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To cite this article: M Kamaruddin *et al* 2021 *IOP Conf. Ser.: Earth Environ. Sci.* **679** 012011

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Screening acetylcholinesterase inhibitors from marine-derived actinomycetes by simple chromatography

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Abstract. Acetylcholinesterase (AChE) is an enzyme involved in the metabolism of Acetylcholine [ACh] cholinergic synapses in the central and peripheral nervous systems. As a result, AChE inhibitors promote an increase in the concentration and length of Ach synaptic activity. Acetylcholinesterase inhibitors (AChEIs) are currently considered the best therapeutic agents for Alzheimer's patients. The potential sources of the current AChE inhibitors are predominantly plants. However, some of these drugs are known to have disadvantages such as limited half-life or side-effect such as hepatotoxicity. Marine microorganisms are a potential new source of the enzyme inhibitors. Since their rich and diverse metabolites have a wide range of biological activities, one of them is an actinomycetes of special interest in antibiotics. The purpose of this study was to screen AChE inhibitors from marine-derived actinomycetes using a simple method, thin layer chromatography (TLC). In addition, TLC was used for the determination of solvents for adsorption of actinomycetes AChE inhibitors. The results showed that the combination of chloroform/methanol = 80/20 had the best AChE inhibitor separation performance, while the combination of DCM/methanol = 90/10 should be considered if the gradient elution was selected during the separation process.

1. Introduction

For thousands of years, natural products have played a major role in the treatment and prevention of human diseases worldwide. Natural medicinal products have been collected from a number of sources including terrestrial plants and microorganisms, marine organisms (invertebrates, plants and microorganisms) [1]. Marine bacteria may produce chemical compounds as bioactive natural products, which may be derived from the primary or, rather, secondary metabolism of these organisms[2,3]. Among such marine bacterial, gram-positive actinomycetes bacteria predominate in soil and are of particular interest, as they are known to produce chemically diverse compounds with a wide range of biological activities, and are of high pharmacological and commercial interest[4]. Actinomycetes are best known for their ability to produce antibiotics that are not limited to antibacterial antibiotics including antifungal, anticancer, and antiviral antibiotics[1].

More than 15,000 antibiotics have been detected in the last 60 years due to their ability to produce antibiotics, of which 70% are actinomycetes and 30% are fungi and other microorganisms[5]. However, there is less concern about the capacity of actinomycetes to generate different enzymes, which is the focus of our study. Our Laboratory has obtained and characterized actinomycetes from various marine samples and, in this study, we analyzed marine-derived actinomycetes for acetylcholinesterase inhibitors of the enzyme potential by thin layer chromatography.



An acetylcholinesterase inhibitor or an anticholinesterase is a chemical that prevents the cholinesterase enzyme from breaking down acetylcholine, thereby enhancing both the degree and duration of activity of the neurotransmitter acetylcholine[6]. Acetylcholinesterase is a tetrameric protein that catalyzes the hydrolysis of acetylcholine. The active site of AChE contains the serine hydroxyl group, which is made more nucleophilic by the proton-acceptor action of the nearby histidine residue[7,8]. Serine residues exert a nucleophilic attack on the carbonyl carbon of ACh. A tetrahedral transition state is reached, resulting in serine acetylation and loss of free choline. The acetyl group binds to histidine as an N-acetate, but is quickly hydrolyzed rapidly to create free choline, acetate, and a free enzyme[9]. Acetylcholinesterase inhibitors exist naturally as venoms and toxins are used as weapons in the form of nerve agents to treat neurological conditions such as Alzheimer's disease, senile dementia, ataxia and myasthenia gravis[10,11].

Thin layer chromatography or TLC is a relatively fast, simple, inexpensive and straightforward chromatography process for separating small molecules on the basis of their relative hydrophobicity[12]. TLC is often used to help the identification of a compound in a mixture when the R_f of a compound is compared to the R_f of a known compound. To use this technique to analyse the products of the decapping reaction needed a small amount of the mixture to be analysed is spotted near the bottom of this plate[12,13]. The TLC plate is then placed in a shallow pool of a solvent in a developing chamber so that only the very bottom of the plate is in the liquid. This liquid as mobile phase that slowly rises up the TLC plate by capillary action[13].

2. Materials and methods

2.1 Cultivation and crude extract preparation

Some strains of isolated actinomycetes will be grown on a Nutrient Agar (NA) medium consisting of a nutrient broth, agar and seawater for 3 days at room temperatures. Colony that grows to be cut into small pieces of agar block and then transferred to each of the four cultivated media [media A consisting of sand, chitin, seaweed and seawater; media B consisting of 100% glycerol, bacto soya and seawater; media C consisting of bio-peptone/polypeptone, yeast extract, soluble starch and seawater; media D consisting of peptone, yeast extract and seawater]. These media will be in the shaker incubator at 200 rpm for 3 days at 25-30°C. 5% v/v of inoculum will be inoculated again in 100 ml of each of the four cultivation media for 5- 7 days at 25-30°C at 200 rpm.

2.2 Actinomycetes extraction

After 5-7 days of cultivation at 25-30°C, 200 rpm, the medium involves the transfer of inoculum to separate funnel and the addition of ethyl acetate with 1 : 1. This work has been performed three times. Ethyl acetate collected and then removed by a rotary evaporator providing a crude extract of actinomycetes and the crude extract stored at -20°C for further screening.

2.3 Screening AChE inhibitors of crude extract

Crude extract obtained from the extraction of marine actinomycetes dissolved in either ethyl acetate or methanol depending on their solubility. The crude extract of actinomycetes samples was spotted using a micro haematocrit tube at different points at the lower limit of the activated TLC plate. Previous results of the solubility test have shown that dichloromethane (DCM) and chloroform have the potential to be the second solvent. In this analysis, we used two chamber glasses each containing chloroform/methanol (80:20) and DCM/methanol (90:10) solutions. The TLC plate was then placed into the chamber glass, the solution travels through the plate, and the plate was removed from the chamber when the solution reached the upper limit of the tip of the plate. Observation of the sample elution results was performed using UV light at wavelengths of 254 nm and 330 nm. The dots that have been displayed were marked with a pencil. As a result, the TLC plate is sprayed with various reagents (ninhydrin, alkaloids, vanillin, and phosphomolybic acid, or PMA). After spraying the reagent, the TLC plate was dried at a high temperature.

3. Results and discussions

The effect of the addition of polar chloroform solvent was very strong in order to change the polarity of the solvent mixture against some crude extract actinomycetes (CNA 147, 153, 156, 161, and 162) with suitable reagents. Whereas the use of DCM polar solvent is very successful in changing polarity only to CNA 148 as shown in Figure 1.

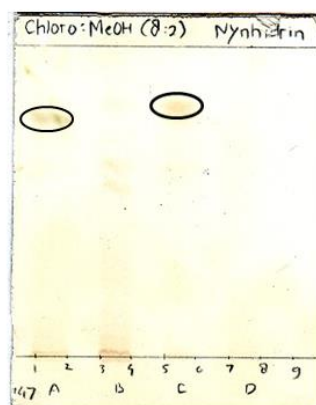


Figure 1. Diagrammatic chromatogram crude extract of Actinomycetes sample CNA 147 with ninhydrin reagent using chloroform : methanol (80:20) solvents

CNA 147 samples sprayed with ninhydrin reagent showed light brown spots [Media A and C], while the use of ninhydrin reagent on the sample revealed reddish purple spots. If the sample shows the right color path, it is probable that the sample contains glycoside compounds as one of the phytoconstituent inhibitors of AChE (Figure 1)[9].

Samples of CNA 153, 156, 161 and 162 with alkaloid reagent spray revealed those samples containing alkaloid compounds. Component of alkaloid with a dark brown color after spraying the alkaloid reagent. As mentioned, alkaloid is one of the AChE inhibitors phytoconstituents (Figures 2a and b)[14]. The CNA 147 sample sprayed with a vanillin reagent showed a vivid green-yellow color for the detection of amines and amino acid compounds. These colors are seen in the media B, C, and D. Whereas, in the media A be streaked, it may be too concentrated (Figure 3)[9,14].

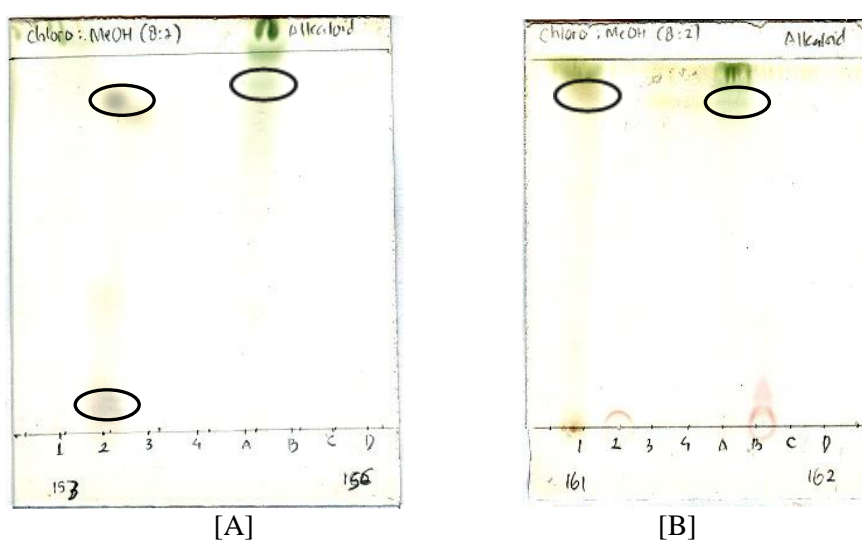


Figure 2. Diagrammatic chromatogram crude extract of Actinomycetes samples with alkaloid reagent using chloroform : methanol (80:20) solvents. [A] Samples of CNA 153 and CNA 156; [B] Samples of CNA 161 and CNA 162

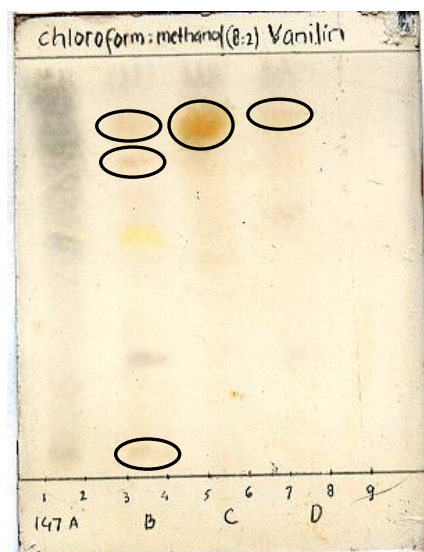


Figure 3. Diagrammatic chromatogram crude extract of Actinomycetes samples of CNA 147 with vanillin reagent using chloroform : methanol (80:20) solvents.

Phosphomolybic acid reagent used for the identification of prostaglandins, hormones, lipids, alkaloids and essential oils. The location of the reagent shall be blue spots when heated at 100 - 110 ° C. The CNA 148 sample showed a deep blue-violet, which caused the TLC plate to overheat. Even so, these spots suggested that the CNA 148 sample contains 2 phytoconstituents of AChE inhibitors (Figure 4)[7].



Figure 4. Diagrammatic chromatogram crude extract of Actinomycetes samples of CNA 148 with PMA reagent using DCM : methanol (90:10) solvents.

4. Conclusion

The addition of polar solvents [chloroform and DCM] to methanol solvents with specific reagents can detect AChE inhibitors from crude actinomycetes extracts. The combination of chloroform : methanol (80:20) resulted in a broader detection of AChE inhibitors than the combination of DCM : methanol (90:10) which only detected one sample (CNA 148).

5. References

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6. Acknowledgement

This research was supported independently.