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## Chemical constituents and anti-tuberculosis activity of ink extracts of cuttlefish, *Sepiella inermis*

Muthusamy Ravichandiran<sup>1</sup>, Selvam Thiripurasalini<sup>2</sup>, Vaithilingam Ravitchandirane<sup>3\*</sup>, Srinivasa Gopalane<sup>4</sup>, Chelladurai Stella<sup>5</sup>

<sup>1</sup>Department of Food and Drug Testing, Puducherry-605 006, India

<sup>2</sup>Department of Chemistry, PRIST University, Thanjavur-613403, India

<sup>3</sup>Department of Zoology, Kanchi Mamunivar Centre for Post-Graduate Studies, Puducherry-605008, India

<sup>4</sup>Department of Forensic Medicine, Omar Al-Mukhtar University, Al-Beida, Libya

<sup>5</sup>Department of Oceanography, Alagappa University, Thondi Campus-623409, India

### PEER REVIEW

#### Peer reviewer

Dr. P. Sampathkumar, Associate Professor, CAS in Marine Biology, Annamalai University, Parangipettai-608 502, India  
Tel: 04144-24322-3225  
E-mail: sampathcas@gmail.com

#### Comments

This is an ideal work to discover the new drug for tuberculosis. Authors are comprehensively worked in anti-tuberculosis using two different solvent extracts of *Sepiella inermis*. The materials and methods are well constructed and findings are well represented in the present study.  
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### ABSTRACT

**Objective:** To study the chemical constituents and the anti-tuberculosis activity of methanol and chloroform ink extracts of *Sepiella inermis*.

**Methods:** Pulverized ink powder was extracted separately with chloroform and methanol. Chemical analysis was carried out by UV-VIS spectrophotometer, FT-IR and GC-MS. Crude extracts were tested *in vitro* for their activity against *Mycobacterium tuberculosis* using Lowenstein Jensen (L-J) medium. Activity in L-J medium was assessed by mean reduction in number of colonies on extract containing bottles as compared to extract free controls.

**Results:** GC-MS of methanol extract revealed four compounds *viz.* hexadecanoic acid, 9, 12-octadecadienoic acid, 9-octadecenoic acid and octadecanoic acid. The chloroform extract containing fourteen compounds. The methanol extract exhibited anti-tuberculosis activity in L-J medium at 64 µg/mL with the observed inhibition of 14 CFU. Chloroform extract displayed a weak activity against *Mycobacterium tuberculosis*.

**Conclusions:** This investigation showed the methanol extract exhibited significant activity against *Mycobacterium tuberculosis* than chloroform extract. Since ink of sepia is available abundantly as a waste material, further studies aimed at isolation and efficacy of active substances pave the way for new anti-tuberculosis drugs.

### KEYWORDS

Anti-tuberculosis, *Mycobacterium tuberculosis*, *Sepiella inermis*, Ink extract

## 1. Introduction

Tuberculosis remains one of the leading infectious killer diseases caused by *Mycobacterium tuberculosis* (*M. tuberculosis*). About one-third of the world's population is infected with tubercle bacillus and it is responsible for

more human mortality than any other single microbial species[1]. This problem has become serious as *M. tuberculosis* developed resistance against both the first line and the second line drugs leading to an emergence of multi drug resistant and extensive drug resistant strains of *M. tuberculosis* all over the world[2,3]. The increasing

\*Corresponding author: Dr. V. Ravitchandirane, Associate Professor, Department of Zoology, Kanchi Mamunivar Centre for Post-Graduate Studies, Puducherry-605008, India.

Tel: 91413-2251094, 9194-4303-7277

E-mail: vairavit@yahoo.co.in

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incidence of multi drug resistant and extensive drug resistant–tuberculosis highlight the urgent need to search for newer anti–tuberculosis drugs.

Marine derived bioactive compounds offer a great hope to fulfill these needs and hold great promise as therapeutics in the treatment of human diseases[4]. Marine organisms have evolved biochemical and physiological mechanisms that include the production of secondary metabolites meant for self protection against infection, predation and competition. *Sepiella inermis* (*S. inermis*), commonly called spineless cuttlefish is one of the major marine food resources exploited in Tamilnadu and Pondicherry coastal waters. The cuttlefish produces a dark ink secreted by its ink gland for its defense[5]. The ink of cuttlefish was identified as a potential source of bioactive compounds and it is a traditional Chinese medicine listed in the Compendium of Materia Medica[6]. Several reports revealed that the ink extract of cuttlefish is found to contain antibacterial, antifungal, antiseptic, platelet aggregation, haem–agglutination and cytotoxic properties[7,8]. But survey of literature discloses that, no studies are available emphasizing the antibacterial activity of ink of *S. inermis* against *M. tuberculosis*. As a result, the present study was conducted to characterize the chemical constituents present in the methanol and chloroform ink extracts of *S. inermis* and their ability to inhibit the growth of *M. tuberculosis*.

## 2. Materials and methods

### 2.1. Fish collection

Fresh cuttlefishes were collected directly from fishing vessels of Pondicherry Coastal Waters (11°46' and 12°03'N; 79°36' and 79°53'E). Fishes were identified as *S. inermis* using the keys given by George[9].

### 2.2. Preparation of extract

The ink–sacs were carefully removed from fresh individuals and the ink was collected and air dried at room temperature. A total of 100 g of air dried ink was subjected to pulverization using mortar and pestle. Pulverized ink powder was soaked in 200 mL chloroform and shaken in a flask shaker (Remi) at room temperature for 8–10 h. The chloroform extract was centrifuged to collect the supernatant and concentrated in vacuum[4]. The residue left after the chloroform extract was then re–extracted in 200 mL methanol for 8–10 h and concentrated as stated above. The

crude extract obtained in each case (1 g for chloroform and 1.6 g for methanol) was used for further studies.

### 2.3. Spectral studies

The column purified chloroform and methanol extracts were scanned in UV–VIS spectrophotometer (Lab India) between 200 and 400 nm. IR spectrum was recorded between 5000 to 400  $\text{cm}^{-1}$  for IR active methanol extract only in FT–IR spectrophotometer (Shimadzu–8300, Japan). Both the methanol and chloroform extracts were subjected to GC–MS (Shimadzu–DP 2010, Japan). And 1  $\mu\text{L}$  of crude extract was injected into DB–5ms Agilent column (30 m, 0.25 mm inner diameter with film thickness 0.25  $\mu\text{m}$ ) with helium (1.5 mL/min) as carrier gas. The temperature was initially maintained at 70 °C for 2 min and gradually increased up to 300 °C with 10 °C per min, holding it for 9 min. The sample injector was maintained at 240 °C throughout the experiment period. The mass spectroscopic analysis was done with –70 CV and between 40–1000  $m/z$  scan range in the duration of 34 min. The compounds separated in succession by GC were compared with NIST–II Library.

### 2.4. Mycobacterium strain and assay protocol

Well characterized strain of *M. tuberculosis* was obtained from the state tuberculosis training and demonstration centre, Government hospital for chest diseases, Pondicherry. Antimicrobial assay was performed in Lowenstein Jensen (L–J) medium[10]. Reagents of L–J medium included monopotassium phosphate, magnesium citrate, magnesium sulphate, asparagine, glycerol and malachite green. An antibiotic supplement like nalidixic acid, lincomycin and cycloheximide also added in the medium to prevent the growth of any non–mycobacterial[11].

One loopful of *M. tuberculosis* was streaked on the sidewall of a McCartney bottle using 3 mm external diameter loop and suspended in 1 mL of sterile distilled water. It was homogenized with glass beads by vortexing and turbidity was adjusted with sterile distilled water to obtain a concentration of 1 mg/mL of tubercle equivalent to McFarland standard 1 ( $10^7$  CFU/mL)[12]. The methanol extract (1, 2, 4, 8, 16, 32 and 64  $\mu\text{g/mL}$ ) and chloroform extract (10, 20, 40, 80, 160, 320 and 640  $\mu\text{g/mL}$ ) were incorporated in the bottles containing medium prior to inspissations. The bottles were incubated at 37 °C for 28 d. Readings were taken weekly. An MIC of 128 or more can be tentatively interpreted as resistant. The results were calculated by mean reduction in number of colonies in bottles containing

extracts. For comparison extract free control slants were used. Each test was done in duplicate.

### 3. Results

The UV spectral analysis of methanol extract showed absorption maxima at 274 and 286 nm and the chloroform extract at 269 nm suggesting the benzenoid nature of the compounds. IR spectrum provides valuable information regarding the functional groups of the crude methanol extract. The vibrational frequencies occurring at 3421.83  $\text{cm}^{-1}$  inferred the O–H stretching and bands at 3100.67 and 2929.97 corresponded to C–H stretching. The signals appearing at 1653.05 attributed to C=O and C=C stretching at 1592.29 and 1559.50. The O–H in plane at 1419.86 indicates the presence of ester groups in the extracts. The GC–MS of methanol extract exhibited four peaks with the retention times ranging from 16.708 to 18.626 min (Figure 1). The fragmentation pattern that resulted from the electron impact mass spectrum of all the four compounds were characterized as hexadecanoic acid, ( $\text{C}_{17}\text{H}_{34}\text{O}_2$ ), 9,12–octadecadienoic acid ( $\text{C}_{19}\text{H}_{34}\text{O}_2$ ), 9–octadecenoic acid ( $\text{C}_{19}\text{H}_{36}\text{O}_2$ ), octadecanoic acid ( $\text{C}_{19}\text{H}_{38}\text{O}_2$ ), similarly chloroform

extract displayed sixteen peaks with the retention times ranging from 11.341 to 27.127 min (Figure 2). The positive electron impact mass spectrum of only thirteen compounds were obtained and characterized as 1–tetradecene ( $\text{C}_{14}\text{H}_{28}$ ), phenol, 3,5–bis (1,1–dimethylethyl) ( $\text{C}_{14}\text{H}_{22}\text{O}$ ), 2–tetradecene ( $\text{C}_{14}\text{H}_{28}$ ), benzene, 1,1'–(1,2–cyclobutanediyl) bis–trans ( $\text{C}_{16}\text{H}_{16}$ ), 1–octadecene ( $\text{C}_{18}\text{H}_{36}$ ), 1H–indole–3–carboxylic acid ( $\text{C}_{10}\text{H}_9\text{NO}_2$ ), nonadecanol–1 ( $\text{C}_{19}\text{H}_{40}\text{O}$ ), methyl stearate ( $\text{C}_{19}\text{H}_{38}\text{O}_2$ ), 1–docosene ( $\text{C}_{22}\text{H}_{44}$ ), 1–heptacosanol ( $\text{C}_{27}\text{H}_{56}\text{O}$ ), 1–benzylindole ( $\text{C}_{15}\text{H}_{13}\text{N}$ ), 1H–indole–2–methyl–3–phenyl ( $\text{C}_{15}\text{H}_{13}\text{N}$ ), 5–methyl–2–phenylindolizine ( $\text{C}_{15}\text{H}_{13}\text{N}$ ).

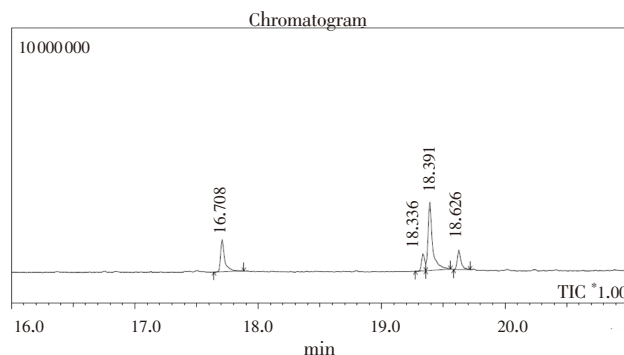


Figure 1. GC–MS of methanol extract of ink of *S. inermis*.

The results of the anti–mycobacterial activity of methanol and chloroform ink extracts of *S. inermis* are shown in Table 1.

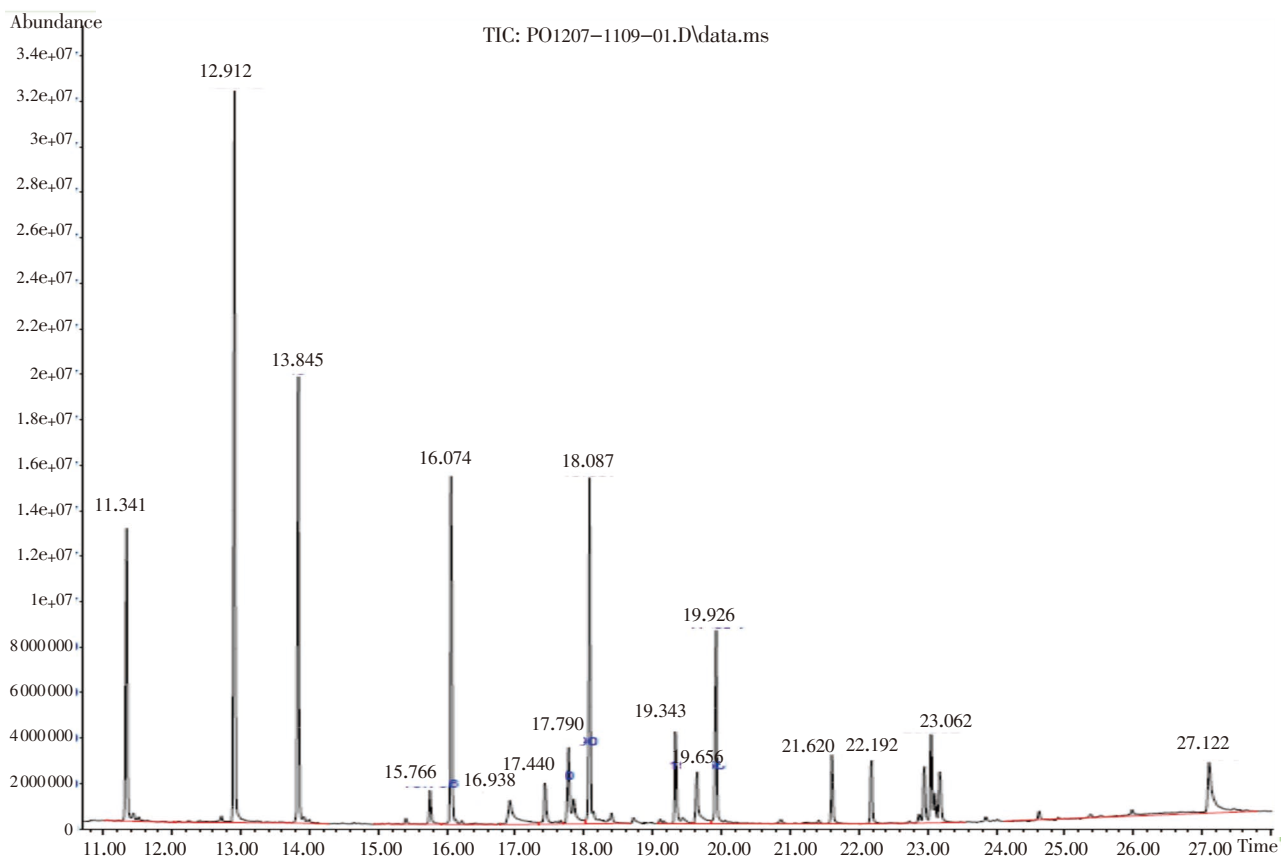


Figure 2. GC–MS of chloroform extract of ink of *S. inermis*.

The results revealed that the methanol extract was more active and exhibited significant inhibitory effect against *M. tuberculosis* at the concentration of 64 µg/mL with the observed inhibition of 14 CFU when compared to chloroform extract.

**Table 1**

Anti-tuberculosis activity using *S. inermis* ink extracts in Lowenstein Jensen (L–J) medium.

Bottles with L–J medium	Methanol extract		Chloroform extract	
	Concentration (µg/mL)	Observed results	Concentration (µg/mL)	Observed results
1	L–J slant without extract	1+	L–J slant without extract	1+
2	1	1+	10	1+
3	2	1+	20	1+
4	4	1+	40	1+
5	8	46 CFU	80	28 CFU
6	16	35 CFU	160	28 CFU
7	32	26 CFU	320	24 CFU
8	64	14 CFU	640	20 CFU

#### 4. Discussion

In the present study, the methanol ink extract of *S. inermis* exhibited significant anti-tuberculosis activity with the MIC values of 64 µg/mL against *M. tuberculosis*. The chloroform extract displayed a weak activity against *M. tuberculosis* with the MIC of 640 µg/mL. As many different methods are available to evaluate anti-tuberculosis activity, no specific cut-off value has been established for reference to analyse the anti-tuberculosis activity<sup>[13]</sup>. However, the inhibitory activity of crude plant/animal extracts were classified as significant if MIC < 100 µg/mL, moderate if MIC ≤ 125 µg/mL or weak if MIC > 625 µg/mL. Crude extracts which did not exhibit inhibition up to 1280 µg/mL were considered not active<sup>[14]</sup>. Considering the above values it is inferred that out of the two extracts tested the methanol extract was found to be more potent than chloroform extract. The GC–MS investigation of methanol ink extract of *S. inermis* revealed the presence of omega fatty acids like hexadecanoic acid, 9, 12-octadecadienoic acid, 9-octadecenoic acid and octadecanoic acid. Both traditionally and scientifically it has been established that omega fatty acids of marine organisms possessed significant medicinal properties like microbial inhibition, platelet agglutination *etc*<sup>[15–17]</sup>.

To the best of our knowledge, the crude ink extracts of *S. inermis* have not been investigated previously for its anti-tuberculosis activity. Therefore, the present investigation may be considered as the first report and this study forms a good preliminary work for further research.

In conclusion, it is to be stressed that the ink of sepia is available abundantly as a waste material while processing sepia for its export as food. If more attention is given on the isolation of bioactive compounds it may pave the way for the development of new anti-tuberculosis drugs from sepia processing industrial waste.

#### Conflict of interest statement

We declare that we have no conflict of interest.

#### Acknowledgements

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#### Comments

##### Background

Marine invertebrate derived bioactive compounds are anticipated outcome of many pharmacological researches world wide. Cuttle fish is found to possess wide antibacterial property. Tuberculosis is a leading infectious killer disease caused by *M. tuberculosis*. Therefore there is a need of searching potent anti-tuberculosis agent.

##### Research frontiers

The author found the chemical constituents of *S. inermis* and its anti-tuberculosis activity of methanolic and chloroform extracts. The methanolic extract contained hexadecanoic acid, 9, 12-octadecadienoic acid, 9-octadecenoic acid and octadecanoic acid. And these compounds exhibited anti-tuberculosis activity. But, chloroform extract containing fourteen compounds and exhibited weak activity against *M. tuberculosis*.

##### Related reports

So far, several works have been done on plant extracts and synthetic chemicals involved in anti-tuberculosis research but for first time the present work revealed the anti-tuberculosis activity of the crude ink extracts of *S. inermis*. Hence, the present investigation is considered

to be the first report. The results also suggested that the methanol extract exhibited anti-tuberculosis activity in L–J medium at 64 µg/mL with the observed inhibition of 14 CFU is basic for further research in the same field.

### Innovations and breakthroughs

Several studies were carried out on ink extract of cuttle fish is found to contain antibacterial, antifungal, antiseptic, platelet aggregation, haem–agglutination and cytotoxic properties. In the present study, authors have extensively worked the anti-tuberculosis activity against *M. tuberculosis*.

### Applications

The present investigation demonstrate the anti-tuberculosis activity against *M. tuberculosis* and may lead to further research on responsible exact compounds and their reason.

### Peer review

This is a ideal work to discover the new drug for tuberculosis. Authors are comprehensively worked in ant-tuberculosis using two different solvent extracts of *S. inermis*. The materials and methods are well constructed and findings are well represented.

## References

- [1] WHO. *WHO Report: Global tuberculosis control*. Geneva: WHO Press; 2010.
- [2] Satti M, Faqir F, Sattar A, Abbasi S, Butt T, Karamat KA, et al. Efficacy of amikacin and ciprofloxacin against clinical isolates of *Mycobacterium tuberculosis*. *J Ayub Med Coll Abbottabad* 2010; **22**(1): 101–103.
- [3] Gupta R, Thakur B, Singh P, Singh HB, Sharma VD, Katoch VM, et al. Anti-tuberculosis activity of selected medicinal plants against multi-drug resistant *Mycobacterium tuberculosis* isolates. *Indian J Med Res* 2010; **131**: 809–813.
- [4] Gomathi P, Nair JP, Sherief PM. Antibacterial activity in the accessory nidamental gland extracts of the Indian squid, *Loligo duvauceli* Orbigny. *Indian J Mar Sci* 2010; **39**(1): 100–104.
- [5] James BW, Amy EM. Caribbean reef squid use ink as a defense against predatory French grunts. *J Exp Mar Biol Ecol* 2010; **388**: 20–27.
- [6] Zhong JP, Wang G, Shang JH, Pan JQ, Li K, Huang Y, et al. Protective effects of squid ink extract towards hemopoietic injuries induced by cyclophosphamine. *Mar Drugs* 2009; **7**: 9–18.
- [7] Lei M, Wang JF, Wang YM, Pang L, Wang Y, Xu W, et al. Study of the radio-protective effect of cuttle fish ink on hemopoietic injury. *Asia Pac J Clin Nutr* 2007; **16**: 239–243.
- [8] Vennila R, Rajesh Kumar RK, Kanchana S, Arumugam M, Balasubramanian T. Investigation of antimicrobial and plasma coagulation property of some molluscan ink extracts: Gastropods and cephalopods. *Afr J Biochem Res* 2011; **5**(1): 14–21.
- [9] George VI. *An illustrated guide on commercial fishes and shellfishes of India*. Kochi, India: The Marine Products Export Development Authority; 2008, p. 162–163.
- [10] Piersimoni C, Scarparo C, Callegaro A, Tosi CP, Nista D, Bornigia S, et al. Comparison of MB/BacT ALERT 3D system with radiometric BACTEC system and Lowenstein–Jensen medium for recovery and identification of mycobacteria from clinical specimens: A multicenter study. *J Clin Microbiol* 2001; **39**: 651–657.
- [11] Carpenter CD, O’Neill T, Picot N, Johnson JA, Robichaud GA, Webster D, et al. Anti-mycobacterial natural products from the Canadian medicinal plant *Juniperus communis*. *J Ethinopharmacol* 2012; **143**: 695–700.
- [12] Tekwu EM, Askun T, Kuete V, Nkengfaek AE, Nyasse B, Etoa FX, et al. Antibacterial activity of selected cameroonian dietary spices ethno-medically used against strains of *Mycobacterium tuberculosis*. *J Ethinopharmacol* 2012; **142**: 374–382.
- [13] Kuete V. Potential of Cameroonian plants and derived products against microbial infections: A review. *Planta Med* 2010; **76**: 1479–1491.
- [14] Luo X, Pires D, Aínsa JA, Gracia B, Mulhovo S, Duarte A, et al. Antimycobacterial evaluation and preliminary phytochemical investigation of selected medicinal plants traditionally used in Mozambique. *J Ethinopharmacol* 2011; **137**: 114–120.
- [15] Kurishara H, Goto Y, Aida M, Hosokawa M, Takahashi K. Antibacterial activity against cariogenic bacteria and inhibition of insoluble glucan production by free fatty acids obtained from dried *Gloiopeltis furcata*. *Fish Sci* 1999; **65**(1): 129–132.
- [16] Sherief PM, George MC, Nair JR, Devika P, Sophia MJ, Priya SV. Antibacterial activity in the extract of accessory nidamental glands of squid and cuttlefish. In: Saiyed Asif Husain Abidi, National Institute of Ocean Technology (Madras, India), Society of Biosciences (India). Proceedings of the National Seminar on New Frontiers in Marine Bioscience Research, January 22–23, 2004. Chennai: Allied Publishers; 2004, p. 47–51.
- [17] Ramasamy P, Barwin Vino A, Saravanan R, Subhapradha N, Vairamani S, Shanmugam A. Screening of antimicrobial potential of polysaccharide from cuttlebone and methanolic extract from body tissue of *Sepia prashadi* Winkworth, 1936. *Asian Pac J Trop Biomed* 2011; **1**(Suppl 2): S244–248.