UNIVERSITI PUTRA MALAYSIA

IN VITRO AND IN VIVO STUDIES ON ANTI-WITHDRAWAL PROPERTIES ON ERYTHROXYLUM CUNEATUM LEAVES ALKALOID EXTRACT

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By

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IN VITRO AND _IN VIVO_ STUDIES ON ANTI-WITHDRAWAL PROPERTIES OF _ERYTHROXYLUM CUNEATUM_ LEAVES ALKALOID EXTRACT

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November 2015

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Erythroxylum cuneatum (EC) is locally known as the 'Chinta Mula' plant. Its leaves are used by the native traditional healers as an anti-addiction treatment. However, its effects were not fully explored scientifically, resulting in lack of documented information on its therapeutic anti-addiction effects. The objectives of this study are to produce a standard extract of EC, examining the efficacy of alkaloid extract of EC on cyclic adenosine monophosphate (cAMP) production in SK-N-SH cell after chronic morphine treatment and to investigate the effect of EC extract on anti-withdrawal properties in the morphine-addicted rats. The alkaloid crude extract of EC underwent two extraction processes, namely the Soxhlet and the acid-base extraction. The alkaloid crude extract of EC was obtained using acid-base extraction and the yield was 0.19% from 1 kg leaves. *The in-vitro* studies was performed separately as two different tests (co-treatment and pre-treatment) whereas *in-vivo* study used 6 groups (n=8) of Wistar rats (male: 180-220 g) which were treated with morphine at 10-30 mg/kg for 5 consecutive days. Withdrawal signs exhibited by the morphine-dependent rats were measured by 9 counts and checking of parametric signs. The rats were then treated with two different interventions which are Methadone (5 mg/kg), and crude alkaloid extract of EC (5, 25 and 50 mg/kg) respectively and the withdrawal signs were re-evaluated again. Co-treatment for 24 h between morphine sulphate with alkaloid extract of EC significantly reduced ($p<0.05$) the production of cyclic AMP at lower concentration (0.1 mg). Similarly pre-treatment with morphine sulphate for 24 h then treated with alkaloid extract of EC for 6 h significantly reduced the production of cyclic AMP ($P<0.05$). *In-vivo* results also showed that administration of alkaloid extracts of EC caused significant reduction ($p<0.05$) in all withdrawal signs. The results obtained from the study suggested that the administration alkaloid extract of EC caused significant decrease in the withdrawal signs of morphine addicted both *in vitro* and *in vivo* studies.

Keywords: *Erythroxylum cuneatum* (EC), Cyclic adenosine monophosphate (cAMP)

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah

KAJIAN *IN VITRO* DAN *IN VIVO* KE ATAS SIFAT SELEPAS KETAGIHAN TERHADAP EKSTRAK ALKALOID DAUN *ERYTHROXYLUM CUNEATUM* (EC)

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Erythroxylum cuneatum (EC) ataupun lebih dikenali dengan nama tempatan Chinta Mula, telah dilapor penggunaanya di kalangan pengamal perubatan tradisional sebagai rawatan anti-ketagihan. Walaubagaimanapun kesannya masih belum tidak diterokai sepenuhnya secara saintifik, menyebabkan kurang maklumat terdokumentasi tentang kesan terapeutik ekstrak alkaloid dari daun EC telah dikaji untuk mengetahui dengan lebih mendalam mengenai ciri-ciri sifat anti-ketagihannya. Objektif kajian adalah untuk menghasilkan ekstrak alkaloid EC, menentukan kesan ekstrak alkaloid EC pada pengeluaran kitaran adenosine monofosfat (cAMP) selepas rawatan morfin secara kronik dalam sel SK-N-SH dan menilai kesan ekstrak EC pada anti-ketagihan pada tikus yang ketagihan morfin. Ekstrak alkaloid diperolehi melalui proses pengekstrakan soxhlet, methanol dan pengekstrakan asid-bes. Ekstrak alkaloid EC diperolehi menerusi pengekstrakan asid-bes dan hasilnya adalah 0.19% daripada 1 kg EC. Kajian sel terbahagi kepada dua; rawatan bersama dan pra-rawatan. Dalam kajian menggunakan tikus, 6 kumpulan (n = 8) tikus Wistar (jantan; 180-220 g), telah diberikan Morfin (10 hingga 30 mg/kg) selama 5 hari. Selepas itu, 9 tanda ketagihan morfin tikus direkod. Semua tikus yang telah dirawat dengan Methadone 5 mg/kg dan ekstrak alkaloid EC (5, 25 dan 50 mg/kg) akan dinilai semula tanda ketagihan mereka. Rawatan bersama selama 24 jam antara morfin sulfat dengan ekstrak alkaloid EC menunjukkan pengurangan ketara ($P < 0.05$) pada dos rendah (0.1 mg). Begitu juga pra-rawatan dengan morfin sulfat untuk 24 jam kemudian dirawat dengan ekstrak alkaloid EC selama 6 jam juga menunjukkan pengurangan pengeluaran kitaran AMP ($P < 0.05$). Hasil kajian melibatkan haiwan pula, menunjukkan bahawa ekstrak alkaloid EC menyebabkan pengurangan ketara ($P < 0.05$) dalam semua ciri-ciri. Hasil kajian mencadangkan bahawa ekstrak alkaloid EC dapat mengurangkan ciri-ciri ketagihan morfin secara *in vivo* dan *in vitro*.

Kata Kunci: *Erythroxylum cuneatum* (EC), Kitaran adenosine monophosphate (cAMP).

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APPROVAL

I certify that a Thesis Examination Committee has met on (date of viva voce) to conduct the final examination of (Muhammad Amin bin Ahmad Zaki) on his thesis entitled (In vitro and in vivo studies on anti-withdrawal properties of *erythroxylum cuneatum*'s leaves) in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the (insert the name of relevant degree).

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LIST OF ABBREVIATION

%	Percentage
±	Plus/minus
°C	Degree Celsius
µg	Microgram
µl	Microliter
>	Less than
<	Greater than
ATCC American	Type Cell Culture
Carbon dioxide	CO2
cAMP	Cyclic adenosine monophosphate
DMSO	Dimethyl sulfoxide
e.g	For example
et al	Co workers
EC	*Erythroxylum cuneatum*
FBS	Fetal bovine serum
FRIM	Forest research institute Malaysia
g	Gram
h	Hour
H2O	Water
IACUC	Institutional animal care and use committe
IC50	Inhibition concentration caused 50% cell death
L	Litre
M	Molar
MEM	Minimum essential media
MeOH	Methanol
mg	Miligram
min	Minute
ml	millilitre
mM	Milimolar
n	Number of replicate
NADA	National Anti-Drug Agency
NaOH	Sodium Hydroxide
O	Oxygen
PBS	Phosphate buffer saline
pH	Negative logarithm of H+ concentration
Rpm	Revolution per minute
s	Second
SD	Standard deviation
SK-N-SH	Human neuroblastoma cell
TLC	Thin layer chromatography
UV	Ultraviolet
v/v	Volume by volume
w/v	Weight by volume

CHAPTER 1

INTRODUCTION

1.1 Introduction

The effort to explore alternatives to synthetic drugs has long been undertaken by medicinal practitioners. Synthetic drugs are generally known to elicit various side effects to the consumers. Despite the massive number of natural products used as the herbal medicines to treat medical illness, there are still more which potentials are not discovered. Although we are progressing with advancements to produce new and better drugs, traditional-ethnic drugs still has its own place in pharmaceutical treatments.

Many plants are being widely used for their therapeutic qualities in the pharmaceutical industry due to the increasing interest in finding substitutes for synthetic products. In Malaysia, there are few plants which were once used as ingredients in treating drug addiction in traditional way such as *Limacia oblongata* (Akar Kunyit), *Moringaoleifera* (Daun Kelor), *Eryhrina subumbrans* (Daun Dedap), *Gomphandra species* (Akar Hempedu Jawa), *Gandarusa vulgaris*, *Mimosa pudica* (Semalu),*Aquilaria malacencis*, *Cassia alata* (Gelenggang), *Randia species* (Duri Randa), and *Acanthus bracteaus* (Kayu Jeruju) according to Supathan (1988). Unlike other plants which have been extensively studied, there is less study conducted on *Erythroxylum cuneatum* (EC) (Chinta Mula). Therefore, this study aims to investigate this plant's anti-withdrawal properties for therapeutic uses.

Drug abuse is the recurrent use of illegal drugs, or the misuse of prescription or over-the-counter drugs with negative consequences. These consequences may involve problems at work, school, home or in interpersonal relationships; problems with the law and the physical risks that come with using drugs in dangerous situations (Samet, J.H., 2007). Drug abuse and addiction is known to run in families that do not have any genetic predisposition in affecting certain individuals to abuse drugs. Drug abuse begins in individuals due to the practice of the same behavior by the people around them, which is peer pressure or influence. Substance abuse may also begin as a bad habit, but when and if the addiction grows, it manifests as a chronic disease of addiction.

In Malaysia, drug addiction is an offence which is placed under Drug Dependency Act 1983 (Treatment and Rehabilitation). This problem had become severe since 1970s which prompted the government to declare the drug as the 'number one public enemy' in 1983. The National Anti-Drug Agency (NADA) is the agency that accounted for addressing problems related to drugs. Cases of drug addiction in the last 5 years showed an interesting but stable pattern. The highest number of drug addicts recorded in 2010 with a total of 23,462 people which decreased to 7,864 people in 2013. Registered drug addicts detected an average decreased to 8.67% from 2009 to 2013 (National Anti-Drug Agency, 2013).

Erythroxylum cuneatum is rarely known in traditional Malay medicine unless brought up anecdotally by local residents on how the leaves have been used in Philippines as fish poison and may be used as a tonic for women who miscarry in Pahang (Burkill, 1935).

In addition, local medical practitioners claimed that the water decoction of leaves from EC is able to reduce the signs of addiction to opioid drugs, particularly heroin.
In recent years, a report from FRIM on the impact of anti-withdrawal signs in morphine-dependent rats have demonstrated the ability to suppress withdrawal signs exhibited by morphine addicted rats 24 hours after cessation of morphine by using both aqueous and methanol extracts of the leaves of EC (Ilham *et. al.*, 2010). It was suggested that this plant might serve as an essential component to reduce withdrawal signs, followed by enforced drug cessation, but up to now, such an effect has not been reported (Kumarnsit *et al*., 2006).

However, the detailed mechanism involving anti-withdrawal properties of this plant remains unknown. Therefore, the design of the study is to understand the anti-withdrawal properties of EC's extracts. This plant can be developed into a new drug for the treatment of drug addiction.

1.2 Problem statement

Reports of drug abuse increases annually in Malaysia. Even though many programs were carried out by the Malaysian government in order to treat drug abuse, the patient's compliance towards the program are doubtful and treatment therapy cost is high. Thus, this study provides other alternatives using locally-available and cost-effective natural products to treat drug abuse especially on withdrawal effects of the drugs.

1.3 Hypothesis

The extracts of *Erythroxylum cuneatum* is able decrease withdrawal signs exhibited by morphine addicted rats.

1.4 Objectives

1.4.1 General objective

The aim of this study is to determine the anti-withdrawal properties effect of *Erythroxylum cuneatum* leaves extract on morphine addicted rats.

1.4.2 Specific objectives

i. To produce a standard extract of *Erythroxylum cuneatum* by using acid base extraction.

ii. To determine the effect of alkaloid extract of *Erythroxylum cuneatum* on cAMP production in SK-N-SH cell after chronic morphine treatment.

iii. To evaluate the effects of alkaloid extract of *Erythroxylum cuneatum* against withdrawal properties in morphine addicted rats.

1.5 Significance of the study

This study will provide a detailed preliminary information regarding the anti-withdrawal properties of *Erythroxylum cuneatum*'s leaf extracts which can be used as a stepping stone for future studies. Furthermore, the study results will be a testament of the feasibility of alternative, natural treatments for synthetic drugs on substance addiction. It is hoped that new avenues of research on this plant extract will open, and due to availability of this plant in Malaysia it can further boost the local economy by promoting and cultivating this plant as a profitable natural product due to its importance in the rehabilitation field.

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