
Isolation of N- Methyl Formamide from Red Algae *Portieria hornemannii* (Lyngbye) P.Silva Active against Two Plant Pathogenic Bacteria

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Abstract: In the present investigation, a purified compound exhibiting antibacterial activity isolated in 1:1 (v/v) chloroform: methanol of red alga *Portieria hornemannii* collected along the coast of Pamban, Rameshwaram (Gulf of Mannar), Tamilnadu, India during February 2007 was identified based on the HPLC,IR, NMR(1H &13C) spectral data. This compound is colorless crystalline nature and yield of purified compound was 53.3mg-1 Kg shade dried alga 0.00533% dry alga. The purified compound of 50 µg showed 14.3±0.56mm and 15.5±0.25mm diameter zone of inhibition, whereas 100µg of commercial antibiotic Streptomycin sulphate showed 37.6±0.56mm diameter zone of inhibition against the test bacterium *Xanthomonas axonopodis* pv *citri* and *Xanthomonas campestris* pv *malvacearum* through agar diffusion technique.

Keywords: Seaweed- *Portieria hornemannii*, N-methylformamide isolation, Spectral data, antibacterial activity against plant pathogens.

1. INTRODUCTION

Xanthomonas is a genus that includes numerous phytopathogenic species, characterized by a narrow range of hosts. However, members of this genus are able to infect a wide variety of plants, distributed among the monocotyledonous and dicotyledonous plant species of worldwide. Angular leaf spot caused by *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*) [*Sin. X. campestris* pv.*malvacearum* (*Smith*) *Dye*]] is one of the most important diseases of tetraploid cotton (*Gossypium hirsutum* L. and *G.barbadense* L.) in many countries, including Brazil. The disease can cause heavy yield losses depending on the year and the cultivar. Vehicle of *Xam* and a source of primary inoculum Cotton seed are considered to be an important severe losses in commercial cotton fields in India, Brazil, Sudan and in the USA.

Citrus canker is a contagious disease of citrus (and some other plant species of the Rutaceae family) caused by the bacteria *Xanthomonas axonopodis* pathovar *citri*. Infected trees display unsightly lesions which can form on leaves, fruit and stems. Trees infected with the disease may suffer from low vigour and a reduction in fruit quality and quantity. Citrus canker is a serious disease impacting on citrus production in India, Pakistan, Sri Lanka, Brazil, USA, and is the subject of a number of control and eradication programs around the world. Experiences with this disease in other countries suggest that yield losses will range from five to 30 percent, depending upon the scion (variety).

Intensive application of synthetic pesticides in agriculture caused damage to the ecological state of the agricultural system (Abetz and young 1983) whereas, pesticides of biological origin are less toxic, generally affect only the target pest and closely related organisms, effective in very small quantities and decompose quickly, thereby resulting in lower exposures and largely avoiding the pollution problems caused by conventional pesticides. Though literature speaks diverse studies of bioactivity of seaweeds, works on testing the antibacterial efficacy of seaweeds against plant pathogens are comparatively a new concept and not much attempt have been made earlier in this line (Kulik,1995;Ara et al;1998 ,1999,2002ab, Arunkumar et al;2005;Kkumar et al;2008). Compared to

the search for new pharmaceutical compounds, very little effort has been devoted to the exploration of agrochemical compounds from marine natural products (Fenical, 1997). Hence in the present invitro study we used two Plant Pathogens against isolated N-Methyl formamide from the red algae *Portieria hornemannii* as biocontrol.

2. MATERIALS AND METHODS

2.1. Isolation of Plant Pathogenic Bacteria

2.1.1. *Xanthomonas axonopodis* pv. *citri* (Hasse) Vauterin et al. (syn. *X. citri* pv. *citri* Gabriel et al., 1989):

The *X. axonopodis* pv. *citri* causing the disease called citrus canker on leaf and fruit of citrus. The infected leaf showing canker spot collected from the field using clean polythene bag brought to the laboratory. The leaf was surface sterilized using 0.1% HgCl₂ for 1 min. and washed with sterilized distilled H₂O at least thrice. Then the leaf was cut into small pieces and soaked in 5 ml of sterilized distilled H₂O for 12 hrs for exudation of bacterium. The bacterial exudates were streaked on peptone-sucrose agar (PSA) medium using the inoculation loop and incubated at 28°C. **Composition of PSA medium** (Jin et al., 2001) Peptone - 5.0 g, Na₂HPO₄ .12H₂O- 2.0 g, Ca(NO₃)₂.4H₂O- 0.5 g, Sucrose- 15 g, Agar- 20 g, Distilled H₂O- 1 litre, pH- 6.8.

2.1.2. *Xanthomonas campestris* pv. *malvacearum* (Smith 1901) Dye 1978b

The *X. c.* pv. *malvacearum* causing angular spot disease (pink color in appearance) on the leaf and balls of cotton. The infected leaf showing the symptom collected from the field using clean polythene bag was brought to the laboratory. The leaf was surface sterilized using 0.1% HgCl₂ for 1 min. Then the leaf was cut into small pieces and soaked in 5 ml sterile distilled H₂O for 12 h for bacterial exudation. The bacterial exudates were streaked on Yeast Peptone Sucrose Agar (YPSA) medium and incubated at 28°C. Composition of YPSA medium (Schaad et al, 1988), Yeast extract- 5g, Peptone- 10g, Sucrose- 20g, Agar- 20g, Distilled H₂O- 1 liter, pH- 7.4.

Colonies of both bacteria appeared on the media were subsequently sub-cultured to isolate pure single colony. Observations were made at every 12 h for 3 days. Pure bacterial colonies were transferred to slants containing its specific media and incubated at 28 °C for 48 h and stored at 4 °C until further work.

2.2. Pathogenicity Test

The final step in the identification of a bacterial plant pathogen was the pathogenicity test. In this step, the isolated bacteria were put back into the host plant to check if it is pathogenic and produces symptoms identical to the ones observed in the original diseased plant sample. To carry out the pathogenicity test, susceptible seedlings of three months old citrus and one month old cotton were raised in pots with garden soil, sand and farmyard manure with the ratio of 3:1:1 v/v/v in botanical garden. Both the plant pathogens *Xanthomonas axonopodis* pv. *citri* and *Xanthomonas campestris* pv. *malvacearum* causing canker in citrus and angular spot in cotton, respectively identified based on colony morphology, Gram's staining and biochemical characteristics isolated from the diseased parts of the host plants were grown on their specific medium at 28 °C for 48 h harvested, re-suspended in sterilized distilled H₂O and adjusted to 0.3 O.D at 460nm (10⁷ -10⁸ cfu/ml bacterial cell concentration) using spectrophotometer. An aliquot of 3ml of the each bacterial suspension was inoculated by spraying on the adaxial side of punctured first three innermost leaves of citrus as well as cotton with their respective pathogenic bacteria with 3 replications. Sterilized distilled H₂O was also inoculated as negative control for each plant. Development of symptoms as a result of infection was observed in every week till the appearance of typical disease. From the infected parts of citrus and cotton, pathogenic bacteria were re-isolated using PSA medium and confirmed as *Xanthomonas axonopodis* pv. *citri* and *Xanthomonas campestris* pv. *malvacearum*, respectively were cultured and maintained at 28°C until further work. Virulence of both the bacteria was maintained by infecting with their respective host on every 6 months and re-isolated from them.

Antibacterial assay through agar diffusion technique. (Arunkumar and Rengasamy, 2000a).

The antibacterial assay was carried out using the agar diffusion technique with 5.0 mm diameter Whatman #1 paper discs. The assay was carried out on 1.5 % nutrient agar medium. Composition of

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nutrient agar medium: Peptone– 10 g, Beef extract – 10 g, Na Cl– 5 g, Distilled H₂O –1000 ml, agar powder – 15 g, pH -7.0.

Sterile paper discs loaded with 50 µl (100 µg of the substances) of different crude extracts using micropipettes were allowed to dry thoroughly under aseptic condition. Then the discs were impregnated in petri plates of 100 mm diameter containing ca. 20 ml of nutrient agar medium smeared with 0.05 ml of bacterial culture in exponential phase of 1.0 OD at 590 nm and incubated at 28 °C for 48 h. The diameters of the agar clear zones of bacterial inhibition around the disc as a result of diffusion of active substances were measured including disc as mm dia. and recorded for antibacterial activity. For the antibacterial assay, three replicates were maintained for each experiment and mean values expressed. The solvents used for reconstituting the crude extracts loaded on paper discs were treated as control and did not show any inhibition zone. The bioassay for each crude extracts was also conducted in nutrient broth for confirmation and the results were expressed. Antibacterial assay through agar diffusion technique: (Arunkumar and Rengasamy, 2000a)

The antibacterial assay was carried out using agar diffusion technique with 5.0mm diameter Whatmann # 1 paper discs. The assay was carried out on 1.5% nutrient agar medium. Peptone- 10g, beef extract- 10 g ,NaCl – 5g , distilled H₂O- 100 ml, agar powder – 15g , pH-7.0

Sterile paper discs loaded with 50 µl (100µg of the substance) of extracts using micropipettes were allowed to dry thoroughly under aseptic condition. Then the discs were impregnated in petriplates of 10mm diameter containing calcium 20 ml of nutrient agar medium smeared with 0.05ml of bacterial culture in exponential phase of 10 OD at 590nm and incubated at 28^oc for 48h. The diameter of the agar clear zones of bacterial inhibition around the disc as a result of diffusion of active substance were measured including disc as mm dia and recorded for antibacterial activity. For the antibacterial assay, three replicates were maintained for each experimental and mean values expressed. The solvents used for reconstituting the extracts loaded on paper discs were treated as control and did not show any inhibition zone.

Isolating and characterization of N-methyl formamide from red alga *Portieria hornemannii*.

2.3. Extraction

About 4Kg of live, healthy and disease free specimens of red alga *Portieria hornemannii* found along the coast of Pamban, Rameshwaram (Gulf of Mannar), Tamilnadu, India was collected during February 2007. After cleaning extraneous particles, seaweeds sample was cleanly washed with sterilized tap water for 3 times. Then the specimen was air dried under shade for 3 days. Shade dried sample was pulverized and ground as fine powder. The powdered 300g of algal sample was extracted with 700ml chloroform: methanol 1:1 (v/v) in air tight in 1 liter Erlenmeyer conical flask at room temperature in dark for 1 month. Extract was transferred to 500ml open conical flask and filtered using Whatmann no.1 filter paper. Then to the filtrate extract anhydrous MgSO₄ was added and shook well in the separating funnel. The extract was kept for 1-2 months under dark condition without any disturbance in the 500 ml beaker covered with aluminum foil.

After one month, colorless crystals started forming in the bottom of the flask leaving thick brownish liquid layer on the top. Observation on the day's progress noted increasing the number and size of the crystals settled at the bottom.

After 2 months upper liquid layer was decanted and the white crystal stored at 0°C. Sixteen mg of white crystals were obtained and stored in desiccates in the laboratory at room temperature. Antibacterial activity of crystal compound.

A crystal weighing 1mg dissolved in methanol was used for bioassay by agar diffusion technique.

The efficacy of pure compound isolated from the red alga *Portieria hornemannii* was compared with the commercial antibiotics used to control the two bacterial pathogen using agar diffusion technique *in vitro*. Each 50µg of isolated compound and antibiotic were loaded on the disc was used for the study.

2.4. Chemical Characterization

The crystal compound was subjected to several studies in order to characterize the chemical nature. The color of the compound was recorded. The purity of the compound was checked by analytical

HPLC using shimadzo machine (Japan), column:ODS-18column (125mm×4.5mm) as the stationary phase. The element consists of methanol: water (80:20 v/v) at a rate of 1 ml/ minute. Samples were analyzed at a wave length of 254nm with a uv detector. The FT-IR spectrum was recorded on a Bruker-model tensor 27. NMR spectra (¹³C and ¹H) on Bruker machine using CDCl₃. The spectral data of FT-IR and NMR (¹H) of authentic compound was taken for confirmation of the isolated compound.

2.5. Discussion and Summary

2.5.1. Solid state N-Methyl formamide

The isolated white crystalline solid state substance N-Methylformamide is confirmed. (Joshua et al., 1954B., J. M. Adams, 1979., Salah Nasr and Louis Bosio., 1998., J. Perepetuo et al., 1999., Hitoshi Ohtaki et al., 2000., S. Lenza and W.L. Vasconcelos., 2001., R.F. R.K. Khanna et al., 2002., Bulent Caglar et al., 2010., Ayesha Jacobs et al., 2011., Sivaraman., et al., 2012G., F. Hammami et al., 2015.,

2.5.2. FTIR Analysis

Our results of isolated N-Methyl formamide FTIR are in coincidence with that of Alstair J. Lees and Brian P. Straughan, 1979., F. Fillaux and M.H. Baron., 1981., Richard R. Izac et al., 1982., S. Aataka et al., 1984., S.V. Snorek et al., 1989., Don Mc Naughton et al., 1999., B.P. Kelleher et al., 2002., D.N. Sayhyanarayana and E. Ganeshsrinivas., 2003., Xiaofei Ma, Jiugao Yu., 2004., J.R. Brucato et al., 2008, G.J. Branislav Jovic et al., 2010., Dugandzie, Vand E. Kahrovie., 2012., Zhen Yang et al., 2014., F. Hammami et al., 2015., and confirmed.

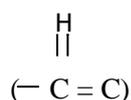
N-methyl formamide (also known as monomethyl formamide) is a clear, colorless liquid with a slight amine odor (Clagett-carr et al., 1988). Finar (1995) reported that except formamide, which is liquid, all the amides are colorless crystalline solids, and those of low molecular weight are soluble in water. In the present investigation, the isolated compound was colorless crystal dissolved in water.

The N-methyl formamide proved to be capable of enhancing the cytotoxic potential of antitumoral compounds, both invitro and invivo. In many cases, thin ability depended on the sequence of treatment, and the enhancement of the cytotoxic effect occurred only when N-methyl formamide administration succeeded anticancer drug treatment (Calcabrini et al., 1997; Valenti et al; 1997). It shows various types of bioactivities such as cytotoxic (Kroger et al; 1983), carcinogenic (Cob et al; 200), hepatotoxicity (Hyland et al; 1992) etc., N-methyl formamide with adriamycin on human melanoma cell line recorded a decrease of adriamycin- induced cytotoxicity and intracellular adriamycin content (Arancia et al; 1944).

In the present investigation, compound isolated in the extract in 1:1 (v/v) chloroform: methanol of red alga portieria hornmannii was probably identified as N-methyl formamide based on the spectral data exhibited antibacterial activity. IR data of the isolated compound showed a peak at 342cm⁻¹ indicates the presence of N-H stretching and carbonyl group presence close to -NH₂ group indicated by the peak formed at 1693 cm⁻¹ (C = O stretching). The presence of aldehyde proton is confirmed by C - H stretching band at 3100-2800 cm⁻¹. Appearance of strong absorption between 3500 and 3200 cm⁻¹ indicated the presence of secondary amine

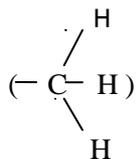
($\begin{matrix} \diagup \\ \text{---} \\ \diagdown \end{matrix}$ NH). (NH). This data is in confirmation with the IR data of reference sample. (Fig.1)

In the ¹H NMR spectra there is no peak in the region of δ=7-8, hence it shows the absence of proton belongs to benzene ring. The integral value of peak appeared in the ¹H NMR spectrum is as δ8.3=4.8 ~5 corresponds to 1H, δ8.5=5.6 ~ 5 corresponds to 1H and δ2.3 =13.1~15 corresponds to 3H. The prominent peak at δ=8.3 shows the presence of aldehyde proton in the compound. (Fig.2)

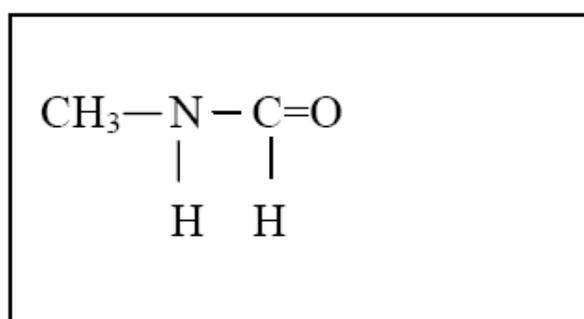


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The broad peak at δ 5.8 indicates presence of either alcohol or amine, corresponds to a single proton probably a single hydrogen bonded to nitrogen. A prominent peak appears at δ 2.3 indicates the presence of three proton in a methyl group



and there is no other pronounceable peak present in the ^1H NMR peak appears at δ 44.29 and δ 49.09 ppm for NCH_2 of N-methylformamide. (Fig.3) This data is in confirmation with the findings of Kelleher et al. (2002). From the data of HPLC, IR and NMR (^1H and ^{13}C), isolated colorless crystalline compound was identified as N-methyl formamide. The molecular formula for N-methyl formamide: $\text{C}_2\text{H}_5\text{NO}$ Molecular structure of N-methyl formamide



The plate shows the colorless crystal isolated in the extract of 1:1(v/v) chloroform: methanol of red alga *Portieria hornemannii*



3. CONCLUSION

It is concluded that a colorless crystal compound isolated from 1: 1 (v/v) chloroform:methanol extract of red alga *Portieria hornemannii* showing promising source of bioactive compounds to control the plant pathogenic bacteria such as *Xanthomonas axonopodis pv.citri* and *X.campestris pv. malvacearum* causing cancer in citrus and angular spot in cotton, respectively.

Table1. FTIR, ^1H and ^{13}C NMR Spectroscopic data of the N-methyl formamide compound from *Portieria hornemannii*.

δH	δC	FTIR cm^{-1}	Functional group
8.39	174	2854	C-H
5.87	51	3423	N-H
2.3	29	2921	N- CH_3
		1634	C=O

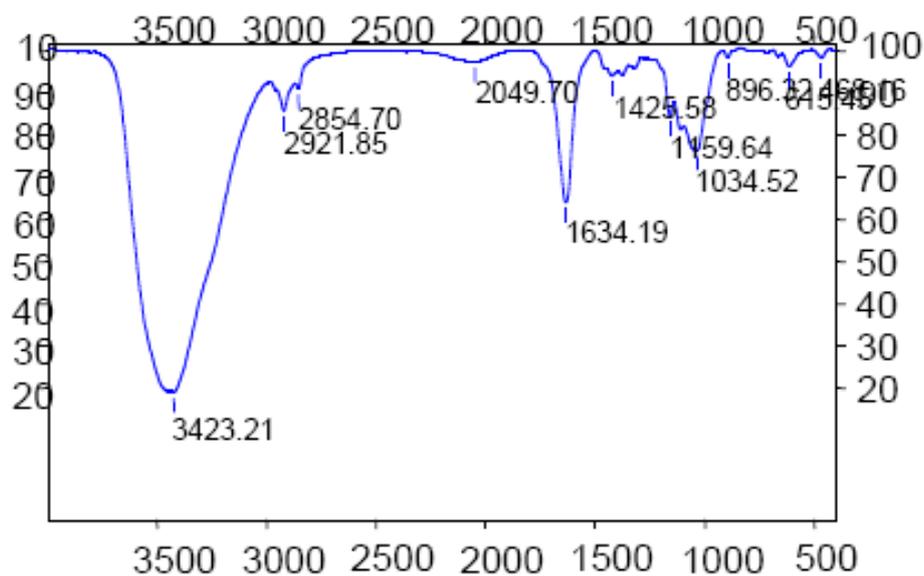


Fig1. FT IR data for the N-methyl formamide compound from red algae *Portieria*

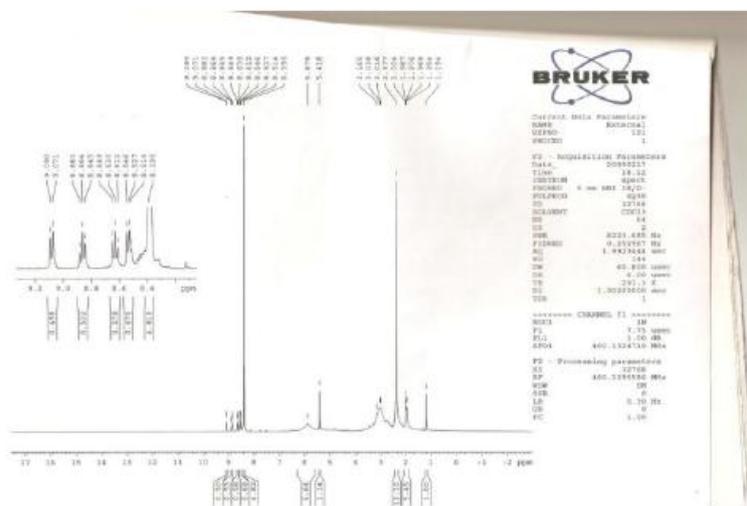


Fig2. ¹H NMR of the isolated N-Methyl formamide from red algae *Portieria hornemannii*.

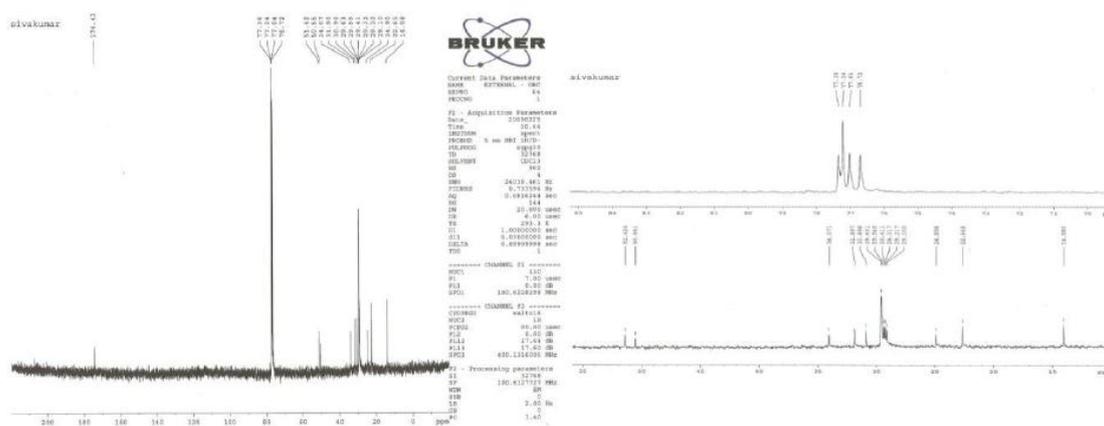


Fig3. ¹³C NMR of the isolated N-Methyl formamide from red algae *Portieria hornemannii*



Fig4. The plate shows the growth of *Xanthomonas axonopodias* pv *citri* (a) and *Xanthomonas campestris* pv. *malvacearum*(b) on peptone sucrose agar medium and artificially developed canker on citrus(c) and artificially infected cotton plant(d).

Table2. Isolated crystalline compound and Standard antibiotic Zone of inhibition against two plant pathogens.

S.No	Concentrations	X.cam.pv.citri	X.cam.pv.malvacearum
		Zone of inhibition in mm	Zone of inhibition in mm
1	50µg/ml of N-Methyl formamide	14.3±0.56	15.5±0.25
2	100µg/ml of Streptomycin sulphate	37.6±0.56	37.6±0.56

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