mass spectrometry (GC-MS) investigation was carried with the Perkin Gas Chromatography-Mass spectrum analyser. The program of the GC was maintained at the temperature of 60ºC with a hold for 5 minutes, followed by a gradual increase rate to 300ºC at 5ºC per minute for 15 minutes. Then 1 µl of sample was injected in the split mode at the ratio of 10:1. The compounds were recognized based on the retention time on the capillary column and matching them with the NIST library database.

Fourier Transform Infrared Spectroscopy [FT-IR]

The Fourier Transform Infrared Spectroscopy (FT-IR, Shimadzu, IR Affinity1 Japan), analysis used to detecting the functional groups (Chemical bonds). The extract powder was compressed with KBr pellet (ratio of 1:10). Then the prepared sample was placed in FTIR Spectrum with a scan range from 400 to 4000 cm^{-1}.

RESULTS AND DISCUSSION

Phytochemical Analysis

In the current investigation, qualitative phytochemical analysis was conducted in *P.hornemannii* with hexane, ethyl acetate, acetone and methanol. The outcomes exposed the profile of metabolites such as alkaloids, terpenoids, steroids, tannins, saponins, flavonoids, phenols, coumarins carbohydrates and glycosides. Among the ten extracts, hexane extract indicated the existence of five phytoconstituents such as terpenoids, tannins, flavonoids and phenols and glycosides. The ethyl acetate extract exhibited the presence of seven compounds such as alkaloids, terpenoids, tannins, saponins, phenols, coumarins and glycosides. Acetone extract showed six compounds without tannins, phenols, carbohydrates and glycosides. The methanolic extract showed the seven compounds such as terpenoids, tannins, saponins, flavonoids, phenols, coumarins, and carbohydrates (Table 1).

Table 1. *Phytochemical analysis of Portieria hornemannii using different solvent extract [M-Methanol, H- hexane, EA-Ethyl Acetate, A-Acetone, present [+], absent [-]*

Sample. No	Phytochemicals	Solvents			
		M	H	EA	A
1.	Alkaloids	+	-	+	+
2.	Terpenoids	-	+	+	+
3.	Steroids	-	-	-	+
4.	Tannins	+	+	+	-
5.	Saponins	-	-	+	+
6.	Flavonoids	+	+	-	+
7.	Phenols	+	+	+	-
8.	Coumarins	+	-	+	+
9.	Carbohydrates	+	-	-	-
10.	Glycosides	+	+	+	-

Seaweeds are known to contain rich phytochemicals include alkaloids, glycosides, saponins, tannins and steroids related active metabolites which are of great medicinal and economical value [48-51]. Many reports discovered the presence of phytochemicals in marine algae have been investigated for their antimicrobial activity [52-54]. Similarly, Prabhakaran et al., 2012 also reported the phytochemical of the seaweed must potential of anti-biofilm activity [55].

In-vitro bacterial Activity

The bactericidal efficacy of hexane, ethyl acetate, acetone and methanol extract of *Portieria hornemannii* were investigated against biofilm forming bacteria (Fig. 1). Among the four extracts, Hexane, showed maximal bactericidal efficacy against the MBF1 strain (13.5 mm) and MBF2 strain (13 mm) and minimal zone of clearance was noted for BF5 strain (8.9 mm). Ethyl acetate extract, showed maximal antibacterial efficiency against the MBF1 strain (13.1 mm) and MBF3 strain (12.9 mm), minimal inhibition was noted for MBF9 strain (8.5 mm). Acetone extract, showed maximal antibacterial activity against MBF3 (13 mm) and MBF5 (12mm), minimum zone of inhibition was observed against MBF7 (8.3 mm). Finally, the Methanol extract presented the maximal antibacterial activity against MBF8 (15.4 mm) and minimum zone of inhibition was observed against MBF4 (9.9 mm).

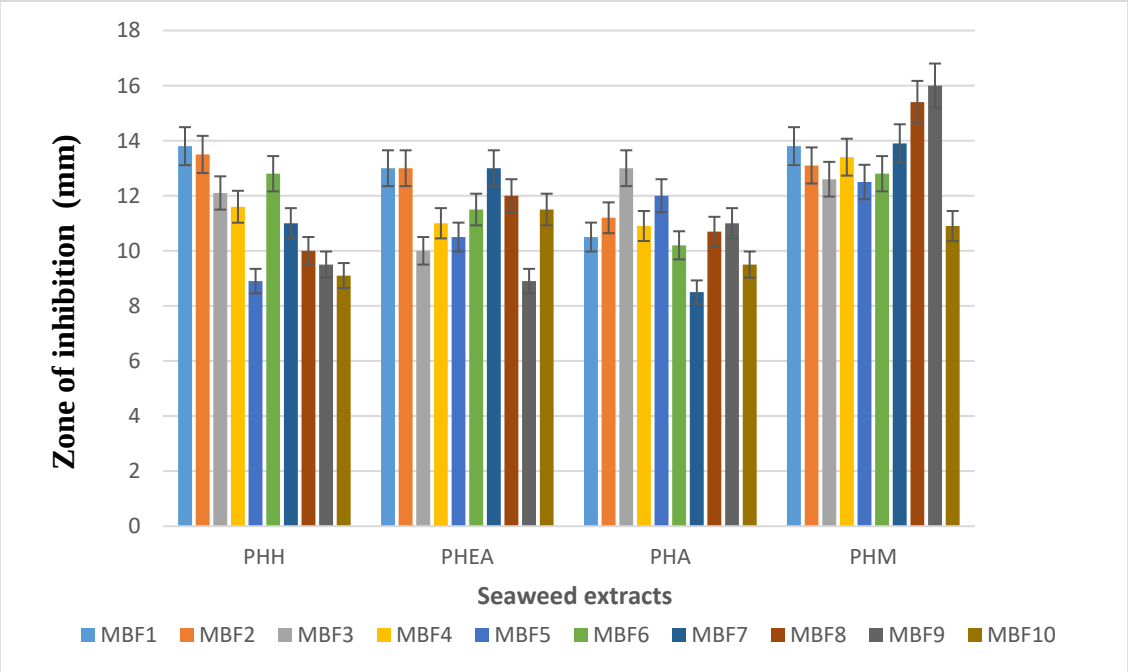


Fig.1. *In-vitro antibacterial potential of different solvent extract of P. hornemannii [PHH- Hexane extract of P. hornemannii, PHEA- Ethyl Acetate extract of P. hornemannii, PHA- Acetone extract of P. hornemannii, PHM-Methanol extract of P. hornemannii].*

In general, seaweed species belonging to Rhodophyceae have a significant antibacterial activity [56]. As the result of the bacterial growth inhibition assay, methanol extract display potential efficacy against all gram-positive bacteria, which is also correlated with previous reports [57-59]. Similarly, Sasidharan., *et al*., 2009 reported that higher antimicrobial activities using methanol extraction yielded seaweed extract [60]. However, the red seaweed of methanol extract was unable to exhibit efficacy against *Bacillus* sp (0mm) [61]. Nevertheless, in this study, methanol extract of red seaweed of *P.hornemannii* exhibits good bactericidal efficacy against *Bacillus* sps (>8mm).

In vitro toxicity assay

In the cytotoxic activity assay, different solvent extracts of *P. hornemannii* seaweed was tested using *Artemia* nauplii (50 numbers). In the control group, no mortality was recorded. At a similar time the percentage of mortality rates against *Artemia* using hexane, ethyl acetate, acetone and methanol solvent at different concentrations (1, 10, 100, 500, 750 and 1000 µg mL^{-1}). The LC_{50} value was registered at more than 500 µg mL^{-1} (Fig. 2). This experiential lethality analysis point out the existence of effective toxicity. Based on the outcomes, the hexane, ethyl acetate, acetone and methanol extract of *Portieria hornemannii* are low toxic.

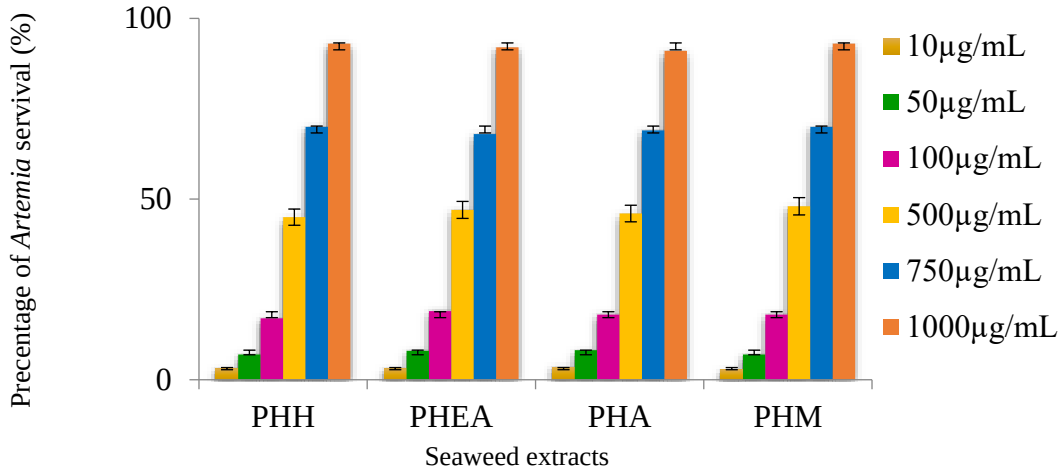


Fig.2. *Toxicity analysis using different solvent extracts of P. hornemannii seaweed was tested using Artemia nauplii.*

Different model systems such as fathead minnows, zebrafish embryos, copepod, Daphnia and rainbow trout have been reported [62]. In addition, this is essential to establish better, rapid and convenient methods to predict the toxic effects of biomolecules on biological organisms. Till now, a few investigations had been described the toxicity effect of seaweed on *Artemia* [63,64].

Finally, evidence of antimicrobial efficiency against a wide range of biofouling bacteria from the methanol extracts obtained from the *P. hornemannii*, along with the lack of toxic effects against *Artemia* sp.

Fourier Transform Infrared Spectroscopy

In the present study, FT-IR is used to identify the functional group in the seaweed (Fig 3 and Table 2).They showed the presence of prominent peaks at 3504 cm^{-1}, 1767 cm^{-1}, 1532 cm^{-1}, 903 cm^{-1}, 839 cm^{-1}, 708 cm^{-1} and 618 cm^{-1}. The peak at 3504 cm^{-1} represents the O-H stretching vibration of hydroxyl groups. The spectral peak at 1767 cm^{-1} was assigned to C=O stretching of a carboxyl group. The peak at 1532 cm^{-1} was assigned to C=C stretching vibration on the aromatic group. The peak 839 cm^{-1} was response for C-H of aromatic ring. The signal 708 cm^{-1} belongs to C=Cl stretching vibration of alkyl halides and 618 cm^{-1} corresponding for C-Br stretching of alkyl halides (65-67)

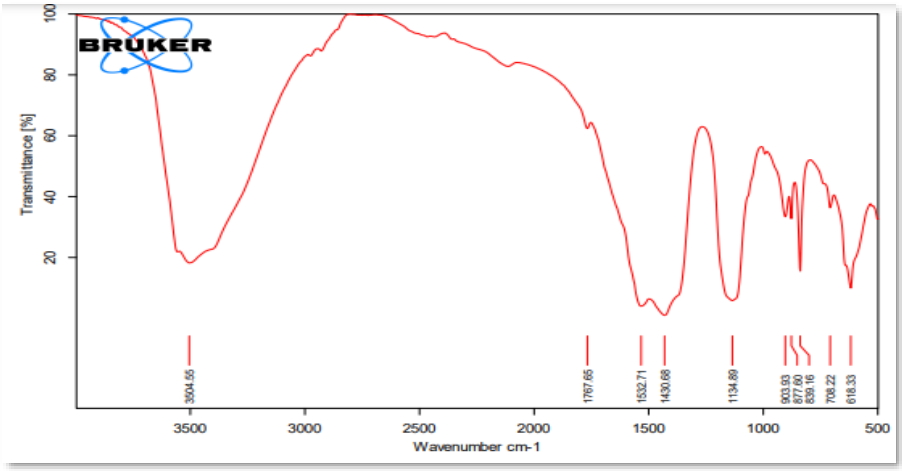


***Fig. 3**. FTIR analysis of methanolic extract of Portieria hornemannii*

***Table 2.** Functional group of methanol extract of Portieria hornemannii obtained through FTIR.*

S. No	Wave Number	Bond	Functional Group
1.	3504	OH Stretch	H-bond
2.	1767	C=O Stretch	Carboxyl
3.	1532	C=C Stretch	Aromatic
4.	839	C-H	Aromatic
5.	708	C-Cl Stretch	Alkyl halides
6.	618	C-Br Stretch	Alkyl halides

Gas Chromatography-Mass Spectrometry

The presence of bioactive compounds in the methanol extract of *P. hornemannii* was identified through the GC MS [Fig. 3 and Table 2].

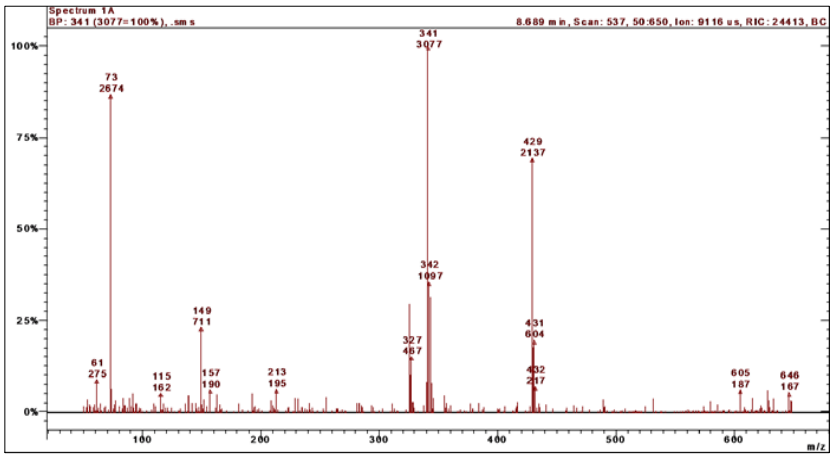


***Fig. 3.** GC-MS analysis of methanol extract of P. hornemannii*

Table 2. *Chemical constituents of methanol extract of Portieria hornemannii obtained through GC-MS*

S.No	Retention time	Name of the compound	Molecular formula	Peak area%
1.	3.4	Oxirane	$C_{19}H_{38}O_2$	24.2
2.	9.82	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	99.9
3.	10.1	Heptadecanoic acid, 25-methyl-methyl ester	$C_{29}H_{58}O_2$	42.8
4.	12.3	Lauroyl peroxide	$C_{24}H_{46}O_2$	67.3

In this study, Oxirane, n-Hexadecanoic acid, Heptacosanic acid, 25-methyl-methyl ester and Lauroyl peroxide are present in the methanol extract of *P. hornemannii*. Previously reported the antibacterial activities of seaweeds were reported because of the presence of bioactive compounds [68,69]. Oxirane was well reported in the red seaweeds with good biological efficacy and evaluated the antimicrobial activity of synthesised oxirane [70]. Oxirane and n-Hexadecanoic acid were revealed superior inhibition activity against microbes and good antioxidant properties [71,72].

CONCLUSION

In summary, this study demonstrated that hexane, ethyl acetate, acetone and methanol extract of *P. hornemannii* have numerous phytochemicals. Nevertheless, it has defensive effects against biofilm-forming bacteria. Thus, this marine alga might be a proper source of potential bioactive compounds. Moreover, this study concludes that the methanol extract has a potential antibacterial efficacy with low toxic effects. Therefore, advanced studies should be performed on the isolation and identification of the potential bioactive compounds. So they may be utilized in antifouling paint formulation.

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Conflict of Interest. The authors declare no potential conflicts of interest with regard to this research, authorship, and/or publication of this article.

Authorship Contributions. K.S., Data collection, analysis and interpretation; P.A., Literature search and corrections.

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