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PHYTOCEUTICALS FROM HEMEROCALLIS FLOWERS AND ROOTS WITH ANTIOXIDANT, ANTICANCER, MOSQUITOCIDAL, AND SCHISTOSOME INHIBITORY ACTIVITIES

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Horticulture

Major professor

Date 8/8/02

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By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Horticulture

2002

ABSTRACT

PHYTOCEUTICALS FROM *HEMEROCALLIS* FLOWERS AND ROOTS WITH ANTIOXIDANT, ANTICANCER, MOSQUITOCIDAL, AND SCHISTOSOME INHIBITORY ACTIVITIES

By

Robert Henry Cichewicz

Daylilies (*Hemerocallis* spp., Hemerocallidaceae) are widely consumed in eastern Asia as both a traditional food and medicine. In this study, *Hemerocallis* flowers and roots were examined in order to identify bioactive constituents that may exhibit antioxidant, anticancer, mosquitocidal, and schistosome inhibitory properties. An investigation of *Hemerocallis* cv. Stella de Oro flowers led to the isolation of nine kaempferol, quercetin, and isorhamnetin 3-*O*-glycosides (**1**-**9**), phenethyl β-D-glucopyranoside (**10**), orcinol β-D-glucopyranoside (**11**), phloretin 2′-*O*-β-D-glucopyranoside (**12**), phloretin 2′-*O*-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside (**13**), a new napthalene-glycoside, stelladerol (**14**), and an amino acid (longitubanine A) (**15**). An examination of *Hemerocallis fulva* 'Kwanzo' Kaempfer roots led to the isolation of seven new anthraquinones, kwanzoquinones A (**16**), B (**17**), C (**19**), D (**20**), E (**21**), F (**22**), and G (**24**), two known anthraquinones, 2-hydroxychrysophanol (**18**) and rhein (**23**), one new naphthalene glycoside, 5-hydroxydianellin (**26**), one known naphthalene glycoside, dianellin (**25**), one known flavone, 6-methylluteolin (**27**), and α-tocopherol.

Several new compounds such as kwanzoquinones A (**16**), B (**17**), C (**19**), E (**21**), and kwanzoquinone A and B monoacetates (**16a** and **17a**, respectively) exhibited promising cancer cell growth inhibitory activity. In addition, the known compounds 2-hydroxychrysophanol (**18**) and rhein (**23**) inhibited cancer cell growth. Three compounds, kwanzoquinone D (**20**), stelladerol (**14**), and 5-hydroxydianellin (**26**), demonstrated remarkable antioxidant activity by inhibiting lipid oxidation more than 90% in an in vitro assay system. Three compounds, 2-hydroxychrysophanol (**18**) and kwanzoquinones C (**19**) and E (**21**), exhibited mosquitocidal properties against fourth instar *Ades aegyptii* larvae. Two compounds, 2-hydroxychrysophanol (**18**) and kwanzoquinone E (**21**), were discovered as novel agents for the prevention and treatment of schistosomiasis. These compounds were found to inhibit the motility and induce mortality in *Schistosoma mansoni* cercariae and adults. The bioactive constituents from daylilies are being further investigated in order to determine their modes of action and for development as new phytoceuticals.

ACKNOWLEDGMENTS

Partial funding of this project was provided by the MSU Agricultural Experiment Station and the Center for Plant Products and Technologies. The MSU Mass Spectrometry Facility is partially supported by a grant from the NIH (DRR-00484). Purchase of the NMR instruments used in this study was made possible by grants from the NIH (1-S10-RR04750) and NSF (CHE-88-00770 and CHE-92-13241). Travel funding for the presentation of this work was provided by the MSU Graduate Studies Program and Dr. S. K. Ries.

I would like to thank Dr. L. S. Barnes, Mr. J. C. Halinar, and Mr. D. Walters for providing the daylilies used in this study. I would like to acknowledge Dr. J. H. McKerrow and Mr. K.-C. Lim for performing the schistosome inhibitory studies. I am grateful for assistance from Dr. E. Walker for providing the *Aedes aegyptii* eggs and for post-mortem observations of the larvae. I would also like to thank Mr. K. Johnson and Dr. L. Le for technical assistance conducting selected NMR experiments. In addition, I would like to acknowledge Dr. B. Borhan and Mr. B. Travis for assistance obtaining IR and optical rotation data. Furthermore, I would like to thank past and present members of the Bioactive Natural Products and Phytoceuticals Laboratory for their kind assistance. I am especially indebted to Dr. L. J. Clifford for her unremitting support. Finally, I would like to acknowledge my major professor and committee chair, Dr. M. G. Nair, and the other committee members, Dr. L. D. Bourquin, Dr. R. E. Schutzki, and Dr. G. M. Strasburg, for their time and guidance in the preparation of this document.

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KEY TO ABBREVIATIONS

Ac_2O	Acetic anhydride
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BuOH	Butanol
$CHCl_3$	Chloroform
CH_2Cl_2	Dichloromethane
CH_3CN	Acetonitrile
COSY	Correlation spectroscopy
cv.	Cultivar
DEPT	Distortionless enhancement by polarization transfer
DMSO	Dimethyl sulfoxide
DQF-COSY	Double-Quantum Filtered Correlation Spectroscopy
D_2O	Deuterium oxide
EIMS	Electron impact ionization mass spectrometry
EtOH	Ethanol
EtOAc	Ethyl acetate
FABMS	Fast atom bombardment mass spectrometry
FT	Fourier transform
Gal	Galactose
Glc	Glucose
HCl	Hydrochloric acid
HCOOH	Formic acid
HMBC	Heteronuclear multiple bond coherence
HMQC	Heteronuclear correlation through multiple quantum coherence
HOAc	Acetic Acid
HPLC	High performance liquid chromatography
HREIMS	High resolution electron impact ionization mass spectrometry
HRFABMS	High resolution fast atom bombardment mass spectrometry
H_2O	Water

H_2SO_4	Sulfuric acid
IR	Infrared
MeOH	Methanol
mp	Melting point
MPLC	Medium pressure liquid chromatography
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
NOESY	Nuclear Overhauser spectroscopy
ODS	Octadecyl silica
PTLC	Preparative thin layer chromatography
Si	Silica
spp.	Species
TBHQ	*tert*-Butylhydroquinone
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
UV	Ultraviolet
Xly	Xylose

INTRODUCTION

In the last century, perennial gardens across the United States have been transformed by the addition of the now ubiquitous daylily (*Hemerocallis* spp.). Generally regarded by gardeners as relatively vigorous, low-maintenance, and pest-free plants, daylilies add an appreciable degree of color and beauty to the garden landscape with their brilliant floral hues and prominent cascading foliage. However, elsewhere in the world, daylilies are valued for much more than just their ornamental qualities. For millennia, daylilies have been cultivated in their native land of Asia where they are still widely regarded as an important source of food and medicine. Despite a long and rich history of utilization by humans, science still possesses a paltry understanding of the pharmacological potential and phytochemical constituents of daylilies.

One of the earliest references to daylilies can be found in an ancient Chinese materia medica penned for the Emperor Huang Ti nearly 5000 years ago (Schabell, 1990). Further references to the medicinal uses of *Hemerocallis* spp. can be found throughout the pages of recorded Chinese and Japanese history in which daylilies are referred to by a variety of colloquial names. One of the most prevalent of the terms ascribed to daylilies is *wangyoucao* (Chinese) or *wasure-gusa* (Japanese), which translates as 'forget sorrow plant' (Carr, 1997). As these names literally imply, daylilies were widely regarded for their reputed antidepressant properties. In addition, *Hemerocallis* spp. have been extensively

used throughout Asia to treat a variety of other ailments including anemia, fever, insomnia, and schistosomiasis.

Daylilies first appeared in Europe during the late sixteenth century where they were cultivated solely for ornamental purposes (Schabell, 1990). These first *Hemerocallis* spp. were rather unremarkable in terms of their orange and yellow ephemeral flowers, and for years daylilies lingered in the shadows of garden recesses as inconspicuous perennials. It was not until the early twentieth century that plant breeders in Europe and the United States (Schabell, 1990) took an interest in daylilies and embarked upon intensive breeding programs that lead to the extraordinary variety and beauty in floral form and color for which daylilies are now highly regarded. Today, several national and international societies such as the American *Hemerocallis* Society and the International European Daylily Society have been established by daylily enthusiast in order to promote, propagate, and educate the public about the more than 40,000 named daylily cultivars that now exist.

Throughout humankind's existence, plants have served as an important source of bioactive compounds. Today, researchers continue to exploit the chemical diversity of the botanical world as a source of novel compounds that possess interesting biological activities. Based on the extensive use of *Hemerocallis* spp. as an important phytomedicinal agent in Asian cultures as well as their many reported pharmacological properties, it is conceivable that daylilies possess a number of new biologically active constituents. Therefore, the objective of this study was to isolate and elucidate the structures of bioactive

constituents from *Hemerocallis* spp. that may have phytoceutical applications. Crude extracts and their respective isolates were subjected to a panel of bioassays in order to evaluate their antioxidant, anticancer, cyclooxygenase inhibitory, mosquitocidal, nematocidal, schistosome inhibitory, and topoisomerase inhibitory activities.

This dissertation is composed of a series of chapters detailing the results of this research. **Chapter 1** is a literature review in which the botany, chemical constituents, traditional uses, and pharmacological properties of daylilies are outlined. In **Chapter 2**, the results of the isolation and structure elucidation study performed on daylily (*Hemerocallis* cv. Stella de Oro) flowers are presented. An accounting of the isolation and structure elucidation of compounds obtained from the roots of *Hemerocallis fulva 'Kwanzo'* Kaempfer is detailed in **Chapter 3**. All of the compounds obtained from the flowers and roots of *Hemerocallis* spp. were investigated in order to evaluate their antioxidant, anticancer, cyclooxygenase inhibitory, mosquitocidal, nematocidal, schistosome inhibitory, and topoisomerase inhibitory activities. The results of these studies and the methods used for these experiments are presented in **Chapter 4**. **Chapters 2-4** provide data that are derived from published and submitted peer reviewed journal articles and a patent application, and are arranged here as manuscripts each with an abstract, introduction, materials and methods, and results and discussion sections.

CHAPTER ONE

LITERATURE REVIEW

Introduction

In Europe and North America, daylilies are regarded as common garden perennials that are grown for their intriguingly shaped and richly colored flowers. However, in Asia daylilies are utilized as both a food item and medicinal agent. The botany, chemical constituents, traditional uses, and pharmacological properties of daylilies are outlined in this chapter.

Botany of *Hemerocallis*

Daylilies are herbaceous, clump-forming, spreading, perennial monocots whose familial affiliation remains in question, although evidence now suggests that daylilies should be placed in their own family, the Hemerocallidaceae. The generic name for daylilies, *Hemerocallis*, is derived from the Greek words for 'beauty' and 'day' in reference to the fact that daylily flowers bloom and subsequently senesce over the period of a single day.

Daylilies are indigenous to Asia; however, they can now be found growing wild throughout portions of Europe and North America as a result of having escaped from cultivation. Debate remains regarding the number of species that comprise the genus *Hemerocallis* with estimates ranging from approximately 12-30 or more (Grenfell, 1998). Some of the reported species of daylilies include

Hemerocallis altissima Stout, *Hemerocallis aurantiaca* Baker, *Hemerocallis citrina* Baroni, *Hemerocallis disticha* Donn., *Hemerocallis esculenta* Koidzumi, *Hemerocallis fulva* L., *Hemerocallis lilioasphodelus* L. (syn. *Hemerocallis flava* L. and *Hemerocallis lilio-ashpodelus* L.), *Hemerocallis longituba* Miq., *Hemerocallis minor* Miller, and *Hemerocallis thunbergii* Barr ex Baker. Intensive breeding programs have led to the development of thousands of cultivars with some estimates indicating the existence of over 40,000 named cultivars at the present time (Grenfell, 1998). The following description of daylily characteristics has been adopted from Jones and Luchsinger (1986), Zomlefer (1994), and from personal observations.

Hemerocallis spp. are typically observed bearing rich green fans of alternate, linear leaves that are lanceolatus in character. Numerous, tall, arching simple leaves arise from a subsurface crown bearing a pattern of linear venation and appearing to fold along the midrib. The leaves typically dieback in autumn; however, semi-evergreen varieties may continue to bear leaves year-around.

The rhizomatous roots of the daylilies display a range of morphologies, although they can generally be characterized as having a tapered appearance with numerous fleshy tuberous or spindle-shaped swellings. The root thickness may range from nearly fibrous to thick and cylindrical. A variety of colors are also observed for the interior of the roots ranging from white to reddish in nature.

Hemerocallis flowers are characterized as bisexual and actinomorphic bearing six tepals composed of three petals and three sepals. The funnel-form lily-like flowers are cymose and borne on a leafless scape. Stamens possess

elongated filaments with linear anthers opening via a lengthwise slit. The pistil is composed of three united carpels and three locules bearing numerous ovules. The daylily fruit is a capsule that contains a number of small, iridescent, black seeds.

Chemical Constituents of *Hemerocallis*

Phytochemical investigations have revealed the presence of a diverse array of chemical constituents in *Hemerocallis* spp. (Yang and Li, 2002). A summary of compounds previously reported from *Hemerocallis* is provided in Table 1.1. The known chemical constituents of *Hemerocallis* can be divided into four general groups based on their biosynthetic origins. These groups include the amino acids and their derivatives, fatty acids and related aliphatic compounds, phenolic constituents, and terpenoid and steroid compounds (Table 1.1).

Many common amino acids have been identified in *Hemerocallis* spp. (Takemoto and Kusano, 1966) (Table 1.1). However, some uncommon amino acids isolated from daylilies include pinnatanine (Grove et al., 1973; Yoshikawa et al., 1994) and oxypinnatanine (Grove et al., 1973; Kruger et al., 1976; Inoue et al., 1990) from the leaves, flowers, stems, and seeds of *H. fulva* and the roots of *H. longituba* (Figure 1.1). In addition, longitubanines A and B (Yoshikawa et al., 1994) have been found in the roots of *H. longituba* (Figure 1.1). A series of unique amino acid derivatives, fulvanines A-E (Figure 1.2), have been isolated

Table 1.1. *Chemical constituents reported from Hemerocallis spp.*

Table 1.1. Chemical constituents reported from *Hemerocallis* spp.

Compound	Source	Part of Plant	Identification Method	Reference
AMINO ACIDS AND DERIVATIVES				
alanine	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966
alloisoleucine	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966
arginine	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966
asparagine	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966
aspartic acid	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966
cysteic acid	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966
cystine	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966
fulvanine A	*H. fulva*	aerial portion	MS, NMR	Inoue et al., 1990
fulvanine B	*H. fulva*	aerial portion	MS, NMR	Inoue et al., 1990
fulvanine C	*H. fulva*	aerial portion	MS, NMR	Inoue et al., 1990
fulvanine D	*H. fulva*	aerial portion	MS, NMR	Inoue et al., 1990

Table 1.1. (cont'd)

Table 1.1. (cont'd)

Compound	Source	Part of Plant	Identification Method	Reference
fulvanine E	*H. fulva*	aerial portion	MS, NMR	Inoue et al., 1990
glutamic acid	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966
glycine	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966
L-4-hydroxyglutamic acid	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966
isoleucine	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966
leucine	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966
longitubanine A	*H. longituba*	root	MS, NMR	Yoshikawa et al., 1994
longitubanine B	*H. longituba*	root	MS, NMR	Yoshikawa et al., 1994
lysine	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966
methionine	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966
oxypinnatanine	*H. fulva*	aerial portion	MS, NMR, X-Ray	Grove et al., 1973; Kruger et al., 1976; Inoue et al., 1990
phenylalanine	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966

Table 1.1. (cont'd)

Compound	Source	Part of Plant	Identification Method	Reference
pinnatanine	*H. longituba*	root	MS, NMR	Grove et al., 1973; Yoshikawa et al., 1994
proline	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966
serine	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966
threonine	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966
tyrosine	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966
valine	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966
FATTY ACIDS AND RELATED ALIPHATIC CONSTITUENTS				
capric acid	*H. fulva*	root	GC	Sarg et al., 1990
caprylic acid	*H. fulva*	root	GC	Sarg et al., 1990
decane	*H. citrina*	flower	GC-MS	Wang et al., 1994
decanoic acid	*H. citrina*	flower	GC-MS	Wang et al., 1994

Table 1.1 (cont'd)

Table 1.1. (cont'd)

Compound	Source	Part of Plant	Identification Method	Reference
3,3-dimethyl butanoic acid	*H. citrina*	flower	GC-MS	Wang et al., 1994
2,4-dimethyl heptane	*H. citrina*	flower	GC-MS	Wang et al., 1994
2,6-dimethyl nonane	*H. citrina*	flower	GC-MS	Wang et al., 1994
3,3-dimethyl nonane	*H. citrina*	flower	GC-MS	Wang et al., 1994
3,7-dimethyl-1-octanol	*H. citrina*	flower	GC-MS	Wang et al., 1994
heneicosane	*H. citrina*	flower	GC-MS	Wang et al., 1994
1-hexadecanol	*H. citrina*	flower	GC-MS	Wang et al., 1994
hexanoic acid	*H. citrina*	flower	GC-MS	Wang et al., 1994
isopalmitic acid	*H. fulva*	root	GC	Sarg et al., 1990
lauric acid	*H. fulva*	root	GC	Sarg et al., 1990
linoleic acid	*H. fulva*	root	GC	Sarg et al., 1990
2-methyl-6-ethyl octane	*H. citrina*	flower	GC-MS	Wang et al., 1994

Table 1.1. (cont'd)

Compound	Source	Part of Plant	Identification Method	Reference
5-methyl-1-heptane	*H. citrina*	flower	GC-MS	Wang et al., 1994
myristic acid	*H. fulva, H. citrina*	flower, root	GC-MS	Sarg et al., 1990; Wang et al., 1994
nonadecane	*H. citrina*	flower	GC-MS	Wang et al., 1994
2-nonanal	*H. citrina*	flower	GC-MS	Wang et al., 1994
nonanol	*H. citrina*	flower	GC-MS	Wang et al., 1994
cis-2-nonen-1-ol	*H. citrina*	flower	GC-MS	Wang et al., 1994
palmitic acid	*H. fulva*	root	GC	Sarg et al., 1990
octadecane	*H. citrina*	flower	GC-MS	Wang et al., 1994
octanoic acid	*H. citrina*	flower	GC-MS	Wang et al., 1994
7-octen-4-ol	*H. citrina*	flower	GC-MS	Wang et al., 1994
oleic acid	*H. fulva*	root	GC	Sarg et al., 1990
palmitic acid	*H. citrina*	flower	GC-MS	Wang et al., 1994

Table 1.1. (cont'd)

Table 1.1. (cont'd)

Compound	Source	Part of Plant	Identification Method	Reference
pentadecane	*H. citrina*	flower	GC-MS	Wang et al., 1994
2-propyl-1-heptanol	*H. citrina*	flower	GC-MS	Wang et al., 1994
tricosane	*H. citrina*	flower	GC-MS	Wang et al., 1994
4,6,8-trimethyl-1-nonene	*H. citrina*	flower	GC-MS	Wang et al., 1994
PHENOLIC COMPOUNDS				
aloe-emodin	*H. citrina*	root	MS, NMR	He et al., 1982
ethyl benzoate	*H. longituba*	root	IR, NMR	Takemoto and Kusano, 1966
3-carbomethoxy 1,8-dihydroxyanthraquinone	*H. fulva*	root	chemical methods, comparison with authentic samples	Sarg et al., 1990
chrysophanol	*H. citrina, H. fulva, H. minor*	root	chemical methods, comparison with authentic sample, IR, MS, NMR	He et al., 1982; Xiu et al., 1982; Sarg et al., 1990
dimethylterephthalate	*H. disticha*	flower	chemical methods, IR, NMR	Lin et al., 1973

Table 1.1. (cont'd)

Table 1.1. (cont'd)

Compound	Source	Part of Plant	Identification Method	Reference
hemerocal	*H. citrina*	root	MS, NMR	He et al., 1982
hemerocallone	*H. minor*	root	IR, MS, NMR	Xiu et al., 1982
2-methoxy obtusifolin	*H. citrina*	root	MS, NMR	He et al., 1982
3-methoxy 1,8-dihydroxy anthraquinone	*H. fulva*	root	chemical methods, comparison with authentic samples	Sarg et al., 1990
mi-hemerocallin	*H. minor*	root	IR, MS, NMR	Xiu et al., 1982
obtusifolin	*H. citrina*	root	MS, NMR	He et al., 1982
rhein	*H. fulva, H. minor*	root	chemical methods, comparison with authentic sample, IR, MS, NMR	Xiu et al., 1982; Sarg et al., 1990
stypandrol (hemerocallin)	*H. esculenta, H. citrina, H. lilio-ashpodelus, H. minor*	root	comparison with authentic sample, IR, MS, NMR	Xiu et al., 1982; Wang et al., 1989
PIGMENTS				
antheraxanthin	*H. flava*	flower	not defined	Valadon and Chapman, 1984

Table 1.1. (cont'd)

Compound	Source	Part of Plant	Identification Method	Reference
all-*trans*-β-carotene	*H. disticha*	flower	HPLC, UV	Tai and Chen, 2000
9-*cis*-β-carotene	*H. disticha*	flower	HPLC, UV	Tai and Chen, 2000
β-carotene-5,6:5′,6′-diepoxide	*H. flava*	flower	not defined	Valadon and Chapman, 1984
ξ-carotene	*H. flava*	flower	not defined	Valadon and Chapman, 1984
β-cryptoxanthin	*H. disticha*	flower	HPLC, UV	Tai and Chen, 2000
13-*cis*-β-cryptoxanthin	*H. disticha*	flower	HPLC, UV	Tai and Chen, 2000
cis-β-cryptoxanthin	*H. disticha*	flower	HPLC, UV	Tai and Chen, 2000
cryptoxanthin-5,6-monoepoxide	*H. flava*	flower	not defined	Valadon and Chapman, 1984
cyanidin 3-glucoside	*Hemerocallis* cultivar, *H. fulva*	flower	chemical methods, co-paper and thin layer chromatography	Asen and Arisumi, 1968; Yoshitama et al., 1980
cyanidin 3-rutinoside	*Hemerocallis* cultivar, *H. fulva*	flower	chemical methods, co-paper and thin layer chromatography, HPLC	Asen and Arisumi, 1968; Yoshitama et al., 1980, Griesbach and Batdorf, 1995

Table 11 (continued)

Table 1.1. (cont'd)

Compound	Source	Part of Plant	Identification Method	Reference
delphinidin 3-glucoside	*Hemerocallis* cultivar	flower	chemical methods, co-paper chromatography	Asen and Arisumi, 1968
delphinidin 3-rutinoside	*Hemerocallis* cultivar	flower	chemical methods, co-paper chromatography, HPLC	Asen and Arisumi, 1968; Griesbach and Batdorf, 1995
lutein	*H. flava*	flower	HPLC	Valadon and Chapman, 1984; Griesbach and Batdorf, 1995
13-*cis*-lutein 5,6-epoxide	*H. disticha*	flower	HPLC, UV	Tai and Chen, 2000
lutein 5,6-epoxide	*H. disticha*	flower	HPLC, UV	Tai and Chen, 2000
13-*cis*-lutein	*H. disticha*	flower	HPLC, UV	Tai and Chen, 2000
all-*trans*-lutein	*H. disticha*	flower	HPLC, UV	Tai and Chen, 2000
9-*cis*-lutein	*H. disticha*	flower	HPLC, UV	Tai and Chen, 2000
cis-lycopene	*H. flava*	flower	not defined	Valadon and Chapman, 1984
trans-lycopene	*H. flava*	flower	not defined	Valadon and Chapman, 1984
neoxanthin	*H. disticha*	flower	HPLC, UV	Valadon and Chapman, 1984; Tai and Chen, 2000

Table 1.1. (cont'd)

Compound	Source	Part of Plant	Identification Method	Reference
phytofluene	*H. flava*	flower	not defined	Valadon and Chapman, 1984
violaxanthin	*H. disticha*	flower	HPLC, UV	Tai and Chen, 2000
violeoxanthin	*H. disticha*	flower	HPLC, UV	Valadon and Chapman, 1984; Tai and Chen, 2000
β-zeacarotene	*H. flava*	flower	not defined	Valadon and Chapman, 1984
zeaxanthin	*H. disticha*	flower	HPLC, UV	Griesbach and Batdorf, 1995; Tai and Chen, 2000
13-*cis*-zeaxanthine	*H. disticha*	flower	HPLC, UV	Tai and Chen, 2000
OTHER TERPENOIDS AND STEROIDS				
bornyl acetate	*H. citrina*	flower	GC-MS	Wang et al., 1994
camphor	*H. citrina*	flower	GC-MS	Wang et al., 1994
farnesyl acetate	*H. citrina*	flower	GC-MS	Wang et al., 1994
farnesyl butyrate	*H. citrina*	flower	GC-MS	Wang et al., 1994

Table 1.1. (cont'd)

Compound	Source	Part of Plant	Identification Method	Reference
friedelin	*H. longituba*	root	elemental analysis, IR, melting point	Takemoto and Kusano, 1966
geraniol	*H. citrina*	flower	GC-MS	Wang et al., 1994
hemeroside A	*H. fulva*	aerial portion	MS, NMR	Konishi et al., 2001
hemeroside B	*H. fulva*	aerial portion	MS, NMR	Konishi et al., 2001
limonene	*H. citrina*	flower	GC-MS	Wang et al., 1994
α-pinene	*H. citrina*	flower	GC-MS	Wang et al., 1994
β-sitosterol	*H. fulva, H. longituba*	root	elemental analysis, IR, compare to authentic sample, MS, NMR	Takemoto and Kusano, 1966; Xiu et al., 1982, Sarg et al., 1990
β-sitosterol glucoside	*H. longituba*	root	elemental analysis, IR, compare to authentic sample	Takemoto and Kusano, 1966
γ-sitosterol	*H. minor*	root	IR, MS, NMR	Xiu et al., 1982
α–terpineol	*H. citrina*	flower	GC-MS	Wang et al., 1994
α-thujene	*H. citrina*	flower	GC-MS	Wang et al., 1994

Table 1.1 (cont'd)

Table 1.1. (cont'd)

Compound	Source	Part of Plant	Identification Method	Reference
MISCELLANEOUS COMPOUNDS				
choline	*H. fulva*	root	chemical methods, comparison with authentic samples	Sarg et al., 1990
fulvanol	*H. fulva*	aerial portion	MS, NMR	Konishi et al., 1996
lactic acid	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966
succinic acid	*H. longituba*	root	HPLC	Takemoto and Kusano, 1966

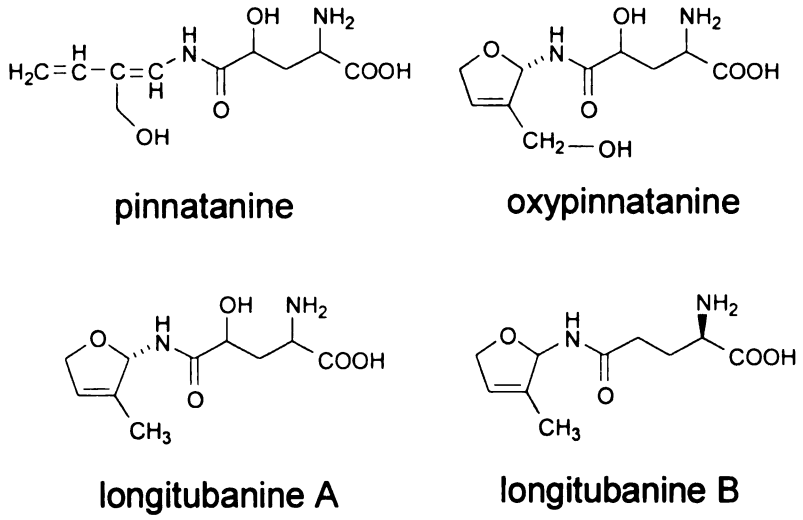


Figure 1.1. Some unique amino acids found in *Hemerocallis* spp.

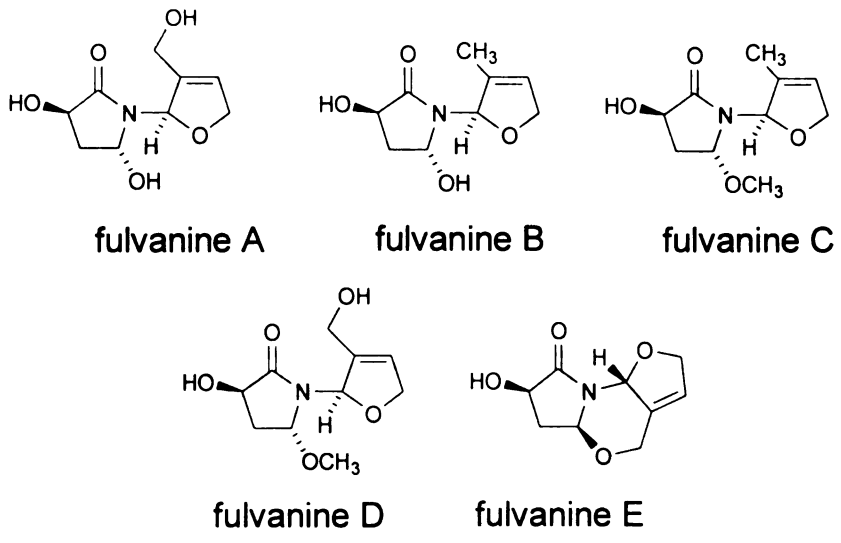
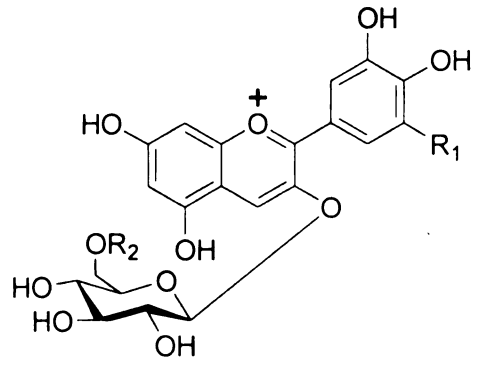


Figure 1.2. Fulvanines A-E found in the aerial portion of *H. fulva*.

from the aerial portion of *H. fulva*. It has been proposed that these 2,5-dihydrofuryl-γ-lactams are metabolites of oxypinnatanine (Inoue et al. 1990).

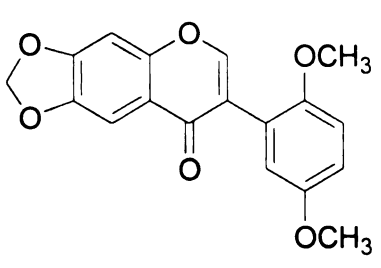
Gas chromatography has been used to analyze various daylily tissues providing evidence for the presence of numerous long-chain fatty acids, alcohols, aldehydes, and alkenes (Isono et al., 1976; Sarg et al., 1990; Wang et al., 1994) (Table 1.1). Most of these compounds are common, unbranched lipophilic constituents such as capric, decanoic, lauric, linoleic, myristic, and palmitic acids. Other constituents include branched species such as 3,3-dimethyl butanoic acid, 3,7-dimethyl-1-octanol, 2-methyl-6-ethyl octane, and 2-propyl-1-heptanol (Wang et al., 1994).

A variety of phenolic pigments have been identified in daylilies (Table 1.1). For example, four anthocyanins, cyanidin 3-glucoside, cyanidin 3-rutinoside, delphinidin 3-glucoside, and delphinidin 3-rutinoside (Asen and Arisumi, 1968; Yoshitama et al., 1980; Griesbach and Batdorf, 1995) (Figure 1.3), have been identified as constituents in the flowers of *H. fulva* and other daylily cultivars. Other phenolic compounds include a unique isoflavone, hemerocallone, and a substituted naphthalene, mi-hemerocallin (Figure 1.4), have also been isolated from the roots of *H. minor* (Xiu et al., 1982). Another compound, a naphthalene dimer known as stypandrol (Figure 1.4), is a potent neurotoxin that has been identified in the roots of several *Hemerocallis* spp. (Xiu et al., 1982; Wang et al., 1989). Early investigations of stypandrol from daylilies resulted in an incorrect structural assignment for this compound and as a result, it was identified as a novel structure and given the generic name hemerocallin (Wang et al., 1989).

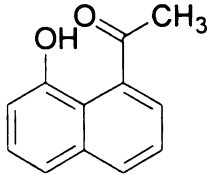


cyanidin 3-glucoside: R_1=H, R_2=H
cyanidin 3-rutinoside: R_1=H, R_2=α-L-rhamnose
delphinidin 3-glucoside: R_1=OH, R_2=H
delphinidin 3-rutinoside: R_1=OH, R_2=α-L-rhamnose

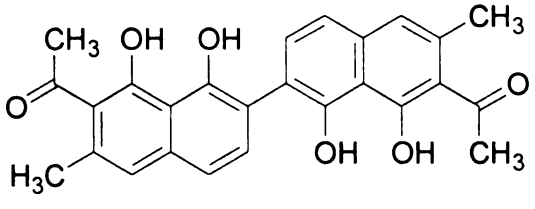
Figure 1.3. Anthocyanins found in the flowers of *Hemerocallis* spp.



hemerocallone

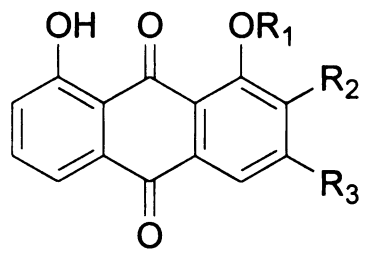


mi-hemerocallin



stypandrol

Figure 1.4. Phenolic compounds found in *Hemerocallis* spp.



	R_1	R_2	R_3
chrysophanol	H	H	CH_3
obtusfolin	CH_3	OH	CH_3
2-methoxy-obtusfolin	CH_3	OCH_3	CH_3
aloe-emodin	H	H	CH_2OH
3-carbomethoxy 1,8-dihydroanthraquinone	H	H	$OCOCH_3$
3-methoxy 1,8-dihydroanthraquinone	H	H	OCH_3
rhein	H	H	COOH
hemerocal	CH_3	OH	CH_2OH

Figure 1.5. Anthraquinones found in *Hemerocallis* spp.

Several anthraquinones have been identified in the roots of *Hemerocallis* (Table 1.1) (Figure 1.5). These include many known compounds such as chrysophanol, obtusfolin, 2-methoxy-obtusfolin, aloe-emodin, 3-carbomethoxy 1,8-dihydroxyanthraquinone, 3-methoxy 1,8-dihydroxy anthraquinone, and rhein (Yang and Li, 2002). A new anthraquinone, hemerocal, was also isolated from the roots of *H. citrina* and identified as 3-hydroxymethyl 1-methoxy 2,8-hydroxyanthraquinone (He et al. 1982).

Many other compounds have been identified in the leaves, root, and flowers of daylilies including a number of terpenoids and steroids (Table 1.1). For example, several carotenoid pigments (Figure 1.6) have been identified in *Hemerocallis* flowers including derivatives of β-carotene, β-cryptoxanthin, lutein, lycopene, and zeaxanthin (Valadon and Chapman, 1984; Griesbach and Batdorf, 1995; Tai and Chen, 2000). In addition, two new steroidal saponins, hemerosides A and B, were obtained from the aerial portion of *H. fulva* (Konishi et al., 2001) (Figure 1.7).

A unique 2,5-dimethoxytetrahydrofuran named fulvanol (Figure 1.8) was isolated from the aerial portion of *H. fulva* (Konishi et al., 1996). It was proposed that this compound, a 4-methoxy methyl-α-L-apioside, might be related to other branched aldofuranose compounds that play important regulatory roles in plants.

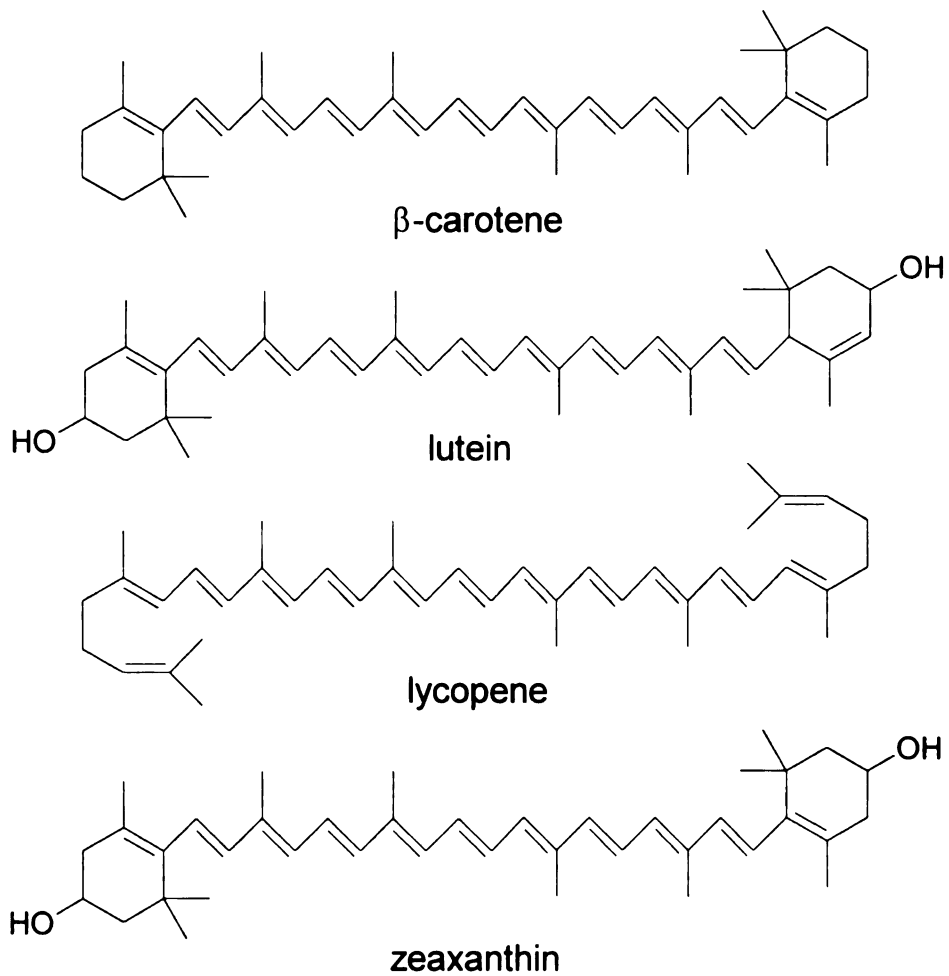
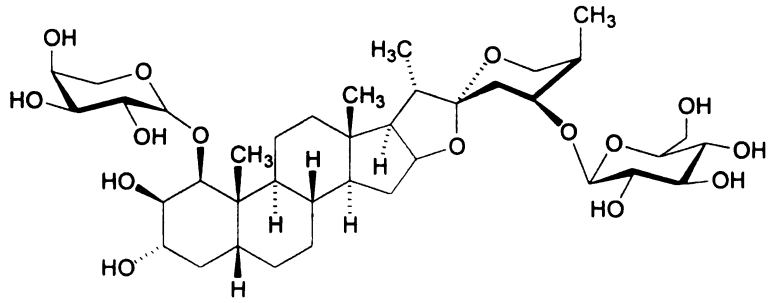
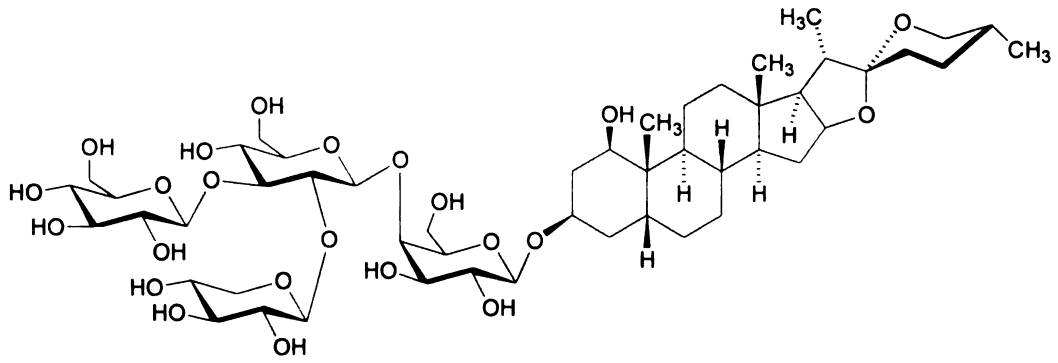


Figure 1.6. Carotenoids found in the flowers of *Hemerocallis* spp.

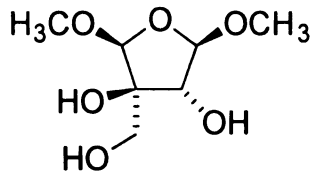


hemeroside A



hemeroside B

Figure 1.7. Structures of hemerosides A and B obtained from the aerial portion of *H. fulva*.



fulvanol

Figure 1.8. A unique 2,5-dimethoxytetrahydrofuran, fulvanol, from the aerial portion of *H. fulva*.

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Biological Activity of *Hemerocallis*

Plants are widely used across Asia as traditional medicines. One group of medicinal plants that are encountered throughout eastern Asia is daylilies. Daylilies have been reportedly used for treating a host of diseases including depression, inflammation, insomnia, and schistosomiasis (Table 1.2). A limited number of tests have been carried out to examine the effects of *Hemerocallis* extracts on biological systems. For example, it had been reported that daylily flowers were capable of alleviating insomnia (Uezu, 1998). Based on these anecdotal claims, Uezu (1998) assessed the effect that freeze-dried *H. fulva* flowers had on sleep behavior in mice. It was determined that mice fed a diet containing daylily flowers exhibited a significant increase in the duration of slow wave and paradoxical sleep as compared to control animals. In another study conducted by Hsieh et al. (1996), the effects of chloroform, ethyl acetate, *n*-butanol, and aqueous extracts of *Hemerocallis flava* L. roots were examined for their impact on motor activity in rats. These researchers found that the aqueous daylily root extract significantly inhibited the motor activity of the test animals. Upon further examination, it was determined that this extract significantly decreased levels of norepinepherine in the cortex as well as reduced the concentrations of dopamine and serotonin in the brain stem tissues of the experimental rats.

Hemerocallis spp. have been used throughout Asia for the treatment of schistosomiasis (Shiao et al., 1962a; Wang et al., 1989). Studies have shown that a preparation containing *H. thunbergii* roots exhibited a

Table 1.2. Conditions treated and reputed beneficial effects of *Hemerocallis* spp.

Condition	Reference	Condition	Reference
anemia	Uezu, 1997	lung trouble	Zhou et al., 1995
chest troubles	Zhou et al., 1995	mumps	Zhou et al., 1995
diarrhea	Uezu, 1997	promote digestion	Uezu, 1997
diuretic	Yoshikawa et al., 1994; Xui et al., 1982	promote emotional well being	Uezu, 1997
dizziness	Zhou et al., 1995	schistosomiasis	Wang et al., 1989; Shu-Hwa et al., 1962
edema	Uezu, 1997; Zhou et al., 1995; Xui et al., 1982	sore throat	Zhou et al., 1995
fever	Uezu, 1997; Yoshikawa et al., 1994	swelling	Uezu, 1997
hemorrhage	Zhou et al., 1995	tinnitus	Zhou et al., 1995
increase appetite	Uezu, 1997	tonic	Uezu, 1997
inflammation	Uezu, 1997	urethral calculi	Zhou et al., 1995
insomnia	Uezu, 1997		

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schistosomistatic effect in mice; however, toxic side-effects of this treatment regime were noted including a degeneration of spinal and optic nerve tissues and liver damage. Other researchers had previously noted the same toxicity in a variety of other animals and had gone further to provide compelling evidence implicating that the polyaromatic compound stypandrol was responsible for these effects (Wang et al., 1989; Yunping and Kangnan, 1989; Huaitao et al., 1987).

A number of other studies have presented information suggesting that *Hemerocallis* spp. possess a variety of other biological activities. For example, Sarg and colleagues (1990) as well as Roia and Smith (1977) reported that daylily flowers and roots exhibited modest antimicrobial properties. It has also been found that daylilies have diuretic properties (Xui et al., 1982). Others found that *Hemerocallis* was able to inhibit fibroblast proliferation (He, 1994) and to induce cancer cells to undergo differentiation (Hata et al., 1998). Furthermore, it was recently reported by Hsieh and colleagues (1996) that daylilies possessed antimalarial properties.

Despite a long and rich history of utilization by humans, science still possesses a paltry understanding of the pharmacological potential and phytochemical constituents of daylilies. Therefore, this research focused on the isolation, structure elucidation, and biological testing of compounds from daylily roots and flowers. The following chapters provide details of the results of these studies and provide a foundation upon which the development of new daylily-based phytoceutical entities can be based.

CHAPTER TWO

ISOLATION AND CHARACTERIZATION OF STELLADEROL, A NEW NAPHTHALENE GLYCOSIDE AND OTHER GLYCOSIDES FROM EDIBLE DAYLILY (*HEMEROCALLIS*) FLOWERS

Abstract

Daylily (*Hemerocallis* spp.) flowers are utilized as an important ingredient in traditional Asian cuisine and are also valued for their reputed medicinal effects. Studies of the bioactive (antioxidant) methanol and aqueous methanol extracts of lyophilized *Hemerocallis* cv. Stella de Oro flowers, lead to the isolation of kaempferol, quercetin, and isorhamnetin 3-*O*-glycosides (**1**-**9**), phenethyl β-D-glucopyranoside (**10**), orcinol β-D-glucopyranoside (**11**), phloretin 2′-*O*-β-D-glucopyranoside (**12**), phloretin 2′-*O*-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside (**13**), a new napthalene-glycoside, stelladerol (**14**), and an amino acid (longitubanine A) (**15**).

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Introduction

Daylilies (*Hemerocallis* spp., Hemerocallidaceae) have been harvested in eastern Asia for thousands of years where they have been utilized as both a food item (Tai and Chen, 2000) and medicinal agent (Tiejun and Tao, 1997; Uezu, 1997) for the treatment of a host of diseases. Daylilies have been reported to possess antidepressant properties, reduce inflammation, and promote digestion. Both fresh and dried daylily flowers are widely consumed as an important component in traditional eastern Asian cuisine. Pharmacological studies have shown that daylilies can facilitate neurological changes in sleeping mice (Uezu, 1998) and impact motor activity in rats as a result of alteration to the normal levels of several central nervous system neurotransmitters (Hsieh et al., 1996). Phytochemical investigations of *Hemerocallis* spp. have identified an assortment of chemical constituents including carotenoids (Tai and Chen, 2000), fulvanine lactams (Inoue et al., 1990; Inoue et al., 1994), anthocyanins (Asen and Arisumi, 1968; Griesbach and Batdorf, 1995), and anthraquinones (He et al., 1982). Unfortunately, very little is known regarding the chemical composition of edible daylily flowers.

This investigation was undertaken in order to examine the bioactive antioxidant chemical constituents of edible daylily flowers. Specifically, this research focuses on the bioactive phenolic glycosides since these compounds are known to have a significant impact on the status of human health and disease prevention. The isolation and structure elucidation of 14 phenolic

glycosides and one amino acid from lyophilized *Hemerocallis* cv. Stella de Oro flowers are reported in this chapter.

Materials and Methods

General Experimental Procedures. ^{1}H NMR spectra were recorded at 300, 500, and 600 MHz on Varian (Palo Alto, CA) INOVA (for 300 and 600 MHz) or VRX (for 500 MHz) instruments. ^{13}C NMR spectra were obtained at 75 and 125 MHz on Varian INOVA and VRX instruments, respectively. All spectra were recorded in DMSO-d_6. Standard pulse sequences were employed for all NMR experiments. FAB mass spectra were acquired at the Michigan State University Mass Spectrometry Facility using a JEOL HX-110 double-focusing mass spectrometer (Peabody, MA) operating in the positive ion mode. The UV spectra were recorded in MeOH using a Shimadzu UV-260 recording spectrophotometer (Kyoto, Japan). Sephadex LH-20 was purchased from Sigma-Aldrich (St. Louis, MO). Si gel PTLC plates (20 × 20 cm; 250, 500, and 1000 μm thick) were obtained from Analtech, Inc. (Newark, DE). Preparative HPLC was performed on a Japan Analytical Industry Co. model LC-20 recycling preparative HPLC with tandem JAIGEL-C_{18} columns (10 μm, 20 mm × 250 mm). All solvents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were of ACS analytical grade.

Plant Material. Approximately 12,000 *Hemerocallis* cv. Stella de Oro (Hemerocallidaceae) flowers (24.6 kg) were hand-harvested from Walters

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Gardens, Inc. (Zeeland, MI) on September 3 and 10, 1999 and frozen at –20 °C. The frozen flowers were lyophilized and ground in a Waring blender, yielding 2.8 kg of fine yellow powder that was stored at –20 °C until extracted.

Extraction and Bioassay Guided Isolation of Compounds 1, 3, 5-7, and 9. Powdered daylily flowers (1.8 kg) were successively extracted with hexane (5 × 6 L) (27 g), EtOAc (6 × 6 L) (20 g), and MeOH (8 × 8 L) (584g). Four 146-g portions of the bioactive MeOH extract, were each applied to XAD-16 resin and eluted with H_2O (2 L) followed by MeOH (1.5 L). The total MeOH eluate (14.5 g) was divided into eight 1.8-g fractions and further fractionated by C_{18} MPLC under isocratic conditions with CH_3CN-H_2O (3:2). The bioactive constituents from each MPLC column were eluted as a single, dark, UV-absorbing (λ 366 nm) band and pooled (12 g). This material was dissolved in EtOH (3 × 300 mL) and the soluble portion (9 g) was again applied to a column of XAD-16 resin and sequentially eluted with H_2O (2 L) followed by 30 (2 L), 60 (2.5 L), and 100 % (2 L) MeOH affording 2.9, 2.7, 1.8, and 1.2 g fractions, respectively.

The 60% MeOH eluate from XAD-16 (1.8 g) was subjected to C_{18} MPLC under a 50-100% MeOH-H_2O gradient. Eleven-milliliter fractions were collected and pooled based on their TLC ($CHCl_3$-EtOAc saturated with H_2O-MeOH-HCOOH, 1:8:2:0.1) profiles. Fractions A-C, composed of subfractions 20-25, 29-37, and 50-64, respectively, were determined to be bioactive and subjected to further purification. PTLC of fraction A (44 mg) with CH_2Cl_2-MeOH-toluene (22:5:1) yielded one fraction (35 mg) that was further purified by C_{18} preparative

HPLC under a 40-60% MeOH-H_2O (with 0.1% TFA) gradient to give compound **9** (beige amorphous solid; 8.3 mg). Using C_{18} preparative HPLC under a 40-60% MeOH-H_2O (with 0.1% TFA) gradient, fractions B (68 mg) and C (45 mg) provided compounds **6** (yellow powder; 43.1 mg) and **5** (yellow powder; 28.0 mg), respectively.

The 100% MeOH eluate (1.2 g) from XAD-16 was subjected to C_{18} MPLC under a 40-60% MeOH-H_2O gradient. Eleven-milliliter fractions were collected and pooled based on their TLC ($CHCl_3$-EtOAc saturated with water-MeOH-HCOOH, 1:8:2:0.1) profiles. Bioactive fractions D and E, composed of subfractions 41-70 and 71-100, respectively, were subjected to further purification. PTLC of fraction D (235 mg) with CH_2Cl_2-MeOH-toluene (130:15:2) provided fractions D1 (10 mg) and D2 (60 mg). Further purification of fractions D1 and D2 by C_{18} preparative HPLC under a 40-60% MeOH-H_2O (with 0.1% TFA) gradient afforded compounds **3** (yellow-brown amorphous solid; 3.0 mg) and **7** (yellow amorphous solid; 5.2 mg), respectively. Fraction E (36 mg) was also subjected to PTLC with CH_2Cl_2-MeOH-toluene (130:15:2) yielding fraction E1 (25 mg) that was subjected to C_{18} preparative HPLC under a 40-60% MeOH-H_2O (with 0.1% TFA) gradient yielding compound **1** (yellow amorphous solid; 2.0 mg).

Extraction and Isolation of Compounds 2, 4, 8, and 10-15. A 1.0 kg portion of the lyophilized flowers was exhaustively extracted with 1:1 MeOH-H_2O (6 × 5 L) and the extract reduced *in vacuo* yielding 390 g of gummy amber extract. The extract was divided into three, 130 g portions and 500 mL water was added to

each. Each portion was partitioned with hexane (3 × 200 mL) and then chloroform (3 × 250 mL). The resultant aqueous extracts were combined, concentrated *in vacuo*, and applied to a XAD-16 column. The column was eluted with water (2 L) followed by 20% MeOH (2 L) and 100% MeOH (2.5 L).

The 20% MeOH eluate (11 g) was subjected to C_{18} MPLC under a 10-40% CH_3CN-H_2O gradient and 200 mL fractions were collected affording fractions F and G. Fraction F (320 mg) was dissolved in 15 mL of warm MeOH and left on the bench-top for 14 days. Upon standing, fraction F yielded 176 mg of a powdery off-white precipitate. The precipitate was analyzed by HPLC (MeOH-H_2O, 3:7) and determined to be composed of an unresolved mixture of several compounds. The mother liquor (144 mg) was subjected to repeated isocratic preparative HPLC (MeOH-H_2O, 3:7) to give compound **15** (white powder; (34.0 mg).

Fraction G (400 mg) was applied to a Sephadex LH-20 column and eluted with MeOH and 15-mL fractions were collected. Fractions 9-11 were pooled based on their TLC (*n*-BuOH-HOAc-$CHCl_3$-H_2O, 5:1:1:4, upper phase) profiles providing a 220-mg fraction that exhibited a strong UV absorption at λ 254 nm. This fraction was further purified by PTLC with *n*-BuOH-HOAc-$CHCl_3$-H_2O (5:1:1:4, upper phase) and gradient preparative HPLC under 5-30% CH_3CN affording compound **11** (clear, glass-like amorphous solid; 13.9 mg).

The 100% MeOH eluate from XAD-16 (20 g) was repeatedly purified by C_{18} MPLC under a 20-100% MeOH-H_2O gradient giving fractions H and I.

Fraction H (600 mg) was applied to Sephadex LH-20 and eluted with 70% MeOH giving 15 mL fractions that were pooled based on their TLC (*n*-BuOH-HOAc-$CHCl_3$-H_2O, 5:1:1:4, upper phase) profiles affording fractions H1-H3. Fraction H1 (230 mg) was subjected to PTLC with CH_2Cl_2-MeOH-toluene-HCOOH (15:6:0.2:0.2) to yield fractions H1A-H1C. Fractions H1A (20 mg) and H1C (17 mg) were further purified by PTLC with $CHCl_3$-EtOAc-MeOH-HCOOH (3:7:1.5:0.1) (yields 13 and 5 mg, respectively) and isocratic C_{18} preparative HPLC (10% CH_3CN) to give compounds **10** (clear glass-like amorphous solid; 9.0 mg) and **13** (clear glass-like amorphous solid; 4.0 mg), respectively. Fraction H1B (15.0 mg) was purified by isocratic C_{18} preparative HPLC (10% CH_3CN), yielding compound **14** (yellow amorphous solid; 12.0 mg).

PTLC of fraction H2 (130 mg) with CH_2Cl_2-MeOH-toluene-HCOOH (15:6:0.2:0.2) afforded a major dark, UV-absorbing band (λ 366 nm) (120 mg) that was applied to Sephadex LH-20 (MeOH) to give fractions H2A (85 mg) and H2B (17 mg). Fractions H2A and H2B were both further purified by gradient preparative HPLC under 40-60% MeOH with 0.1% TFA (yields were 10 and 5 mg, respectively) followed by additional gradient preparative HPLC under 10-30% CH_3CN affording compounds **4** (yellow-brown amorphous solid; 4.2 mg) and **2** (yellow amorphous solid; 2.5 mg), respectively.

Fraction H3 (100 mg) was subjected to further purification by gradient preparative HPLC under 40-60% MeOH with 0.1% TFA (yield was 15 mg) followed by gradient preparative HPLC under 10-30% CH_3CN yielded compound **8** (yellow-brown amorphous solid; 2.3 mg).

Fraction I (1 g) was purified by repeated column chromatography on Sephadex LH-20 eluted with 70 and 100% MeOH, respectively. This provided a 120 mg fraction that was further purified by PTLC with $CHCl_3$-EtOAc-MeOH-HCOOH (3:7:1.5:0.1) (yield 20 mg) and gradient C_{18} preparative HPLC (5-35% CH_3CN) to give compound **12** (clear glass-like amorphous solid; 13.0 mg).

Stelladerol (14) (1-(1,5,8-trihydroxy-3-methyl-napthalen-2-yl)-ethanone-8-*O*-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside): yellow amorphous solid; UV (MeOH) λ_{max} (log ε) 223 (4.97), 313 (4.15), 345 (4.18) nm; ^{1}H NMR (600 MHz, DMSO-d_6) δ_H 9.84 (1H, s, -OH, exchange with D_2O), 9.63 (1H, s, -OH, exchange with D_2O), 7.41 (1H, s, *J*=1.5, H-4), 7.27 (1H, d, *J*=8.3, H-7), 6.76 (1H, d, *J*=8.3, H-6), 4.85 (1H, d, *J*=7.5, H-1′), 4.23 (1H, d, *J*=7.5, H-1″), 4.02 (1H, d, *J*=10.5, H-6′), 3.69 (1H, dd, *J*=5.3, 11.3, H-5″), 3.58 (1H, m, H-6′), 3.31 (2H, m, H-2′, H-5′), 3.29 (1H, m, H-4″), 3.18 (1H, t, *J*=9.0, H-4′), 3.11 (2H, m, H-3′, H-3″), 3.02 (2H, m, H-2″, H-5″), 2.51 (3H, s, -$COCH_3$), 2.25 (3H, d, *J*=1.5, -CH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ_C 204.9 (s, C=O), 150.2 (s, C-1), 148.2 (s, C-5), 146.8 (s, C-8), 131.3 (s, C-3), 126.2 (s, C-10), 125.5 (s, C-2), 114.0 (s, C-9), 113.9 (d, C-4), 112.1 (d, C-7), 109.0 (d, C-6), 104.2 (d, C-1″), 103.4 (d, C-1′), 76.5 (d, C-3′, C-3″), 76.2 (d, C-5′), 73.4 (d, C-2′, C-2″), 70.0 (d, C-4′), 69.6 (d, C-4″), 68.6 (t, C-6′), 65.6 (t, C-5″), 32.0 (q, -$COCH_3$), 19.4 (q, -CH_3); FABMS *m/z* 549 $[M+Na]^+$, 527 $[M+H]^+$, 395 $[M\text{-Xyl}+2H]^+$, 233 $[M\text{-Xyl-Glc}+2H]^+$; HRFABMS *m/z* 527.1756 $[M+H]^+$ (calcd for $C_{24}H_{31}O_{13}$, 527.1765).

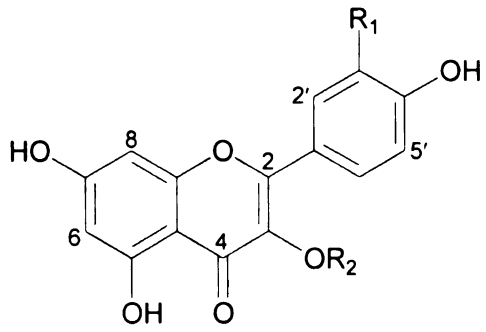
Results and Discussion

Methanol and aqueous methanol extracts of edible *Hemerocallis* cv. Stella de Oro flowers were subjected to a series of chromatographic procedures, including C_{18} MPLC and preparative HPLC, silica gel PTLC, and Sephadex LH-20 column chromatography, affording 15 compounds (Table 2.1). The structures of these compounds, including nine flavonol-3-*O*-glycosides (**1**-**9**) (Figure 2.1), phenethyl β-D-glucopyranoside (**10**), orcinol β-D-glucopyranoside (**11**), two dihydrochalcone-glycosides, phloretin 2′-*O*-β-D-glucopyranoside and phloretin 2′-*O*-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside (**12** and **13**, respectively), one new naphthalene-glycoside, stelladerol (**14**), and one amino acid, longitubanine A (**15**) (Figure 2.2) were established based on UV, NMR (^{1}H, ^{13}C, DEPT, difference NOE, DQF-COSY, HMQC, and HMBC), and MS experiments and by comparisons with literature data (Table 2.1). All of these compounds are reported here for the first time as components of edible daylily flowers. The ^3J coupling constants of the anomeric protons were used to determine the absolute α-(L-arabinose and L-rhamnose) or β-(D-galactose, D-glucose, and D-xylose) configuration of the common, naturally-occurring sugar residues found in each of the glycosides.

Examination of the ^{1}H and ^{13}C NMR spectra of compound **14** indicated that it was composed of a highly substituted naphthalene moiety conjugated with a disaccharide. The aglycone spins in the ^{1}H NMR spectrum were represented by three doublets at δ_H 7.41 (1H, J=1.5), 7.27 (1H, J=8.3), and 6.76 (1H, J=8.3).

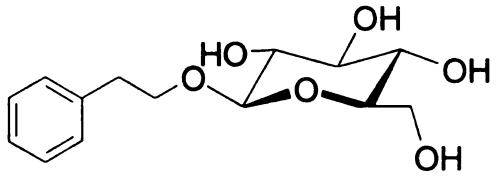
Table 2.1. Yield of 15 compounds isolated from methanol and aqueous methanol extracts of edible *Hemerocallis* cv. Stella de Oro flowers and literature sources containing comparative spectroscopic data

	compound	yield (mg/kg dry material)	reference
1	kaempferol 3-*O*-α-L-arabinopyranoside	1.1	Vasange et al., 1997
2	quercetin 3-*O*-β-D-xylopyranoside	2.5	Dick et al., 1987
3	kaempferol 3-*O*-β-D-glucopyranoside	1.7	Markham et al., 1982
4	quercetin 3-*O*-β-D-glucopyranoside	4.2	Markham et al., 1982
5	kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside	15.6	Markham et al., 1982
6	quercetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside	23.9	Markham et al., 1982
7	quercetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside	2.9	Agrawal and Bansal, 1989
8	quercetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside	2.3	Siewek et al., 1984; Webby and Boase, 1999
9	isorhamnetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside	4.6	Masterova et al., 1991
10	phenethyl β-D-glucopyranoside	9.0	Kitajima et al., 1998
11	orcinol β-D-glucopyranoside	13.9	Chung et al., 1999; Kuster et al., 1996
12	phloretin 2′-*O*-β-D-glucopyranoside	13.0	Lu and Foo, 1997
13	phloretin 2′-*O*-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside	4.0	Lu and Foo, 1997
14	stelladerol (1-(1,5,8-trihydroxy-3-methyl-napthalen-2-yl)-ethanone-8-*O*-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside)	12.0	new compound
15	longitubanine A	15.0	Yoshikawa et al., 1994

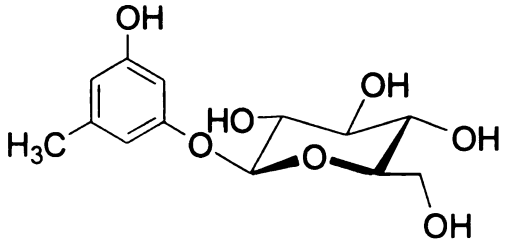


	R_1	R_2
1	H	α-L-arabinopyranoside
2	OH	β-D-xylopyranoside
3	H	β-D-glucopyranoside
4	OH	β-D-glucopyranoside
5	H	α-L-rhamnosyl-(1→6)-β-D-glucopyranoside
6	OH	α-L-rhamnosyl-(1→6)-β-D-glucopyranoside
7	OH	α-L-rhamnosyl-(1→6)-β-D-galactopyranoside
8	OH	α-L-rhamnopyranosyl-(1→6)-[α-L-rhamnosyl-(1→2)]-β-D-glucopyranoside
9	OMe	a-L-rhamnopyranosyl-(1→6)-[α-L-rhamnosyl-(1→2)]-β-D-glucopyranoside

Figure 2.1. Structures of kaempferol, quercetin, and isorhamnetin 3-*O*-glycosides (compounds **1-9**) isolated from *Hemerocallis* cv. Stella de Oro flowers.



10



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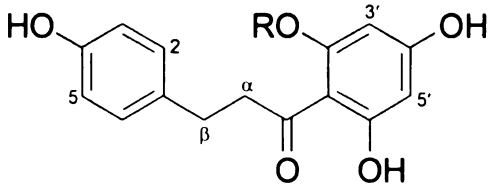
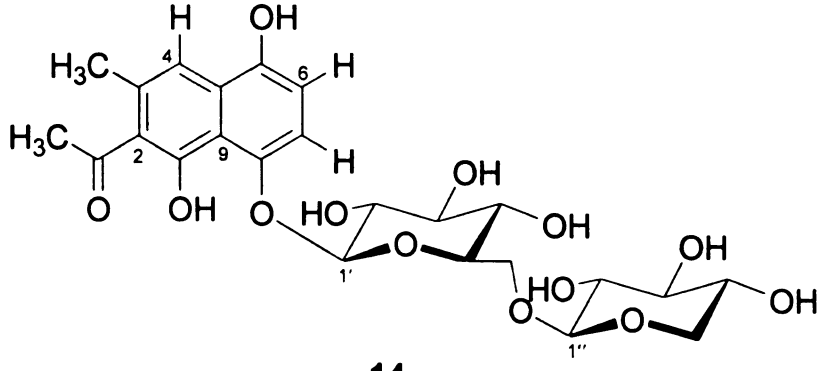


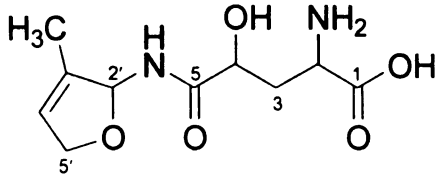
R

12 β-D-glucopyranoside

13 β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside



14



15

Figure 2.2. Structures for compounds **10-15** isolated from *Hemerocallis* cv. Stella de Oro flowers.

Analysis of the DQF-COSY spectrum confirmed the correlation between the *ortho* coupled protons at δ_H 7.27 and 6.76, while the proton at δ_H 7.41 couple weakly with the methyl doublet at δ_H 2.25. The multiplicities of the aglycon spins were determined by DEPT demonstrating that the naphthalene nucleus was composed of three methine carbons (δ_C 113.9, 112.1, and 109.0) and seven quaternary spins (δ_C 150.2, 148.2, 146.8, 131.3, 126.2, 125.5, and 114.0). In addition, two methyls (δ_C 32.0 and 19.4) and one carbonyl (δ_C 204.9) were observed. Based on these data, HMQC and HMBC experiments were used to establish the structure of the aglycon as shown in Figure 2.3.

Additional methylene and methine spins were observed in compound **14** between δ_C 65.6 and 104.2 that were assigned to xylopyranose and glucopyranose residues. A 1→6 linkage was confirmed between the two sugar moieties based on ^3J HMBC correlations between the H-6′ protons (δ_H 3.58 and 4.02) and C-1″ (δ_C 104.2) (Figure 3). Further confirmation of the structure for compound **14** was obtained as a result of 1D difference NOE experiments in which reciprocal NOE enhancements were observed between H-7 and H-6, H-7 and H-1′,–CH_3 (δ_H 2.25) and H-4, as well as –CH_3 (δ_H 2.25) and –$COCH_3$ (δ_H 2.51) (Figure 2.3). Therefore, the structure of the new naphthalene glycoside **14** was established as that illustrated in Figure 2.2. Compound **14** has been given the trivial name stelladerol in recognition of its biogenic source.

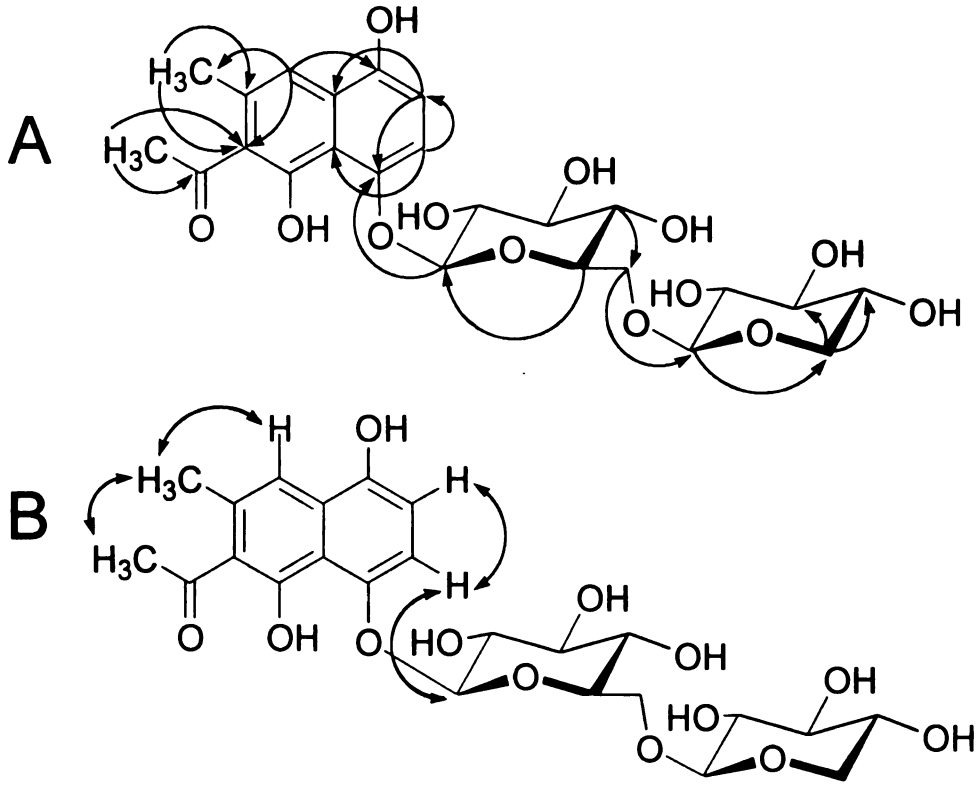


Figure 2.3. Selected HMBC (A) and difference NOE (B) correlations used to determine the structure of stelladerol (**14**).

Conclusions

Daylily flowers have been used extensively throughout eastern Asia as an important traditional food item and medicinal agent. Yet despite this rich history of use, very little was known regarding the chemical composition of the flowers. In this study of *Hemerocallis* cv. Stella de Oro flowers, a number of compounds were reported here for the first time as constituents of daylily flowers. These compounds include kaempferol, quercetin, and isorhamnetin 3-*O*-glycosides (**1-9**), phenethyl β-D-glucopyranoside (**10**), orcinol β-D-glucopyranoside (**11**), phloretin 2′-*O*-β-D-glucopyranoside (**12**), phloretin 2′-*O*-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside (**13**), a new napthalene-glycoside, stelladerol (**14**), and an amino acid (longitubanine A) (**15**). The biological of activities of these compounds have been investigated and are reported in **Chapter Four**.

CHAPTER THREE

KWANZOQUINONES A-G AND OTHER CONSTITUENTS OF *HEMEROCALLIS FULVA* 'KWANZO' ROOTS

Abstract

Daylilies (*Hemerocallis* spp.) have been used in Asia for the treatment of schistosomiasis; however, the active principles have not been fully characterized. In this study of *Hemerocallis fulva* 'Kwanzo' Kaempfer roots, several compounds were isolated including seven new anthraquinones, kwanzoquinones A (**16**), B (**17**), C (**19**), D (**20**), E (**21**), F (**22**), and G (**24**), two known anthraquinones, 2-hydroxychrysophanol (**18**) and rhein (**23**), one new naphthalene glycoside, 5-hydroxydianellin (**26**), one known naphthalene glycoside, dianellin (**25**), one known flavone, 6-methylluteolin (**27**), and α-tocopherol. The structures of the compounds were elucidated by spectroscopic and chemical methods.

Introduction

Daylily roots (*Hemerocallis* spp., Hemerocallidaceae) have been used in Asia to treat schistosomiasis (Shiao et al., 1962a; Shiao et al., 1962b). However, this method of treatment has fallen into disfavor due to a host of toxic side effects and deaths associated with the administration of *Hemerocallis* root extracts to humans (Wang et al., 1989). Previous efforts to identify the active constituent responsible for the therapeutic properties of *Hemerocallis* roots led to the isolation of a neurotoxic binaphthalenetetrol known as stypandrol (Wang and Yang, 1993) which had been shown to cause paralysis, blindness and death in mammals (Main et al., 1981; Colegate et al., 1985). In another report (Chen et al., 1962), researchers obtained a yellow powdery isolate to which was ascribed both the biological activity against schistosomes, as well as the toxic side effects associated with the use of *Hemerocallis* roots; however, its structure was never identified. While other studies have described additional compounds found in daylilies, none of these efforts have addressed the need to fully characterize the bioactive schistosome inhibitory chemical constituents from *Hemerocallis* roots.

In this study of the roots of *Hemerocallis fulva* 'Kwanzo' Kaempfer roots, a series of seven new and two known anthraquinones, one new and one known naphthalene glycosides, and one flavone were obtained. The isolation and structure elucidation of these compounds is reported in this chapter.

Materials and Methods

General Experimental Procedures. ^{1}H NMR spectra were recorded at 500 and 600 MHz on Varian (Palo Alto, CA) VRX (500 MHz) and INOVA (600 MHz) instruments, respectively. ^{13}C NMR spectra were obtained at 125 MHz on a Varian VRX instrument. NMR spectra of compounds **16** and **17** were obtained in $CDCl_3$ while all other spectra were recorded in DMSO-d_6 (Cambridge Isotope Laboratories, Inc., Andover, MA). Standard pulse sequences were employed for all 1D (^{1}H, ^{13}C, DEPT, selective ^{1}H decoupling, and difference NOE) and 2D (DQF-COSY, long-range COSY, NOESY, HMQC, and HMBC) NMR experiments. Mass spectra were acquired at the Michigan State University Mass Spectrometry Facility using a JOEL AX-505H double-focusing mass spectrometer operating at 70 eV for EIMS analysis and a JEOL HX-110 double-focusing mass spectrometer (Peabody, MA) operating in the positive ion mode for FABMS experiments. The UV spectra were recorded in EtOH using a Shimadzu UV-260 recording spectrophotometer (Kyoto, Japan). IR spectra were obtained on a Mattson Galaxy Series FTIR 3000 using WinFIRST software (Thermo Nicolet, Madison, WI). Optical rotations were measured with a Perkin-Elmer Polarimeter 341 (Shelton, CT). Melting points were determined using a Thomas Model 40 Hot Stage (Philadelphia, PA). Sephadex LH-20 was purchased from Sigma-Aldrich (St. Louis, MO). Si gel (particle size 40-63 μm) was obtained from Fischer Scientific (Pittsburgh, PA). Amberlite XAD-16 resin was purchased from Supelco (Bellefonte, PA). LC-SORB SP-A-ODS gel (particle size 25-40 μm) was obtained from Dychrom (Santa Clara, CA). Si gel

PTLC plates (20 × 20 cm; 250, 500, and 1000 μm thick) were acquired from Analtech, Inc. (Newark, DE). Preparative HPLC was performed on a Japan Analytical Industry Co. model LC-20 recycling preparative HPLC with a JAIGEL-C_{18} column (10 μm, 20 mm × 250 mm). Standards (α-tocopherol, D-glucopyranose and L-rhamnopyranose) were purchased from Sigma-Aldrich (St. Louis, MO). All other solvents and chemicals were purchased from Spectrum Laboratory Products, Inc. (New Brunswick, NJ) and were of ACS analytical grade.

Plant Material. *Hemerocallis fulva* 'Kwanzo' plants were purchased from Dr. Linda Sue Barnes, Perennial Patch (Wade, North Carolina) in August 1999. The plants were grown on the Michigan State University campus before being harvested in April 2001. The leaves were removed and the roots and crowns of 124 plants (10 kg) were washed and frozen at –4 °C. The frozen roots were lyophilized and ground in a Waring blender, yielding 2.2 kg of fine, light-brown powder.

Extraction and Isolation of Compounds 16-28. The lyophilized, powdered roots (2.0 kg) were sequentially extracted with 3 × 8 L portions of hexane, EtOAc, and MeOH yielding 25, 23, and 130 g of extracts, respectively. The hexane extract was redissolved in 500 mL of hexane and partitioned with 3 × 500 mL portions of MeOH. The MeOH fractions were pooled, yielding 15 g of extract that was applied to Si gel VLC and eluted with 4 L hexane, 3 L hexane-acetone (9:1),

and 3 L hexane-acetone (3:2). The hexane eluate (8.5 g) was subjected to Si gel MPLC under gradient conditions with 100% hexane to 100% acetone, and a total of 30 fractions, each 200 mL, were collected. All fractions were analyzed by TLC and pooled according to similarities in their profiles, yielding fractions A1-A4.

The hexane-acetone (9:1) eluate from the Si gel VLC (4.5 g) was subjected to Si gel MPLC under gradient conditions with 100% hexane to hexane-acetone (1:1) providing 900 mL fractions B1-B4. Fraction B2 (1.5 g) was rechromatographed by Si gel MPLC under gradient conditions with 100% hexane to 100% EtOAc and a total of 18 fractions, each with a volume of 200 mL, were collected and pooled based on TLC profiles giving fractions C1-C4. Fractions A3 (1g), A4 (1g), C2 (300 mg), and C3 (300mg) were pooled based on further examination by TLC and applied to Si gel MPLC. Elution was carried-out under gradient conditions with 100% hexane to 100% $CHCl_3$ to $CHCl_3$-ethanol (1:1) and 18 mL fractions, D1-D90, were collected. Fractions D1-D10 were pooled (500 mg) and further subjected to Si gel MPLC under gradient conditions with 100% hexane to hexane-acetone (97:3) and 15 mL fractions E1-E40 were collected. Fractions E6-E20 (200 mg) were composed of primarily one major component and thus were pooled and subjected to sequential Si gel PTLC with hexane-EtOAc (10:1) (72 mg), hexane- diethyl ether (6:1) (51 mg), and benzene-$CHCl_3$ (20:1), yielding 30 mg of α-tocopherol as a clear oil that exhibited spectral characteristics matching those reported in the literature (Baker and Myers, 1991), and was found identical in all respects to an authentic standard.

Fractions D12-D45 (300 mg) were combined, applied to Si gel PTLC plates, and developed twice in benzene-$CHCl_3$ (10:1). A bright yellow band (44 mg) was obtained and following extraction from the Si gel, it was dissolved in a minimal volume of $CHCl_3$, and hexane was added drop-wise until a slight degree of turbidity was noted. The solution was stored at –20 °C, yielding an inseparable 1:1 mixture (based on ^1H NMR) of compounds **16** and **17** as fine yellow needles (12 mg). Compounds **16** and **17**, and their monoacetates **16a** and **17a**, were subjected to a variety of chromatographic techniques including further Si gel TLC and MPLC, as well as, ODS MPLC and ODS preparative HPLC, but failed to separate them as single entities.

The MeOH extract of the roots was dissolved in 800 mL MeOH-H_2O (3:1) and left at 4 °C until a precipitate formed. The mixture was centrifuged (16,000 × *g*, 15 min, 4 °C) and the supernatant decanted to give 30 g of extract. The extract was applied to a column of XAD-16 resin and eluted with 10 L H_2O, 6 L 25% aqueous MeOH, and 8 L 100% MeOH. The MeOH eluate (18 g) was dissolved in 500 mL H_2O and partitioned with $CHCl_3$ (3 × 300 mL). The $CHCl_3$ fractions were pooled and evaporated under reduced pressure, yielding 2 g of extract that was applied to ODS MPLC, eluted with 50-100% MeOH, and 16 mL fractions F1-F166 were collected. Fractions F116-F125 were pooled giving 100 mg of residue that was dissolved in MeOH-acetone (3:1) and stored at –20 °C, yielding 7 mg of compound **23** as a yellow powder. Compound **23** was identified as rhein based on comparisons of its physical and spectral data to those reported in the literature (Danielsen and Aksnes, 1992).

The aqueous phase (16 g), from partitioning with $CHCl_3$, was dissolved in 50 mL of MeOH and 450 mL of acetone was slowly added while stirring, and the mixture was left at 4 °C. The supernatant (14 g) was applied to ODS MPLC and eluted with 45-100% MeOH under gradient conditions, yielding 750-mL fractions G1-G6. Fraction G3 (1 g) was again applied to ODS MPLC and eluted with CH_3CN-MeOH-H_2O-TFA (25:25:50:0.1 to 30:30:40:0.1) under gradient conditions yielding fractions H1-H6. Fraction H5 (170 mg) was applied to Sephadex LH-20 with MeOH. The major component eluted as a yellow band (25 mg) and was further purified by ODS preparative HPLC with CH_3CN-MeOH-H_2O-TFA (50:20:30:0.1), yielding 16 mg of compound **24** as a yellow powder.

Fraction G1 (10 g) was applied to ODS MPLC with 10-50% CH_3CN under gradient conditions and 550 mL fractions (I1-I7) were collected. Fraction I3 (410 mg) was chromatographed on Sephadex LH-20 with MeOH, yielding 80 mg of yellow amorphous solid. This material was further purified by successive Si gel PTLC chromatography with EtOAc-$CHCl_3$-MeOH-H_2O-HCOOH (65:25:10:0.8:0.1) (75 mg) followed by $CHCl_3$-MeOH- H_2O (8:2:1) (70 mg). Final purification by ODS preparative HPLC with 60% MeOH gave 61 mg of compound **25** as a clear-yellow, glass-like solid.

Fraction I4 (1.5 g) was applied to Sephadex LH-20 and eluted with MeOH giving 150-mL fractions J1-J6. Fractions J3-J6 (400 mg), I7 (300 mg), and H2-H4 (700 mg) were pooled and subjected to ODS MPLC with CH_3CN-MeOH-H_2O-TFA (20:20:60:0.1-40:40:20:0.1) under gradient conditions and 16-mL fractions K1-K105 were collected. Fractions K22-K38 (430 mg) were combined and

chromatographed on Sephadex LH-20 with MeOH, giving fractions L1-L2. Fraction L1 (300 mg) was applied to Si gel PTLC and developed twice with $CHCl_3$-MeOH-H_2O (8:2:0.1) giving a single band that was further purified by ODS preparative HPLC with 60% MeOH to yield 31 mg of compound **26** as a clear, glass-like solid.

Fraction L2 (130 mg) was applied to Sephadex LH-20 and eluted with MeOH to give 80 mg of a yellow amorphous solid. This material was dissolved in MeOH and placed at –20 °C yielding 62 mg of precipitate. The precipitate was chromatographed twice by ODS preparative HPLC with CH_3CN-MeOH-H_2O-TFA (40:15:45:0.1) to give 30 mg of yellow amorphous solid. Further purification of it was achieved by using 60-100% MeOH as the solvent under gradient conditions, yielding a single fraction. It was reduced in vaccuo and kept at –20 °C yielding 1 mg of compound **22** as a yellow powder.

Fractions K50-K55 were combined (98 mg), subjected to Sephadex LH-20 chromatography using MeOH as the eluant, and 125-mL fractions (M1-M5) were collected. Fraction M5 (40 mg) was dissolved in MeOH and left at room temperature, whereupon 25 mg of compound **19** was obtained as fine, yellow needles.

Fractions K56-K62 were pooled (130 mg), applied to Sephadex LH-20 and eluted with MeOH to yield fractions N1-N3. Fraction N1 (50 mg) was subjected to further Sephadex LH-20 chromatography with MeOH, giving a fraction (35 mg) that was chromatographed again on ODS preparative HPLC using CH_3CN-MeOH-H_2O-TFA (50:20:30:0.1). A single fraction was collected, reduced in

vaccuo, and placed at –20 °C, yielding 6 mg of compound **20** as golden-yellow needles. Fraction N2 (7 mg) was further purified by ODS preparative HPLC using CH_3CN-MeOH-H_2O-TFA (50:20:30:0.1) as the mobile phase to yield 1 mg of compound **27** as a yellow glass-like solid.

Fractions K63-K77 were pooled and subjected to Sephadex LH-20 chromatography using MeOH as the mobile phase, and 100 mL fractions (O1-O5) were collected. Fraction O3 (30 mg) was applied to ODS preparative HPLC using CH_3CN-MeOH-H_2O-TFA (50:20:30:0.1) as the mobile phase and yielded an amorphous yellow solid (6 mg). This material was further purified by ODS preparative HPLC under the same conditions, and the resultant fraction was reduced in vaccuo and placed at –20 °C to yield 4 mg of compound **21** as fine yellow needles.

Fractions K94-K100 were reduced in vaccuo to dryness, yielding 13 mg of an orange amorphous solid. This material was dissolved in a minimal volume of MeOH and left at –20 °C providing 7 mg of compound **18** as orange needles.

Kwanzoquinones A and B (16 and 17). Yellow needles; 165-167 °C; UV λ_{max} (EtOH) 212, 262, 287, 403 nm; IR (KBr) ν_{max} 3438, 1700, 1696, 1691, 1685, 1670, 1652, 1630, 1595, 1559 cm^{-1}; ^1H NMR ^{13}C NMR data, see Table 3.1; HRFABMS *m/z* 295.0971 $[M+H]^+$ (calcd for $C_{18}H_{15}O_4$, 295.0970).

Acetylation of Compounds 16 and 17. A portion (4 mg) of the 1:1 mixture of compounds **16** and **17** was dissolved in 1 mL of pyridine and 1 mL of Ac_2O was

added ,and the solution was stirred at room temperature for 16 h. Deionized H_2O was added to the reaction mixture, and it was subsequently partitioned with $CHCl_3$ (× 3). The $CHCl_3$ fractions were reduced in vaccuo and applied to a silica gel PTLC plate. The plate was repeatedly developed (× 3) in hexane-diethylether-$CHCl_3$ (5:1:0.1) giving two UV active bands. Band 1 (R_f = 0.4) (0.7 mg) was found to be identical to kwanzoquinones A and B (1 and 2) while band 2 (R_f = 0.2) (3.6 mg) was crystallized from MeOH to give yellow needles that were an inseparable mixture of kwanzoquinone A and B monoacetates (**16a** and **17a**, respectively).

Kwanzoquinone A and B Monoacetates (16a and 17a). Yellow needles; IR (KBr) ν_{max} 1773, 1706, 1675, 1592, 1457, 1438, 1368, 1328, 1251, 1187 cm^{-1}; ^1H NMR ($CDCl_3$) δ_H 8.10 (4H, m, H-5's and H-8's), 8.02 and 7.99 (2H, s, H-4's), 7.55 (2H, m, H-6 and H-7 for compounds **17a** and **16a**, respectively), 2.49 (12H, brs, H-12's and –$OCOCH_3$'s), 2.45 (6H, s, H-14's), 2.41 (6H, s, H-13's); HRFABMS at *m/z* 337.1068 $[M+H]^+$ (calcd for $C_{20}H_{17}O_5$, 337.1076).

2-Hydroxychrysophanol (18). Orange needles; mp 239-240 °C; UV λ_{max} (EtOH) (logε) 208 (4.19), 235 (4.05), 258 (4.11), 426 (3.73) nm; IR (KBr) ν_{max} 3408, 1653, 1620, 1560, 1473, 1456, 1434, 1310, 1271, 1190 1023 cm^{-1}; ^1H NMR (DMSO-d_6) δ_H 12.04 (1H, brs, 1-OH), 11.90 (1H, s, 8-OH), 10.34 (1H, brs, 2-OH), 7.76 (1H, dd, 8.0, 7.5, H-6), 7.66 (1H, dd, 7.5, 1.0, H-5), 7.55 (1H, s, H-4), 7.31 (1H, dd, 8.0, 1.0, H-7), 2.26 (1H, s, 3-CH_3); ^{13}C NMR, see Table 3.2; EIMS

Table

positior
1
2
3
4
4a
5
6
7
8
8a
9
9a
10
10a
11
12
13
14
1-O

[a]All s
diss
[b]Rec
[c]Rec
[d]HM
exhi
[e-h]As

Table 3.1. NMR spectral data for kwanzoquinones A (**16**) and B (**17**) in $CDCl_3$[a]

	16			**17**		
position	δ_H (J in Hz)[b]	δ_C[c]	HMBC[d]	δ_H (J in Hz)[b]	δ_C[c]	HMBC[d]
1	-	159.6 (s)	1-OH	-	159.6 (s)	1-OH
2	-	114.4[e] (s)	1-OH, H-13, H-4	-	114.5[e] (s)	1-OH, H-13, H-4
3	-	144.7[f] (s)	H-4, H-13	-	144.9[f] (s)	H-4, H-13
4	7.61 (s)	121.5 (d)	H-13	7.61 (s)	121.5 (d)	H-13
4a	-	133.1 (s)	H-4	-	133.1 (s)	H-4
5	8.04 (s)	127.8 (d)	H-14	8.13 (d, 7.5)	127.7 (d)	H-6
6	-	146.2 (s)	H-8, H-14	7.58 (d, 7.5)	135.6 (d)	H-8, H-14
7	7.58 (d, 7.5)	135.1 (d)	H-5, H-14	-	145.6 (s)	H-5, H-14
8	8.15 (d, 7.5)	127.1 (d)	H-7	8.05 (s)	127.2 (d)	H-14
8a	-	133.4 (s)	H-8	-	131.2 (s)	H-8
9	-	188.1 (s)	H-8	-	188.5 (s)	H-8
9a	-	135.7[g] (s)	1-OH, H-4	-	135.8[g] (s)	1-OH, H-4
10	-	182.3 (s)	H-4, H-5	-	181.9 (s)	H-4, H-5
10a	-	130.9 (s)	H-5	-	133.0 (s)	H-5
11	-	203.0 (s)	H-12	-	203.0 (s)	H-12
12	2.59 (s)	31.9 (q)	-	2.59 (s)	31.9 (q)	-
13	2.37 (s)	20.2 (q)	H-4	2.37 (s)	20.2 (q)	H-4
14	2.51 (s)	21.9[h] (q)	H-5, H-7	2.51 (s)	22.0[h] (q)	H-6, H-8
1-OH	12.95 (s)	-	-	12.90 (s)	-	-

[a]All spectra were recorded using 12 mg of a 1:1 mixture of compounds **16** and **17** dissolved in 1 mL of $CDCl_3$ with a 5 mm probe at 25 °C.

[b]Recorded at 500 MHz.

[c]Recorded at 125 MHz. Multiplicities were determined by DEPT experiment.

[d]HMBC data were recorded using a J_{CH} = 8 Hz and are expressed as protons exhibiting $^{2\text{-}3}J_{CH}$ couplings to the carbons as indicated.

[e-h]Assignments bearing the same letter may be interchanged.

Table 3.2. ^{13}C NMR assignments for compounds **18-22** and **24**[a]

position	**18**	**19**	**20**	**21**	**22**	**24**
1	149.4 s	153.9 s	153.9 s	149.1 s	153.4 s	158.6 s
2	150.2 s	147.7 s	147.7 s	148.4 s	146.0 s	131.2 s
3	132.3 s	141.4 s	141.5 s	136.7 s	145.3 s	140.4 s
4	122.8 d	121.5 d	121.4 d	119.0 d	117.9 d	112.0 d
4a	123.1 s	128.0 s	128.0 s	123.3 s	128.2 s	136.2 s
5	119.0 d	119.0 d	119.0 d	119.1 d	119.1 d	118.1 d
6	137.3 d	137.1 d	137.1 d	137.4 d	137.2 d	135.9 d
7	123.7 d	124.1 d	124.0 d	123.7 d	124.0 d	124.2 d
8	161.2 s	161.2 s	161.2 s	161.3 s	161.2 s	161.3 s
8a	115.9 s	115.9 s	115.9 s	116.0 s	116.1 s	116.7 s
9	192.2 s	191.5 s	191.4 s	192.3 s	191.7 s	189.2 s
9a	114.3 s	115.2 s	115.2 s	114.6 s	115.7 s	122.4 s
10	180.1 s	180.8 s	180.6 s	180.2 s	180.8 s	181.8 s
10a	133.7 s	133.2 s	133.1 s	133.8 s	133.3 s	132.3 s
11	16.4 q	17.2 q	17.2 q	57.8 t	58.1 t	19.5 q
12	-	-	-	-	-	167.8 s
1′	-	102.9 d	102.8 d	-	102.7 d	-
2′	-	74.2 d	74.0 d	-	74.1 d	-
3′	-	76.3 d	76.0 d	-	76.2 d	-
4′	-	69.7 d	69.7 d	-	69.7 d	-
5′	-	77.3 d	73.8 d	-	77.2 d	-
6′	-	60.8 t	63.7 t	-	60.7 t	-
1″	-	-	166.4 s	-	-	-
2″	-	-	41.1 t	-	-	-
3″	-	-	167.4 s	-	-	-

[a]Data recorded in DMSO-d_6 at 125 MHz at 25 °C. Multiplicities were determined by DEPT experiment and confirmed by analysis of HMQC spectra.

m/z 270 $[M]^+$ (100), 253 (2), 242 (8), 213 (4), 196 (3), 185 (2), 168 (5), 139 (11); HREIMS *m/z* 270.0532 $[M]^+$ (calcd for $C_{15}H_{10}O_5$, 270.0528) (for literature values refer to Li and McLaughlin, 1989; Midiwo and Arot, 1993).

Kwanzoquinone C (19). Fine yellow needles; mp 233-234 °C; $[\alpha]^{20}_D$ –46° (*c* 0.031, EtOH); UV λ_{max} (EtOH) (logε) 206 (4.20), 227 (4.23), 260 (4.17), 429 (3.78) nm; IR (KBr) ν_{max} 3433, 1671, 1624, 1559, 1473, 1382, 1373, 1293, 1263, 1067 cm^{-1}; ^1H NMR (DMSO-d_6) δ_H 12.04 (1H, brs, 8-OH), 12.00 (1H, s, 1-OH), 7.79 (1H, dd, *J* = 7.5, 8.0 Hz, H-6), 7.70 (1H, dd, *J* = 1.0, 7.5 Hz, H-5), 7.61 (1H, s, H-4), 7.36 (1H, dd, *J* = 1.0, 8.0 Hz), 5.07 (1H, d, *J* = 7.5 Hz, H-1′), 3.60 (1H, ddd, *J* = 2.0, 5.5, 12.0 Hz, H-6a′), 3.42 (1H, ddd, *J* = 6.0, 11.5, 11.5 Hz, H-6b′), 3.31 (1H, m, H-2′), 3.25 (1H, m, H-3′), 3.16 (1H, m, H-4′), 3.13 (1H, m, H-5′), 2.42 (3H, s, H-11); ^{13}C NMR data, see Table 3.2; HRFABMS *m/z* 433.1139 $[M+H]^+$ (calcd for $C_{21}H_{21}O_{10}$, 433.1135).

Kwanzoquinone D (20). Golden-yellow needles; mp 174-175 °C; $[\alpha]^{29}_D$ –313° (*c* 0.008, EtOH); UV λ_{max} (EtOH) (logε) 205 (4.28), 227 (4.35), 260 (4.31), 290 *sh* (3.91), 430 (3.96) nm; IR (KBr) ν_{max} 3430, 1734, 1717, 1699, 1670, 1653, 1559, 1457, 1268, 1066 cm^{-1}; ^1H NMR (DMSO-d_6) δ_H 12.57 (1H, brs, 1-OH), 11.96 (1H, s, 8-OH), 7.77 (1H, dd, *J* = 7.5, 8.0 Hz, H-6), 7.67 (1H, dd, *J* = 1.0, 7.5 Hz, H-5), 7.57 (1H, s, H-4), 7.33 (1H, dd, *J* = 1.0, 8.0 Hz), 5.06 (1H, d, *J* = 7.5 Hz, H-1′), 4.27 (1H, dd, *J* = 2.5, 11.9 Hz, H-6a′), 4.12 (1H, dd, *J* = 6.5, 11.9 Hz, H-6b′), 3.38 (1H, m, H-5′), 3.33 (1H, m, H-2′), 3.28 (1H, m, H-3′), 3.23 (2H, s, H-2″), 3.21 (1H,

m, H-4′), 2.37 (3H, s, H-11); ^{13}C NMR data, see Table 3.2; HRFABMS *m/z* 519.1139 $[M+H]^+$ (calcd for $C_{24}H_{23}O_{13}$ 519.1151).

Kwanzoquinone E (21). Fine yellow needles; mp 196-197°C; UV λ_{max} (EtOH) (logε) 209 (4.32), 235 (4.10), 258 (4.27), 354 (3.72), 426 (3.76) nm; IR (KBr) ν_{max} 3469, 1652, 1619, 1559, 1473, 1458, 1382, 1321, 1273, 1092 cm^{-1}; ^1H NMR (DMSO-d_6) δ_H 12.06 (1H, brs, 1-OH), 11.92 (1H, s, 8-OH), 10.47 (1H, brs, 2-OH), 7.87 (1H, d, *J* = 0.5 Hz, H-4), 7.78 (1H, dd, *J* = 7.8, 7.8 Hz, H-6), 7.70 (1H, dd, *J* = 0.5, 7.8 Hz, H-5), 7.33 (1H, dd, *J* = 0.5, 7.8 Hz, H-7), 5.40 (1H, brs, 11-OH), 4.59 (2H, s, H-11); ^{13}C NMR data, see Table 3.2; EIMS *m/z* 286 $[M]^+$ (62), 268 (89), 240 (56), 212 (100), 184 (50), 155 (14), 128 (19), 120 (19); HREIMS *m/z* 286.0479 $[M]^+$ (calcd for $C_{15}H_{10}O_5$, 286.0477).

Kwanzoquinone F (22). Yellow powder; mp 204-206°C; $[\alpha]^{29}_D$ –38° (*c* 0.01, EtOH); UV λ_{max} (EtOH) (logε) 228 (4.04), 259 (4.03), 291 (3.57), 432 (3.68) nm; IR (KBr) ν_{max} 3450, 1698, 1684, 1652, 1635, 1559, 1540, 1457, 1262, 1027 cm^{-1}; ^1H NMR (0.75 mL DMSO-d_6/2 drops D_2O) δ_H 7.88 (1H, s, H-4), 7.79 (1H, dd, *J* = 7.5, 8.0 Hz, H-6), 7.71 (1H, dd, *J* = 1.0, 7.5 Hz, H-5), 7.36 (1H, dd, *J* = 1.0, 8.0 Hz, H-7), 5.07 (1H, d, *J* = 7.5, H-1′), 4.37 (1H, d, *J* = 16.0 Hz, H-11a), 4.65 (1H, d, *J* = 16.0 Hz, H-11b), 3.60 (1H, d, *J* = 3.0, 12.5 Hz, H-6a′), 3.40 (1H, dd, *J* = 5.5, 12.0 Hz, H-6b′), 3.26 (1H, m, H-2′), 3.25 (1H, m, H-3′), 3.15 (1H, m, H-4′), 3.12 (1H, m, H-5′); ^{13}C NMR data, see Table 3.2; HRFABMS *m/z* 433.1132 $[M+H]^+$ (calcd for $C_{21}H_{21}O_{10}$, 433.1135).

Kwanzoquinone G (24). Yellow powder; mp 235-236°C; λ_{max} (EtOH) (logε) 219 (4.25), 283 (4.19), 413 (3.63) nm; IR (KBr) ν_{max} 3420, 1717, 1700, 1670, 1634, 1577, 1365, 1320, 1261, 1223 cm^{-1}; ^1H NMR (DMSO-d_6) δ_H 12.82 (1H, s, 8-OH), 12.81 (2H, brs, 1-OH and 12-OH), 7.67 (1H, dd, *J* = 8.1, 8.1 Hz, H-6), 7.57 (1H, dd, *J* = 1.2, 8.1 Hz, H-5), 7.56 (1H, s, H-4), 7.28 (1H, dd, *J* = 1.5, 8.1, H-7), 2.67 (3H, s, H-11); ^{13}C NMR data, see Table 3.2; HRFABMS *m/z* 299.0547 $[M+H]^+$ (calcd for $C_{16}H_{11}O_6$, 299.0556).

Dianellin (25). White needles; mp 156-157 °C; $[\alpha]^{29}_D$ –137° (*c* 0.01, EtOH); UV λ_{max} (EtOH) (logε) 225 (4.75), 301 (3.80), 334 (3.78) nm; IR (KBr) ν_{max} 3416, 2923, 1651, 1633, 1579, 1467, 1443, 1356, 1270, 1067 cm^{-1}; ^1H NMR (DMSO-d_6) δ_H 9.53 (1H, brs, 1-OH), 7.47 (1H, dd, *J* = 1.0, 8.0 Hz, H-5), 7.40 (1H, dd, *J* = 8.0, 8.0 Hz, H-6), 7.30 (1H, dd, *J* = 1.0, 8.0 Hz, H-7), 7.21 (1H, s, H-4), 5.04 (1H, d, *J* = 7.5 Hz, H-1′), 4.62 (1H, d, *J* = 1.5 Hz, H-1″), 3.93 (1H, dd, *J* = 1.5, 11.0 Hz, H-6a′), 3.68 (1H, m, H-2″), 3.59 (1H, m, H-5′), 3.50 (2H, m, H-6b′ and H-3″), 3.49 (1H, m, H-5″), 3.39 (1H, m, H-2′), 3.36 (1H, m, H-3′), 3.20 (1H, m, H-4″), 3.18 (1H, m, H-4′), 2.52 (3H, s, H-12), 2.25 (3H, s, H-13), 1.12 (3H, d, *J* =6 Hz, H-6″); ^{13}C NMR (DMSO-d_6) δ_C 204.4 (s, C-11), 154.2 (s, C-8), 150.2 (s, C-1), 135.7 (s, C-10), 132.8 (s, C-3), 127.3 (d, C-6), 125.2 (s, C-2), 122.3 (d, C-5), 119.4 (d, C-4), 113.2 (s, C-9), 110.7 (d, C-7), 102.6 (d, C-1′), 100.7 (d, C-1″), 76.2 (d, C-3′), 76.0 (d, C-5′), 73.3 (d, C-2′), 71.9 (d, C-4″), 70.7 (d, C-3″), 70.4 (d, C-2″), 70.1

(d, C-4′), 68.4 (d, C-5″), 66.6 (t, C-6′), 31.9 (q, C-12), 19.0 (q, C-13), 17.7 (q, C-6″); HRFABMS *m/z* 525.1970 $[M+H]^+$ (calcd for $C_{25}H_{33}O_{12}$, 525.1972).

5-Hydroxydianellin (26). Yellow amorphous solid; mp 152-153 °C; $[\alpha]^{29}_D$ –212° (*c* 0.01, EtOH); UV λ_{max} (EtOH) (logε) 224 (4.92), 318 (4.13), 346 (4.15) nm; IR (KBr) ν_{max} 3420, 1698, 1684, 1653, 1635, 1559, 1457, 1364, 1257, 1059 cm^{-1}; ^1H NMR (DMSO-d_6) δ_H 9.71 (2H, brs, 1-OH and 5-OH), 7.43 (1H, s, H-4), 7.16 (1H, d, *J*=8.0, H-7), 6.76 (1H, d, *J*=8.0, H-6), 4.87 (1H, d, *J*=7.5, H-1′), 4.61 (1H, m, H-1″), 3.92 (1H, m, H-6a′), 3.69 (1H, brs, H-2″), 3.52 (1H, m, H-6b′), 3.51 (1H, m, H-5′), 3.50 (1H, m, H-3″), 3.48 (1H, H-5″), 3.34 (2H, m, H-2′ and H-3′), 3.21 (1H, m, H-4″),3.18 (1H, m, H-4′), 2.51 (3H, s, H-12), 2.26 (3H, s, H-13), 1.14 (3H, d, *J* = 6 Hz, H-6″); ^{13}C NMR (DMSO-d_6) δ_C 204.7 (s, C-11), 150.2 (s, C-1), 148.3 (s, C-5), 146.7 (s, C-8), 131.3 (s, C-3), 126.4 (s, C-10), 125.6 (s, C-2), 114.2 (s, C-9), 113.8 (d, C-4), 111.9 (d, C-7), 108.6 (d, C-6), 103.5 (d, C-1′), 100.7 (d, C-1″), 76.3 (d, C-3′), 75.9 (d, C-5′), 73.3 (d, C-2′), 72.0 (d, C-4″), 70.8 (d, C-3″), 70.5 (d, C-2″), 70.0 (d, C-4′), 68.4 (d, C-5″), 66.6 (t, C-6′), 31.9 (q, C-12), 19.3 (q, C-13), 17.7 (q, C-6″); HRFABMS *m/z* 541.1910 $[M+H]^+$ (calcd for $C_{25}H_{33}O_{13}$, 541.1921).

6-Methylluteolin (27). Yellow glass-like solid; UV and IR data were identical to literature values (Milovanovic et al., 1996); ^1H NMR (DMSO-d_6) δ_H 10.92 (1H, s, 5-OH), 9.71 (1H, s, 7-OH), 9.55 (1H, s, 4′-OH), 9.23 (1H, s, 3′-OH), 7.40 (1H, d, *J* = 2.0 Hz, H-2′), 7.16 (1H, dd, J = 2.0, 8.5 Hz, H-6′), 6.80 (1H, d, J = 8.5 Hz, H-5′),

6.47 (1H, s, H-3), 6.32 (1H, s, H-8), 1.92 (3H, s, -CH_3); ^{13}C NMR (DMSO-d_6) δ_C 180.1 (s, C-4), 165.0 (s, C-5), 164.2 (s, C-9), 154.5 (s, C-7), 147.4 (s, C-4′), 145.6 (s, C-3′), 145.3 (s, C-2), 123.9 (d, C-6′), 123.5 (s, C-1′), 117.5 (d, C-2′), 115.8 (d, C-5′), 109.8 (d, C-3), 105.8 (s, C-6), 102.8 (s, C-10), 90.2 (d, C-8), 7.5 (q, -CH_3); HRFABMS *m/z* 301.0709 $[M+H]^+$ (calcd for $C_{16}H_{13}O_6$, 301.0712).

Hydrolysis of Compounds 19, 20, 22, 25, and 26. Approximately 0.5 mg of compounds **19**, **20**, **22**, **25**, and **26** were each combined with 2.5 mL of 15% aqueous HCl and left at 50 °C for about 6 h with constant stirring. The mixtures were each neutralized with the drop-wise addition of 5% aqueous NaOH and partitioned with EtOAc. The EtOAc fractions were reduced in vaccuo, the resultant residues were spotted on an analytical silica gel TLC plate along with compounds **18** and **21**, and the plate was developed in toluene-EtOAc-HOAc (4:2:0.1). After development, the plate was dried, lightly sprayed with 10% aqueous H_2SO_4, and charred with a heat-gun. The hydrolysate from compounds **19** and **20** exhibited a bright pink spot (R_f = 0.8) that was identical to that observed for 2-hydroxychrysophanol (**18**). Similarly, the hydrolysate of compound **22** yielded a pink spot (R_f = 0.4) that was identical to kwanzoquinone E (**21**). The aqueous portions from compounds **19**, **20**, **22**, **25**, and **26** were also reduced in vaccuo and the residues spotted on analytical silica gel TLC plates along with D-glucopyranose and L-rhamnopyranose. The plate was developed in *n*-ButOH-HOAc-H_2O (3:1:1), air dried, sprayed with 10% aqueous H_2SO_4, and charred with a heat-gun. The hydrolysate from compounds **19**, **20**, **22**, **25**, and

26 each exhibited a black spot (R_f = 0.6) that was identical with that observed for D-glucopyranose. In addition, compounds **25** and **26** also exhibited a second dark greenish-black spot (R_f = 0.7) that matched L-rhamnopyranose.

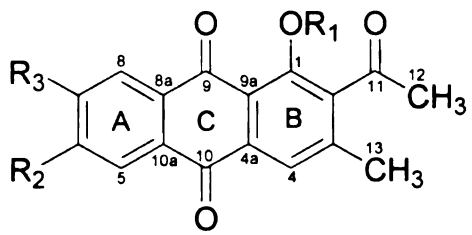
Results and Discussion

The roots of *H. fulva* 'Kwanzo' were successively extracted with hexane, EtOAc, and MeOH. The hexane and MeOH extracts were selected for further study and subsequently subjected to a combination of chromatographic procedures including Si gel MPLC and PTLC, ODS MPLC and preparative HPLC, and crystallization. This work led to the isolation of seven new anthraquinones, kwanzoquinones A-G (**16**, **17**, **19-22**, **24**), and a new naphthalene glycoside (**26**). The structures and complete ^{1}H and ^{13}C NMR spectral assignments for these new compounds, as well as those for compounds **18**, **25**, and **27**, were defined based on extensive 1D and 2D NMR studies and are reported here for the first time and are presented in Figure 3.1. The yield of the compounds obtained from the *H. fulva* 'Kwanzo' roots is presented in Table 3.3.

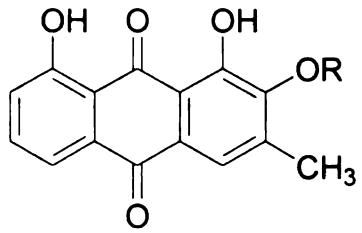
The hexane extract was subjected to a series of chromatographic procedures, leading to the isolation of 12 mg of fine, yellow needles following crystallization from $CHCl_3$-hexane. Initial inspection of the ^{1}H and ^{13}C NMR spectra of this product indicated a doubling of most proton and carbon signals that suggested it was perhaps a large dimeric compound composed of more than 31 unique carbon nuclei. However, positive FABMS indicated a major signal at

Table 3.3. Yield of 12 compounds isolated from *Hemerocallis fulva* 'Kwanzo' roots

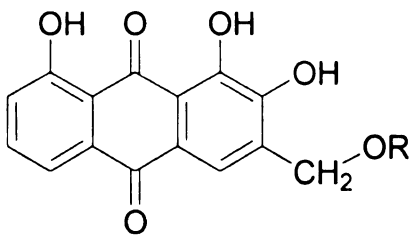
	compound	yield (mg/kg dry material)
16 and 17	kwanzoquinones A and B	9.5
18	2-hydroxychrysophanol	11.1
19	kwanzoquinone C	18.0
20	kwanzoquinone D	5.7
21	kwanzoquinone E	3.8
22	kwanzoquinone F	0.9
23	rhein	4.7
24	kwanzoquinone G	8.2
25	dianellin	15.5
26	5-hydroxydianellin	30.5
27	6-methylluteolin	0.5



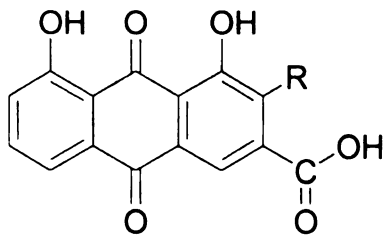
16 R_1=H, R_2=CH_3, R_3=H
16a R_1=Ac, R_2=CH_3, R_3=H
17 R_1=H, R_2=H, R_3=CH_3
17a R_1=Ac, R_2=H, R_3=CH_3



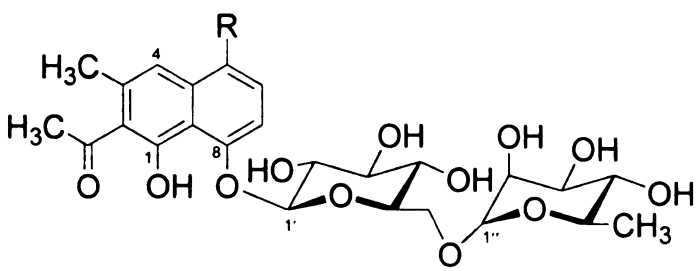
18 R=H
19 R=β-D-glucopyranoside
20 R=malonyl-(1→6)-β-D-glucopyranoside



21 R=H
22 R=β-D-glucopyranoside



23 R=H
24 R=CH_3



25 R=H
26 R=OH

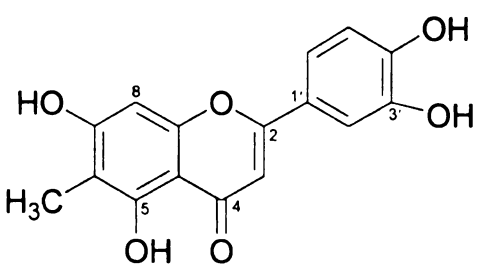


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Figure 3.1. Structures for compounds **16-27** isolated from *Hemerocallis fulva* 'Kwanzo' roots.

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m/z 295 $[M+H]^+$ that suggested the product was a mixture of two structurally related isomers each with a formula of $C_{18}H_{14}O_4$. This was supported by the presence of a significant fragment ion at *m/z* 273 $[M+H-H_2O]^+$. Further evidence was also provided by HMBC experiment that showed two sets of contours representing the $^{2\text{-}3}J_{CH}$ connectivities for two compounds, each composed of 18 carbon and 14 proton spins. Extensive efforts to separate these two compounds by Si gel MPLC and TLC, ODS MPLC and preparative HPLC, and crystallization with a variety of solvent systems proved unsuccessful. Further attempts were made to separate the acetylated products (**16a** and **17a**) from one another, but this method also failed. Therefore, the structure elucidation and full ^1H and ^{13}C NMR assignments of compounds **16** and **17** (Table 3.1) were performed on the inseparable 1:1 mixture of these two constitutional isomers.

Compounds **16** and **17** were determined to each be composed of substituted 1-hydroxyanthraquinone moieties. Evidence for this came from a combination of HRFABMS with m/z 295.0971 $[M+H]^+$ (calc. 295.0970) and spectroscopic studies. The IR spectrum of compounds **16** and **17** exhibited a number of diagnostic absorption bands at 3438 (broad, O-H stretch), 1670 (C=O stretch, non-chelated), and 1633 cm^{-1} (C=O stretch, chelated). The UV spectrum showed a λ_{max} at 403 nm that suggested the presence of a single *peri*-hydroxyl functionality (Schripsema et al. 1999). This was supported by the ^1H NMR spectrum that revealed two sharp singlets at δ_H 12.90 and 12.95 for compounds **17** and **16**, respectively, that were both exchanged upon addition of D_2O. Further evidence for the presence of a single hydroxyl functionality in compounds **16** and

17 came from their acetylation products **16a** and **17a**. Both of these acetyl derivatives exhibited the same molecular ion with HRFABMS at *m/z* 337.1068 $[M+H]^+$ (calcd for $C_{20}H_{17}O_5$, 337.1076) confirming the addition of a single acetate to **16** and **17**. The ^1H NMR spectrum of **16a** and **17a** no longer displayed downfield peaks between δ_H 12 and 13 while the ^{13}C NMR spectrum exhibited new signals at δ_C 19.6 (-OCO<u>C</u>H_3) and 169.0 (-O<u>C</u>OCH_3).

^1H NMR and DEPT experiments revealed the presence of two aromatic (δ_C 20.2 q × 2, 21.9 q, and 22.0 q) and one acetyl (δ_C 31.9 q × 2) methyl groups in both compounds **16** and **17**. Data from the HMBC experiment (Table 3.2) provided evidence for the assignment of these functionalities for compounds **16** and **17**. Further support in favor of this conclusion was obtained from long-range COSY and difference NOE experiments (Fig. 3.2). Both compounds **16** and **17** exhibited reciprocal NOE correlations upon irradiation of the methyl protons of C-12 (both δ_H 2.59) and 1-OH's (δ_H 12.95 and 12.90, respectively). In addition, NOE enhancements and long-range COSY correlations were noted between the methyl protons of C-13 (both δ_H 2.37) and the H-4 aromatic singlet (both δ_H 7.61). Together, these data confirmed the proposed ring B assignments for compounds **16** and **17**.

Compound **16** exhibited reciprocal NOE enhancements and COSY correlations Fig. 3.2) amongst H-7 (δ_H 7.58 d, *J*=7.5 Hz) and H-8 (δ_H 8.15 d, *J*=7.5 Hz), as well as between the methyl protons of C-14 (δ_H 2.51 s) and protons at positions H-7 and H-5 (δ_H 8.04 s). This evidence confirmed that the aromatic methyl C-14 (δ_H 21.9) was attached at position 6 on ring A of compound **16**.

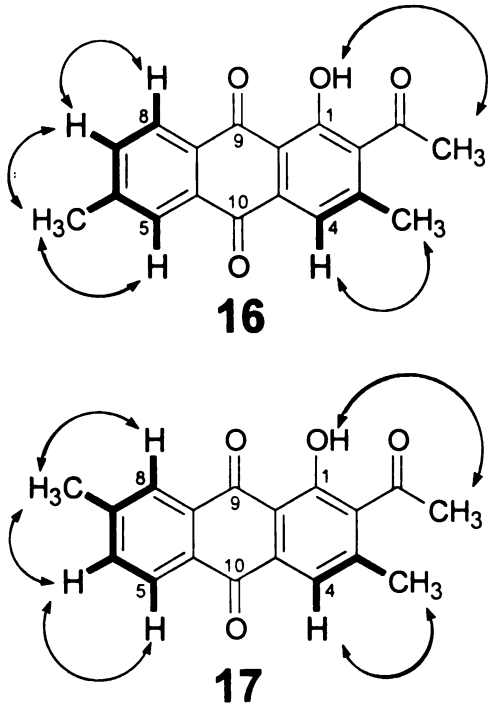

Figure 3.2. Difference NOE (→) and long-range COSY (—) correlations used to establish the structures of kwanzoquinones A (**16**) and B (**17**).

Compound **17** differed from compound **16** by displaying reciprocal NOE enhancements and long-range COSY correlations between the methyl protons of C-14 (δ_H 2.51 s) and protons H-6 (δ_H 7.58 d, *J*=7.5 Hz) and H-8 (δ_H 8.05 s). Similar NOE and COSY correlations were noted between H-6 and H-5 (δ_H 8.13 d, *J*=7.5 Hz). Therefore, the assignment of the aromatic methyl C-14 (δ_C 22.0) was confirmed at position 7 on ring A of compound **17**. Compounds **16** and **17** have been named kwanzoquinones A and B, respectively, in recognition of their biogenic source.

The MeOH extract was subjected to repeated ODS and Sephadex LH-20 gel column chromatography yielding compounds **18-27**. Following purification, compound **18** was obtained from MeOH as orange needles. HREIMS (*m/z* 270.0532 $[M]^+$ (calcd for $C_{15}H_{10}O_5$, 270.0528)) and spectral evidence (IR, UV, 1D and 2D NMR) confirmed that compound **18** (1,2,8-trihydroxy 3-methylanthraquinone) had been previously isolated from *Myrsine africana* L. (Myrsinaceae) and was given the trivial name 2-hydroxychrysophanol (Li and McLaughlin, 1989). Previous studies had only given partial ^1H and no ^{13}C NMR assignments for this compound; therefore, we undertook a thorough NMR investigation of **18** in order to confirm its proposed structure. This is the first report of compound **18** from daylilies and its complete ^{13}C NMR spectral data are presented in Table 3.3.

Compound **19** was obtained as yellow needles and exhibited many spectral characteristics similar to **18**. The IR spectrum of **19** revealed absorption bands at 3455 (broad, O-H stretch), 1671 (C=O stretch, non-chelated), and 1624

cm^{-1} (C=O stretch, chelated). The UV spectrum presented a λ_{max} at 429 nm that was accordant with the presence of two *peri*-hydroxyl functionalities (Schripsema et al., 1999; Brauers et al., 2000; Li et al., 2000). In addition, the ^1H NMR spectrum showed two downfield peaks (δ_H 12.00 and 12.04) that were exchanged with D_2O. Together this evidence supported the presence of a 1,8-dihydroxyanthraquinone moiety for compound **19**.

FABMS gave *m/z* 433 $[M+H]^+$ that represented a molecular composition of $C_{21}H_{21}O_{10}$. ^1H NMR provided important evidence for the substitution pattern of rings B and A in compound **19**. Three protons representing an ABC spin system at δ_H 7.70 (dd, J = 1.0, 7.5 Hz), 7.79 (dd, J = 7.5, 8.0 Hz), and 7.36 (dd, J = 1.0, 8.0 Hz) were assigned to C-5, C-6, and C-7, respectively, on ring A of compound **19**. ^{13}C NMR and DEPT experiments (Table 3.2) provided further evidence for the identity of the substituents attached to ring B of compound **19** with one methine (δ_C 121.5), one *C*-linked (δ_C 141.4) methyl (δ_C 17.2), and two quaternary carbon (δ_C 147.7 and 153.9) linked with a hetero-atom. These carbons were assigned positions in ring B of compound **19** based on their respective chemical shifts and the results from HMBC and HMQC experiments. Five additional methine (δ_C 69.7, 74.2, 76.3, 77.3, and 102.9) and one methylene (δ_C 60.8) spins were observed that exhibited chemical shift values that coincided with those for a glucopyranose moiety. Further evidence of the presence of a glucopyranose moiety in compound **19** was obtained by comparative TLC of the hydrolysate with authentic D-glucopyranose. The glucopyranose was assigned a β-configuration based on the coupling of H-1′ (δ_H 5.07, d, J = 7.5 Hz). The complete structure of

compound **19** was confirmed by HMBC experiment. Compound **19** is a new anthraquinone glycoside and has been given the name kwanzoquinone C.

The molecular formula of compound **20** was determined to be $C_{24}H_{22}O_{13}$ based on FABMS analysis that exhibited *m/z* 519 $[M+H]^+$. The spectral data of **20** were very similar to those obtained for compound **19**. The most significant difference was observed in the ^1H and ^{13}C NMR (Table 3.2) spectra with the addition of three new carbon signals at δ_C 41.1, 166.4, and 167.4 and a new proton resonance at δ_H 3.23 integrating for two hydrogens. These chemical shifts were characteristic of those expected for a malonyl moiety. The linkage of the malonyl group in compound **20** was established as malonyl-(1→6)-β-D-glucopyranoside based on the observed downfield shift of C-6′ to δ_C 63.7 verses that observed for compound **19** (Δ = +2.9 ppm). This was verified by HMBC analysis (Figure 3.3) which exhibited weak $^4J_{CH}$ correlations from H-6a′ (δ_H 4.12) and H-6b′ (δ_H 4.27) to C-1″ (δ_C 41.1) and H-2″ (δ_H 3.23) to C-6′ (δ_C 63.7). This confirmed that compound **20** was a new anthraquinone malonyl-glucoside named kwanzoquinone D.

EIMS analysis of compound **21** gave a molecular ion of *m/z* 286 $[M]^+$ indicating a molecular formula of $C_{15}H_{10}O_6$. The UV (λ_{max} at 426 nm) and IR (absorption bands at 3469 (broad, O-H stretch), 1667 (C=O stretch, non-chelated), and 1620 cm^{-1} (C=O stretch, chelated)) spectra suggested a 1,8-dihydroxyanthraquinone chromaphore for compound **21**. The ^1H NMR spectrum provided evidence for four exchangeable protons at δ 12.06, 11.92, 10.47, and 5.40 representing three aromatic and one aliphatic hydroxyl functionalities. An

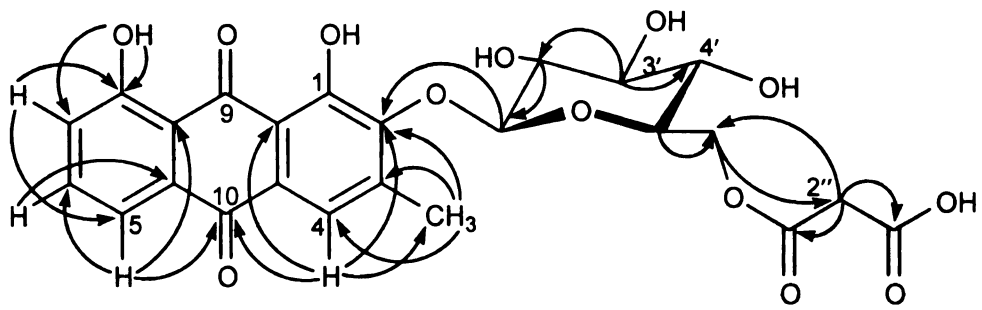

Figure 3.3. Selected HMBC correlations used to determine the structure of kwanzoquinone D (**20**).

ABC spin system was observed with protons at δ_H 7.70 (dd, J = 0.5, 7.8 Hz), 7.78 (overlapping dd, J = 7.8, 7.8 Hz), and 7.33 (dd, J = 0.5, 7.8 Hz) which occupied contiguous positions attached to C-5, C-6, and C-7, respectively, on ring A of compound **21**.

^1H and ^{13}C (Table 3.2) NMR and DEPT experiments of compound **21** gave evidence that ring B possessed quaternary carbons with *ortho*-hydroxyl functionalities (δ 149.1 s and 148.4 s), a hydroxy-methylene moiety (δ_H 4.59 s, 2H and δ_C 57.8 t) attached to a quaternary carbon (δ_C 136.7), and a methine (δ_C 119.0). An HMBC experiment was used to make full assignments of these proton and carbon spins as shown for compound **21** (Figure 3.4). Compound **21** was identified as a new anthraquinone and has been named kwanzoquinone E.

Compound **22** exhibited spectral data similar to **21** with the addition of five methine (δ_C 69.7, 74.1, 76.2, 77.2, and 102.7) and one methylene (δ_C 60.7) spins that coincided with those for a glucopyranose moiety. The addition of a glucopyranose moiety in compound **22** was confirmed by HRFABMS which gave a molecular ion of *m/z* 449.1082 $[M+H]^+$ (calcd 449.1084 for $C_{21}H_{21}O_{11}$) that represented a molecular formula of $C_{21}H_{20}O_{11}$. The glucopyranose moiety was determined to be *O*-linked at position 11 due to the downfield shift of this carbon signal to δ_C 58.1 (Δ = +0.4) and the change in the splitting pattern of the attached protons. While the enantiotopic C-11 protons of compound **21** (δ_H 4.59, 2H) appeared as a singlet, the diastereotopic C-11 protons of compound **22** (δ_H 4.65 , 1H and 4.73, 1H) were each a doublet (J = 16.0 Hz) in achiral solvent (0.75 mL DMSO-d_6 with 2 drops of D_2O). Further evidence in support of the composition

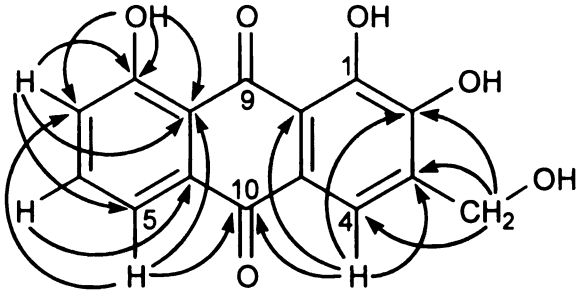

Figure 3.4. Selected HMBC correlations used to determine the structure of kwanzoquinone E (**21**).

of compound **22** was obtained from acid hydrolysis that yielded kwanzoquinone E and D-glucopyranose based on co-TLC with authentic sugar samples. The assignments of all proton and carbon (Table 3.2) spins in compound **22** were confirmed by HMBC experiment. Compound **22** is a new conjugated anthraquinone glucoside and has been given the name kwanzoquinone F.

Compound **23** was obtained as an amorphous yellow powder and its spectral data were found to match those reported for rhein, a known anthraquinone (Danielsen and Aksnes, 1992). Compound **24** exhibited spectral data similar to **23** with the main differences in the ^{1}H NMR spectrum being the loss of an aromatic doublet (*ca* δ_H 8.15, 1H, J = 1.5 Hz) and the concomitant loss of splitting in the proton signal at δ_H 7.59 (s, 1H). This indicated that position 2 in ring B of compound **24** was substituted. These observations coincided with the appearance of an aromatic methyl (δ_H 2.67 s, 3H and δ_C 19.5 q) and the downfield shift of C-2 in compound **23** from δ_C 124.2 to 131.2 (Δ = +7.0 ppm) in compound **24** (Table 3.2). HMBC experiment was able to confirm that this methyl was a substituent of C-2 based on the long-range coupling of the C-11 methyl protons to C-2 (δ_C 131.2) and C-3 (δ_C 140.4). Compound **24** is a new anthraquinone and has been named kwanzoquinone G.

FABMS of compound **25** provided a molecular ion of 525 $[M+H]^+$ and the ^{13}C NMR spectrum exhibited ten sp^2 carbon signals between 110 and 155 ppm along with 12 additional sp^3 carbon signals that were characteristic of a rutinose moiety. In light of the presence of three additional carbon signals that represented an aromatic methyl (δ_C 19.0) and an acetyl moiety (δ_C 31.9 and

204.4), it was determined that compound **25** was a substituted naphthalene diglycoside. Acid hydrolysis of compound **25** yielded D-glucopyranose and L-rhamnopyranose based on co-TLC with authentic sugar samples. HMQC and HMBC experiments established the aglycone portion of compound **25** as 2-acetyl-3-methyl-1,8-dihydroxynaphthalene, dianellidin. Further scrutiny of the HMBC data provided for the assignment of an 8-*O*-linkage to the rutinoside moiety based on a correlation from H-1′ (δ_H 5.04, d, J = 7.5 Hz) to C-8 (δ_C 154.2 s). According to these data, compound **25** was identified as dianellin, previously isolated from *Dianella* spp. (Liliaceae) (Batterham et al., 1961). This is the first account of compound **25** from daylilies and the first report detailing its ^{1}H and ^{13}C NMR spectral data.

The ^{1}H, ^{13}C and DEPT NMR data of compound **26** were very similar to those observed for **25**. The loss of one aromatic methine spin in compound **25** was replaced by a quaternary carbon (δ_C 148.3) that was linked to a hetero-atom. The FABMS analysis of compound **26** yielded a molecular ion at *m/z* 541 $[M+H]^+$ indicating a molecular formula of $C_{25}H_{32}O_{13}$. A comparison of the ^{1}H and ^{13}C NMR spectral data for the aglycone portion of compound **26**, with the reported values for the naphthalene glycoside stelladerol (Cichewicz and Nair, 2002), demonstrated that both possessed the same aglycone moiety. However, these compounds differed with respect to their glycosidic moiety. Acid hydrolysis of the new naphthalene glycoside revealed the presence of D-glucopyranose and L-rhamnopyranose moieties in compound **26**. HMBC correlation data for compound **26** (Figure 3.5) showed that it possessed an 8-*O*-β-D-

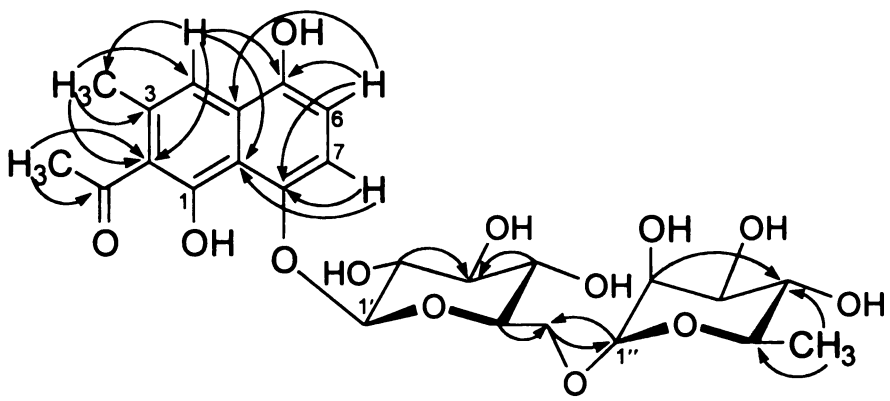

Figure 3.5. Selected HMBC correlations used to determine the structure of 5-hydroxydianellin (**26**).

rhamnopyranosyl-(1→6)-β-D-glucopyranoside moiety. Significant HMBC correlations that were used to deduce these connectivities included those observed for H-1′ (δ_H 4.87) to C-8 (δ_C 146.7), and H-6a′ (δ_H 3.92) and H-6b′ (δ_H 3.52) to C-1″ (δ_C 100.7), as well as, from H-1″ (δ_H 4.61) to C-6′ (δ_C 66.6). Based on these data, compound **26**, 5-hydroxydianellin (1-(1,5,8-trihydroxy-3-methyl-napthalen-2-yl)-ethanone-8-*O*-β-D-rhamnopyranosyl-(1→6)-β-D-glucopyranoside), was identified as a new naphthalene glycoside.

Compound **27** was obtained as a yellow, clear, glass-like solid and identified as 5,7,3,4-tetrahydroxy-6-methylflavone (6-methylluteolin) that was previously reported from *Salvia nemorosa* L. (Lamiaceae) (Milovanovic et al., 1996). The structure of compound **27** was confirmed based on detailed 1D and 2D NMR studies and by comparisons of its UV and IR spectral data with those reported. This is the first report of compound **27** from daylilies and the first detailed account of its ^{1}H and ^{13}C NMR spectral properties.

Conclusions

Daylily roots have been used extensively throughout eastern Asia as a traditional treatment for schistosomiasis. However, the bioactive constituents have never been fully characterized. In this study of *Hemerocallis fulva* 'Kwanzo' Kaempfer roots, several compounds were isolated including seven new anthraquinones, kwanzoquinones A (**16**), B (**17**), C (**19**), D (**20**), E (**21**), F (**22**), and G (**24**), two known anthraquinones, 2-hydroxychrysophanol (**18**) and rhein (**23**), one new naphthalene glycoside, 5-hydroxydianellin (**26**), one known naphthalene

glycoside, dianellin (**25**), one known flavone, 6-methylluteolin (**27**), and α-tocopherol. The biological of activities of these compounds have been investigated and are reported in **Chapter Four**.

CHAPTER FOUR

BIOLOGICAL ACTIVITIES OF COMPOUNDS ISOLATED FROM *HEMEROCALLIS* CV STELLA DE ORO FLOWERS AND *HEMEROCALLIS FULVA* 'KWANZO' ROOTS

Abstract

Daylilies (*Hemerocallis* spp.) have been used in eastern Asia for the treatment of a variety of medical conditions. Isolation studies conducted with *Hemerocallis* cv. Stella de Oro flowers and *H. fulva* 'Kwanzo' roots yielded a variety of compounds that have been evaluated for their biological activities. These compounds were assayed for anticancer, antioxidant, cyclooxygenase inhibitory, mosquitocidal, nematocidal, schistosome inhibitory, and topoisomerase inhibitory effects. The new anthraquinones, kwanzoquinones A (**16**), B (**17**), C (**19**), E (**21**), and kwanzoquinone A and B monoacetate analogues (**16a** and **17a**, respectively), inhibited the proliferation of human cancer cells in vitro. In addition to these new anthraquinones, the known compounds 2-hydroxychrysophanol (**18**) and rhein (**23**) inhibited cancer cell growth. Three compounds, kwanzoquinone D (**20**), stelladerol (**14**), and 5-hydroxydianellin (**26**), demonstrated remarkable antioxidant activity by inhibiting lipid oxidation by more than 90% in an in vitro assay system. Two compounds, 2-hydroxychrysophanol (**18**) and kwanzoquinone E (**21**), were discovered as novel agents for the prevention and treatment of schistosomiasis. These compounds were found to

inhibit the motility and induce mortality of *Schistosoma mansoni* cercariae and adults. Kwanzoquinones C and E and 2-hydroxychrysophanol exhibited moderate mosquitocidal properties. These compounds induced mortality of fourth instar *Aedes aegyptii* larvae within 24 h. None of the compounds demonstrated nematocidal, cyclooxygenase inhibitory, or topoisomerase inhibitory activities.

Introduction

Hemerocallis spp. have been used extensively throughout eastern Asia as a traditional food item and medicinal agent for treating a variety of conditions (Table 1.2). A small number of pharmacological studies performed using crude *Hemerocallis* extracts have indicated the presence of bioactive components in daylilies; however, the chemical constituents responsible for these effects have not been conclusively determined. Phytochemical investigations of daylilies have revealed the presence of a limited number of chemical constituents in the flowers and roots of these plants, but the biological activities of these components have not been determined. Additional work is needed to further evaluate the bioactivities of compounds from *Hemerocallis* spp.

Materials and Methods

Cancer Cell Growth Inhibition Assay. Compounds **1-21** and **23-26** were tested for their activity against breast, central nervous system (CNS), colon, and lung human tumor cell lines. The breast (MCF-7), CNS (SF-268), and lung (NCI-H460) cultures were purchased from the National Cancer Institute (Bethesda, MD) while the colon culture (HCT-116) was purchased from the American Type Culture Collection (Rockville, MD). All cell lines were maintained in a humidified chamber at 37 °C with 5% CO_2 in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (1 unit/100mL), and streptomycin (1 μg/100mL) . All cell lines were sub-cultured according to their individual growth profiles in order to ensure exponential growth throughout the experiments. Breast (10,000

cells/well), CNS (15,000 cells/well), colon (7,500 cells/well), and lung (7,500 cells/well) cancer cells were aliquoted (100 μL) into 96-well plates and allowed to grow for 24 h before the addition of test compounds to the media. Compounds were dissolved in DMSO and diluted in sterile media as necessary to obtain the appropriate concentration. The test compounds were added to the sample wells in 100-μL aliquots so that the final concentration of DMSO did not exceed 0.25%. Test compounds, standards, and DMSO control were incubated for 48 h, after which the assay was terminated via the addition of cold trichloroacetic acid. The plates were incubated for one hour at 4 °C, washed with deionized water (× 5), and air-dried. A 100-μL aliquot of 0.4% sulforhodamine B stain in 1% acetic acid was added to each well and the plates were incubated for 30 min at room temperature. Following incubation, the wells were rinsed with 1% acetic acid (× 5) and the bound stain was dissolved in 100 μL of 10 mM Trizma base. The plates were shaken for five minutes on a gyrorotary shaker after which the absorbance of each well was recorded with an automated microplate reader (model EL800, Bio-Tek Instruments, Inc., Winooski, VT) at 515 nm. Three independent experiments were performed in triplicate using at least five drug concentrations inclusive of the 50% growth inhibitory concentration. Results are expressed as the concentration of compound required to inhibit cellular growth 50% (GI_{50}) ± SE Further details regarding these procedures have been reported (Boyd and Paull, 1995; Skehan et al. 1990).

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Antioxidant Assay. Compounds **1-21** and **23-26** were tested in vitro for their ability to inhibit the oxidation of large unilamellar vesicles (LUVs). The vesicles were prepared by combining the phospholipid 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids, Inc., Alabaster, AL) with the fluorescent probe 3-[*p*-(6-phenyl)-1,3,5-hexantrienyl]phenyl-propionic acid (Molecular Probe, Inc., Eugene, OR) (in a molar ration of 350:1 (lipid:probe). A buffer maintained in Chelex resin and composed of 0.15 M NaCl, 0.01 M MOPS (pH 7.0), and 0.1 mM EDTA (all purchased from Sigma-Aldrich, St. Louis, MO) was used to suspend the lipid-probe mixture and this was exposed to ten freeze-thaw cycles in a dry ice-ethanol bath. The resultant material was passed through a 100 nm polycarbonate filter 29 times to give the LUVs.

Experiments were conducted by combining LUVs, 100 mM NaCl, and 50 mM Tris-HEPES (pH 7.0) and test compound in DMSO (final concentration of 10 μM test compound in 2 mL). Oxidation of the lipid-probe substrate was initiated by the addition of 20 μL of a 0.5 mM $FeCl_2$ solution. Data represent the relative fluorescence intensity of the probe-lipid-test compound mixture as compared to a probe-lipid control. All compounds were tested in triplicate and results are reported as the mean ± one standard deviation after fifteen minutes of incubation. The antioxidant standards *tert*-butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and vitamin E (α-tocopherol), were purchased from Sigma-Aldrich (St. Louis, MO). Full experimental details have been previously reported (Arora et al., 1997; Wang et al., 1999).

Cyclooxygenase Inhibition Assay. Compounds **1-21** and **23-26** were tested for their ability to inhibit cyclooxygenase I and II enzymes in vitro. These experiments were performed and components obtained from sources as previously described (Wang et al., 1999). Briefly, cyclooxygenase enzyme preparations were incubated at 37 °C with arachidonic acid and test compound (100 μM) was delivered in DMSO. The initial rates of oxygen consumption were recorded and compared to controls. All compounds were tested in triplicate.

Mosquito Larvicidal Assay. Compounds **16-21** and **23-26** were tested for their activity against fourth instar larvae of *Aedes aegyptii* (Rockefeller strain) as previously described (Roth et al., 1998) with minor modifications. Larvae were reared from eggs in deionized water at 27 °C with the addition of bovine liver extract (25 mg/L). Approximately 10-15 fourth instar larvae were placed in test tubes in 990 μL of deionized water. The test compounds were dissolved in DMSO and 10 μL of each sample were added to the larvae so as to give a final concentration of 50, 25, or 12.5 μg/mL of test compound. The larvae were observed at 15 and 30 min and 1, 2, 4, 12, 24, and 36 h for changes in motility and mortality. Compounds were tested in replicate (n=5) with DMSO controls. No mortality was observed among the control larvae up to 36 h.

Nematicidal Assay. Compounds **1-21** and **23-26** were tested for activity against two nematode strains, *Caenorhabditis elegans* and *Panagrellus redivivus*, as previously described (Nair et al., 1989) with minor modifications. *Caenorhabditis elegans* was maintained in a mixed culture with *Escherichia coli* in a medium containing NaCl (3 g), peptone (1.25 g), agar (8.5 g), KH_2PO_4 (1.5 g), K_2HPO_4 (0.3 g), $CaCl_2$ (0.1 g), $MgSO_4$ (0.1 g), cholesterol (2.5 mg), and deionized water (500 mL). *Panagrellus redivivus* was grown in a medium containing peptone (2.5 g), glucose (5 g), molasses (10 g), agar (9 g), and deionized water (500 mL). Tests were performed by placing 10-15 nematodes in 48 μL of media into each well of a 96-well plate. Two microliters of test compound dissolved in DMSO were added to each test well such that the well contained a final concentration of 25 μg/mL of test compound in a volume of 50 μL. The nematodes were observed at 15 and 30 min and 1, 2, 4, 12, 24, and 48 h for changes in motility and mortality. Tests were performed in triplicate with DMSO controls.

***Schistosoma mansoni* Cercaricidal Inhibition Assay.** Compounds **16-26** were examined for their effects on *S. mansoni* (Puerto Rican strain) cercariae motility and mortaility. Cercariae were obtained from infected *Biomphalaria glabrata* snails by light induction. Details regarding the methods used for the maintenance of both *S. mansoni* and *B. glabrata* cultures have been previously reported (Salter et al., 2000). A total of 50-100 cercariae in 100-μL distilled H_2O were collected and placed in 96-well vinyl assay plates (Costar, Acton, MA). Stock solutions of compounds **1-11**, including **1a** and **2a**, were prepared by

dissolving 1 mg of test compound in 100 μL of DMSO and 19.9 mL of distilled H_2O. This stock solution was further diluted as needed and 100 μL aliquots were added to each well. Cercariae motility (i.e. tail movement and swimming behavior) was observed under a dissecting microscope. Viability of the cercariae was determined by removing the test compounds after ten hours and replacing it with fresh water. Recovery from exposure to the test compounds was assessed after 24 h.

***Schistosoma mansoni* Schistosomulicidal Inhibition Assay.** Compounds **16-26** were tested for their effects on *S. mansoni* schistosomula motility and mortality. Schistosomula were prepared from *S. mansoni* cercariae by shearing the tails and incubating the organisms for 2 days in RPMI-1640 media with fetal bovine serum plus antibiotics in flat-bottomed 96-well culture plates. Test compounds were added to the media as described for the cercariae assay and the schistosomula were observed for changes in movement, feeding, and viability.

***Schistosoma mansoni* Adult Schistosomicidal Assay.** Compounds **16-26** were tested for their ability to alter the motility and mortaility of *S. mansoni* adults. Adult worms were perfused from Syrian Golden hamsters as described (Davies et al., 2001). Twenty male and female adult worms were cultured in 24 well Falcon plates at 37 °C in one milliliter of RPMI-1640 media supplemented with 2 g/L glucose, 0.3 g/L L-glutamate, and 2 g/L $NaHCO_3$, 15% fetal bovine serum

(heat inactivated), antibiotics, and 15 μL of hamster red blood cells (washed with RPMI). Five-microliter aliquots of test compounds in DMSO or DMSO control were added to each well. The movement, feeding, and viability of the adult worms were monitored for 24 h. The media was removed and replaced with fresh media to which the test compounds were added again and the adult worms observed for another 24 h. Finally, the media was again removed and replaced with fresh media without test compounds and the recovery of the adult worms was monitored for another 24 h.

Topoisomerase Inhibition Assay. Compounds **1-21** and **23-26** were tested for their ability to inhibit the activity of topoisomerase I and II enzymes in vitro as previously described (Roth et al., 1998). Three mutant strains of *Saccharomyces cerevisae* were provided by Dr. John Nitiss (St. Jude Children's Hospital, Memphis, TN) that possess recombinant forms of topoisomerase I and II enzymes. The *S. cerevisae* strain $JN394_{t-1}$ lacks the topoisomerase I enzyme and is resistant to the topoisomerase inhibitor camptothecan. The *S. cerevisae* strain $JN394_{t-2-5}$ possesses a mutated topoisomerase II enzyme and is resistant to etoposide and other topoisomerase II inhibitors, but is sensitive to topoisomerase I inhibitors. Another strain of *S. cerevisea*, JN394, contains recombinant forms of both topoisomerase I and II and is sensitive to both topoisomerase I and II inhibitors. Organisms were grown in a liquid culture medium composed of peptone (10.0 g), yeast extract (5.0 g), glucose (10.0 g), and deionized water (500 mL) at 25 °C. The organisms were lawned onto the

surface of an agar plate. Compounds were dissolved in DMSO and applied to the surface of the cultured plates such that 2 μL contained 25 μg of test compound. The plates containing the test compounds and DMSO controls were incubated at 30 °C for 72 h and were observed every 24 h for signs of growth inhibition. All experiments were performed in triplicate.

Results and Disscussion

Overview of Results. The compounds obtained from the flowers and roots of *Hemerocallis* were tested for a range of biological activities against a panel of bioassays. These tests included an examination of the anticancer, antioxidant, cyclooxygenase inhibitory, mosquitocidal, nematocidal, schistosome inhibitory, and topoisomerase inhibitory effects of the daylily-derived compounds. A summary of the results of these studies is presented in Table 4.1. Several compounds exhibited promising anticancer, antioxidant, mosquitocidal, and schistosome inhibitory effects. Details of these results are noted below. None of the compounds demonstrated, nematocidal, cyclooxygenase inhibitory, or topoisomerase inhibitory activities.

Anticancer Activity. It was previously reported that crude *Hemerocallis* extracts inhibited fibroblast proliferation (He, 1994) and induced cancer cells to undergo differentiation (Hata et al., 1998); however, the active constituents were never identified. In these studies, several compounds were obtained from *H. fulva* roots that exhibited growth inhibitory effects against human breast, CNS, colon,

Table 4.1. Summary of the results of bioassays performed on compounds obtained from *Hemerocallis* cv. Stella de Oro flowers and *H. fulva 'Kwanzo'* roots[a]

compound	assay						
	anticancer	antioxidant	cyclooxygenase inhibition	mosquitocidal	nematocidal	schistosome inhibition	topoisomerase inhibition
1	-	-	-	nt	-	nt	-
2	-	+	-	nt	-	nt	-
3	-	-	-	nt	-	nt	-
4	-	+	-	nt	-	nt	-
5	-	+	-	nt	-	nt	-
6	-	+	-	nt	-	nt	-
7	-	-	-	nt	-	nt	-
8	-	-	-	nt	-	nt	-
9	-	-	-	nt	-	nt	-
10	-	-	-	nt	-	nt	-
11	-	-	-	nt	-	nt	-
12	-	-	-	nt	-	nt	-
13	-	-	-	nt	-	nt	-
14	-	++	-	nt	-	nt	-
15	-	-	-	nt	-	nt	-

Table 4.1. (cont'd)

compound	assay						
	anticancer	antioxidant	cyclooxygenase inhibition	mosquitocidal	nematocidal	schistosome inhibition	topoisomerase inhibition
16 and **17**	++	-	-	-	-	-	-
16a and **17a**	++	-	-	-	-	-	-
18	++	+	-	++	-	++	-
19	++	+	-	++	-	-	-
20	-	++	-	-	-	-	-
21	++	+	-	++	-	++	-
22	nt	nt	nt	nt	nt	-	nt
23	+	+	-	-	-	-	-
24	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-
26	-	++	-	-	-	-	-
27	nt	nt	nt	nt	nt	nt	nt

[a]- (not active), nt (not tested), + (weak to moderate activity), ++ (strong activity)

and lung cancer cell lines. The GI_{50} concentrations of these compounds are presented in Table 4.2. The mixtures of compounds **16** and **17,** as well as, **16a** and **17a** exhibited strong growth inhibitory effects against breast cancer cells with GI_{50} values of 2.6 ± 0.6 and 1.8 ± 0.2 mg/mL, respectively. In contrast, the GI_{50} concentration of these compounds was approximately three to six times higher against the other three cell lines (Table 4.2).

Compounds **18**, **19**, and **21** exhibited consistent activity against all four cancer cell lines (Table 4.2). The in vitro cytotoxicity of compound **18** against three tumor cell lines including lung (A-549, ED_{50} 3.1 μg/mL), nasal (KBMRI, ED_{50} 5.7 μg/mL), and colon (HT-29, ED_{50} 2.8 μg/mL) had been previously described (Li and McLaughlin, 1989). The results obtained here for compound **18** are consistent with the previously reported findings. Compounds **19** and **21** are both new compounds that represent 2-*O*-glucopyranose conjugated and 3-hydroxymethyl derivatives, respectively, of compound **18**. Both of these compounds were found to possess cancer cell growth inhibitory properties against all cell lines tested at concentrations similar to that of compound **18**. Compound **23** exhibited moderate activity against all four cancer cell lines.

In light of the growth inhibitory activity exhibited by these compounds against a number of cancer cell lines and the current cancer chemotherapeutic application of other anthraquinone derivatives, compounds **16-19**, **21**, and **23** warrant further investigation to determine their mode of action and potential clinical applications.

Table

Heme

cor

16

16a

ad

[a]Va

Table 4.2. Growth inhibitory effects of anthraquinones isolated from *Hemerocallis fulva* 'Kwanzo' roots against four human cancer cell lines

compound	cell line (GI_{50}, μg/mL ± SE)			
	MCF-7 (breast)	SF-268 (CNS)	HCT-116 (colon)	NCI-H460 (lung)
16 and **17**	2.6 ± 0.6	14.7 ± 2.5	13.5 ± 0.9	10.3 ± 1.2
16a and **17a**	1.8 ± 0.2	5.3 ± 0.8	10.5 ± 0.7	8.5 ± 0.6
18	6.5 ± 1.2	2.4 ± 1.8	6.3 ± 0.8	6.3 ± 0.8
19	6.7 ± 0.4	6.1 ± 1.0	7.4 ± 0.6	3.8 ± 0.3
21	2.8 ± 0.3	3.8 ± 0.7	5.0 ± 0.3	7.3 ± 0.7
23	17.2 ± 0.8	16.3 ± 1.8	21.1 ± 1.8	15.4 ± 1.7
adriamycin[a]	1.7 ± 0.2	1.9 ± 0.7	2.1 ± 0.6	1.7 ± 0.4

[a]Values for adriamycin are expressed in μM.

Antioxidant Activity. Compounds **1-15**, **18-21**, and **23-26** were evaluated for their potential antioxidant activity at 10 μM, while the mixtures of compounds **16** and **17** and **16a** and **17a** were tested at 10 μg/mL. The results of these experiments are presented in Figure 4.1. Under these experimental conditions, stelladerol (**14**) and 5-hydroxydianellin (**26**) exhibited strong antioxidant activity (94.6 ± 1.4 and 99.6 ± 2.0% inhibition, respectively) that was more pronounced than that of the commercial synthetic antioxidants TBHQ, BHA, and BHT (81.8 ± 1.2, 80.0 ± 1.0, and 86.4 ± 1.3%, respectively) and vitamin E (15.7 ± 0.6%). Both compounds **14** and **26** share a common aglycon portion composed of 2-acetyl-1,5-dihydroxy-3-methyl naphthalene moieties. This structural feature of compounds **14** and **26** allows for them to function as antioxdants via the formation of oxidized resonance stabilized quinone radicals and stable naptho-quinone products. It is interesting to note that compound **25** is structurally similar to compounds **14** and **26**, but lacks a 5-hydroxy moiety and did not exhibit any pronounced antioxidant effects.

A number of the anthraquinones demonstrated moderate antioxidant activity. These include compounds **18**, **19**, **21**, and **23** that exhibited 49.9 ± 4.1, 42.0 ± 4.8, 28.6 ± 3.1, and 25.8 ± 2.0% inhibition of oxidation, respectively. The most striking activity was noted for compound **20**, an anthraquinone 2-*O*-malonyl-(1→6)-β-D-glucopyranoside, which inhibited oxidation by 99.9 ± 2.0%. This represents an approximate 58% increase in activity compared to compound **19**, which lacks a 6′-malonyl moiety. The reason for the pronounced difference in activity between these two compounds remains unclear.

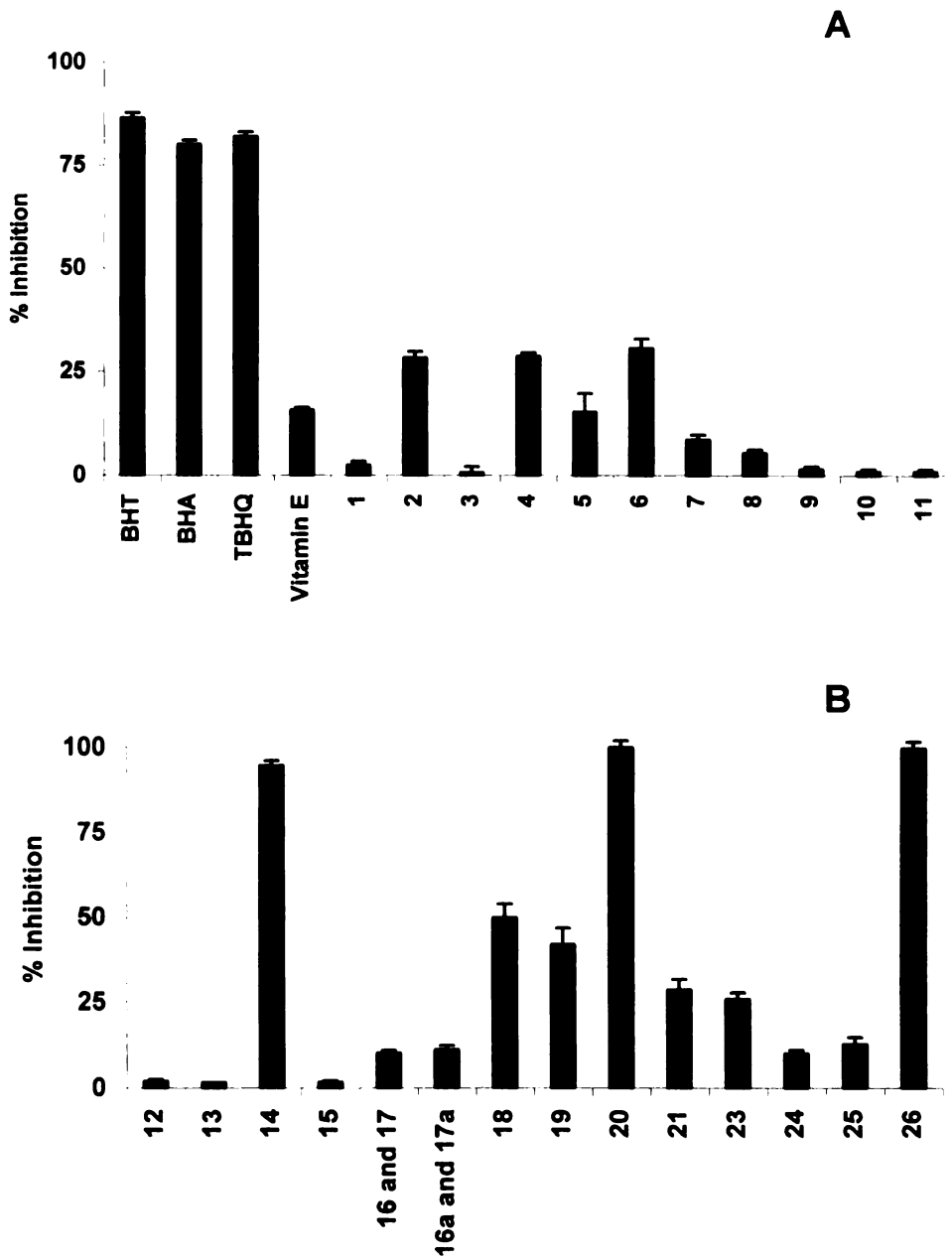

Figure 4.1. Inhibition of LUV phospholipid oxidation by synthetic antioxidants (panel A) and compounds **1-11** (panel A) and compounds **12-21** and **23-26** (panel B). Compounds were tested in triplicate at 10 μM (except the mixtures of **16-17** and **16a-17a** at 10 μg/mL). Results are expressed as the mean percent inhibition ± one standard deviation.

Several of the flavonol 3-*O*-glycosides in which quercetin represented the aglycon moiety, such as compounds, **2**, **4**, and **6**, exhibited more modest antioxidant effects with 28.2 ± 1.5, 28.6 ± 0.8, and 31 ± 2.3% inhibition, respectively. In comparison, the flavonol 3-*O*-glycosides that possessed a kaempferol or isorhamnetin aglycon moiety generally exhibited lower antioxidant inhibitory effects at the same concentration. Our results are in agreement with previously published studies demonstrating that substitutions to the B-ring of flavonoids, such as the hydroxyl substituents at C-3′, 4′ in quercetin, make this flavonol a more effective antioxidant than kaempferol (C-4′ hydroxyl) or isorhamnetin (C-3′ methoxy and C-4′ hydroxyl) due to their comparatively hindered ability to chelate metal ions (Arora et al., 1998; Rice-Evans, 1999).

Phenolic compounds are highly regarded for their important dietary roles as chemopreventive agents (Bravo, 1998). The noted beneficial effects of these bioactive compounds are mitigated in part by means of their antioxidant effects as free radical scavengers or metal ion chelators (Arora et al., 1998; Rice-Evans et al., 1997; Gordon and Roedig-Penman, 1999). Today, in vivo oxidative events are widely recognized as factors affecting the onset and progression of various diseases such as cancer, arteriosclerosis, and neural degenerative disorders (Bland, 1995). In light of the complex array of phenolic compounds observed in daylily flowers, in addition to the host of antioxidant carotenoids present in these tissues, it can be conjectured that the dietary consumption of *Hemerocallis* flowers may convey a variety of beneficial chemopreventive effects to humans.

Mosquitocidal Activity. Compounds **16-26**, including **16a** and **17a**, were tested their activity against fourth instar *A. aegyptii* larvae. Compounds **18**, **19**, and **21** exhibited mosquitocidal activity at 50 μg/mL (Figure 4.2). Within 15-30 min of exposure to compounds **18**, **19**, and **21**, the mosquito larvae began to exhibit a darkening of the gastric caeca and Malpighian tubules. After approximately 1 h, the midgut region and anus were also darkened. However, after 12-24 h the darkening of the midgut was greatly reduced in size and intensity. The significance of these observations remains unclear. The active compounds were further tested at 25 and 12.5 μg/mL (Figure 4.3). At 25 mg/mL, compound **18** (71.5 ± 5.6% mortality) exhibited the greatest activity, followed by compounds **21** and **19** (42.3 ± 6.1and 24.1 ± 7.2% mortality, respectively). Post-mortem dissection of the larvae treated with compounds **18**, **19**, and **21** revealed an enlargement of the still darkened gastric caeca and a general dissolution of the alimentary canal.

Schistosome Inhibitory Activity. Schistosomiasis is a disease caused by parasitic digenetic trematodes of the genus *Schistosoma*. The World Health Organization estimates that *Schistosoma* species currently infect 200 million people, while another 600 million are at risk (Chitsulo et al., 2000). A large number of schistosomes are known; however, only five appear to be primarily responsible for human infections. These include *Schistosoma haematobium*, *Schistosoma intercalatum*, *Schistosoma japonicum*, *Schistosoma mansoni*, and

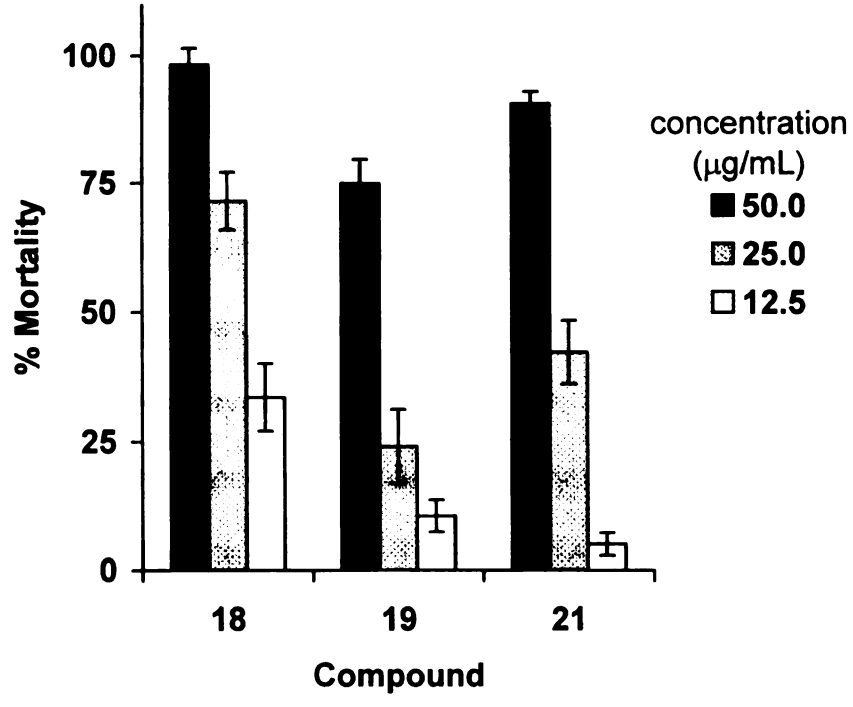

Figure 4.2. Dose-response effect of 2-hydroxychrysophanol (**18**), kwanzoquinone C (**19**), and kwanzoquinone E (**21**) on fourth instar *A. aegyptii* larvae mortality. Results are expressed as the percent mortality ± one standard deviation of the larvae following 24 h of incubation with test compounds at three different concentrations (μg/mL). Experiments were performed with replicates (*n*=5) of test tubes containing 10-15 larvae.

Schistosoma mekongi (Elliot, 1996; Schafer and Hale, 2001). Schistosomes pass through a complex life-cycle (Figure 4.3) in which free-swimming cercariae emerge from their intermediate freshwater snail hosts and infect humans by attaching to the skin via mucus secretions. Cercariae then penetrate the skin by releasing proteolytic enzymes (McKerrow and Salter, 2002). Concurrently, the cercariae shed their tails and transform into schistosomula that enter the venous vascular system where they are carried to the heart and lungs, before reaching the systemic circulation. Ultimately, the schistosomula arrive at the liver where they grow into sexually mature adults. Male and female adults form copulating pairs in the portal venous system. Later, they migrate to the mesenteric or vesical veins depending on the specific species of schistosome, and begin laying eggs for a period of typically 3 to 5 years. The eggs evoke a host immune response that results in the formation of granulomas leading to fibrosis and the sequelae of clinical manifestations (Bica et al., 2000; Elliot, 1996; Morris and Knauer, 1997; Schafer and Hale, 2001). These clinical manifestations of schistosomiasis may include bloody diarrhea and hematuria, portal and pulmonary hypertension, hepatosplenomegaly, and death. There are limited options available for the chemotherapeutic treatment for *Schistosoma* infections with the drug-of-choice being the pyrazionoisoquinoline, praziquantel (Elliot, 1996). Unfortunately, the long-term, worldwide application of the drug coupled with the recent discovery of praziquantel-tolerant schistosomes has generated concern over the development of drug-resistant *Schistosoma* strains (Cioli, 1998, 2000; William et al., 2001).

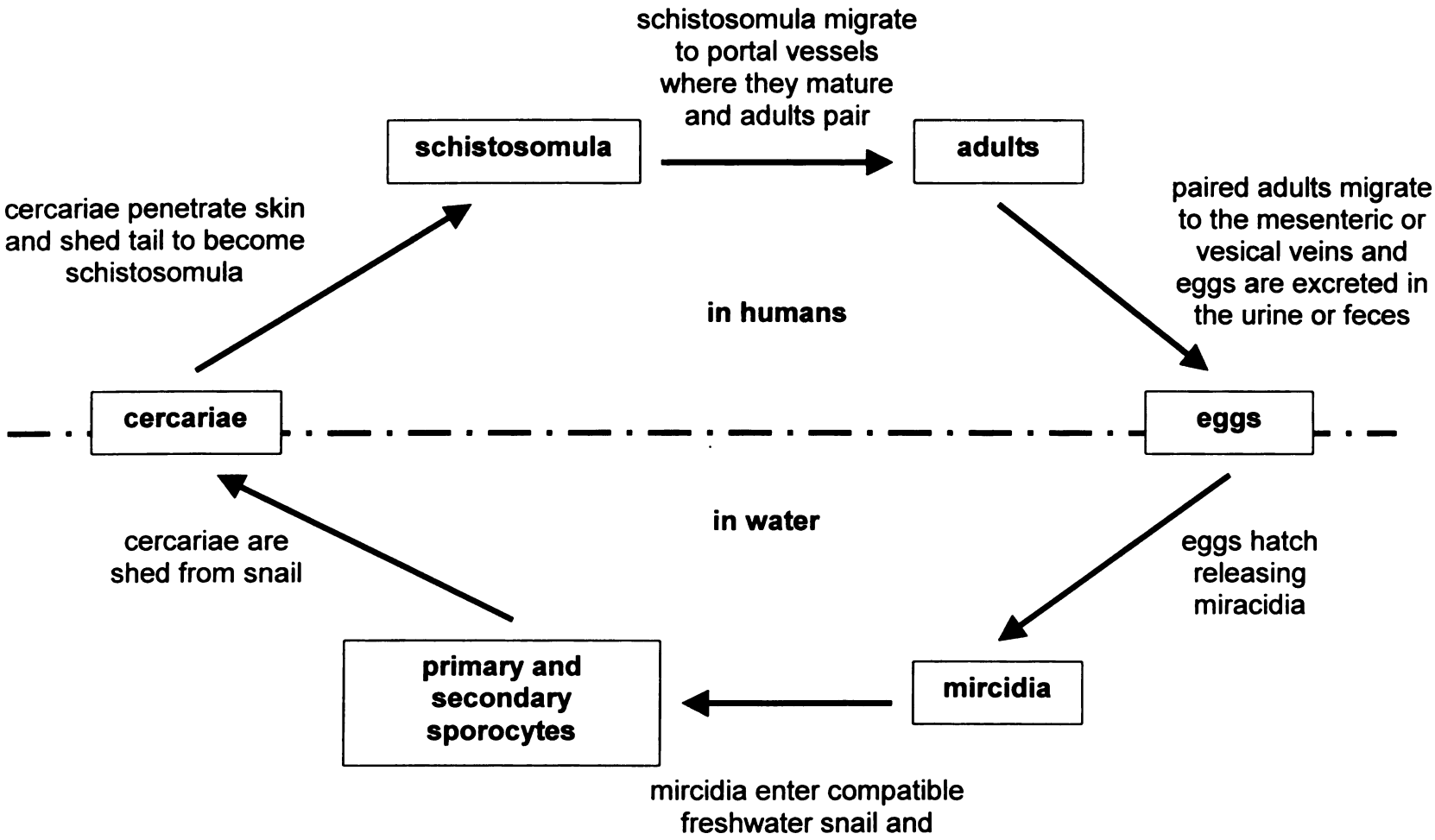

Figure 4.3. Schistosome life-cylce.

With few other options available for combating schistosomiasis, there is an urgent need to develop new methodologies for the treatment and prevention of *Schistosoma* infections (Cioli, 1998, 2000).

Daylily roots (*Hemerocallis* spp., Hemerocallidaceae) have been used in Asia to treat schistosomiasis (Shiao et al., 1962a; Shiao et al., 1962b). However, this method of treatment has been disfavored due to a host of toxic side effects and deaths associated with the administration of *Hemerocallis* root extracts to humans (Wang et al., 1989). Previous efforts to identify the active constituents responsible for the therapeutic properties of *Hemerocallis* roots led to the isolation of a neurotoxic binaphthalenetetrol known as stypandrol (Wang and Yang, 1993) which had been shown to cause paralysis, blindness and death in mammals (Main et al., 1981; Colegate et al., 1985). In another report (Chen et al., 1962), researchers obtained a yellow powdery isolate to which was ascribed both the biological activity against schistosomes, as well as the toxic side effects associated with the use of *Hemerocallis* roots; however, its structure was never identified. While other studies have described additional compounds found in daylilies, none of these efforts have addressed the need to fully characterize the bioactive anti-schistosome chemical constituents from *Hemerocallis* roots.

Compounds **16-26**, including **16a** and **17a**, were tested in vitro for their activity against multiple life-stages (cercariae, schistosomula, adult) of the human pathogenic trematode *S. mansoni*. At a concentration of 25 μg/mL, compounds **18** (2-hydroxychrysophanol) and **21** (kwanzoquinone E) exhibited significant activity by completely immobilizing all cercariae within 15 s and 14 min,

respectively. The dose-response effect of these compounds is shown in Figure 4.4. The potency of compound **18** was not diminished even when diluted to a concentration of 3.1 μg/mL. After 30 min of exposure to test compounds, the test solution was removed and replaced with fresh medium. Cercariae treated with compound **18** exhibited 80% mortality after 24 h while those exposed to compound **21** were all dead. None of the other compounds isolated from *H. fulva* roots, including the glycosides of compounds **18** and **21**, compounds **19** and **22**, respectively, exhibited any activity at 25 μg/mL. The adult worms were also immobilized within 16 h by compounds **18** and **21** at 50 μg/mL. Following removal of the compounds, 35 and 55% of the adults exposed to compounds **18** and **21**, respectively, were dead. In contrast to the effects on the cercariae and adults, the intermediate schistosomula stage was refractory to all compounds at 25 μg/mL. Based on these promising results, compounds **18** and **21** are being investigated further in order to determine their mode of action and for potential development as topical anti-cercarial agents for the prevention of *Schistosoma* infection.

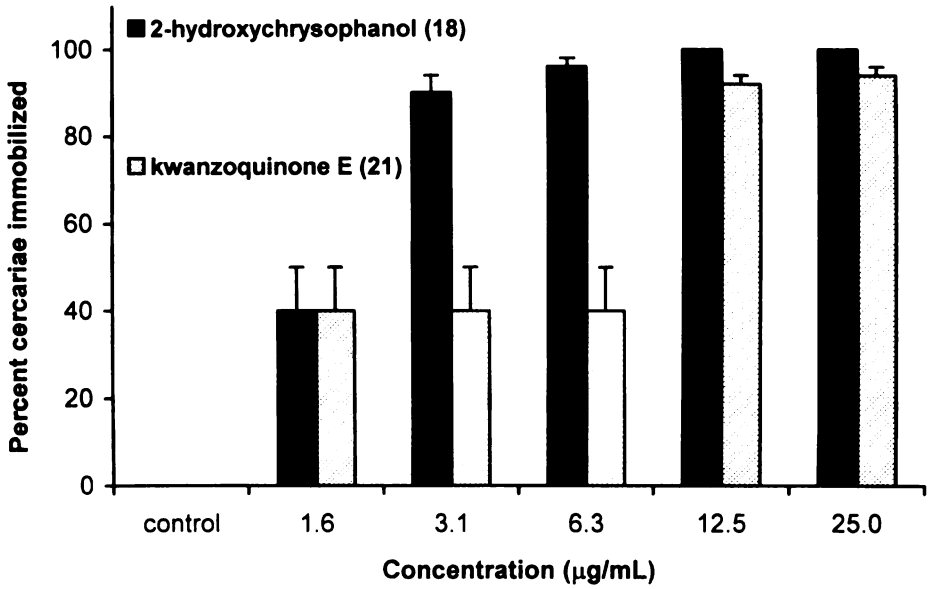

Figure 4.4. Dose-response effect of 2-hydroxychrysophanol (**18**) and kwanzoquinone E (**21**) on *S. mansoni* cercariae mobility. Motility was accessed based on the movement and swimming behavior of the invasive aquatic larval stage 10 min after the addition of test compound. Data are expressed as the mean ± one standard deviation of the percent of immobilized cercariae (n=10).

CHAPTER FIVE

SUMMARY AND CONCLUSIONS

Daylilies (*Hemerocallis* spp.) have been used for thousands of years in eastern Asia as an important food item and medicinal agent for the treatment of a host of diseases. Yet, despite a long and rich history of use, very little was known about the bioactive components that are present in this plant. In **Chapter One**, a summary of all known research conducted prior to these studies regarding the chemistry and pharmacological activity of *Hemerocallis* spp. was presented. Based on this review, it was determined that daylilies should be investigated as a source of new bioactive compounds for the treatment of a variety of diseases.

The flowers of *Hemerocallis* cv. Stella de Oro were subjected to a series of extraction and isolation procedures that led to the procurement of fifteen compounds that were tested for biological activity. The compounds obtained from the flowers are presented in **Chapter Two**. These compounds included kaempferol, quercetin, and isorhamnetin 3-*O*-glycosides (**1-9**), phenethyl β-D-glucopyranoside (**10**), orcinol β-D-glucopyranoside (**11**), phloretin 2′-*O*-β-D-glucopyranoside (**12**), phloretin 2′-*O*-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside (**13**), a new napthalene-glycoside, stelladerol (**14**), and an amino acid (longitubanine A) (**15**). The structures of these compounds were determined based on thorough spectral and physical analyses including UV, MS,

and NMR experiments. This is the first report of these compounds as components of edible daylily flowers.

It had been widely reported that daylily roots were used throughout Asia as a traditional treatment for schistosomiasis. However, the active components responsible for the root's therapeutic properties had never been fully characterized. The results of an isolation study performed on *H. fulva* 'Kwanzo' roots are presented in **Chapter Three**. As a consequence of this work, a number of compounds were discovered in daylily roots including seven new anthraquinones, kwanzoquinones A (**16**), B (**17**), C (**19**), D (**20**), E (**21**), F (**22**), and G (**24**), two known anthraquinones, 2-hydroxychrysophanol (**18**) and rhein (**23**), one new naphthalene glycoside, 5-hydroxydianellin (**26**), one known naphthalene glycoside, dianellin (**25**), one known flavone, 6-methylluteolin (**27**), and α-tocopherol. The structures of the compounds were elucidated by a combination of spectroscopic and chemical methods. All of these compounds are reported here for the first time as components of the daylily roots.

The compounds obtained from the flowers and roots of daylilies were subjected to a variety of bioassays in order to determine their potential as new anticancer, antioxidant, cyclooxygenase inhibitory, mosquitocidal, nematocidal, schistosome inhibitory, and topoisomerase inhibitory agents. The results of these studies are presented in **Chapter Four**. Based on this work, several compounds reported in **Chapters Two** and **Three** exhibited promising cancer cell growth inhibitory, antioxidant, mosquitocidal, and schistosome inhibitory activities. Kwanzoquinones A (**16**), B (**17**), C (**19**), E (**21**), and kwanzoquinone A

and B monoacetates (**16a** and **17a**, respectively) and the known anthraquinones, 2-hydroxychrysophanol (**18**) and rhein (**23**), exhibited cancer cell growth inhibitory properties against a panel of four human tumor cell lines. Additional studies are currently underway in order to further characterize their mode of action. Three new compounds exhibited strong antioxidant properties in this investigation. These compounds were stelladerol (**14**), kwanzoquinone D (**20**), and 5-hydroxydianellin (**26**). All three compounds inhibited lipid oxidation by more than 90%. Three compounds exhibited mosquitocidal properties. Compounds **18**, **19**, and **21** were found to induce mortality in fourth instar *A. aegyptii* larvae. Two compounds were discovered to inhibit schistosome activity. 2-Hydroxychrysophanol (**18**) and kwanzoquinone E (**21**) were found to inhibit the motility and induce mortality in *S. mansoni* cercariae and adults. Both of these compounds are being further investigated in order to determine their mode of action and for development as potential agents for prevention and treatment of schistosomiasis.

Together, these studies have significantly expanded the available body of information regarding the chemical composition and phytoceutical potential of *Hemerocallis* flowers and roots. Both the flowers and roots are widely consumed in eastern Asia as a medicinal agent and component of traditional cuisine. A variety of new and known compounds were reported here for the first time as components of the edible flowers and roots of *Hemerocallis*. Several of these compounds can serve as models for the development of new anticancer, antioxidant, mosquitocidal, and schistosome inhibitory agents. The schistosome

inhibitory properties of compounds **18** and **21** are particularly intriguing. At this time, more that 200 million people worldwide are infected by *Schistosoma* spp. Recently, signs of schistosomes resistant to praziquantel, have emerged underscoring the urgent need for the creation of new agents for the treatment of schistosomiasis. It is hoped that compounds **18** and **21** will provide new avenues for the development of a novel, safe, and effective treatment for schistosomiasis.

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