UNIVERSITI PUTRA MALAYSIA

PHOTOPROTECTIVE PROPERTIES, CHEMICAL, BIOLOGICAL AND PRODUCT FORMULATION STUDIES ON Zanthoxylum rhetsa (Roxb.) DC.

RAMESHKUMAR SANTHANAM

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PHOTOPROTECTIVE PROPERTIES, CHEMICAL, BIOLOGICAL AND PRODUCT FORMULATION STUDIES ON *Zanthoxylum rhetsa* (Roxb.) DC.

By

RAMESHKUMAR SANTHANAM

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

November 2016

DEDICATION

This thesis is dedicated to my beloved family, Professor and friend

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Doctor of Philosophy

PHOTOPROTECTIVE PROPERTIES, CHEMICAL, BIOLOGICAL AND PRODUCT FORMULATION STUDIES ON *Zanthoxylum rhetsa* (Roxb.) DC.

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RAMESH KUMAR SANTHANAM

November 2016

Chairman : Professor Khozirah Shaari, PhD
Institute : Bioscience

Protection against photo oxidative damages is essential for living organisms. Humans especially need to counteract oxidative stress and damages caused by harmful UV radiations. *Zanthoxylum rhetsa* (Indian Prickly Ash) is an aromatic tree, commonly found in tropical regions. Various parts of this plant species have been used medicinally to treat health disorders such as diabetes, cholera, microbial infections and rheumatism. Recently, the seeds of *Z. rhetsa*, has been investigated for its sunscreen potential with promising results. However the photo protective properties of other parts of this plant species remains unexplored. The objective of this study is to evaluate the bark material of *Z. rhetsa* for its photo protective and anti-aging properties. The crude methanolic extract of *Z. rhetsa* bark and its various solvent fractions were preliminarily screened, *in vitro*, for their UV protection properties. In comparison with other fractions, the ethyl acetate fraction revealed the highest SPF value (13.36 at 100 µg/ml), significant UVA/UVB absorption, free radical scavenging, anti-collagenase and anti-elastase properties.

Subsequently, the extract and fractions were tested for their cytotoxic effect against human dermal fibroblasts (HDF) and mouse melanoma (B16-F10), representing normal and cancer cell lines, respectively. All the test samples were found to be non-toxic to HDF cells. However, the chloroform fraction revealed cytotoxicity towards the melanoma B16-F10 cells with an IC_{50} value of 132.7 µg/ml. Diverse array of compounds present in the solvent fractions were identified using GC-MS analysis. Compounds such as lupeol, yangambin, kobusin, columbamine and hesperidin were isolated and identified from the bioactive fractions of *Z. rhetsa*. All the isolated compounds were tested for their cytotoxic effect against HDF and melanoma B16-F10 cell lines. The compounds were all found to be non-toxic to HDF cells. Meanwhile, two of the isolated compounds, kobusin and columbamine, were observed to have significant cytotoxic effect against the melanoma B16-F10 cells, with IC_{50} value of 112.2 µg/ml and 195.6 µg/ml, respectively.

Furthermore the isolated compounds were tested for their SPF value, where hesperidin showed an SPF value of 13.38 at 100 µg/ml, which is almost the same as the SPF value of the ethyl acetate fraction. This indicated that the UV protection property of the solvent fraction is largely due to the presence of hesperidin. The ethyl acetate fraction and hesperidin were further tested for UVB induced cytotoxicity, and for inhibition of inflammatory cytokines (IL-1β, IL-6 and TNF-α) and of MMPs (collagenase; MMP1, stromelysin-1; MMP3 and gelatinase; MMP9). The ethyl acetate fraction and hesperidin significantly prevent cell death, inhibited the expressions of pro-inflammatory cytokines and MMPs.

Due to the favourable UV protection properties of the ethyl acetate fraction, it was further evaluated for its suitability as an active ingredient (10% w/w) in two sunscreen formulations (F1 & F2). The F1 and F2 cream, respectively showed an SPF value of 3.60 ± 0.28 and 6.90 ± 0.57, a UVA/UVB ratio of 0.469 and 0.538 and critical wavelength of 365.3 and 360, with moderate boot star rating, pseudo plastic behaviour and high microbial growth resistance. Altogether, these results support the photo protective property of the Z. *rhetsa* bark and its extract, particularly the ethyl acetate fraction of the methanolic extract has the potential to be developed further as an active ingredient in sunscreen and other cosmeceutical products. To the best of our knowledge this is the first report on the photo protective properties of the bark material of *Z. rhetsa* in extract as well as in cream formulation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

SIFAT FOTOPROTEKTIF, KIMIA DAN BIOLOGI SERTA PENGAJIAN FORMULASI PRODUK DARIPADA *Zanthoxylum rhetsa*

Oleh

RAMESH KUMAR SANTHANAM

November 2016

Pengerusi : Profesor Khozirah Shaari, PhD
Institut : Biosains

Perlindungan terhadap kerosakan oksidatif foto adalah penting untuk semua organisma hidup. Manusia terutamanya perlu mengatasi tekanan oksidatif dan kerosakan yang disebabkan oleh sinaran UV yang berbahaya. *Zanthoxylum rhetsa* (Hantu Duri or Batang berduri) adalah sejenis tumbuhan beraroma, yang biasanya dijumpai di kawasan tropika. Pelbagai bahagian dari spesis tumbuhan ini telah digunakan dalam perubatan untuk merawat gangguan kesihatan seperti kencing manis, taun, jangkitan mikrob dan penyakit sendi. Baru-baru ini, biji benih *Z. rhetsa*, telah dikaji kerana berpotensi sebagai pelindung matahari yang menjanjikan keberkesanan yang baik. Walau bagaimanapun sifat-sifat pelindung foto untuk bahagian lain daripada spesies tumbuhan ini masih belum diterokai. Objektif kajian ini adalah untuk mengkaji potensi ekstrak dan sebatian di dalam kulit *Z. rhetsa* yang bermanfaat untuk perlindungan foto dan anti-penuaan. Ekstrak metanol mentah kulit *Z. rhetsa* dan pelbagai pecahan pelarut awalnya telah disaringkan, *in vitro*, untuk sifat perlindungan UV mereka. Dalam perbandingan dengan pecahan lain, pecahan etil asetat mendedahkan nilai SPF tertinggi (13.36 pada 100 µg / ml), penting dalam penyerapan UVA / UVB, radikal bebas skaveng, anti-kolagenase dan anti-elastin.

Ekstrak mentah dan pecahan pelarut telah diuji untuk kesan sitotoksik terhadap sel kulit fibroblas manusia (HDF) dan melanoma tikus (B16-F10), yang masing-masing mewakili bahagian sel normal dan kanser. Semua sampel tumbuhan yang diuji didapati tidak toksik kepada sel-sel HDF. Walau bagaimanapun, pecahan kloroform mempamerkan kesan toksik terhadap sel-sel melanoma B16-F10 dengan nilai IC_{50}, 132.7 µg/ml. Kepelbagaian sebatian yang hadir dalam pecahan pelarut telah dikenal pasti melalui analisis GC-MS. Sebatian seperti lupeol, yangambin, kobusin, columbamin telah diasingkan dan dikenal pasti serta diuji untuk kesan sitotoksik terhadap HDF dan sel melanoma B16-F10. Kesemua sebatian didapati tidak toksik kepada sel-sel HDF. Sementara itu, dua daripada sebatian terpencil, kobusin dan columbamin, dilihat mempunyai kesan sitotoksik yang kuat terhadap sel melanoma B16-F10, dengan masing-masing memberikan nilai IC_{50} 112.2 µg/ml dan 195.6 µg/ml.

Tambahan pula, sebatian terpencil telah disaringkan untuk nilai SPF, di mana hesperidin menunjukkan nilai SPF 13.38, yang hampir sama dengan nilai SPF bagi pecahan etil asetat. Ini menunjukkan bahawa sifat perlindungan UV dari pecahan pelarut sebahagian besarnya adalah disebabkan oleh kehadiran hesperidin. Pecahan etil asetat dan hesperidin selanjutnya diuji untuk kesan sitotoksik aruhan UVB, dan seterusnya penghambatan radang sitokina (IL-1β, IL-6 dan TNF-α) dan MMPs (kollagenase; MMP1, stromelisin-1; MMP3 dan gelatinase; MMP9). Pecahan etil asetat dan hesperidin menghalang kematian sel secara signifikan,menghambat penglahiran sitokina pro-keradangan dan MMPs.

Oleh kerana sifat perlindungan UV yang menggalakkan dari pecahan etil asetat, ia telah dikira sebagai sesuai untuk dijadikan sebagai bahan aktif dalam formulasi pelindung matahari. Satu percubaan awal telah dijalankan di mana 10% pecahan etil asetat telah dimasukkan ke dalam dua formulasi krim pelindung matahari (F1 dan F2). Formulasi F1 dan F2 krim, masing-masing menunjukkan nilai SPF sebanyak 3.60 ± 0.28 dan 6.90 ± 0.57, nisbah UVA/UVB 0.469 dan 0.538, dan panjang gelombang kritikal 365.3 dan 360, dengan penarafan sederhana bagi bintang Boot , tingkah laku semuplastik dan kesan rintangan pertumbuhan mikrob yang tinggi. Keseluruhannya, keputusan ini menyokong sifat perlindungan foto daripada kulit *Z. rhetsa* dan ekstrak, terutamanya pecahan etil asetat dari ekstrak methanol, yang mempunyai potensi untuk dibangunkan sebagai bahan aktif dalam produk pelindung matahari dan produk kosmeseutikal yang lain. Dalam pengetahuan kami, ini adalah laporan pertama untuk sifat-sifat pelindungan foto bahan kulit *Z. rhetsa* dalam ekstrak serta dalam formulasi krim.

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I certify that a Thesis Examination Committee has met on 14 November 2016 to conduct the final examination of Rameshkumar Santhanam on his thesis entitled "Photoprotective Properties, Chemical, Biological and Product Formulation Studies on *Zanthoxylum rhetsa* (Roxb.) DC." 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 Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Yaya Rukayadi, PhD
Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairman)

Gwendoline Ee Cheng Lian, PhD
Professor
Faculty of Science
Universiti Putra Malaysia
(Internal Examiner)

Faridah binti Abas, PhD
Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Internal Examiner)

Satyajit Dey Sarker, PhD
Professor
Liverpool John Moores University
United Kingdom
(External Examiner)

NOR AINI AB. SHUKOR, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 26 January 2017

This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Khozirah Shaari, PhD
Professor
Institute of Bioscience
Universiti Putra Malaysia
(Chairman)

Syahida Ahmad, PhD
Senior Lecturer
Faculty of Biotechnology and Biomolecular Science
Universiti Putra Malaysia
(Member)

Sharida Fakurazi, PhD
Associate Professor
Institute of Bioscience
Universiti Putra Malaysia
(Member)

ROBIAH BINTI YUNUS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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Signature: _____
Name of Member of Supervisory Committee: Dr. Syahida Ahmad

Signature: _____
Name of Member of Supervisory Committee: Associate Professor Dr. Sharida Fakurazi

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LIST OF ABBREVIATIONS

AAAPVN	N-Succinyl-Ala-Ala-Ala-p-nitroanilide
AKT	AKT8 virus oncogene cellular homolog
AO/EB	Acridine Orange/Ethidium Bromide
AP-1	Activator protein -1
APCI	Atmospheric Pressure Chemical Ionization
APPI	Atmospheric Pressure Photo Ionization
CFU	Colony Forming Unit
CI	Chemical Ionization
COX-2	Cyclooxygenase-2
CPDs	Cyclobutane Pyrimidine Dimers
CYR	Cysteine-Rich Protein
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOPA	Dihydroxyphenylalanine
DPPH	Diphenyl-1-Picrylhydrazyl
EA	Ethyl Acetate
ECM	Extracellular matrix
EGCG	Epigallocatechin Gallate
EI	Electron Ionization
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular signal-Regulated Kinase
ESI	Electrospray Ionization
EU	European Union

EWG	Environmental Working Group
FAB	Fast Atom Bombardment
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GAE	Gallic Acid Equivalents
GC	Gas Chromatography
GC-MS	Gas Chromatography mass spectrometry
GSH	Glutathione reductase
HAE	Hydroxy alkenals
HPLC	High Performance Liquid Chromatography
HRP	Horse Radish Peroxidase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
I-κB	Inhibitor of NF-κB
JAK	Janus-family Tyrosine Kinase
JNK	Jun N-terminal Kinase
LC	Liquid Chromatography
LC-MS	Liquid chromatography mass spectrometry
LPS	Lipopolysaccharides
MAD	Malondialdehyde
MAPK	Mitogen Activated Protein Kinase
MMPs	Matrix Metalloproteinases
MS	Mass spectrometry
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide

NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NF-κB	Nuclear Factor-kappa B
NMR	Nuclear Magnetic Resonance
NO	Nitric oxide
Nrf2-ARE	Nuclear factor –erythroid 2-Antioxidant Response Element
OHDG	Hydroxy-deoxyguanosine
P13K	Phosphoinositide 3 kinase
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDA	Photo Diode Array
PGE2	Prostaglandins
PMMA	Poly Methyl Methacrylate
PP	Pyrimidine-Pyrimidone
PPD	Persistent Pigment Darkening
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
PVDF	Polyvinylidene Fluoride
QE	Quercetin Equivalent
qRT-PCR	Quantitative Reverse-Transcriptase Polymerase Chain Reaction
Rf	Retention factor
RI	Retention Index
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SA-β-gal	senescence-associated β-galactosidase
SDS	Sodium Dodecyl Sulphate

SI	Similarity Index
SIM	Selected Ion Montioring
SMAD	Suppressor of Mothers Against Decapentaplegic (Contraction of Sma and Mad)
SPF	Sun Protection Factor
STAT	Signal Transducer and Activator of Transcription
TFC	Total Flavonoid Content
TGF-β	Transforming Growth Factor- β
TIMP	Tissue Inhibitor of Metalloproteinase
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
TNF-α	Tumor Necrosis Factor-α
TOF	Time of Flight
TPC	Total Phenolic Content
UCA	Urocanic Acid
UK	United Kingdom
USA	United States of America
USD	United States Dollar
UV	Ultraviolet
UVA	Ultraviolet A
UVB	Ultraviolet B
UVC	Ultraviolet C
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Introduction

Aging is the natural process of getting older. Genetically, aging cannot be avoided but it can be slowed down by modulating or altering the environmental factors. In general, aging is classified into two types i.e., “intrinsic aging and extrinsic aging". Intrinsic aging is a natural aging process which occurs in all living things. It is due to genetic inheritance and is the result of deterioration in cell function and metabolism over the passage of time (Puizina-Ivic, 2008). Extrinsic aging, also known as photo aging, occurs as a result of various environmental factors, notably due to ultraviolet (UV) radiation. Natural aging can be slowed down but it is unalterable whereas extrinsic aging is alterable by preventing the UV exposure (Sjerobabski-Masnec and Šitum, 2010). Ultraviolet radiation is the part of electromagnetic radiation that reaches the earth’s surface in the form of sunlight. UV radiation from the Sun is divided into three types based on the magnitude of their wavelength, i.e., UVA (320-400 nm), UVB (290-320 nm) and UVC (200-290nm). About 95% of UV radiation reaching the earth surface is UVA. In comparison, far less UVB reaches the surface although the radiation is more intense. Meanwhile UVC is prevented from reaching the earth's atmosphere by the ozone layer (Balakrishnan and Narayanasamy, 2011; Chen et al.,2012).

Skin is the primary organ that is directly exposed to the sun and is therefore affected by the UV radiation. Absorbtion of this radiation results in DNA damage and excessive discharge of reactive oxygen species (ROS). These ROS deliberately activates the receptors and modulates the signaling pathways such as transforming growth factor (TGF-β), activator protein 1(AP-1) and NF-κB. These, in turn, lead to the release of several pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, and finally, promote the expression of matrix metalloproteinases (MMPs) (Vicentini et al., 2011; Chen et al., 2012). Out of 19 MMPs in normal human skin, collagenase; MMP1, stromelysin-1; MMP3 and gelatinase; MMP9 are significantly expressed by UV radiation (; Fisher et al., 2002; Quan et al., 2009). The skin cells in all living organisms characteristically possess a natural repairing mechanism as a response to the harmful effects of UV radiation. Particularly in humans, protection from UV radiation is naturally possible due to the presence of biological chromophores such as nucleic acids, amino acids, urocanic acid, and melanin precursors in the layers of skin (Antony, 1997). Due to ozone depletion and other environmental factors, the amount of UVA/UVB radiation reaching the Earth’s surface atmosphere has increased significantly over recent decades (World Health Organization, 2016). Prolonged exposure to UV radiation results in various acute and chronic effects. Acute effect results in the skin being sunburned, reddened and tanned. Meanwhile, chronic effect results in skin cancer, cataract and aging. According to WHO, 2 to 3 million non-melanoma and 132,000 melanoma cancers occur globally, every year. In addition, it has been predicted that a 10% decrease in the stratospheric ozone layer may result in 300,000 and 4500 additional cases, annually, of non-melanoma and melanoma skin

cancers, respectively (World Health Organization, 2016). These estimated figures, indicated that the normal immune response and repairing mechanism of the human body is no longer sufficient to overcome the onslaught of the resulting UV-induced molecular damage. Hence, humans will need an additional external means of protection from UV radiations. Efficient protection from UVA/UVB radiation can help reduce irrefutable signs of premature aging, deep wrinkles and skin cancers.

Globally, many health authorities and regulatory bodies unanimously recommended sunscreen as one of the option to protect against the harmful effects of UV radiation. Many are increasing their efforts at creating awareness and setting guidelines on proper use of sunscreen as well as conducting researches to develop effective sunscreen products. In fact, research has shown that proper application of sunscreen offers almost 100 percent protection against squamous cell carcinoma, basal cell carcinoma and malignant melanoma cancer *via* shielding the p53 gene (Hacker et al., 2013). The global market for suncare products is expected to reach USD 11.1 billion by the year 2020 (The Global Sun Care Products Market, 2015). Transparent Market Research, USA, in its report also stated that the global market value of skin care products is expected to reach USD 155.4 billion by 2021, (Transparent Market Research, 2015). For ages, synthetic-based sunscreen products have dominated the market. Recently however, many of these synthetic ingredients were shown to be toxic. In 2015, the leading US based non-profit environmental research organization known as the Environment Working Group (EWG) released a report stating that out of 1700 sunscreen products (USA market) screened, about 80% of the products contain toxic chemicals and offers less sun protection. These toxic chemicals can cause skin allergy, hormone disruption and various other problems by penetrating into the skin and blood (Environmental working group, 2015). It also pollutes the environment and coastal waters by releasing free radicals (Tovar-Sánchez et al., 2013), overall threatens the public to use synthetic sunscreens. Thus, in order to avoid the dangers and side effects of using toxic chemicals, consumers are placing greater preference for 'natural sunscreen' products containing plant-based ingredients which are believed to be safer and yet effective. Recently Zion research, the leading market research and social research company based in India, reported that the demand for organic personal care products (include oral care, skin care, hair care and other cosmetics) are increasing. In 2014 the market value was around USD 9.2 billion and is expected to reach USD 16 billion in 2020 (Globe Newswire, 2015). Many research studies are being conducted on evaluation and development of natural sunscreens derived from traditional medicine and other medicinal plants (Jung et al., 2014; Pallela et al., 2010). In fact, well known botanicals such as green tea, aloe vera, neem and cucumber are already in use as active ingredients in many personal care products (Korać and Khambholja, 2011). For natural sunscreens, the plant extract and their phytochemical constituents are tested for their toxicity, sunscreen protection factor (SPF) value, UVA/UVB absorption spectrum, free radical scavenging, anti-inflammatory response and MMP inhibition properties (Reis Hinneburg et al., 2006; Khazaeli and Mehrabani, 2010; Mansur et al., 2016). In this research the well-known traditional medicinal plant *Zanthoxylum rhetsa*, particularly its bark material, was investigated for its photoprotective properties. A simple sunscreen cream formulation was further developed and its properties evaluated.

1.2 Problem statement and Justification

The use of natural compounds and botanical extracts are becoming more common although they have not completely replaced the dominance of synthetic materials. Interests in natural ingredients in sunscreen formulation is mainly driven by the 'back to nature' movement and the promise of equal or greater efficiency with lesser side effects by using these materials. Despite recent findings illustrating good sunscreen activity of the methanolic extract of *Z. rhetsa* seeds (Kale et al., 2011), very little is known about the photoprotective and anti-oxidative effects of the plant as a whole. Preliminary investigation on the methanolic bark extract of *Z. rhetsa* against UV protection properties gave very interesting and encouraging results. The bark extract, specifically the ethyl acetate soluble constituents, showed excellent Sunscreen Protection Factor (SPF) value. This is much better in comparison to other natural sunscreen phytoingredients such as *Camellia sinesis* (green tea) and *Aloe vera*. These results strongly indicated that *Z. rhetsa* bark extract could be further developed as a natural active ingredient for an effective broad spectrum sunscreen and anti-ageing cream. The extract of *Z. rhetsa* may be useful as a broadly effective UVB- and UVA-screening agent for topical application to skin and other surfaces where enhanced UV-protection against damaging effects of solar ultraviolet radiation is desired.

1.3 Objective

1. To evaluate the photoprotective properties of *Z. rhetsa* bark extract through several primary antioxidant and anti-photo aging bioassays.
2. To determine the toxic and cytotoxic effect of the extract/fractions against Human Dermal Fibroblasts (HDF) and B16-F10 mouse melanoma cells, respectively.
3. To identify the chemical constituents present in the extract/fractions of *Z. rhetsa* using GC-MS analysis.
4. To isolate and identify the compounds from the bioactive fractions of *Z. rhetsa* using column chromatography, mass spectroscopy and NMR analysis.
5. To identify the UVB induced toxicity, inflammatory cytokines and MMP inhibition properties of the bioactive fraction /compound of *Z. rhetsa* against HDF cells.
6. To formulate topical sunscreen creams using the bioactive fractions and determine the physicochemical and biological properties of the formulated cream by *in vitro* analysis.

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LIST OF PUBLICATIONS

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