PHYTOCHEMICALS FROM ROOT BARKS OF *GARCINIA COWA* ROXB. AND ANTIOXIDANT ACTIVITY

PONGSAN KORNANAN

Graduate School Srinakharinwirot University

2022

พฤกษเคมีจากเปลือกรากชะมวงและฤทธิ์ ยับยั้งการเกิดออกซิเดชัน

พงศ์สันต์ กรอานันท์

ปริญญานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตร
วิทยาศาสตรมหาบัณฑิต สาขาวิชาเคมี
คณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ
ปีการศึกษา 2565

PHYTOCHEMICALS FROM ROOT BARKS OF *GARCINIA COWA* ROXB. AND ANTIOXIDANT ACTIVITY

PONGSAN KORNANAN

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of MASTER OF SCIENCE

(Chemistry)

Faculty of Science, Srinakharinwirot University

2022

THE THESIS TITLED

PHYTOCHEMICALS FROM ROOT BARKS OF *GARCINIA COWA* ROXB. AND ANTIOXIDANT ACTIVITY

BY

PONGSAN KORNANAN

HAS BEEN APPROVED BY THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT

OF THE REQUIREMENTS FOR THE MASTER OF SCIENCE

IN CHEMISTRY AT SRINAKHARINWIROT UNIVERSITY

(Assoc. Prof. Dr. Chatchai Ekpanyaskul, MD.)

Dean of Graduate School

ORAL DEFENSE COMMITTEE

..... Major-advisor

(Prof. Dr.Sunit Suksamrarn)

..... Chair

(Assoc. Prof. Dr.Boon-ek Yingyongnarongkul)

..... Co-advisor

(Dr.Kulvadee Dolsophon)

..... Committee

(Asst. Prof. Dr.Nuttapon Apiratikul)

Title	PHYTOCHEMICALS FROM ROOT BARKS OF *GARCINIA COWA* ROXB. AND ANTIOXIDANT ACTIVITY
Author	PONGSAN KORNANAN
Degree	MASTER OF SCIENCE
Academic Year	2022
Thesis Advisor	Professor Dr. Sunit Suksamrarn
Co Advisor	Dr. Kulvadee Dolsophon

Garcinia cowa Roxb (Cha-muang) is one of the well-known medicinal plants belong to the Clusiaceae family. Many parts of *G. cowa* have been investigated for their interesting phytochemicals. In this work, the root barks of *G. cowa* were extracted with EtOAc and then with MeOH, and the extracts were fractionated and purified by column chromatographic techniques. From the MeOH extract obtained, four biflavonoids were identified as (+) volkensiflavone (BIF1), (+) morelloflavone (BIF2), (+) spicataside (BIF3), and (+) fukugiside (BIF4). The structure of all isolated compounds was elucidated by spectroscopic techniques, especially IR, 1D- and 2D- NMR including by comparison of their spectroscopic data with those reported in the literature. The antioxidant activity of the extracts and the isolated compounds were tested using DPPH radical scavenging assay. The isolated compounds BIF2 and BIF4 as well as the MeOH extract exhibited strong activity with IC_{50} values of 8.85, 19.65, and 47.40 μg/mL, respectively.

Keyword : Garcinia cowa Biflavonoids DPPH radical scavenging activity

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my supervisor, Prof. Dr. Sunit Suksamrarn, for her kind and helpful supervision, hearty encouragement, and research assistantship support throughout this work.

I would like to thank all my committee members, Asst. Prof. Dr. Nuttapon Apiratikul my co-advisors, Dr. Kulvadee Dolsophon, for their endless kindness, thoughtful advice, valuable time, patient reading and warm encouragement. In addition, I feel grateful to Assoc. Prof. Dr.Boon-ek Yingyongnarongkul, Ramkhamhaeng University, for his useful comments and encouragement.

This work was partially supported by the Center of Excellence for Innovation in Chemistry (PERCH-CIC).

I am grateful to Department of Chemistry, Faculty of Science, Ramkhamhaeng University for recording the optical rotations.

Many special thanks also go to my teachers, friends, colleagues and staff of the Department of Chemistry, Faculty of Science, Srinakharinwirot University for their friendship, kind support and encouragement.

I would like to thank to Dr. Jantana Yahuafai and Dr. Suratsawadee Piyaviriyakul, Section of Clinical Research, Division of Research and Academic Support, National Cancer Institute for kindly providing the assistance with some in vitro studies.

Finally, I wish to express my profound gratitude to my parents and family for their love, unconditional support and encouragement throughout my whole life.

PONGSAN KORNANAN

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

Chapter 1

Introduction

1.Background

Garcinia species are genus in Clusiaceae were founded in tropical Asia, Africa, New Caledonia, Polynesia and Brazil. These plants contain a wild range of biologically active metabolites which, in the last few decades, have received considerable attention with advantages to treat several diseases (Aruoma, 1998; Santo, Santana, Figueiredo, & Junior, 2020). Recently, Jawaharlal Nehru Tropical Botanic Garden & Research Institute reported *Garcinia* species have received important interest globally from the scientific as well as industry, and several novel skeletons, bioactivities, and potential utilities have been reported. (Aravind, Menon, & Rameshkumar, 2017).

Garcinia cowa Roxb. or Cha-Muang in Thailand belongs to the Clusiaceae family. It grows widely in the tropical rainforest area of Southeast Asia, West and East Africa, Central, and South America. Many parts of *G. cowa* have been used in traditional folk medicine (Ritthiwigrom, Laphookhieo, & G.S., 2013). For example, the barks are used as an antipyresis agent, fruits and leaves are used for the improvement of blood circulation, treatment of coughs, indigestion and as a laxative; the roots and latex are used for fever relief (Pattalung, Thongtheeraparp, & Wiriyachitra, 1994).

Our research group has been interested in exploring new chemical constituents isolated from certain *Garcinia* plants and reported some biological activities, such as cholinesterase inhibitory activity of prenylated xanthone from *G. fusca* (Saenkham et al., 2020). In this research, *G. cowa* root barks have been investigated to isolate and purify for the phytochemical compounds with some biological activities.

2.Objectives

In this study, the work has been set the following objectives:

1. To isolate, purify, and identify the chemical structure of the isolated compounds from the root barks of *G. cowa* extracts.

2. Investigate the biological activities of the compounds obtained.

Chapter 2

Literature review

G. cowa commonly known as Cha-muang in Thai. It is a small to medium sized tree found widely in tropical rainforest area. Many parts of *G. cowa* have been used in traditional folk medicine. For example, the barks, latex and roots have been used as an antifever agent while the fruits and leaves for indigestion, improvement of blood circulation and as an expectorant (Pattalung et al., 1994).

Figure 1 *G. cowa* (Cha-muang)

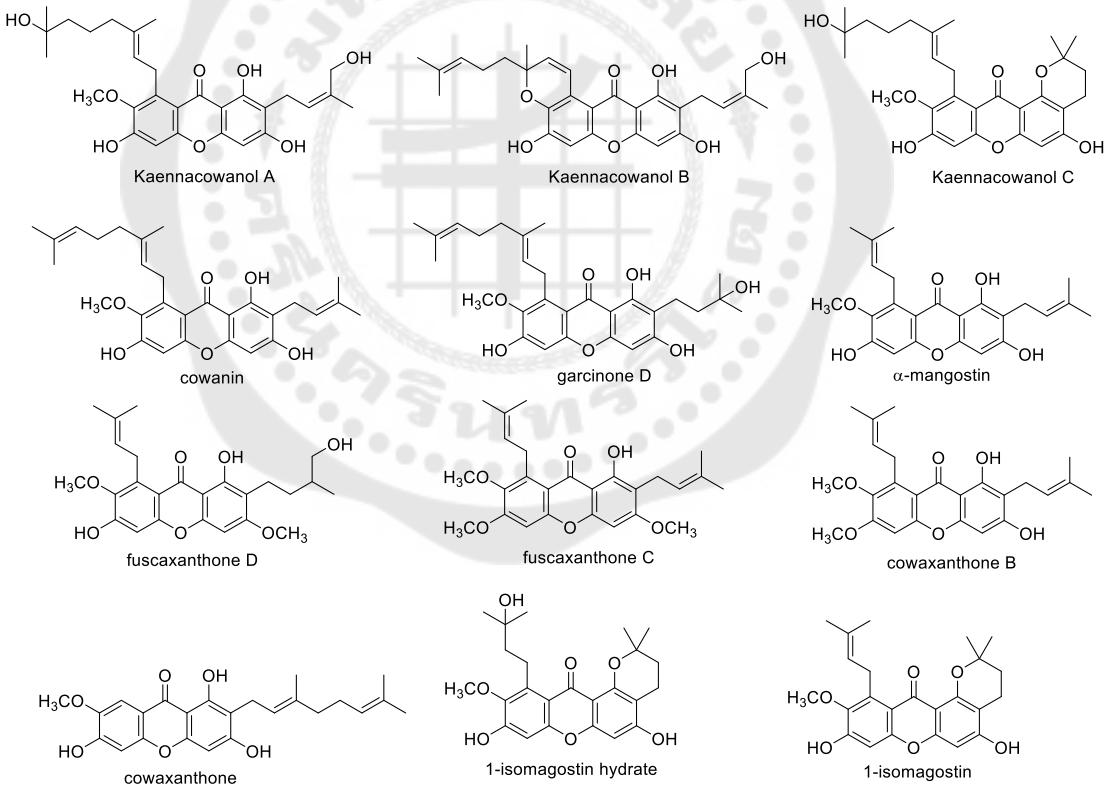
In recent years different parts of *G. cowa* were investigated by solvent extraction with different polarities and further chromatographed to isolate phytochemical compounds and investigated their biological activities.

1. Chemical constituents from *G. cowa*

1.1.Roots

The part of roots of *G. cowa* was reported in several research. The roots were collected from different places such as Kalasin Province, Thailand (Kaennakam, Siripong, & Tip-pyang, 2015), Sarasah Bonta, Harau Valley, and West Sumatra Indonesia (Wahyuni, Shaari, Stanslas, & Hamidi, 2016), and Trang Province Thailand (Tayana, Suteerapatarnon, & Deachathai, 2017).

In 2015, Kaenakam et al. studied the dichloromethane extract of roots in the successful isolation of the 3 new xanthones, kaennacowanols A–C, and other 19 known compounds fuscaxanthone I, cowanol, cowanin, garcinone D, α-mangostin, pruniflorone C, β-mangostin, fuscaxanthone D, fuscaxanthone C, cowaxanthone B, fuscaxanthone F, norcowanin, cowaxanthone, 1-isomagostin hydrate, 1-isomagostin, 9-hydroxycalabaxanthone, 5-hydroxy-8,9-dimethoxy-2,2-dimethyl-7-(3-methyl-2-butenyl)-2H,6Hpyrano[3,2-b]xanthen-6-one, fuscaxanthone A and jacareubin. The isolates were evaluated for their cytotoxicity against KB (human epidermoid carcinoma) and HeLa (human cervical carcinoma) cell lines. The isolated compounds 1-isomagostin hydrate and jacareubin showed IC_{50} value of 7.97 μM and 9.10 μM against KB cell and norcowanin showed IC_{50} value of 9.34 μM against HeLa cell (Kaennakam et al., 2015).



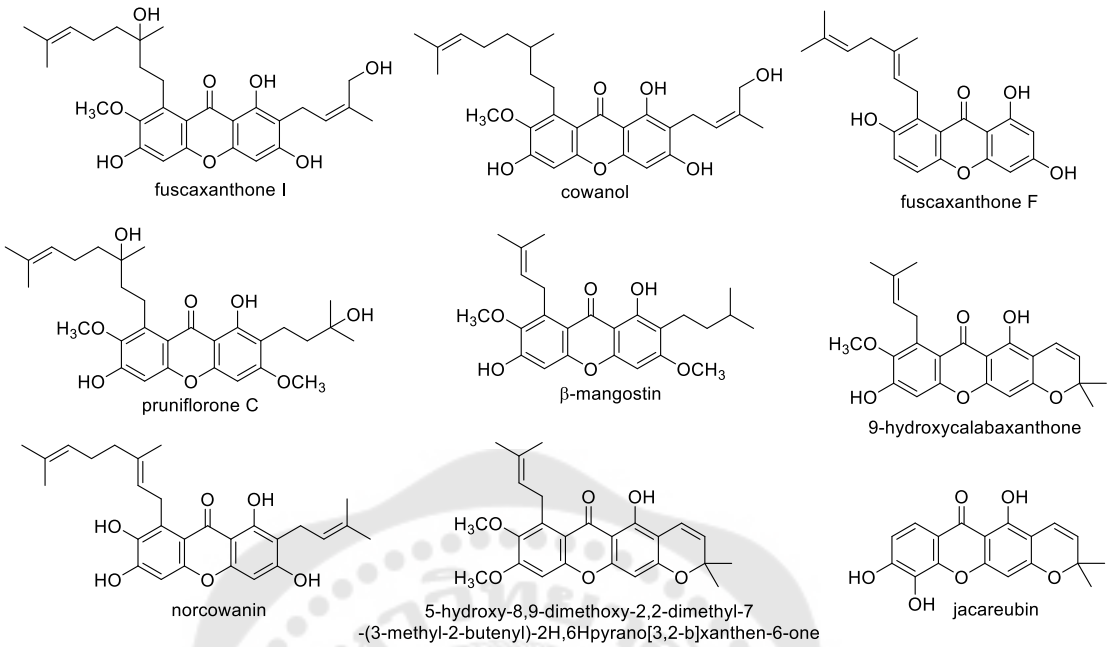


Figure 2 Isolated compounds of Keanakham et al., 2015

From: Keanakham, S. (2015) Kaennacowanols A–C, three new xanthones and their cytotoxicity from the roots of *Garcinia cowa*. *Fitoterapia*. P. 172

In 2016, Wahyuni et al. described that 5 known compounds 14-hexadecatetraenyl-2-cyclohexen-1-one, 2-(3-methyl-2-butenyl)-1,5,6-trihydroxy-3-methoxy-4-(1,1-dimethyl-2-propenyl)-9H-xanthen-9-one, rubraxanthone, cowanine, and 1,5-dihydroxyxanthone were isolated from roots of *G. cowa*. Among them, cowanin showed IC_{50} value of 4.10 μM, 5.40 μM, and 11.30 μM against MCF-7, H-460, and DU-145 cancer cell line (Wahyuni et al., 2016).

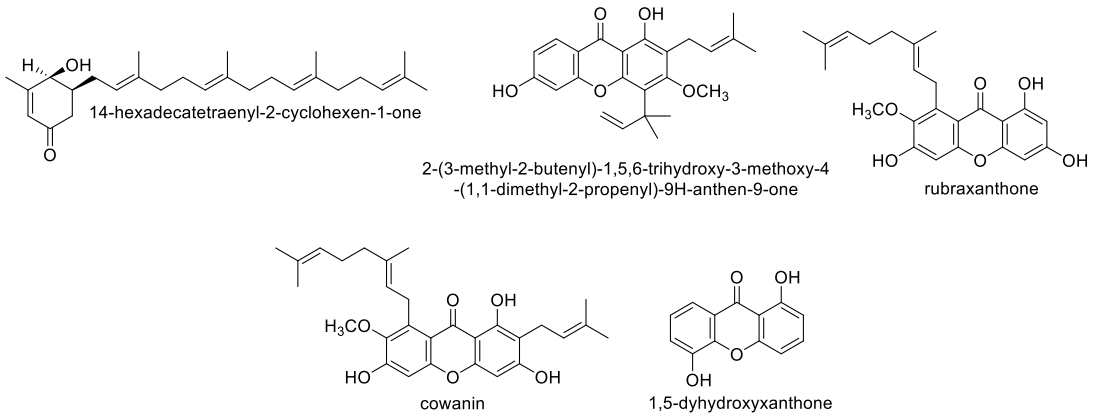
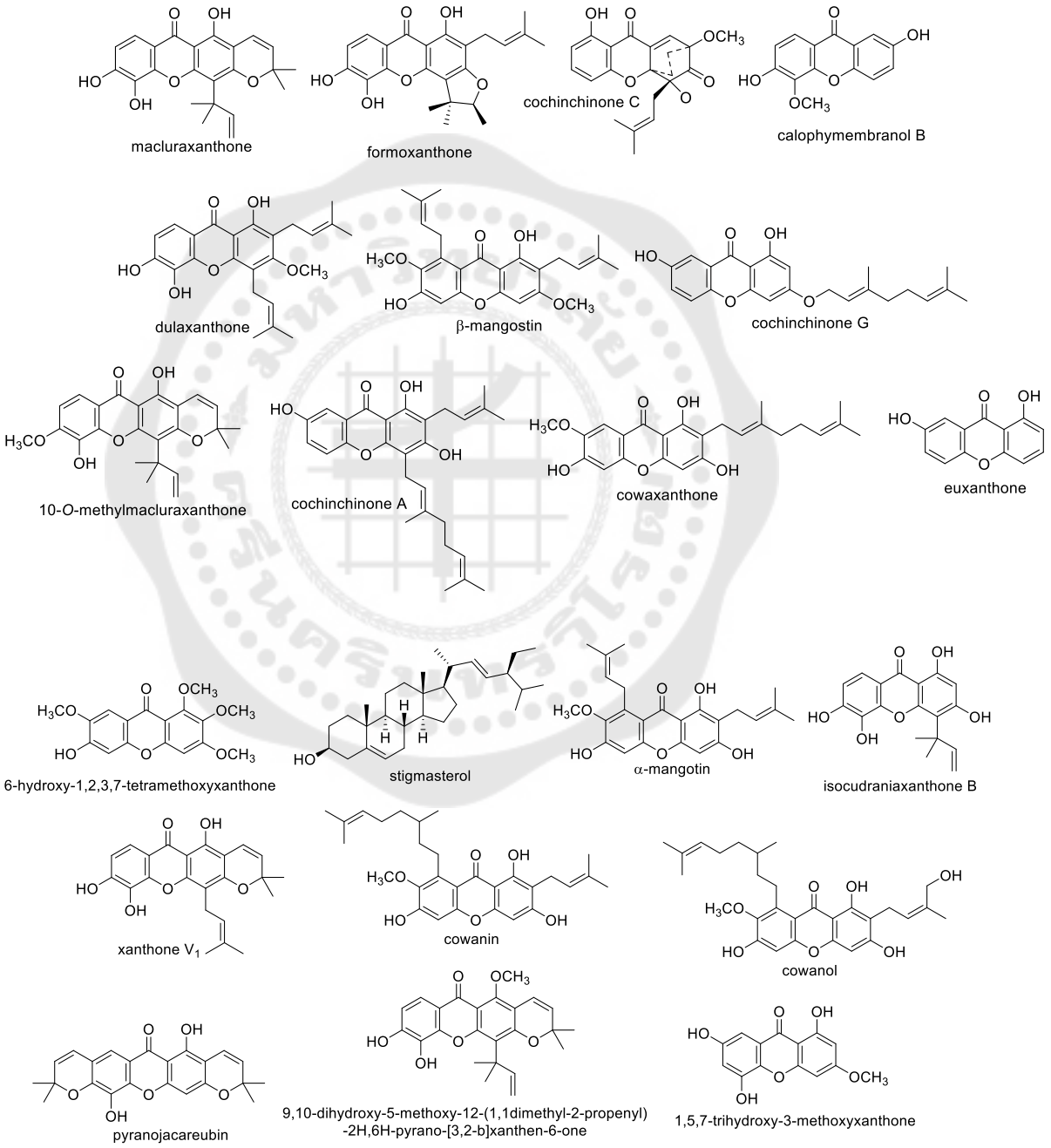


Figure 3 Isolated compounds of Wahyuni et al., 2016

From: Wahyuni, F. (2016) Cytotoxic properties and complete nuclear magnetic resonance assignment of isolated xanthones from the root of *Garcinia cowa* Roxb. *Pharmacognosy Magazine*. P. 53

In 2017, Tayana et al. isolated 44 compounds from acetone extract of roots. They enabled to isolate of 35 xanthones, 2 anthraquinones, 2 flavonoids, and 5 terpenes including macluraxanthone, formoxanthone C, cochinchinone C, calophymembranol B, dulxanthone B, β-mangostin, cochinchinone G, 10-*O*-methylmacluraxanthone, cochinchinone A, cowaxanthone, euxanthone, 6-hydroxy-1,2,3,7-tetramethoxyxanthone], stigmasterol, α-mangostin, isocudraniaxanthone B, xanthone V1, cowanin, cowanol, pyranojacareubin, 9,10-dihydroxy-5-methoxy-12-(1,1dimethyl-2-propenyl)-2*H*,6*H*-pyrano-[3,2-*b*]xanthen-6-one, 1,5,7-trihydroxy-3-methoxyxanthone, norathyriol, 7-geranyloxy-1,3-dihydroxyxanthone, gartanin, morusignin I, parvixanthone B, 1,3,7 trihydroxy-2-(3-methylbut-2-enyl)xanthone, 5-*O*-methylxanthone V1, friedelin, lupenone, lupane, damnacanthal, 2,3-dihydroxy 1-methoxylanthraquinone, cochinchinone E, 1,3,6-trihydroxy-7- methoxy-2,5-*bis*(3-methyl-2-butenyl)xanthone, *b*-sitosterol, kaempferol, assiguxanthone B, 1,6- dihydroxy-5-methoxyxanthone, 3,4-dihydro-6,11-dihydroxy-2,2-dimethyl-pyrano-[3,2-*c*]-xanthene-7(2*H*)-one, morelloflavone, cowagarcinone B, 6-hydroxy-2,3-dimethoxyxanthone and assiguxanthone). The isolated compounds α-mangostin showed stronger activity against *B. cereus* TISTR 687 and MRSA-SK1 (MIC

0.5 μg/mL) than that of vancomycin (MIC 1 μg/mL), Cowanin and cowanol showed strong antibacterial activities against *B. cereus* TISTR 687, MRSA-SK1, and *S. aureus* TISTR 1466 with MICs range of 2-4 μg/mL. Isocudraniaxanthone B, xanthone V1, and kaempferol showed IC_{50} values of 19.75, 19.70, and 11.67 μM, respectively (Tayana et al., 2017).



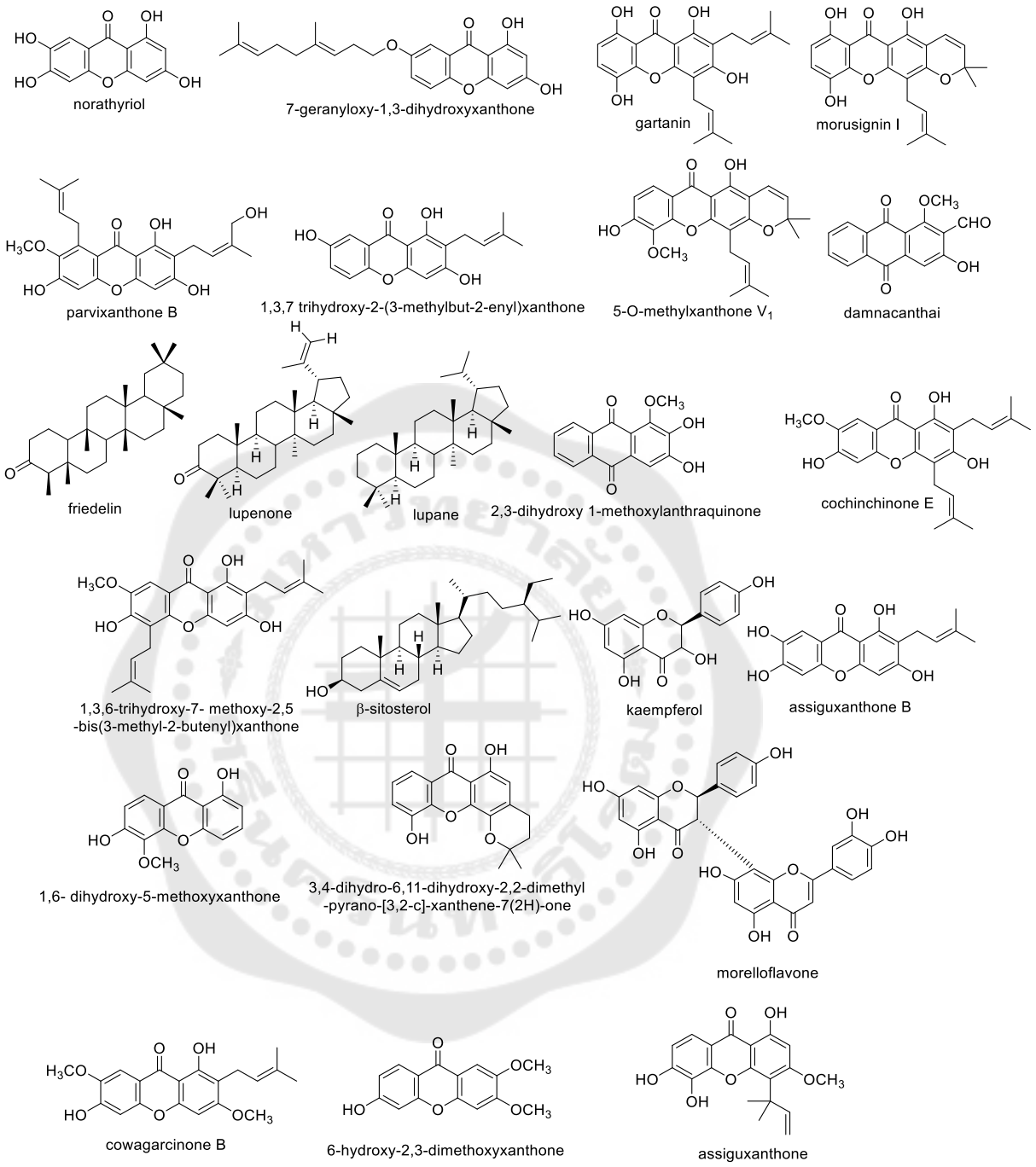


Figure 4 Isolated compounds of Tayana et al., 2017

From: Tayana, N. (2017) Phytochemistry and bioactive compounds from *Garcinia cowa* roxb. *Asia-Pacific Journal of Science and Technology*. P. 5

1.2. Stem barks

The part of barks or stem barks was collected from different places such as Bangkok Province Chulalongkorn University, Thailand (Lihitwitayawuid, Phadungcharoen, Mahidol, & Ruchirawat, 1997) & (Likhitwitayawuid , Phadungcharoen, & Krungkrai, 1998), Nong khai Province of Thailand (Siridechakorn et al., 2012), and Indonesia (Husni, Nahari, Wirasti, Wahyuni, & Dachriyanus., 2015) & (Wahyuni, Fatma, Shaari, & Stanslas, 2015).

In 1997, a new xanthone 7-*O*-methylgarcinone E was isolated, and the structure was elucidated with the NMR technique (Lihitwitayawuid et al., 1997).

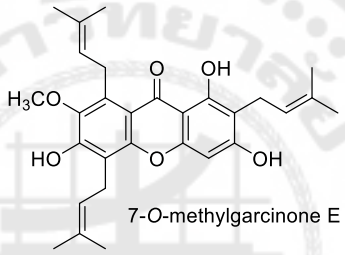


Figure 5 Isolated compound of Lihitwitayawuid et al., 1997

From: Lihitwitayawuid, K. (1997) 7-O-Methylgarcinone E from *Garcinia cowa*. *Phytochemistry*, P. 1299

In 1998, Lihitwitayawuid et al. isolated 5 known xanthones including 7-*O*-methylgarcinone E, cowanin, cowanol, cowaxanthone, and β-mangostin from ethanol extract in previous work. Cowaxanthone show an IC_{50} value of 1.50 µg/mL and other compounds showed IC_{50} values 1.50 - 3.00 µg/mL against the antimalarial activity (Likhitwitayawuid et al., 1998).

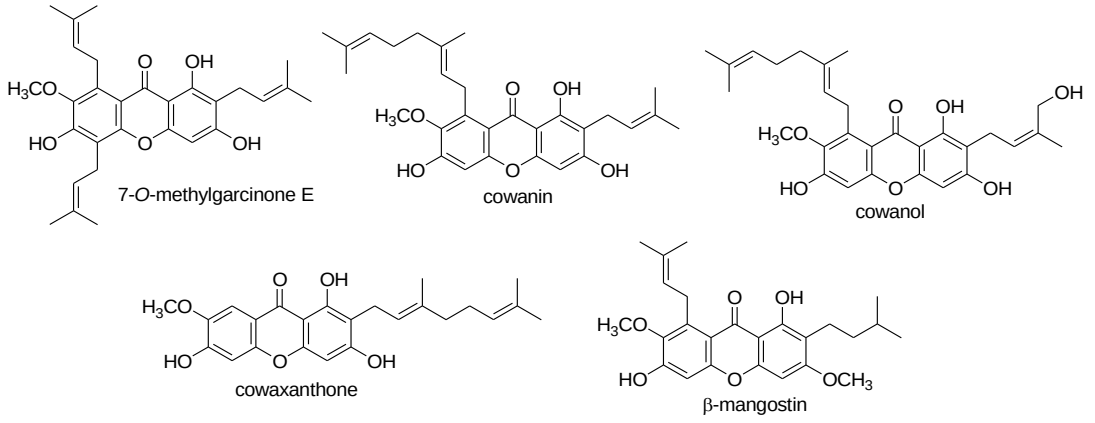


Figure 6 Isolated compounds of Lihitwitayawuid et al., 1998

From: Lihitwitayawuid, K. (1998) Antimalarial xanthones from *Garcinia cowa*. *Planta Medica* P. 70

In 2012, Siridechakorn et al. isolated of the two new compounds, garciniacowol and garciniacowone along with 15 known compounds parvifoliol F, α-mangostin, β-mangostin, cowaxanthone, norcowanin, cowanin, cowanol, cowagarcinone B, cowagarcinone D, cowagarcinone E, fuscaxanthone A, fuscaxanthone C, 6-*O*-methylmangostanin, cowaxanthone D, and 1,7 dihydroxyxanthone from acetone extract. Four compounds, garciniacowone, cowanin, cowanol, and cowagarcinone E showed MIC values of 2, 4, 2, and 8 µg/mL against MRSA SK1, respectively. Only five compounds garciniacowone cowaxanthone, norcowanin, cowanin and cowanol were active against *S. aureus* (Siridechakorn et al., 2012).

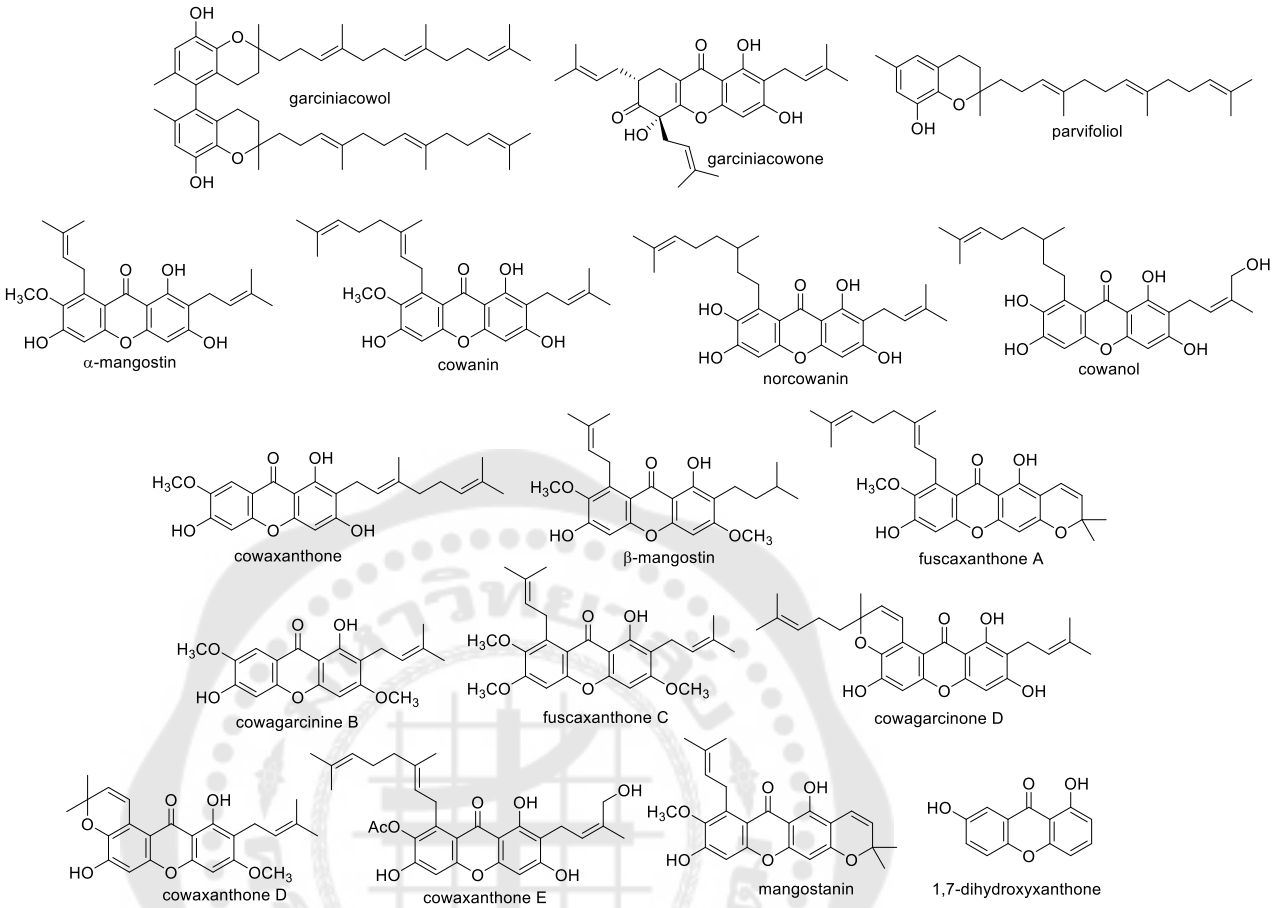


Figure 7 Isolated compounds of Siridechakorn et al. 2012

From: Siridechakorn, I. (2012) Antibacterial dihydrobenzopyran and xanthone derivatives from *Garcinia cowa* stem barks. *Fitoterapia,* P. 1432

In 2015, Husni et al. reported that the ethanol extract of the stem barks of *G. cowa* displayed a significant cytotoxic effect on T47D (human breast cancer) with IC_{50} value of 5.10 µg/mL (Husni et al., 2015).

In 2015, Wahyuni et al. investigated the methanol extract of stem barks in the successful isolation of the six xanthones 6-hydroxycalabaxanthone, 2-(3-methyl-2-butenyl)-1,5,6-trihydroxy-3-methoxy-4-(1,1-dimethyl-2-propenyl)-9H-xanthen-9- one, rubraxanthone, α-mangostin, 1,3,6-trihydoxy-7-methoxy-4-(4-acetoxy-3-methyl-2-butenyl)-8-(3,7-dimethyl-2,6-octadienyl)xanthone and cowanin. α-Mangostin and

cowanin showed potent activities against MCF-7 with IC_{50} value 4.10 and 5.30 μM. α-Mangostin also had potent activity against H-460 with IC_{50} value 5.40 μM, while 6-hydroxycalabaxanthone potent against DU-145 with IC_{50} value 6.40 μM (Wahyuni, Fatma, et al., 2015).

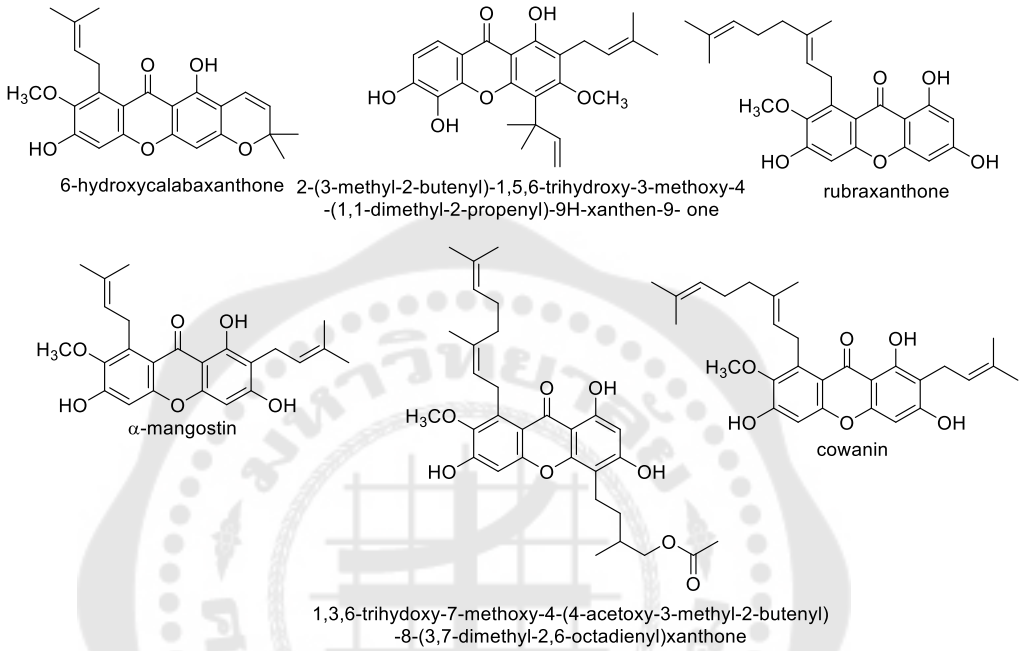


Figure 8 Isolated compounds of Wahyuni et al., 2015

From: Wahyuni, F. (2015) Cytotoxic xanthones from the stem bark of *Garcinia cowa* Roxb. *Journal of Chemical and Pharmaceutical Research,* P. 228

1.3. Stem

The part of stems were collected from Yunnan Province China (Shen & Yang, 2006), (Shen, Tian, & Yang, 2007), and (Tian et al., 2008)

In 2006, Shen et al. investigated the ethanol extract of stems and isolated two new xanthones, 1,5,6-trihydroxy-3-methoxy-4-(3-hydroxyl-3-methylbutyl)xanthone and 1,5-dihydroxy-3-methoxy-6',6'-dimethyl-2*H*-pyrano(2',3':6,7)-4-(3-methylbut-2 enyl)xanthone and other six known compounds, 1,3,5-trihydroxy-6',6'-dimethyl-2*H* pyrano(2',3':6,7)xanthone, dulxanthone A, 1,5,6-trihydroxy-3,7-dimethoxyxanthone, 1,7-

dihydroxyxanthone, 1,3,5-trihydroxy-6- methoxyxanthone, 1,3,6,7-tetrahydroxyxanthone and structure elucidate with NMR technique (Shen & Yang, 2006).

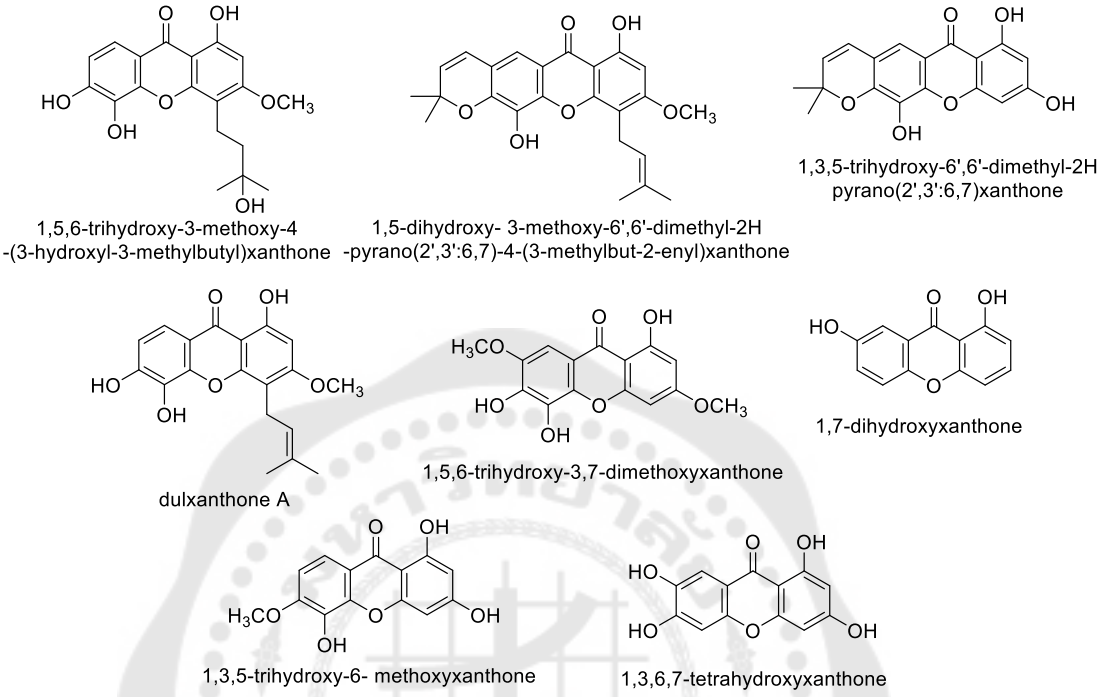


Figure 9 Isolated compounds of Shen et al., 2006

From: Shen, J. (2006) Two new xanthone from stem *G cowa*. *Chemical and Pharmaceutical Bulletin*, P. 127

In 2007, Shen et al. isolated three new flavanone glycosides garccowaside A, garccowaside B and garccowaside C, and three other known compounds, S-(-)-5,7,3',5'-tetrahydroxyflavanone, (+)-3,5,7,3',5'-pentahydroxyflavanone, and quercetin from ethanol extract in previous work. The isolated compounds showed higher IC_{50} than betulanic acid on HepG2, MCF-7 and SF268 cancer cell lines (Shen et al., 2007).

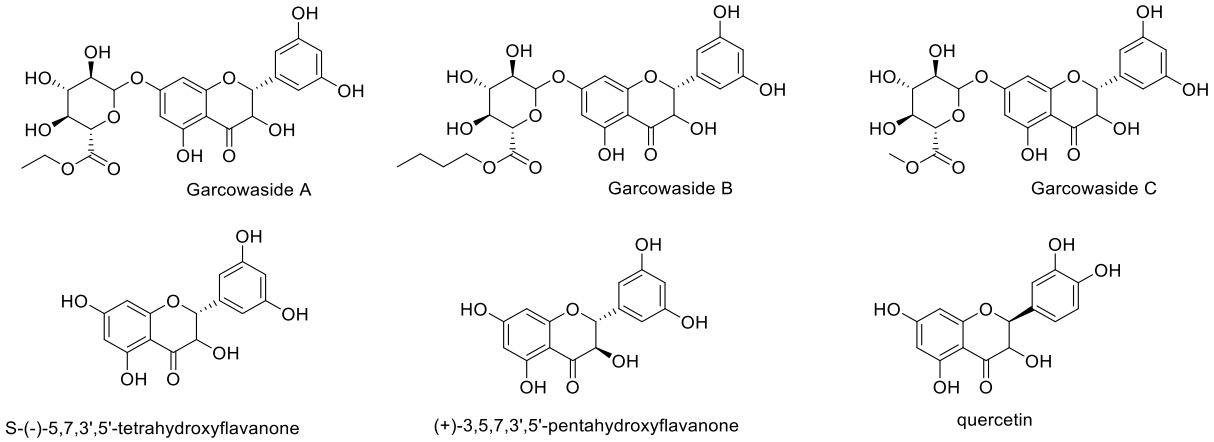


Figure 10 Isolated compounds of Shen et al., 2007

From: Shen, J. (2007) The constituents from the stems of *Garcinia cowa*. *Pharmazie*, P. 31

In 2008, Tian et al. studied dulaxanthone A in ethanol extract from previous work to investigate the cytotoxicity against HepG2 cells. Dulxanthone A showed the more effective IC_{50} value of 20 µg/mL against HepG2 cells (Tian et al., 2008).

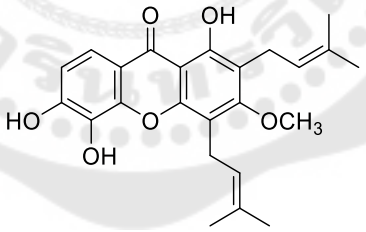


Figure 11 Isolated compound of Tian et al., 2008

From: Tian, Z. (2008) (2008). Dulxanthone A induces cell cycle arrest and apoptosisvia up-regulation of p53 through mitochondrial pathway in HepG2 cells. *International Journal of Cancer,* P. 32

1.4. Stem latexes

The part of latexes were collected from different places such as Songkhla province, Thailand (Pattalung et al., 1994), Nakorn Sri Thammarat Province Thailand (Mahabusarakam, Chairerk, & Taylor, 2005), and Yunnan Province, China (Na, Song, & Hu, 2013).

In 1994, Pattalung et al. described that five xanthone, cowanin, cowanol, cowaxanthone, 1,3,6- trihydroxy- 7-methoxy-2,5-bis(3-methyl-2-butenyl)xanthone, and norcowanin were isolated from methanol extract. Among them cowanol and cowaxanthone showed moderate antimicrobial activity against ATCC25923 *S aureus*. (Pattalung et al., 1994).

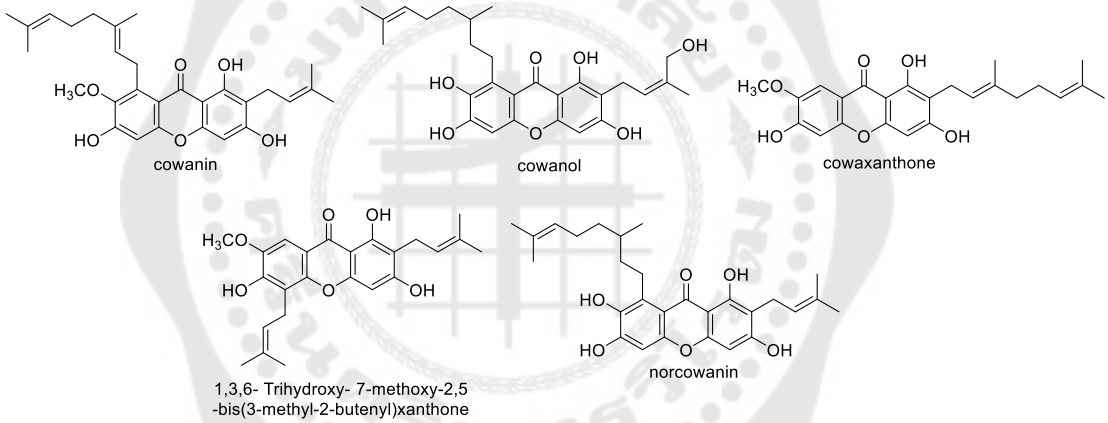


Figure 12 Isolated compounds of Pattalung et al., 1994

From: Pattalung, P. (1994) Xanthones of *Garcinia cowa*. *Planta Medica*, P. 367

In 2005, Mahabusarakam et al. isolated five new xanthones cowagarcinone A–E and six known xanthones, cowaxanthone, cowanin, cowanol, 1,3,6-trihydroxy-7-methoxy-2,5-bis(3-methyl-2-butenyl)xanthone, mangostinone, and fuscaxanthone A from acetone extract of latexes. The crude latexes showed an IC_{50} value of 13.20 µg/mL against DPPH radical scavenging activity. However, cowaxanthone, cowanin, cowanol, 1,3,6-trihydroxy-7-methoxy-2,5-bis(3-methyl-2-butenyl)xanthone, fuscaxanthone A,

cowagarcinone A and cowagarcinone E the IC_{50} values being over 200 μM (Mahabusarakam et al., 2005).

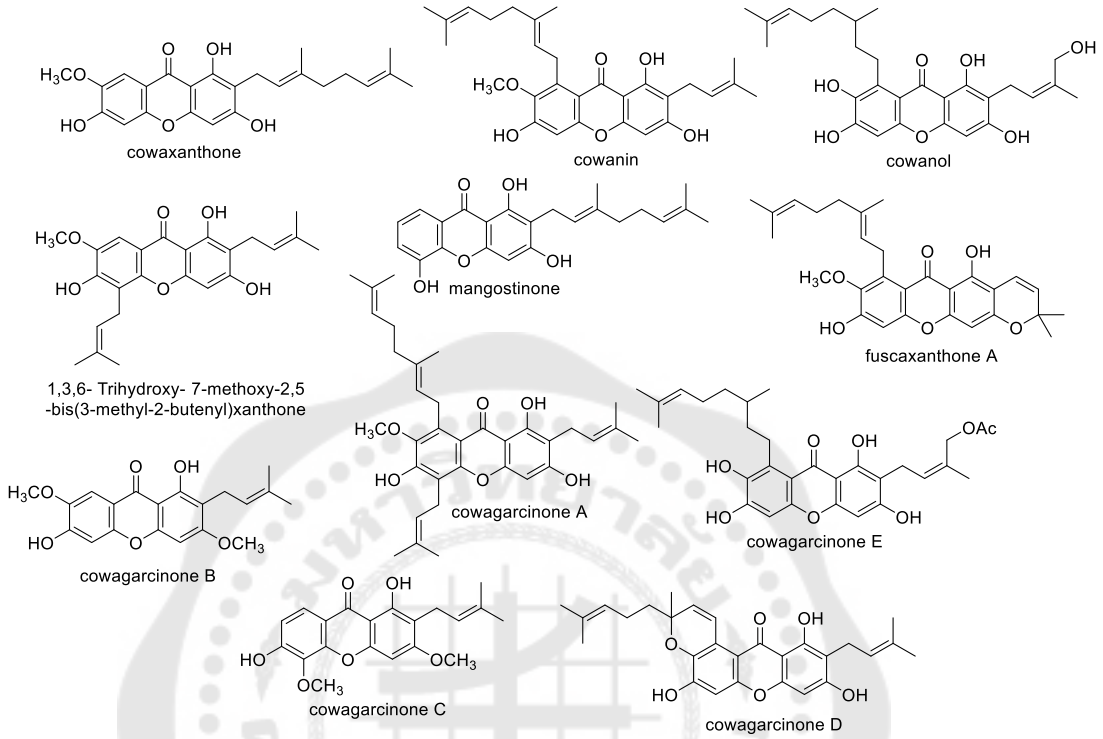


Figure 13 Isolated compounds of Mahabusarakam et al., 2005

From: Mahabusarakam, W. (2005) Xanthones from *Garcinia cowa* Roxb. latex. *Phytochemistry*, P. 1149

In 2013, Na et al. investigated the ethanol extract of latexes in the successful isolation of the new prenylated xanthone, 3-*O*-methylcowaxanthone, together with four known xanthones, cowaxanthone, 7-*O*-methylgarcinone, α-mangostin and γ-mangostin. 3-*O*-Methylcowaxanthone was evaluated with five human cancer cell lines, HL-60 (human leukemia cell), SMMC-7721 (liver cancer cell), A-549 (lung cancer cell), MCF-7 (human breast cancer), and SW480 (human colon cancer), but it was inactive (Na et al., 2013).

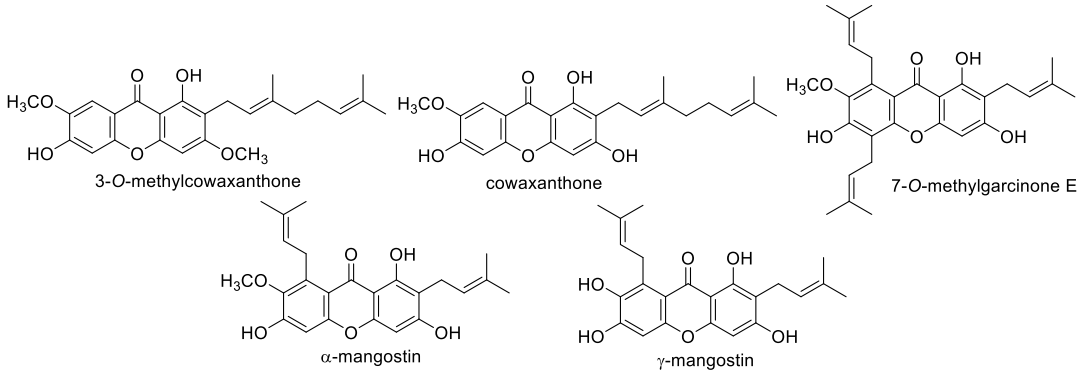


Figure 14 Isolated compounds of Na et al., 2013

From: Na, Z. (2013) A new prenylated xanthone from latex of *Garcinia cowa* Roxb. *Records of Natural Products*, P. 221

1.5.Twigs

The part of twigs were collected from different places such as Songkhla Province (Panthong, Hutadilok-Towatana, & Panthong, 2009) and Nong Khai Province, Thailand (Cheenpracha, Phakhodee, Ritthiwigrom, Prawat, & Laphookhieo, 2011).

In 2009, Panthong et al. studied the acetone extract of twigs to the isolation of the new xanthone cowaxanthone F and other known compounds, 1,6-dihydroxyxanthone, volkensiflavone, morelloflavone, and fukugiside. Two biflavonoids morelloflavone and fukigiside showed a good IC_{50} in the radical scarvenging activity. (Panthong et al., 2009).

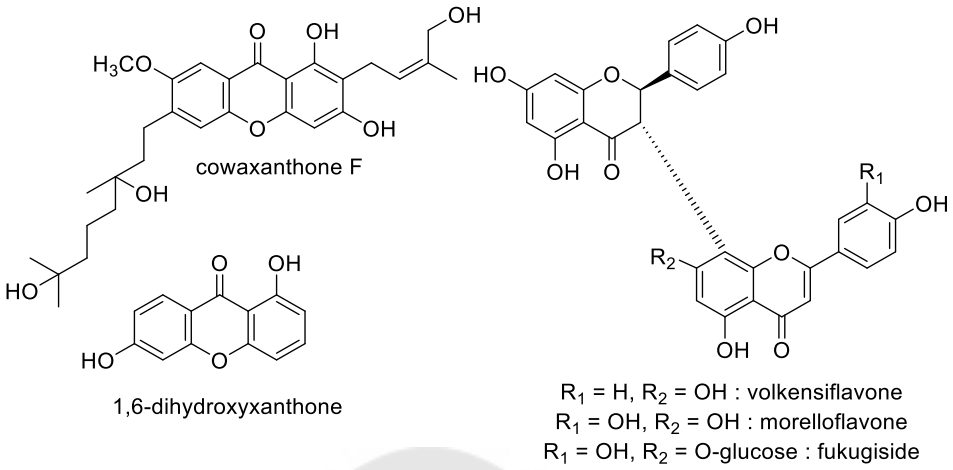


Figure 15 Isolated compounds of Panthong et al., 2009

From: Panthong, K. (2009). Cowaxanthone F, a new tetraoxygenated xanthone, and other anti-inflammatory and antioxidant compounds from *Garcinia cowa*. *Canadian Journal of Chemistry*, P. 1637

In 2011, Cheenpracha et al. found a new depsidone cowadepsidone and six known xanthones, β-mangostin, cowanin, 3,6-di-*O*-methyl-γ-mangostin, cowanol, norcowanin, and cowaxanthone from *n*-hexane extract of twigs. The isolates compounds cowanin, 3,6-di-*O*-methyl-γ-mangostin and norcowanin exhibited strong cytotoxicity against KB cancer cell line with the IC_{50} value of 7.36, 6.64, and 6.43 μg/mL, respectively. Cowanin, 3,6-di-*O*-methyl-γ -mangostin, norcowanin and cowaxanthone exhibited strong cytotoxicity against NCI-H187 cancer cell line with the IC_{50} value of 7.03, 8.58, 5.92, and 3.87 μg/mL, respectively. (Cheenpracha et al., 2011).

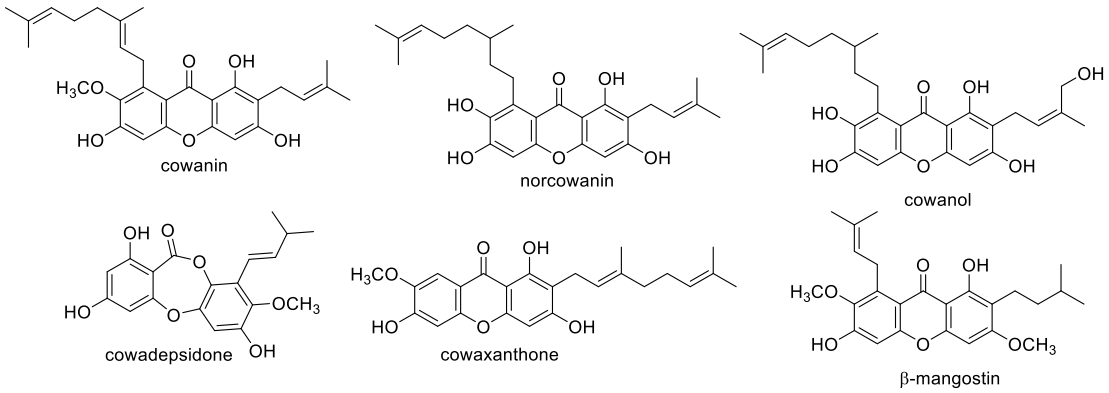


Figure 16 Isolated compounds of Cheenpracha et al., 2011

From: Cheenpracha, S. (2011) A new depsidone from the twigs of *Garcinia cowa*. *Heterocycles*, P. 1140

1.6.Leaves

The part of leaves were collected from different places such as Songkhla province Thailand (Sakunpak & Panichayupakaranant, 2012) & (Sae-Lim et al., 2020), and Yunnan Province, China (Xia et al., 2015), and Indonesia (Wahyuni, Triastuti, & Arifin, 2015).

In 2012, Sakunpak et al. reported the ethyl acetate extract of *G. cowa* exhibited the antibacterial activity against *H. pylori* and chamuangone was showed antibacterial activity against *Streptococcus pyogene*, *Streptococcus viridans H. pylori*, *Enterococcus sp,* and *Staphylococcus* with IC_{50} values of 7.80, 15.60, 15.60, 31.20, and 31.20 µg/mL, respectively. (Sakunpak & Panichayupakaranant, 2012).

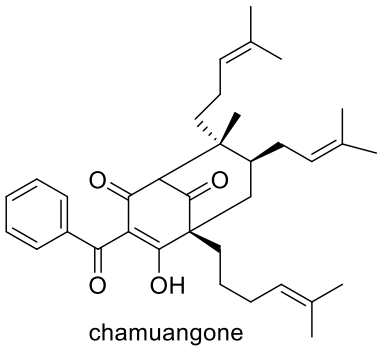


Figure 17 chemical structure of chamuangone Sakunpak et al., 2012

From: Sakunpak, A. (2012) Antibacterial activity of Thai edible plants against gastrointestinal pathogenic bacteria and isolation of a new broad spectrum antibacterial polyisoprenylated benzophenone, chamuangone. *Food Chemistry*, P. 829

In 2015, Wahyuni et al. described three compounds, methyl 2,4,6- trihydroxy-3-(3-methylbut-2-enyl)benzoate, garcinisidone-A and methyl 4,6-dihydroxy-2-(4-methoxy-5- (3-methylbut-2-enyl)-3,6-dioxocylohexa-1,4-dienyloxy)-3-(3-methylbut-2-enyl)benzoate isolated from dichloromethane extract of leaves. All of them had IC_{50} values of 21.00 μM, 21.20 μM, and 17.20 μM against MCF-7, while only garcinisidone-A was active against H-460 with IC_{50} value of 18.1 μM (Wahyuni, Shaari, Stanslas, Lajis, & Hamidi, 2015).

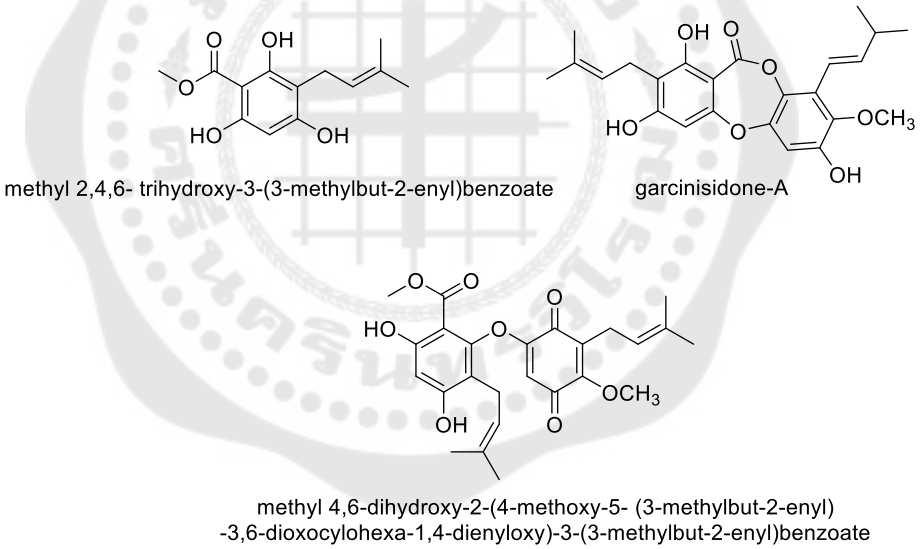


Figure 18 Isolated compounds of Wahyuni et al., 2015

From: Wahyuni, F., Shaari, K., Stanslas, J., Lajis, N., & Hamidi, D. (2015). Cytotoxic compounds from the leaves of *Garcinia cowa* Roxb. *Journal of Applied Pharmaceutical Science*, P. 8

In 2015, Wahyuni et al. reported that the ethanol extract of *G. cowa* leaves showed the IC_{50} of value 6.13 µg/mL against T47D breast cancer cells (Wahyuni, Triastuti, et al., 2015).

In 2015, Xia et al. found the two new xanthones, cowaxanthones G and H, and 23 known compounds, isojacareubin, 1,3,5-trihydroxy-6′,6′-dimethyl-2H-pyrano-(2′,3′:6,7)xanthone, 1,5,6-trihydroxy-2-prenyl-6′,6′-dimethyl-2H-pyrano(2′,3′:3,4)xanthone, dulxanthone A, cambogin, garcimultiflorones E and F, oblongifolin C, guttiferone F, garciniagifolone A, garcicowins C and D, symphonone H, jacareubin, xanthone V1a, isoprenylxanthone, garcinexanthone C, xanthone V1a, 1,3,5-trihydroxyxanthone, ugaxanthone, 1,5,6-trihydroxy-3-methoxyxanthone, 1,3,7-trihydroxylxanthone, and 1,4,5-trihydroxyxanthone from the dichloromethane extract of leaves. The isolated compounds cowaxanthones G, 1,5,6-trihydroxy-2-prenyl-6′,6′-dimethyl-2H-pyrano(2′,3′:3,4)xanthone, jacareubin, and xanthone V1a showed significant inhibition on cell viability (IC_{50} < 10 µM) which could be used for lead compounds to development of anticancer drugs (Xia et al., 2015).

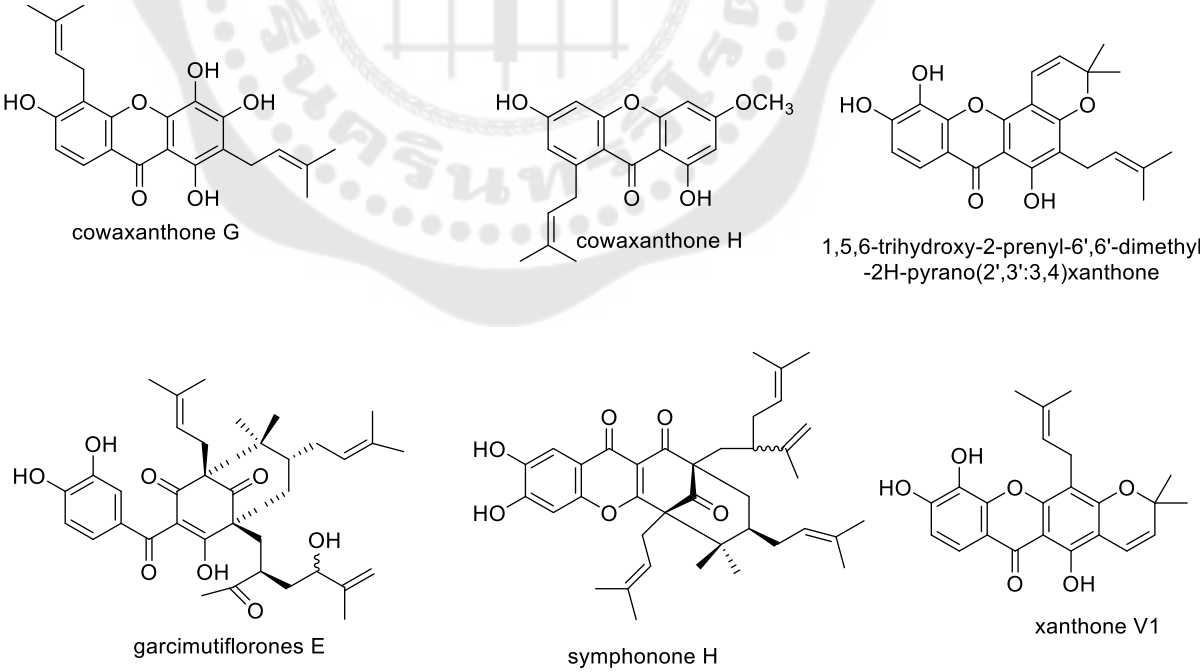


Figure 19 Isolated compounds of Xia et al., 2015

From: Xia, Z., (2015) Xanthones from the leaves of *Garcinia cowa* induce cell cycle arrest, apoptosis, and autophagy in cancer cells. *Molecules*, P. 11388

In 2020, Sae-Lim et al. isolated chamuangone from the *n*-hexane extract of leaves. Chamuangone exhibited strong inhibitory of HeLa cells proliferation with IC_{50} values of 3.59 μM and also inhibited EGFR-TK with the IC_{50} value of 2.85 nM (Sae-Lim et al., 2020).

1.7.Flowers

In 2015, Sriyatep et al. collected fresh flowers from Chiang Rai Province, Thailand, and investigated the methanol extract of young fresh flower in the successful isolation of the five new xanthones, garciniacowones A–E and fourteen known xanthone cowaxanthone, 3-*O*-methylmangostenone D, garcinone A, garcinone B, mangostanin, 6-*O*-methylmangostanin,fuscaxanthone A, fuscaxanthone C, 7-*O*-methylgarcinone E, cowaxanthone D, α-mangostin, β-mangostin, 3,6-di-O-methyl-γ-mangostin, and rubraxanthone. The isolates compounds garcinianone A, garcinianone B, and rubraxanthone showed antibacterial activity against *Bacillus subtilis* TISTR 088, while garcinone A, mangostanin and rubraxanthone exhibited antibacterial activity against *Bacillus cereus* TISTR 008. The isolated compounds, garciniacowone A, garciniacowone B, garcinone A, garcinone B, mangostanin, α-mangostin, β-mangostin, and rubraxanthone showed antibacterial activity against TISTR 088 (B. subtilis), with MIC values ranging from 2 to 8 μg/mL. Garcinone A, mangostanin, and rubraxanthone also demonstrated antibacterial activity against TISTR 688 (*B. cereus*), with MIC value of 4 μg/mL. On the other hand α-mangostin and β-mangostin showed the α-glucosidase inhibition with IC_{50} values of 7.80 and 8.70 μM, respectively (Sriyatep et al., 2015).

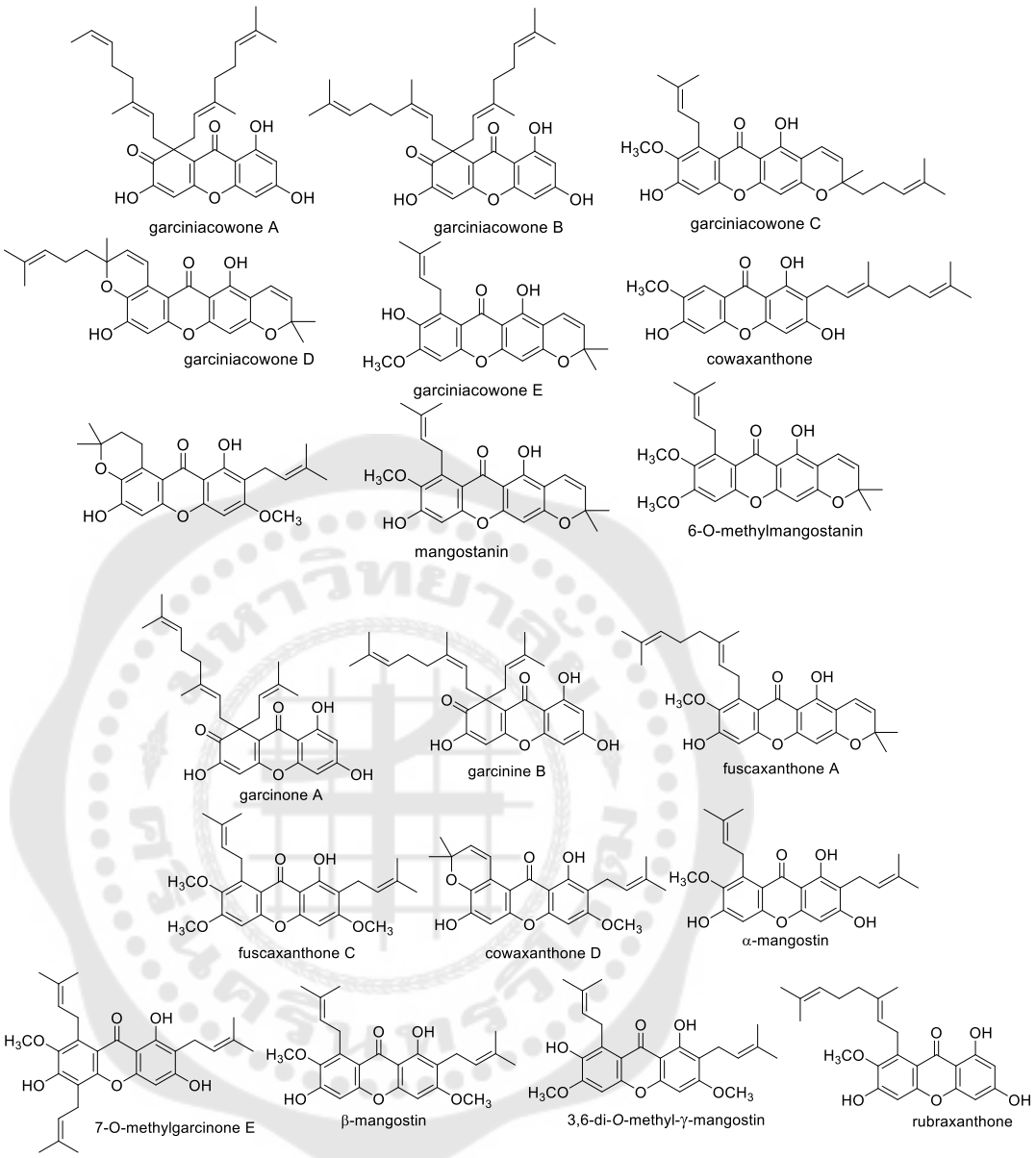


Figure 20 Isolated compounds from Sriyatep et al., 2015

From: Sriyatep, T. (2015) Bioactive prenylated xanthones from the young fruits and flowers of *Garcinia cowa*. *Journal of Natural Products*, P. 266

1.8.Fruits

The part of fruits were collected from different places such as Songkhla province, Thailand (Panthong, Pongcharoen, Phongpaichit, & Taylor, 2006), Nong Khai

Province, Thailand (Sriyatep et al., 2014), India (Sarma, Sarmah, Kashyap, & Kalita, 2014) & (Gupta et al., 2021), and Indonesia (Wahyuni, Febria, & Arisanty, 2017).

In 2006, Panthong et al. found the five new xanthones, cowaxanthones A–E, and another ten known compounds, l,6-dihydroxy-3,7-dimethoxy-2-(3-methyl-2-butenyl)xanthone, fuscaxanthone C, 7-*O*-methylgarcinone E, β-mangostin, cowanol, mangostanin, 6-*O*-methylmangostanin, cowanin, α-mangostin and cowaxanthone from hexane extract of fruits. Mangostanin and α-mangostin showed the activity against ATCC 25923 and MRSA SK1, with MIC values of 4 and 8 μg/mL (Panthong et al., 2006).

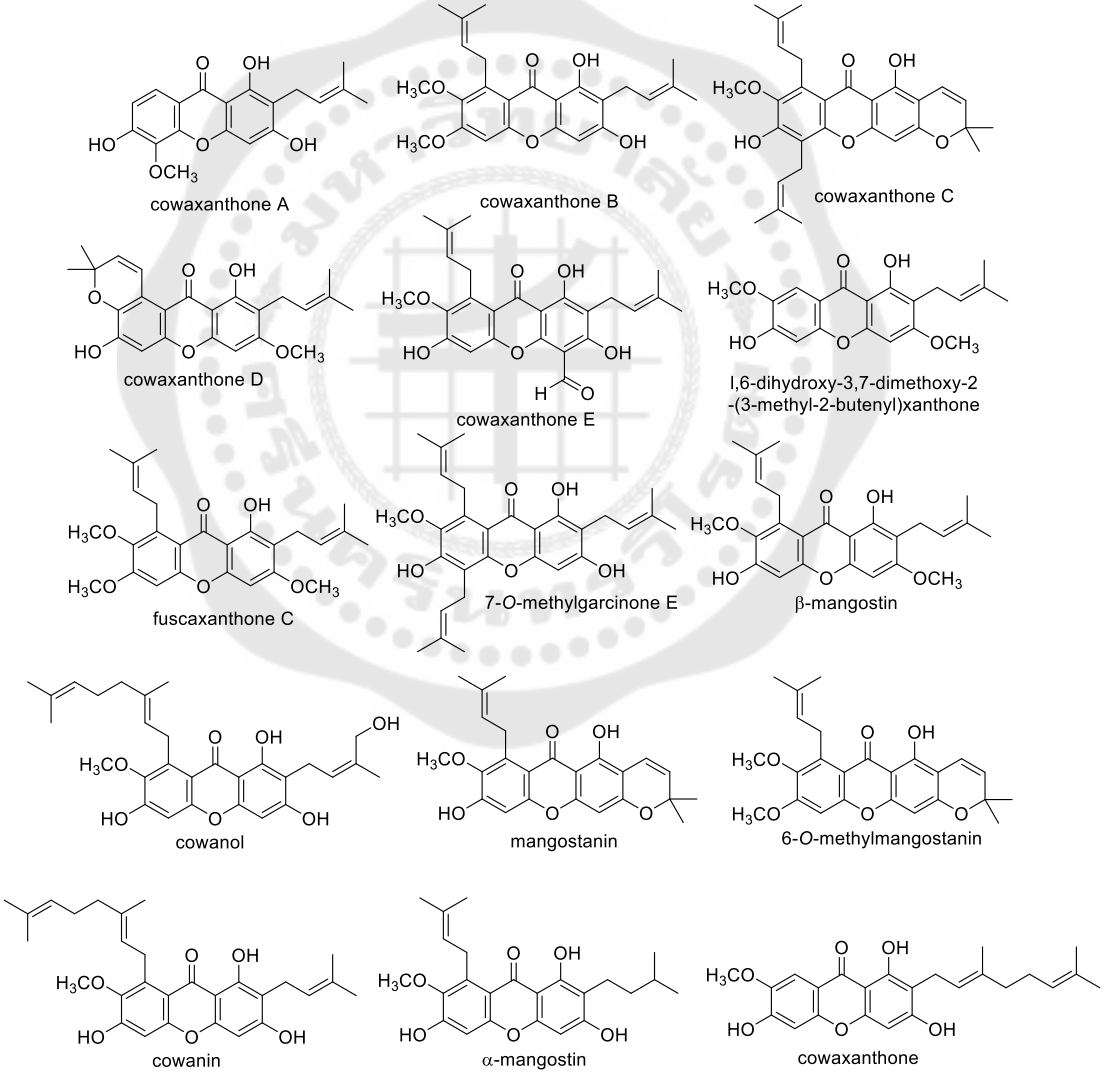


Figure 21 Isolated compounds from Panthong et al., 2006

From: Panthong, K. (2006) Tetraoxygenated xanthones from the fruits of *Garcinia cowa*. *Phytochemistry*, P. 1000

In 2014 Sarma et al. reported that the methanol extract of fresh fruits from *G. cowa* showed a good IC_{50} value of 33.15 µg/mL against DPPH radical scavenging activity (Sarma et al., 2014).

In 2014, Sriyatep et al. isolated two new tetracyclo[7.3.3.33,11.03,7] tetradecane-2,12,14-trione derivatives, cowabenzophenones A and B from methanol extract of ripe fruits. Cowabenzophenones A and cowabenzophenones B showed IC_{50} values of 1.12 and 4.54 µM, respectively against KB cancer cell line (Sriyatep et al., 2014).

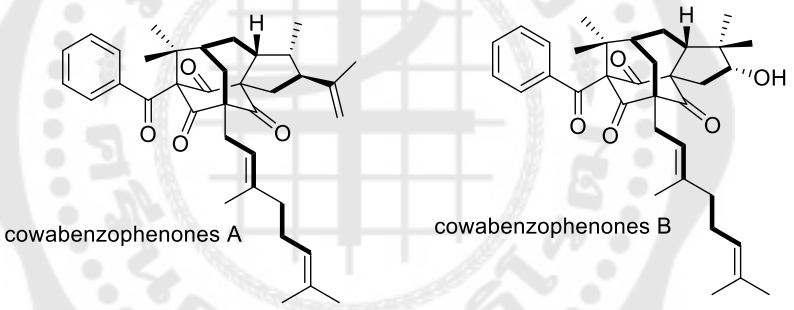


Figure 22 Isolated compounds from Sriyatep et al., 2014

From: Sriyatep, T. (2014) Cowabenzophenones A and B, two new tetracyclo[7.3.3.33,11.03,7]tetradecane-2,12,14-trione derivatives from ripe fruits of *Garcinia cowa*. *Fitoterapia*, P. 287

In 2017, Wahyuni et al. described that the dichloromethane extract of fresh rind fruits from *G. cowa* induces apoptosis in HeLa cervical cancer cells and showed a good IC_{50} value of 5.7 µg/mL (Wahyuni et al., 2017).

2. Biological activities of *Garcinia* plants

Garcinia have many broad biological activities are as follow; antibacterial, anti-inflammatory, antiviral, anticancer, antifungal, antidepressant, anti-HIV, anti-diabetic and antioxidant (Santo et al., 2020).

2.1 Oxidative stress & Antioxidant activity

“Oxidative stress” is the term that refers to not equal between the generation of (ROS) and the activity of the antioxidant defenses and can cause disease. (Aruoma, 1998). Oxidative stress is a relatively new concept, widely used in medical sciences in the past three decades. It causes of common diseases, such as preeclampsia, diabetes, atherosclerosis, high blood pressure, acute renal failure, Alzheimer’s, and Parkinson’s (Munteanu & Apetrei, 2021). Many studies show that antioxidants play an important role in human health, and in treating diseases, due to reducing oxidative stress. Therefore, xanthones, biflavonoids, and benzophenones were reported to possess remarkable levels of bioactivities, the antioxidant activity of biflavonoids and benzophenones have good significance because these antioxidants can reduce the cause of diseases including heart disease, inflammation, immune system decline, arthritis and cancer (Aravind et al., 2017).

Chapter 3

Experiment

1.Plant materials

The air-dried root barks of *G. cowa* were collected from Chanthaburi Province, Thailand, in January, 2007. A voucher specimen has been deposited at the Laboratory of Natural Product Research Unit, Chemistry Department of Srinakharinwirot University.

2.General experimental procedures

^{1}H- and ^{13}C-NMR spectra are determined on a Bruker Ascend^{TM}500 –FT-NMR spectrometer operating at 500 MHz (^{1}H) and 125 MHz (^{13}C).

IR spectra were obtained using a Perkin-Elmer UATR TWO spectrophotometer.

Column chromatography (CC) performed on silica gel 60 having either a particle size less than 0.063 mm (Merck 1.07729) or a particle size is 70-230 mesh (SiliCycle, SILIAFLASH G60).

Sephadex LH-20 (GE Health care) also used as an absorbent in size exclusion chromatography.

Thin layer chromatography (TLC) monitored using Merck precoated silica gel 60 F_{254} and visualized under UV light (at wavelengths of 254 and 365 nm) and by spraying with anisaldehyde H_2SO_4 reagent followed by heating.

Specific optical rotations were taken on a Jasco-1020 polarimeter.

3.Extraction of the root barks of *G. cowa*

The air-dried root barks of *G.cowa* (530 g) were extracted successively with EtOAc (3 × 2.5 L) and then with MeOH (3 × 2.5 L) at room temperature for each one week and the solvents were evaporated to yield the EtOAc (brownish residue, 116 g) and MeOH (reddish brown sticky, 120 g) extracts, respectively, as shown in Figue 23.

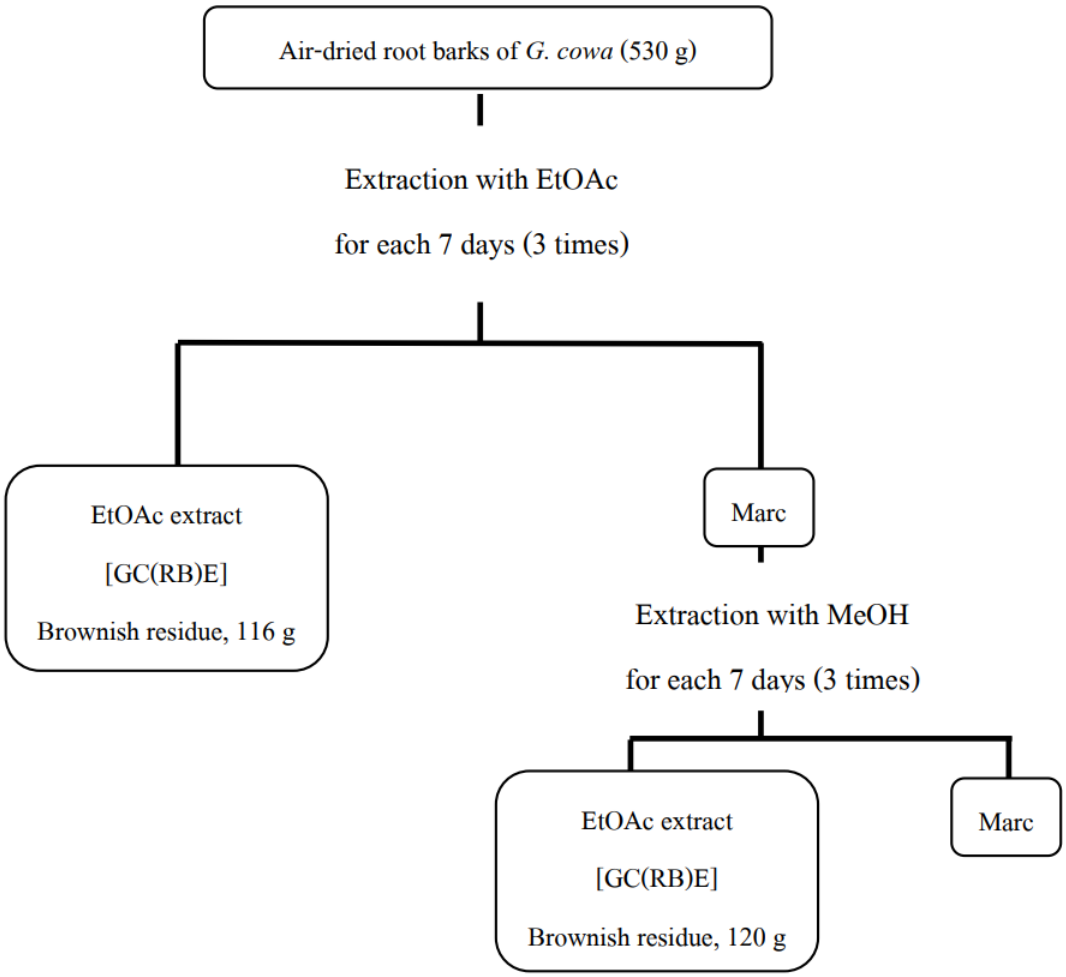


Figure 23 Extraction procedure of the root barks of *G. cowa*

The chemical screening of the EtOAc and MeOH extracts was monitored by TLC. The EtOAc extract showed purple, green, and orange color after treating with an anisaldehyde-H_2SO_4 reagent, indicating the presence of fatty acid, triterpene, xanthone, and biflavonoid respectively. The MeOH extract showed orange and brown, indicating the presence of biflavonoids and tannin.

4.Separation the EtOAc extract

A portion of EtOAc extract (73.5 g, brownish residue) was fractionated by CC (Ø10 x 12 cm) eluting with a gradient of hexane–acetone (98:2 to 0:100), acetone–MeOH (95:5–0:100), and H_2O-MeOH (50:50) to afford 6 main fractions F1-F6 (Figure 24).

From TLC observation of F1-F6, F3 and F5 were shown many complexes of the light purple and green spot with an anisaldehyde-H_2SO_4 reagent and compare with authentic compounds while, F5 have many xanthones similar to main xanthones such as; cowanin, cowaxanthone, and cowanol. Then this fraction was chromatographed and structure elucidated in the next step. Fraction F5 (1.7 g, brown sticky) was fractionated by column chromatography (silica gel, 80 g), employing *n*-hexane–acetone (92:8 to 0:100) to yield 19 sub-fractions (F5.1 – F5.19). Sub-fractions F5.12 (86 mg, sticky yellow) and F5.13 (72 mg, sticky yellow) were yielded as a mixture of cowanin and cowaxanthone. Sub-fraction of F5.15 (625 mg) was separated by CC eluting with *n*-hexane–acetone (96:4 to 0:100) to afford **XAN1** (179 mg, yellow sticky solid, sss7311) and **XAN2** (246 mg, pale brown sticky solid, sss7312).

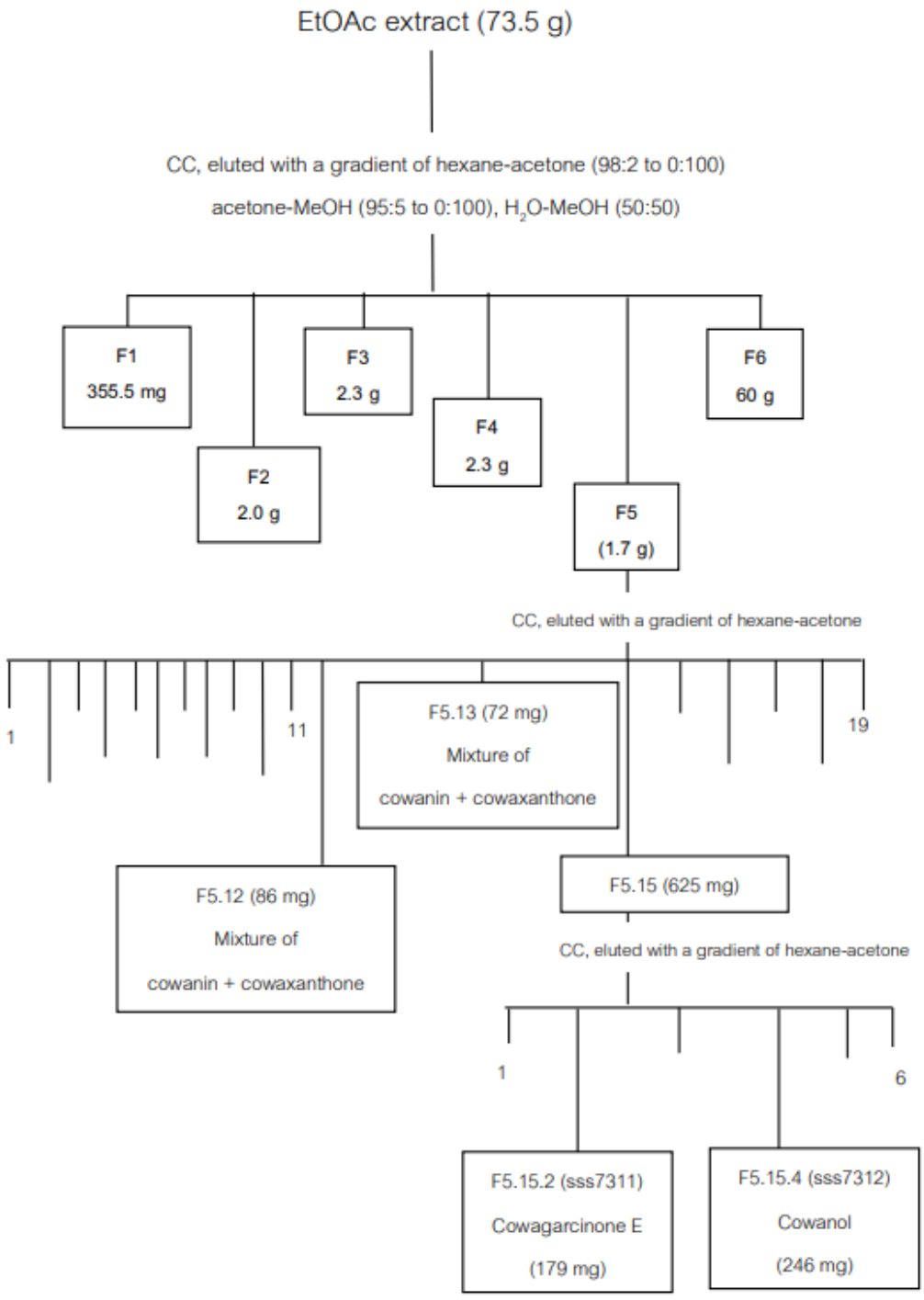


Figure 24 Separation procedure of EtOAc extract of the root barks of *G. cowa*

5.Separation the MeOH extract

A portion of MeOH extract (110 g, brownish residue) was fractionated by CC (Ø 10 x 10 cm) eluting with a gradient of DCM–MeOH (92:8 to 20:80) to afford 11 main fractions (F1-F11). Sub-fractions F5, F8 and F10 showed orange spots with different Rf values on TLC. Fraction F5 (1.7 g, dark brown sticky) was fractionated by column chromatography (CC) (silica gel, 80 g), using *n*-hexane–acetone (92:8 to 0:100) to yield 9 sub-fractions (F5.1 – F5.9). Sub-fractions of F5.5 (293 mg, pale brown solid) was separated by a Sephadex LH-20 column using MeOH to afford **BIF1** (15 mg, sss7269, yellow solid). Sub-fractions F5.(7+8) (251 mg, pale brown sticky) was repeated CC of eluting with DCM-MeOH-H_2O (9:0.5:0.5 to 8:1:0.5), to give 8 sub-fractions (F5.(7+8).1 – F5.(7+8).8), **BIF2** was obtained from sub-fractions F5.(7+8).6 as a yellow solid (21 mg, sss7262). Fraction F8 (3.1 g, dark brown sticky) was purified by CC (Ø 5x 50 cm), eluting with DCM–MeOH (96:5 to 0:100) to obtain 8 subfractions (F8.1 – F8.8). Fraction F8.6 (1.15 g, brown sticky) was separated by CC (DCM–MeOH–H_2O, 8.5:1:0.5 to 6:3:1) to give 7 subfractions (F8.6.1 – F8.6.7). Repeated CC of subfraction F8.6.5 (235.3 mg, brown sticky) eluting with DCM –MeOH–H_2O (8.5:1:0.5) afforded **BIF3** (36 mg, sss7257, pale brown solid). A portion of F10 (5 g, brown sticky) was further chromatographed over silica gel (Ø 5x 50 cm), eluting with a gradient of DCM–MeOH (92:8 to 75:25) to provide 18 sub-fractions and **BIF4** was yielded (0.75 g) as a pale brown solid from sub-fraction F10.15.

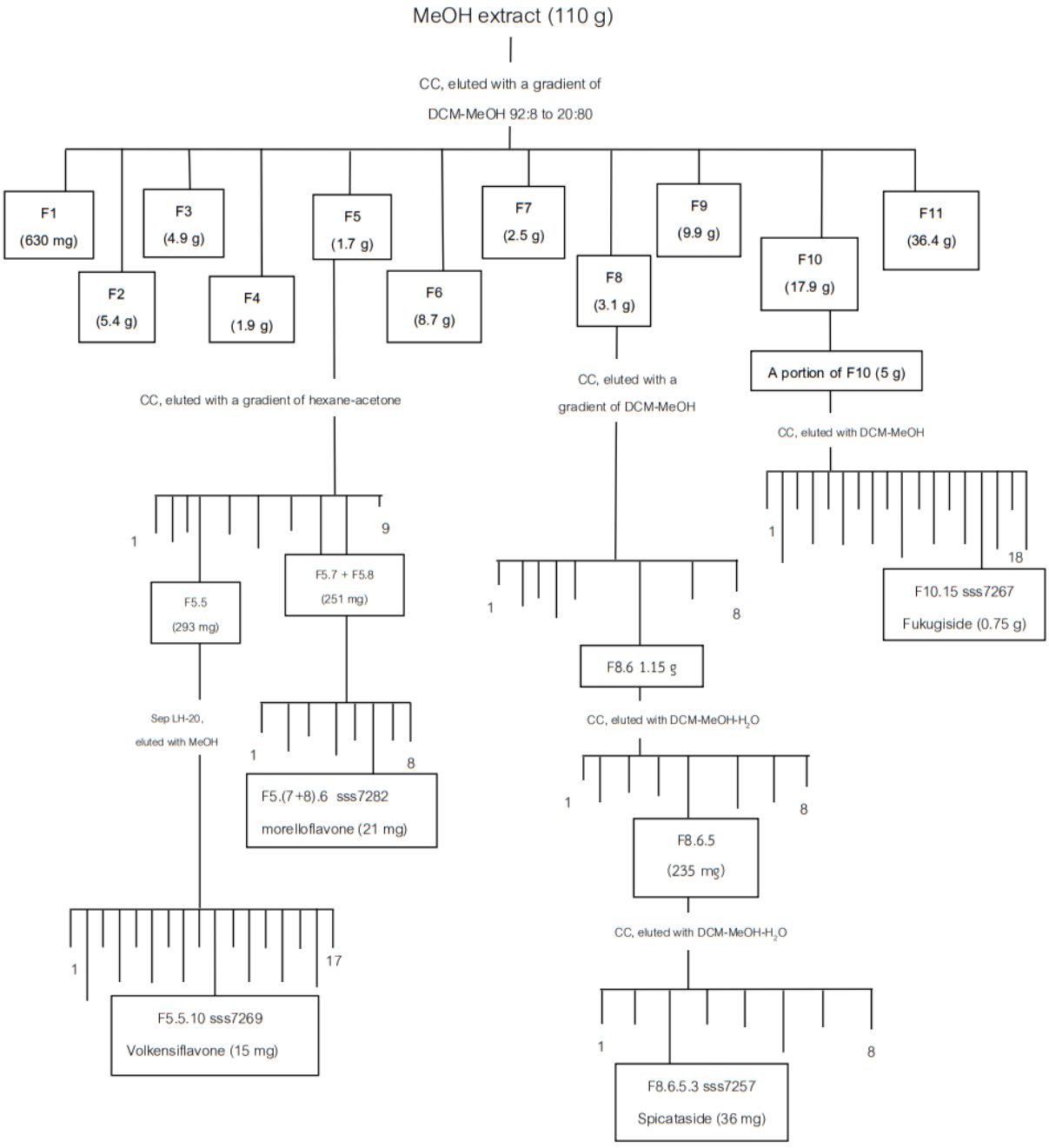


Figure 25 Separation procedure of MeOH extract of the root barks of *G. cowa*

6.DPPH scavenging assay

The DPPH radical scavenging activity of samples was determined by reaction with 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals in a microplate format (Gerhauser et al., 2003) & (van Amsterdam, Roveri, Maiorino, Ratti, & Ursini, 1992). DPPH solution was prepared in ethanol. The plant extract at various concentrations was diluted with DMSO to get sample solution. 5 μL of sample solution was treated with 195 μL of 100 μM DPPH solution and reacted at room temperature for 30 min in dark. The absorbance was measured at 515 nm with a blank containing DPPH and ethanol. Ascorbic acid (0.78 - 100 μg/mL) was used as a positive control. The ability to scavenge the DPPH radical was expressed as percentage inhibition and calculated according to the equation (Adebiyi, Olayemi, Ning-Hua, & Guang-Zhi, 2017):

$$\text{DPPH radical scavenging activity (\%)} = (A_{blank} - A_{sample}) / A_{blank} \times 100$$

where A_{blank} is the absorbance of the control and A_{sample} is the absorbance of the sample

The half-maximal inhibitory concentration (IC_{50}) of the sample against DPPH radical was calculated by ANOVA and, Dunnett's test for individual differences using SPSS program version 25.

7.Physical data of isolated compounds

1. **XAN1** (cowagarcinone E, sss7311)

Pale yellow sticky solid*, Rf* : 0.45 (30% acetone-hexane)

^{1}H NMR (500 MHz, $CDCl_3$); δ_H 13.78 (s, 1H, 1-OH), 6.82 (s, 1H, H-5), 6.32 (s, 1H, H-4), 5.33 (brt, J ca 6.2 Hz, 1H, H-12), 5.25 (brt, J ca 6.2 Hz, 1H, H-17), 5.01 (brt, J ca 6.2 Hz, 1H, H-21), 4.74 (s, 3H, H-14), 4.08 (d, J = 6.2 Hz, 2H, H-16), 3.82 (s, 3H, 7-OCH_3), 3.55 (d, J = 6.2 Hz, 2H, H-11), 2.12 (s, 3H, OAc), 1.23 – 1.99 (m, 4H, H-19-20), (s, 9H, H-23-25), 1.80 (s, 3H, H-15).

2. **XAN2** (cowanol, sss7312)

Pale brown sticky solid, *Rf* : 0.30 (30% acetone-hexane)

^{1}H NMR (500 MHz, $CDCl_3$); δ_H 13.82 (s, 1H, 1-OH), 6.80 (s, 1H, H-5), 6.29 (s, 1H, H-4), 5.44 (t, J ca 7.9 Hz, 1H, H-12), 5.24 (t, J = 6.1 Hz, 1H, H-17), 5.00 (t, J = 7.8 Hz, 1H, H-21), 4.33 (s, 2H, H-14), 4.07 (d, J = 6.1 Hz, 2H, H-16), 3.78 (s, 3H, 7-OCH_3), 3.50 (d, J = 7.9 Hz, 2H, H-11), 1.53 – 2.00 (m, 4H, H-19-20), (s, 9H, H-23-25), 1.76 (s, 3H, H-15).

3. **BIF1** (volkensiflavone, sss7269)

Yellow solid, *Rf* : 0.70 (DCM:MeOH:H_2O 8:1.5:0.5)

Mp : 225-226 °C, [lit 290-292°C (Chen, Lin, & Hung, 1975), 219-220°C (Masuda, Yamashita, Takeda, & Yonemori, 2005)

Optical rotation: $[\alpha]_D^{22}$ +165.5 (*c* 0.23, MeOH), [lit [(Chen, Lin, & Hung, 1975) $[\alpha]_D^{25}$ +1.6

IR: ν_{max} 3175, 1634, 1605, 1574, 1504, 1422, 1361, 1236, 1158 cm^{-1}

^{1}H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6), see **Table 1**

4. **BIF2** (morelloflavone, sss7262)

Yellow solid, *Rf* : 0.60 (DCM:MeOH:H_2O 8:1.5:0.5)

Mp : 223-224 °C, [lit 249-250°C (Chen, Lin, & Hung, 1975), 290-292°C (Li et al., 2002)

Optical rotation: $[\alpha]_D^{22}$ +234.8 (*c* 0.20, MeOH), (Chen, Lin, & Hung, 1975) $[\alpha]_D^{20}$ +17

IR: ν_{max} 3199, 1638, 1600, 1578, 1509, 1361, 1258, 1160 cm^{-1}

^{1}H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6), see **Table 2**

4. **BIF3** (spicataside, sss7257)

Yellow solid, *Rf* : 0.40 (DCM:MeOH:H_2O 8:1.5:0.5)

Mp : 221-222°C, [lit 241-243°C (Konoshima, Ikeshiro, & Miyahara, 1970)

Optical rotation: $[\alpha]_D^{23}$ +73.7 (*c* 0.20, MeOH), [lit (Brusotti et al., 2016) $[\alpha]_D^{25}$ +1.0 (*c* 0.1, MeOH)

IR: ν_{max} 3264, 1641, 1598, 1518, 1448, 1368, 1258, 1167 cm^{-1}

^1H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6), see **Table 3**

5. **BIF4** (fukugiside, sss7267)

Yellow solid, *Rf* : 0.30 (DCM:MeOH:H_2O 8:1.5:0.5)

Mp : 223-224 °C, [lit 249-250°C (Chen, Lin, & Hung, 1975), 290-292°C (Li et al., 2002)

Optical rotation: $[\alpha]_D^{22}$ +96.4 (*c* 0.21, MeOH), [lit [Brusotti 2016 $[\alpha]_D^{25}$ +6.1 (*c* 0.1, MeOH)

IR: ν_{max} 3264, 1641, 1598, 1518, 1448, 1368, 1258, 1167 cm^{-1}

^1H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6), see **Table 4**

CHAPTER 4

Result and Discussion

The air–dried root barks of *Garcinia cowa* were extracted with EtOAc and then with MeOH to obtain the EtOAc and MeOH extracts, respectively. The chemical screening of the extracts was carefully monitored by TLC technique. The EtOAc extract showed many green spots on TLC after treating with an anisaldehyde–H_2SO_4 reagent indicating the presence of the xanthone compounds (Pratiwi, Fudholi, Martien, & Pramono, 2017), whereas the MeOH soluble fraction gave orange spots with this reagent, indicating the presence of the biflavonoids content. The EtOAc F5 fraction was further separated by column chromatography to provide 19 subs-fraction (F5.1 – F5.19). The isolated compounds **XAN1** and **XAN2** were provided from F5.15 (625 mg), together with a mixture of cowanin and cowaxanthone were found from the F5.12 and F5.13 by comparing their TLC *Rf* values with authentic compounds. The MeOH extract was chromatographed over silica gel to give 11 main fractions (F1–F11). The isolated biflavonoids **BIF1**, **BIF2**, **BIF3** and **BIF4** were provided from F5.5 (293 mg), F5.(7+8) (251 mg), F8.6.5 (235 mg) and a portion of F10 (0.75 g), respectively.

1.Structural determination of XAN1 – XAN2

1.1.XAN1(cowagarcinone E, sss7311)

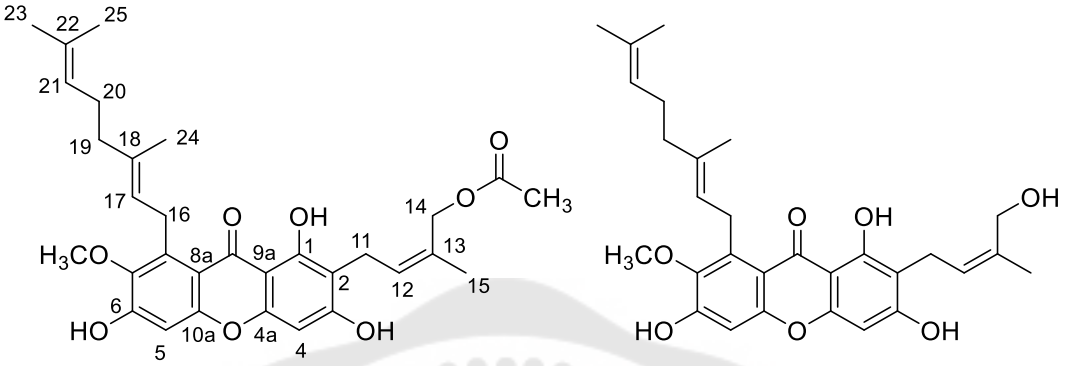


Figure 26 Structures of XAN1 and XAN2

The ^{1}H-NMR data of **XAN1** shown characteristic resonance for *Garcinia* xanthone: a singlet of a chelated hydroxy moiety at δ_H 13.78 (1-OH); a singlet of methoxy protons at δ_H 3.82 (3H); two singlet of isolated aromatic protons at δ_H 6.32 (1H) and δ_H 6.82 (1H), respectively. The signals of the geranyl unit appeared as two olefinic protons at δ_H 5.24 (t, J ca 6.2 Hz, 1H, H-17) and 5.00 (t, J = 7.8 Hz, 1H, H-21), a doublet methylene protons at δ_H 4.08 (J = 6.2 Hz, 2H, H-16), two multiplets of H-19 and H-20, including three methyl singlets H-23-H-25 at δ_H ca 1.23 – 1.99. The signals of an isoprenyl appeared an olefinic proton at δ_H 5.33 (brt, J ca 6.2 Hz, 1H, H-12), methylene protons at δ_H 3.55 (d, J = 6.2 Hz, 2H, H-11), an oxymethylene protons at δ_H 4.74 (s, 2H, H-14) a singlet acetate moiety at δ_H 2.12 and a methyl singlet at δ_H 1.80 (H-15). The ^{1}H-NMR spectrum of **XAN2** was very similar to that of **XAN1** but without an acetoxy group. Comparison of the NMR data of both compounds with the literature values, **XAN1** and **XAN2** were deduced to have structures of cowagarcinine E and cowanol, respectively.

2.Biflavonoid

Biflavonoids are compounds containing two flavonoids monomer and linked by a C-C or C-O-C bond, most of them containing C-C linked monomer. The biosynthesis of biflavonoids involves the radical pairing of two flavonoids units, and different combinations of flavonoid dimers such as flavanone-flavone, flavones-flavone, flavone-flavonol. In *Garcinia* plants, the 3-8" linked biflavonoids are most found (Aravind et al., 2017). At room temperature biflavonoids exhibit duplicate NMR signals, while at high temperature only one set of resonance is obtain (Jamila, Khairuddean, Khan, & N., 2014).

2.1.BIF1 (Volkensuflavone, sss7269)

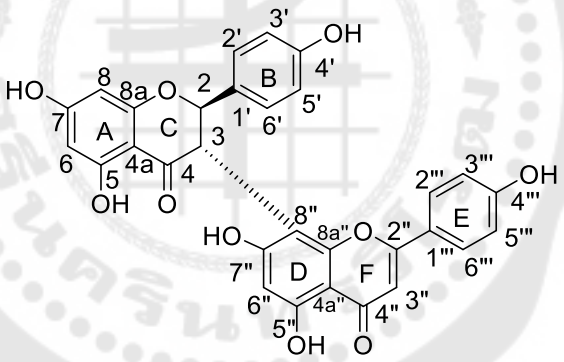


Figure 27 Structure of BIF1

BIF1 was obtained as a yellow solid. The IR spectrum showed the presence of hydroxy groups at 3700-3000 cm^{-1}, conjugated carbonyl groups at 1634 cm^{-1} and 1574 cm^{-1}. Its ^{1}H-NMR data showed the ratio of major to minor signal as 1:0.6 of their respective pairs, indicating of a biflavonoid system (Table 1). The ^{13}C-NMR and DEPT spectra displayed 30 major signals attributable to 14 methines and 14 quaternary carbons and 2 conjugated carbonyl carbons (Table 1). The flavanone unit was found two methine doublets of H-2 at (δ_H 5.66, J = 12.0 Hz) and H-3 at (δ_H 4.98, J = 12.0 Hz) on ring C, together with a COSY correlation was observed between these protons. The

HMBC correlations observed between H-6 and C-8 as well as H-8 and C-6, then the aromatic protons (ring A) at δ_H 5.93 (H-6/8, s, 2H) were assigned to be located at C-6 (δ_C 95.3) and C-8 (δ_C 96.2) positions, and the small $J_{6,8}$ value (1.7 Hz) of their minor pair confirmed the *meta*-coupling among them. The hydroxy groups were attached at C-5 (δ_C 163.9) and C-7 (δ_C 166.6) by HMBC correlations of chelated hydroxy (OH-5, δ_H 12.26) to C-5, C-6 and C-4a (δ_C 101.7). The AM pattern of aromatic protons on ring B appearing at δ_H 7.09 (H-2', $J_{2,3}$ = 8.4 Hz) and δ_H 6.34 (H-3', $J_{5,6}$ = 8.4 Hz), indicated the 1,4-disubstitution which was confirmed by HMBC correlations between H-2' and C-6' as well as H-3' and C-5'. For flavone unit, the HMBC correlations observed between two aromatic singlets H-3'' (δ_H 6.63) to C-6''' (δ_C 129.0) and H-6'' (δ_H 6.22) to C-8'' (δ_C 100.9), together with the chelated hydroxy OH-5'' (δ_H 13.04) to C-5'' (δ_C 160.6) and C-6'' (δ_C 98.8). In addition, ring E was also observed a 1,4-disubstitution same as ring B at δ_H 7.91 (H-2''', $J_{2,3}$ = 8.7 Hz) and δ_H 6.93 (H-3''', $J_{5,6}$ = 8.7 Hz) and confirmed by HMBC correlation among them.

Connections among the ring A/C/B and of D/F/E, including the linkage between flavanone and flavone units were provided by analysis of their HMBC and NOESY (Figure 28). The NOESY correlations of H-2 and H-3 to aromatic protons H-2'/6' (δ_H 7.09) as well as HMBC correlations of H-2 to C-2' (δ_C 128.6), and of H-3 to C-1' (δ_C 128.4) indicating of rings B/C connection. The HMBC correlation of the chelated hydroxy OH-5, H-6, and H-8 to C-4a confirming the rings A/C connection. Linkage between rings D, E, and F of flavone subgroup were confirmed by connectivity between H-3'' to C-1'''; H-2'''/6''' to C-2'' in HMBC spectra together with NOESY correlations of H-3'' to H-6''' suggesting rings E/F link. The HMBC spectrum, correlations displayed from H-6'' and OH-5'' to C-4a'' indicating of rings D/F connection. Furthermore, HMBC interactions seen between the methine protons at H-3 and C-8'', C-8a'' and C-7'' supporting the linkage of the flavanone and flavone subunits via C-3 and C-8'' positions. By comparison, the NMR data of **BIF1** was similar to those of volkensiflavone (Jamila et al., 2014) (Table 1), suggested they have the same chemical structure.

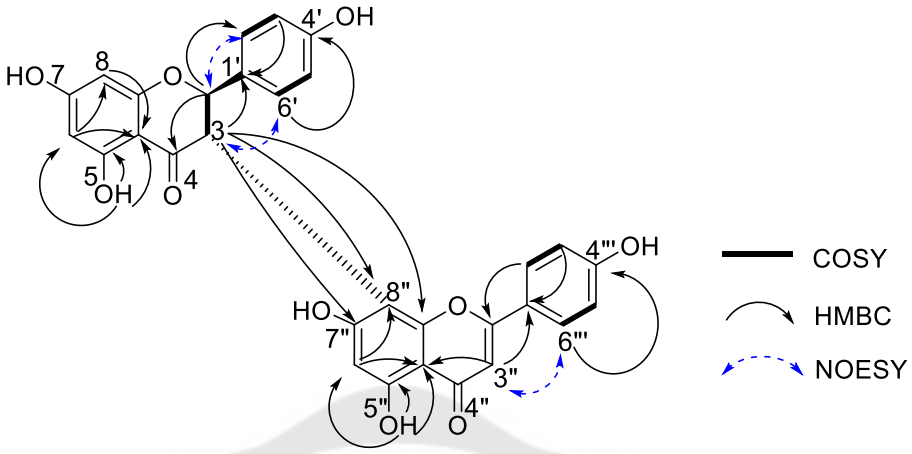


Figure 28 HMBC and NOESY correlation of BIF1

Table 1 Comparison of ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) data of BIF1 with Volkensiflavone in DMSO-d_6

Position	δ_H (mult., *J* in Hz)				δ_C			
	Volkensiflavone [a]		BIF1		Volkensiflavone [a]		BIF1	
	Major	Minor	Major	Minor	Major	Minor	Major	Minor
2	5.70 (d, 11.5, 1H)	5.57 (d, 12.5, 1H)	5.66 (d, 12.0, 1H)	5.53 (d, 12.3, 1H)	81.8	80.8	81.0	82.0
3	4.99 (d, 12.0, 1H)	4.98 (br s, 1H)	4.98 (d, 12.0, 1H)	4.97 (d, 12.3, 1H)	48.0	47.3	48.3	47.5
4					196.5	196.3	196.5	196.7
4a					100.6	100.1	101.7	101.9
5					163.4	162.8	163.9	-
6	5.99 (d, 2.0, 2H)	6.04 (d, 1.5, 1H)	5.93 (s, 1H)	5.98 (d, 1.7, 1H)	96.3	95.1	95.3	95.6
7					167.0	166.3	166.6	167.3
8	5.99 (d, 2.0, 2H)	6.01 (d, 1.5, 1H)	5.93 (s, 1H)	6.02 (d, 1.7, 1H)	98.5	97.9	96.2	96.5
8a					162.8	161.7	161.2	
1'					128.0	127.6	128.4	127.9
2'	7.11 (d, 8.0, 2H)	7.11 (d, 8.0, 2H)	7.09 (d, 8.4, 2H)	7.07 (d, 8.3, 2H)	128.4	128.2	128.6	129.1
3'	6.38 (d, 8.0, 2H)	6.66 (m, 2H)	6.34 (d, 8.4, 2H)	6.59 (d, 8.4, 2H)	114.3	114.5	114.5	114.7
4'					157.5	157.2	157.4	157.7
5'	6.38 (d, 8.0, 2H)	6.66 (m, 2H)	6.34 (d, 8.4, 2H)	6.59 (d, 8.4, 2H)	114.3	114.5	114.5	114.7
6'	7.11 (d, 8.0, 2H)	7.11 (d, 8.0, 2H)	7.09 (d, 8.4, 2H)	7.07 (d, 8.3, 2H)	128.4	128.2	128.6	129.1
2''					163.8	163.3	163.7	164.0
3''	6.63 (s, 1H)	6.78 (s, 1H)	6.63 (s, 1H)	6.75 (s, 1H)	103.1	102.9	102.4	102.2
4''					181.7	181.6	181.9	181.9
4a''					103.1	103.6	103.2	103.2
5''					161.7	161.1	160.6	160.4
6''	6.24 (s, 1H)	6.66 (s, 1H)	6.22 (s, 1H)	6.04 (s, 1H)	98.5	97.9	98.8	98.2
7''					160.9	160.0	163.0	-
8''					101.5	102.2	100.9	100.4
8a''					155.2	154.4	155.5	154.6
1'''					120.7	121.1	120.9	121.3
2'''	7.92 (d, 8.5, 2H)	7.62 (d, 8.5, 2H)	7.91 (d, 8.7, 2H)	7.59 (d, 8.6, 2H)	128.8	128.7	129.0	128.3
3'''	6.93 (d, 8.5, 2H)	6.66 (m, 2H)	6.93 (d, 8.7, 2H)	6.62 (d, 8.6, 2H)	115.8	115.6	116.1	115.8
4'''					160.9	160.5	161.3	-
5'''	6.93 (d, 8.5, 2H)	6.66 (m, 2H)	6.93 (d, 8.7, 2H)	6.62 (d, 8.6, 2H)	115.8	115.6	116.1	115.8
6'''	7.92 (d, 8.5, 2H)	7.62 (d, 8.5, 2H)	7.91 (d, 8.7, 2H)	7.59 (d, 8.6, 2H)	128.8	128.7	129.0	128.3
5-OH	12.20 (s, 1H)	12.10 (s, 1H)	12.26 (s, 1H)	12.15 (s, 1H)				
5''-OH	13.00 (s, 1H)	12.90 (s, 1H)	13.04 (s, 1H)	12.93 (s, 1H)				

[a](Jamila et al., 2014)

2.2.BIF2 (Morelloflavone)

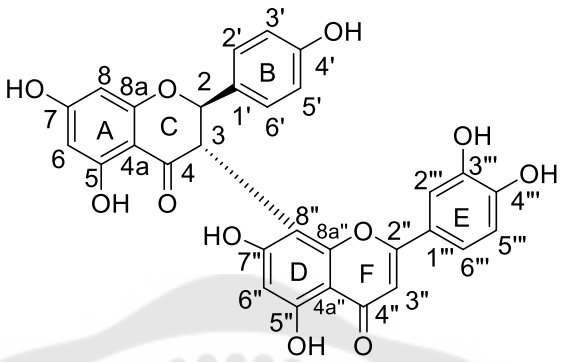


Figure 29 Structure of BIF2

BIF2 was obtained as a pale yellow solid. The IR adsorption was appeared at wave number 3500-2900 cm^{-1}, 1638 cm^{-1} and 1600 cm^{-1}. Its ^{1}H-NMR data was suggested a biflavonoid scaffold same as **BIF1**, and the ratio of major to minor signal as 1:0.3 of their respective pairs. The ^{13}C-NMR and DEPT displayed 30 major signals attributable to 13 methines and 15 quaternary carbons two conjugated carbonyl carbons at δ_C 196.2 and 181.6 ppm (Table 2). By comparing with the NMR chemical shifts of **BIF1** and **BIF2**, they shared the same flavanone-flavone system. The difference was a tri-substitution on the ring E. The ^{1}H NMR data of ring E were observed an ABX pattern for aromatic protons at δ_H 7.41 (H-2''', s, 1H) and two doublets at δ_H 6.89 (H-5''', J = 8.1 Hz, 1H) and δ_H 7.43 (H-6''', J = 8.1 Hz, 1H) which was confirmed by 2D NMR techniques. Only H-5''' and H-6''' show their COSY correlations, and only H-5''' show HMBC correlation to C-3''', this suggested their tri-substitution on ring E.

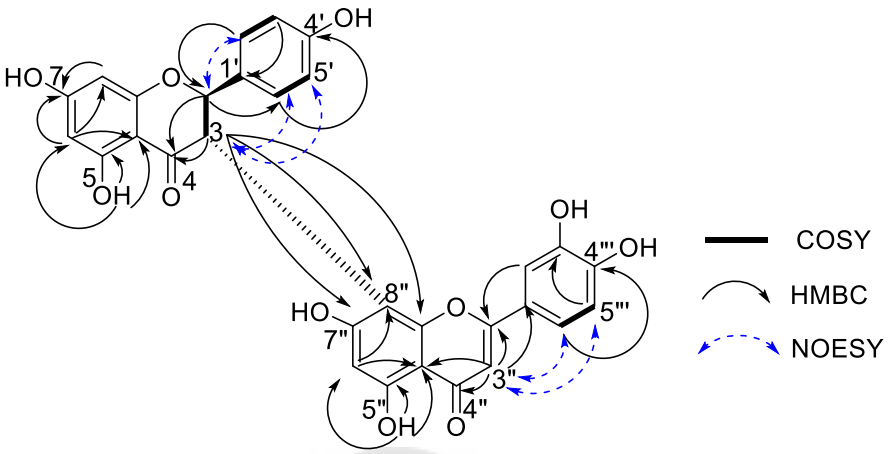


Figure 30 HMBC and NOESY correlation of BIF2

.

Table 2 Comparison ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) data of BIF2 with morelloflavone in DMSO-d_6

Position	δ_H (mult., *J* in Hz) DMSO-d_6				δ_C			
	morelloflavone [a]		BIF2		morelloflavone [a]		BIF2	
	Major	Minor	Major	Minor	Major	Minor	Major	Minor
2	5.74 (d, 12.0, 1H)	5.61 (d, 12.5, 1H)	5.69 (d, 12.0, 1H)	5.87 (d, 12.1, 1H)	80.8	81.7	80.9	81.8
3	4.92 (d, 12.0, 1H)	5.04 (d, 12.5, 1H)	4.88 (d, 12.0, 1H)	4.97 (d, 12.1, 1H)	48.2	48.5	48.2	47.3
4	-	-	-	-	196.1	196.3	196.2	
4a	-	-	-	-	101.4	1016	101.5	101.6
5	-	-	-	-	161.7	162.4	163.8	163.9
6	6.00 (dd, 2.0, 1H)	6.03 (s, 1H)	5.96 (s, 1H)	6.00 (s, 1H)	95.2	95.2	95.3	
7	-	-	-	-	163.4	163.4	160.5	160.2
8	6.00 (dd, 2.0, 1H)	6.03 (d, 1.5, 1H)	5.96 (s, 1H)	5.96 (s, 1H)	96.1	96.3	96.2	96.3
8a					166.5	166.9	166.6	167.0
1’					128	127.5	128.1	-
2’	7.17 (d, 8.0, 2H)	7.12 (d, 8.0, 2H)	7.13 (d, 7.8, 1H)	7.07 (d, 7.6, 1H)	128.4	128.8	128.5	128.8
3’	6.41 (d, 8.0, 2H)	6.63 (d, 8.0, 2H)	6.37 (d, 7.7, 1H)	6.59 (d, 7.6, 1H)	114.3	114.5	114.4	114.6
4’	-	-	-	-	157.2	157.5	157.3	157.6
5’	6.41 (d, 8.0, 2H)	6.63 (d, 8.0, 2H)	6.37 (d, 7.7, 1H)	6.59 (d, 7.6, 1H)	114.3	114.3	114.4	114.6
6’	7.17 (d, 8.0, 2H)	7.12 (d, 8.0, 2H)	7.13 (d, 7.8, 1H)	7.07 (d, 7.6, 1H)	128.4	128.4	128.5	128.8
2’’	-	-	-	-	163.7	163.8	163.4	-
3’’	6.58, (s, 1H)	6.63, (s, 1H)	6.57, (s, 1H)	6.61 (s, 1H)	102.1	102.9	102.2	-
4’’	-	-	-	-	181.6	181.6	181.6	-
4a’’	-	-	-	-	103.0	103.6	103.1	-
5’’					160.4	160.2	163.8	163.9
6’’	6.28, (s, 1H)	6.08, (s, 1H)	6.21, (s, 1H)	6.05 (s, 1H)	98.5	97.9	98.6	98.0
7’’		-		-	162.8	162.7	162.8	162.8
8’’					100.5	100.0	100.5	100.0
8a’’					155.3	154.5	155.2	154.6
1’’’					121.0	121.4	121.0	121.5
2’’’	7.45, (s)	7.26, (s)	7.41, (s, 1H)	7.23 (s, 1H)	113.2	113.7	113.3	113.8
3’’’					145.6	145.9	145.6	146.0
4’’’					149.6	149.6	149.7	-
5’’’	6.93, (d, 8.0, 1H)	6.52, (d, 5.0, 1H)	6.89, (d, 8.1, 1H)	6.95, (d, 8.4, 1H)	116.1	115.1	116.1	115.1
6’’’	7.43, (br s, 1H)	6.99 (dd, 2.0, 7.5,	7.43, (d, 8.1, 1H)		119.2	117.9	119.3	118.0
5-OH	12.20 (s, 1H)	12.10 (s, 1H)	12.25 (s, 1H)	12.10 (s, 1H)				
5’’-OH	13.00 (s, 1H)	12.90 (s, 1H)	13.07 (s, 1H)	12.97 (s, 1H)				

[a](Jamila et al., 2014)

2.3.BIF3 (Spicataside)

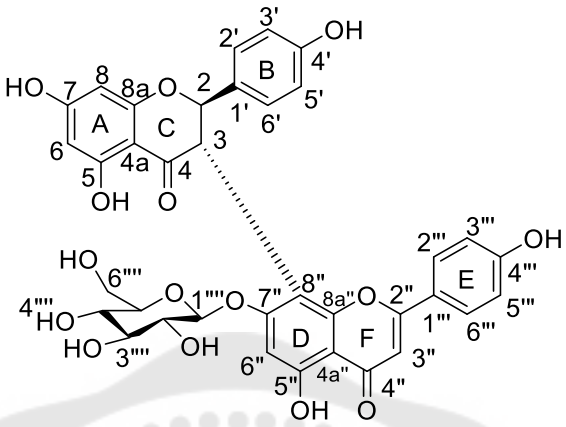


Figure 31 Structure of BIF3

BIF3 was obtained as a pale brown solid. The IR bands were found at 3500-3000 cm^{-1}, 1636 $cm^{-1}$and 1596 cm^{-1} similar to those of BIF1 and BIF2. Its ^{1}H-NMR data was confirmed a biflavonoid skeleton same as BIF1 and the ratio of major to minor signal as 1:0.5 of their respective pairs. The ^{13}C-NMR and DEPT displayed 34 major signals attributable to 19 methines, 1 methylene and 14 quaternary carbons and two conjugated carbonyl carbons at δ_C 196.5 and 181.9 ppm (Table 3). Its NMR data were similar to those of BIF1 except for the presence of an additional of glucose unit, in which an anomeric (δ_C 99.9), 4 oxymethines (δ_C 69.6 (C-4''''), 73.1 (C-2''''), 76.1 (C-3''''), 77.1 (C-5'''')) and one methylene carbons (δ_C 60.5) were suggested from its carbon and DEPT spectra (Table 3). ^{1}H NMR data also supported as shown at δ_H 4.74 (d, J = 7.1 Hz, H-1'''') and δ_H 3.66 (m, 1H, H-6''''). The HMBC (H-1''''/C-7'') and NOESY (H-1''''/H-6'') connections in their spectra suggested the glucose unit attached to ring D at C-7''. In addition, the observed coupling constant value of 7.1 Hz for the anomeric proton indicated the H-1'''' and H-2'''' were *trans* diaxial configuration (Figure 32), and hence the sugar was β-D-glucose (Roslund et al., 2008 & Aravinda et al., 2015 & Mountessou et al., 2018).

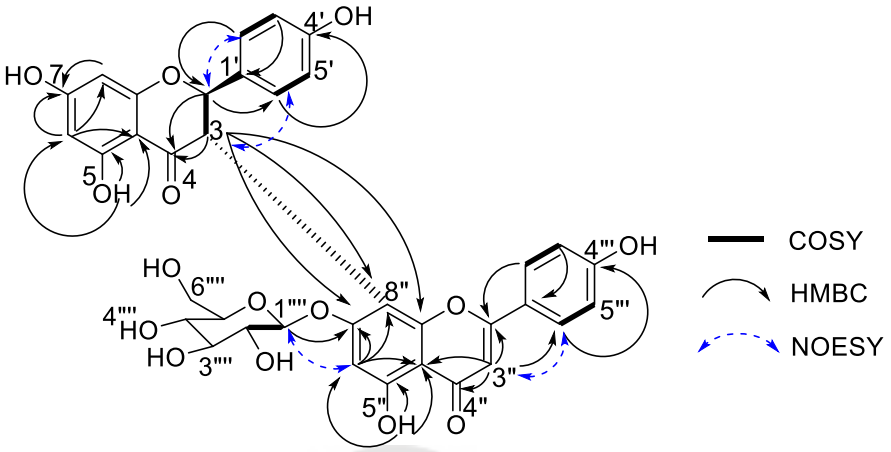


Figure 32 HMBC and NOESY correlation of of BIF3

Table 3 Comparison ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) data of BIF3 with spicataside in DMSO-d_6

Position	δ_H (mult., *J* in Hz) DMSO-d_6				δ_C			
	spicataside [a]		BIF3		spicataside [a]		BIF3	
	Major	Minor	Major	Minor	Major	Minor	Major	Minor
2	5.64 (d, 12.0, 1H)	5.51 (d, 12.5, 1H)	5.66 (d, 12.4, 1H)	5.75 (d, 11.8, 1H)	81.9	81.1	82.3	-
3	5.33 (d, 12.0, 1H)	5.00 (d, 12.5, 1H)	5.38 (d, 12.4, 1H)	5.01 (d, 11.8, 1H)	48.3	47.8	46.9	-
4					196.7	195.9	196.8	195.3
4a					101.5	101.1	101.5	101.6
5					163.2	163.7	163.0	162.9
6	5.89 (d, 2.0, 2H)	5.81 (s, 2H)	5.97 (s, 1H)	5.91 (br s)	96.4	95.2	95.4	94.9
7					167.4	166.2	167.2	166.2
8	5.89 (d, 2.0, 2H)	5.81 (s, 2H)	6.04 (s, 1H)	5.91 (br s)	96.4	95.2	96.4	96.1
8a					162.5	162.3	163.8	-
1’					127.6	127.3	126.7	-
2’	7.21 (d, 8.5, 2H)	7.14 (d, 8.5, 2H)	7.21 (d, 7.8, 1H)	7.13 (d, 7.7, 1H)	128.2	128.1	129.7	128.6
3’	6.56 (d, 8.0, 2H)	6.34 (d, 8.0, 2H)	6.53 (d,7.8, 1H)	6.31 (d, 7.7, 1H)	114.5	114.4	115.0	114.3
4’					157.3	157.1	157.7	157.2
5’	6.56 (d, 8.0, 2H)	6.34 (d, 8.0, 2H)	6.53 (d,7.8, 1H)	6.31 (d, 7.7, 1H)	114.5	114.4	115.0	114.3
6’	7.21 (d, 8.5, 2H)	7.14 (d, 8.5, 2H)	7.21 (d, 7.8, 1H)	7.13 (d, 7.7, 1H)	128.2	128.1	129.7	128.6
2’’					164.0	163.8	164.0	164.1
3’’	6.88 (s, 1H)	6.73 (s, 2H)	6.84 (s, 1H)	6.72 (s, 1H)	103.0	102.2	103.3	103.1
4’’					181.7	181.6	181.9	-
4a’’					104.8	104.3	104.9	104.7
5’’					161.2	160.9	160.4	160.4
6’’	6.48 (s, 1H)	6.73 (s, 2H)	6.48 (s, 1H)	6.72 (s, 1H)	97.7	97.1	98.8	
7’’					159.6	159.3	160.8	160.7
8’’					102.2	102.0	102.2	102.5
8a’’					154.3	153.5	153.6	-
1’’’					120.7	120.3	121.0	120.6
2’’’	7.70 (d, 8.5, 2H)	7.98 (d, 9.0, 2H)	7.66 (d, 8.2, 1H)	7.96 (d, 8.3, 1H)	129.1	128.8	128.3	129.0
3’’’	6.69 (d, 9.0, 2H)	6.97 (d, 9.0, 2H)	6.66 (d, 8.2, 1H)	6.94 (d, 8.3, 1H)	115.9	115.6	115.8	116.0
4’’’					160.8	160.3	161.3	161.5
5’’’	6.69 (d, 9.0, 2H)	6.97 (d, 9.0, 2H)	6.66 (d, 8.2, 1H)	6.94 (d, 8.3, 1H)	115.9	115.6	115.8	116.0
6’’’	7.70 (d, 8.5, 2H)	7.98 (d, 9.0, 2H)	7.66 (d, 8.2, 1H)	7.96 (d, 8.3, 1H)	129.1	128.8	128.3	129.0
1’’’’	5.16 (d, 7.5, 1H)	4.98 (m, 1H)	4.74 (d, 7.1, 1H)	5.14 (d, 7.4, 1H)	100.8	99.9	99.9	100.1
2’’’’	3.58 (br s, 1H)	overlap	Obscured signal	3.07 (m, 1H)	72.8	72.5	73.1	73.6
3’’’’	3.73 (s, 1H)	With H_2O peak	Obscured signal	-	77.2	77.0	76.1	76.8
4’’’’	3.40 (m, 1H)	3.30 (m, 1H)	3.17 (t, 9.0, 1H)	3.07 (m, 2H)	73.7	73.2	69.6	-
5’’’’	3.49 (m, 1H)	3.30 (m, 1H)	Obscured signal	-	69.6	69.6	77.1	77.4
6’’’’	3.56 (d, 7.0, 2H)	3.30 (d, 7.0, 2H)	3.66 (m, 1H)	3.71 (m, 1H)	60.8	60.5	60.5	-
5-OH	12.26 (s, OH)	12.19 (s, OH)	Obscured signal	-				
5’’-OH	13.16 (s, OH)	12.94 (s, OH)	12.07 (s, 1H)					

[a](Jamila et al., 2014)

2.4.BIF4 (fukugiside)

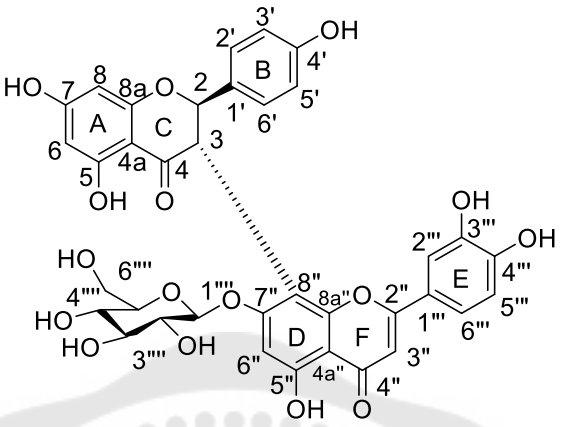


Figure 33 Structure of BIF4

BIF4 was obtained as a pale brown solid. The IR band were shown at 3600-3000 cm^{-1}, 1641 $cm^{-1}$and 1598 cm^{-1}. Its ^{1}H-NMR data was confirmed a biflavonoid skeleton, and the ratio of major to minor signal as 1:0.5 of their respective pairs same as BIF3. The ^{13}C-NMR and DEPT spectra displayed 34 major signals attributable to 18 methines, 1 methylene and 15 quaternary carbons and two conjugated carbonyl carbons at δ_C 196.7 and 181.9 ppm (Table 4). Its NMR data were similar to those of BIF2 except for the presence of an additional of glucose unit. The ^{1}H NMR data of glucose moiety was observed at an anomeric proton δ_H 4.74 (d, J = 6.9 Hz, H-1'''') and δ_H 3.65 (m, 1H, H-6'''') together with an anomeric carbon (δ_C 100.1), 4 oxymethine carbons (δ_C 69.7 (C-4''''), 73.2 (C-3''''), 76.8 (C-2''''), 77.4 (C-5'''')) and one methylene carbon (δ_C 60.8) were suggested from its carbon and DEPT spectra (Table 4). The HMBC (H-1''''/C-7'') and NOESY (H-1''''/H-6'') connections in their spectra suggested the glucose unit attached to ring D at C-7''. In addition, the observed coupling constant value of 6.9 Hz for the anomeric proton indicated the H-1'''' and H-2'''' were *trans* diaxial configuration (Figure 34), and hence the sugar was β-D-glucose (Roslund et al., 2008 & Aravinda et al., 2015 & Mountessou et al., 2018).

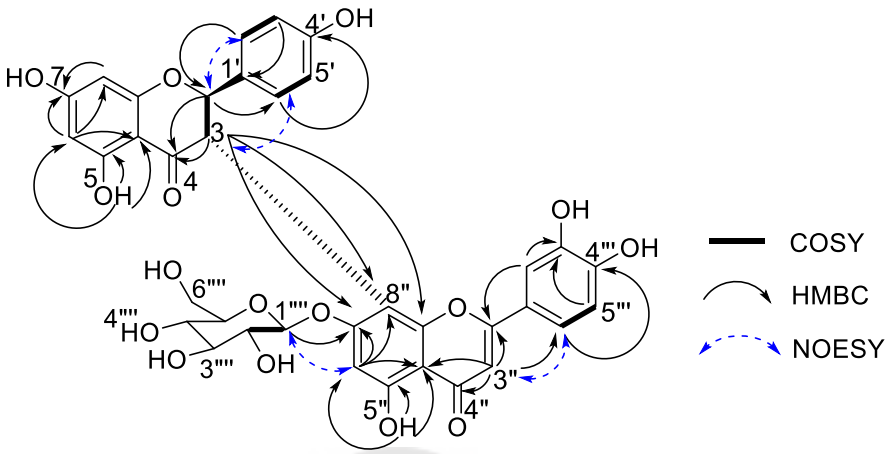


Figure 34 HMBC and NOESY correlation of BIF4

3.Stereochemical detemination

The large coupling constant values of 12.0-12.4 Hz presented between H-2 and H-3 in the ^{1}H NMR data of the **BIF1-BIF4** flavanone units suggested their relative configurations as biaxial arrangements (Osorio, Londoño, & Bastida, 2013), (Mountessou et al., 2018). All isolated biflavonoids gave positive specific optical rotations:

BIF1 $[\alpha]_D^{22}$ +165.5 (*c* 0.23, MeOH), [lit [(Chen, 1975) (+) volkensiflavone $[\alpha]_D^{25}$ +1.6;

BIF2 $[\alpha]_D^{22}$ +234.8 (*c* 0.20, MeOH), [lit [(Chen, 1975) (+) morelloflavone $[\alpha]_D^{20}$ +17;

BIF3 $[\alpha]_D^{23}$ +73.7 (*c* 0.20, MeOH), [lit (Brusotti, 2016) (+) spicataside $[\alpha]_D^{25}$ +1.0;

BIF4 $[\alpha]_D^{22}$ +96.4 (*c* 0.21, MeOH), [lit (Brusotti, 2016) (+) fukugiside $[\alpha]_D^{25}$ +6.1.

Therefore, all biflavonoids obtained were assigned to be (+) volkensiflavone (**BIF1**), (+) morelloflavone (**BIF2**), (+) spicataside (**BIF3**), and (+) fukugiside (**BIF4**).

Table 4 Comparison ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) data of BIF4 with fukugiside in DMSO-d_6

Position	δ_H (mult., *J* in Hz) DMSO-d_6				δ_C			
	fukugiside [a]		BIF4		fukugiside [a]		BIF4	
	Major	Minor	Major	Minor	Major	Minor	Major	Minor
2	5.80 (d, 12.0, 1H)	-	5.71 (d, 12.3, 1H)	5.78 (d, 11.9, 1H)	82.5	-	82.3	80.9
3	4.91 (d, 12.0, 1H)	-	5.38 (d, 12.3, 1H)	4.89 (d, 11.9, 1H)	50.7	-	46.9	48.6
4	-	-	-	-	195.0	-	196.7	195.2
4a	-	-	-	-	103.5	-	101.5	101.6
5	-	-	-	-	164.5	-	163.0	162.9
6	5.94 (d, 4.6, 1H)	-	5.95 (s, 1H)	5.93 (s, 1H)	96.5	-	95.3	95.1
7	-	-			165.7	-	163.8	164.4
8	5.96 (d, 4.0, 1H)	-	6.01 (s, 1H)	5.91 (s, 1H)	97.7	-	96.4	96.3
8a		-			167.0	-	166.4	167.2
1’		-			130.3	-	126.8	128.3
2’	7.17 (d, 8.4, 2H)	-	7.19 (d, 8.1, 1H)	7.17 (d, 8.1, 1H)	129.6	-	129.7	128.7
3’	6.53 (d, 8.4, 2H)	-	6.53 (d, 8.1, 1H)	6.34 (d, 8.1, 1H)	115.5	-	115.0	114.4
4’	-	-	-	-	158.0	-	157.3	157.8
5’	6.53 (d, 8.4, 2H)	-	6.53 (d, 8.1, 1H)	6.34 (d, 8.1, 1H)	115.5	-	115.0	114.4
6’	7.17 (d, 8.4, 2H)	-	7.19 (d, 8.1, 1H)	7.17 (d, 8.1, 1H)	129.6	-	129.7	128.7
2’’	-	-	-	-	165.8	-	164.0	164.1
3’’	6.47 (s, 1H)	6.73 (s, 1H)	6.64, (s, 1H)	6.68 (s, 1H)	103.5	-	103.0	103.3
4’’	-	-	-	-	182.0	-	181.9	181.9
4a’’	-	-	-	-	106.4	-	104.7	104.9
5’’		-			162.0	-	160.4	-
6’’	6.48, (s, 1H)	-	6.48, (s, 1H)	6.71, (s, 1H)	100.0	-	98.3	-
7’’		-			161.2	-	160.8	160.7
8’’		-			103.5	-	102.1	102.5
8a’’		-			155.0	-	153.8	154.6
1’’’		-			123.7	-	120.9	121.3
2’’’	7.25 (s, 1H)	-	7.25, (s, 1H)	7.40, (s, 1H)	114.9	-	113.4	113.9
3’’’		-			146.0	-	146.2	145.8
4’’’		-			152.5	-	150.1	150.2
5’’’	6.93 (d, 8.4, 1H)	-	6.90 (d, 7.8, 1H)	6.55 (d, 8.3, 1H)	114.9	-	115.4	116.3
6’’’	7.59 (d, 8.0, 1H)	-	7.02 (d, 7.8, 1H)	7.43 (d, 8.3, 1H)	120.6	-	118.3	119.6
1’’’’	5.15 (d, 8.0, 1H)	-	4.74 (d, 6.9, 1H)	5.13 (d, 7.6, 1H)	101.6	-	100.1	100.1
2’’’’	3.3-3.8 (m, 5H)	-	Obscured signal	3.08 (m, 1H)	76.1	-	76.8	76.2
3’’’’	3.3-3.8 (m, 5H)	-	Obscured signal	Obscured signal	77.5	-	73.2	73.7
4’’’’	3.3-3.8 (m, 5H)	-	3.16 (t, 8.9, 1H)	3.08 (m, 1H)	69.6	-	69.7	-
5’’’’	3.3-3.8 (m, 5H)	-	Obscured signal	Obscured signal	79.1	-	77.4	77.2
6’’’’	3.3-3.8 (m, 5H)	-	3.65 (d, 11.2, 1H)	3.71 (d, 10.9, 1H)	60.9	-	60.8	60.6
5-OH	12.65 (s, 1H)	-	12.00 (s, 1H)	-				
5’’-OH	12.08 (s, 1H)	-	12.90 (s, 1H)	13.10 (s, 1H)				

[a](Aravinda, Asha, & Rameshkumar, 2015)

4.Antioxidant activity of BIF1 – BIF4

The antioxidant activity of the two extracts and **BIF1 – BIF4** was evaluated by DPPH radical scavenging activity assay and compared with ascorbic acid. As shown in Table 5, Bioactive xanthones and biflavonoids are main contents in the respective less polar (EtOAc) and more polar (MeOH) soluble fractions of Garcinia extracts. The xanthone constituents were reported as weak radical scavenging substances, the MeOH extract expressed approximately 4-time more potent than that of the less polar one (IC_{50} 182.01 µg/mL). Morelloflavone (**BIF2**) displayed the highest effect with IC_{50} 8.85 µg/mL and the activity of which was comparable to that of positive control, ascorbic acid, followed by **BIF4** (IC_{50} 19.65 µg/mL). From the preliminary SAR observations, the absence of a phenolic hydroxyl at C-3 on ring E of these biflavonoid systems suggested for the weak activity for compounds **BIF1** and **BIF3**. Thus, both hydroxyls at positions 3 and 4 on ring E enhance the activity.

Table 5 The antioxidant activity of the extracts and compounds determined with DPPH radical scavenging.

Results are expressed as mean ± SD (n = 2-3)

Sample	DPPH radical scavenging activity IC_{50} (µg/mL)
Ascorbic acid	6.24 ± 0.86
EtOAc extract	182.01 ± 81.49
MeOH extract	47.40 ± 15.12
Volkensiflavone (**BIF1**)	>100
Morolelllavone (**BIF2**)	8.85 ± 3.00
Spicataside (**BIF3**)	> 100
Fukugiside (**BIF4**)	19.65 ± 1.24

CHAPTER 5

Conclusion

Investigation of the chemical constituents of the EtOAc extract obtain from the root barks of *G. cowa* led to the isolation of two xanthones, cowagarcinone E (**XAN1**) and cowanol (**XAN2**). The structures of both compounds were determined by their NMR data analysis, as well as by comparing their TLC *Rf* values with authentic compounds.

Chromatographic separation of the *G. cowa* MeOH extract gave four 3,8" linked biflavonoids, **BIF1 – BIF4**. By extensive 1D- and 2D- NMR data analysis of these biflavonoids offered their structure, as well as by comparison with their literature values, the chemical structures of these biflavonoids were deduced as volkensiflavone (**BIF1**), morelloflavone (**BIF2**), spicataside (**BIF3**) and fukugiside (**BIF4**).

We discovered that the MeOH extract is a good source of antioxidant compounds. **BIF2** and **BIF4** are strong antioxidant agents. **BIF2** exhibited the strongest activity which was comparable to that of the positive control, ascorbic acid.

APPENDIX

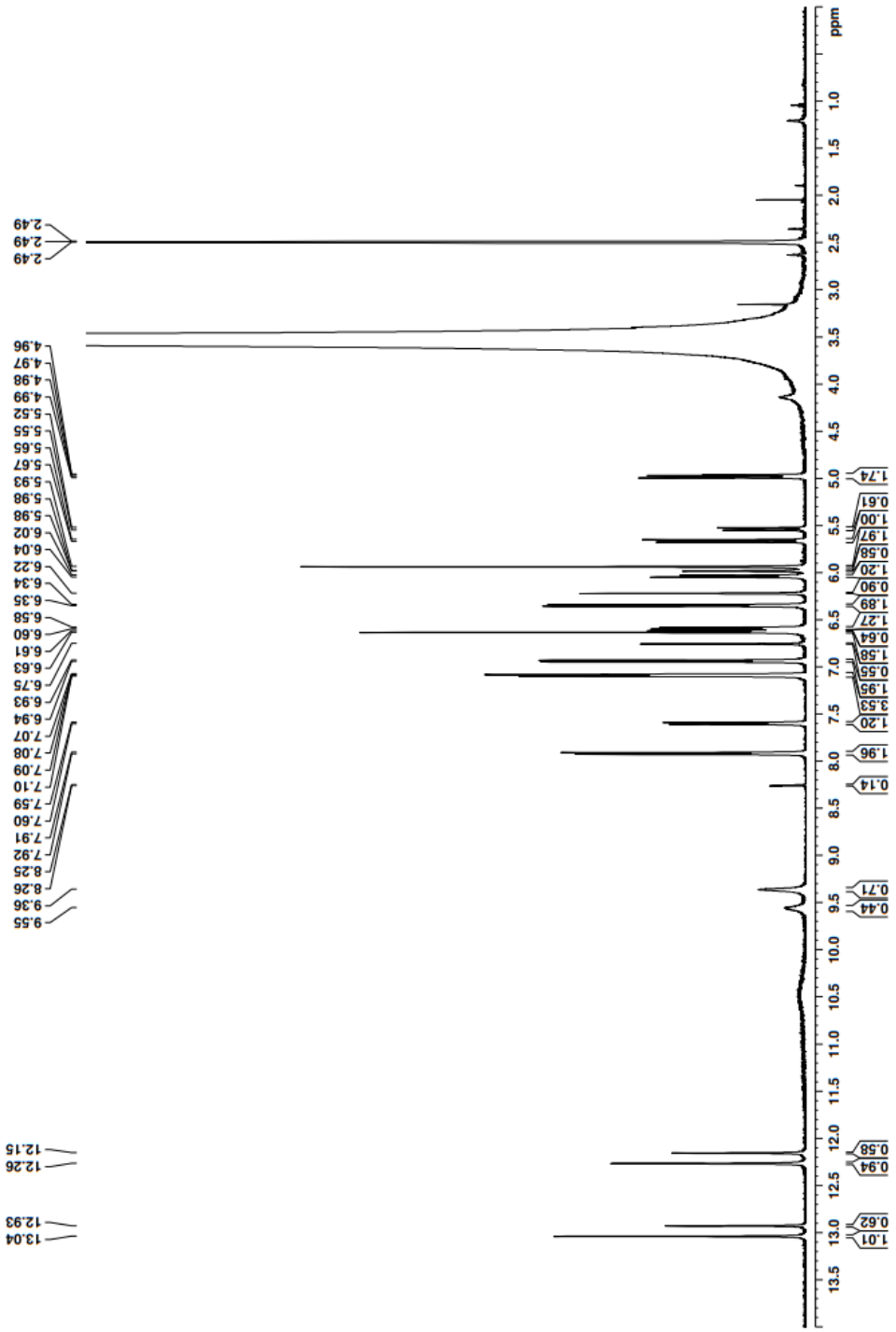


Figure 35 ^{1}H NMR Spectrum of BIF1 (volkensiflavone) in DMSO-d_6

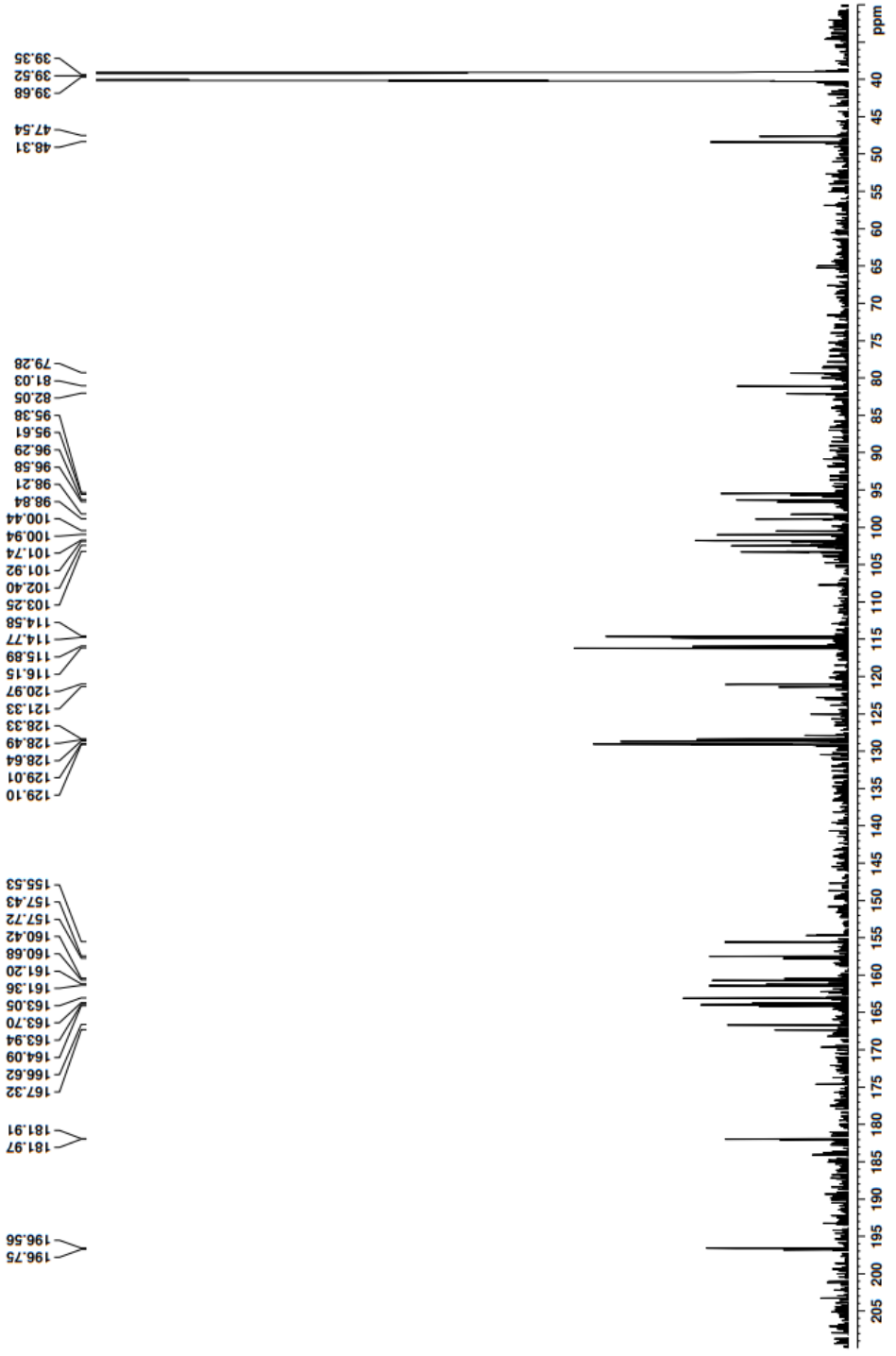


Figure 36 ^{13}C NMR Spectrum of BIF1 (volkensiflavone) in DMSO-d_6

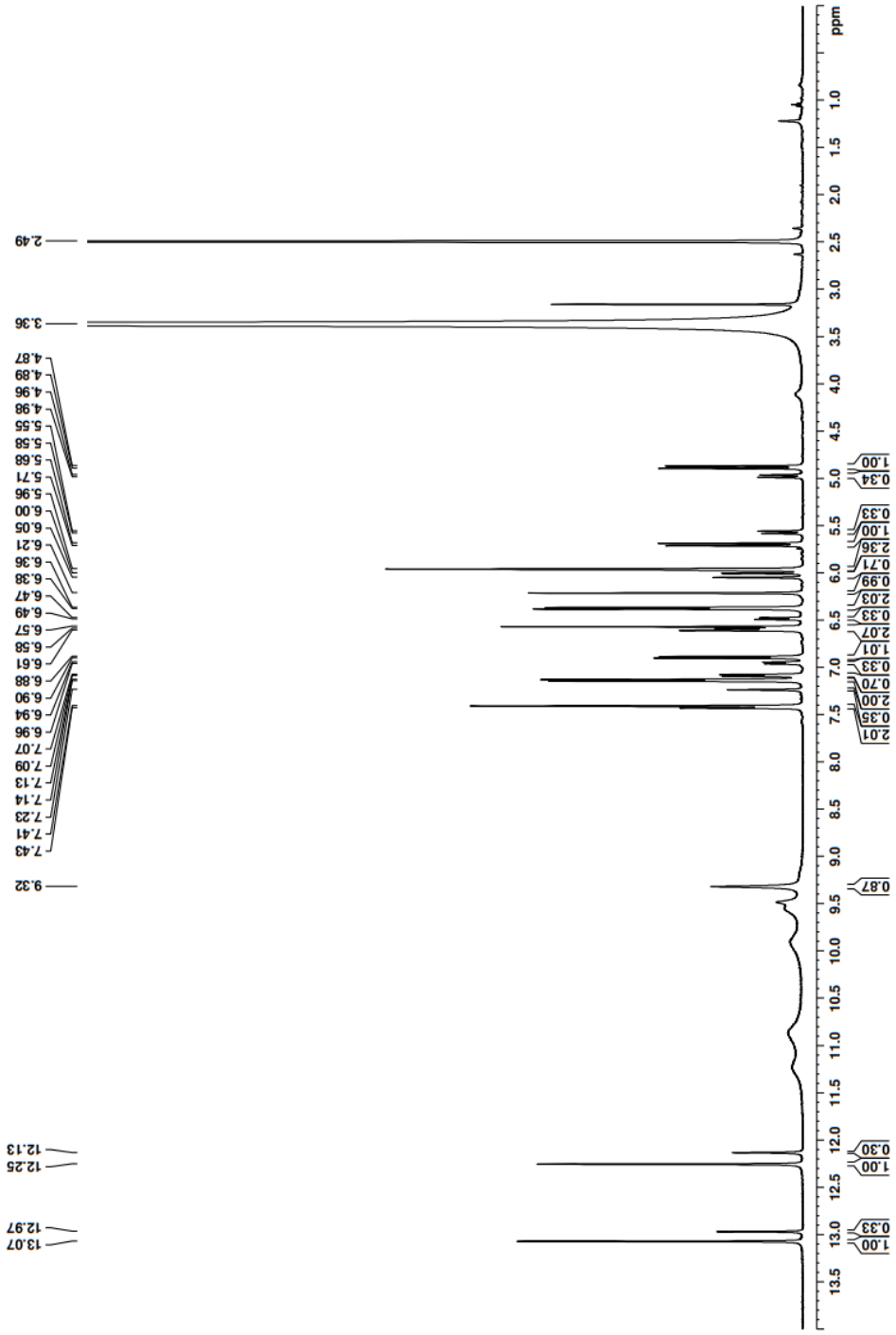


Figure 37 ^{1}H NMR Spectrum of BIF2 (morelloflavone) in DMSO-d_6

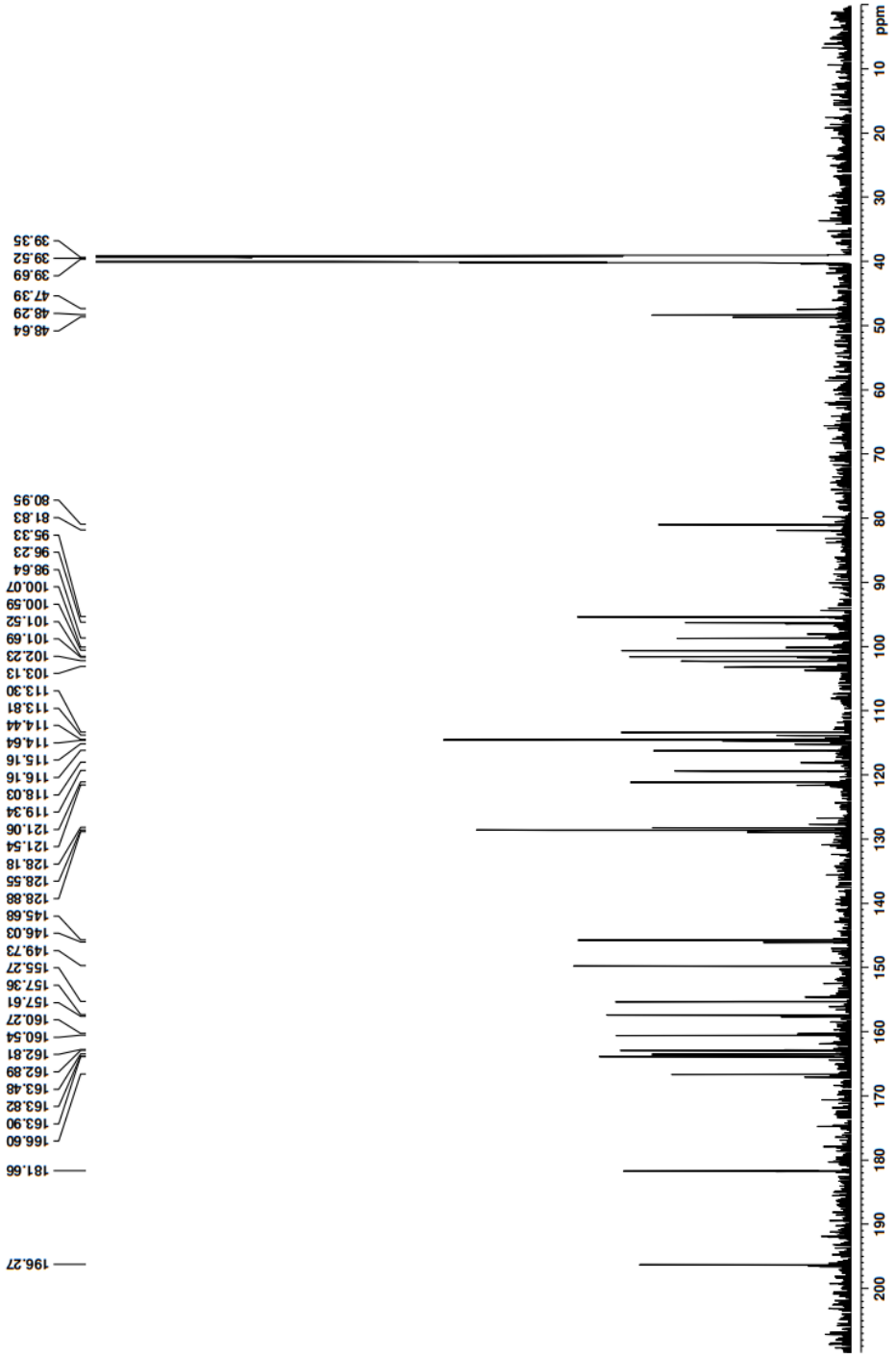


Figure 38 ^{13}C NMR Spectrum of BIF2 (morelloflavone) in DMSO-d_6

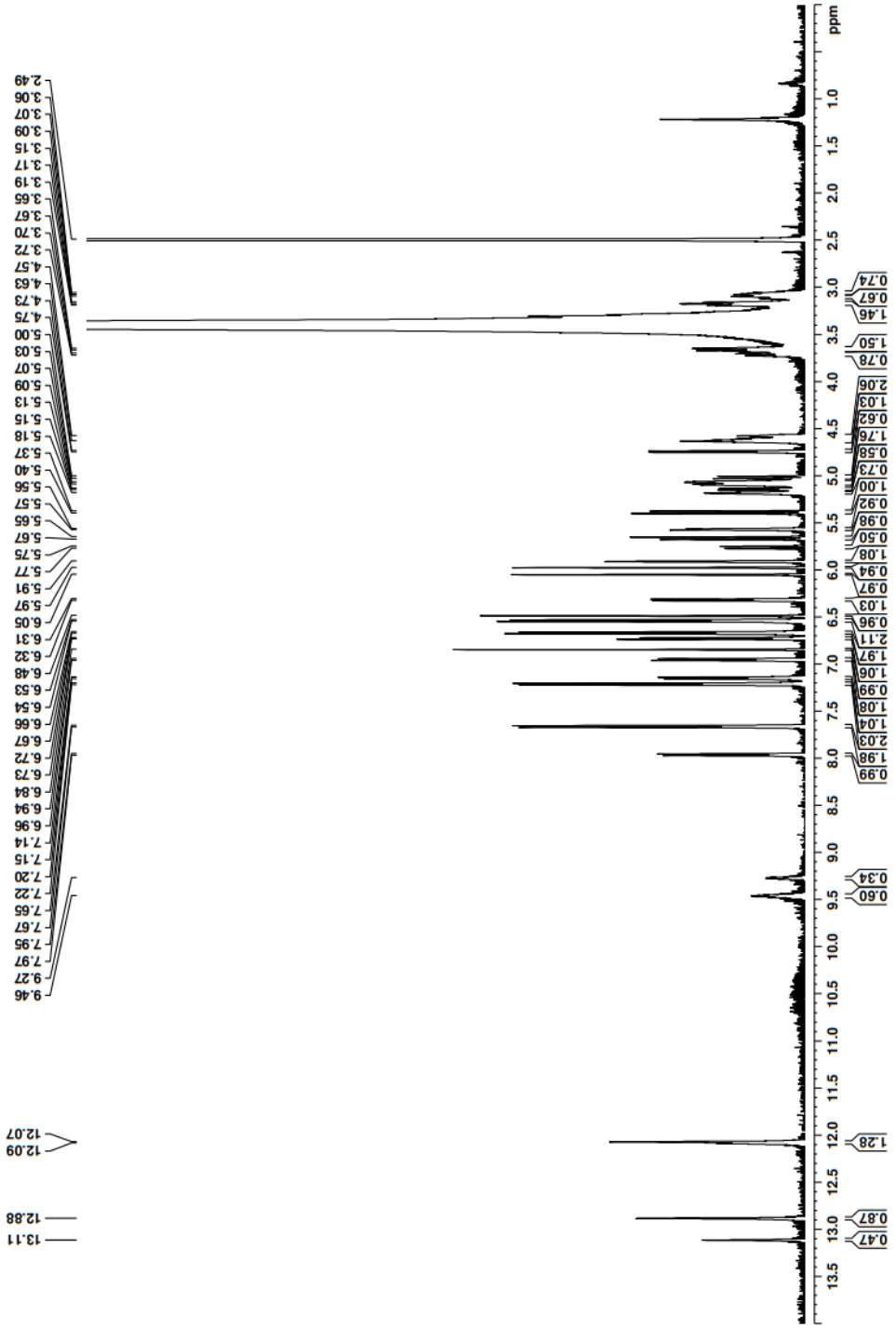


Figure 39 ^{1}H NMR Spectrum of BIF3 (spicataside) in DMSO-d_6

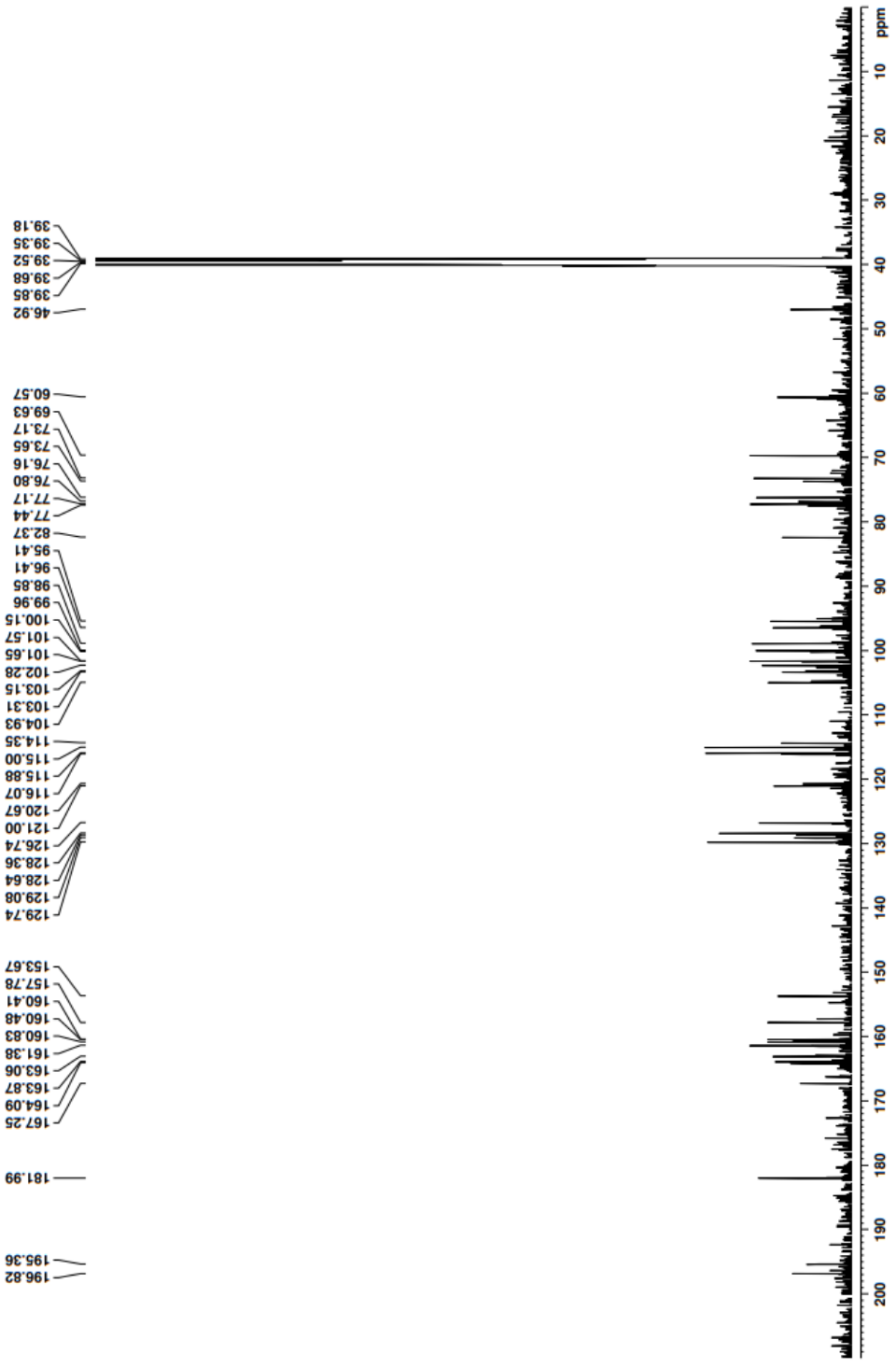


Figure 40 ^{13}C NMR Spectrum of BIF3 (spicataside) in DMSO-d_6

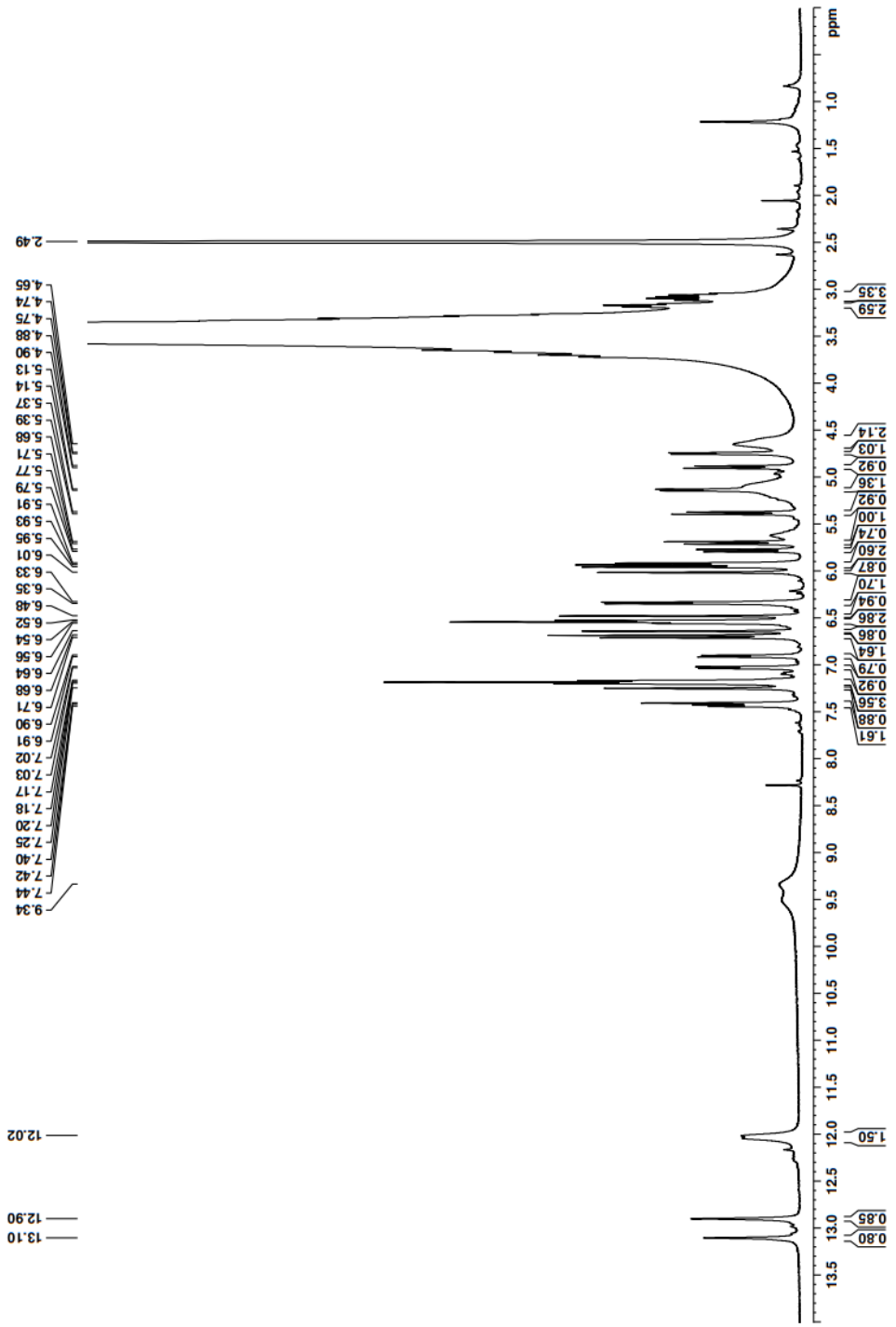


Figure 41 ^{1}H NMR Spectrum of BIF4 (fukugiside) in DMSO-d_6

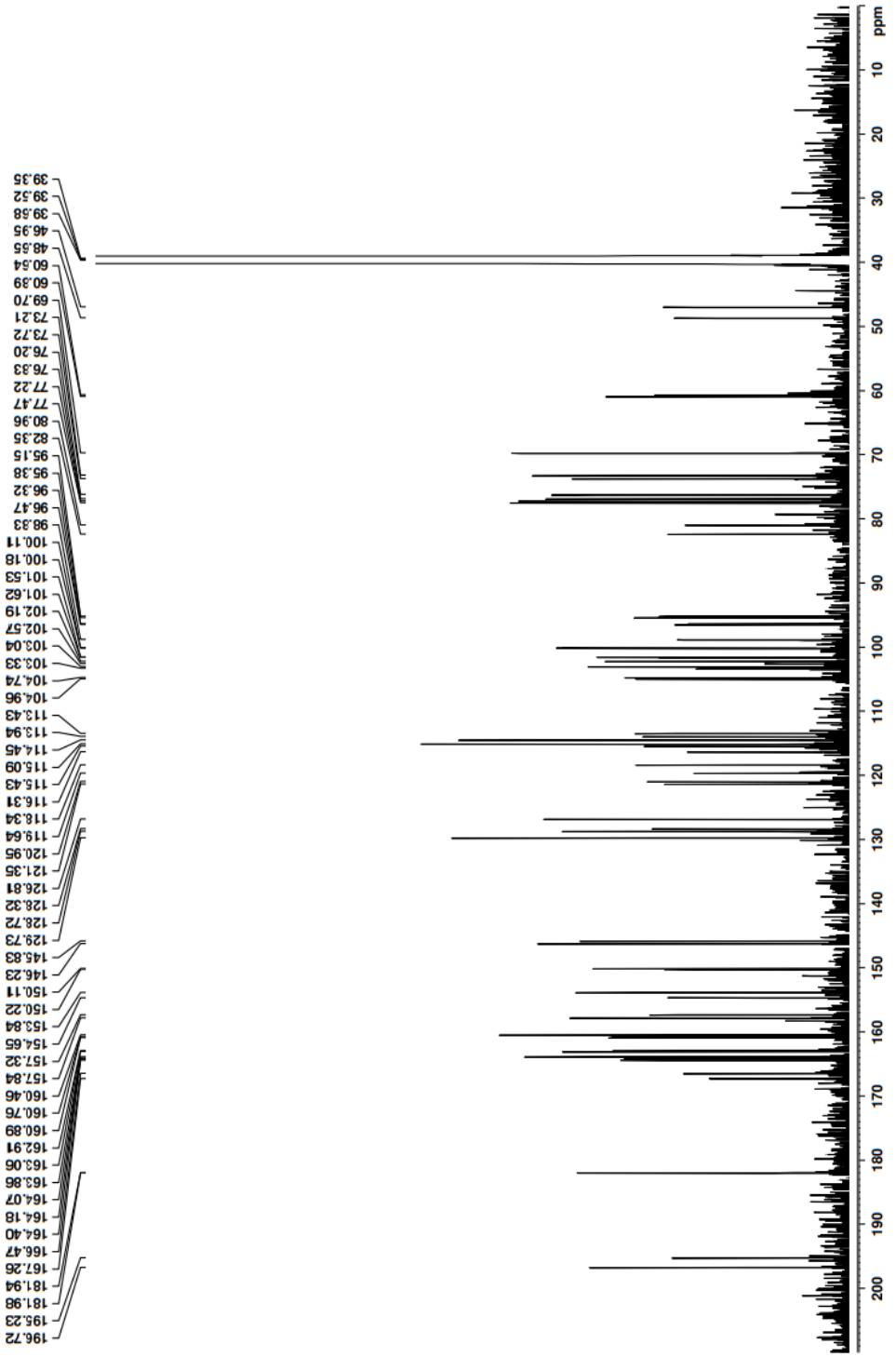


Figure 42 ^{13}C NMR Spectrum of BIF4 (fukugiside) in DMSO-d_6

Research Article

Biflavonoids from Root Barks of *Garcinia cowa* with Radical Scavenging Activity

Pongsan Kornanan[1], Audchara Saenkham[1], Jannarin Nontakham[2], Prayumat Onsrisawat[2], Kulvadee Dolsophon[1], Prasert Pattanaprateeb[1] and Sunit Suksamrarn[1]*

Received: 15 October 2022
Revised: 2 December 2022
Accepted: 2 December 2022

ABSTRACT

Plants of the Clusiaceae family have received considerable attention due to their availability of interesting secondary metabolites. We have previously collected a number of phytochemicals from the *Garcinia* species. In continuation of the search for new bioactive substances from Thai natural resources, we have found that the MeOH extract obtained from the root barks of *Garcinia cowa* Roxb., exhibited a significant antioxidative activity. Subsequently isolation, four compounds of the 3,8" linked biflavonoids were yielded and identified as (+) volkensiflavone (**1**), (+) morelloflavone (**2**), (+) spicataside (**3**), and (+) fukugiside (**4**). Their chemical structures were mainly elucidated by NMR data analysis and by comparison with the reported values. The antioxidant capacity of the isolates was tested using DPPH scavenging assay and compounds **2** and **4** exhibited strong activity with IC_{50} values of 8.85 and 19.65 μg/mL, respectively. The highest activity of compound **2** which was comparable to that of the positive control, revealed the importance of both phenolic hydroxyls at C-3 and C-4 on ring E of the biflavonoid framework.

Keywords: *Garcinia cowa*, biflavonoids, antioxidation

[1] Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Srinakharinwirot University, Thailand

[2] Section of Clinical Research, Division of Research and Academic Support, National Cancer Institute, Bangkok, Thailand

* Corresponding author, email: sunit@g.swu.ac.th

Introduction

Garcinia is a genus of the family Clusiaceae found worldwide such as in tropical Asia, Africa, New Caledonia, Polynesia, and Brazil [1], of which Thailand exhibits a diversity of 29 species [2]. *Garcinia* plants are well known for their bioactive constituents. *Garcinia cowa* Roxb. or Cha-Muang in Thai has been used in food preparation and as traditional folk medicine. The barks are used as an antipyresis agent, fruits and leaves are used for the improvement of blood circulation, treatment of coughs, indigestion and as a laxative; the roots and latex are used for fever relief [2]. All plant parts: roots, stem barks, twigs, latex, leaves and fruits of *G. cowa* have been examined for their bioactive constituents, notably high content of xanthones, phloroglucinols, and flavonoids/biflavonoids [2]. For examples, phloroglucinol benzophenones and xanthones isolated from the leaves and roots inhibited nitric oxide production and α-glucosidase effects [3-4]; xanthones from the roots showed antibacterial activities against *B. cereus* and MRSA-SK1 [5] and cytotoxicity property towards KB and HeLa cells [6]; tetraoxygenated xanthones of the barks and latex were found to possess interesting antimalarial activity against *Plasmodium falciparum* [7-8] but poor radical scavenging action [9] and in addition biflavonoids of the twigs expressed good antioxidative activity [10]. To date, four biflavonoids were obtained from roots [5], twigs [10] and branches [11]. Therefore, phytochemical investigations of *G. cowa* root barks led to the isolation of four biflavonoids: volkensiflavone (**1**), morelloflavone (**2**), spicataside (**3**) and fukugiside (**4**) from the MeOH extract and compound **3** is reported for the first time from this plant species. The antioxidant property of the extracts and isolated compounds was also evaluated based on the principle of scavenging the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical.

Materials and Methods

General experimental procedures

Optical rotations were measured using a Jasco-1020 polarimeter using a 10 mm microcell in MeOH. 1D and 2D NMR experiments were recorded on Bruker NEO 500 FT-NMR spectrometer in dimethyl sulfoxide-d_6 (DMSO-d_6). Chemical shifts were reported using residual DMSO-d_6 (δ_H 2.49 and δ_C 39.52 ppm) as internal standard. IR spectra were obtained using a Perkin-Elmer UATR TWO spectrophotometer. Specific optical rotations were taken on a Jasco-1020 polarimeter. The spots were monitored using TLC sheet precoated with UV fluorescent Merck silica gel 60 F_{254} and were visualized under UV light of 254 nm, and upon staining with anisaldehyde–H_2SO_4 reagent followed by heating. Column chromatography was carried out using Silicycle silica gel 60 (< 0.063 mm) and Sephadex LH-20 (GE Health care).

Plant material

The root barks of *G. cowa* were collected from Chanthaburi Province, Thailand, in January, 2007. A voucher specimen has been deposited at the Laboratory of Natural Product Research Unit, Chemistry Department of Srinakharinwirot University.

Extraction and isolation

The air-dried root barks (530 g) were powdered and extracted with EtOAc (3 × 2.5 L) and then with MeOH (3 × 2.5 L) at room temperature for one week in each extraction and the filtered combined solution of each solvent extraction was evaporated to yield the EtOAc (brownish residue, 98 g) and MeOH (reddish brown sticky, 120 g) extracts, respectively. The MeOH extract exhibited stronger antioxidation activity was further chromatographed. It should be noted that an intense orange coloration upon staining with an anisaldehyde-H_2SO_4 reagent on TLC was the key biflavonoid detection during chromatographic process.

The MeOH extract (110 g) which gave TLC of many orange spots was hence chromatographed over silica gel (80 g) eluting with CH_2Cl_2, gradually increasing the polarity with MeOH to give 11 main fractions (M1–M11). Fraction M5 (1.7 g) was fractionated by column chromatography (CC) (silica gel, 80 g), employing *n*-Hexane–Acetone (92:8 to 0:100) to yield 9 sub-fractions (M5.1–M5.9). Sub-fractions of M5.4 and M5.5 (293 mg) were separated by a Sephadex LH-20 column using MeOH to afford volkensiflavone (**1**, 15 mg,) as a yellow solid. Fractions M6 (8.7 g) was subjected to CC eluting with *n*-Hexane–Acetone (60:40) to provide morelloflavone (**2**, yellow solid, 1.0 g). Fraction M8 (3.1 g) was purified by CC eluting with CH_2Cl_2–MeOH (96:5 to 0:100) to obtain 8 subfractions (M.8.1–M.8.9). Fraction M8.6 (1.1 g) was separated by CC (CH_2Cl_2-MeOH-H_2O, 8.5:1:0.5 to 6:3:1) to give 7 subfractions (M8.6.1– M8.6.7). Repeated CC of subfraction M8.6.5 (235 mg) eluting with CH_2Cl_2-MeOH-H_2O (8.5:1:0.5) afforded spicataside (**3**, 36 mg) as a pale brown solid. Fukugiside (**4**, yellow solid 0.75 g) was successfully yielded by repeated CC of fraction M10 (5 g) using a gradient of CH_2Cl_2-MeOH, 92:8 to 75:25).

Volkensiflavone (**1**): Yellow solid; $[\alpha]_D^{22}$ +165.5 (*c* 0.23, MeOH), lit [12] $[\alpha]_D^{25}$ +1.6; IR: ν_{max} 3175, 1634, 1605, 1574, 1504, 1422, 1361, 1236, 1158 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6) data, see Table 1 and Table 2, respectively.

Morelloflavone (**2**): Pale yellow solid; $[\alpha]_D^{22}$ +234.8 (*c* 0.20, MeOH), lit [12] $[\alpha]_D^{20}$ +17; IR: ν_{max} 3199, 1638, 1600, 1578, 1509, 1361, 1258, 1160 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6) data, see Table 1 and Table 2, respectively.

Spicataside (**3**): Pale brown solid; $[\alpha]_D^{23}$ +73.7 (*c* 0.20, MeOH), lit [13] $[\alpha]_D^{25}$ +1.0 (*c* 0.1, MeOH); IR: ν_{max} 3226, 1636, 1596, 1446, 1363, 1270, 1238, 1164, 1068 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6) data, see Table 1 and Table 2, respectively.

Fukugiside (**4**): Yellow solid; $[\alpha]_D^{22.5}$ +96.4 (*c* 0.21, MeOH), lit [13] $[\alpha]_D^{25}$ +6.1 (*c* 0.1, MeOH); IR: ν_{max} 3264, 1641, 1598, 1518, 1448, 1368, 1258, 1167 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6) data, see Table 1 and Table 2, respectively.

Table 1 ^{1}H NMR Spectroscopic Data for Compounds **1**–**4** in DMSO-d_6

No.	1	2	3	4
	δ_H Major, Minor (multiplicity, J in Hz)			
2	5.66 (d, 12.0), 5.53 (d, 12.3)	5.69 (d, 12.0), 5.87 (d, 12.1)	5.66 (d, 12.4), 5.75 (d, 11.8)	5.78 (d, 11.9), 5.79 (d, 12.3)
3	4.98 (d, 12.0), 4.97 (d, 12.3)	4.88 (d, 12.0), 4.97 (d, 12.1)	5.38 (d, 12.4), 5.01 (d, 11.8)	5.38 (d, 11.9), 4.89 (d, 12.3)
6	5.93 (s), 5.98 (d, 1.7)	5.96 (s), 6.05 (s)	5.97(s), 5.90 (br s)	5.95 (s), 5.91(s)
8	5.93 (s), 6.02 (d, 1.7)	5.96 (s), 5.96 (s)	6.04 (s), 5.90 (br s)	6.01 (s), 5.91 (s)
2'	7.09 (d, 8.4), 7.07 (d, 8.4)	7.13 (d, 7.8), 7.07 (d, 7.6)	7.21 (d, 7.8), 7.14 (d, 7.7)	7.17 (d, 8.1), 7.17 (d, 8.1)
3'	6.34 (d, 8.4), 6.59 (d, 8.4)	6.37 (d, 7.7), 6.57 (d, 7.6)	6.53 (d, 7.8), 6.31 (d, 7.7)	6.53 (d, 8.1), 6.34 (d, 8.1)
5'	6.34 (d, 8.4), 6.59 (d, 8.4)	6.37 (d, 7.7), 6.57 (d, 7.6)	6.53 (d, 7.8), 6.31 (d, 7.7)	6.53 (d, 8.1), 6.34 (d, 8.1)
6'	7.09 (d, 8.4), 7.07 (d, 8.4)	7.13 (d, 7.8), 7.07 (d, 7.6)	7.21 (d, 7.8), 7.14 (d, 7.7)	7.17 (d, 8.1), 7.17 (d, 8.1)
3''	6.63 (s), 6.75 (s)	6.57 (s), 6.61 (s)	6.84 (s), 6.72 (s)	6.64, (s), 6.68, (s)
6"	6.22 (s), 6.04 (s)	6.21 (s), 6.05 (s)	6.48 (s), 6.72 (s)	6.48, (s), 6.71, (s)
2"'	7.91 (d, 8.7), 7.59 (d, 8.6)	7.43 (br s), 7.23 (s)	7.66 (d, 8.2), 7.96 (d, 8.3)	7.20 (d 7.8), 7.43, (d, 8.3)
3"'	6.93 (d, 8.7), 6.62 (d, 8.6)		6.66 (d, 8.2), 6.94 (d, 8.3)	
5"'	6.93 (d, 8.7), 6.62 (d, 8.6)	6.89 (d, 8.1) 6.95 (d, 8.4)	6.66 (d, 8.2), 6.94 (d, 8.3)	6.90 (d, 7.8), 6.55 (t, 8.3)
6"'	7.91 (d, 8.7), 7.59 (d, 8.6)	7.43 (d, 8.1)	7.66 (d, 8.2), 7.96 (d, 8.3)	7.02 (d, 7.8), 7.43 (d, 8.3)
1""	-	-	4.75 (d, 7.3), 5.14 (d, 7.3)	4.74 (d, 6.9) 5.13 (d, 7.6)
2""	-	-	Obscured signal, 3.05 (m)	Obscured signal, 3.08 (m)
3""	-	-	Obscured signal	Obscured signal
4""	-	-	3.17 (m), 3.07 (m)	3.16 (m), 3.08 (m)
5""	-	-	Obscured signal	Obscured signal
6""	-	-	3.66 (m), 3.71 (m)	3.67 (d, 10.9)
5-OH	12.26 (s), 12.15 (s)	12.25 (s), 12.10 (s)	12.07 (s)	12.00 (s)
5"-OH	13.04 (s), 12.93 (s)	13.07 (s), 12.97 (s)	12.88 (s), 13.11 (s)	12.90 (s), 13.10 (s)

Table 2 ^{13}C NMR Spectroscopic Data for Compounds **1** - **4** in DMSO-d_6

No.	1	2	3	4
	δ_C Major, Minor			
2	81.0, 82.0	80.9, 81.8	82.3, 82.9	82.3, 80.9
3	48.3, 47.5	48.2, 47.3	46.9, 48.5	46.9, 48.6
4	196.5, 196.7	196.2,	196.8, 196.3	196.7, 195.2
4a	101.7, 101.9	101.5, 101.6	101.5, 101.6	101.5, 101.6
5	163.9	163.8, 163.9	163.0, 162.9	163.0, 162.9
6	95.3, 95.6	95.3	95.4, 94.9	95.3, 95.1
7	166.6, 167.3	160.5, 160.2	167.2, 166.5	163.8, 164.4
8	96.2, 96.5	96.2, 96.3	96.4, 96.1	96.4, 96.3
8a	161.2	166.6, 167.0	163.8	166.4, 167.2
1'	128.3, 127.9	128.1	126.7	126.8, 128.3
2'	128.6, 129.1	128.5, 128.8	129.7, 128.6	129.7, 128.7
3'	114.5, 114.7	114.4, 114.6	115.0, 114.3	115.0, 114.4
4'	157.4, 157.7	157.3, 157.6	157.7, 157.2	157.3, 157.8
5'	114.5, 114.7	114.4, 114.6	115.0, 114.3	115.0, 114.4
6'	128.6, 129.1	128.5, 128.8	129.7, 128.6	129.7, 128.7
2''	163.7, 164.0	163.4	164.0	164.4, 164.1
3''	102.4, 102.2	102.2	103.3, 103.1	103.0, 102.5
4''	181.9, 181.9	181.6	181.9	181.9
4a''	103.2, 103.2	103.1	104.9, 104.7	104.7, 104.9
5''	160.6, 160.4	163.8, 163.9	160.4, 160.4	160.4
6''	98.8, 98.2	98.6	98.8	98.3
7''	163.0, 163.0	162.8, 162.8	160.8, 160.7	160.8, 160.7
8''	100.9, 100.4	100.5, 100.0	102.2, 102.5	102.1, 103.3
8a''	155.5, 154.6	155.2, 154.6	153.6	153.8, 154.6
1'''	120.9, 121.3	121.0, 121.5	121.0, 120.6	120.9, 121.3
2'''	129.0, 128.3	113.3, 113.8	128.3, 129.0	113.4, 113.9
3'''	116.1, 115.8	145.6, 146.0	115.8, 116.0	146.2, 145.8
4'''	161.3	149.7	161.3, 161.5	150.1, 150.2
5'''	116.1, 115.8	116.1, 115.1	115.8, 116.0	115.4, 116.3
6'''	129.0, 128.3	119.3, 118.0	128.3, 129.0	118.3, 119.6
1''''			99.9, 100.1	100.1, 100.1
2''''			73.1, 73.6	76.8, 76.2
3''''			76.1, 76.8	73.2, 73.7
4''''			69.6	69.7
5''''			77.1, 77.4	77.4, 77.2
6''''			60.5	60.8, 60.6

DPPH scavenging assay

The DPPH radical scavenging activity of samples was determined by reaction with 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals in a microplate format [14-15]. DPPH solution was prepared in ethanol. The plant extract at various concentrations was diluted with DMSO to get sample solution. 5 µL of sample solution was treated with 195 µL of 100 µM DPPH solution and reacted at room temperature for 30 min in dark. The absorbance was measured at 515 nm with a control containing DPPH and ethanol. Ascorbic acid (0.78-100 µg/mL) was used as a positive control. The ability to scavenge the DPPH radical was expressed as percentage inhibition and calculated according to the equation [16]:

$$\text{DPPH radical scavenging activity (\%)} = (A_{control} - A_{sample}) / A_{control} \times 100$$

where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the sample.

The half-maximal inhibitory concentration (IC_{50}) of the sample against DPPH radical was calculated based on the liner regression of the percentage of remaining DPPH radical against the sample concentration. Assays were performed in triplicate and results are shown as mean ± standard deviation. The difference significance was assessed using one-way ANOVA followed by Dunnett's test for individual differences using SPSS program version 25.

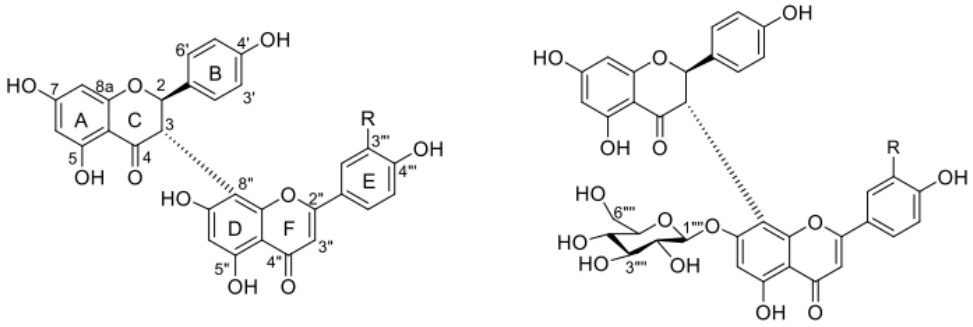


Figure 1 Chemical structures of compounds **1**–**4**.

Results and Discussion

The dried powder of the *G. cowa* root barks was thoroughly extracted with EtOAc and then with MeOH. The resulting fractions were screened for antioxidation activity using DPPH radical scavenging activity procedure and the MeOH extract, which found much interesting activity (Table 3) with IC_{50} of 47.40 µg/mL than the less polar EtOAc fraction (IC_{50} 182.01 µg/mL), was subjected to further investigation for bioactive substituents. Chromatographic separation of this MeOH soluble extract yielded four biflavonoids of the 3,8" linked type, **1**–**4** (Figure 1). Several common chemical and

spectroscopic characteristics are evident for the isolated compounds. They display an intense orange color with anisaldehyde–H_2SO_4 reagent on TLC. Their IR spectra show absorption bands for hydroxyl (3175–3264 cm^{-1}), conjugated carbonyl (1634–1641 cm^{-1}) and aromatic (1605–1574 cm^{-1}) functional groups. Their ^1H and ^{13}C NMR spectra exhibit two sets of resonances which is a characteristic of biflavonoid system and agree well with the published data for flavanone-flavone type [17].

Compound **1** was obtained as a yellow solid with $[\alpha]_D^{22}$ +165.5 (*c* 0.23, MeOH) and its IR data showed strong absorption bands at 3175 (OH), 1634 (conjugated CO), 1605 and 1574 (aromatic) cm^{-1}. The ^1H- and ^{13}C-NMR spectra recorded in DMSO-d_6 (Tables 1 and 2) exhibited two sets of signals in a relative 1.6:1 ratio. The ^{13}C NMR, DEPT and HMQC data offered the presence of 30 carbons attributable to 14 methines and 14 quaternary carbons including two conjugated carbonyl carbons at δ_C 196.5 and 181.9 ppm, suggesting of a biflavonoid system for **1**. Two sets of methine doublet at δ_H 5.66 (J = 12.0 Hz) and 4.98 (J = 12.0 Hz) on ring C were shown in the ^1H NMR spectrum including COSY correlations observed between these protons indicating the presence of a flavanone unit. By HMQC and HMBC correlations the aromatic protons (ring A) at δ_H 5.93 (H-6/8, s, 2H) was assigned to be located at C-6 (δ_C 95.3) and C-8 (δ_C 96.2) positions. The doublets of *ortho* coupled aromatic protons (rings B and E) appearing at δ_H 7.09 (H-2'/6', d, J = 8.4 Hz, 2H) and δ_H 6.34 (H-3'/5', d, J = 8.4 Hz, 2H) of ring B, together with δ_H 7.91 (H-2'''/6''', d, J = 8.7 Hz, 2H) and δ_H 6.93 (H-3'''/5''', d, J = 8.6 Hz, 2H) of ring E were attributed to the position C-2'/6' (δ_C 128.6), C-3'/5' (δ_C 114.5), C-2'''/6''' (δ_C 129.0), and C-3''',5''' (δ_C 116.1), respectively by HMQC connectivity. Two singlets at δ_H 6.63 and 6.22 were assigned to H-3" and H-6" of rings F and D, respectively, together with the two singlet signals of chelated hydroxy at δ_H 13.04 (OH-5$''$) and 12.26 (OH-5) were observed.

Connections among the rings A/B/C and D/E/F, including the linkage between flavanone and flavone units were provided by analysis of its HMBC and NOESY spectra (Figure 2). The NOESY correlations of methine proton at δ_H 5.66 (H-2) and δ_H 4.98 (H-3) to aromatic protons H-2'/6' (δ_H 7.09), as well as HMBC correlations of H-2 to C-2'/6' (δ_C 128.6), and of H-3 to C-2'/6' indicating of rings B/C connection. The HMBC correlation of the chelated hydroxy OH-5 and H-6 to C-4a (δ_C 101.7) confirming rings A/C connection. Connectivity between H-3" (δ_H 6.63) to C-2"(δ_C 163.7); H-2'''/6''' (δ_H 7.91) to C-2" (δ_C 163.7) and C-3" (δ_C 102.4) in HMBC spectra together with NOESY correlations of H-3" (δ_H 6.63) to H-2'''/6''' (δ_H 7.91) suggesting rings E/F link. The HMBC spectrum, correlations displayed from H-6" to C-5", and from OH-5" to C-4a" indicating of rings D/F connection. Furthermore, HMBC interactions seen between the methine protons at H-3 and C-8", C-8a" and C-7" supporting the linkage of the flavanone and flavone subunits via C-3 and C-8" positions.

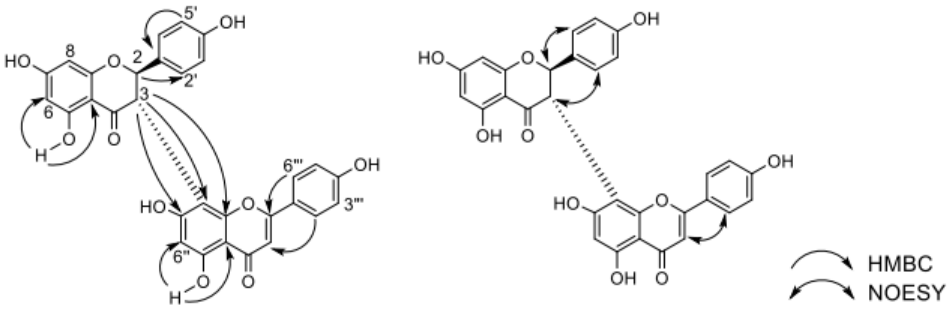


Figure 2 HMBC and NOESY correlations for volkensiflavone (**1**)

The chemical structures of the other biflavonoids **2**–**4** were also determined in the same manner as for **1**. Compounds **2**–**4** were isolated as yellow amorphous and their NMR data measured in DMSO-d_6 (as shown in Table 1 and Table 2) also revealed as two sets of resonances. By comparison of their IR and NMR spectroscopic data with the literature value and with those of compound **1** together with the extensive 1D- and 2D-NMR data analysis, compounds **2**–**4** shared the same flavanone-flavone scaffold as for **1** (Figure 1). In fact, compounds **3** and **4** are glucoside analogs of volkensiflavone (**1**) and morelloflavone (**2**), respectively. The metabolites **1**, **2** and **4** have been found previously from the same plant of *G. cowa* [10], whilst spicataside (**3**) was obtained from other *Garcinia* species [17-20].

The relative configuration of the two stereogenic centers at C-2 and C-3 was suggested from the large coupling constant value of 11.9–12.4 Hz presented between H-2 and H-3 in the ^{1}H NMR spectra of the flavanone unit in compounds **1**–**4** indicated for their *trans* diaxial arrangement [19, 21]. Furthermore, the positive specific optical rotation values for **1**–**4** (**1** : $[\alpha]_D^{22}$ +165.5; **2** : $[\alpha]_D^{22}$ +234.8; **3** : $[\alpha]_D^{23}$ +73.7; and **4** : $[\alpha]_D^{22.5}$ +96.4) are in good agreement with earlier reports. All isolated metabolites are therefore characterized as (+) volkensiflavone (**1**), (+) morelloflavone (**2**), (+) spicataside (**3**), and (+) fukugiside (**4**).

The antioxidant activity of the two extracts and compounds **1**–**4** was evaluated by DPPH radical scavenging activity assay and compared with those of well-known antioxidant, ascorbic acid. Bioactive xanthones and biflavonoids are main contents in the respective less polar (EtOAc) and more polar (MeOH) soluble fractions of *Garcinia* extracts [2-12]. The xanthone constituents were reported as weak radical scavenging substances [9]. As shown in Table 3, the MeOH extract expressed approximately 4-time more potent than that of the less polar one (IC_{50} 182.01 µg/mL). Morelloflavone (**2**) displayed the highest effect with IC_{50} 8.85 µg/mL and the activity of which was comparable to that of the positive control, ascorbic acid, followed by **4** (IC_{50} 19.65 µg/mL). From the preliminary SAR observations, the absence of a phenolic hydroxyl at C-3 on ring E of these biflavonoid systems suggested for the weak activity for compounds **1** and **3**. Thus, both hydroxyls at positions 3 and 4 on ring E enhance the activity.

Table 3 The antioxidant activity of the extracts and compounds determined with DPPH radical scavenging. Results are expressed as mean ± SD (n = 2-3)

Sample	DPPH radical scavenging activity IC_{50} (µg/mL)
Ascorbic acid	6.24 ± 0.86
EtOAc extract	182.01 ± 81.49*
MeOH extract	47.40 ± 15.12
Volkensiflavone (**1**)	>100
Morolelllavone (**2**)	8.85 ± 3.00
Spicataside (**3**)	> 100
Fukugiside (**4**)	19.65 ± 1.24

*Indicates that this value is significantly different compared with the positive control (ascorbic acid) at $p < 0.05$.

Conclusions

We discovered that the MeOH extract of *G. cowa* root barks is a good source of antioxidant compounds. Four biflavonoids of the 3,8" linked type were obtained and two of them were strong antioxidant agents. Compound **2** exhibited the strongest activity which was comparable to that of the positive control, ascorbic acid.

Acknowledgements

This work was supported by the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Ministry of Higher Education, Science, Research and Innovation and by Srinakharinwirot University (Grant number 029/2564). The authors would like to thank Department of Chemistry, Faculty of Science, Srinakharinwirot University for the laboratory facilities. Moreover, the authors also would like to thank to Dr. Jantana Yahuafai and Dr. Suratsawadee Piyaviriyakul, Section of Clinical Research, Division of Research and Academic Support, National Cancer Institute for kindly providing the assistance with some *in vitro* studies.

References

1. Santo BLSE, Santana LF, Junior WHKJ, Araújo FO, Bogo D, et al. Medicinal potential of *Garcinia* species and their compounds. Molecules. 2020;25:4513-42.
2. Ritthiwigrom T, Laphookhieo S, Pyne SG. Chemical constituents and biological activities of *Garcinia cowa* Roxb. Maejo Int J Sci Technol. 2013;7(2):212-31.

3. Raksat A, Phukhatmuen P, Yang J, Maneerat W, Charoensup, R., Andersen, R.J., et al. Phloroglucinol benzophenones and xanthones from the leaves of *Garcinia cowa* and their nitric oxide production and α-glucosidase inhibitory activities. J Nat Prod. 2020;83:164-8.
4. An NTK, Hien NV, Thuy NT, Phuong DL, Bach HG, Tra NT., et. al. Garcicowanones C-E, three new hydrated-geranylated xanthones from the roots of *Garcinia cowa* Roxb. ex Choisy, and their α-glucosidase inhibition activities. Nat Prod Res. 2022;1-9.
5. Tayana N, Suteerapataranon S, Deachathai S. Phytochemistry and bioactive compounds from *Garcinia cowa* roxb. Asia-Pac J Sci Technol. 2017;22(03):1-8.
6. Kaennakam S, Siripong P, Tip-pyang S. Kaennacowanols A–C, three new xanthones and their cytotoxicity from the roots of *Garcinia cowa*. Fitoterapia. 2015;102:171-6.
7. Likhitwitayawuid K, Phadungcharoen T, Krungkrai J. Antimalarial xanthones from *Garcinia cowa*. Planta Med. 1998;64:70-2.
8. An NTK, Ha DT, Long PQ, Thuy TTT. Tetraoxygenated xanthones from the latex of *Garcinia cowa* growing in Vietnam. Vietnam J Sci Techno. 2018;56(5):560-6.
9. Mahabusarakam W, Chairerk P, Taylor WC. Xanthones from *Garcinia cowa* Roxb. latex. Phytochemistry. 2005; 66:1148-53.
10. Panthong K, Hutadilok-Towatana N, Panthong A, Cowaxanthone F. A new tetraoxygenated xanthone, and other anti-inflammatory and antioxidant compounds from *Garcinia cowa*. Can J Chem. 2009;87:1636-40.
11. Shen J, Yang Y. Chemical constituents of branch of Garcinia cowa Roxb. Zhongcaoyao. 2007; 38:993-4.
12. Chen FC, Lin YM, Hung JC. Phenolic compounds from heartwood of *Garcinia multiflora*. Phytochemistry. 1975;14:300-3.
13. Brusotti G, Papetti A, Serra M, Temporini C, Marini E, Orlandini S, et al. *Allanblackia floribunda* Oliv: An aphrodisiac plant with vasorelaxant properties. J Ethnopharmacol. 2016; 192:480-5.
14. Gerhäuser C, Klimo K, Heiss E, Neumann I, Gamal-Eldeen A, Knauft J, et al. Mechanism-based in vitro screening of potential cancer chemopreventive agents. Mutat Res. 2003;523:163-72.
15. Van Amsterdam, F.T.M., Roveri, A., Maiorino, M., Ratti, E. & Ursini, F. Lacidipine: a dihydropyridine calcium antagonist with antioxidant activity. Free Radic Biol Med. 1992; 12:183-7.
16. Adebiyi OE, Olayemi FO, Ning-Hua T, Guang-Zhi Z. *In vitro* antioxidant activity, total phenolic and flavonoid contents of ethanol extract of stem and leaf of *Grewia carpinifolia*. Beni-Suef Univ J Basic Appl Sci. 2017;6:10-4.
17. Jamila N, Khairuddean M, Khan S, Khan N. Complete NMR assignments of bioactive rotameric (3⟶8) biflavonoids from the bark of *Garcinia hombroniana*. Magn Reson Chem. 2014; 52:345-52.

18. Chen FC, Lin YM, Hung JC. New biflavanone glucoside from *Garcinia multiflora*. Phytochemistry. 1975;14:818-20.
19. Osorio E, Londoño J, Bastida J. Low-density lipoprotein (LDL)-antioxidant biflavonoids from *Garcinia madruno*. Molecules. 2013;18:6092-100.
20. Salleh WMNHW, Sazali, NSAN, Ahmad F, Taher M. Biflavonoids from the leaves and stem bark of *Garcinia griffithii* and their biological activities. Marmara Pharm J. 2017;21(4):889-7.
21. Mountessou BYG, Tchamgoue J, Dzoyem JP, Tchuenguem RT, Surup F, Choudhary, MI, et al. Two xanthones and two rotameric (3⟶8) biflavonoids from the cameroonian medicinal plant *Allanblackia floribunda* Oliv. (Guttiferae). Tetrahedron Lett. 2018;59:4545-50.

REFERENCES

Adebiyi, O. E., Olayemi, F. O., Ning-Hua, T., & Guang-Zhi, Z. (2017). In vitro antioxidant activity, total phenolic and flavonoid contents of ethanol extract of stem and leaf of *Grewia carpinifolia*. *Beni-Suef University Journal of Basic and Applied Sciences, 6*(1), 10-14.

Aravind, A., Menon, L., & Rameshkumar, K. (2017). Structural diversity of secondary metabolites in *Garcinia* species. *Jawaharlal Nehru Tropical Botanic Garden and Research Institute, 2*, 19-75.

Aravinda, A., Asha, K., & Rameshkumar, K. (2015). Phytochemical analysis and antioxidant potential of the leaves of *Garcinia travancorica* Bedd. *Natural Product Research, 30*(2), 232-236.

Aruoma, O. (1998). Free radicals, oxidative stress, and antioxidants in human health and disease. *Journal of the American Oil Chemists' Society, 75*, 199-212.

Brusotti, G., Papetti, A., Serra, M., Temporini, C., Marini, E., Orlandini, S., Sanda, A.K., Watcho, P. & Kamtchouing, P. (2016). *Allanblackia floribunda* Oliv.: An aphrodisiac plant with vasorelaxant properties. *Journal of Ethnopharmacology, 192*, 480-485.

Cheenpracha, S., Phakhodee, W., Ritthiwigrom, T., Prawat, U., & Laphookhieo, S. (2011). A new depsidone from the twigs of *Garcinia cowa*. *Heterocycles, 83*(5), 1139-1143.

Chen, F. C., Lin, Y. M., & Hung, J. C. (1975). Phenolic compounds from heartwood of *Garcinia multiflora*. *Phytochemistry, 14*, 300-303.

Gerhauser, C., Klimo, K., Heiss, E., Neumann, I., Gamal-Eldeen, A., Knauft, J., Liu, G., Sitthimonchai, S., & Frank, N. (2003). Mechanism-based in vitro screening of potential cancer chemopreventive agents. *Mutation Research, 523-524*, 163-172.

Gupta, P., Kar, A., Sharma, N., Singh, P., Goswami, N., & Kumar, S. (2021). Protective effect of standardised fruit extract of *Garcinia cowa* Roxb. ex Choisy against ethanol induced gastric mucosal lesions in Wistar rats. *Annals of Medicine, 53*(1), 1696-1708.

Husni, E., Nahari, F., Wirasti, Y., Wahyuni, F., & Dachriyanus. (2015). Cytotoxicity study of ethanol extract of the stem bark of asam kandis (*Garcinia cowa* Roxb.) on T47D breast cancer cell line. *Asian Pacific Journal of Tropical Biomedicine, 5*(3), 249-252.

Jamila, N., Khairuddean, M., Khan, S. N., & Khan, N. K. (2014). Complete NMR assignments of bioactive rotameric (3⟶ 8) biflavonoids from the bark of *Garcinia hombroniana*. *Magnetic Resonance in Chemistry, 52*, 345-352.

Kaennakam, S., Siripong, P., & Tip-pyang, S. (2015). Kaennacowanols A–C, three new xanthones and their cytotoxicity from the roots of *Garcinia cowa*. *Fitoterapia, 102*, 171-176.

Konoshima, M., Ikeshiro, Y., & Miyahara, S. (1970). The constitution of biflavonoids from *Garcinia* plants. *Tetrahedron Letters, 48*, 4203-4206.

Li, X. C., Joshi, A. S., Tan, B., ElSohly, H. N., Walker, L. A., Zjawiony, J. K., & Ferreira, D. (2002). Absolute configuration, conformation, and chiral properties of flavanone-(3-->8")-flavone biflavonoids from *Rheedia acuminata*. *Tetrahedron Letters, 58*, 8709-8717.

Lihitwitayawuid, K., Phadungcharoen, T., Mahidol, C., & Ruchirawat, S. (1997). 7-O-Methylgarcinone E from *Garcinia cowa*. *Phytochemistry, 45*, 1299-1301.

Likhitwitayawuid , K., Phadungcharoen, T., & Krungkrai, J. (1998). Antimalarial xanthones from *Garcinia cowa*. *Planta Medica, 64*, 70-72.

Mahabusarakam, W., Chairerk, P., & Taylor, W. C. (2005). Xanthones from *Garcinia cowa* Roxb. latex. *Phytochemistry, 66*(10), 1148-1153.

Mountessou, B., Tchamgoue, J., Dzoyem, J., Tchuenguem, R., Surup, F., Choudhary, M., Green, I., & Kouam, S. (2018). Two xanthones and two rotameric (3⟶ 8) biflavonoids from the Cameroonian medicinal plant *Allanblackia floribunda* Oliv. (Guttiferae). *Tetrahedron Letters, 59,* 4545-4550.

Masuda, T., Yamashita, D., Takeda, Y., & Yonemori, S. (2005). Screening for tyrosinase inhibitors among extracts of seashore plants and identification of potent

inhibitors from *Garcinia subelliptica*. *Bioscience, Biotechnology, and Biochemistry, 69*(1), 197-201.

Munteanu, I., & Apetrei, C. (2021). Analytical methods used in determining antioxidant activity:A review. *International Journal of Molecular Sciences, 22*(3380), 1-30.

Na, Z., Song, Q., & Hu, H. (2013). A new prenylated xanthone from latex of *Garcinia cowa* Roxb. *Records of Natural Products, 7*(3), 220-224.

Osorio, E., Londoño, J., & Bastida, J. (2013). Low-density lipoprotein (LDL)-antioxidant biflavonoids from *Garcinia madruno*. *Molecules, 18*, 6092-6100.

Panthong, K., Hutadilok-Towatana, N., & Panthong, A. (2009). Cowaxanthone F, a new tetraoxygenated xanthone, and other anti-inflammatory and antioxidant compounds from *Garcinia cowa*. *Canadian Journal of Chemistry, 87*(11), 1636-1640.

Panthong, K., Pongcharoen, W., Phongpaichit, S., & Taylor, W. C. (2006). Tetraoxygenated xanthones from the fruits of *Garcinia cowa*. *Phytochemistry, 67*(10), 999-1004.

Pattalung, P., Thongtheeraparp, W., & Wiriyachitra, P. (1994). Xanthones of *Garcinia cowa*. *Planta Medica, 60*, 365-368.

Pratiwi, L., Fudholi, A., Martien, R., & Pramono, S. (2017). Development of TLC and HPTLC method for determination α- mangostin in mangosteen peels (*Garcinia Mangostana* L.,). *International Journal of Pharmacognosy and Phytochemical Research, 9*(3), 297-302.

Ritthiwigrom, T., Laphookhieo, S., & G.S., P. (2013). Chemical constituents and biological activities of *Garcinia cowa* Roxb. *Maejo International Journal of Science and Technology, 7*(2), 212-231.

Roslund, M., Tähtinen, P., Niemitzc, M., & Sjöholm, R. (2008). Complete assignments of the 1H and 13C chemical shifts and JH,H coupling constants in NMR spectra of D-glucopyranose and all D-glucopyranosyl-D-glucopyranosides. *Carbohydrate Research 343, 343*, 101-112.

Sae-Lim, P., Seetaha, S., Tabtimmai, L., Suphakun, P., Kiriwan, D., Panichayupakaranant, P., & Choowongkomon, K. (2020). Chamuangone from *Garcinia cowa* leaves inhibits cell proliferation and migration and induces cell apoptosis in human cervical cancer in vitro. *Journal of Pharmacy and Pharmacology, 72*(3), 470-480.

Saenkham, A., Jaratrungtawee, A., Siriwattanasathien, Y., Boonsri, P., Chainok, K., Suksamrarn, A., Namsa-aid, M., Pattanaprateeb, P., & Suksamrarn, S. (2020). Highly potent cholinesterase inhibition of geranylated xanthones from Garcinia fusca and molecular docking studies. *Fitoterapia, 146*, 104637-104651.

Sakunpak, A., & Panichayupakaranant, P. (2012). Antibacterial activity of Thai edible plants against gastrointestinal pathogenic bacteria and isolation of a new broad spectrum antibacterial polyisoprenylated benzophenone, chamuangone. *Food Chemistry, 130*(4), 826-831.

Santo, E., Santana, B., Figueiredo, L., & Junior, K. (2020). Medicinal potential of *Garcinia* species and their compounds. *Molecules, 25*(19), 4513-4542.

Sarma, A., Sarmah, P., Kashyap, D., & Kalita, A. (2014). Evaluation of nutraceutical properties andantioxidant activity of *Garcinia cowa* roxb. ex choisy fruits found in assam (India). *World Journal of Pharmacy and Pharmaceutical Sciences, 3*(12), 853-859.

Shen, J., Tian, Z., & Yang, J. (2007). The constituents from the stems of *Garcinia cowa*. *Pharmazie, 62*(7), 31-38.

Shen, J., & Yang, J. (2006). Two new xanthone from stem *G cowa*. *Chemical and Pharmaceutical Bulletin, 54(1)*, 126-128.

Siridechakorn, I., Phakhodee, W., Ritthiwigrom, T., Promgool, T., Deachathai, S., Cheenpracha, S., Prawat, U., & Laphookhieo, S. (2012). Antibacterial dihydrobenzopyran and xanthone derivatives from *Garcinia cowa* stem barks. *Fitoterapia, 83*(8), 1430-1434.

Sriyatep, T., Maneerat, W., Sripisut, T., Cheenpracha, S., Machan, T., Phakhodee, W., & Laphookhieo, S. (2014). Cowabenzophenones A and B, two new

tetracyclo[7.3.3.33,11.03,7]tetradecane-2,12,14-trione derivatives from ripe fruits of *Garcinia cowa*. *Fitoterapia, 92*, 285-289.

Sriyatep, T., Siridechakorn, I., Maneerat, W., Pansanit, A., Ritthiwigrom, T., Andersen, R. J., & Laphookhieo, S. (2015). Bioactive prenylated xanthones from the young fruits and flowers of *Garcinia cowa*. *Journal of Natural Products, 78*(2), 265-271.

Tayana, N., Suteerapatarnon, S., & Deachathai, S. (2017). Phytochemistry and bioactive compounds from *Garcinia cowa* roxb. *Asia-Pacific Journal of Science and Technology, 22*(03), 1-8.

Tian, Z., Shen, J., Moseman, A., Yang, Q., Yang, J., Xiao, P., Wu, E., & Kohane, I. (2008). Dulxanthone A induces cell cycle arrest and apoptosisvia up-regulation of p53 through mitochondrial pathway in HepG2 cells. *International Journal of Cancer, 122*(1), 31-38.

van Amsterdam, F. T. M., Roveri, A., Maiorino, M., Ratti, E., & Ursini, F. (1992). Lacidipine: a dihydropyridine calcium antagonist with antioxidant activity. *Free Radical Biology and Medicine, 12*, 183-187.

Wahyuni, F., Fatma, S., Shaari, K., & Stanslas, J. (2015). Cytotoxic xanthones from the stem bark of *Garcinia cowa* Roxb. *Journal of Chemical and Pharmaceutical Research, 7*(1), 227-236.

Wahyuni, F., Febria, S., & Arisanty, D. (2017). Apoptosis induction of cervical carcinoma HeLa cells line by dichloromethane fraction of the rinds of *Garcinia cowa* Roxb. *Pharmacognosy Journal, 9*(4), 475-478.

Wahyuni, F., Shaari, K., Stanslas, J., & Hamidi, D. (2016). Cytotoxic properties and complete nuclear magnetic resonance assignment of isolated xanthones from the root of *Garcinia cowa* Roxb. *Pharmacognosy Magazine, 12*(45), 52-56.

Wahyuni, F., Shaari, K., Stanslas, J., Lajis, N., & Hamidi, D. (2015). Cytotoxic compounds from the leaves of *Garcinia cowa* Roxb. *Journal of Applied Pharmaceutical Science, 5*(2), 006-011.

Wahyuni, F., Triastuti, D., & Arifin, H. (2015). Cytotoxicity study of ethanol extract of the leaves of asam kandis (*Garcinia cowa* Roxb.) on T47D breast cancer cell line. *Pharmacognosy Journal, 7*(6), 369-371.

Xia, Z., Zhang, H., Xu, D., Lao, Y., Fu, W., Tan, H., Cao, P., Yang, L., & Xu, H. (2015). Xanthones from the leaves of *Garcinia cowa* induce cell cycle arrest, apoptosis, and autophagy in cancer cells. *Molecules, 20*(6), 11387-11399.

VITA