

121693
183
21/07

CHEMISTRY AND IMMUNOMODULATORY PROPERTIES
OF AEGLE MARMELLOS, L. CORREA.

BY

PATHMASIRI ANANDA KUMARA SENEVIRATNE

B.Sc. (SPECIAL), HONS.

THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY OF
THE FACULTY OF APPLIED SCIENCE, UNIVERSITY OF SRI
JAYEWARDENEPURA, NUGEGODA, SRI LANKA.

MAY, 1992

121693

TABLE OF CONTENTS	PAGE
TABLE OF CONTENTS	i
LIST OF TABLES	vii
LIST OF FIGURES	xii
ACKNOWLEDGEMENTS	xviii
ABBREVIATIONS	xxi
ABSTRACT	xxiii
CHAPTER 1. INTRODUCTION	1
1.1. Ayurvedic principles and introduction to chemical and immunological studies.	1
1.2. The complement system and polymorphonuclear leucocytes.	4
1.2.1. Immunological activity test models.	
1.3. Ethnopharmacognostic survey of <u>Aegle marmelos</u> , L. Correa including critical evaluation of the field survey.	17
1.3.1. Field survey.	
1.3.1.1. Scope.	
1.3.1.2. Strategy.	
1.3.1.3. Questionnaire.	
1.3.2. Vernacular names.	

1.3.3.	Botany.	
1.3.4.	Ethnobotany.	
1.3.5.	Chemistry.	
1.3.6.	Pharmacology.	
1.3.7.	Ethnopharmacology.	
1.3.8.	Biological activity.	
1.3.9.	Toxicology.	
1.3.10.	Fruit ripening changes.	
1.4.	Outline of the thesis.	88
CHAPTER 2.	RESULTS AND DISCUSSION	90
2.1.	Anticomplementary activities and inhibition of Chemiluminescence generated by polymorphonuclear leucocytes of various fractions of the unripe fruit.	90
2.2.	Polysaccharides.	91
2.2.1.	Activity guided fractionation and isolation.	
2.2.2.	Chemical characterization	
2.2.3.	Mechanistic studies.	
2.2.4.	In vivo studies for immunological adjuvant activity.	
2.3.	Proanthocyanidin.	131
2.3.1.	Activity guided fractionation and isolation.	

- 2.3.2. Characterization and structure elucidation
- 2.3.3. Mechanistic studies.
- 2.3.4. Inhibition of Chemiluminescence.
- 2.3.5. Immunomechanistic study for the scavenging activity.
- 2.3.6. Immunomechanistic study for the cytotoxicity.
- 2.4. Activity guided fractionation and isolation of low molecular weight compounds on chemiluminescence assay. **169**
 - 2.4.1. Characterization and structure elucidation
 - 2.4.2. Immunomechanistic study for the scavenging activity.
 - 2.4.3. Immunomechanistic study for the cytotoxicity.

- CHAPTER 3. MATERIALS AND METHODS **191**
 - 3.1. Instruments and Apparatus. **191**
 - 3.2. Immunological assays. **192**
 - 3.2.1. Buffers and reagents for haemolytic complement assays.
 - 3.2.2. Microtitre haemolytic complement assay.
 - 3.2.3. Luminol dependent chemiluminescence assay.

3.2.3.1. Buffers and reagents for chemiluminescence assay.

3.2.3.2. Chemiluminescence assay for phagocytic activity.

3.3. Isolation and Characterization.

202

3.3.1. Plant material and extraction.

3.3.2. Activity guided fractionation and isolation of crude aqueous extract.

3.3.3. Purification and isolation of polysaccharides from the crude aqueous extract.

3.3.3.1. Chemical characterization of polysaccharide.

3.3.3.2. Anticomplementary activity of polysaccharide.

3.3.3.3. Mechanistic complement assays to study the mode of action of polysaccharide.

3.3.3.4. In vivo assays of polysaccharide for the adjuvant activity.

3.3.4. Purification and isolation of proanthocyanidins.

3.3.4.1. Chemical characterization of proanthocyanidin, SE-1.

- 3.3.4.2. Haemolytic complement assays of proanthocyanidin.
 - 3.3.4.3. Mechanistic complement assays of proanthocyanidin.
 - 3.3.4.4. Inhibition of luminol dependent chemiluminescence of proanthocyanidin.
 - 3.3.4.5. Immunomechanistic assay for the scavenging activity.
 - 3.3.4.6. Immunomechanistic assay for the cytotoxicity.
- 3.3.5. Activity guided fractionation and isolation of low molecular weight compounds.
- 3.3.5.1. Inhibition of luminol dependent chemiluminescence of low molecular weight constituents.
 - 3.3.5.2. Immunomechanistic assay for the scavenging activity.
 - 3.3.5.3. Immunomechanistic assay for the cytotoxicity.

CHAPTER 4.	GENERAL DISCUSSION	255
4.1.	Conclusions	256
4.2.	Future work	261
REFERENCES		263

LIST OF TABLES.

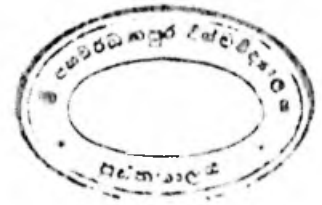
TABLE.	PAGE
1.1. Net chemical reactions of the formation and conversion of reactive oxygen species (ROS) during the phagocytosis.	15
1.2. Yield percentage of some coumarins	40
1.3. Coumarins of <u>Aegle marmelos</u> .	41
1.4. Alkaloids of <u>Aegle marmelos</u> .	52
1.5. Steroids of <u>Aegle marmelos</u> .	50
1.6. Terpenes of <u>Aegle marmelos</u> .	57
1.7. Synthetic mixture composition of Bael fruit flavor.	60
1.8. Amino acids in Bael fruit.	68
1.9. Mineral nutrient content of Bael fruit.	70
1.10. Pharmacologically active phytochemicals of <u>Aegle marmelos</u> .	70
1.11. Prepared drugs and their uses in indigenous medicine.	76
1.12. Comparative study of various parts and their uses in ayurvedic medicine.	77
2.1. Inhibitory effects of different fractions on complement mediated haemolysis and on luminol dependent chemiluminescence by PMNL.	91

TABLE.	PAGE
2.2. Anticomplementary activities of various polysaccharide sub fractions on CP and AP complement assays.	93
2.3. Anticomplementary activities of SA-12 polysaccharide sub fraction on CP and AP complement assays.	93
2.4. Retention times and peak areas of GL chromatogram of sugars of SA-12a-1.	105
2.5. AP anticomplementary activities of SA-12a-1, Dx.S. and zymosan.	108
2.6. Effect of Mg^{2+} concentration on the AP complement activity of polysaccharides SA-12A-1 .	112
2.7. Anticomplementary and chemiluminescence activities of the proanthocyanidin fraction.	132
2.8. The wave lengths at the absorption maxima of reference anthocyanidins and the sample.	141
2.9. ^{13}C - NMR spectral data of proanthocyanidin polymer SE-1.	155
2.10. Anticomplementary activities of SE-1 in various Ca^{2+} concentrations.	164
2.11. The influence of proanthocyanidin on scavenging of O_2^- generated in a cell free system.	168

TABLE	PAGE
2.12. Cytotoxic effects of proanthocyanidin SE-1 on CL _{lum} generated by PMNL.	169
2.13. Inhibitory activities of two low molecular weight fractions	170
2.14. Inhibition of Chemiluminescence by sub fractions of fraction F.	172
2.15. Inhibition of Chemiluminescence by sub fractions of fraction 5.	173
2.16. TLC analysis results and inhibitory activities on CL _{lum} of pure compounds.	174
2.17. Inhibition of Chemiluminescence by sub fractions of fraction 8.	175
2.18. Inhibition of Chemiluminescence by sub fractions of fraction 3.	176
2.19. TLC analysis results and inhibitory activities of pure compounds on CL _{lum} produced by PMNL.	177
2.20. The influence of isolated compounds on scavenging of O ₂ ⁻ generated in a cell free system.	188
2.21. Cytotoxic effects of isolated compounds on CL ^{lum} generated by PMNL.	190
3.1. Sample concentrations and corresponding lysis percentages of SA-12a-1.	196

TABLE	PAGE
3.2. Inhibition of chemiluminescence of xanthotoxol 200 at various concentrations.	
3.3. Adjuvant activity of SA-12a-1; DTH response (Foot pad swelling in mm) after IP administration.	218
3.4. Adjuvant. activity of SA-12a-1) ; Antibody response (serum titres, -2log dilution) after IP administration.	219
3.5. Adjuvant activity of SA-12a-1; DTH response (Foot pad swelling in mm) after IC administration.	221
3.6. Adjuvant activity of SA-12a-1; Antibody response (serum titres, -2log dilution). after IC administration.	222
3.7. Adjuvant activity of SA-12a-1;DTH response (Foot pad swelling in mm after IP administration of 1,3,10 µg of sample.	224
3.8. Adjuvant activity of Dx.S.;DTH response (Foot pad swelling in mm) after IP administration of 1,3,10 µg of sample.	225
3.9. Adjuvant activity of SA-12a-1; Antibody response (serum titres,-2log dilution) after IP administration.	226

TABLE	PAGE
3.10. Adjuvant activity of Dx.S.;	226
Antibody response (serum titres, -2 log dilution) after IP administration	
3.11. Body weights of mice (in g) used for Experiment 1.	227
4.1. Yield percentage and the activity of isolated pure compounds.	259



LIST OF FIGURES

FIGURE	PAGE
1.1. Classical and alterantive complement pathways.	5
1.2. Activation of the complement cascade via the alternative and classical pathways.	7
1.3. Schematic representation of phagocytosis.	13
1.4. Structural formulae of simple coumarins of <u>A. marmelos</u> .	42
1.5. Structural formulae of furano coumarins of <u>A. marmelos</u> .	43
1.6. Structural formulae of pyrano coumarins of <u>A. marmelos</u> .	44
1.7. Some of the proposed Biosynthetic pathways of coumarins.	46
1.8. Structural formulae of rutin (quercetin-3- -rutinoside).	49
1.9. Structural formulae of furanoquinoline alkaloids of <u>A. marmelos</u> .	53
1.10. Structural formulae of phenyl ethyl amine alkaloids of <u>A. marmelos</u> .	54
1.11. Structural formulae of 5-phenyloxazole alkaloids of <u>A. marmelos</u> .	54
1.12. Structural formulae of steroids of <u>A. marmelos</u> .	56

FIGURE.	PAGE
1.13. The average repeating unit of the degraded gum polysaccharide.	63
1.14. The average repeating unit of the polysaccharide of the whole gum.	64
1.15. Possible partial structure for the carbohydrate moiety of glycoprotein.	66
2.1. Fractionation of Polysaccharide SA-12. UV 220 nm elution profile of the fractions obtained from Sepharose CL-2B column.	95
2.2. Inhibition of AP haemolysis by the fractions collected from Sepharose CL-2B column of SA-12.	96
2.3. Fractionation of Polysaccharide SA-12a. UV 220 nm elution profile of the fractions obtained from Sepharose CL-2B (with SDS) column.	97
2.4. AP anticomplementary activities of various SA-12 polysaccharide fractions.	98
2.5. AP anticomplementary activities of various polysaccharide fractions.	99
2.6. CP anticomplementary activities of various polysaccharide fractions.	100
2.7. Gas liquid chromatogram of trimethylsilylated Monosaccharides from SA-12a-1	104

FIGURE.	PAGE
2.8. AP anticomplementary activity of SA-12a-1 with two known AP active agents.	107
2.9. Effects of preincubation condition on inhibitory activity of SA-12a-1.	110
2.10. Effect of SA-12a-1 on Ra.E. in AP complement assay.	111
2.11. Effect of Mg^{2+} concentration on AP inhibitory activity of SA-12a-1.	113
2.12. Effect of C3 on AP anticomplementary activity of SA-12a-1. (Absorbance at 405 nm).	116
2.13. Effect of factor B on AP anticomplementary activity of SA-12a-1. (Absorbance at 405 nm).	117
2.14. Adjuvant activity of SA-12a-1. DTH response (Foot pad swelling in mm) after intraperitoneal administration.	121
2.15. Adjuvant activity of SA-12a-1. DTH response (Foot pad swelling in mm) after intracutaneous administration.	122
2.16. Adjuvant activity of SA-12a-1. Antibody response (Serum titres, -2log dilution) after intraperitoneal administration.	123

FIGURE	Page
2.17. Adjuvant activity of SA-12a-1. Antibody response (Serum titres, -2log dilution) after intracutaneous administration.	124
2.18. Adjuvant activity of SA-12a-1 and Dx.S.. DTH response (Foot pad swelling in mm) after intraperitoneal administration.	127
2.19. Adjuvant activity of SA-12a-1 and Dx.S. Antibody response (Serum titres, -2log dilution) after intraperitoneal administration.	128
2.20. 254 nm elution profile of fraction E on Sephadex LH - 20.	133
2.21. Acid catalysed decomposition of the dimeric propelargonidin.	136
2.22. Diagramatic representation of TLC profiles of proanthocyanidin hydrolysate.	139
2.23. UV - visible absorption spectra of SE-1 with reference pelargonidin chloride.	142
2.24. UV - visible absorption spectra of SE-1 with reference anthocyanidins.	143
2.25. UV - visible absorption spectra of the hydrolysate of SE-1 in the presence and absence of $AlCl_3$.	144

FIGURE	PAGE
2.26. Chemical degradation of proanthocyanidin polymer using toluene- α -thiol.	146
2.27. Calibration curve for determination of \overline{MP} of the proanthocyanidin polymer SE-1.	149
2.28. ^{13}C - NMR spectrum of proanthocyanidin SE-1.	152
2.29. ^1H - NMR spectrum of proanthocyanidin SE-1.	153
2.30. Tentative structure of the proanthocyanidin polymer SE-1.	157
2.31. Effect of preincubation condition on the inhibitory activity of SE-1.	160
2.32. The influence of proanthocyanidin on C1.	161
2.33. The influence of proanthocyanidin on C4.	161
2.34. The influence of proanthocyanidin on C2.	163
2.35. Effect of preincubation of proanthocyanidin with Sh.EA. on CP activation.	163
2.36. Effect of Ca^{2+} concentration on CP anticomplementary activity of SE-1.	165
2.37. Structural formulae of isolated compounds from ethyl acetate fraction.	180
2.38. ^1H - NMR spectrum of An-3.	182
2.39. ^1H - NMR spectrum of An-6.	183
2.40. Structural formulae of some $\text{CL}_{1\text{um}}$ active phenolic compounds.	186

FIGURE	PAGE
3.1. Inhibition of AP complement activation (Absorbance at 405 nm)by SA-12a-1 polysaccharide fraction.	195
3.2. The graph of Lysis percentage vs Concentration of test compound and determination of IC ₅₀ value of SA-12a-1.	197
3.3. Inhibition of Luminol dependent chemiluminescence by xanthoxol at different concentrations.	201
3.4. Activity guided fractionation and isolation scheme of <u>A. marmelos</u> crude extract.	203
3.5. Fractionation of crude polysaccharides using ultrafiltration membranes.	205
3.6. Purification and isolation of proanthocyanidin fraction.	228
3.7. Activity guided fractionation and isolation scheme of low molecular weight constituents.	242

ACKNOWLEDGEMENT

I wish to express my profound gratitude to Professor K.T.D.de Silva, former Director of Sri Lankan team of Sri Lanka-Netherlands Medicinal Plant project and former Dean and Professor of Chemistry, Faculty of Applied Science, University of Sri Jayewardenepura for giving me this opportunity to work under this Research Programme for my Ph.D. degree.

I express my gratitude to Professor.A.M.Abeyssekera, Present Director of Sri Lankan team of Sri Lanka-Netherlands Medicinal plant project and Associate Professor, Department of Chemistry, University of Sri Jayewardenepura for his encouragement, constructive criticism and valuable suggestions made, through out the research project.

I wish to express my heartfelt thanks to my supervisor Dr.(Mrs) S.Samarasinghe, Senior Lecturer, Department of Chemistry, University of Sri Jayewardenepura for her invaluable advice, encouragement and continuous guidance given to me during the past four years.

It is my obligation to extend my gratitude to Professor dr.R.P.Labadie, Director of the Netherlands team of Sri Lanka-Netherlands Medicinal plant research project and Head, Department of Pharmacognosy, University of Utrecht, the Netherlands for giving me an opportunity to work at the Department of Pharmacognosy and for his valuble suggestions and continuous guidance during my stay in the Netherlands.

I also wish to thank Dr.A.J.J.Van den Berg for his methodical guidance, supervision and valuble criticism. My grateful thanks are also due to Dr.W.G.Van der Sluis for obtaining spectroscopic data and for his immense help in elucidating the structures of the compounds.

I extend my thanks to Dr.H.Van dijk, Section of Experimental Immunology, Laboratory of Microbiology, Medical Faculty of the State University of Utrecht, the Netherlands for his kind assistance and guidance for the *in vivo* assays.

I appreciate very much the invaluble help given to me by Miss Linda Quarles Van ufford, Department of Pharmacognosy, University of Utrecht, the Netherlands especially carrying out the *in vivo* assays.

I express my deep appreciation to the Ministry of Development co-operation, the Netherlands for financing this project as this Ph.D. programme was carried out under this project.

I take this opportunity to thank Drs. Stephen Horsten, Dr. Burt kroes, Drs. France Hofhuis and all other members of the Department of Pharmacognosy for their hospitality and assistance given to me during my stay in the Netherlands.

I also wish to thank Professor. A. Bamunuarachchi, Professor. W. S. Fernando and all other staff members of the Department of Chemistry, University of Sri Jayewardenepura for their support and encouragement during the last few years. I wish to thank Mr. Srilal Rangoda for his assistance to carry out my laboratory work during this research project.

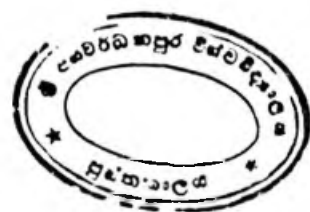
I heartily thank my mother and brothers for their encouragement and all the help given to me during my long period of study. Finally I would like to thank my wife Dhehinie for her patience, kindness and encouragement during the preparation of this thesis.

I DEDICATE THIS THESIS TO MY PARENTS.

ABBREVIATIONS

AP	Alternative pathway
CL _{lum}	Luminol dependent chemiluminescence
CP	Classical pathway
DTH	Delayed type hypersensitivity
Dx.S	Dextran sulphate
EGTA-VB	Ethylene bis(oxyethylenitrilo)tetraacetic acid-supplemented vermol saline buffer containing 2.5mM of Mg ²⁺ .
FAB-MS	Fast atom bombardment - Mass spectroscopy
GC-MS	Gas chromatography - Mass spectroscopy
GLC	Gas liquid chromatography
GPC	Gel permeation chromatography
HBSS	Hank's balanced salt solution
HPLC	High performance liquid chromatography
HPS	Human pooled serum
HX	Hypoxanthine
IC ₅₀	50% Inhibition concentration
IC	Intra cutaneous
IP	Intra peritoneal
I.STD.	Internal standard
MAC	Membrane attack complex
MAF	Molar adjustment factor
\bar{M}_n	Number average molecular weight

\bar{M}_p	Peak average molecular weight
MPO	Myeloperoxidase
NMR	Nuclear magnetic resonance
PBS	Posphate buffered saline
PMNL	Polymorphonuclear leucocytes
Ra.E.	Rabbit erythrocytes
RF	Response factor
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
Sh.E	Sheep erythrocytes
SOD	Superoxide dismutase
STZ	Serum treated zymosan
TFA	Tri fluoro acetic acid
THF	Tetra hydro furan
TLC	Thin layer chromatography
UV	Ultra violet
VSB	Vernol saline buffer containing 0.5 mM Mg ²⁺ and 0.15 mM Ca ²⁺
XO	Xanthine oxidase



CHEMISTRY AND IMMUNOMODULATORY PROPERTIES OF AEGLE
MARMELOS, L. CORREA.

Pathmasiri Ananda Kumara Seneviratne

ABSTRACT

In the initial screening of immunomodulating compounds from Sri Lankan medicinal plants, it was pronounced that Aegle marmelos was one of the most effective plants on our test models. At the initial stage of this research programme a three month field survey was carried out and the various medicinal uses, applications and prepared drugs of A. marmelos were collected. These ethnopharmacological results collected from both field work and literature survey are described in Chapter 1.

The broad range of application of the unripe fruit in Ayurveda and in other indigenous systems of medicine lead us to carry out investigations on the unripe fruit. In the experimental immunopharmacognostic phase, immunomodulatory compounds were isolated, purified and characterized from the aqueous extract, through action guided fractionation procedures.

In this thesis, the chemistry and the immunomodulatory properties of isolated compounds are discussed. The results described in this dissertation refer to activities found on human complement activation and on Polymorphonuclear leucocytes (PMNL) activation.

Two polymeric compounds namely, a polysaccharide and a proanthocyanidin were isolated from the aqueous extract and the polysaccharide showed potent alternative pathway (AP) anticomplementary activity while the latter showed classical pathway (CP) anticomplementary activity. Further, mechanistic complement assays revealed that the anticomplementary activities are not due to the chelation of bivalent ions but due to the enzymatic activity related complement consumption. It is much interesting to report that the polysaccharide showed potent immunological adjuvant activity on intraperitoneal administration to mice. Both polymers were characterized using various chromatographic and spectroscopic methods of analysis. These results suggested that the structure of the proanthocyanidin is more likely to be a new compound. The proanthocyanidin was a polymer of afzelechin (as the monomer unit) with -C- glucosyl moieties.

The PMNL are particularly involved in acute inflammatory responses and are able to perform phagocytosis. The ethyl acetate fraction which was obtained by partitioning the aqueous extract, showed strong inhibition of luminol dependent chemiluminescence (CL_{lum}) produced by zymosan activated human polymorphonuclear leucocytes (PMNL). CL_{lum} is measured by means of a luminometer. The ethyl acetate fraction was subjected to further fractionation and purification based on the CL_{lum} activity. Various chromatographic methods were used and consequently, five low molecular weight constituents were isolated. Four out of five constituents were coumarins and the other was a flavonoid compound. Two coumarins, namely xanthotoxol and scopoletin, showed strong CL_{lum} activity. The flavonoid namely rutin (Quercetin-3-rutinoside), was not earlier reported from A. marmelos fruit. ¹H-, ¹³C-NMR, GCMS and MS spectroscopic data of the pure compounds were used for their characterization and structure elucidation.

The mechanistic assays for cytotoxicity and for scavenging of reactive oxygen species were performed as control experiments and results showed that the activity is not due to either the cytotoxicity or scavenging of ROS.

On a weight basis, the proanthocyanidin (0.84 %) and the polysaccharides (0.125 %) isolated from the unripe fruit were found to be the most active immunomodulatory constituents. These two polymeric constituents are the compounds most responsible for the total activity of the aqueous extract of the unripe fruit. Xanthoxol, scopoletin and rutin are the most active compounds in the CL_{lum} assay.