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BIOACTIVE CONSTITUENTS AND ANTI-INFLAMMATORY EFFECT OF AGARWOOD LEAF EXTRACT AND ITS NANO-ENCAPSULATED AS HALAL THERAPEUTIC ALTERNATIVE

BY

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ABSTRACT

The serious adverse effects associated with the prolonged use of synthetic anti-inflammatory medications have raised concerns in the pharmaceutical field. In response, the pharmaceutical industry has begun to embrace the halal built-in approach to meet the desired unified attributes of safety and high quality. This work is focused on *Aquilaria malaccensis* (Family Thymelaeaceae), an agarwood-producing tree geographically distributed in Indomalesian rainforests. Different parts of the tree have been traditionally used to treat several medical disorders including inflammatory-related conditions. However, the scientific evaluation of the anti-inflammatory related traditional claims of this plant has been very scarce. In this study, *A. malaccensis* ethanolic leaf extract (ALEX-M) is subjected to characterization via phytochemical screening, FTIR, GC-MS and LC-MS. The extract was also subjected to isolation of bioactive compounds with anti-inflammatory potential using column chromatography. The structures of the isolated compounds were elucidated using HREIMS, IR, UV, 1D and 2D NMR spectroscopy. Polymeric nanocapsules were fabricated using water-in-oil-in-water (w/o/w) emulsion method uing Tragacanth gum as a natural and Halal polymer to encapsulate the extract. The toxicity and anti-inflammatory activity of the free extract (ALEX-M) and nanoencapsulated extract (ALEX-M-PNCs) were assessed in-vitro on RAW264.7 macrophages and in Zebrafish embryos. The findings revealed that a total of 58 compounds were tentatively identified in the extract using LC-Q-TOF-MS, some of which have been previously reported to exhibit anti-inflammatory activity. The dichloromethane and ethyl acetate fractions yielded eight compounds including one terpenoid, four flavonoids and three benzophenones. DCM-C2, DCM-C4, EA-C5 and EA-C7 demonstrated the highest inhibition of NO production in LPS-stimulated RAW 264.7 macrophages with EC_{50}=27.75, 35.25, 16.65 and 27.78 μg/mL, respectively. The extract-loaded nanocapsules (ALEX-M-PNCs) were spherical in shape with desirable size (167.13±1.24 nm) and polydispersity index (0.29±0.01) and demonstrated relatively high encapsulation efficiency (87.36±1.81 %). ALEX-M demonstrated high viability on RAW 264.7 cells and towards zebrafish embryos at high concentrations in contrast to nanocapsules that showed relatively higher cytotoxicity. ALEX-M and ALEX-M-PNCs demonstrated ability to reduce NO production in LPS-stimulated macrophages in a dose-dependent manner with EC_{50} values of 164.790±6.318 μg/mL and 5.50 ± 0.31 μg/mL, respectively. A significant reduction in the amount of NO was also observed with ALEX-M and ALEX-M-PNCs in LPS-treated zebrafish embryos with EC_{50} values of 43.09±7.67 μg/mL and 11.92±2.61 μg/mL, respectively. The results confirmed the traditional use of *A. malaccensis* in the management of inflammation and supports its use as a safe and Halal therapeutic alternative. In addition, nano-encapsulation of the extract potentiated its anti-inflammatory effect. Further in-depth research can be conducted to elucidate the mechanisms underlying the anti-inflammatory effect of the extract.

خلاصة البحث

ان الاستخدام طويل الأمد للعقاقير الطبية المضادة للالتهابات وما يترتب عليها من أعراض جانبية قد أثار الكثير من المخاوف مؤخرا. ومن هنا، بدأت الصناعات الدوائية في تبني منهج الحلال لتحقيق المعايير القياسية للسلامة والجودة. وفي هذا البحث، تم اختيار شجرة *Aquilaria malaccensis* ، من عائلة Thymelaeaceae، وهي احدى أشجار العود المتركزة جغرافيا في الغابات المطيرة الاندومالیزية، حيث سبق استخدام أجزاء مختلفة من الشجرة في العلاج التقليدي للعديد من المشاكل الصحية بما في ذلك علاج الالتهابات، إلا أن الاثبات العلمي لهذا الاستخدام التقليدي للنبات كمضاد للالتهابات لا يزال مطلوباً. في هذه الدراسة، يخضع المستخلص الإيثانولي للنبات (ALEX-M) إلى دراسة شاملة للخصائص باستخدام الفحص الكيميائي ومطياف متحول فوريه في مجال الضوء تحت الأحمر (FTIR) ومطياف الكتلة للكروماتوغرافيا الغازية (GC-MS) و مطياف الكتلة للكروماتوغرافيا السائلة (LC-MS) بالاضافة إلى فصل المركبات النشطة بيولوجيا ذات الفاعلية المضادة للالتهابات باستخدام عمود الكروماتوغرافيا (CC) ثم توضيح هياكلها باستخدام طرق التحليل المختلفة مثل HREIMS و IR و UV و NMR أحادي وثنائي الأبعاد. ومن خلال استخدام طريقة المستحلب المركب (w / o / w) واختيار الصمغ Tragacanth كبوليمر طبيعي وحلال، تم تصنيع الكبسولات النانوية البوليمرية لتغليف المستخلص.في المعمل (in-vitro)، تم اجراء اختبار السمية الخلوية والنشاط المضاد للالتهابات للمستخلص الحر (ALEX-M) أو المستخلص داخل الحبوب النانوية (ALEX-M-PNCs) في خلايا RAW 264.7 وباستخدام أجنة سمك الزيبرا. أظهرت النتائج أنه عن طريق استخدام مطياف الكتلة للكروماتوغرافيا السائلة (LC-Q-TOF-MS)، تم الكشف عن وجود ما يعادل ٥٨ مركبا بصورة مبدئية في المستخلص، وقد تم إثبات فعالية بعض هذه المركبات في أبحاث سابقة كمركبات مضادة للالتهابات. و تم استخلاص ثمانية مركبات من مستخلص المذيبات العضوية - مثل ثنائي كلورو ميثان وأسيتات الإيثيل - تشمل تربينويد واحد وأربعة من مركبات الفلافونويد وثلاثة من بنزوفينونات . واتضح أن المركبات (DCM-C2, DCM-C4, EA-C5, EA-C7) أثبتت فعالية في تثبيط انتاج أكسيد النيتريت (NO) في الخلايا RAW 264.7 المحفزة بـ LPS وكان التركيز الذي ثبط ٥٠٪ (EC_{50}) من أكسيد النيتريت (NO) لهذه المركبات يعادل 27.75 و 35.25 و 16.65 و 27.78 ميكروغرام / مل ، على التوالي. كما وجد أن الكبسولات النانوية المحملة بالمستخلص كروية الشكل وذات حجم نانوي (167.13±1.24 nm) بمعامل التشتت المتعدد polydispersity (0.29±0.01) ، كما أظهرت كفاءة تغليف عالية نسبيا (87.36 ± 1.81٪). أظهرت الكبسولات النانوية ALEX-M-PNCs سمية خلوية أعلى نسبيا من ALEX-M في خلايا RAW 264.7 واتجاه أجنة سمك الزيبرا. وكذلك أظهر كلا من المستخلص الحر والمستخلص داخل الحبوب النانوية القدرة على تقليل إنتاج أكسيد النيتريت (NO) المحفزة عن طريق LPS في خلايا RAW 264.7 وتزداد طرديا بزيادة الجرعة. و بحساب قيمة التركيز الذي ثبط ٥٠٪ من أكسيد النيتريت (EC_{50}) المستخلص الحر والمستخلص داخل الحبوب النانوية ، كانت الجرعات 164.790±6.318 ميكروغرام/مل و 5.50±0.31 ميكروغرام/مل ، على التوالي. كما لوحظ أيضاً انخفاض كبير في كمية NO في أجنة سمك الزيبرا المعالجة بالمستخلص الحر والمستخلص داخل الحبوب النانوية بعد تحفيزها بمركب LPS وبلغ التركيز الذي ثبط ٥٠٪ (EC_{50}) من أكسيد النيتريت 43.09±7.67 ميكروغرام/مل و 11.92±2.61 ميكروغرام / مل ، على التوالي. وخلصت نتائج البحث إلى دعم الاستخدام التقليدي لـ *Aquilaria malaccensis* في علاج الالتهابات كبديل علاجي آمن وحلال. بالإضافة إلى ذلك ، فإن تحميل المستخلص في حبوب نانوية يعزز من تأثيره المضاد للالتهابات كما ظهر في خلايا RAW 264.7 وأجنة سمك الزيبرا. ويمكن إجراء مزيد من البحوث المتعمقة لتوضيح الآليات الكامنة وراء التأثير المضاد للالتهابات اللمستخلص.

APPROVAL PAGE

The thesis of Manar Abdullah Eissa has been approved by the following:

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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I dedicate my dissertation to my family and my beloved husband. My achievements were made possible by their constant love and encouragement. Their immeasurable support has sustained me throughout my journey.

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ALEX, ALEX-M-PNCs and B-PNCs on LPS-induced NO production.

LIST OF EQUATIONS

LIST OF ABBREVIATIONS

NSAIDs	Non-steroidal anti-inflammatory drugs
OECD	Organization for Economic Cooperation and Development
$AlCl_3$	Aluminum Chloride
NO	Nitric oxide
PGE2	Prostaglandin E2
NF-$\kappa\beta$	Nuclear factor kappa B
TNFα	Tumor necrosis factor-α
ILs	Interleukins
PLA2	Phospholipase A2
COX	Cyclooxygenase
LOX	Lipoxygenase
PGs	Prostaglandins
TXs	Thromboxanes
LTs	Leukotrienes
BSA	Bovine Serum Albumin
iNOS	Induced nitric oxide synthase
LPS	Lipopolysaccharide
WHO	World Health Organization
FTIR	Fourier Transformed Infrared Spectroscopy
IR	Infrared
GC-MS	Gas Chromatography Mass Spectrometry
LC-MS	Liquid Chromatography Mass Spectrometry
HPLC	High Performance Liquid Chromatography
MS	Mass Spectrometry
LC	Liquid Chromatography
ESI	Electron Spray Ionization
CC	Column Chromatography
TLC	Thin layed Chromatography
UV	Ultraviolet
NMR	Nuclear Magnetic Resonance
^{1}H-NMR	Proton nuclear magnetic resonance
^{13}C-NMR	Carbon-13 nuclear magnetic resonance
COSY	Correlation Spectroscopy
HMBC	Heteronuclear Multiple Bond Correlations
HMQC	Heteronuclear Multiple Quantum Coherence
GLUT4	Glucose transporter type 4
GRAS	Generally recognized As Safe
PLA	poly lactic acid
PGA	poly (D, L-glycolic acid)
PLGA	poly lactide-co-glycolide
o/w	Oil-in-water emulsion
w/o	Water-in-oil emulsion
w/o/w	Water-in-oil-water emulsion
o/w/o	Oil-in-water-in-oil emulsion

PLA-TPGS	Polylactide Tocopheryl Polyethylene Glycol Succinate
TG	Tragacanth Gum
FDA	Food and Drug Administration
CC_{50}	Cytotoxic Concentration that causes death of 50% of cells
DLS	Dynamic Light Scattering
PDI	Polydispersity Index
ZP	Zeta potential
EE	Encapsulation Efficiency
LC	Loading Capacity
TEM	Transmission Electron Microscope
UV-Vis	Ultraviolet Visible
TGA	Thermogravimetric Analysis
Hpf	Hours Post Fertilization
Dpf	Days Post Fertilization
DMSO	Dimethyl Sulfoxide
ROS	Reactive Oxygen Species
LC_{50}	Lethal concentration that causes death to 50% of the test subjects
KAED	Kulliyah of Architecture and Environmental Design
DMEM	Dulbecco's Modified Eagle's Medium
FRIM	Forest Research Institute Malaysia
WST-1	4-[3-(4iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
BUE	British university in Egypt
DAF-FM-DA	4-Amino-5-methylamino-2',7'-difluorofluorescein diacetate
OEC	Oral Epithelial Cells
CREAM	Central Research and Animal Facility
UPM	Universiti Putra Malaysia
rpm	Rotation per minute
TIC	Total Ion Chromatography
NIST	National Institute of Standards and Technology
LC-Q-TOF-MS	Liquid Chromatography Quadrupole Time of Flight Mass Spectrometry
HRESIMS	High-Resolution Electrospray Ionization Mass Spectrometry
CCHE	Cancer Children Hospital Egypt
MP-WS	Mobile Phase Working Solution
PCDL	Personal Compound Databases and Library
MoNA	MassBank of North America
HMDB	Human Metabolome Database
FBS	Fetal Bovine serum
PBS	Phospahe Buffer Saline
VLC	Vaccum Liquid Chromatography
IFN-γ	Interferon-γ
IC_{50}	Inhibitory concentration that causes inhibition of the effect by 50%
EC_{50}	Effective concentration that gives 50% of maximum response
Kcps	Kilo count per second
ALEX-M	Aquilaria Leaf Extract obtained by Macceration

ALEX-M-PNCs	Aquilaria Leaf Extract obtained by Macceration Polymeric Nanocapsules
B-PNCs	Blank nanocapsules
ZFET	Zebrafish Embryo Toxicity Test
LC_{50}	Lethal concentration that causes death to 50% of the test subjects
bpm	beat per minute
SD	Standard Deviation
ANOVA	Analysis of Variance
RT	Retention Time
SFE	Supercritical Fluid Extraction
NDGA	Nordihydroguaiaretic Acid
HRF	Heterocyclic Ring Fission
BFF	Benzofuran Ring Fission
RDA	Retro-Diels Alder
HPETEs	Hydro peroxy eicosa tetraenoic acids
ppm	Part per minute
Rf	Retention factor
DCM	Dichloromethane
EA	Ethyl Acetate
UPSI	Universiti Pendidikan Sultan Idris
DCM-C1	Dichloromethane-compound 1
DCM-C2	Dichloromethane-compound 2
DCM-C3	Dichloromethane-compound 3
DCM-C4	Dichloromethane-compound 4
EA-C5	Ethyl acetate-compound 5
EA-C6	Ethyl acetate-compound 6
EA-C7	Ethyl acetate-compound 7
EA-C8	Ethyl acetate-compound 8
HLB	Hydrophillic Lipophillic Balance
FESEM	Field Emission Scanning Electron Microscope