STUDIES ON ANTIHYPERTENSIVE AND ANTICANCER PROPERTIES OF *PERISTROPHE BICALYCULATA* (*RETZ*) NEES EXTRACTS AND THE MOLECULAR BASIS OF THE ACTIVITIES

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DEPARTMENT OF BIOCHEMISTRY

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A DISSERTATION SUBMITTED TO THE POSTGRADUATE SCHOOL, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY

AHMADU BELLO UNIVERSITY,

ZARIA, NIGERIA

JANUARY, 2013

DECLARATION

I hereby declare that the work reported in this dissertation was carried out and written by me under the joint supervision of Dr. Sani Ibrahim, Prof. D.A. Ameh of the Department of Biochemistry, Faculty of Sciences, and Prof. J.O. Ayo of the Department of Physiology and Pharmacology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria. I hereby declare that no part of this work has been presented elsewhere for award of higher degree. The works of other investigators have been acknowledged and referred to accordingly.

Abdulazeez Mansurah

CERTIFICATION

This dissertation entitled "STUDIES ON ANTIHYPERTENSIVE AND ANTICANCER PROPERTIES OF *PERISTROPHE BICALYCULATA* (*RETZ*) NEES EXTRACTS AND THE MOLECULAR BASIS OF THE ACTIVITIES" By Abdulazeez Mansurah meets the regulations governing the award of the degree of Doctor of Philosophy (PhD) of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

To my late parents, Alhaji Mas'ud and Hajiya Hafsat Abdulazeez; and my late grandma, Hajiya Aishatu Tsoho Yakawada; without whose exemplary lifestyles and tremendous contributions, I wouldn't be who I am today. May your souls rest in Jannatul Firdaus, Amin.

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ABSTRACT

In the present study, the antihypertensive, anticancer and melanogenesis inhibitory effects of leaf extracts of *Peristrophe bicalyculata* (*Retz*) Nees, a plant used in South West Nigeria for the treatment of several cardiovascular diseases, including hypertension were investigated. The antioxidant activities of the extract were also investigated. Results of the radical scavenging, tyrosinase inhibition and metal ion chelating activities of extracts of *P. bicalyculata* demonstrated its antioxidant activity. Induction of hypertension in rats with L-NAME significantly ($P < 0.05$) increased systolic blood pressure (from 113 ± 3.71 mm Hg to 212 ± 2.08 mm Hg by the fourth week), which significantly ($P < 0.05$) reduced when rats were given 5 mg/kg captopril (74.00 ± 4.36 mm Hg), the partially-purified extract of *P. bicalyculata* at 2.5 mg/kg (103.33 ± 8.11 mm Hg) and at 25 mg/kg (94 ± 1.73 mm Hg). Hypertension increased the activity of angiotensin converting enzyme (ACE) and decreased that of endothelial nitric oxide synthase significantly ($P < 0.05$) in the serum, kidney and aorta of rats. Treatment with 5 mg/kg captopril, the extract at 2.5 mg/kg and at 25 mg/kg significantly ($P < 0.05$) decreased the activity of angiotensin converting enzyme and increased that of endothelial nitric oxide synthase (eNOS) in the kidney, serum and aorta. At the level of gene expression, ACE mRNA levels increased significantly ($P < 0.05$) in kidneys and aorta of hypertensive rats. This significantly ($P < 0.05$) decreased in the kidneys of rats given 5 mg/kg captopril and 25 mg/kg of the extract, but not in the aorta of these rats. The expression of eNOS, which decreased significantly ($P < 0.05$) in the kidneys and aorta of hypertensive rats, increased significantly in aorta of hypertensive rats given captopril and the extract at both doses. However, treatment did not increase eNOS mRNA levels in the kidneys. In rat aortic endothelial cells, captopril (0.52 ± 0.03) significantly ($P < 0.05$) decreased ACE expression compared to cells treated with 2

mmol/L L-NAME (0.79 ± 0.05 fold). There was no significant difference in ACE mRNA expression between control cells (0.55 ± 0.03), cells treated with captopril and the partially purified extract (0.64 ± 0.08 fold). The eNOS expression in RAOECs treated with L-NAME (0.48 ± 0.03 fold) was significantly ($P<0.05$) lower than control cells (1.71 ± 0.15 fold). This increased significantly ($P < 0.05$) when cells were treated with captopril (1.01 ± 0.10 fold), 10 µg/ml (1.05 ± 0.09 fold) and 100 µg/ml (0.95 ± 0.03 fold) of the purified extract. The levels of thiobarbituric acid-reactive substances present in the serum, kidney and aorta also increased significantly ($P < 0.05$) in hypertensive rats compared to rats in the control group. This reduced significantly ($P < 0.05$) when rats were given captopril, the extract at 2.5 mg/kg and 25mg/kg. The induction of apoptosis by the partially purified anticancer fraction on human mouth epidermal carcinoma (KB) cells was significantly ($P < 0.05$) higher than control cells after 24 and 48 hours. The percentage necrotic cells increased significantly ($P < 0.05$) after 48 hours when compared to 24 hours, showing a dose-dependent effect. Results of the melanogenesis inhibitory effect of the extract showed that the methanolic butanol fraction of methanol extract was capable of inhibiting melanin formation. The GC-MS analysis of the partially-purified antihypertensive extract indicated an abundance of P,P,P-triphenyl-Imino(triphenyl)phosphorane while the partially purified anticancer extract of *Peristrophe bicalyculata* contained andrographolide in abundance. In conclusion, this study has provided scientific evidence that *Peristrophe bicalyculata* reduces blood pressure possibly via the renin angiotensin system by decreasing the expression of ACE mRNA and increasing eNOS expression. It has also demonstrated that the partially-purified anticancer fraction of *Peristrophe bicalyculata* is a potential source of valuable anticancer therapy. This finding has also established the potential use of *Peristrophe bicalyculata* for the treatment of melanoma.

TABLE OF CONTENTS

Content **Page**

TITLE PAGE ---------- i

DECLARATION ---------- ii

CERTIFICATION ---------- iii

DEDICATION ---------- iv

ACKNOWLEDGEMENT ---------- v

ABSTRACT ---------- vii

TABLE OF CONTENTS ---------- ix

LIST OF FIGURES ---------- xvii

LIST OF TABLES ---------- xx

LIST OF PLATES ---------- xxi

LIST OF APPENDICES ----------xxiii

LIST OF ABBREVIATIONS ---------- xxv

CHAPTER ONE ----------1

1.0 Introduction ----------1

1.1 Statements of Research Problem ---------- 4

1.2 Null Hypothesis ---------- 5

1.3 Justification ---------- 5

1.4 Aims and Objectives of Study ----------6

CHAPTER TWO ---------- 8

2.0 Literature Review ---------- 8

2.1 Taxonomy and History of *Peristrophe bicalyculata* ---------- 8

2.2 Uses of *Peristrophe bicalyculata* ---------- 8

2.3 Hypertension ---------- 9

2.3.1 Definition, Classification and Aetiology ---------- 9

2.3.1.1 Definition ---------- 9

2.3.1.2 Classification ---------- 10

2.3.1.3 Aetiology ---------- 11

2.3.2 Treatment of Hypertension ---------- 12

2.3.2.1 Diuretics ---------- 12

2.3.2.2. Sympathoplegic Agents ---------- 13

2.3.2.3 Angiotensin Converting Enzyme Inhibitors ---------- 14

2.3.2.4 Angiotensin II Receptor Blockers ---------- 14

2.3.2.5 Vasodilators ---------- 15

2.3.2.6 Direct Rennin Inhibitors ---------- 15

2.3.3 Experimental Model of Hypertension ---------- 16

2.4 Chronic Nitric Oxide Inhibition-Induced Hypertension ---------- 16

2.4.1 Synthesis of Nitric Oxide ---------- 18

2.4.2 Transport and Mechanisms of Action of Nitric Oxide ---------- 19

2.4.3 Role of RAAS in Nitric Oxide-Induced Hypertension---------- 21

2.4.3.1 Interactions between angiotensin converting enzyme, kinnins and nitric oxid-- 23

2.4.3.2 Effect of angiotensin converting enzyme inhibition on endothelial nitric oxide synthase and nitric oxide ----------24

2.5 Cancer ---------- 26

2.5.1 Definition and Prevalence ---------- 26

2.5.2 Stages of Cancer Development ---------- 27

2.5.2.1 Initiation ----------27

2.5.2.2 Promotion ---------- 28

2.5.2.3 Progression ---------- 28

2.5.3 Risk Factors of Cancer ---------- 30

2.5.3.1 Smoking ---------- 30

2.5.3.2 Infections ---------- 30

2.5.3.3 Dietary-Related Factors ---------- 32

2.5.3.4 Genetic Predisposition ---------- 34

2.5.4 Epidemiology and Pathogenesis of Selected Cancers ---------- 34

2.5.4.1 Hepatocellular Carcinoma ---------- 34

2.5.4.2 Cervical Cancer ---------- *35*

2.5.4.3 Colon Cancer ---------- *36*

2.5.4.4 Mouth Epidermal Carcinoma ---------- *37*

2.6 Hypertension and Cancer ---------- 38

2.6.1 Mediators between Cancer and Hypertension ---------- *38*

2.6.1.1 Nitric Oxide ---------- *38*

2.6.1.2 Rennin Angiotensin System ---------- *40*

2.7 Melanogenesis---------- 42

2.8 Free-radical Scavenging Activity By 2,2-diphenyl-2-picryl-hydrazine ---------- 43

2.9 Metal Iron Chelation ---------- 44

CHAPTER THREE ---------- 45

3.0 Materials and Methods ---------- 45

3.1 Materials ---------- 45

3.1.1 Chemicals and Reagents ---------- 45

3.1.2 Instruments ---------- 45

3.1.3 Plant Material ---------- 46

3.2 Methods ---------- 46

3.2.1 *Preparation and Fractionation of Extracts of Peristrophe bicalyculata* --------- *46*

3.3 Biological Properties of Extracts of *Peristrophe bicalyculata* ---------- *48*

3.3.1 Determination of Total Phenolic and Phytochemial Content ---------- 48

3.3.2 2,2-Diphenyl-2-picryl-hydrazine Radical Scavenging Assay ---------- 48

3.3.3 Tyrosinase Inhibition Assay ---------- 49

3.3.4 Metal Ion Chelating Assay ----------50

3.3.5 Lipid Peroxidation Inhibitory Effect (Ferric Thiocyanate Method) ----------51

3.3.6 Determination of Angiotensin-Converting Enzyme Inhibitory Activity--------- 52

3.4 Bioassay-Directed Isolation of the Antihypertensive Component(s) of *Peristrophe bicalyculata* ---------- 53

3.4.1 Animals ---------- 53

3.4.2 Preparation of P. bicalyculata Extracts for Antihypertensive Screening ------- 53

3.4.3 Preparation of Partially-Purified Fractions of Peristrophe bicalyculata on Hypertensive Rats ---------- 54

3.4.4 Experimental Design ---------- 55

3.4.4.1 Induction of Acute Hypertension ---------- 55

3.4.4.2 Induction of Chronic Hypertension ---------- 58

3.4.4.3 Partial Purification of Cold Water Extract of Peristrophe bicalyculata -------- 61

3.5 Determination of Angiotensin Converting Enzyme and Endothelial Nitric Oxide Synthase Expression ---------- 62

3.5.1 Rat Aortic Endothelial Cell Isolation ---------- 62

3.5.1.1 Rat aortic endothelial cell culture---------- 63

3.5.1.2 Cytotoxic studies ---------- 63

3.5.1.3 Protocol of incubation studies ---------- 64

3.5.2 Protocols for RNA Isolation and Quantitation ----- ---------- 64

3.5.2.1 Harvesting of cells ---------- 64

3.5.2.2 Isolation of total RNA from kidneys and aorta of rats---------- 64

3.5.2.3 RNA Quantitation, Integrity and Purity---------- 65

3.5.2.4 Primer Design---------- 65

3.5.2.5 Reverse Transcription and Real Time Reverse Transcriptase Polymerase Chain Reaction (RTRT-PCR) ---------- 66

3.6 Anticancer Activity of *Peristrophe bicalyculata* ---------- *69*

3.6.1 Cell culture ---------- 69

3.6.1.1 Preparation of Media ---------- 69

3.6.1.2 Sample preparation ---------- 69

3.6.1.3 Cell lines---------- 69

3.6.1.4 Cell culturing ---------- 70

3.6.1.5 The sulphorhodamine assay ---------- 71

3.6.1.6 Partial purification of Ethyl Acetate Fraction of P.bicalyculata ---------- 76

3.6.1.7 Acridine orange/ethidium bromide (AO/EB) staining ---------- 76

3.7 Melanogenesis Inhibitory Effect of *Peristrophe bicalyculata*---------- *77*

3.7.1 Cell Culture---------- *77*

3.7.2 Inhibitory Effect of Melanin Biosynthesis of $B_{16}F_{10}$ Melanoma Cells ---------- *77*

3.7.2.1 Determination of melanin content in $B_{16}F_{10}$ melanoma cells ---------- *78*

3.8 Gas Chromatography-Mass Spectrometric Analysis of Partially Purified Fractions

of *Peristrophe bicalyculata* ---------- *78*

3.8.1 Sample Preparation for GC-MS ---------- *78*

3.8.2 GC-MS Procedure ---------- *79*

3.9 Statistical Analysis ---------- 79

CHAPTER FOUR ---------- *80*

4.0 Results ---------- 80

4.1 Yield and Biological Activities of *Peristrophe bicalyculata* ---------- 80

4.1.1 Percentage Yield of Extracts of Peristrophe bicalyculata ---------- *80*

4.1.2 Phytochemical and Total Phenolic Content of Peristrophe bicalyculata ------- *82*

4.1.3 Antioxidant and Biological Activities of Peristrophe bicalyculata ---------- *82*

4.2 Antihypertensive Effects of *Peristrophe bicalyculata* ---------- *89*

4.2.1 ACE Inhibitory Activity of Peristrophe bicalyculata ---------- *89*

4.2.2 Effect of P. *bicalyculata* on L-NAME-induced Hypertensive Rats ---------- *91*

4.2.3 *Partial Purification of Cold Water Extract of Peristrophe bicalyculata* ------- *93*

4.2.4 Effect of the Partially Purified Fractions of Peristrophe bicalyculata on Angiotensin converting enzyme activity ---------- *100*

4.3 Antihypertensive Effects of Partially Purified Extract of *Peristrophe bicalyculata in vivo* ---------- *102*

4.3.1 *Effect of Partially Purified Extract of* P. *bicalyculata on Body Weight of L-NAME-induced Hypertensive Rats* ---------- *102*

4.3.2 Effect of Partially Purified Extract of P. *bicalyculata on Blood Pressure of L-NAME-induced Hypertensive Rats* ---------- *104*

4.3.3 Effect of Partially-Purified Extract of P. *bicalyculata on the Activity of Angiotensin Converting Enzyme in the Serum, Kidney and Aorta of L-NAME-induced Hypertensive Rats* ---------- *106*

4.3.4 Effect of the Partially Purified Extract of Peristrophe bicalyculata on the Activity of Endothelial Nitric Oxide Synthase in the Serum, Kidney and Aorta of L-NAME-induced Hypertensive Rats ---------- *110*

4.3.5 Effect of the Partially-Purified Extract of Peristrophe bicalyculata on the Levels of Thiobarbituric Acid-Reactive Substances in the Serum, Kidneys and Aorta of L-NAME-induced Hypertensive Rats ---------- *114*

4.4 Molecular Effect of Partially Purified Extract of *P. bicalyculata* ---------- 118

4.4.1 Effect of Partially Purified Extract of Peristrophe bicalyculata on the Expression of Angiotensin Converting Enzyme in the Aorta and Kidney of L-NAME-induced Hypertensive Rats ---------- *118*

4.4.2 Effect of Partially-Purified Extract of Peristrophe bicalyculata on the Expression of Endothelial Nitric Oxide Synthase in L-NAME-induced Hypertensive Rats ---------- 121

4.4.3 Effect of Partially Purified Extract of Peristrophe bicalyculata on the Expression of Angiotensin Converting Enzyme in rat aortic endothelial cells ----- ---------- 124

4.4.4 Effect of Partially Purified Extract of Peristrophe bicalyculata on the Expression of Endothelial Nitric Oxide Synthase in rat aortic endothelial cells -- ---------- 126

4.5 Anticancer Activity of Extracts of *Peristrophe bicalyculata* ---------- *129*

4.5.1 Partial Purification of Ethyl Acetate Fraction of P. bicalyculata ---------- 132

4.5.2 Acridine Orange/Ethidium Bromide Assay Staining of Human Mouth Epidermal Carcinoma (KB) ---------- 139

4.5.2.1 Apoptotic Effect of Partially Purified Extract of Peristrophe bicalyculata --- 139

4.5.2.2 Necrotic Effect of Partially-Purified Extract of Peristrophe bicalyculata --- 139

4.6 Melanogenesis Inhibitory Effect of *Peristrophe bicalyculata* ---------- *144*

4.7 Gas Chromatograpy and Mass Spectometry Analysis of Partially Purified Antihypertensive and Anticancer Fractions of *Peristrophe bicalyculata* ----- *150*

4.7.1 Gas Chromatography and Mass Spectrometry Analysis of the Partially Purified Antihypertensive Fraction of Peristrophe bicalyculata ---------- 150

4.7.2 Gas Chromatography and Mass Spectrometry Analysis of the Partially Purified Anticancer Fraction of Peristrophe bicalyculata ---------- 153

CHAPTER FIVE ---------- 156

5.0 Discussion ---------- 156

5.1 Antioxidant Activities of Peristrophe bicalyculata ---------- 156

5.2 Antihypertensive Effects of Peristrophe bicalyculata ---------- 158

5.3 Anticancer Activity of Peristrophe bicalyculata ---------- 162

5.4 Melanogenesis Inhibitory Effect of Peristrophe bicalyculata ---------- 163

5.5 Gas Chromatography and Mass Spectrometry Analysis of the Partially Purified Antihypertensive and Anticancer Fractions of Peristrophe bicalyculata ----- 165

CHAPTER SIX ---------- *167*

6.0 Summary, Conclusion and Recommendations ---------- *167*

6.1 Summary ---------- *167*

6.2 Conclusion ---------- *168*

6.3 Recommendations ---------- *169*

LIST OF FIGURES

Figure 2.1: Outcomes of carcinogen metabolic activation ----------29

Figure 2.2: Mechanisms of development of hypertension due to anti-angiogenic therapy ---------- 39

Figure 3.1: Extraction and fractionation steps of *Peristrophe bicalyculata* --------- *47*

Figure 4.1: Lipid peroxidation inhibitory activity of different extracts of P. *bicalyculata* at different incubation times ---------- *88*

Figure 4.2: Yields and fractions obtained from cold water extract of *Peristrophe bicalyculata* ---------- *97*

Figure 4.3: Effect of partially-purified extract of *Peristrophe bicalyculata* on body weight of L-NAME-induced hypertensive rats ---------- *103*

Figure 4.4: Effect of partially-purified extract of *Peristrophe bicalyculata* on systolic blood pressure of L-NAME-induced hypertensive rats ---------- *105*

Figure 4.5: Effect of partially-purified extract of *Peristrophe bicalyculata* on activity of angiotensin converting enzyme (ACE) in the serum of L-NAME hypertensive rats --*107*

Figure 4.6: Effect of partially purified extract of *Peristrophe bicalyculata* on activity of angiotensin converting enzyme (ACE) in the kidneys and aorta of L-NAME hypertensive rats ---------- *109*

Figure 4.7: Effect of partially-purified extract of *Peristrophe bicalyculata* on activity of endothelial nitric oxide synthase (eNOS) in the serum of L-NAME-induced hypertensive rats ----------*111*

Figure 4.8: Effect of partially-purified extract of *Peristrophe bicalyculata* on activity of endothelial nitric oxide synthase (eNOS) in kidney and aorta of L-NAME- induced hypertensive rats ---------- *113*

Figure 4.9: Effect of partially purified extract of P. *bicalyculata* on thiobarbituric acid reactive substances (TBARS) in serum of L-NAME-induced hypertensive rats -------*115*

Figure 4.10: Effect of partially purified extract of P. *bicalyculata* on thiobarbituric acid-reactive substances (TBARS) in the kidneys and aorta of L-NAME-induced hypertensive rats ---------- *117*

Figure 4.11: Effect of *Peristrophe bicalyculata* on the expression of angiotensin-converting enzyme (ACE) in the kidneys and aorta of L-NAME-induced hypertensive rats ---------- *119*

Figure 4.12: Effect of *Peristrophe bicalyculata* on the expression of endothelial nitric oxide synthase (eNOS) in the kidneys and aorta of L-NAME-induced hypertensive rats -
---------- *122*

Figure 4.13: Effect of *Peristrophe bicalyculata* on the expression of angiotensin-converting enzyme (ACE) in rat aortic endothelial cells ---------- *125*

Figure 4.14: Effect of *Peristrophe bicalyculata* on the expression of endothelial nitric oxide synthase (eNOS) in rat aortic endothelial cells ---------- *127*

Figure 4.15: Yield and fractions obtained from ethyl acetate extract of *Peristrophe bicalyculata* ---------- *136*

Figure 4.16: Percentage apoptotic human mouth epidermal carcinoma cells after 24 and 48 hours treatment with *Peristrophe bicalyculata* ---------- *142*

Figure 4.17: Percentage necrotic human mouth epidermal carcinoma cells after 24 and 48 hours treatment with *Peristrophe bicalyculata* ---------- *143*

Figure 4.18: Cytotoxic effects of extracts and methanolic fractions of *Peristrophe bicalyculata* on $B_{16}F_{10}$ melanoma cells---------- *147*

Figure 4.19: The melanin content of $B_{16}F_{10}$ melanoma cells after treatment with Kojic acid, butanol and cold water extracts of *Peristrophe bicalyculata* at various concentrations ---------- *149*

Figure 4.20: Gas chromatogram of partially-purified antihypertensive fraction of *Peristrophe bicalyculata* ---------- *151*

Figure 4.21: Mass spectrum of P,P,P-triphenyl-Imino(triphenyl)phosphorane Retention time: 47.33 minutes; $C_{18}H_{15}OP$; mol. Wt: 278 g) ---------- *152*

Figure 4.22: Gas chromatogram of partially-purified anticancer fraction of *Peristrophe bicalyculata* ---------- *154*

Figure 4.23: Mass spectrum of andrographolide 2(3H)-furanone (Retention time: 58.63 minutes; $C_{20}H_{30}O_5$; mol. Wt: 350 g) ---------- *155*

LIST OF TABLES

Table 4.1: Yield and percentage yield of extracts of *Peristrophe bicalyculata* -------- *81*

Table 4.2: Phytochemical composition of extract of *Peristrophe bicalyculata* -------- *83*

Table 4.3: Total phenolic content of extracts of *Peristrophe bicalyculata* ---------- *84*

Table 4.4: Scavenging, tyrosinase inhibition and metal chelating concentrations of different extracts of *Peristrophe bicalyculata* ---------- *86*

Table 4.5: ACE inhibitory effect of extracts of P. *bicalyculata* ---------- *90*

Table 4.6: Effect of P. *bicalyculata* on L-NAME-induced hypertension in rats ------- *92*

Table 4.7: Fractions and yields of cold water extract of *Peristrophe bicalyculata* obtained by column chromatographic separation ---------- 95

Table 4.8: Effect of partially-purified fractions of P. *bicalyculata* on hypertensive rats -- ---------- 99

Table 4.9: IC_{50} values of partially purified fractions of cold water extract of *Peristrophe bicalyculata* on Angiotensin converting enzyme activity ---------- *101*

Table 4.10: The IC_{50} (µg/mL) values for anti-cancer activity of extracts of *Peristrophe bicalyculata* ---------- *131*

Table 4.11: Fractions and yields obtained from column chromatographic separation of ethyl acetate extract of *Peristrophe bicalyculata* ---------- *134*

Table 4.12: Anticancer activity of fractions of P. *bicalyculata* ---------- *138*

Table 4.13: Percentage viabilities of $B_{16}F_{10}$ melanoma cells treated with varying concentrations of extracts and fractions of P. *bicalyculata* ---------- *14*

LIST OF PLATES

Plate I: A rat with cannulated jugular vein and artery attached to 1 mm polyethylene tube filled with saline and heparin sodium ---------- 57

Plate II: A filter under suction attached to a culture bottle in the laminar flow -------- 73

Plate III: Plating of cells under a sterilized hood in preparations for the SRB assay - 74

Plate IV: A cell culture incubator containing cell culture flasks and plates ---------- 75

Plate V: Thin layer chromatographic separation of the cold water extract of *Peristrophe bicalyculata* for its antihypertensive activity ---------- 94

Plate VI: Thin layer chromatographic separation of fractions obtained from cold water extract of *Peristrophe bicalyculata* ---------- *96*

Plate VII: Agarose gel electrophoregram of angiotensin-converting enzyme (ACE) and β-actin from the kidneys and aorta of control, untreated and treated hypertensive rats ---- ---------- *120*

Plate VIII: Agarose gel electrophoregram of endothelial nitric oxide synthase (eNOS) and β-actin from the kidneys and aorta of control, treated and untreated hypertensive rats ----------*123*

Plate IX: Agarose gel electrophoregram of angiotensin-converting enzyme, endothelial nitric oxide synthase and β-actin from rat aortic endothelial cells following treatment with L-NAME, captopril and partially purified extract of *Peristrophe bicalyculata* ------ ---------- *128*

Plate X: Thin layer chromatographic separation of the ethyl acetate fraction of methanol extract of *Peristrophe bicalyculata* for its anticancer activity ---------- *133*

Plate XI: Thin layer chromatographic separation of fractions obtained from ethyl acetate fraction of methanol extract of *Peristrophe bicalyculata* ---------- *135*

Plate XII: Effect of *Peristrophe bicalyculata* on KB cells showing characteristic normal (N), necrotic (Ne) and apoptotic (A) morphology when stained with ethidium bromide and acridine orange stain ----------*141*

LIST OF APPENDICES

Appendix 1: Lipid peroxidation inhibitory activity of different extracts of *P. bicalyculata* at different incubation times ---------- 185

Appendix 2: Effect of partially-purified extract of *Peristrophe bicalyculata* on body weight of L-NAME-induced hypertensive rats ---------- 186

Appendix 3: Effect of partially-purified extract of *Peristrophe bicalyculata* on systolic blood pressure of L-NAME-induced hypertensive rats ---------- 187

Appendix 4: Effect of partially-purified extract of *Peristrophe bicalyculata* on activity of angiotensin-converting enzyme in the serum, kidney and aorta of L-NAME hypertensive rats ---------- 188

Appendix 5: Effect of partially-purified extract of *Peristrophe bicalyculata* on activity of endothelial nitric oxide synthase (eNOS) in the serum, kidney and aorta of L-NAME-induced hypertensive rats ---------- 189

Appendix 6: Effect of partially purified extract of P. *bicalyculata* on thiobarbituric acid reactive substances (TBARS) in serum, kidneys and aorta of L-NAME-induced hypertensive rats ---------- 190

Appendix 7: Effect of *Peristrophe bicalyculata* on the expression of angiotensin-converting enzyme in the kidneys and aorta of L-NAME-induced hypertensive rats- 191

Appendix 8: Effect of *Peristrophe bicalyculata* on the expression of endothelial nitric oxide synthase (eNOS) in the kidneys and aorta of L-NAME-induced hypertensive rats -
----------192

Appendix 9: Effect of *Peristrophe bicalyculata* on the expression of angiotensin-converting enzyme and endothelial nitric oxide synthase in rat aortic endothelial cells ---------- 193

Appendix 10: Percentage apoptotic human mouth epidermal carcinoma cells after 24 and 48 hours treatment with *Peristrophe bicalyculata* ----------194

Appendix 11: Percentage necrotic human mouth epidermal carcinoma cells after 24 and 48 hours treatment with *Peristrophe bicalyculata* ---------- 195

Appendix 12: Cytotoxic effects of extracts and methanolic fractions of *Peristrophe bicalyculata* on $B_{16}F_{10}$ melanoma cells ---------- 196

Appendix 13: The melanin content of $B_{16}F_{10}$ melanoma cells after treatment with Kojic acid, butanol and cold water extracts of *Peristrophe bicalyculata* at various concentrations---------- 197

Appendix 14: Mass spectrum of Ethyl Propanoic acid, 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one and 1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy) tetrasiloxane ---------- 198

Appendix 15: Mass spectrum of 2,4,5-trimethoxybenzaldehyde, diazoprogesterone and cis-Z-.alpha.-bisabolene epoxide ---------- 199

Appendix 15: Mass spectrum of aromadendrene oxide ---------- 200

LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
BHA	Butylated hydroxyanisole
DMEM	Dulbecco's modified eagle's medium
DPPH	2,2-Diphenyl-2-picryl-hydrazine
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
HeLa	Human cervical cancer
HepG2	Human hepatocellular liver carcinoma
HT-29	Human mouth epidermal carcinoma
L-NAME	N-nitro- L-arginine methyl ester
NO	Nitric oxide
NOS	Nitric oxide synthase
PBS	Phosphate buffered saline
RAS	Rennin angiotensin system
RAAS	Rennin angiotensin aldosterone system
RAOEC	Rat aortic endothelial cells
ROS	Reactive oxygen species
TLC	Thin layer chromatography
GC-MS	Gas chromatography mass spectrometry

CHAPTER ONE

1.0 INTRODUCTION

Coronary artery diseases present some of the major health problems across the globe today, with stroke and hypertension being the most common. Hypertension is usually called a "silent killer" because persons with hypertension are often asymptomatic for years (Holm *et al*., 2006). The renin-angiotensin aldosterone system (RAAS) is an important regulator of sodium and water balance, as well as blood pressure homeostasis in man. Angiotensin-converting enzyme (ACE; peptidyldipeptide hydrolase, EC 3.4.15.1), which is part of the system found in the plasma and endothelial cells of animals and humans, acts on the decapeptide angiotensin I to form a highly active vasopressor, angiotensin II. It also degrades the potent vasodilator, bradykinin to an inactive heptapeptide (Reeves and O'Dell, 1986; Schmieder *et al*., 2007).

Angiotensin-converting-enzyme inhibitors block the activation of the RAAS by preventing the conversion of angiotensin-I to angiotensin-II and could retard the progression of both heart failure and atherosclerosis. Also, studies have demonstrated that alterations in the expression or activity of nitric oxide synthase (NOS) or the availability of nitric oxide (NO) plays a significant role in the development of hypertension (Augustyniak *et al.,* 2005) and cancer (Ray *et al*., 2004). Epidemiological evidence from prospective studies points to the possibility that there may be association between hypertension and cancer because of the pathophysiological similarity between the two. For example, the incidence of hypertension and cancer increases with age. Also both diseases have similar risk factors including obesity, alcohol consumption and smoking, which cause elevated blood pressure and also some form of cancers. Both

diseases have some common epidemiological factors and pathophysiological pathways (Ray *et al*., 2004). The search for more efficient agents to replace the current ones in the management of the two diseases has continued for years (Zhu *et al.,* 2009; Abdulazeez *et al.,* 2010; Wirtz *et al.,* 2011).

Indeed, there have been worldwide efforts to discover new anticancer and antihypertensive agents from plants, especially with information obtained from ethnomedical data, in which selection of a plant is based on prior knowledge about its use in folk medicine. However, in the case of cancer, which is a complicated and heterogenous disease, the use of such data alone may not be reliable, prompting the need to determine the cytotoxic effects of various plants on different types of carcinoma (Slambrouck *et al*., 2007).

Majority of anticancer studies done to determine the cytotoxic effects of plant extracts and other drugs are first done *in vitro* using tissue or cell cultures, before determining their effects *in vivo*. Cell culture can be defined as the process whereby cells removed from animal tissues or whole animals continue to grow when supplied with nutrients and growth factors. This *in vitro* culture process allows single cells to act as independent units much like any microorganism such as bacteria or fungi. The cells are capable of division by mitosis and the cell population can continue to grow until limited by some parameter such as nutrient depletion, cell accumulation and cell-to-cell contact. Cell culture is one of the major tools used in life sciences to investigate the normal physiology or biochemistry of cells for example; metabolic pathways can be investigated by applying radioactively labeled substrates and subsequently examining the products; to test the effects of the compounds on specific cell types. Such

compounds may be metabolites, hormones or growth factors. Similarly, potentially toxic or mutagenic compounds may be evaluated in cell culture; to produce artificial tissue by combining specific cell types in sequence and to synthesize valuable products (biologicals) from large-scale cell cultures (Butler, 2005; Freshney, 2005).

The antihypertensive and ACE inhibitory effect of extracts of *Peristrophe bicalyculata* was demonstrated in two kidney-1-clip hypertensive rats compared to control rats (Abdulazeez *et al*., 2010). The extract also reduced plasma triacylglycerols and total cholesterol and increased high-density lipoprotein in Polaxamer 407-induced hyperlipidaemic rats compared to control rats; and was more effective than the standard drug, Atorvastatin (Abdulazeez, 2011). This decrease in blood pressure, ACE activity and hypolipidemic effects exhibited by the plant shows that there is a scientific support for its traditional use in the management of hypertension. Studies by Ogunwande *et al*. (2010) demonstrated the anticancer activity of oils from *Peristrophe bicalyculata* using MCF-7 (human breast tumour) and MDA-MB-468 (human breast tumour) cells. The anticancer activity of crude extracts of the plant against EAC cell lines have also been reported (Ashokan and Muthuraman, 2011; Tanavade *et al*., 2012).

The present study was aimed at specifically confirming the efficacy of *Peristrophe bicalyculata* in the treatment of hypertension; determine the antihypertensive principle(s) and molecular mechanisms underlying their pharmacological action, by investigating their effects on the expression and/or control of ACE and eNOS in hypertensive rats. Also, the cytotoxic activity of *Peristrophe bicalyculata* on five cancer cell lines was investigated. It was conceivable that the investigation may shed more

light on the current understanding of the principle component(s) and the mechanism of action of *Peristrophe bicalyculata* in the prevention and treatment of hypertension. The components to be identified in the extracts of the plant may be additional arsenal for the management of hypertension and cancer.

1.1 STATEMENTS OF RESEARCH PROBLEM

1. Hypertension is a common cardiovascular disease which has become a worldwide problem of epidemic proportions, affecting 15-20% of all adults, and 11.2% Nigerians (about 17.92 million); with ailments such as arteriosclerosis, stroke, myocardial infarction and end-stage renal disease (Akinkugbe, 1997; Ulasi *et al*., 2011).

2. Cancer causes 10% of all deaths worldwide. It is second only to cardiovascular disease as the main cause of death in developed countries. According to the World Health Organization (WHO), about 57 million deaths occurred in 2008, with death from cardiovascular diseases (above 17 million) being the highest. Death from cancer (7.6 million) was second with lung, trachea and bronchial cancers being most common. However, majority of cancer patients have coexistent diseases, with more than fifty percent of them reporting cardiovascular diseases including hypertension (WHO, 2008).

3. Although cancer is reported more in the developed world, about two-thirds of all cancers occur in developing countries with many of them either not reported or wrongly diagnosed.

4. The RAAS and NO are believed to be the possible mediators linking hypertension and cancer (Carrizo *et al*., 1998). The inhibition of RAAS has been

an important strategy for the treatment of hypertension, and has been shown to reduce the risk of cancer in patients. However, there is a need for more effective and safer drugs as cancer is not a single disease, and the need for seeking remedy for both diseases cannot be overemphasized.

1.2 NULL HYPOTHESES

1. *Peristrophe bicalyculata* extract does not reduce blood pressure by inhibiting the rennin angiotensin system.

2. *Peristrophe bicalyculata* is not cytotoxic against some cancer cells.

1.3 JUSTIFICATION

1. *Peristrophe bicalyculata* is the most common plant used in treatment of hypertension in many parts of Nigeria, but there is paucity of information on its efficacy, active principle(s) and possible mode of action.
2. Most of the antihypertensive and anticancer drugs so far available do not possess complete curative properties, and research into herbs used by traditional healers has led to the discovery of several antihypertensive and anticancer drugs; clearly showing how the study of natural products may result in the discovery of valuable drugs, many of which are yet to be discovered.
3. Studies have demonstrated that alterations in the expression or activity of endothelial nitric oxide synthase (eNOS) or the availability of NO plays a significant role in the development of hypertension (Augustyniak *et al.,* 2005) and cancer (Ray *et al*., 2004). Both eNOS system and RAAS are mediators linking hypertension and cancer, hence are referred to as “gatekeepers” because their actions are wide-ranging and fine-tune or override other systems, and

perturbations in these systems are linked to dysfunction and disease (Linz *et al*., 1999).

4. Accumulating data from *in vitro* animal and clinical studies indicate that antagonism of the RAAS suppresses tumour growth, metastasis and angiogenesis in a broad range of experimental models of malignancy, and retrospective studies in humans provide evidence that long-term use of antihypertensive drugs such as ACE inhibitors may protect against cancer (Amee *et al*., 2010).
5. Drugs that are potent ACE inhibitors have been shown to also inhibit melanogenesis, hence the interest in determining the melanogenesis inhibitory activity of *Peristrophe bicalyculata* (Lee *et al.,* 2012).

1.4 AIMS AND OBJECTIVES OF STUDY

The general objective of this study was to determine the antihypertensive effect of leaf extract of *Peristrophe bicalyculata,* its mechanism of action and cytotoxic effect on some cancer cells. The specific objectives were:

1. To carry out phytochemical analysis of leaf extract of *Peristrophe bicalyculata.*
2. To determine the effect of *Peristrophe bicalyculata* extract on the activity of ACE and eNOS in N-nitro- L-arginine methyl ester (L-NAME) induced hypertensive rats.
3. To evaluate the effect of filter-sterile partially purified antihypertensive component(s) on the *in vivo* and *in vitro* expression of ACE and eNOS.
4. To investigate the cytotoxic effect of *Peristrophe bicalyculata* extract on four cancer cell lines; Human cervical cancer (HeLa), human colon adenocarcinoma

(HT-29), human hepatocellular liver carcinoma (HepG2) and human mouth epidermal carcinoma (KB).

5. To evaluate the melanogenesis-inhibitory effect of the extract of *Peristrophe bicalyculata* on mouse melanoma cells ($B_{16}F_{10}$).
6. To identify potent antihypertensive and anticancer components(s) present in the plant.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 TAXONOMY AND HISTORY of *Peristrophe bicalyculata*

Peristrophe bicalyculata (Retz) Nees belongs to the Kingdom: Plantae; Phylum: Magnoliophyta; Class: Magnoliopsida; Order: Lamiales; Family: Acanthaceae, and to the Genus: *Peristrophe*. It is native to warm tropical regions of Africa, in the Sahel part of the region from Mauritania to Niger and Northern Nigeria, India, Burma and Thailand (Burkill, 1985). The Hausas in Northern Nigeria call it 'tubanin dawaki' meaning 'flower of the horse'. In 'Berer' and 'Wolof' languages of Senegal, it is called 'buben' and 'moto', respectively (Burkill, 1985). In the Indore district of India, the local name is 'chotiharjori'. The plant is relished for grazing by cattle in Senegal and provides fodder in many other countries (Dwivedi, 2002). *Peristrophe bicalyculata* is a perennial herb with about 1.50 m high stems that are sparingly setose-pilose. The leaves are sub-equal ovate-lanceolate and narrowed into winged petiole of up to 10 cm long and 4 cm broad, with conspicuous rod-like cystoliths on both sides. The flowers are two-lipped, and very numerous in a lax leafy panicle. The pedicels are angular and scabrid, the calyx-segments linear lanceolate and the corolla is 1.5 cm long. It has two stamens, two-celled anthers and 1cm long fruit (Burkill, 1985).

2.2 USES OF *Peristrophe bicalyculata*

Peristrophe bicalyculata (Retz.) Nees is used by the honey collectors in India, due to the repelling smell and taste of its juice, which when applied on the body drives bees away. It is used by the traditional healers in the treatment of many skin related ailments,

an antidote for snake poison when macerated in an infusion of rice, and as an insect repellant. It is also used as horse feed, and ploughed into the soil as green manure (Chopra, 1959). The seeds carry a certain amount of mucilage which on drying can be stretched out into a fine thread used to remove foreign bodies, and treatment of eye and ear infections. A yellowish-brown essential oil extracted from the seeds by steam-distillation has been shown to have tuberculostatic activity *in vitro*, inhibiting the growth of various strains of *Mycobacterium tuberculosis* (Burkill, 1985). The leaves of the plant have analgesic, antipyretic, anti-inflammatory activities (Rathi *et al*., 2003), and antibacterial, fungistatic and bacteriostatic properties (Burkill, 1985, Janakiraman *et al.*, 2012). In South-west Nigeria, the plant is used as green manure as well as the treatment of hypertension and cardiovascular-related diseases (Abdulazeez *et. al*., 2010).

2.3 HYPERTENSION

2.3.1 Definition, Classification and Aetiology

2.3.1.1 Definition

Hypertension, also called high blood pressure, is one of the most common diseases of modern times. Blood pressure is the pressure that the flowing blood exerts on the walls of blood vessels; and may be diastolic or systolic blood pressure. Diastolic blood pressure refers to the lowest blood pressure value during the diastole (rest phase) of the heart, while Systolic is the highest value of the blood pressure during the systole, when blood is ejected from the left ventricle of the heart during circulation (Kaplan, 1998). The WHO has described hypertension as a state in which systolic blood pressure is 140 mmHg or more and diastolic pressure is 90 mmHg or more (Aftab, 1995). Hypertension

is an important risk factor for cardiovascular accidents, coronary heart disease, cardiac hypertrophy with heart failure (hypertensive heart disease), aortic dissection, and renal failure. It can also accelerate atherogenesis and induce changes favorable for aortic dissection and cerebrovascular haemorrhage (Holm *et al.*, 2006).

2.3.1.2 Classification

Based on the Seventh Report of the Joint National Committee on the Prevention, Detection, Evaluation and Treatment of High Blood Pressure (JNC-7), hypertension can be classified into three stages (Herman *et al*., 2004):

1. Pre-hypertension - Systolic blood pressure 120-139 mm Hg or diastolic blood pressure 80-89 mm Hg
2. Stage I hypertension - Systolic blood pressure 140-159 mm Hg or diastolic blood pressure 90-99 mm Hg
3. Stage II hypertension - Systolic blood pressure $\geq$ 160 mm Hg or diastolic blood pressure > 100 mm Hg

According to the JNC-7 report, the correlation between blood pressure and the risk of cardiovascular diseases has been shown to be continuous, consistent and independent of other risk factors. As blood pressure increases, so does the possibility of heart attack, stroke and kidney disease. For patients between 40 and 70 years old, each increment of 20 mmHg in Systolic blood pressure or 10 mmHg in diastolic blood pressure doubles the risk of cardiovascular diseases across the entire range from 115/75 mmHg to 185/115 mmHg (Herman *et al.*, 2004).

2.3.1.3 Aetiology

Studies have demonstrated that nearly 90-95 % of cases of hypertension are idiopathic or primary and are classified as essential hypertension, while the remaining 5-10% is classified as secondary hypertension. By definition, essential hypertension has no identifiable cause (Lifton, 1996). The pathogenesis of primary hypertension is multifactorial, with genetic and environmental factors playing important roles. Increased salt intake and obesity have long been incriminated. Although these factors alone are, apparently, not sufficient to raise blood pressure to abnormal levels, they are synergistic with a genetic predisposition. Other factors that may be involved in the pathogenesis of essential hypertension are sympathetic nervous system hyperactivity, abnormal cardiovascular development, renin-angiotensin system activity, and defect in natriuresis, intracellular sodium and calcium, as well as exacerbating factors (including obesity, alcohol, cigarette smoking, and polycythaemia) (Haslam and James, 2005).

Secondary hypertension results from an identifiable cause, which when treated can resolve without the need for antihypertensive medications. Renal disease is the most common cause of secondary hypertension as the long-term regulation of arterial pressure is intimately linked to the ability of the kidneys to excrete sufficient sodium chloride to maintain normal sodium balance, extracellular fluid volume, and blood volume at normotensive arterial pressures. Furthermore, disorders in renal function from subtle to overt are probably involved in the pathogenesis of most, if not all, cases of essential hypertension (Luke, 1993). Other causes of secondary hypertension include sleep apnoea, tumours, Cushing's syndrome, coartation of the aorta, drugs, apparent mineralocorticoid excess syndrome, glucocorticoid remediable aldosteronism, polycystic kidney disease and pregnancy (Haslam and James, 2005).

2.3.2 Treatment of Hypertension

There are many classes of medications for the treatment of hypertension called antihypertensives; which by varying means act by lowering blood pressure. Drug trials to lower moderately elevated blood pressure have failed to reduce overall death rates, and the type of medication to be used at the early stage of hypertension has been a subject of several studies and various national guidelines. However, unless hypertension is severe, lifestyle changes such as weight reduction and regular aerobic exercise improve blood flow and help to reduce resting heart rate and blood pressure (Elley and Arroll, 2002). Reducing dietary sugar and sodium (salt) intake in the diet are also strongly recommended before initiation of drug therapy (Appel *et al*., 1997). Antihypertensive drugs are classified into six categories: diuretics, sympathoplegic agents, ACE inhibitors, angiotensin II inhibitors, direct vasodilators and rennin inhibitors.

2.3.2.1 Diuretics

Diuretics are listed as the current first-line antihypertensives in the JNC 7 guidelines for the treatment of hypertension (Herman *et al*., 2004). Their primary therapeutic effect is a decrease in the plasma and extracellular fluid volumes resulting in reduced preload; which leads to a decrease in cardiac output and total peripheral resistance, all of which reduce the workload of the heart. Diuretics include thiazides; for example, hydrochlorothiazide, which acts on distal convoluted tubule to inhibit Na^+-Cl^- symport; Loop diuretics, such as fusosemide and bumetanide, increase urine Ca^{2+} content, and are often used for the treatment of severe hypertension when direct vasodilators are administered and Na^+ and H_2O retention becomes a problem; K-sparing diuretics such as triamterene and amiloride (Na^+ channel inhibitors); and spironolactone (aldosterone

antagonist) used to enhance the natriuretic effects of other diuretics (for example, thiazides) and to counteract their K^+-depleting effects.

2.3.2.2 Sympathoplegic Agents

These drugs can be divided into centrally-acting adrenergic drugs and drugs that act on peripheral nervous system (Lilley *et al*., 2010)

a. *Centrally-acting adrenergic drugs*

The centrally acting adrenergic drugs, clonidine and methyldopa both act by modifying the function of the sympathetic nervous system. This is because stimulation of the sympathetic nervous system leads to an increase in heart rate and force of contraction, the constriction of blood vessels, and the release of renin from the kidney, which results in hypertension. These drugs act by stimulating the $alpha_2$-adrenergic receptors in the brain to reduce sympathetic outflow from the central nervous system, preventing noradrenaline production, which reduces blood pressure. This stimulation of the $alpha_2$ -adrenergic receptors also affects the kidneys, by reducing the activity of renin.

b. *Antihypertensive agents that act on peripheral nervous system*

These drugs include:

Beta-adrenergic blockers reduce heart rate through beta-1 receptor blockade and also reduce the secretion of the hormone, *renin*, which in turn reduces both Angiotensin II-mediated vasoconstriction and aldosterone-mediated volume expansion. Long-term use of beta-blockers also reduces peripheral vascular resistance; for example, propranolol, metoprolol and nadolol (Lilley *et al.*, 2010).

Alpha$_1$ adrenergic blockers also modify the function of the sympathetic nervous system by inhibiting α_1-adrenergic receptors and, thus, noradrenaline, which causes dilation of arteries and veins, reduces peripheral vascular resistance and, subsequently, decreases blood pressure. They also increase urinary flow rates and decrease outflow obstruction by preventing smooth muscle contractions in the bladder neck and urethra. Examples include doxazosin, prazosin and terazosin (Lilley *et al*., 2010).

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Two dual-action α_1 and β-adrenergic receptor blockers have the dual antihypertensive effects of reduction in heart rate (beta$_1$ receptor blockade) and vasodilation (alpha$_1$ receptor blockade); for example, labetalol and carvedilol (Lilley *et al*., 2010).

2.3.2.3 Angiotensin Converting Enzyme inhibitors

Angiotensin I analogues competitively inhibit the enzyme ACE. ACE inhibitors result in reduced cardiac stimulation, with resultant lowering of cardiac output. Examples include enalapril and captopril. Inhibition of ACE results in the decreased formation of angiotensin II and decreased metabolism of bradykinin, leading to systematic dilation of the arteries and veins, and consequently, a decrease in arterial blood pressure. In addition, inhibiting angiotension II formation diminishes angiotensin II-mediated aldosterone secretion from the adrenal cortex, leading to a decrease in water and sodium reabsorption and a reduction in extracellular volume (Klabunde, 2009).

2.3.2.4 Angiotensin II receptor blockers

Angiotensin II receptor blockers (ARBs) affect primarily vascular smooth muscle and the adrenal gland by selectively blocking the binding of Angiotensin II to the type 1 Angiotensin II receptors in these tissues, thus preventing vasoconstriction and the

secretion of aldosterone. Unlike ACE inhibitors that block the breakdown of bradykinins, which accumulate and cause adverse effects such as cough, ARBs do not have such adverse effects. They also do not adversely affect lipid profiles or cause rebound hypertension after discontinuation. Examples include losartan, valsartan, Irbesartan and candesartan. These drugs antagonize the angiotensin II-induced biologic actions such as smooth muscle contraction, sympathetic pressor mechanisms and aldosterone secretion, associated with hypertension (Kirk, 1999).

2.3.2.5 Vasodilators

Vasodilators act directly on arteriolar and/or venous smooth muscle to cause relaxation. They do not work through adrenergic receptors. They include hydralazine, which dilates arterioles but not veins; minoxidil and diazoxide, known to open K^+ channels in smooth muscles and stabilize membrane at its resting potential; sodium nitroprusside, which works by increasing intracellular cGMP and dilates both arterial and venous vessels (Lilley *et al.*, 2010).

Calcium channel blockers also cause smooth muscle relaxation by blocking the binding of calcium to its receptors, thereby preventing contraction. Examples are nifedipine, amlodipine (Norvasc), diltiazem and verapamil (Kragten and Dunselman, 2007).

2.3.2.6 Direct renin inhibitors

They are analogues of angiotensinogen that inhibit rennin; for example, aliskiren. Renin inhibitors block the conversion of angiotensinogen to angiotensin I. Thus, they decrease the level of angiotensin II and activate the renin-angiotensin-aldosterone system to further decrease the release of rennin (Lilley *et al.*, 2010).

2.3.3 Experimental Model of Hypertension

Human essential hypertension is a complex, multifactorial, quantitative trait under polygenic control. Various models of experimental hypertension have been primarily developed to obtain information on the aetiopathogenesis of hypertension and also in the pharmacological screening of potential antihypertensive agents. They include renovascular hypertension, such as two kidney one clip (2K2K) and one kidney one clip (1K1C) hypertension; hypertension induced by external compression of renal parenchyma, examples are page hypertension, dietary, endocrine, neurogenic, psychologenic, chronic nitric oxide inhibition-induced hypertension, obesity-related and genetic hypertension (Badyal *et al*., 2003).

These models of hypertension share many features which are common to human hypertension. Many of the models have been developed using the aetiological factors presumed to be responsible for human hypertension such as excessive salt intake, hyperactivity of RAAS and genetic factors. Since regulation of blood pressure is multifactorial, the effectiveness of an antihypertensive agent in one model does not necessarily mean that the mechanism of action of the agent in that given model is related to the pathogenesis of elevated blood pressure (Badyal *et al*., 2003).

2.4 CHRONIC NITRIC OXIDE INHIBITION-INDUCED HYPERTENSION

The advent of the chronic NOS inhibition model of hypertension has portayed NO as an indispensable and irreplaceable element in the maintenance of circulatory integrity, regulating diverse functions as vascular tone, renal salt excretion and renin secretion. Inhibition of NO has been shown to induce vasoconstriction and arterial hypertension in experimental animals. One of the most frequently used experimental models to analyse

the role of NO in hypertension is that induced by the oral administration of NOS inhibitors, such as N^Gnitro-l-arginine methyl ester (L-NAME) in rats (Badyal *et al.*, 2003). This results in vasoconstriction, enhanced sodium and water renal reabsorption, renal vasoconstriction, elevation in oxidative stress and enhanced calcium signalling in smooth muscle cells. Nitric oxide inhibition also affects the structure of different organs, including blood vessels, renal, myocardial and nervous tissues (Garcia-Estan *et al*., 2006).

Substantial evidence demonstrates that NO plays a critical role in the maintenance of blood pressure homeostasis as treatment with eNOS inhibitors produced intense renal vasoconstriction with marked reduction of renal plasma flow, a smaller reduction in glomerular fitration rate and significantly increased blood pressure in rats and mice. Baylis *et al.* (1992) reported that chronic administration of L-NAME promoted persistent hypertension and renal damage. Also, higher doses of L-NAME have been shown to cause severe and progressive form of hypertension associated with glomerular ischaemia, glomerulosclerosis and renal interstitial expansion (Ribeiro *et al*., 1992). More recently, disruption of the gene encoding eNOS, for example, knockout mice lacking the gene for the eNOS, developed systemic and pulmonary hypertension, whereas deletion of the genes encoding the neuronal NOS and inducible NOS isoforms, respectively led to no detectable circulatory changes (Laubach *et al*., 1995; Nelson *et al*., 1995). The findings indicate that NO is a fundamental and irreplaceable element in the regulation of blood pressure and they gave birth to a new model of arterial hypertension (Baylis *et al*., 1992; Ribeiro *et al*., 1992).

2.4.1 Synthesis of Nitric Oxide

Nitric oxide is synthesized from L-arginine by the action of NOS. The enzyme, NOS exists in three isoforms: brain NOS (bNOS) or neuronal NOS (nNOS or NOS1), endothelial NOS (eNOS or NOS3) or constitutive NOS (cNOS) and inducible NOS (iNOS or NOS2). Both nNOS and eNOS are responsible for the continuous basal release of NO and require calcium/calmodulin for activation, while iNOS is calcium-independent and is expressed only in response to inflammatory cytokines and lipopolysaccharides. The three isoforms of NOS are products of separate genes that share 50-60% amino acid homology (Nathan and Xie, 1994; Silver, 2011). The cytochromes function as a homodimer during activation. They have five enzyme co-factors, including flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, tetrahydrobiopterin (BH_4) and calmodulin (Cho *et al*., 1995, Silver, 2011).

An increase in intracellular calcium triggers a cascade of events, leading to activation of eNOS and NO synthesis. Intracellular calcium binds to calmodulin to form a calcium-calmodulin complex and regulates the binding of calmodulin to the 'latch domain', which permits electron transfer from NADPH via flavin groups within the reductase domain to a haem-containing active site, thereby facilitating the conversion of O_2 and L-arginine to NO and L-citrulline (Abu-Soud *et al.*, 1994). In contrast to eNOS, iNOS does not require an increase in intracellular calcium as it contains calmodulin tightly bound to each subunit of the enzyme, which results in the permanent activation of the enzyme (Silver, 2011). This explains why calcium chelators are capable of reducing eNOS activity significantly; however, the role of calcium with regard to iNOS activity remains unclear. Geller *et al.* (1993) showed that calcium chelators significantly

reduced iNOS activity, whereas Charles *et al*. (1993) reported that iNOS activity was not influenced by calcium chelators.

L-Arginine is the only physiological nitrogen donor for NOS-catalysed reactions; hence its availability determines the rate of NO generation. Arginine synthesis as well as its transport into the cells can also influence NO synthesis. Arginine is synthesized from citrulline by the actions of arginosuccinate synthetase and arginosuccinate lyase and catabolized by arginase (Albina *et al.*, 1988). The synthesis and transport represent a potential target for experimental and therapeutic manipulation of cellular NO synthesis. It has been shown that endogenous arginine synthesis is coupled to NO synthesis and that induction of arginosuccinate synthetase is not secondary to increased NOS activity (Morris and Billiar., 1994). Apart from synthesis, transport of arginine into the cells can also regulate NOS activity. Competitive inhibition of arginine uptake by L-lysine, L-ornithine (naturally occurring amino acids) and L-NAME or N^G-monomethyl-L-arginine (L-NMMA, arginine analogues) results in decreased NO synthesis (Inoue *et al.*, 1993). Nitric oxide synthase generate hydrogen peroxide, superoxide and NO (Bredt *et al.*, 1992). In L-arginine-depleted cells, superoxide generation is increased, whereas the presence of L-arginine reduces superoxide generation. Superoxide ($.O_2^-$) generated at low concentrations of L-arginine reacts with NO, resulting in increased generation of peroxynitrite ($ONOO^-$) and cell toxicity (Xia *et al.*, 1996).

2.4.2 Transport and Mechanism of Action of Nitric Oxide

Initially, it was believed that NO reaches its intracellular targets by freely diffusing through the cell membranes. However, because of its ultra-short half-life (< 30 s) and high reactivity with oxygen radical ($.O_2^-$), haem and non-haem-iron, the concept of free

diffusion of NO was hard to accept. NO reacts with thiol (-SH) groups of proteins and can form stable, biologically active, *S*-nitrosyl compounds which possess biological activities identical to those of NO *in vitro* and *in vivo* (Keaney *et al.*, 1993). Also, since iron–nitrosyl compounds are associated with thiol-containing ligands, the formation of dinitrosyl iron cysteine has been suggested as a second pathway for NO transport (Mulsch *et al.*, 1993).

Nitric oxide mediates multiple biological effects, although initially its effects were thought to be solely mediated via activation of soluble guanylate cyclase and guanosine 3',5'-cyclic monophosphate (cGMP). Generation of cGMP is known to mediate relaxation, inhibit vascular smooth muscle cell growth, prevent platelet aggregation and adherence of neutrophils to endothelial cells (Murad, 1994). Recent studies suggest that NO can also induce its biological effects via non-cGMP-dependent pathways. Other relevant mechanisms include *S*-nitrosylation of thiols, formation of nitrotyrosines, NO binding to iron–sulphur clusters and NO binding to haem-containing proteins of the respiratory chain (Mohr *et al.*, 1996). NO can also act as a free-radical scavenger and inactivate $.O_2^-$, thereby preventing cell toxicity (Cook and Tsao, 1993). However, under appropriate conditions, reaction of NO with $.O_2^-$ can also result in the generation of peroxynitrite ($ONOO^-$), a potent oxidant which decomposes to form the reactive hydroxyl radical HOONO. Moreover, peroxynitrite and its metabolites are capable of inducing cytotoxicity by inducing lipid peroxidation, nitrosation of several tyrosine molecules that regulate enzyme function and signal transduction as well as Na^+ channel inactivation. Together, these findings suggest that the actions of NO in a cell depend on its concentration, the cellular redox state, and the abundance of metals, protein thiols

and low-molecular weight thiols (glutathione), as well as other nucleophil targets (Beckman and Crow, 1993).

Nitric oxide (NO) is now known to be produced by various cells in different organs, including smooth muscle cells, mesangial cells, neurones, platelets, hepatocytes, macrophages, fibroblasts and epithelial cells. It regulates smooth muscle cell tone, platelet aggregation and adhesion, cell growth, apoptosis, neurotransmission and injury as well as infection-induced immune reactions. NO also serves as an endogenous vasodilator, platelet inhibitor, antioxidant, and regulator of vascular endothelium by sustaining its anticoagulant and antithrombogenic properties (Zatz and Baylis, 1998).

2.4.3 Role of RAAS in Nitric Oxide Inhibition-Induced Hypertension

RAAS is a hormone system that regulates blood pressure and water balance. When blood volume or blood pressure is low, the kidney secretes rennin, which stimulates the production of angiotensin I from angiotensinogen; angiotensin I is then converted to angiotensin II by ACE. Angiotensin II is the major bioactive product of the rennin angiotensin system, binding to receptors on intraglomerular mesangial cells, causing these cells to contract along with the blood vessels surrounding them, and causing the release of aldosterone from the *zona glomerulosa* in the adrenal cortex. Aldosterone causes the tubules of the kidneys to retain sodium and water. This increases the volume of fluid in the body, which also increases blood pressure (Solomon and Anavekar, 2005).

Acute inhibition of angiotensin II and α-adrenergic receptors in L-NAME–hypertensive rats almost completely reversed hypertension, raising the possibility that the state of generalized vasoconstriction characteristic of the chronic NOS inhibition model is maintained by an interaction between the sympathetic nervous system and the RAAS. Also, Ribeiro *et al.* (1992) showed that concomitant chronic administration of losartan, an angiotensin II receptor antagonist to rats chronically treated with L-NAME prevented both hypertension and renal injury associated with this model, suggesting a key participation of the RAAS in these events. These findings have been corroborated in several subsequent studies that involved chronic administration of either angiotensin II receptor antagonists or ACE inhibitors (Takase *et al*., 1996). In addition, chronic angiotensin II blockade reversed established hypertension and reduced persistent hypertension after discontinuation of NOS inhibition (Morton *et al*., 1993), raising the possibility that the RAAS plays a causative or permissive role in L-NAME hypertension, even after NO inhibition is interrupted. The angiotensin II dependence of chronic NOS inhibition hypertension may be associated with normal or increased tissue angiotensin II levels (Garcia *et al*., 1997), increased sensitivity to angiotensin II and/or an interaction between Ang II and the sympathetic nervous system (Conrad and Whittemore, 1992).

Plasma rennin activity is unpredictable and bears no consistent relationship with the protective action of chronic angiotensin II blockade as it may be increased (Ribeiro *et al.*, 1992) unchanged or decreased (Arnal *et al*., 1992) in this model of hypertension. A vast amount of experimental data provides evidence that ACE inhibitors increase NO and decrease vascular O_2^- production, partly by inhibiting the generation of angiotensin II and prolonging the half-life of bradykinin. Moreover, reports of an impressive

protective effect of ACE inhibitors on cardiovascular complications in patients and in animal models (Hornig *et al*., 1997) without decreasing blood pressure suggests there is a crosstalk between ACE and the B_2 kinin receptor, such that the ACE inhibitor group of compounds, independent of ACE inhibition, appears to be able to modulate B_2 kinin receptor signalling (Marcic *et al*., 1999).

2.4.3.1 Interactions between angiotensin converting enzyme, kinnins and nitric oxide

Changes in the synthesis of ACE, bradykynin and NO have been associated with a number of cardiovascular conditions, including hypertension, atherosclerosis or coronary heart disease. ACE inhibitors are able to treat these diseases by both accumulation of endothelium-derived kinins and the inhibition of angiotensin II (Linz *et al*., 1995). ACE is a transmembrane zinc metallopeptidase that cleaves carboxy-terminal and is expressed in great amounts in vascular endothelial cells. A soluble form of the enzyme is found in plasma, which is presumably derived from the membrane-bound form by proteolytic cleavage. ACE plays a major role in the regulation of vascular tone by converting the biological inactive decapeptide angiotensin I into the vasoconstrictor and proliferative octapeptide angiotensin II. In a similar manner, ACE inactivates the vasodilatory nonapep-tide bradykinin, which derives from a number of different sources (Gohlke *et al.*, 1992).

Endothelium-derived or exogenously added bradykinin exerts its vasodilatory action by stimulating endothelial B-kinin receptors thereby causing the synthesis and release of vasodilator substances such as endothelium-derived hyperpolarizing factor, prostacyclin and NO (Wiemer *et al*., 1991). Many of the effects of NO on platelets, smooth muscle cells, and cardiac myocytes are mediated by activation of soluble guanylyl cyclase to

synthesize cyclic GMP. The biological function of soluble guanylyl cyclase and NO/cyclic GMP NO in endothelial cells is not yet completely understood, although one function of endothelial cyclic GMP may be a negative feed-back mechanism to turn off further NO synthesis (Martin *et al.*, 1988).

Several studies have demonstrated the ability of NO to modulate the activity or expression of ACE (Linz *et al*., 1999). Michel *et al.* (1996) showed that ACE activity is upregulated in L-NAME-induced hypertensive rats. Also, removal of endothelium from rat aorta increased arterial ACE activity, possibly due to the presence of ACE in the adventitia and in the vascular smooth muscle layer. In contrast, it was demonstrated that enhanced NO or NO releasing compounds were capable of inhibiting the activity of ACE in a concentration-dependent and competitive manner. Also, stimulated endothelial NO release from rat carotid arteries physiologically reduced conversion of angiotensin I to angiotensin II. This inverse relationship between ACE expression or activity and the NO system was also found in hypertensive rats after long-term ACE inhibition, where inhibited vascular and cardiac ACE expression and activity were associated with an upregulated eNOS expression and increased vascular NO release (Linz *et al*., 1999).

2.4.3.2 Effect of angiotensin converting enzyme inhibition on endothelial nitric oxide synthase and nitric oxide

Studies have demonstrated the relative contribution of NO and significance of the endothelium to the dilator response of bradykinin and ACE inhibitors (Wiemer *et al.*, 1996; Linz *et al*., 1999). According to Linz *et al*. (1999), the inhibition of ACE using ramiprilat in cultured bovine aortic endothelial cells stimulates NO synthesis, and induces eNOS expression with enhanced production of cyclic GMP after maximal

stimulation with the calcium ionophore. Wiemer *et al*. (1996), also demonstrated that ACE inhibition and high bradykinin levels enhance accumulation of endothelial cyclic GMP, an index for NO synthesis and release.

Although the mechanism by which ACE inhibition upregulates eNOS expression is unclear, it is conceivable that ACE inhibitors induce accumulation of endogenous kinins to mediate this effect. This was borne out of the fact that the enhanced aortic cyclic GMP content observed after treatment with ramipril was suppressed by co-treatment with an antagonist of badykinin receptor, icatibant (Gohlke *et al*., 1993). Also, cultured bovine aortic endothelial cells incubated for 48 h with 8-bromo-cyclic GMP had higher levels of cyclic GMP, eNOS mRNA and protein (Ravichandran and John, 1995).

Another candidate contributing to the upregulation of eNOS expression induced by ACE inhibition is cyclic AMP. It is known that ACE inhibitors and bradykinin enhance the turnover of endothelial prostacyclin via increased intracellular calcium, which in turn stimulates the formation of cyclic AMP (Schroder and Schror, 1993), known to attenuate intracellular calcium increase and NO release (Luckhoff *et al*., 1990). Although there are no available data on the effect of cyclic AMP on the regulation of eNOS expression, it has been shown that elevated cyclic AMP activated gene transcription via binding to nuclear factor-1 (NF-1), which is present in the promoter region of eNOS (Zhang *et al.,* 1995). This may explain why forskolin, a strong cyclic AMP elevating compound was able to upregulate eNOS protein in cultured bovine endothelial cells (Schroder and Schror, 1993). This intracellular feedback regulation on eNOS expression by cyclic AMP is believed to be more potent and rapid when compared to cyclic GMP (Ravichandran and John, 1995).

2.5 CANCER

2.5.1 Definition and Prevalence

Cancer is the uncontrolled growth and spread of abnormal cells. It starts in one organ or tissue called the primary site; and if undetected or cannot be controlled through treatment, it spread (metastasizes) to other organs throughout the body. Cancer is a genetic disease that involves alterations in three types of genes, known to be responsible for tumorigenesis: oncogenes, tumor suppressor genes and stability genes. Mammalian cells are able to protect themselves against the lethal effects of gene mutation; however, if mutations occur from several genes, they contribute to the development of cancer (Vogelstein and Kinzler, 2004).

In the year 2000, cancer was diagnosed in 10 million people worldwide and caused 6.2 million deaths, an increase of about 22% since 1990. It causes 10% of all deaths worldwide and is second only to cardiovascular disease as the main cause of death in developed countries. Although cancer is usually regarded as a problem of the developed world, about two-thirds of all cancer occurs in the three-quarters of the world's population who live in developing countries. Worldwide, there are about 22 million people living with cancer at any one time. Further, the number of cases of cancer worldwide is predicted to increase by 5 million to 15 million new cases each year by 2020. This is largely due to the increasing longevity of many populations, medical advances in the treatment of other non-communicable diseases, and also current trends in the prevalence of smoking and unhealthy lifestyles, leading to an increase in the incidence of certain forms of cancer (Stewart and Kleihues, 2003).

2.5.2 Stages of Cancer Development

Carcinogenesis (the process of cancer development) is a multistage process divided into three distinct stages: initiation, promotion, and progression. Initiation involves an irreversible genetic change, usually a mutation in a single gene. Promotion is generally associated with increased proliferation of initiated cells, which increases the population of initiated cells. Progression involves accumulation of more genetic mutations leading to the acquisition of the malignant or invasive phenotype (Kinzler and Vogelstein, 1998).

2.5.2.1 Initiation

Initiation is an irreversible event that occurs when a genotoxic chemical, or its reactive metabolite, causes a DNA mutation in a critical growth controlling gene such as Ha-ras (Nelson *et al.,* 1995). Outwardly, initiated cells seem normal, but they remain indefinitely susceptible to promotion and further neoplastic development. DNA mutations that occur in initiated cells confer growth advantages, which allow them to evolve and grow faster bypassing normal cellular growth controls. Three important steps involved in initiation are carcinogen metabolism, DNA repair and cell proliferation. Chemical agents must be metabolically activated before they become carcinogenic. These carcinogens or their active metabolites, are strong electrophiles that bind to DNA to form adducts, which must be removed by DNA repair mechanisms to prevent DNA damage. Failure to repair chemical adducts, followed by cell proliferation, results in permanent alterations or mutation(s) in the genome, leading to oncogene activation or inactivation of tumuor suppressor genes (Minamoto *et al*., 1999).

2.5.2.2 Promotion

Promotion is a reversible process in which chemical agents stimulate proliferation of initiated cells. Typically, promoting agents are non-genotoxic; that is, they are unable to form DNA adducts or cause DNA damage but are able to stimulate cell proliferation. Hence, exposure to tumour promoting agents results in rapid growth of the initiated cells and the eventual formation of non-invasive tumours. Importantly, tumour formation is dependent on repeated exposure to the tumor promoter. Halting application of the tumour promoter prevents or reduces the frequency with which tumours form. The sequence of exposure is important because tumours do not develop in the absence of an initiating agent, even if the tumour promoting agent is applied repeatedly. Therefore, the genetic mutation caused by the initiating agent is essential for further neoplastic development under the influence of the promoting agent (Minamoto *et al*., 1999).

2.5.2.3 Progression

Progression refers to the process of acquiring additional mutations that lead to malignancy and metastasis. Further mutations by agents such as benzo(a)pyrene, β-napthylamine, 2-acetylaminofluorene, aflatoxin B_1, dimethylnitrosamine, 2-amino-3-methylimidazo(4,5-*f*)quinoline (IQ), benzidine, vinyl chloride, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone are usually needed for cells to acquire the phenotypic characteristics of malignant tumor cells (Martinez *et al*., 2003). These chemicals are converted into positively charged metabolites that bind to negatively charged groups on molecules like proteins and nucleic acids, resulting in the formation of DNA adducts which, if not repaired, lead to mutations. The result of these mutations enables the tumours to grow, invade surrounding tissues, and metastasize (Martinez *et al*., 2003).

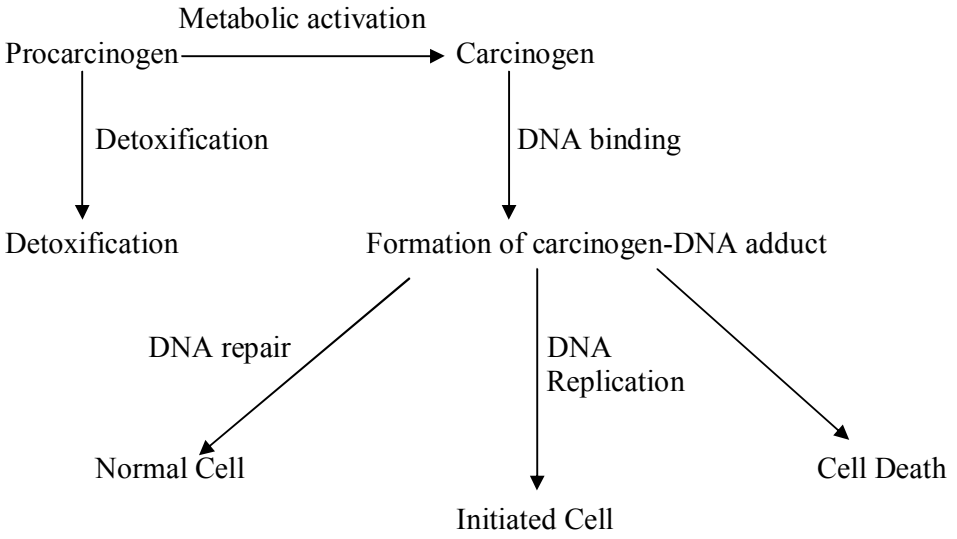


Figure 2.1: Outcomes of carcinogen metabolic activation (Martinez *et al.*, 2003).

2.5.3 Risk Factors of Cancer

Although the major and specific causes of some cancers are known; for example, smoking for lung cancer, reproductive factors for breast cancer, and human papillomavirus (HPV) infection for cervical cancer, little is understood about the causes of other common cancers (Allen *et al.*, 2005).

2.5.3.1 Smoking

It is estimated that tobacco use causes about 4 million deaths each year (Stewart and Kleihues, 2003). Tobacco causes a range of fatal diseases, the most important of which are ischaemic heart disease, chronic obstructive lung disease, and cerebrovascular disease. Tobacco consumption is also the major preventable cause of cancer and a direct and avoidable cause of an enormous cancer burden. This is because smoking accounts for about a third of all cancers, including cancer of the lungs, pancreas, bladder, kidneys, larynx, mouth, pharynx (except nasopharynx) and oesophagus. Chewing betel quid and tobacco, which is common in some parts of South-East Asia, is also an important risk factor for cancer of the mouth and pharynx (World Cancer Research Fund, 1997). Carcinogens such as polycyclic aromatic hydrocarbons and aromatic amines, and other compounds including N-nitrosamines and heavy metals present in tobacco smoke are thought to cause cancer in humans (Allen *et al.*, 2005).

2.5.3.2 Infections

Infections are believed to be the most important established cause of cancer after tobacco smoking. Approximately 18% of cancers worldwide (about 1.6 million cases per year) are attributable to viral (13%), bacterial (5%), and helminth (0.1%) infections (WHO, 2008), the majority of which occur in the developing world. In theory, if these infectious diseases were controlled, up to one in four cancers in developing countries,

and 1 in 10 cancers in developed countries could be prevented (Allen *et al*., 2005). They include:

Human papillomaviruses: The HPV infection is a sexually-transmitted virus and the major causative agent for invasive cervical cancer. The most common HPV subtypes identified are HPV16, 18, 31, 33 and 45, although in some Asian countries the subtypes HPV52 and 58 are more common. HPV infection may also cause some cancers of the head and neck, particularly cancers of the oral cavity and squamous cell carcinomas of the skin. It has been suggested that HPV infection may also be related to cancers of the bladder and prostate, although the evidence remains scanty (Allen *et al*., 2005).

Hepatitis viruses: Chronic infection with the hepatitis B virus is responsible for causing up to 250,000 liver cancers each year, which corresponds to about 60% of all primary liver cancers across the world. The virus can be transmitted from a mother to her child, from person to person during childhood, and via sexual or parenteral transmission during adulthood. Hepatitis C virus is also involved in the aetiology of hepatocellular carcinoma and may cause about 25% of all liver cancers, with particularly high proportions in Africa (41%), Japan (36%), and Oceania (33%) (Allen *et al*., 2005).

Herpes viruses: Infection with the Epstein Barr virus is involved in the aetiology of several types of lymphoma, including Burkitt's lymphoma, Hodgkin's disease, and immunosuppression-related lymphomas and nasopharyngeal carcinoma, and may contribute up to 100,000 cancers per year worldwide. Human herpesvirus-8 (HHV-8), or Kaposi's sarcoma-associated herpesvirus, is closely related to the Epstein Barr virus. It is considered the principal cause of Kaposi's sarcoma. HHV-8 is the most prevalent in populations at highest risk of developing Kaposi's sarcoma, such as homosexual men

infected with the human immunodeficiency virus in Western countries and in African populations where the tumour has long been endemic (Allen *et al*., 2005).

Other infections known to cause cancer are Human T-cell leukaemia virus type 1 (HTLV-1) a causal agent of adult T-cell leukaemia/lymphoma; human immunodeficiency virus (HIV), whose immunosuppressive effect appears to facilitate the development of Kaposi's sarcoma; non-Hodgkin lymphoma, squamous cell carcinoma of the conjunctiva, and probably also Hodgkin's disease and leiomyosarcoma in children. *Helicobacter pylori* increases the risk of gastric adenocarcinoma, and to a lesser extent, gastric non-Hodgkin lymphoma (Allen *et al*., 2005).

2.5.3.3 Dietary-related Factors

The possible role of diet in the aetiology of cancer was highlighted in the 1970s, when it was noted that Western countries that have diets high in animal products, fat, and sugar, have high rates of cancers of the colorectum, breast, and prostate. In contrast, developing countries with diets that are typically based on one or two starchy staple foods, with low intakes of animal products, fat, and sugar have low rates of these cancers (Allen *et al*., 2005).

Despite extensive research over the last two decades, few specific dietary determinants of cancer risk have been established, even for cancers such as colorectal cancer where dietary factors are the most obvious candidate risk factors. This is due to various reasons, the most important being the difficulty in accurately measuring dietary intake in epidemiological studies. Other problems with epidemiological studies of diet and cancer include the relatively narrow range of dietary exposures within one population,

and the changes in dietary patterns over time, so that it is very difficult to determine whether dietary habits at a young age may affect cancer risk later in life (Allen *et al*., 2005).

Obesity: Obesity is defined as an excess accumulation of adipose tissue. It occurs in mammalian species, when calorific intake exceeds energy expenditure. Obesity is associated with an increased frequency of many cancers. Epidemiological data suggest a significant association between increased body mass index (BMI) and several haematological cancers, pancreatic cancer, prostate cancer, post-menopausal breast cancer and other cancers (Khandekar *et. al*., 2011). Several evidence suggests that combinations of factors are secreted by the adipocyte. These factors include increased leptin, decreased adiponectin and increased inflammatory cytokine secretion, along with contributions from the secondary effects of obesity, especially hyperinsulinaemia and hyperlipidaemia, result in the increased incidence of cancer. It is likely that many of these factors collaborate with other environmental factors to result in tumourigenesis. However, there are many unanswered questions as to the precise role of each of these influences (Khandekar *et. al*., 2011).

Alcohol: A high intake of alcoholic beverages increases the risk of cancers of the upper respiratory and digestive tracts (oral cavity, tongue, pharynx, larynx, and oesophagus). These cancers are also caused by smoking, and the increase in risk is particularly great for people who both smoke and drink heavily. Heavy and prolonged alcohol consumption is also associated with liver cancer via the development of cirrhosis and alcoholic hepatitis (Allen *et al*., 2005).

2.5.3.4 Genetic Predisposition

The exploration of the human genome has presented new opportunities to identify the genetic basis underlying cancer development. The evidence for a genetic predisposition to cancer derives originally from observations of cancer clustering in families. Most cancers show some degree of familial clustering and, in general, a person who has a sibling or a parent with cancer has an increased risk of also developing that cancer. A family history of cancer may be partly due to common exposure to environmental agents, as well as shared genetic susceptibility. Some affected families may have an increased susceptibility to several types of cancer; for example, those with multiple endocrine neoplasia syndrome have a substantially increased risk for developing cancers of the pituitary, parathyroid, adrenal, pancreas, and other endocrine tissues. However, it should be noted that family history does not account for the majority of cases of cancer (Allen *et al*., 2005).

2.5.4 Epidemiology and Pathogenesis of Selected Cancers

2.5.4.1 Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related mortality worldwide. Although the majority of HCC cases occur in developing countries of Asia and Africa, the prevalence of liver cancer has risen considerably in Japan, Western Europe as well as the United States. HCC most commonly develops in patients with chronic liver disease, the aetiology of which includes viral hepatitis (B and C), alcohol, obesity, iron overload and dietary carcinogens, including aflatoxins and nitrosamines (Bishayee *et al*., 2010).

The pathogenesis of hepatic carcinogenesis remains incompletely understood, but is believed to involve sequential events, including chronic inflammation, hepatocyte hyperplasia, dysplasia and ultimately malignant transformation. Compelling evidence provides an insight in the role of inflammation in initiation, promotion and progression of HCC. Hepatic inflammation, due to exposure to infectious agents (hepatotropic viruses) as well as toxic compounds represents an early step in the development of malignancy with genetic and epigenetic events, occurring as a later manifestation of a prolonged inflammatory process. An expanding body of evidence suggests that inflammation-mediated processes, including the production of cytokines, chemokines, reactive oxygen and nitrogen species (ROS and RNS), and mediators of the inflammatory pathways may contribute to hepatic neoplasia. Oxidative stress, through generation of ROS, including singlet oxygen, superoxide anion, hydrogen peroxide and hydroxyl radical, acts as a predisposing factor to hepatocarcinogenesis and is the common driving force of HCC in chronic liver diseases (Bishayee *et al*., 2010).

2.5.4.2 Cervical Cancer

Cancer of the cervix is the second most common life-threatening cancer among women worldwide, with incidence rates ranging from 4.8 per 100,000 women per year in the Middle East to 44.3 per 100,000 in East Africa. Epidemiologic and clinical data demonstrate that HPV, especially HPV-16 and HPV-18, play a major role in the aetiology of cervical cancer. However, many investigators acknowledge that HPV is not sufficient to induce cervical cancer and that a multifactorial aetiology which may include herpes simplex virus type 2 infections, cigarette smoking, vaginal douching, nutrition, and use of oral contraceptives, have been proposed as contributing factors (Haverkos, 2005).

Integration of HPV-DNA in the host's genome disrupts or deletes the E2 region, which results in the loss of its expression. This interferes with the function of E2, which normally down-regulates the transcription of the E6 and E7 genes. The interference leads to increased expression of E6 and E7 genes, which subverts the cell growth-regulatory pathways, and modifies the cellular environment in order to facilitate viral replication. The E6 and E7 gene products deregulate the cell growth cycle by binding and inactivating the tumour suppressor protein (p53) and the retinoblastoma gene product (pRb), thus increasing proliferation rate and genomic instability. As a consequence, the host cell accumulates more and more damage DNA that cannot be repaired, leading to transformed cancerous cells (Gomez and Santos, 2007).

2.5.4.3 Colon Cancer

Colorectal cancer is the fourth most common cancer and the second most common cause of cancer death in the United States, with approximately 130, 000 new cases and 55, 000 deaths per year. Compelling experimental and epidemiological evidence indicates that components of Western diets are important factors in the modulation of colon cancer (Potter, 1999). Calcium and vitamin D have emerged as promising chemopreventive agents, and an inverse relationship between dietary intake of folate (and of other one carbon donors) and risk of colorectal carcinogenesis has been shown in humans and experimental animals (Lamprecht and Lipkin, 2003).

The inappropriate expression of β-catenin has been indicated as the hallmark of colon cancer. In normal cells, the wild-type APC protein controls the steady-state levels of β-catenin and has an important role in targeting the protein for degradation. Loss or mutation of APC function results in the translocation of β-catenin to the nucleus, where

it interacts with TCF-family transcription factors and activates the transcription of several genes, including those encoding cyclin D1, c-MYC and the anti-apoptotic protein survivin, which is overexpressed by colorectal tumours (Potter, 1999; Lamprecht and Lipkin, 2003).

2.5.4.4 Mouth Epidermal Carcinoma

Oral cancer is the sixth most common malignancy with about 400,000 new cases annually. Oral cancer is the commonest cancer in the South East Asia. In India, oral cancer comprises 32% to 40% of total malignancy, whereas in western countries it is only 3% to 5% of total cancers (Williams, 2000).

Various researchers using microsatellite markers have demonstrated that alterations in certain regions of chromosomes 3p, 9p, 17p and 18q are related to the development of oral cancer. The most common genetic change and an early event is the loss of the 9p21-22 chromosomal region (containing gene $p16^{INK4a}$/$p14^{ARF}$/CDKN2A). The p53 gene is inactivated in approximately half of oral cancer cases, but there is debate about the timing of this change (Williams, 2000).

2.6 HYPERTENSION AND CANCER

Hypertension and cancer are two leading diseases in the world. Hypertension is the most common cardiovascular disease and a major public health problem in both developed and developing countries. It is a major risk factor for stroke, coronary heart disease, cardiac and renal failure (Benowitz, 2001). Both diseases have similar epidemiological and pathophysiological pathways as well as risk factors, such as age, obesity, smoking and alcohol consumption. Also, majority of cancer patients have coexistent diseases, with more than 50% of cancer patients reporting cardiovascular disorders, including hypertension (Grossman *et al*., 2002).

2.6.1 Mediators between Cancer and Hypertension

2.6.1.1 Nitric Oxide

Nitric oxide (NO) plays an important pathophysiological role in both hypertension and cancer. Several studies have demonstrated the development of hypertension in cancer patients treated with anti-angiogenic drugs such as bevacizumab (Kamba and McDonald, 2007; Pande *et al*., 2007). This is because vascular endothelial growth factor (VEGF) increases expression of eNOS by activation of kinase domain receptor (KDR) tyrosine kinase and protein kinase C signaling; thus, inhibition of VEGF decreases eNOS expression and hence NO levels, which increases mean arterial pressure (fig. 2.2) (Pande *et al*., 2007). Thus, it is clear that NO enhances angiogenesis as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) induce and require NO to elicit their effects (Carrizo *et al*., 1998). Anti-angiogenic therapy-induced hypertension may be caused by increased microvascular resistance and vascular regression resulting from endothelia cell apoptosis (Levy *et al*., 2001).

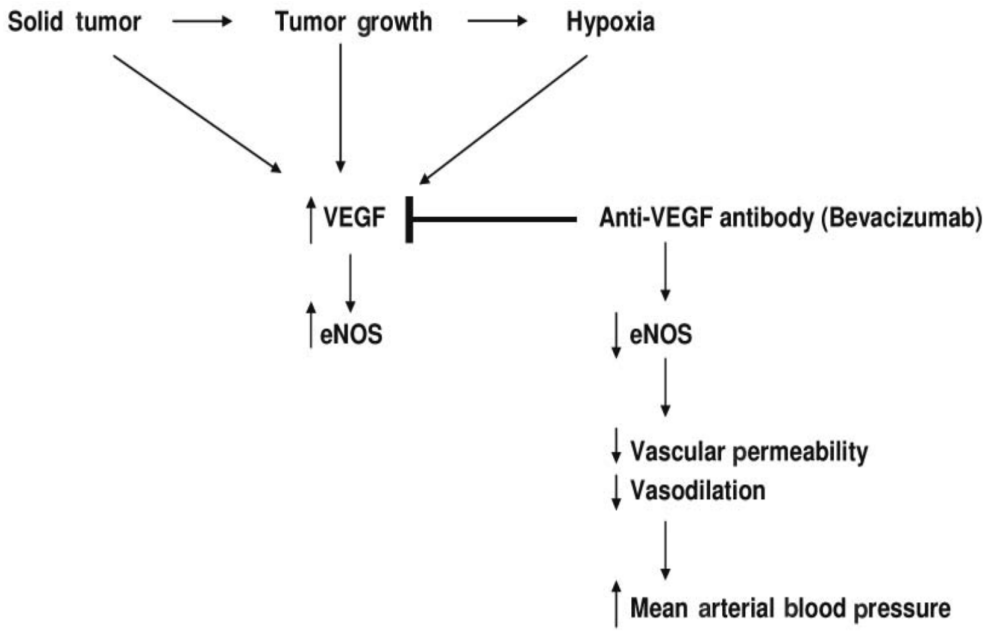


Figure 2.2: Mechanisms of development of hypertension due to anti-angiogenic therapy (Pande *et al.,* 2007).

2.6.1.2 Renin angiotensin system

There is accumulating molecular and *in vivo* evidence to indicate that the expression and actions of the rennin angiotensin system influence malignancy and also predict that renin angiotensin system inhibitors, which are currently used to treat hypertension and cardiovascular disease, might augment cancer therapies. Studies indicate that rennin angiotensin system is frequently dysregulated in malignancy, and this correlates with poor patient outcomes. Antagonism of the rennin angiotensin system suppresses tumour growth, metastasis and angiogenesis in a broad range of experimental models of malignancy, and retrospective studies in humans provide evidence that long-term use of ACE inhibitors may protect against cancer (George *et al*., 2010).

The association between cancer and hypertension is based on specific rennin angiotensin system antagonism rather than on a general antihypertensive effect. This is because patients who take ACE inhibitors and angiotensin receptor blockers had a decreased risk of developing some types of cancers, while there were dysregulation of rennin angiotensin system components in human malignancies (George *et al*., 2010). Although the role of the rennin angiotensin system in tumour angiogenesis is not well understood, both angiotensin II and angiotensin II type-1 receptors have been shown to stimulate angiogenesis. Angiotensin II induces VEGF, thereby playing an important role in VEGF-mediated tumour development and angiogenesis. Similarly, the angiotensin II type-1 receptors receptor pathway supports tumour-associated macrophage infiltration, which results in enhanced tissue VEGF protein levels, angiogenesis and growth of tumour cells (Egami *et al*., 2003).

Several investigators have demonstrated the positive role of angiotensin II and angiotensin II type-1 receptor in tumour angiogenesis, and the antiangiogenic effects of ACE inhibitors and angiotensin II type-1 receptor antagonists. For example, captopril and perindopril have been shown to inhibit angiogenesis. Perindopril, which was shown to be a potent inhibitor of the growth of hepatocellular carcinoma and angiogenesis, also suppresses VEGF at clinically relevant doses. On the contrary, some ACE inhibitors reportedly promote angiogenesis; for example, a very low dose combination of perindopril and indapamide (a non-thiazide sulfonamide diuretic) induces neovascularization (Abali *et al.*, 2002).

2.7 MELANOGENESIS

Melanogenesis can simply be defined as the formation of melanin in living cells. Melanin in human skin is a natural solar filter absorbing and reflecting most of the UV radiation on the skin and thus plays a significant role in skin colour and pigmentation. However, overproduction and accumulation of melanin results in several skin disorders, including freckles, melasma, age spots and other hyperpigmentation syndromes (Huang *et al.,* 2011). Upon exposure of the skin to UV radiation, melanogenesis is initiated through an enzyme called tyrosinase. Tyrosinase (EC 1.14.18.1), a dinuclear type-3 copper-containing mixed function oxidase, catalyzes melanin synthesis via the hydroxylation of a monophenol and the oxidation of o-diphenols into o-quinones, which then polymerize to form brown and black pigments. The enzyme is universally distributed in animals, plants, fungi and bacteria but much is still unknown about its biological function. In higher plants, it protects the plant against insects and microorganisms, catalyzing the formation of a melanin scab impervious to further attack (Kowalski *et al*., 1992; Whitaker, 1995). Tyrosinase is also responsible for the browning of certain fruits and vegetables during handling, processing and storage; and both browning of fruit and skin darkening can be suppressed, at least partially, by inhibition of tyrosinase (Yu and Kim, 2010). The study of tyrosinase inhibitory activity became of interest in recent years because of the significant industrial and economic impact of the inhibitors of this protein (Kowalski *et al*., 1992).

Several studies (Chang, 2009; Wu et al., 2009; Huang *et al.,* 2011) have demonstrated that plants containing high amounts of phenols, flavonols and flavones are good antioxidants and are capable of inhibiting melanogenesis by inhibiting tyrosinase.

2.8 Free-radical Scavenging Activity By 2,2-diphenyl-2-picryl-hydrazine

The model of scavenging the stable 2,2-diphenyl-2-picryl-hydrazine (DPPH) radical is a widely used method to evaluate the free radical scavenging ability of various samples (Chang, 2009; Wu et al., 2009; Huang *et al.,* 2011). DPPH is a stable nitrogen-centered free radical, the colour of which changes from violet to yellow upon reduction by either the process of hydrogen or electron donation. In the presence of hydrogen donors, DPPH is reduced and a stable free radical is formed from the scavenger. The reaction of DPPH is monitored by the decrease of the absorbance of its radical at 515 nm, but upon reduction by an antioxidant, the absorption disappears. Substances able to perform this reaction can be considered as antioxidants and therefore radical scavengers (Hebbel *et al*., 1990).

2.9 Metal Iron Chelation

Bivalent transition metal ions are known to act as catalysts of oxidative processes, leading to the formation of hydroxyl radicals and hydroperoxide decomposition via Fenton Chemistry (Halliwell, 1997). Ferrous ion, known as the most important lipid oxidizing pro-oxidant among the transition metals due to its high reactivity have been demonstrated to catalyze the production of oxyradicals, such as lipid and hydroxyl radicals in its redox-active form (Halliwell and Gutteridge, 1999). These processes can be delayed by iron chelators that mobilize tissue iron by forming soluble and stable complexes that are excreted in feaces and urine. They also reduce iron-related complications in human, thus improving the quality of life and overall survival in some complicated diseases such as thalassemia major, Alzheimer's and cardiovascular diseases (Grazul and Budzisz, 2009) due to the protection against oxidative damage. Ferrozine forms red-colored complexes with ferrous ions, but in the presence of

chelating agents, the ferrozine complex formation is disrupted with a resultant decrease in the red coloured complex formed.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals and Reagents

Gallic acid, vitamin E, vitamin C (L-(+)-ascorbic acid), 2,2-diphenyl-1-picryhydrazyl radical (DPPH), EDTA, sulphorodamine B (SRB), dimethyl sulfoxide (DMSO), ammonium thiocyanate, ferrozine, ferric chloride ($FeCl_2$), Hippuryl-histidyl-leucine (HHL), angiotensin converting enzyme (ACE) from rabbit lung were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tyrosinase from mushroom (4187 U/mg) and L-tyrosine were purchased from Fluka (Switzerland), linoleic acid (Wako Pure Chemical Industrial Ltd., Osaka, Japan), N^G-nitro-L-arginine methyl (L-NAME) and Captopril (Sigma Chemical Co., St. Louis, MO, USA), NucleoSpin RNA kit (Macherey-Nagel GmbH & Co. KG) Attachment factor solution (AFS), Rat aortic endothelial cells and culture medium (Cell Applications, Inc., San Diego, U.S.A). All other chemicals were of analytical grade.

3.1.2 Instruments

Rotary evaporator (Buchi, Switzerland)

Freeze dryer (Christ Alpha 1-2 LD, Germany)

Microplate reader (Bio-Rad, model 680 microplate reader, USA)

Spectrophotometer (Jenway 6100, Dunmow, Essex, U.K)

Molecular image FX (Bio-Rad Laboratories, Hertfordshire, UK, Ltd.)

Cell culture incubator (Shel Lab, model 2123TC, USA)

Fluorescent light microscope (Olympus CK 40, Postfach, Hamburg, Germany)

3.1.3 Plant Material

The leaves of *Peristrophe bicalyculata* (Retz) Nees were harvested at maturity in the month of June, 2010 at Ibadan, Oyo State, Nigeria and in the month of February, 2011. The plant was identified and authenticated by the botanist at the herbarium of the Department of Biological Science, Ahmadu Bello University (ABU), Zaria, with a voucher number 2863.

3.2 METHODS

3.2.1 Preparation and Fractionation of Extracts of *Peristrophe bicalyculata*

The powdered leaves of *Peristrophe bicalyculata* (500 g) was extracted in n-hexane (defatting) using Soxhlet apparatus, before extracting with methanol (70%). The extract was then suction filtered, concentrated under reduced pressure using a rotary evaporator (Buchi, Switzerland) and lyophilized. The methanol extract obtained was then dissolved in distilled water (1000 mL) and partitioned in ethyl acetate and n-butanol using separating funnel, to obtain methanol, ethyl acetate of methanol, butanol and water fractions of methanol extract which were concentrated and lyophilized. Another 50 g of *Peristrophe bicalyculata* was extracted in distilled water (500 mls) by stirring (Harmony Hot Plate Stirrer, Japan) for thirty (30) minutes to obtain cold water extract. Another 50 g was boiled for 30 minutes in hot water to obtain hot water extract. The extracts were sieved using a muslin cloth and then filtered under suction pressure with a Whatman's filter paper (No. 1). They were then concentrated under reduced pressure using a rotary evaporator (Buchi, Switzerland), lyophilized (Christ Alpha 1-2 LD, Germany) and stored at 4^0C until needed.

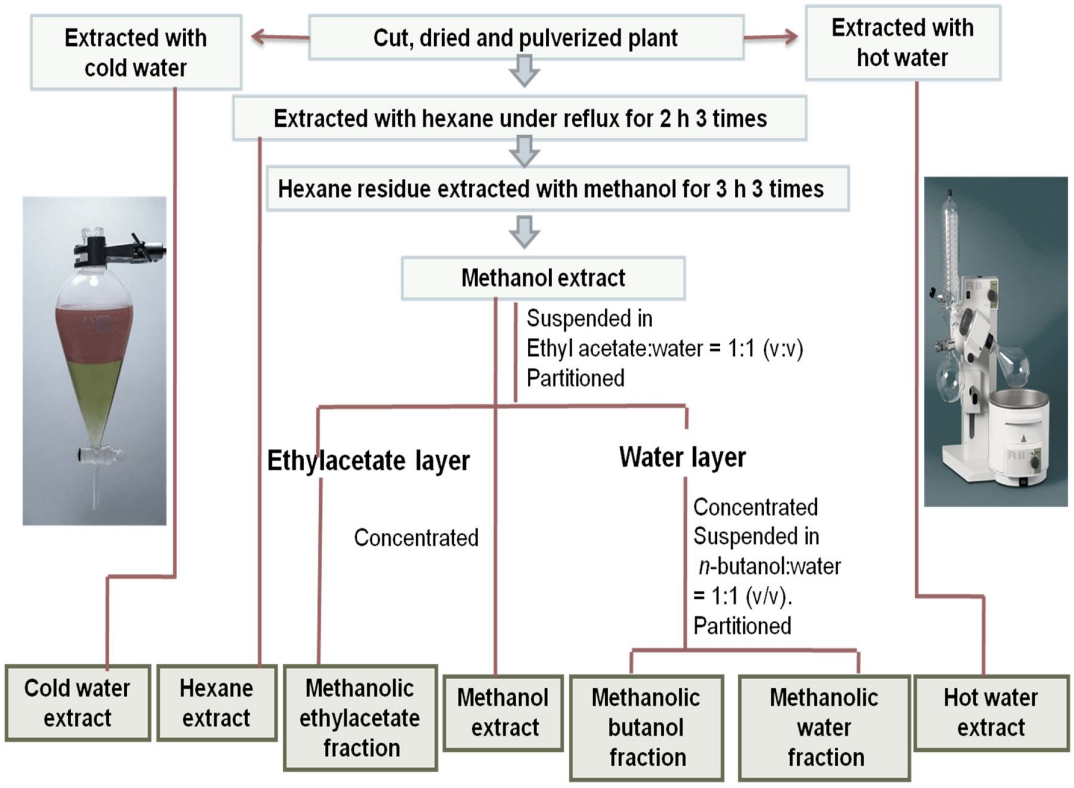


Figure 3.1: Extraction and fractionation steps of *Peristrophe bicalyculata*

3.3 Biological Properties of Extracts of *Peristrophe bicalyculata*

3.3.1 Determination of Total Phenolic and Phytochemical Content

Total phenolic content of the extracts was determined as described by Chandler and Dodds (1993) using gallic acid as a standard phenolic compound. The Folin-Ciocalteu method is based on the reduction of a phosphotungstate-phosphomolybdate complex by phenolic compounds to a blue complex. Briefly, the sample (1 mL) was mixed with 1 mL of ethanol (95%), 5 mL distilled water and 0.5 mL of 50% Folin-Ciocalteu reagent. The mixture was allowed to stand for 5 minutes before adding 1 mL of Na_2CO_3 (5%). This was thoroughly mixed, placed in the dark for one hour before the absorbance was measured at 725 nm.

The phytochemicals: anthraquinones, flavones, cardiac glycosides, saponins, phenols, tannins, carotenoid, triterpines and alkaloids present in the cold water extract were assayed by standard methods (Sofowora, 1993; Trease and Evans, 2002).

3.3.2 Radical Scavenging Assay using 2,2-Diphenyl-2-picryl-hydrazine

Diphenyl-2-picryl-hydrazine is a kind of stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule which is widely used to investigate radical scavenging activity. In the DPPH radical scavenging assay, antioxidants react with the DPPH radical, which is a stable free radical and exists naturally in deep violet color, to turn into a yellow colored α,α-diphenyl-β-picryl hydrazine. The degree of discoloration indicates the radical-scavenging potential of the antioxidant (Tachibana and Kikuzaki, 2001). The free-radical scavenging activities of the extracts and standard antioxidants (vitamin C and E) were determined by a modified DPPH assay (Tachibana and Kikuzaki, 2001). Briefly, 50 µL of five serial

concentrations (0.01 to 100 mg/mL) of the extracts and standard were dissolved in ethanol (95%). About 50 µL of ethanol solution of DPPH was then placed into each well of a 96-well microplate and the samples added. The reaction mixtures were allowed to stand for 30 minutes at 27 ± 2^0C, and the absorbance was measured at 515 nm by a microplate reader against a blank (95%, v/v ethanol). Vitamins C and E were used as positive controls. The experiments were done in triplicate. The percentage radical scavenging activity was calculated as follows:

Scavenging (%) = [(A−B)/A] × 100

Where, A is the absorbance of the control

B is the absorbance of the sample.

3.3.3 Tyrosinase Inhibition Assay

Tyrosinase enzyme catalyses the hydroxylation of L-tyrosine into L-DOPA which is converted by oxidation to O-dopaquinone. O-dopaquinone is then converted through a series of non-enzymic reactions to melanin, which is measured spectrophotometrically (Yu and Kim, 2010). The tyrosinase inhibition activities of the extracts were assayed by the modified dopachrome method using tyrosine as a substrate (Shimizu *et al.*, 1998). Briefly, 50 µL of five serial concentrations of the extracts (0.01-100 mg/mL), 50 µL of 200 units tyrosinase solution in 0.1M phosphate buffer (pH 6.8), 50 µL of 1 mg/ml tyrosine solution in 0.1M phosphate buffer, and 50 µL of 0.1M phosphate buffer were added into a 96-well plate. The mixture was incubated at 37 ± 2^0C for 60 minutes and the absorbance measured at 450 nm. Kojic acid (0.001–10 mg/mL) was used as positive control. All experiments were performed in triplicate. The percentage tyrosinase inhibition was calculated thus:

Tyrosinase inhibition activity (%) = [(A−B) − (C−D)/(A−B)]×100

Where: A is the absorbance of the blank after incubation;

B is the absorbance of the blank before incubation;

C is the absorbance of the sample after incubation; and

D is the absorbance of the sample before incubation.

The sample concentrations providing 50% inhibition (IC_{50}) were calculated from the graph plotted between the percentages of tyrosinase inhibition activity and the sample concentrations.

3.3.4 Metal Ion Chelating Assay

In the metal ion chelating assay, ferrozine forms red-colored complexes with ferrous ions, but in the presence of chelating agents, the ferrozine complex formation is disrupted with a resultant decrease in the red coloured complex formed (Manosroi *et al.*, 2010). Briefly, 100 µL of five serial concentrations of the extracts (0.01-100 mg/mL) dissolved in 95% (v/v) ethanol were added to a solution of 2 mM $FeCl_2$ (50 µL) in distilled water. The reaction was initiated by the addition of 5 mM ferrozine (50 µL) and the total volume was adjusted to 300 µL with distilled water. Then, the mixture was left at 27 ± 2^0C for 15 minutes. Absorbance was then measured at 570 nm using a microplate reader. Ethylenediaminetetraacetic acid (EDTA) was used as a positive control. The negative control contained $FeCl_2$ and ferrozine, which were complex formation molecules. All experiments were performed in triplicate. The inhibition percentages of ferrozine-Fe_2^+ complex formation were calculated by the following equation:

Metal chelating activity (%) = [(A−B)/A] × 100

Where, A is the absorbance of the control and

B is the absorbance of the sample.

The sample concentrations providing 50% metal chelating activity (CC_{50}) were calculated from the graph plotted between the percentages of metal-chelating activity and the sample concentrations (Manosroi *et al*., 2010).

3.3.5 Lipid Peroxidation Inhibitory Effect (Ferric Thiocyanate Method)

The antioxidant activities of the extracts were determined using the Ferric thiocyanate method (Huda-Faujan *et al*., 2007). In this assay, linoleic acid is used as a model system for measuring the levels of lipid peroxides. The lipid peroxide formed reacts with ferric chloride to form ferric ions. Ferric ions then react with ammonium thiocyanate to form ferric thiocyanate, the concentration of which is measured spectrophotometrically. Briefly, 4 mg of each extract was dissolved in 4.0 mL ethanol (99.5%) and kept in a dark bottle. Each sample was then mixed with 4.1 ml linoleic acid, 8 ml phosphate buffer (0.02 M, pH 7.0) and 3.9 mL distilled water to make up to 20 mL. Butylated hydroxyanisole and vitamin E were used as positive controls and another bottle (no sample) was used as a negative control. The solutions were incubated at 40^0C and 0.1 mL taken from each sample. To each sample, 9.7 mL ethanol (75%) and 0.1 mL NH_4SCN (30%, as a colour reagent) were added and precisely 3 minutes after 0.1 mL of $FeCl_2$ (0.002 M) in HCl 3.5% was added to the reaction mixture. The absorbance of the resulting red colour was measured at 500 nm using a spectrophotometer (Jenway 6100, Dunmow, Essex, U.K.) every 24 hour until a day after the absorbance of the control reached maximum value (day seven). The inhibition of lipid peroxidation was calculated as follows:

$$\text{Percentage (\%) Inhibition} = 100 - [(A_1/A_o) \text{ X } 100]$$

Where A_o is the absorbance of the control reaction and

A_1 is the absorbance in the presence of the sample extracts.

3.3.6 Determination of Angiotensin-Converting Enzyme Inhibitory Activity

Angiotensin converting enzyme converts hippuryl-histidyl-leucine (HHL) to hippuric acid and Histidyl-leucine. The concentration of hippuric acid formed is measured spectrophotometrically and determines the activity of the enzyme. The assay for ACE inhibitory activity was determined using the Cushman and Cheung method (1970) with some modifications on the assay conditions. Briefly, the inhibitor solution (purified extract) was added to a test-tube containing 0.1M potassium phosphate buffer (pH 8.3), 5 mM hippuryl-histidyl-leucine (HHL), 0.1M potassium phosphate and 0.3 M NaCl (pH 8.3). Then the enzyme, ACE was added to the mixture and incubated at 37^0C for 30 minutes. The reaction was terminated by adding 0.25 ml of 1 M HCl, and then 1.5 ml ethyl acetate was added to extract the hippuric acid formed by the action of ACE. The ethyl acetate was removed by heat evaporation, residual hippuric acid (HA) dissolved in 1 ml of deionized water, and absorbance of the solution was taken at 228 nm to determine the hippuric acid concentration. The inhibition was calculated from the equation:

$$\frac{Ec - Es}{Ec - Eb} \quad X \quad 100$$

Where Es is the absorbance of the reaction mixture (absorbance of HA generated in the presence of ACE inhibitor component)

Ec is the absorbance of the buffer (the absorbance of HA generated without ACE inhibitors)

Eb is the absorbance when the stop solution was added before the reaction occurred (the absorbance of HA generated without ACE, corresponding to HHL autolysis in the course of enzymatic assay). The inhibitory activity is the amount of inhibitor solution needed to inhibit the original ACE activity by 50% (IC_{50}).

3.4 BIOASSAY-GUIDED ISOLATION OF THE ANTIHYPERTENSIVE COMPONENT(S) OF *PERISTROPHE BICALYCULATA*

3.4.1 Animals

One hundred (100), apparently, healthy male Sprague-Dawley (SD) rats of about eight (8) weeks' old, weighing between 180 and 230 g were obtained from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakhon Pathom, Thailand, and maintained in an air-conditioned animal house of the Faculty of Pharmacy, Chiang Mai University, Thailand. They were fed normal rat chow and allowed access to clean water *ad libitum*. The rats were allowed to acclimatize for two weeks before the commencement of the experiment.

3.4.2 Preparation of P. *bicalyculata* Extracts for Antihypertensive Screening

To determine the antihypertensive activity of the cold water extract and water fraction of methanol extract, doses were calculated based on the estimated quantity of leaves ingested locally as drug (1 g/70 kg body weight). The active component was assumed to be present in 1 g of the crude extract.

The powdered plant (50 g) yielded 9.03 g cold water extract. Therefore, 1 g of the powdered plant contains 1 x 9.03/50 = 0.1806 g active component locally believed to be effective in reducing blood pressure.

This implies 0.1806 g is taken by a 70 kg adult Nigerian man (0.1806 g/70 kg) =

2.58 $x10^{-3}$ g/kg bodyweight = 2.58 mg/kg bodyweight.

However, the extract was found to be ineffective in rats at 2.58 mg/kg until its dose was increased to 10 mg/kg.

For the water fraction of methanol extract, 41.44 g powdered plant yielded 8.49 g

Therefore, 1 g = 1 x 8.49/41.44 = 0.2049g /70 kg bodyweight = 2.93 x 10^{-3} g/kg bodyweight and it equals 2.93 mg/kg.

Thus, 3.0 mg/kg of water fraction of methanol extract was given to rats and was found to be effective.

3.4.3 Preparation of Partially-Purified Fractions of *Peristrophe bicalyculata* used on Hypertensive Rats

Doses of each fraction given to rats were calculated based on the percentage of initial dose (10 mg/kg) and amount of cold water extract (Figure 4.2) used during column chromatography (3 g):

$$\text{Percentage of fraction} = \frac{\text{Weight of fraction (obtained after fractionation)}}{\text{Weight of extract used for fractionation (3 g)}} \times 100$$

Dosage calculation = Percentage of fraction X Initial dose of extract used (10 mg/kg)

Fraction 1

0.0104/3 x 100 = 0.35%

0.35/100 x 10 = 0.03 mg/kg

Fraction 2

0.2127/3 x 100 = 7.09%

7.09/100 x 10 = 0.709 mg/kg

Fraction 3

1.3438/3 x 100 = 44.79%

44.79/100 x 10 = 4.48 mg/kg

Fraction 4
0.2431/3 x 10 = 8.10 %
8.10/100 x 10 = 0.810 mg/kg

Fraction 5
0.9028/3 x 100 = 30.09%
30.09/100 x 10 = 3.009 mg/kg

3.4.4 Experimental Design

The antihypertensive activity of extracts and fractions of *Peristrophe bicalyculata* was investigated in rats induced with acute and chronic hypertension. Acute hypertension was induced at the preliminary stage of the experiment to determine the most active extract. Rats were divided into five groups of six rats each: group 1 (control group), group 2 (hypertensive group), group 3 (hypertensive and given the standard drug, Captopril at 5 mg/kg), group 4 (hypertensive and given water fraction of methanol extract at 3 mg/kg) and group 5 (hypertensive and given cold water extract at 10 mg/kg).

3.4.4.1 Induction of Acute Hypertension

This was done as described by Magos and Vidrio (1991). Rats were fixed on a supine position on a dissecting table and anaesthetized with an intraperitoneal injection of Pentobarbital sodium (40 mg/kg). A longitudinal mid-tracheal incision, approximately 2 cm long was made in order to expose the trachea, the right jugular vein and both common carotid arteries. The trachea was cannulated with a polyethylene tube (2.75 mm diameter) to maintain a free airway. For the administration of extracts and isotonic saline solution, the right jugular vein was cannulated with a saline-filled polyethylene tube (1 mm diameter). The systemic blood pressure was recorded from the cannulated left carotid artery, connected to a Physiological Pressure Transducer (Statham P23 AC Strain gauge Tansducer, Laboratories, Inc. Hato Rey, Puerto Rico) and displayed on a

Grass 7D Polygraph (Grass Instrument Co., Quincy, Mass, USA). The cannulation of the carotid artery was performed in the same manner as that of the jugular vein, and the polyethylene tube (1 mm diameter) filled with heparin sodium in saline solution was used. Blood pressure and heart rate were monitored until steady base-line levels were obtained. L-NAME at 3 mg/kg was administered via venous cannula to induce hypertension. After the blood pressure was stabilized, the extracts were administered through the jugular vein cannula and changes in blood pressure were determined.



Plate I: A rat with cannulated jugular vein and artery attached to 1 mm polyethylene tube filled with saline and heparin sodium.

3.4.4.2 Induction of Chronic Hypertension

Chronic hypertension was induced in rats after partially purifying the most active extract. Twenty five (25) apparently healthy rats were divided into five (5) groups of five rats each: Group 1 (Control group), given the vehicle only; Group 2 (hypertensive control); group 3 (standard control), administered with captopril at 50 mg/kg; group 4, given the active fraction at 2.5 mg/kg; and group 5, administered with the active fraction at 25 mg/kg. All rats in groups 2 to 5 were induced with hypertension by oral administration of L-NAME at 40 mg/kg body wt/day in drinking water for four (4) weeks (Pechanova *et al.,* 2006). The treatment lasted four weeks also. The SBP and DBP of rats in all the groups were measured weekly, using a tail-cuff with a UR-5000 programmed electro-sphygmomanometer (Wang *et al.,* 2008).

Sample Collection: At the end of the experiment, rats were decapitated under anaesthesia to collect blood and tissue samples for the determination of ACE activity, eNOS activity and thiobarbituric acid-reactive substances (TBARS). Kidneys and aorta were removed, cleaned of fatty and connective tissues and blotted dry. They were flash frozen in liquid nitrogen and stored at -70^{0}C until required (Gallagher *et al*., 1999).

Quantitative Determination of Lipid Peroxidation: This was carried out as described by Okhawa *et al.* (1979). Briefly, 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid solution adjusted to pH 3.5 with NaOH, and 1.5 mL of 0.8% aqueous solution of TBA was added to samples less than 0.2 mL of 10% (w/v) tissue homogenate. The mixture was made up to 4.0 mL with distilled water, and then heated in water bath at 95°C for 60

minutes. After cooling with tap water, 1.0 mL of distilled water and 5.0 mL of the mixture of n-butanol and pyridine (15:1, v/v) were added and shaken vigorously. This was centrifuged at 4000 x g for 10 minutes and the absorbance of the organic layer taken at 532 nm. 1,1,3,3-Tetramethoxypropane was used as standard and results were expressed as nmol of malondyaldehyde (MDA).

Determination of Angiotensin-Converting Enzyme Activity: The ACE activity was determined as described by Cushman and Cheung (1971). Organs were homogenised in cold Trizma-HCl buffer (pH = 7.8) (Sigma) and centrifuged at 4°C for 15 minutes and 5000 x g. The supernatants were used for the assay. Briefly, the sample (0.2 mL) was added to ACE solution (50 µL) and the reaction started by adding 0.2 mL of 5 mmol/L hippuric histidyl leucine. This was incubated at 37°C for 15 minutes. The reaction was terminated by adding 0.25 mL of 1.0 N hydrochloric acid and then 2.0mL ethyl acetate to extract the hippuric acid formed by the action of ACE. This was centrifuged at 3600 x g for 2 min, and 1 mL of upper layer transferred into a microcentrifuge tube and heated by dry bath at 100°C for 15 minutes to remove ethylacetate by evaporation. The resulting hippuric acid was dissolved in 3.0 mL of distilled water, and the absorbance read at 228 nm using a spectrophotometer (Jenway 6100, Dunmow, Essex, U.K.). The activity was determined by:

$$\text{Units/ml enzyme} = \frac{(A228nm\ Test - A228nm\ Blank)\ (2)\ (3)}{(a)(b)(c)(d)}$$

Where; 2 = Conversion factor since the hippuric acid detected was 1/2 of the total amount produced in the assay: 2 mL of ethyl acetate was added and 1 mL of the organic layer containing the product, hippuric acid, was removed.

3 = Total volume of hippuric acid solution

a = Millimolar extinction coefficient of hippuric acid at 228 nm (9.8)

b = Time (in minutes) of the assay as per the unit definition (15 minutes)

c = Extraction efficiency of ethyl acetate (0.91), d = Volume (in milliliter) of enzyme used (0.05)

Serum enzyme activity was expressed in units, which corresponded to 1 µmol of hippuric acid released by hydrolysis of HHL per minute per milliliter serum, while activity in tissues was expressed in units which corresponded to 1 µmol of hippuric acid released by enzymatic hydrolysis of HHL per minute per milligram of tissue.

Determination of Nitric Oxide Synthase Activity: The activity of nitric oxide synthase was determined as described by Ghigo (2006) with some modifications. The principle of this assay is based on the conversion of nitrate to nitrite and the spectrophotometric quantitation of nitrite using Griess reagents. Briefly, tissue samples were homogenized in 1 x PBS (pH 7.4) and centrifuged at 10,000 x g at 4^0C to obtain supernatant. Serum was also obtained from blood samples and pippetted into well-labelled Eppendorf tubes, before adding 80 µL assay buffer, 5 µL substrate, 5 µL NADPH (1 mM), 13 µL nitrate reductase and 1 µL glyceraldehydes-3-phosphate dehydrogense. For sample blank, 10 µL distilled water was used instead of substrate and NADPH. The NOS working reagent (100 µL) was then added to each tube and incubated at 37^0C for 20 minutes, before adding 7 µL zinc sulphate and 7 µL sodium hydroxide. The mixture was vortexed and centrifuged at 14,000 x g for 10 minutes and 100 µL of the clear supernatant was transferred to a clean tube. Nitric oxide formed was determined by adding 100 µL sulphanilamide, and 100 µL N-(1-Naphthyl)-ethylenediamine to all tubes before adding 200 µL of the NO working reagent to each sample and standard tube. This was incubated for 5 min at 60°C, centrifuged to pellet any condensation and 250

µL of each reaction transfered to separate wells in a 96-well plate and absorbance read at 540 nm. Standard curve was prepared using standard nitrate, by subtracting the absorbance of blank from absorbance of standard and plotting this against standard concentrations and slope calculated. Values of NOS activity were obtained as follows:

$$\text{Nitric oxide synthase activity} = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}}{\text{Slope}} \times 1/t$$

$\text{Abs}_{\text{sample}}$ and $\text{Abs}_{\text{blank}}$ are absorbance values of the sample and sample blank respectively and t is the reaction time (20 minutes). One unit of NOS catalysed the production of 1µmole of nitric oxide per minute at pH 7.5 and 37^0C.

3.4.4.3 Partial Purification of Cold Water Extract of Peristrophe bicalyculata

The cold water extract of *Peristrophe bicalyculata,* which had the most antihypertensive activity, was partially purified by column chromatography after thin layer chromatographic separation.

The thin layer chromatography was carried out using ethylacetate:formic acid:methanol (1:1:3) solvent which gave five separate constituents, visible under UV-spectrum (Plate V). Column chromatography using silica gel was then run with ethyl acetate (100%), ethyl acetate:formic:methanol (15:2:0.5), ethyl acetate:methanol (1:1), ethyl acetate:methanol (1:3), ethyl acetate:methanol: formic acid (1:3:1) and methanol: formic acid (9:1) and ten fractions were obtained (Table 4.7). The fractions were reduced to five (Figure 4.2) after thin layer chromatographic separation (Plate VI).

3.5 DETERMINATION OF ANGIOTENSIN CONVERTING ENZYME AND ENDOTHELIAL NITRIC OXIDE SYNTHASE EXPRESSION

3.5.1 Rat Aortic Endothelial Cell Isolation

Rat aortic endothelial cells were isolated from Sprague Dawley rats by the enzymic technique as described by Chen *et al.* (2004) with some modifications. Rats were anesthesized with nebutal (40 mg/kg), washed clean and sterilized by cleaning with 70% ethanol. A midline laparotomy was made, and the aorta isolated and removed carefully. The periadventitial fats around the vessels were carefully cleaned under a dissecting microscope using forceps and iris scissors. Both ends of the aorta were cannulated with Intramedic® PE-20 tubes (inner diameter, 0.04 cm, outer diameter, 0.13 cm) and tied. The aortas were soaked for 20 min at 24^0C in phosphate-buffered saline (PBS), pH 7.4, containing 100 mg/mL penicillin-streptomycin and 50 µg/mL amphotericin B. The aortas were then flushed with 0.5 mL PBS, pH 7.4. After which 50 µL of 0.25% trypsin in PBS was instilled into the aorta, allowed to dwell for 2 min, and drained into a 15-mL tube, containing 10 mL rat aortic endothelial cell culture medium, supplemented with 20% foetal bovine serum (FBS), 50 µg/mL amphotericin B and 100 mg/mL penicillin-streptomycin. This process was repeated five to six times for each aorta. The collected outflow was centrifuged at 300 × g for 5 min. The supernatant was discarded, and the cell pellet resuspended in 1 mL complete rat aortic endothelial cell culture medium with 10% FBS and transferred to a 24-well plate, one well per aorta. The cells were grown at 37°C in 95% O_2 and 5% CO_2. After 10-14 days, trypsin was used to detach cells for further subculture in 25 cm^2 plastic culture flasks. The cells were identified by their cobblestone structure. They were also compared with rat aortic endothelial cells using an Olympus culture microscope.

3.5.1.1 Rat aortic endothelial cell culture

The culture flask was first prepared by adding 7.5 ml attachment factor solution (AFS) to a T-75 flask under a biological safety cabinet, rocked gently to distribute the solution evenly to cover the whole culture surface and incubated for 30 minutes at 37°C or 2 hours. The cryopreserved vial of rat aortic endothelial cell (RAOEC), was then removed from the liquid nitrogen storage tank, using proper protection for the eyes and hands, thawed quickly by placing the lower half of the vial in a 37°C water bath for 1 minute and re-suspended by gently pipetting the cells. The cell suspension (1mL) was transferred into the AFS coated T-75 flask containing 15 ml of rat aortic endothelial cell medium and incubated at 37°C in a humidified 5% CO_2 incubator. The medium was changed after 24 hours to remove all traces of dimethyl sulphuroxide (DMSO). The medium was changed every other day until the cells reached confluent. The cells were sub-cultured.

3.5.1.2 Cytotoxic studies

Cells were seeded at 1 $x10^4$ and dose-response studies performed using the active fraction at concentrations of 1, 10, 100, 1000 µg/ml and captopril at 100, 10, 1 and 0.1 mmol/L to determine the least cytotoxic concentration. The cells were cultured in RAOEC medium at 37°C in a humidified 5% CO_2 incubator. Two concentrations (10 and 100 µg/ml) of the fraction and 1 mmol/L captopril were the least cytotoxic on cells and were used for further experiments.

3.5.1.3 Protocol of incubation studies

Confluent endothelial cells were washed with PBS and seeded at 1 x 10^5 per well into 24-well plates, 20 µl of 2 mmol/L L-NAME added to all groups, except the control before adding test samples. The total volume was adjusted to 1000 µl with RAOEC medium and incubated for 24 h (Morita *et al*., 1995; Seki *et al*., 1997) at 37^0C, 95% O_2 and 5% CO_2.

3.5.2 Protocols for RNA Isolation and Quantitation

3.5.2.1 Harvesting of cells

This was done according to the manufacturer's protocol. Briefly, the cell culture medium was aspirated before adding PBS to wash the cells. Trysin/EDTA (0.1-0.3%) was used to detach the cells, after which culture medium was added to stop trypsinization. This was centrifuged for 5 minutes at 3,000 x g to obtain the cells.

3.5.2.2 Isolation of total RNA from kidneys and aorta of rats

The frozen kidneys and aorta were disrupted by grinding into powder using a mortar and pestle in liquid nitrogen. Buffer containing β-mercaptoethanol was added to the powder, mixed immediately and homogenized with a NucleoSpin filter. This was followed by addition of 350 µL of ethanol (70%) whilst still mixing vigorously, loaded on a column (NucleoSpin RNA column) and centrifuged for 30 s at 11,000 x g. 350 µl of membrane desalting buffer was then added and centrifuged at 11000 x g for 1 minute to dry the membrane. The DNA was digested in 95 µL rDNase reaction and incubated at room temperature (24-26^0C) for 15 minutes. The membrane was then dried and RNA eluted by centrifuging for 1 minute at 11,000 x g.

3.5.2.3 RNA Quantitation, Integrity and Purity

Total RNA was quantified using a NanoDrop 1000 spectrophotometer. The integrity was determined using 2% agarose gel electrophoresis. Briefly, 100 ml of Tris-acetic-EDTA buffer (24.2 g Tris, 5.71 g glacial acetic acid and 0.5 mM EDTA) was added to 2 g of agarose in a 250 mL conical flask. The suspension was heated in the microwave oven to melt the agarose, before pouring into the agarose gel rack with the combs well placed. The gel was then loaded with samples after solidification, placed in the electrophoresis compartment and 1XTAE buffer added. The set up was then connected to a power pack and ran for 45 minutes at 10 V/cm, after which it was soaked in ethidium bromide for 15 minutes before viewing. Discrete and thick 28S: 18S rRNA gel bands with an approximate mass ratio of 2:1 indicated high integrity.

3.5.2.4 Primer Design

The DNA sequence of the genes of interest (ACE and eNOS) and control gene (β-actin) were obtained from NCBI website (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide), before designing appropriate primers. Care was taken to avoid non-coding regions within the sequences. Also, a BLAST search was run on all primers to be sure of their non-annealing to other targets. Primer sequences were as follows: ACE: forward primer 5'-GCCACGTCCCGGAAATACGAAGAATTGC-3', and reverse primer 5'-GCGACATAGTGCCTCGTGGAACTGGAAC-3', (GenBank accession no. NM_012544.1); eNOS forward primer 5'-TCCAGTAACACAGACAGTGCA-3' and reverse primer 5'-CAGGAAGTAAGTGAGAGC-3', (GenBank accession no. RNU_02534) and β-Actin forward primer 5'-ACAACCTTCTTGCAGCTCCTCC-3'

and reverse primer 5'-GAAGCATTTGCGGTGCACGATG-3', (GenBank accession no. NM_031144.2).

Conditions for angiotensin-converting enzyme: They were: cDNA synthesis at 55^0C for 30 minutes, 30 cycles of denaturation at 95^0C, annealing at 60^0C for 30 seconds, elongation at 72^0C and final elongation at 72^0C for 10 minutes.

Conditions for endothelial nitric oxide synthase: They were: cDNA synthesis at 55^0C for 30 minutes, 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 seconds, elongation at 72°C for 1 minutes and final elongation for 10 minutes.

Conditions for β-Actin: They were: cDNA synthesis at 55^0C for 30 minutes, 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 seconds, elongation at 72°C for 1 minute and final elongation for 10 minutes.

3.5.2.5 Reverse Transcriptase and Real Time Reverse Transcriptase Polymerase Chain Reaction (RTRT-PCR)

cDNA was prepared from RNA of cells and tissues and then reverse transcribed using Superscript III one-step RT-PCR system with platinum Taq DNA polymerase (Invitrogen). Briefly, 10 µL of RNA, 1 µL forward and 1 µL reverse primers, 12.5 µL 2X reaction mix buffer (0.4 mM of each dNTPs, 3.2 mM $MgSO_4$) and 0.5 µl of Taq enzyme were added into a PCR reaction tube, and mixed gently. Reverse transcriptase-polymerase chain reaction was then run after setting appropriate conditions on the PCR machine (Kobayashi *et al.*, 2000; Meis and Khanna, 2009). Quantitative real time PCR was run using SYBR green core reagents, as described by the manufacturers (Sigma

Aldrich) and performed with a stepOne real time PCR system. Briefly, 10 µL of cDNA, 0.8 µL forward and 0.8 µL reverse primers, 12.5 µL 2X SYBR Green PCR ReadyMix (containing HotStarTaq *Plus* DNA Polymerase, dNTPs, $MgSO_4$ and SYBR Green dye) and 0.9 µl of RNase-free water were added into a PCR reaction tube, and mixed gently. Melting curve analysis on final PCR products were done by heating to 95^0C and cooling to 50^0C, before gradually increasing temperature. The relative quantification of ACE and eNOS expression was normalized to β-actin and calculated with the Pfaffl method using the Relative Expression Software Tool (REST, http://www.rest.gene-quantification).

Ratio of ACE or eNOS/ β-actin =

$$\frac{(E_{target})^{\Delta Ct\ target\ (control-sample)}}{(E_{reference})^{\Delta Ct\ ref\ (control-sample)}}$$

Where;

E_{target} = Real time PCR efficiency of target gene (ACE or eNOS);

$E_{reference}$ = Real time PCR efficiency of reference gene (β-actin)

ΔCt_{target} and ΔCt_{ref} = Ct deviation of control – sample of target and reference gene transcript respectively

Purification of PCR Product: The PCR products obtained above were purified to remove traces of unused primers using the Nucleospin Extract II kit. Briefly, Buffer NT (200 µL) was diluted in 800 µL sterile water before adding 100 µL of the PCR product. The mixture was loaded on a Nucleospin extract II column and centrifuged for one (1) minute at 11,000 x g and the flow-through discarded. The column was placed in another collection tube and 700 µl of buffer NT3 added. This was centrifuged at 11,000 x g for 1 minute. The silica membrane was dried by discarding the flow through and

centrifuging again to remove all traces of NT3. The column was incubated at 70^0C for 3 minutes to remove all traces of ethanol from buffer NT3. The column was then placed in a new microcentrifuge tube (1.5 mL), 100 µL buffer NE added and incubated at room temperature for 1 minute. This was centrifuged at 11,000 x g for 1 minute to elute DNA from the PCR product (Kobayashi *et al.*, 2000).

Agarose Gel Electrophoresis: PCR products were separated by electrophoresis on a 1.8 % agarose gel using TAE buffer (1X) and ethidium bromide and were visualized by ultraviolet-induced fluorescence. Briefly, the buffer (50X) was prepared by weighing tris base (242 g), glacial acetic acid (57.1 g), 0.5 mM EDTA (pH 8.0) and made up to 1000 mL with distilled water. TAE buffer (1X) was prepared by measuring 20 mL of 20X and made up to m1000 mL. The agarose gel was prepared by adding 100 mL of the buffer (1X) to 1.8 g of agarose in a 250 mL conical flask. The suspension was heated in the microwave oven to melt the agarose, and then poured into the agarose gel rack with the combs well placed. Thereafter, the gel was loaded with samples after solidification, was placed in the electrophoresis compartment and 1XTBE buffer added. The set-up was then connected to a power pack and run for 45 minutes at 12 V/cm, after which it was soaked in ethidium bromide (2 mg/L) for 15 minutes before visualizing by Molecular image FX and amount of mRNA was described as the ratio to β-actin (Kobayashi *et al.*, 2000).

3.6 ANTICANCER ACTIVITY OF *PERISTROPHE BICALYCULATA*

3.6.1 Cell culture

3.6.1.1 Preparation of Media

Incomplete Dulbecco's Modified Eagle's Medium (DMEM) was prepared by mixing 10.43 g of DMEM, 2 g sodium hydrogen carbonate (Na_2HCO_3), 3.57 g 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 1.40 µL β-mercaptoethanol in a flat bottom flask and made up to 1000 mL with distilled water. This was then sterilized by filtering with 0.2 µm pore size cellulose acetate membrane into a sterile bottle. The medium was stored at 4^0C until needed.

Complete Dulbecco's Modified Eagle's Medium (CDMEM) was prepared in a sterile hood by adding 9 mL of fetal bovine serum (FBS) and 1 mL penicillin-streptomycin to 90 mL DMEM. This was adequately labelled and stored at 4^0C.

3.6.1.2 Sample preparation

All extracts were dissolved in DMEM to a final concentration of 10 mg/mL. The solutions were centrifuged at 5,700 x g at room temperature (24-26^0C) for 2 minutes and sterilized by filtration with 0.22 µm pore size cellulose acetate membrane. This was then diluted with the complete DMEM medium to concentrations of 0.1 to 1000 µg/mL. All samples were stored at -20°C until required.

3.6.1.3 Cell lines

HeLa, HT-29, HepG2 and KB were obtained from the American Type Culture Collection and stored in nitrogen tanks.

3.6.1.4 Cell culturing

A stock of each cell line from the nitrogen tank was quickly thawed in 70^0C water by swirling gently. This was transferred into a 25 cm^3 plastic culture flask, containing 5 mL CDMEM under a sterile laminar hood. The cells were incubated in a temperature-controlled and humidified incubator aerated with 95% O_2 and 5% CO_2 at 37^0C (Butler, 2005).

Cell Harvesting: When cells reached confluence, they were washed with PBS and trypsinized to detach cells. Trypsin was discarded and 10 ml of CDMEM added to neutralize any remaining trypsin. The cells were then transferred into 75 cm^3 culture flasks (Freshney, 2005).

Cell counting: Cells were counted using a haemocytometer as described by Butler (2005). Briefly, the cell culture suspension obtained after trypsinization was centrifuged at 2,200 x g for 5 minutes and 4^0C. The cells were then counted and approximately 1×10^6 cells/mL was transferred to a haemocytometer slide and counted using a microscope (Olympus CK 40, Japan). The counting was repeated three times and the average taken (Freshney, 2005).

*Cell plating: A*proximately 10,000 cells were seeded per well into a 96-well plate, total volume adjusted to 200 µL with the CDMEM medium and incubated in a temperature-controlled and humidified incubator (Shel Lab, model 2123TC, USA) aerated with 95% O_2 and 5% CO_2 at 37^0C for 24 hours (Freshney, 2005).

3.6.1.5 The sulphorhodamine assay

The anti-proliferative activity of the extracts on cancer cell lines was determined by the modified SRB assay (Papazisis *et al*., 1998). The assay relies on the ability of SRB to bind protein components of cells that have been fixed to tissue culture plates by trichloroacetic acid. SRB is a bright-pink aminoxanthene dye with two sulfonic groups that bind to basic amino acid residues under mild acidic conditions, and dissociate under basic conditions (Vichai and Kirtikara, 2006). The culture plates were divided into 3 groups: the reference plate (T_0 plate), the treated plate (T_{treat}) and control plate (C). For the reference plate (T_0 plate), after 24 h incubation, the cells were fixed with ice-cold 50% trichloroacetic acid (TCA) solution at 4^0C for 2 h. The plates were then washed with distilled water five times and air-dried overnight. The fixed plate was dyed by adding 50 µL 0.4% SRB solution to each well to allow staining at room temperature for 30 minutes, before washing with 1% acetic acid to remove unbound dye. The plates were then air-dried overnight again. The bound SRB was solubilised by adding 100 µL Tris-solution to each well and shaking for 30 minutes on a shaker platform. The absorbance of all plates was taken at 540 nm with a microplate reader. All experiments were done in triplicate.

For the treated plates (T_{treat}), after the first 24 h of incubation, the cells were treated with 20 µL of the extracts at the different concentrations, while 20 µL complete DMEM was added to control plate (C). The plates were then re-incubated for 24 hours, fixed and dyed using the same steps described above for reference plate. The percentage cell growth (%G) was calculated thus:

$$\%G = (T_{treat} - T_0/C - T_0) \times 100$$

Where; T_{treat} is the absorbance of the extract-treated plates,

C is the absorbance of the control plate, and

T_0 is the absorbance of the reference plate.

The concentration of extract providing 50% inhibition (IC_{50} mg/mL) was calculated from the graph of correlation of percentage growth against concentrations of the extracts. In addition, the cytotoxic level of the extracts was classified based on the IC_{50} value obtained as described by Boyed (1989): Potentially very toxic (<10 µg/mL), potentially toxic (10 to 100 µg/mL), potentially harmful or moderately toxic (100 to 500 µg/mL) and potentially non-toxic (>500 µg/mL).



Plate II: A filter under suction attached to a culture bottle in the laminar flow.



Plate III: Plating of cells under a sterilized hood in preparations for the SRB assay.



Plate IV: A cell culture incubator containing cell culture flasks and plates.

3.6.1.6 Partial Purification of Ethyl Acetate Fraction of Peristrophe bicalyculata

The ethyl acetate fraction of methanol extract of *Peristrophe bicalyculata*, which was most cytotoxic against human mouth epidermal carcinoma (KB) was partially purified by column chromatography after thin layer chromatographic separation.

The thin layer chromatography was carried out using ethylacetate and Hexane (5:5) solvent which gave six separate constituents, visible under UV-spectrum (Plate X). Column chromatography using silica gel was then run with hexane (100%), hexane:ethyl acetate (8:2), hexane:ethyl acetate (5:5), hexane:ethyl acetate (3:7), ethyl acetate:chloroform (9:10), ethyl acetate:methanol (9:1) and ethyl acetate: methanol (1:1) and ten fractions were obtained (Table 4.11). The fractions were reduced to seven after thin layer chromatographic separation (Plate 10). The first fraction from hexane:ethyl acetate (8:2) (fraction 2a) was labelled fraction 1, while fractions 2b and 2c were combined to give fraction 2. The hexane:ethylacetate (5:5), hexane:ethylacetate (3:7) and ethylacetate:chloroform (9:10) solvents each gave three different fractions with different characteristics (fractions 3, 4 and 5). The fractions from ethylacetate: methanol (9:1) were combined to give fraction 6, while fractions 7a and 7b were combined to give fraction 7 (Figure 4.15).

3.6.1.7 Acridine orange/ethidium bromide (AO/EB) staining

Morphological assessment of apoptotic cells was performed using the 96-well-based-AO/EB staining method as described by Ribble *et al*. (2005). Briefly, 10,000 cells were seeded per well in 96-well microtitre plates divided into different groups, based on sample concentration and number of incubation days. After 24 h of incubation in appropriate medium, they were treated with the standard drug, cisplastin at 1000, 100

and 10 µg/mL and the extract at 1000, 500 and 250 µg/mL and incubated for another 24 and 48 h at 37^0C in a 95% O_2 and 5% CO_2 atmosphere. Thereafter, 8 µL of AO/EB dye mix was added to each well. The cells were then viewed and counted under fluorescent light microscope and microphotographs taken.

3.7 MELANOGENESIS INHIBITORY EFFECT OF *Peristrophe bicalyculata*

3.7.1 Cell Culture

Mouse melanoma cells ($B_{16}F_{10}$) were cultured under standard conditions in complete culture medium, containing DMEM supplemented with 10% (v/v) foetal bovine serum, penicillin (100 U/mL) and streptomycin (100 mg/mL). Cells were incubated in a temperature-controlled and humidified incubator with 95% O_2 and 5% CO_2 at 37^0C.

3.7.2 Inhibitory Effect of Melanin Biosynthesis of $B_{16}F_{10}$ Melanoma Cells

The cytotoxic effect of all extracts on $B_{16}F_{10}$ melanoma cells were initially determined using the sulphorhodamine B colorimetric assay (Papazisis *et al*., 1998) before determining the *in vitro* melanin production of the least cytotoxic extracts. The inhibitory effect of melanin biosynthesis was done as described by Arunga *et al*. (2007). Briefly, confluent cultures of $B_{16}F_{10}$ melanoma cells were rinsed in PBS and trypsinized using 0.25% trypsin/EDTA. The cells were placed in 24-well plastic culture plates at a density of 1 x 10^5 cells/well and incubated for 24 h in media prior to being treated with the samples. After 24 h, the media was replaced with 998 µL of fresh media and 2 µL of DMSO were added, with or without (control) the test sample, at various concentrations (n = 3). Kojic acid was used as a positive control. The cells were incubated for an additional 48 h, and then the medium was replaced. After 24 h, the remaining adherent cells were assayed.

3.7.2.1 Determination of melanin content in $B_{16}F_{10}$ melanoma cells

The melanin content of cells after treatment was determined as described by Arunga *et al.* (2007): after removing the medium and washing the cells with PBS, the cell pellet was dissolved in 1.0 mL of 1 N NaOH. The crude cell extracts were assayed using a microplate reader at 405 nm to determine the melanin content. The results from the cells treated with the test samples were analyzed as a ratio of the results from the control culture.

3.8 GAS CHROMATOGRAPHY-MASS SPECTROMETRIC ANALYSIS of PARTIALLY PURIFIED FRACTIONS OF *PERISTROPHE BICALYCULATA*

The gas chromatography-mass spectrometric (GC-MS) analysis was conducted using Shimadzu machine (GC-2010, Shimadzu Scientific Instruments, Tokyo, Japan). The mass analysis machine (GCMS QP2010) was connected to the column RTX-5MS.30 m.0.25 mm internal diameter.

3.8.1 Sample Preparation for GC-MS

Into samples from both fractions, 1 mL Milli-Q water was added, vortexed vigorously and kept at room temperature for 1 h. The resulting solution was vortexed again and centrifuged (MaxSpeed/RT/15'). The supernatant was freeze-dried for 2 h and re-suspended in a solution of Ethanolate:chloroform (9:1).

3.8.2 GC-MS Procedure

The samples were filtered with a microsyringe (20 micra) and 2 μL of each was injected into the GC (on-column injection). Care was taken not to use plastic syringe. Using hexane as blank, the machine was adjusted to detect molecules with molecular mass ranging from 50 to 280 Daltons over a temperature gradient of 50 to 280 ^{o}C. Spectra were generated using the in-built softwares (GC-MS solution version 2.5 SUI). Gas chromatography-mass spectrometric realtime analysis and GC-MS post-run analysis were evaluated using three libraries: NIST, Wiley intergrated and a domestic library using a CAS number of EMBRAPA Genetic Resources and Biotechnology. Each spectrum was confirmed by two replicated readings to ensure reproducibility. All peaks were further analysed for compound identification, based on their similarities with structures available in the libraries.

3.9 STATISTICAL ANALYSIS

Data obtained were expressed as mean ± standard error of mean (mean ± SEM) and analysed using SPSS 17. The significance among groups was determined by one way analysis of variance and LSD post hoc test was applied for multiple comparisons. Values of $P < 0.05$ were regarded as statistically significant.

CHAPTER 4

4.0 RESULTS

4.1 YIELD AND BIOLOGICAL ACTIVITIES OF *PERISTROPHE BICALYCULATA*

4.1.1 Percentage Yield of Extracts of *Peristrophe bicalyculata*

The percentage yields of extracts of *Peristrophe bicalyculata* are presented in table 4.1. The yield from 50 g of the cold and hot water extracts were 9.03 ± 0.44 and 7.79 ± 2.24 g, respectively, while extraction of 500 g with methanol yielded 41.44 ± 5.87 g. Further fractionation of the methanol extract with ethyl acetate, butanol and water yielded 12.28 ± 4.37, 8.61 ± 2.66 and 8.49 ± 3.46 g, respectively.

Table 4.1: Yield and percentage yield of extracts of *Peristrophe bicalyculata*

Extract	**Yield (g)**	**Percentage Yield (%)**
Cold Water (50 g)	9.03 ± 0.44	18.06 ± 0.88
Hot Water (50 g)	7.79 ± 2.24	15.58 ± 4.49
Hexane (500 g)	4.61 ± 1.17	1.00 ± 0.23
Methanol (500 g)	41.44 ± 5.87	8.29 ± 1.15
Ethyl acetate of Methanol	12.28 ± 4.37	2.46 ± 0.88
Butanol of Methanol	8.61 ± 2.66	1.72 ± 0.52
Water of Methanol	8.49 ± 3.46	1.70 ± 0.69

Values are Mean ± SEM

4.1.2 Phytochemical and Total Phenolic Content of *Peristrophe bicalyculata*

Results from the phytochemical analysis (Table 4.2) of cold water extract of *Peristrophe bicalyculata* shows the presence of flavones, cardiac glycosides, tannins, steroids, saponins and alkaloids, with cardiac glycosides, flavones and alkaloids at very high concentrations.

The total phenolic contents of the cold water extract (152.35 ± 14.02 mg/g), methanol extract (150.00 ± 17.25 mg/g) and ethyl acetate fraction of methanol extract (140.00 ± 15.45) were not significantly different, but were significantly ($P < 0.05$) higher than that obtained in any other extract. The hexane extract (40.65 ± 2.09 mg/g) had the least phenolic content (Table 4.3).

4.1.3 Antioxidant and Biological Activities of *Peristrophe bicalyculata*

Results of radical scavenging, tyrosinase inhibition and metal ion chelating activities of extracts of *Peristrophe bicalyculata* were expressed based on the Scavenging concentration (SC_{50}), Inhibition concentration (IC_{50}) and chelating concentration (CC_{50}) values, defined as the concentration of the extract required to inhibit 50% activity, respectively, (Table 4.4). The SC_{50} of butanol fraction of methanol extract (0.530 ± 0.01 mg/mL), methanol extract (0.579 ± 0.02 mg/mL) and ethyl acetate fraction of methanol extract (0.510 ± 0.04 mg/mL) were not significantly different from each other, and were most effective as they inhibited the enzyme at the lowest concentrations. The SC_{50} at which the standard antioxidant, vitamin C (0.705 ± 0.08 mg/mL) inhibited 50% activity of the enzyme was significantly ($P < 0.05$) lower than the water fraction of methanol extract (1.768 ± 0.04 mg/mL), cold water (0.768 ± 0.04 mg/mL), hot water (1.708 ± 0.05 mg/mL) and hexane (0.908 ± 0.03 mg/mL) extracts.

Table 4.2: Phytochemical composition of extract of cold water extract of *Peristrophe bicalyculata*

Phytochemicals	**Observations**
Anthraquinone	-
Cardiac glycoside	+++
Tannin	++
Phenols	++
Carotenoid	-
Flavone	+++
Alkaloid	+++
Triterpenes/steroids	+
Saponnins	++

(+), (++) and (+++) represent intensity observed; (-) represent absent

Table 4.3: Total phenolic content of cold water extracts of *Peristrophe bicalyculata*

Sample	**Total Phenolic Content (mg/g)**
Hot Water Extract	48.80 ± 4.06^{c}
Cold Water Extract	152.35 ± 14.02^{a}
Methanol Extract	$150.00 \pm 17.25^{a,d}$
Hexane Extract	40.65 ± 2.09^{c}
Water Fraction of Methanol Extract	48.43 ± 3.96^{c}
Ethyl acetate fraction of Methanol Extract	$140.00 \pm 15.45^{a,d}$
Butanol Fraction	120.67 ± 11.60b

Values are Mean ± SEM.

^{a,b,c,d} = Values with different superscript letters in the same column are significantly ($P < 0.05$) different.

The concentration required to inhibit activity of tyrosinase by 50% (IC_{50}) were not significantly ($P > 0.05$) different from those of the butanol fraction of methanol extract (0.557 ± 0.04 mg/mL) and ethyl acetate fraction of methanol extract (0.520 ± 0.08 mg/mL), hexane (0.560 ± 0.06 mg/mL) and cold water (0.550 ± 0.05 mg/mL) extracts; but were significantly lower ($P < 0.05$) than the inhibition concentration for water fraction of methanol extract (0.810 ± 0.07 mg/mL), hot water (0.740 ± 0.04 mg/mL) and methanol (0.650 ± 0.07 mg/mL) extracts. However, the inhibition concentration of the water fraction of methanol extract (0.810 ± 0.07 mg/mL) and that of the hot water extract (0.740 ± 0.04 mg/mL) were not significantly different. On the other hand, the tyrosinase inhibition concentration of Kojic acid (1.070 ± 0.05 mg/mL) was significantly ($P < 0.05$) higher than those of all the extracts (Table 4.4).

The chelating concentration at 50% activity (CC_{50}) of EDTA (1.210 ± 0.24 mg/mL) was significantly ($P < 0.05$) higher than that of any of the extracts: ethyl acetate fraction of methanol extract (0.503 ± 0.02 mg/mL), butanol fraction of methanol extract (0.653 ± 0.05 mg/mL), water fraction of methanol extract (1.380 ± 0.15 mg/mL), methanol (1.050 ± 0.02 mg/mL), hexane (0.763 ± 0.05 mg/mL), cold water (0.933 ± 0.06 mg/mL) and hot water (1.460 ± 0.18 mg/mL) extracts.

Table 4.4: Scavenging, tyrosinase inhibition and metal chelating concentrations of different extracts of *Peristrophe bicalyculata*

Sample	SC_{50} (mg/ml)	IC_{50} (mg/ml)	CC_{50} (mg/ml)
Hot Water extract	1.708 ± 0.05^{b}	0.740 ± 0.04^{d}	$1.460 \pm 0.18j$
Cold water extract	0.768 ± 0.04^{c}	0.550 ± 0.05^{c}	0.933 ± 0.06^{h}
Water fraction of Methanol Extract	1.768 ± 0.04^{d}	0.810 ± 0.07^{d}	1.380 ± 0.15^{e}
Vitamin C	0.705 ± 0.08^{a}	-	-
Hexane extract	0.908 ± 0.03^{e}	0.560 ± 0.06^{c}	0.763 ± 0.05^{f}
Butanol fraction of Methanol Extract	0.530 ± 0.01^{f}	$0.557 + 0.04^{c}$	0.653 ± 0.05^{x}
Methanol extract	$0.579 \pm 0.02^{f,h}$	$0.650 + 0.07^{g}$	1.050 ± 0.02^{y}
Ethyl acetate fraction of Methanol Extract	$0.510 \pm 0.04^{f,g}$	0.520 ± 0.08^{c}	0.503 ± 0.02^{z}
Kojic acid	-	1.070 ± 0.05^{ab}	-
EDTA	-	-	1.210 ± 0.25^{r}

Values are Mean ± SEM.

$^{a-z}$ = Values with different superscript letters within the same column are significantly ($P < 0.05$) different

SC_{50} = Scavenging concentration at 50% activity (mg/ml).

CC_{50} = Chelating concentration at 50% activity (mg/ml).

IC_{50} = Tyrosinase inhibition concentration at 50% activity (mg/ml).

EDTA = Ethylenediaminetetraacetic acid

The lipid peroxidation inhibitory activity of extracts of *Peristrophe bicalyculata* (Figure 4.1), expressed based on the absorbance of the samples, is directly proportional to the concentration of lipid peroxides formed. The result demonstrated the ability of all samples to inhibit the formation of peroxides at levels not significantly different ($P > 0.05$) from those of vitamin C and BHA after 24 h of incubation (day 2). However, the inhibition decreased with increase in incubation period in all the extracts, except the cold water extract (CWE) and butanol fraction of methanol extract (BE), whose inhibition also decreased with incubation but was significantly higher ($P < 0.05$) than that of vitamin E or BHA on day 7.

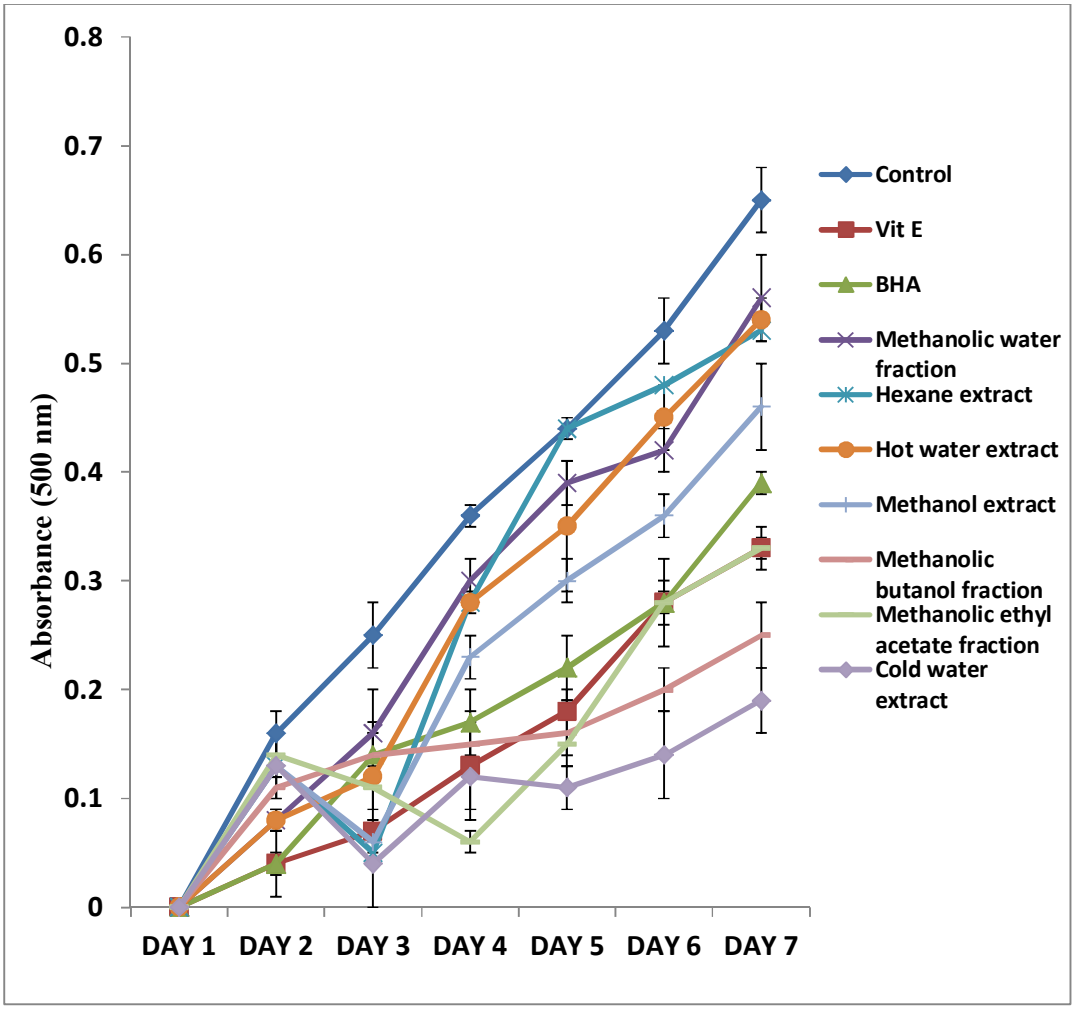


Figure 4.1: Lipid peroxidation inhibitory activity of different extracts of *P. bicalyculata* at different incubation times

Vit E = vitamin E; BHA = butylated hydroxyanisole

(use landscape b4 printing)

4.2 ANTIHYPERTENSIVE EFFECTS of *Peristrophe bicalyculata*

4.2.1 Angiotensin Converting Enzyme Inhibitory Activity of *Peristrophe bicalyculata*

Results of the ACE inhibitory activity of all the extracts are presented in Table 4.5. The standard drug, captopril inhibited ACE activity at a concentration (2.31 ± 0.17 µg/mL) significantly ($P < 0.05$) lower than the extracts. The concentration at which the cold water extract (11.84 ± 0.88 µg/mL) inhibited the enzyme was not significantly different from that of the water fraction of methanol extract (15.16 ± 3.82 µg/mL), but it was significantly ($P < 0.05$) lower than the concentration obtained for the hot water extract (27.03 ± 2.96 µg/mL), butanol fraction of methanol extract (20.26 ± 1.40 µg/mL) and the hexane extract (24.38 ± 1.95 µg/mL). However, the ethyl acetate fraction of methanol extract and methanol extract, with IC_{50} of 34.32 ± 2.06 µg/mL and 34.82 ± 3.58 µg/mL respectively, inhibited the enzyme at the highest concentrations, which were not significantly different from each other.

Table 4.5: Angiotensin-converting enzyme inhibitory effect of extracts of *P. bicalyculata*

Samples	**IC_{50} (µg/ml)**
Captopril	2.31 ± 0.17^{a}
Cold water extract	11.84 ± 0.88^{b}
Hot water extract	27.03 ± 2.96^{c}
Hexane extract	$24.38 \pm 1.95^{c,d}$
Methanol extract	34.82 ± 3.58^{f}
Ethyl acetate fraction of methanol extract	34.32 ± 2.06^{f}
Water fraction of methanol extract	15.16 ± 3.82^{b}
Butanol fraction of methanol extract	20.26 ± 1.40^{d}

Values are Mean ± SEM

$^{a-f}$ = Values with different superscript letters are significantly ($P < 0.05$) different

4.2.2 Effect of *P. bicalyculata* on L-NAME-induced Hypertensive Rats

The effect of *Peristrophe bicalyculata* on L-NAME-induced hypertensive rats is presented on Table 4.6. The administration of L-NAME to rats significantly ($P < 0.05$) increased blood pressure by 65 ± 5.06 % compared to rats in the control group (2.13 ± 0.03%). The increased blood pressure was significantly ($P < 0.05$) reduced by captopril (62.70 ± 5.56%), water fraction of methanol extract (15.67 ± 1.02%) and cold water extract (15.16 ± 2.09%). The reduction in systolic blood pressure was significantly ($P < 0.05$) higher in rats given captopril (62.70 ± 5.56%) than in those given water fraction of methanol extract (15.67 ± 1.02%) and cold water extract (15.16 ± 2.09%). However, the effects of the water fraction of methanol extract and cold water extract on blood pressure of hypertensive rats were not significantly ($P < 0.05$) different.

The heart rate of rats in the control group (0.10 ± 0.02%) was significantly lower ($P < 0.05$) than that in rats given captopril (2.45 ± 1.09%), water fraction of methanol extract (2.67 ± 0.84%) and cold water extract (4.33 ± 0.71%). Heart rate values of rats given captopril, cold water extract and water fraction of methanol extract of *Peristrophe bicalyculata* were not significantly different.

The duration of action of captopril on hypertensive rats lasted for over 480 minutes, and was significantly ($P < 0.05$) higher than those of the water fraction of methanol extract and cold water extract, which lasted for 33.33 ± 4.01 and 150.00 ± 39.50 minutes, respectively. However, the duration of action of the cold water extract in hypertensive rats was significantly higher ($P < 0.05$) than in those given water fraction of methanol extract.

Table 4.6: Effect of *Peristrophe bicalyculata* on L-NAME-induced hypertension in rats.

Extract	**Dose (mg/kg)**	**SBP Change (%)**	**HR Change (%)**	**Duration of action (mins)**
Control	-	2.13 ± 0.03^{c}	0.10 ± 0.02^{f}	Infinite
L-NAME	3	$+ 65 \pm 5.06^{f}$	9.15 ± 2.24	$>720^{i}$
Captopril	5	62.70 ± 5.56^{a}	$2.45\ 1.09^{d}$	$> 500^{h}$
Water fraction of methanol extract	3	15.67 ± 1.02^{b}	2.67 ± 0.84^{d}	33.33 ± 04.01^{g}
Cold Water Extract	10	15.16 ± 2.09^{b}	4.33 ± 0.71^{d}	150.00 ± 39.50^{e}

Values are Mean ± SEM

$^{a-i}$ = Values with different superscripts in the same column are significantly ($P < 0.05$) different

+ : Increase in blood pressure

- : Decrease in blood pressure

L-NAME = N-nitro- L-arginine methyl ester

SBP = Systolic blood pressure

HR = Heart rate

4.2.3 Partial Purification of Cold Water Extract of *Peristrophe bicalyculata*

After thin layer chromatographic separation of the cold water extract (Plate V), column chromatographic separation yielded 10 different fractions (Table 4.7) with the ethyl acetate (100%), ethyl acetate:methanol (1:1), ethyl acetate:methanol (1:3) and methanol: formic acid (9:1) solvents, giving one fraction each (fractions 1, 3, 4 and 6 respectively); while ethyl acetate:formic: methanol (15:2:0.5) and ethyl acetate: methanol:formic (1:3:1) yielded three (3) fractions each: fractions 2a, 2b, 2c and 5a, 5b and 5c, respectively. The yield from fraction 2c was the highest (1.3738 g), which was about 50% of the extract used; and the least was fraction 2a (0.0042 g).

Thin layer chromatographic separation (Plate VI) of all fractions were carried out; and the fractions were reduced to five (Figure 4.2). Fractions 1 and 2a were combined to give R_f spot 1; fractions 2b and 2c gave R_f spots 2 and 3, respectively; fractions 3 and 4 were combined to give R_f spot 4; while fractions 5a, 5b, 5c and 6 gave R_f spot 5. These fractions were then administered to hypertensive rats to determine the most active.

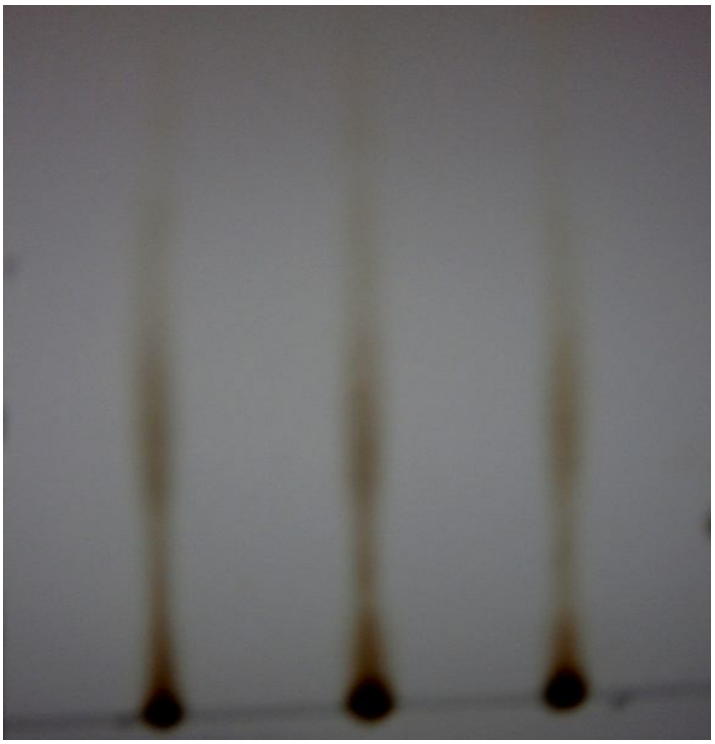


Plate V: Thin layer chromatographic separation of the cold water extract of *Peristrophe bicalyculata* for its antihypertensive activity.

Table 4.7: Fractions and yields of cold water extract of *Peristrophe bicalyculata* obtained by column chromatographic separation

Solvents	Fractions	Yield (g)
Ethyl acetate (100%)	Fraction 1	0.0062
Ethyl acetate:formic: methanol (15:2:0.5)	Fraction 2a	0.0042
	Fraction 2b	0.2127
	Fraction 2c	1.3438
Ethyl acetate:methanol (1:1)	Fraction 3	0.2043
Ethyl acetate:methanol (1:3)	Fraction 4	0.0388
Ethyl acetate:Methanol:formic (1:3:1)	Fraction 5a	0.6499
	Fraction 5b	0.1936
	Fraction 5c	0.0516
Methanol:formic acid (9:1)	Fraction 6	0.0077
Total		**2.7128**

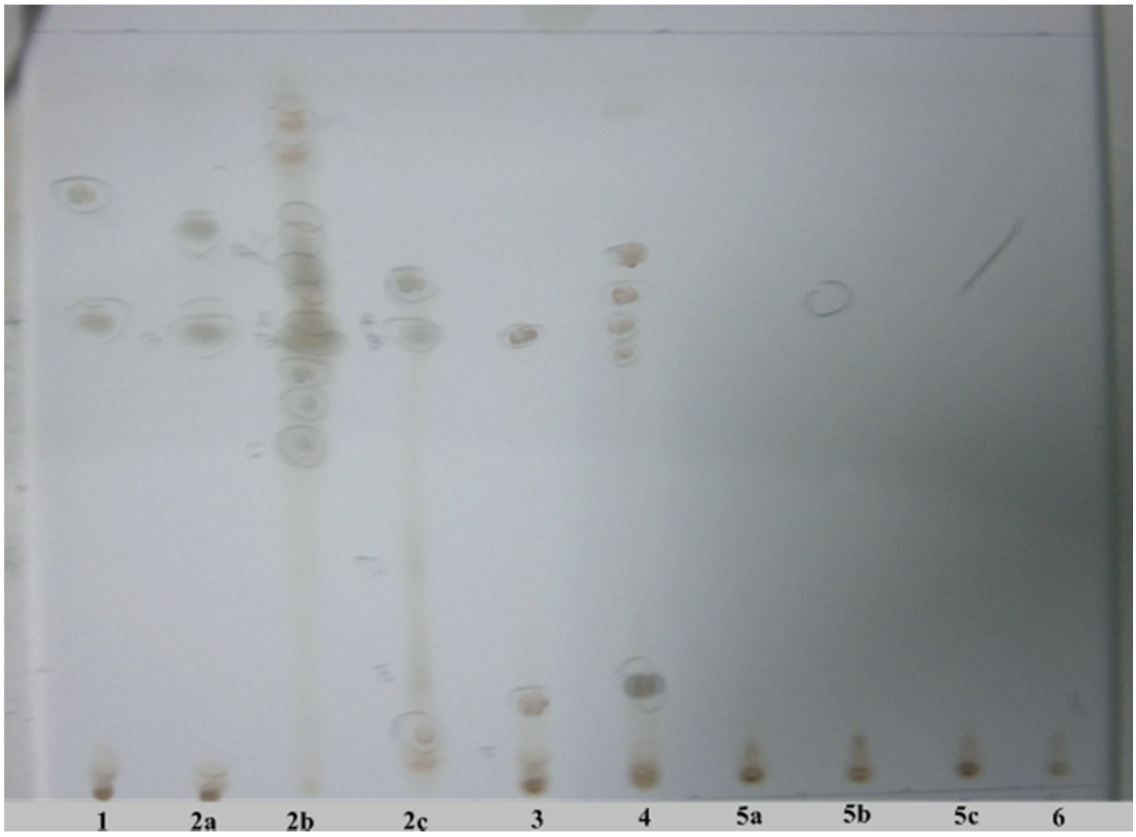


Plate VI: Thin layer chromatographic separation of fractions obtained from cold water extract of *Peristrophe bicalyculata*

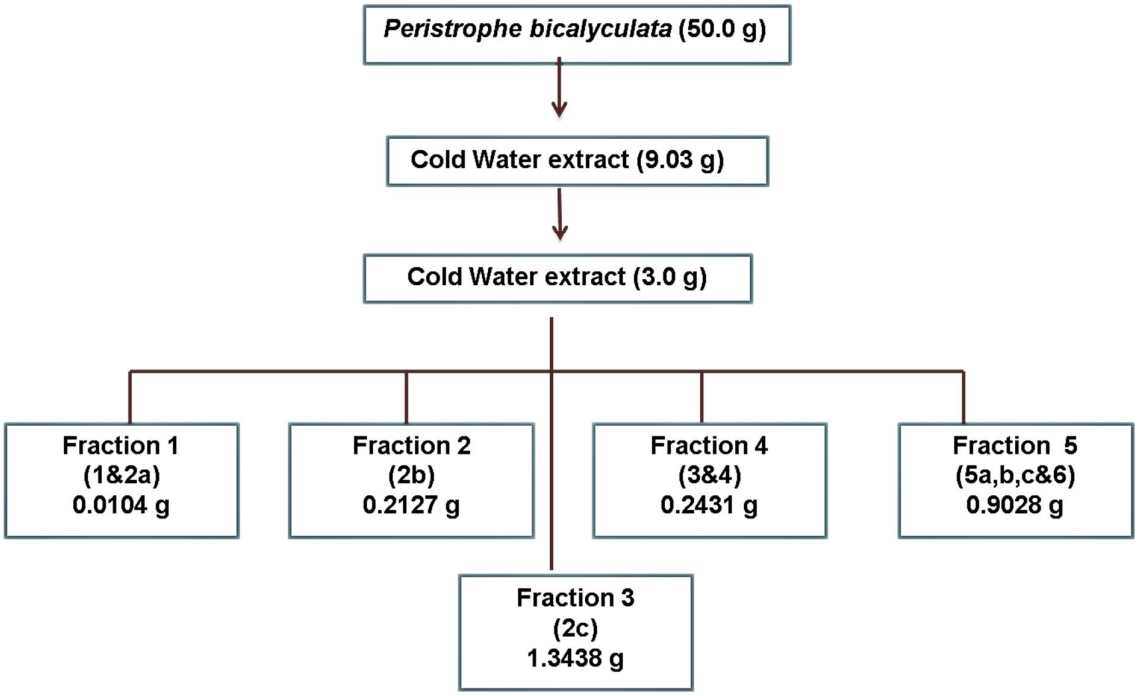


Figure 4.2: Yields and fractions obtained from cold water extract of *Peristrophe bicalyculata*

From the results, blood pressure of L-NAME-induced hypertensive rats increased significantly ($P < 0.05$) from 1.21 ± 0.06% (control rats) to 60.00 ± 9.02%. This was significantly ($P < 0.05$) reduced by 40.14 ± 9.08%, 21.00 ± 2.94%, 12.67 ± 1.60%, 29.33 ± 2.26% and 25.17 ± 1.11 in rats given captopril, extracts from R_f Spots 2, 3, 4 and 5 respectively. The percentage blood pressure reduction in hypertensive rats given captopril (40.14 ± 9.08%) was significantly ($P < 0.05$) higher than rats given other fractions. Extract from R_f spot 4 reduced blood pressure of hypertensive rats by 29.33 ± 2.26%, which was significantly ($P < 0.05$) higher than reduction percentages induced by extracts from R_f spot 2 (21.00 ± 2.94%) and R_f spot 3 (12.67 ± 1.60%), but not significantly ($P < 0.05$) different from the percentage induced by the extract from R_f spot 5 (25.17 ± 1.11%). Conversely, the extract from R_f spot 1 increased blood pressure of hypertensive rats by 13.17 ± 2.43%.

There was a significant ($P < 0.05$) increase in the heart rate of L-NAME-induced hypertensive rats (12.15± 5.62%), compared to those given other fractions. The percentage decrease in rats given extracts from R_f spot 4 (4.33 ± 0.80%) and 5 (2.33 ± 1.11%) were not differ significantly ($P > 0.05$).

The action of captopril on blood pressure of L-NAME hypertensive rats lasted for over 480 minutes, and was significantly ($P < 0.05$) higher than the durations of action recorded for any other fraction. The duration of action of extract from R_f spot 2 (130.83 ± 43.32 min) was significantly higher ($P < 0.05$) than R_f spot 3 (116.67 ± 37.16 min), R_f spot 4 (69.17 ± 13.57 min) and R_f spot 5 (65.00 ± 3.16 min).

Table 4.8: Effect of partially purified fractions of P. *bicalyculata* on hypertensive rats

Groups	Dose (mg/kg)	Change in Blood Pressure (%)	Change in Heart Rate (%)	Duration of action
Control	-	1.21 ± 0.06^{a}	1.67 ± 0.10^{h}	-
L-NAME	3.0	$+60.00 \pm 9.02^{b}$	12.15 ± 5.62^{i}	> 720
Captopril	5.0	-40.14 ± 9.08^{c}	3.13 ± 1.06^{m}	> 500 s
R_f Spot 1	0.03	$+13.17 \pm 2.43^{d}$	9.17 ± 0.65^{k}	480.00 ± 80.50^{x}
R_f Spot 2	0.71	-21.00 ± 2.94^{e}	4.67 ± 0.76^{m}	130.83 ± 43.32^{y}
R_f Spot 3	4.48	-12.67 ± 1.60^{f}	$6.00 \pm 2.62^{k,m}$	116.67 ± 37.16^{y}
R_f Spot 4	0.81	-29.33 ± 2.26^{g}	4.33 ± 0.80^{m}	69.17 ± 13.57^{z}
R_f Spot 5	3.00	$-25.17 \pm 1.11^{e,g}$	2.33 ± 1.11^{m}	65.00 ± 3.16^{z}

Values are Mean ± SEM

$^{a-z}$ = Values with different superscripts in the same column are significantly ($P<0.05$) different

R_f = Retention factor

4.2.4 Effect of the Partially Purified Fractions of *Peristrophe bicalyculata* on Angiotensin Converting Enzyme Activity

As shown in Table 4.9, captopril inhibited 50% of the ACE activity (IC_{50}) at a concentration (2.38 ± 0.19 µg/mL) significantly lower ($P < 0.05$) than that recorded for any other fraction. The ACE inhibitory activities of extracts from R_f spot 5 (11.85 ± 0.88 µg/mL) and R_f spot 3 (15.86 ± 1.78 µg/mL) were not significantly ($P < 0.05$) different; but were significantly lower than those of R_f spots 1 and 2, which inhibited the enzyme at the highest concentrations (24.98 ± 1.73 and 27.23 ± 1.60 µg/mL, respectively).

Table 4.9: IC_{50} values of partially purified fractions of cold water extract of *Peristrophe bicalyculata* on Angiotensin converting enzyme activity

Samples	**IC_{50} (µg/ml)**
Captopril	2.38 ± 0.19^{a}
R_f Spot 1	24.98 ± 1.73^{b}
R_f Spot 2	27.23 ± 1.60^{b}
R_f Spot 3	$15.86 \pm 1.78^{c,d,e}$
R_f Spot 4	19.40 ± 1.58^{d}
R_f Spot 5	11.85 ± 0.88^{e}

Values are Mean ± SEM

$^{a-e}$ = Values with different superscript letters are significantly different ($P < 0.05$)

R_f = Retention factor

4.3 ANTIHYPERTENSIVE EFFECTS OF PARTIALLY PURIFIED EXTRACT OF *PERISTROPHE BICALYCULATA IN VIVO*

4.3.1 Effect of Partially Purified Extract of *P. bicalyculata* on Body Weight of L-NAME-induced Hypertensive Rats

The partially purified extract of *Peristrophe bicalyculata* was administered to L-NAME-induced hypertensive rats for four weeks and body weights and blood pressure of rats recorded weekly. From the results (Figure 4.3), the body weights of rats in all groups rose significantly ($P < 0.05$) in the second week of the experiment, compared to that of the first week. However, the increase in weight of rats in the second, third and fourth week were not significantly different ($P > 0.05$). There was a significant increase in body weight of hypertensive rats than rats in control group, while the increase in body weight of rats given captopril was not significantly different from that recorded in rats given the partially purified extract of P. *bicalyculata.*

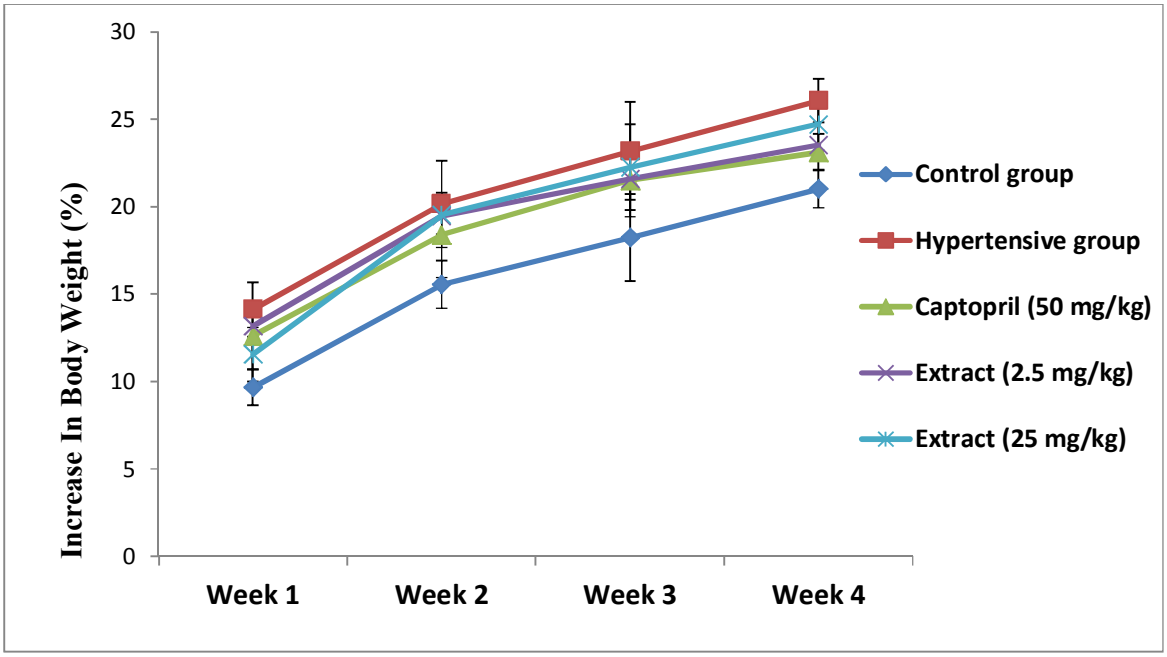


Figure 4.3: Effect of partially-purified extract of *Peristrophe bicalyculata* on percentage change in body weight of L-NAME-induced hypertensive rats.

4.3.2 Effect of Partially Purified Extract of P. *bicalyculata* on Blood Pressure of L-NAME-induced Hypertensive Rats

There was no significant ($P > 0.05$) change in systolic blood pressure of rats in the control group from the first to the fourth week. However, the blood pressure of rats given L-NAME increased significantly ($P < 0.05$) from 113 ± 3.71 mm Hg to 169.3 ± 3.48 mm Hg in the first week and up to 212 ± 2.08 mm Hg by the fourth week. There was no significant ($P > 0.05$) difference in blood pressure of hypertensive rats (169.3 ± 3.48 mm Hg) and rats treated with captopril (172 ± 5.85 mm Hg) and the partially purified extract of P. *bicalyculata* at 2.5 (176.00 ± 5.70 mm Hg) and 25 mg/kg (180 ± 10.12 mm Hg) in the first week of treatment. By the second, third and fourth week of treatment, the blood pressure of rats given captopril and both doses of the partially purified extract of P. *bicalyculata* decreased significantly ($P < 0.05$) when compared to hypertensive rats. Administration of captopril significantly ($P < 0.05$) reduced blood pressure from 172 ± 5.85 mm Hg in the first week to 74.00 ± 4.36 mm Hg by the fourth week. The partially purified extract of P. *bicalyculata* at 2.5 mg/kg significantly ($P < 0.05$) reduced systolic blood pressure of hypertensive rats, from 176.00 ± 5.70 to 103.33 ± 8.11 mm Hg by the fourth week; while rats given the extract at 25 mg/kg had their systolic blood pressure reduced from 180 ± 10.12 to 94 ± 1.73 mm Hg by the fourth week, showing a dose-dependent response (Figure 4.4).

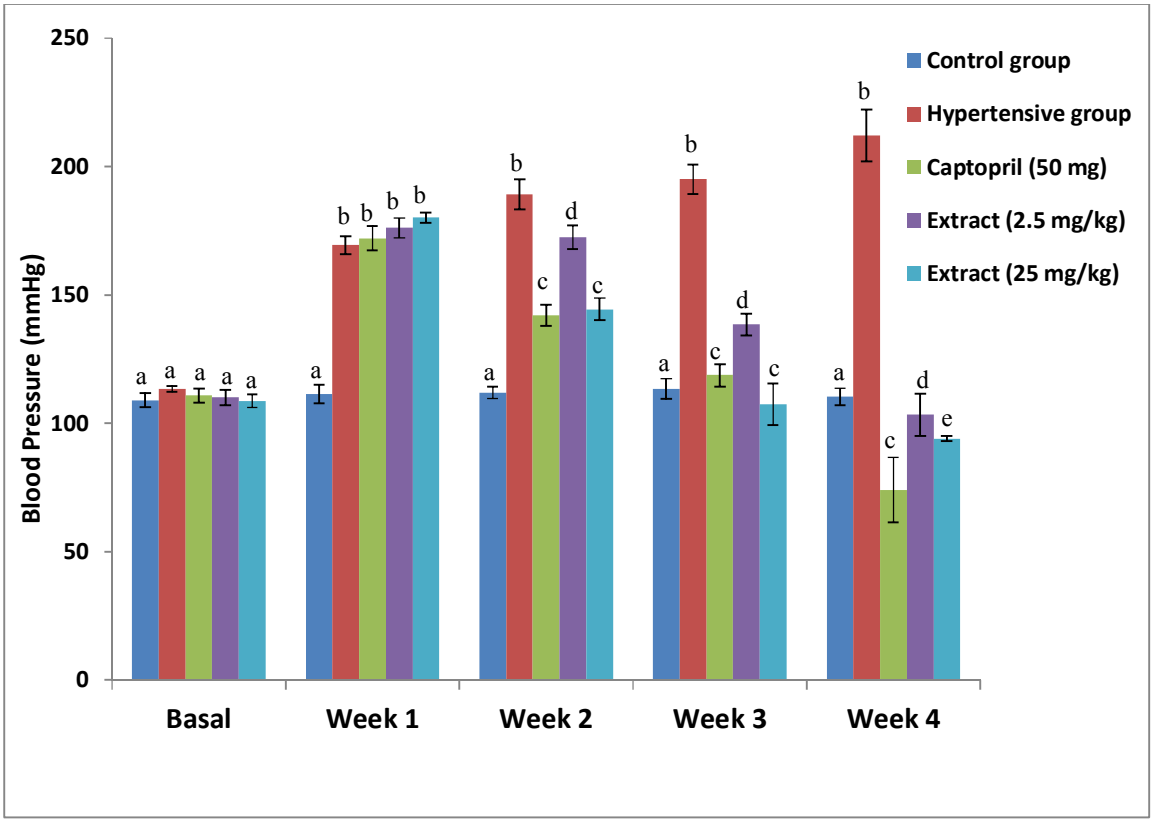


Figure 4.4: Effect of partially-purified extract of *Peristrophe bicalyculata* on systolic blood pressure of L-NAME-induced hypertensive rats.

a, b, c, d, e = Mean values within the same week having different superscript letters are significantly different ($P<0.05$)

L-NAME = N-nitro- L-arginine methyl ester

4.3.3 Effect of Partially-Purified Extract of *P. bicalyculata* on the Activity of Angiotensin Converting Enzyme in the Serum, Kidney and Aorta of L-NAME-induced Hypertensive Rats

There was a significant ($P < 0.05$) increase in the activity of ACE in the serum of L-NAME hypertensive rats compared to rats in the control group (Figure 4.5). The activity decreased significantly ($P < 0.05$) when rats were given captopril (0.11 ± 0.01 µmol/min/mL), and 2.5 mg/kg (0.28 ± 0.03 µmol/min/mL) or 25 mg/kg (0.19 ± 0.05 µmol/min/mL). There was no significant difference in ACE activity in serum of rats given captopril (0.11 ± 0.01 µmol/min/mL), rats in the control group (0.05 ± 0.01 µmol/min/mL) and those given the extract at 25 mg/kg (0.19 ± 0.05 µmol/min/mL).

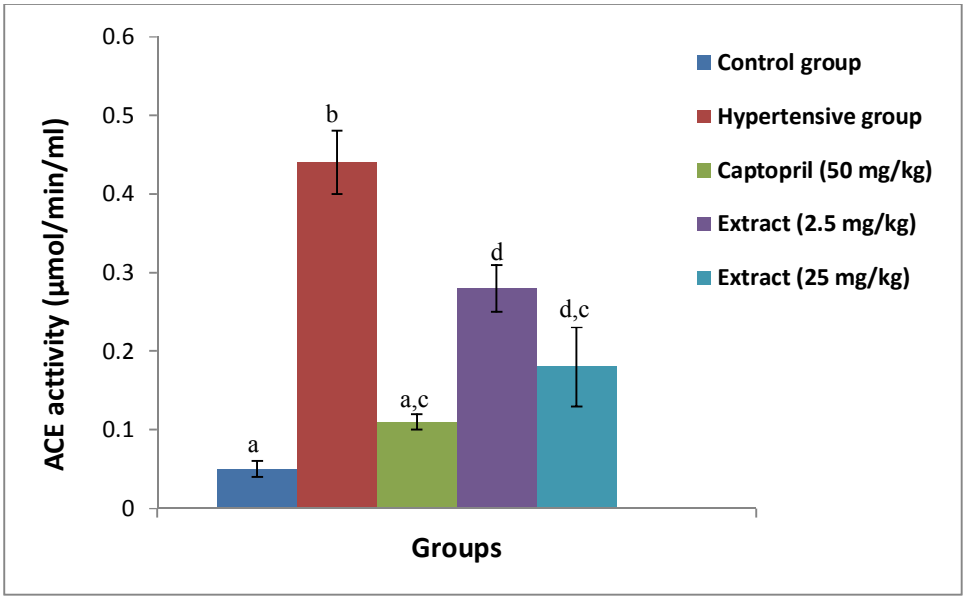


Figure 4.5: Effect of partially-purified extract of *Peristrophe bicalyculata* on activity of angiotensin converting enzyme (ACE) in the serum of L-NAME hypertensive rats (n = 25).

$^{a, b, c, d}$ = Mean values having different superscript letters are significantly different ($P<0.05$)

L-NAME = N-nitro- L-arginine methyl ester

The kidney ACE activity of hypertensive rats (2.3 ± 0.17 µmol/min/mg) was significantly ($P < 0.05$) higher than that recorded in rats in the control group (1.38 ± 0.07 µmol/min/mg). Administration of captopril (1.45 ± 0.04 µmol/min/mg) significantly ($P < 0.05$) reduced ACE activity compared to that obtained in rats given the extract at 2.5 mg/kg (1.66 ± 0.07 µmol/min/mg), but was not significantly different from those administered with the extract at 25 mg/kg (1.56 ± 0.02 µmol/min/mg). However, the kidney ACE activity in rats given the extract at 25 mg/kg (1.56 ± 0.02 µmol/min/mg) was not significantly different ($P < 0.05$) from the activity recorded in those given the extract at 2.5 mg/kg (1.66 ± 0.07 µmol/min/mg), rats given captopril and rats in the control group (Figure 4.6).

Induction of hypertension significantly ($P < 0.05$) increased the activity of ACE in aorta of rats; however, administration of captopril (1.07 ± 0.11 µmol/min/mg) and the extracts at 2.5 mg/kg (1.35 ± 0.05 µmol/min/mg) and 25 mg/kg (1.25 ± 0.09 µmol/min/mg) significantly ($P < 0.05$) reduced the activity. The ACE activities in aorta of rats given the extract at both doses were not significantly different, but the activity was significantly ($P < 0.05$) higher in the aorta of rats in the control group and those given captopril (Figure 4.6).

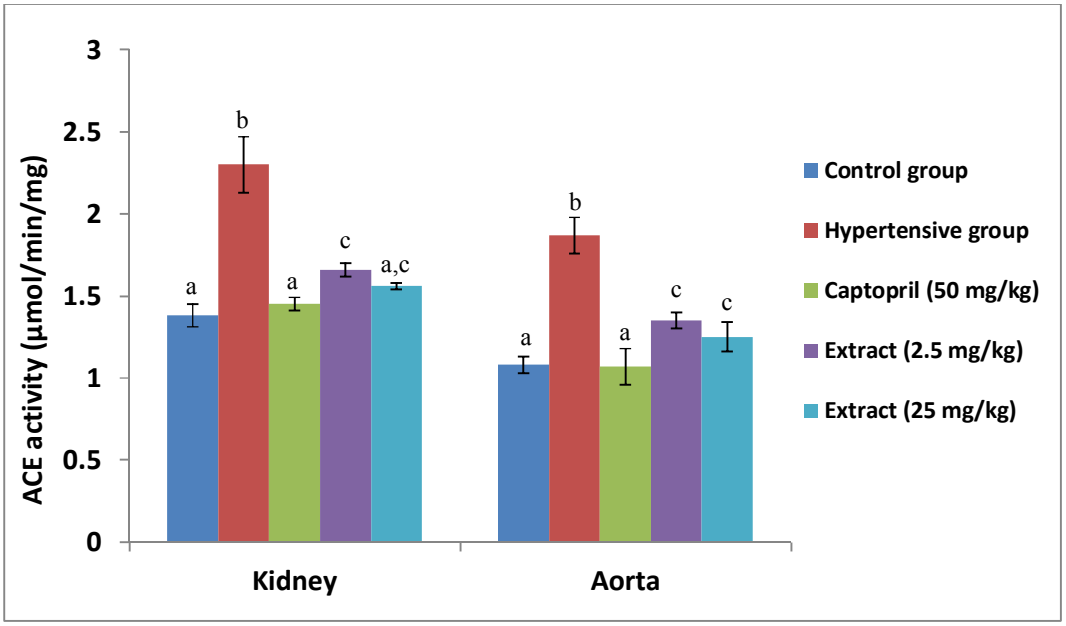


Figure 4.6: Effect of partially purified extract of *Peristrophe bicalyculata* on activity of angiotensin converting enzyme (ACE) in the kidneys and aorta of L-NAME hypertensive rats (n = 25)

$^{a, b, c, d}$ = Mean values within the same group having different superscript letters are significantly different ($P < 0.05$)

L-NAME = N-nitro- L-arginine methyl ester

4.3.4 Effect of the Partially Purified Extract of *Peristrophe bicalyculata* on the Activity of Endothelial Nitric Oxide Synthase in the Serum, Kidney and Aorta of L-NAME-induced Hypertensive Rats

Results of the effect of partially purified extracts of *Peristrophe bicalyculata* on eNOS activity in the kidneys, serum and aorta of L- NAME-induced hypertensive rats are presented in Figures 4.7 and 4.8. The results demonstrated that induction of hypertension significantly ($P < 0.05$) decreased the activity of eNOS in the serum and aorta compared to that recorded in rats in the control group.

Serum eNOS activity increased significantly ($P < 0.05$) in rats given captopril (8.33 ± 0.53 µmol/min/mL) compared to those in the hypertensive group (3.99 ± 0.61 µmol/min/mL), but the difference was not significant when compared to the values recorded in control rats (9.50 ± 1.22 µmol/min/mL) and those given the partially purified extract at 25 mg/kg (7.44 ± 0.74 µmol/min/mL). There was a significant ($P < 0.05$) increase in serum eNOS activity of rats given the partially purified extract at 2.5 mg/kg (5.92 ± 0.67 µmol/min/mL) and 25 mg/kg (7.44 ± 0.74 µmol/min/mL), thus, showing a dose-response effect.

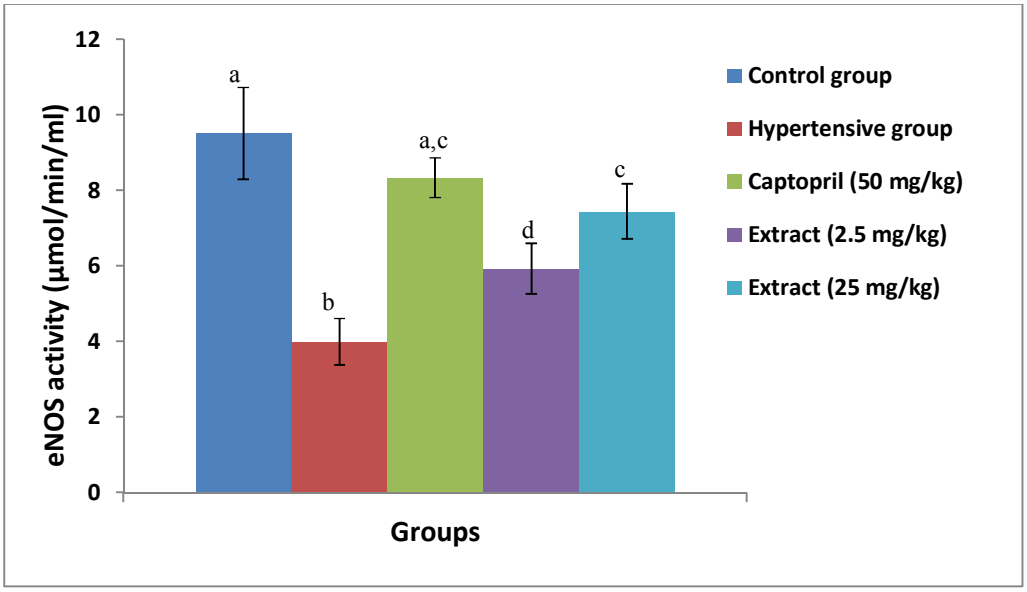


Figure 4.7: Effect of partially-purified extract of *Peristrophe bicalyculata* on activity of endothelial nitric oxide synthase (eNOS) in the serum of L-NAME-induced hypertensive rats (n = 25).

$^{a, b, c, d}$ = Mean values having different superscript letters are significantly different ($P < 0.05$)

L-NAME = N-nitro- L-arginine methyl ester

The eNOS activity in the kidneys of hypertensive rats (5.43 ± 0.23 μmol/min/mg) was not significantly different from that obtained in control rats (6.27 ± 1.25 μmol/min/mg). However, eNOS activity increased significantly ($P < 0.05$) in rats given captopril (7.53 ± 1.15 μmol/min/mg) and the extract at 25 mg/kg (6.44 ± 1.13 μmol/min/mg), compared to hypertensive rats and those given 2.5 mg/kg (4.69 ± 0.50 μmol/min/mg) of the partially-purified extract, demonstrating a dose-dependence effect (Figure 4.8).

Induction of hypertension significantly ($P < 0.05$) decreased aortic eNOS activity in rats (5.40 ± 1.03 μmol/min/mg) compared to the values obtained in rats in the control (11.97 ± 0.81 μmol/min/mg) and all the treated groups. Rats given captopril (9.84 ± 0.67 μmol/min/mg) had significantly ($P < 0.05$) higher aortic eNOS activity than those given the partially-purified extract at 2.5 mg/kg (7.90 ± 1.65 μmol/min/mg), but the difference was not significant when compared to the activity recorded in rats given 25 mg/kg (9.42 ± 0.75 μmol/min/mg) of the partially purified extract. There was no significant difference in the activities of eNOS in aorta of rats given the extract at 2.5 mg/kg (7.90 ± 1.65 μmol/min/mg) and 25 mg/kg (9.42 ± 0.75 μmol/min/mg).

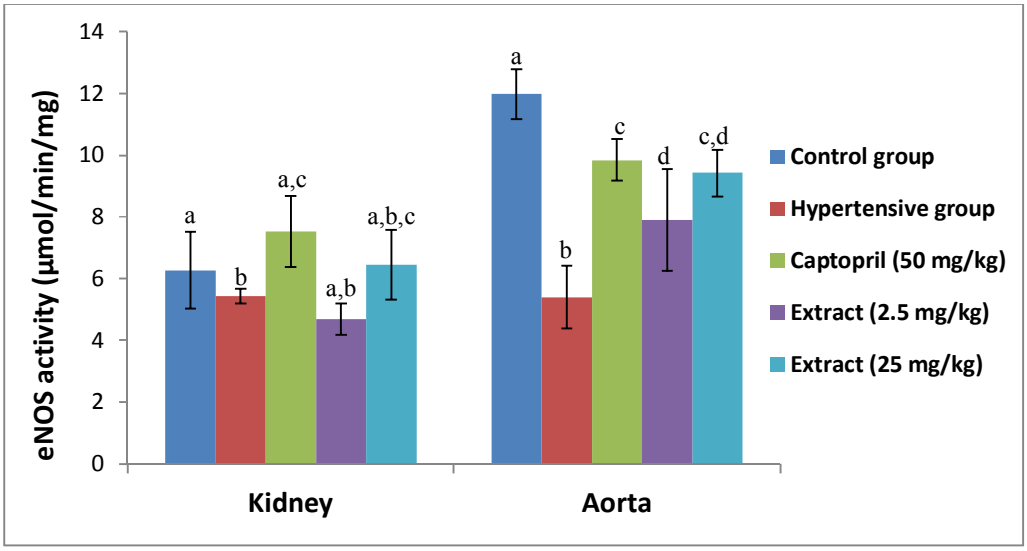


Figure 4.8: Effect of partially-purified extract of *Peristrophe bicalyculata* on activity of endothelial nitric oxide synthase (eNOS) in kidney and aorta of L-NAME- induced hypertensive rats (n = 25).

[a, b, c, d] = Mean values within the same group having different superscript letters are significantly different ($P<0.05$)

L-NAME = N-nitro- L-arginine methyl ester

4.3.5 Effect of the Partially-Purified Extract of *Peristrophe bicalyculata* on the Levels of Thiobarbituric Acid-Reactive Substances in the Serum, Kidneys and Aorta of L-NAME-induced Hypertensive Rats

The levels of TBARS present in the serum (Figure 4.9), kidneys and aorta (Figure 4.10) increased significantly ($P < 0.05$) in L-NAME-induced hypertensive rats, compared to that in rats in the control group. The serum level of TBARS in hypertensive rats (35.43 ± 1.98 mmol/dL) was about five times that in control rats (8.82 ± 0.16 mmol/dL). However, administration of captopril (13.56 ± 0.79 mmol/dL), the partially purified extract at 2.5 mg/kg (23.83 ± 1.65 mmol/dl) and 25mg/kg (17.75 ± 1.08 mmol/dL) reduced serum TBARS levels significantly ($P < 0.05$). The partially-purified extract demonstrated a dose-dependent response.

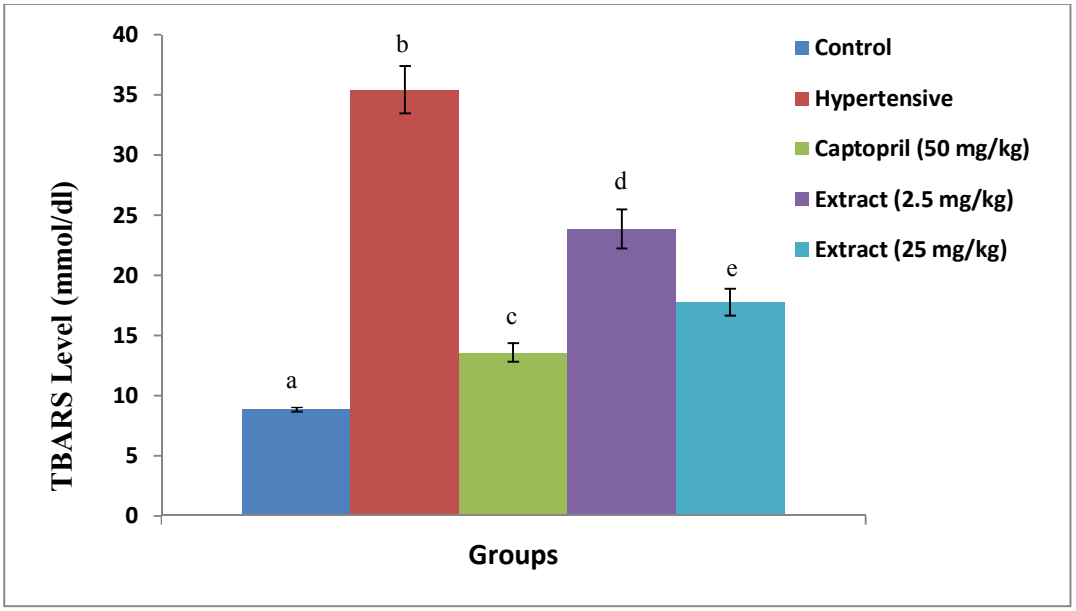


Figure 4.9: Effect of partially purified extract of *P. bicalyculata* on thiobarbituric acid reactive substances (TBARS) in serum of L-NAME-induced hypertensive rats (n = 25).

$^{a, b, c, d, e}$ = Mean values having different superscript letters are significantly different ($P < 0.05$)

L-NAME = N-nitro- L-arginine methyl ester

The TBARS level in the kidneys (79.06 ± 3.59 mmol/100g) of rats increased significantly ($P < 0.05$) to 152.85 ± 6.20 mmol/100 g on induction of hypertension, but administration of captopril (89.20 ± 4.11 mmol/100 g), the partially purified extract at 2.5 mg/kg (129.36 ± 5.17 mmol/100 g) and 25 mg/kg (92.94 ± 4.14 mmol/100 g) reduced the level significantly ($P < 0.05$). The reduction in kidney TBARS level in rats given captopril was not significantly different from that of rats given the partially-purified extract at 25 mg/kg (92.28 ± 4.14 mmol/100g), but was significantly ($P < 0.05$) higher, when compared to those given at 2.5 mg/kg (129.36 ± 5.17 mmol/100 g), showing a dose-dependence response (Figure 4.10).

There was a significant ($P < 0.05$) increase in aortic TBARS level (128.51 ± 8.41 mmol/100 g) of L-NAME-induced hypertensive rats compared to rats in the control group (68.12 ± 2.16 mmol/100 g). The TBARS level was significantly ($P < 0.05$) reduced in rats given captopril (83.05 ± 3.01 mmol/100 g), the partially-purified extract at 2.5 mg/kg (99.56 ± 5.92 mmol/100 g) and 25 mg/kg (93.94 ± 5.20 mmol/100 g).

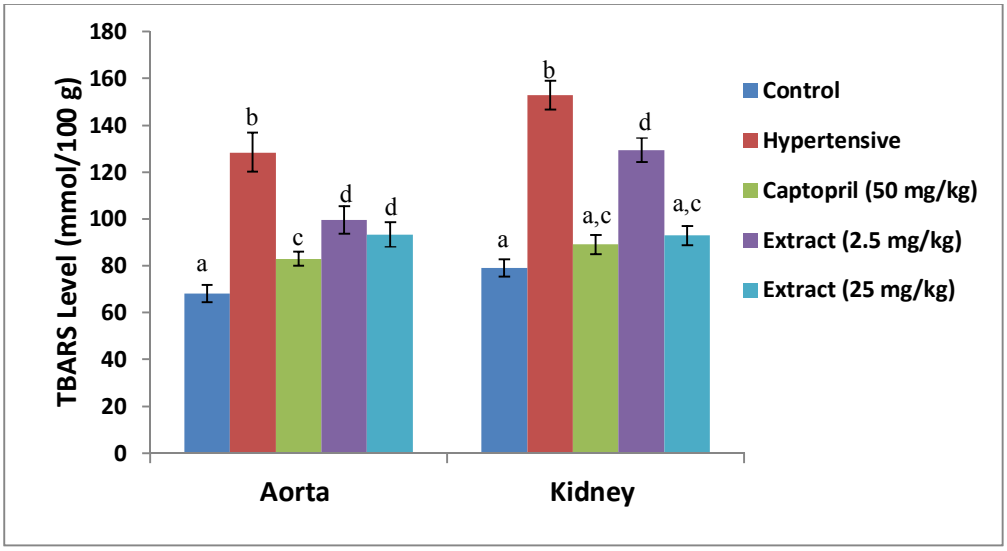


Figure 4.10: Effect of partially purified extract of *P. bicalyculata* on thiobarbituric acid-reactive substances (TBARS) in the kidneys and aorta of L-NAME-induced hypertensive rats (n = 25).

[a, b, c, d] = Mean values within the same group having different superscript letters are significantly different ($P<0.05$)

L-NAME = N-nitro- L-arginine methyl ester

4.4 Molecular Effect of Partially Purified Extract of P. *bicalyculata*

4.4.1 Effect of Partially Purified Extract of *Peristrophe bicalyculata* on the Expression of Angiotensin Converting Enzyme in the Aorta and Kidney of L-NAME-induced Hypertensive Rats

As demonstrated in Figure 4.11 and Plate VII, ACE mRNA was significantly ($P < 0.05$) expressed in kidneys (1.14 ± 0.04) of hypertensive rats, compared to that in the kidneys (0.35 ± 0.09) of rats in control group. After treatment with captopril at 50 mg/kg, ACE mRNA expression reduced significantly ($P < 0.05$) to 0.46 ± 0.03, and the value was not significantly different from that obtained in rats given the partially purified extract at 25 mg/kg (0.53 ± 0.02), but significantly ($P < 0.05$) lower than that in kidneys of rats treated with the extract at 2.5 mg/kg (0.84 ± 0.04), showing a dose-dependent effect.

The aorta of hypertensive rats had significantly ($P < 0.05$) higher level of ACE mRNA (1.04 ± 0.17) than in aorta of rats in the control group (0.69 ± 0.06). Treatment with captopril significantly reduced ($P < 0.05$) ACE mRNA expression to 0.73 ± 0.06, and was not significantly ($P > 0.05$) different from ACE mRNA levels in rats given the partially purified extract at 25 mg/kg (0.90 ± 0.06), but significantly ($P < 0.05$) lower than that in aorta of rats given the partially purified extract at 2.5 mg/kg (1.21 ± 0.03).

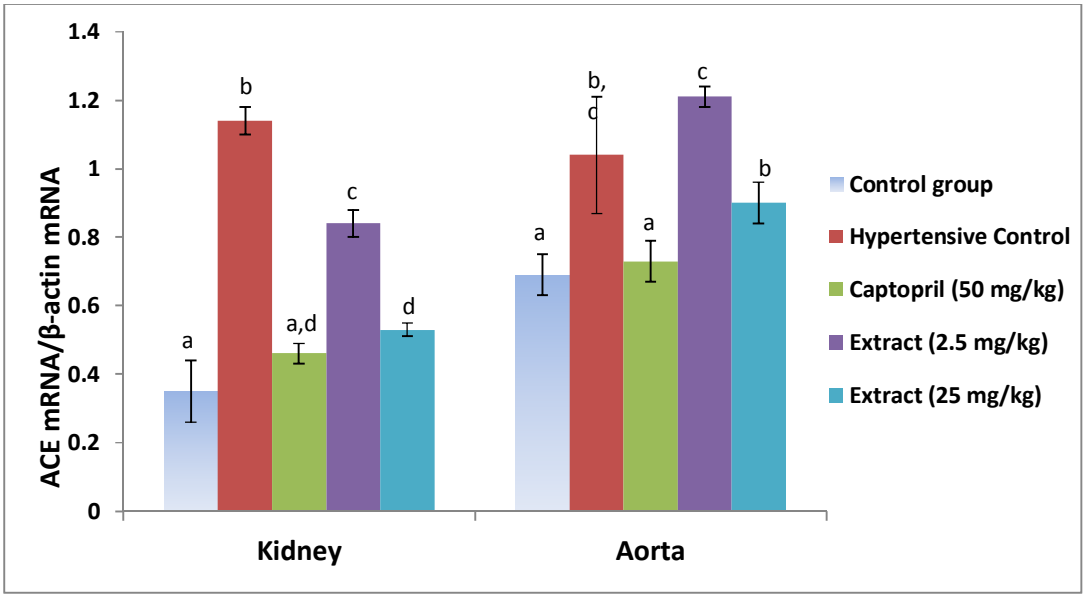


Figure 4.11: Effect of partially purified extract of *Peristrophe bicalyculata* on the expression of angiotensin-converting enzyme (ACE) in the kidneys and aorta of L-NAME-induced hypertensive rats (n = 25).

[a, b, c, d] = Mean values for each tissue/serum having different superscript letters are significantly different ($P<0.05$)

L-NAME = N-nitro- L-arginine methyl ester

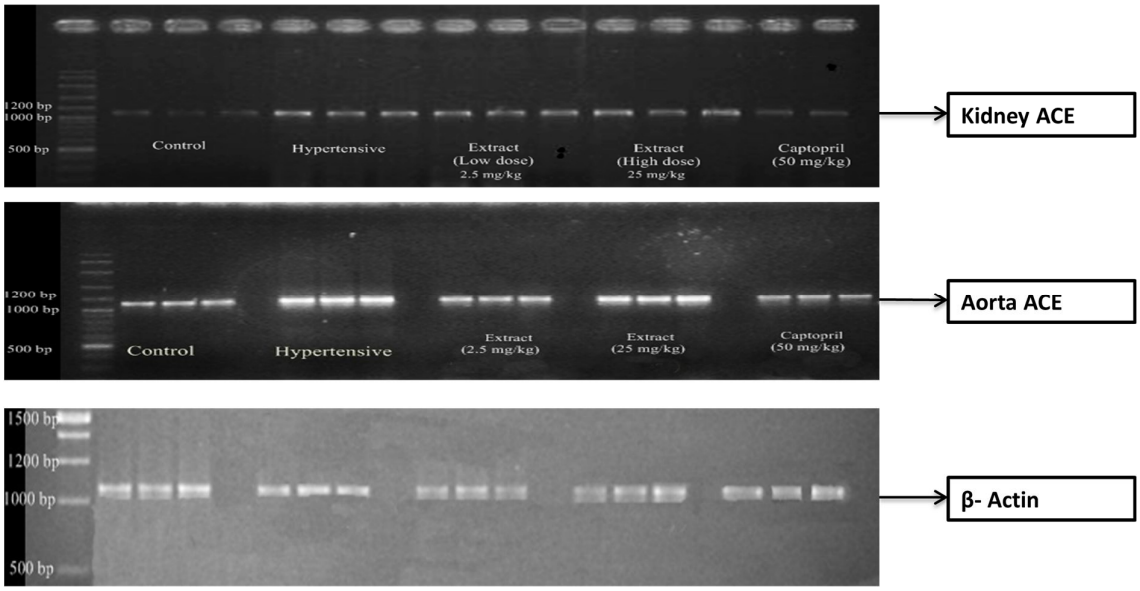


Plate VII: Agarose gel electrophoregram of angiotensin-converting enzyme (ACE) and β-actin from the kidneys and aorta of control, untreated and treated hypertensive rats.

4.4.2 Effect of Partially-Purified Extract of *Peristrophe bicalyculata* on the Expression of Endothelial Nitric Oxide Synthase in L-NAME-induced Hypertensive Rats

As shown in Figure 4.12 and Plate VIII, the induction of hypertension significantly ($P < 0.05$) decreased kidney expression of eNOS mRNA (0.76 ± 0.02) than kidneys (0.94 ± 0.06) of control rats. There was no significant difference in the expression of eNOS mRNA in the kidneys of hypertensive rats (0.76 ± 0.02), rats given captopril (0.84 ± 0.01), the partially-purified extract at 2.5 mg/kg (0.86 ± 0.04) and 25 mg/kg (0.74 ± 0.07).

The expression of eNOS mRNA in the aorta of hypertensive rats (0.49 ± 0.08) also decreased significantly ($P < 0.05$) compared to that in aorta of control rats (0.88 ± 0.01). The eNOS mRNA levels in the aorta of rats given captopril (0.83 ± 0.05), the partially purified extract of at 2.5 mg/kg (0.91 ± 0.03) and 25 mg/kg (0.77 ± 0.03) was not significantly ($P < 0.05$) different from that of control rats, but significantly higher than that in hypertensive rats (Figure 4.12).

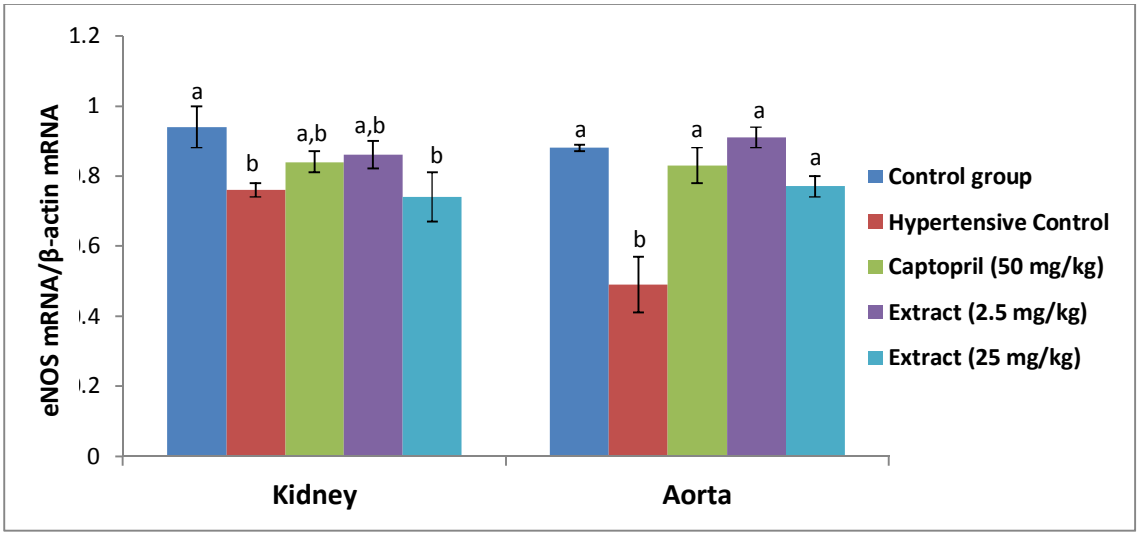


Figure 4.12: Effect of partially purified extract of *Peristrophe bicalyculata* on the expression of endothelial nitric oxide synthase (eNOS) in the kidneys and aorta of L-NAME-induced hypertensive rats (n = 25).

[a, b, c] = Mean values for each tissue/serum having different superscript letters are significantly different ($P < 0.05$)

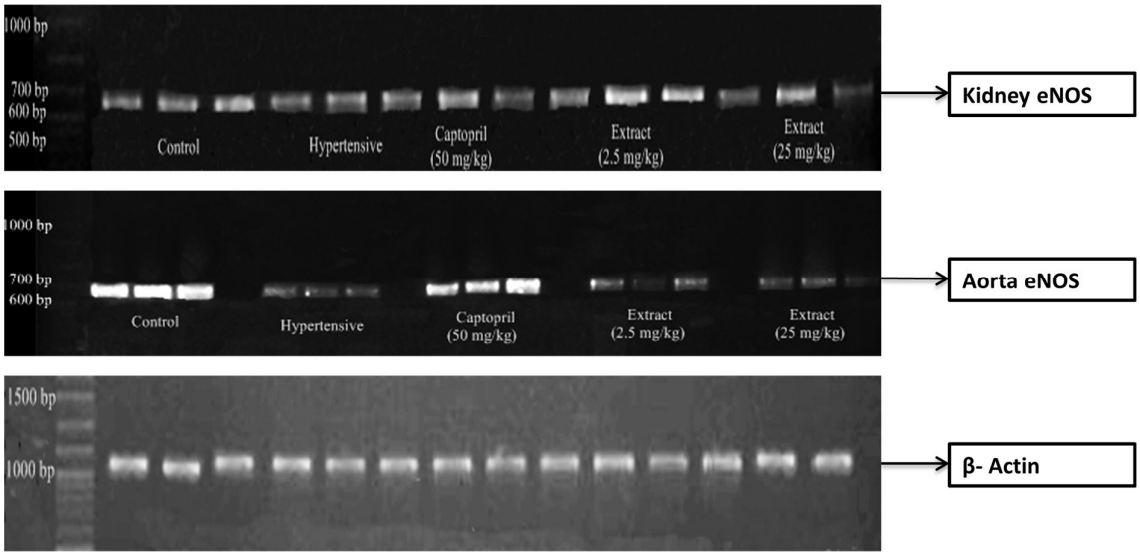


Plate VIII: Agarose gel electrophoregram of endothelial nitric oxide synthase (eNOS) and β-actin from the kidneys and aorta of control, treated and untreated hypertensive rats.

4.4.3 Effect of Partially Purified Extract of *Peristrophe bicalyculata* on the Expression of Angiotensin Converting Enzyme in rat aortic endothelial cells

As demonstrated in Figure 4.13, treatment of rat aortic endothelial cells with 2 mmol/L of L-NAME significantly ($P < 0.05$) increased the expression of ACE mRNA. The expression significantly ($P < 0.05$) reduced when the cells were treated with captopril (1 mmol/L) and the partially-purified extract at 100 µg/ml (0.64 ± 0.08 fold). There was no significant difference in ACE mRNA expression between cells obtained from the control cultures (0.55 ± 0.03), those treated with either captopril (0.52 ± 0.03) or the partially-purified extract at 100 µg/ml (0.64 ± 0.08 fold).

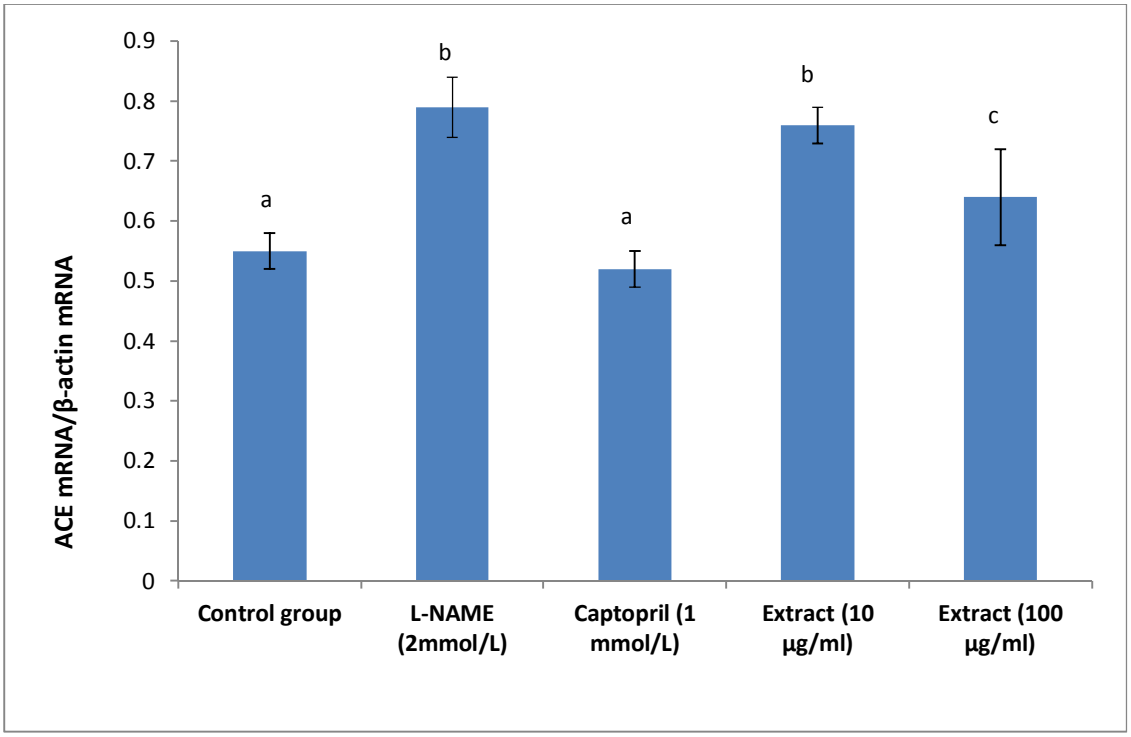


Figure 4.13: Effect of partially purified extract of *Peristrophe bicalyculata* on the expression of angiotensin-converting enzyme (ACE) in rat aortic endothelial cells

[a,b,c] = Mean values having different superscript letters are significantly different ($P < 0.05$)

4.4.4 Effect of Partially Purified Extract of *Peristrophe bicalyculata* on the Expression of Endothelial Nitric Oxide Synthase in rat aortic endothelial cells

The mRNA expression of eNOS reduced significantly ($P < 0.05$) in cells treated with L-NAME (0.48 ± 0.03 fold) compared to control cells (1.71 ± 0.15 fold), but the expression significantly increased in cells treated with captopril (1.01 ± 0.10 fold), the partially purified extract at 10 µg/mL (1.05 ± 0.09 fold) and 100 µg/mL (0.95 ± 0.03 fold). There was no significance difference ($P > 0.05$) in expression of eNOS mRNA between cells treated with captopril and the partially-purified extract at both low and high doses (Figure 4.14 and Plate IX).

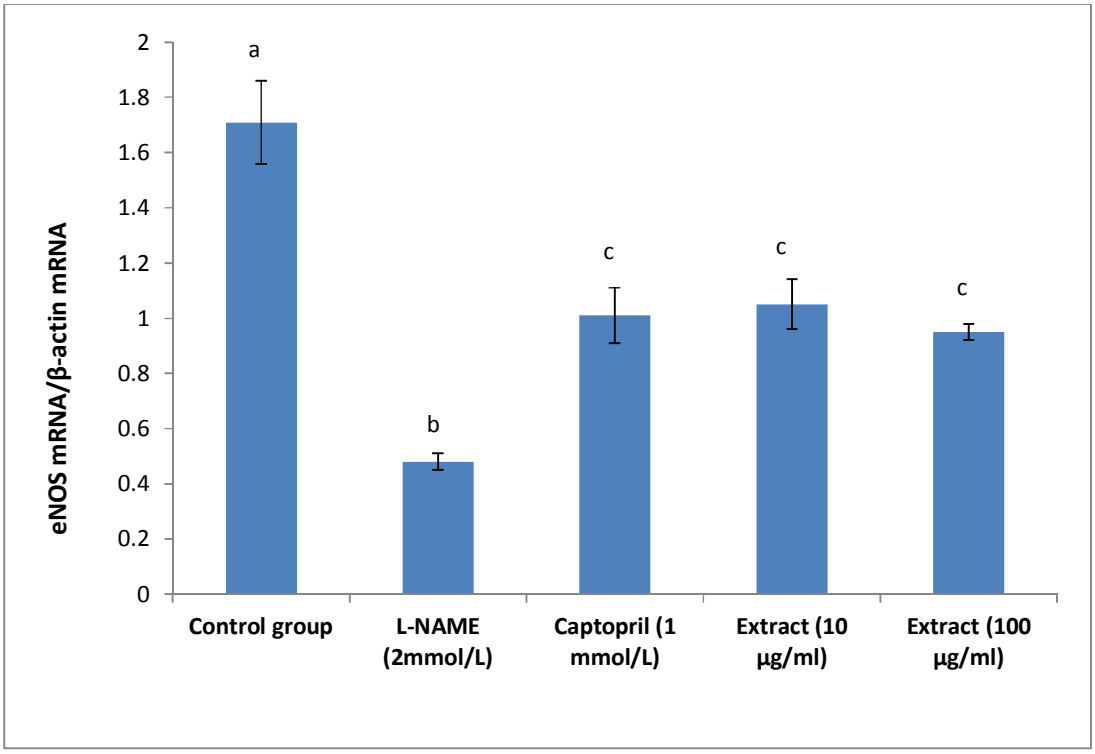


Figure 4.14: Effect of partially purified extract of *Peristrophe bicalyculata* on the expression of endothelial nitric oxide synthase (eNOS) in rat aortic endothelial cells

[a, b, c] = Mean values having different superscript letters are significantly different ($P < 0.05$)

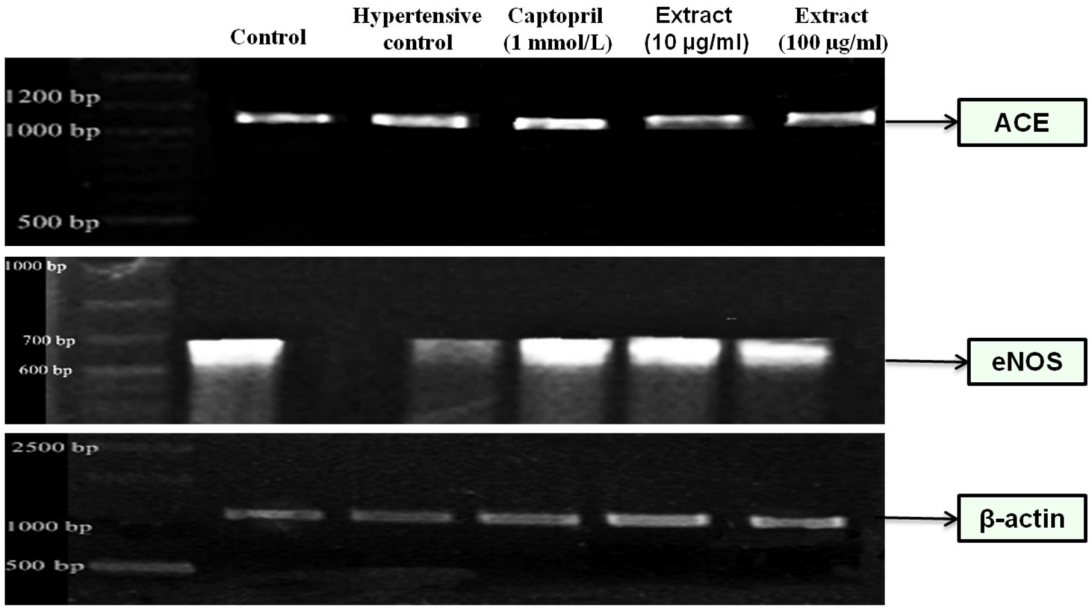


Plate IX: Agarose gel electrophoregram of angiotensin-converting enzyme, endothelial nitric oxide synthase and β-actin from rat aortic endothelial cells following treatment with L-NAME, captopril and partially purified extract of *Peristrophe bicalyculata*

4.5 ANTICANCER ACTIVITY OF EXTRACTS OF *PERISTROPHE BICALYCULATA*

The extracts of *Peristrophe bicalyculata* were tested for their anticancer activity in four cancer cell lines: human cervical cancer (HeLa), human colon adenocarcinoma (HT-29), human hepatocellular liver carcinoma (HepG2) and human mouth epidermal carcinoma (KB). From the results (Table 4.10), the ethyl acetate fraction of methanol extract with an IC_{50} of 15.6 ± 0.52 µg/mL inhibited 50% of KB cancer cells at concentrations significantly lower ($P < 0.05$) than the hexane extract, with an IC_{50} of 328 ± 52.1 µg/mL. The IC_{50} values of the methanol extract, water and butanol fractions of methanol extract were >500 µg/mL. However, the standard drugs, cisplastin (3.32 ± 0.09 µg/mL), doxorubicin (0.43 ± 0.02 µg/mL) and vincristine (0.05 ± 0.01 µg/mL) inhibited 50% of KB cancer cells at concentrations significantly lower ($P<0.05$) than those of the ethyl acetate fraction of methanol extract (15.6 ± 0.52 µg/mL) and hexane (328 ± 52.1 µg/mL) extract.

The IC_{50} value of vincristine (0.02 ± 0.001 µg/ml) against HeLa was significantly lower than the values obtained for cisplastin (3.42 ± 1.95 µg/mL), doxorubicin (2.74 ± 0.15 µg/mL) and all the extracts. There was no significant difference ($P > 0.05$) between the IC_{50} of the methanol extract and ethyl acetate fraction of methanol extract, with the values of 430 ± 34.0 µg/mL and 450 ± 25.60 µg/mL, respectively; while the hexane extract, water and butanol fractions of methanol extract had IC_{50} values above 500 µg/mL.

Also, with respect to HT-29, the IC_{50} value of vincristine (0.07 ± 0.031 µg/ml) was significantly lower than those of cisplastin (1.12 ± 0.56 µg/mL), doxorubicin (1.13 ± 0.24 µg/mL) and all the extracts tested. The IC_{50} value of the butanol fraction of methanol extract (298 ± 20.50 µg/mL) was significantly ($P < 0.05$) lower than the value obtained for the water fraction of methanol extract (490 ± 42.6 µg/mL), while the hexane extract, methanol extract and ethyl acetate fraction of methanol extract had IC_{50} values above 500 µg/mL.

The IC_{50} values of cisplastin (5.52 ± 0.76 µg/mL), doxorubicin (0.42 ± 0.25 µg/mL) and vincristine (2.87 ± 0.71 µg/mL) against HepG2 were significantly ($P < 0.05$) lower than those of the ethyl acetate fraction of methanol extract (395 ± 37.0 µg/mL), hexane extract and butanol fraction of methanol extract (Table 4.10).

Table 4.10: The IC_{50} (µg/mL) values for anti-cancer activity of extracts of *Peristrophe bicalyculata*

Sample	**IC_{50} values (µg/ml ± SD)**			
	KB	**HeLa**	**HT-29**	**Hep G2**
Hexane extract	**328 ± 52.1^{a}**	**>500**	**>500**	**>500**
Methanol extract	**>500**	**430 ± 34.0^{g}**	**>500**	**ND**
Water fraction of methanol extract	**>500**	**>500**	**490 ± 42.6^{i}**	**ND**
Butanol fraction of methanol extract	**>500**	**>500**	**298 ± 20.50^{k}**	**>500**
Ethyl acetate fraction of methanol extract	**15.6 ± 0.52^{b}**	**450 ± 25.60^{g}**	**>500**	**395 ± 37.0^{o}**
Cisplatin	**3.32 ± 0.09^{c}**	**3.42±1.95^{f}**	**1.12 ± 0.56^{j}**	**5.52 ± 0.76^{p}**
Doxorubicin	**0.43 ± 0.02^{d}**	**2.74 ± 0.15^{f}**	**1.13 ± 0.24^{j}**	**0.42 ± 0.25^{m}**
Vincristine	**0.05 ± 0.01^{e}**	**0.02 ± 0.001^{h}**	**0.07 ± 0.031^{l}**	**2.87 ± 0.71^{n}**

Values are mean ± SEM

$^{a\text{-}p}$ = Values with different superscript letters in the same column are significantly different ($P < 0.05$)

> 500 = IC_{50} values is higher than 500 µg/mL (inactive)

4.5.1 Partial Purification of Ethyl Acetate Fraction of *Peristrophe bicalyculata*

Thin layer chromatographic separation of ethyl acetate fraction of methanol extract carried out using ethyl acetate and hexane (5:5) gave six constituents visible under UV-spectrum (Plate X). Column chromatography using silica gel run with hexane (100%), hexane:ethyl acetate (8:2), hexane:ethyl acetate (5:5), hexane:ethyl acetate (3:7), ethyl acetate:chloroform (9:10), ethyl acetate:methanol (9:1) and ethyl acetate: methanol (1:1) yielded ten fractions (Table 4.11). The fractions were reduced to seven after thin layer chromatographic separation (Plate XI).

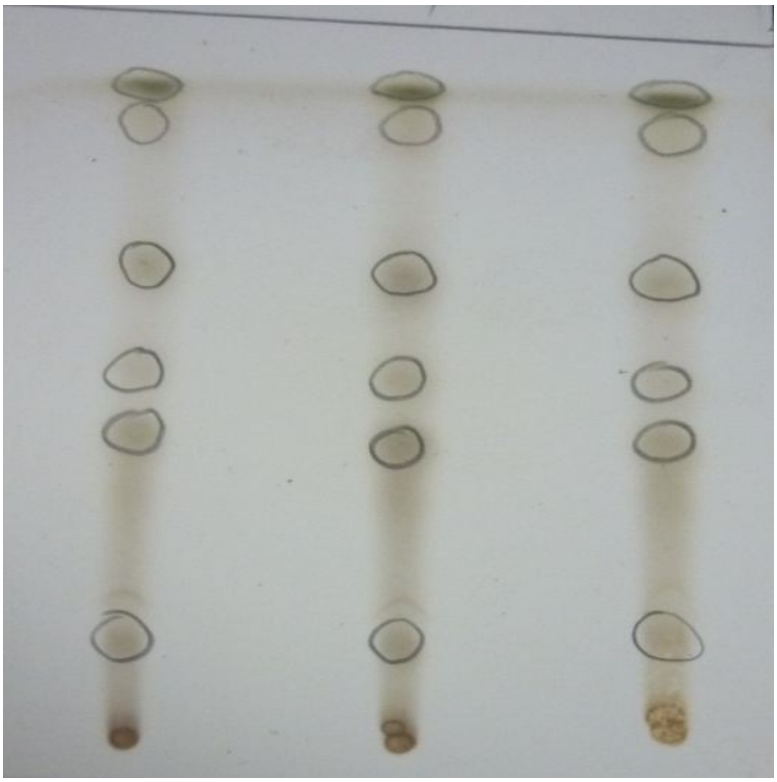


Plate X: Thin layer chromatographic separation of the ethyl acetate fraction of methanol extract of *Peristrophe bicalyculata* for its anticancer activity.

Table 4.11: Fractions and yields obtained from column chromatographic separation of ethyl acetate fraction of methanol extract of *Peristrophe bicalyculata*

Solvents	Fractions	Yield (g)
Hexane (100%)	-	
Hexane:ethyl acetate (8:2)	Fraction 2a	0.0704
	Fraction 2b	0.2104
	Fraction 2c	0.1426
Hexane:ethyl acetate (5:5)	Fraction 3	0.3603
Hexane:ethyl acetate (3:7)	Fraction 4	0.5901
Ethyl acetate:chloroform (9:10)	Fraction 5	0.7139
Ethyl acetate:methanol (9:1)	Fraction 6a	1.2204
	Fraction 6b	0.3771
Ethyl acetate: methanol (1:1)	Fraction 7a	0.3964
	Fraction 7b	0.1413
Total Yield		**4.222**

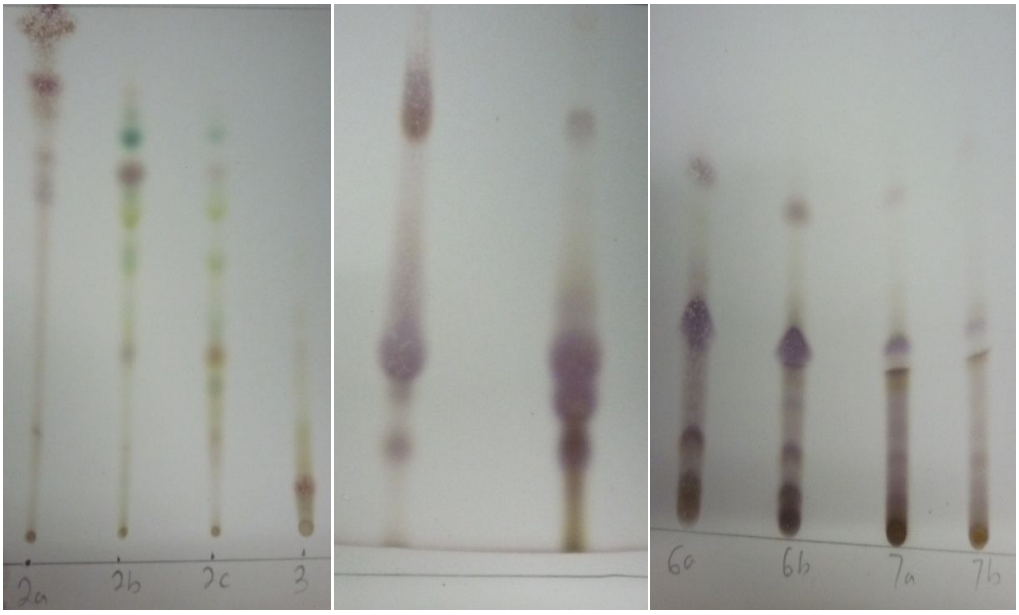


Plate XI: Thin layer chromatographic separation of fractions obtained from ethyl acetate fraction of methanol extract of *Peristrophe bicalyculata*

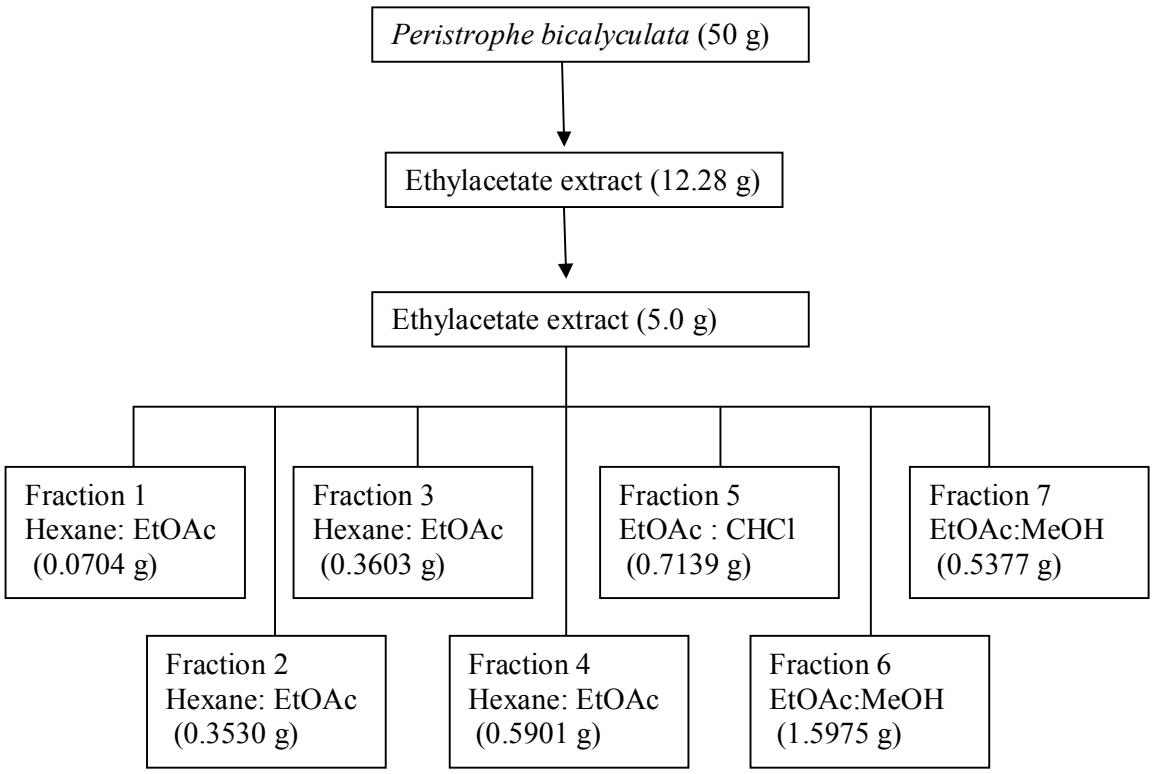


Figure 4.15: Yield and fractions obtained from ethyl acetate extract of *Peristrophe bicalyculata*

As shown in Figure 4.15, the yield from fraction 6 (1.5975 g) was highest followed by fraction 5 (0.7139 g), fraction 4 (0.5901 g), fraction 7 (0.5377 g), fraction 3 (0.3603g), while fraction 1 was the lowest with a yield of 0.0704 g.

All seven (7) fractions were tested for their anticancer activity against human mouth epidermal carcinoma (KB) by the sulphorhodamine B (SRB) assay. From the results (Table 4.12), R_f spots 1, 2, 4, 6 and 7 inhibited 50% of the cancer cells at concentrations above 500 µg/mL, which was significantly ($P < 0.05$) higher than the concentration at which R_f spot 3 (235 ± 33.05 µg/mL) inhibited the cells. R_f spot 5 inhibited the cancer cells at a concentration (3.5 ± 0.21 µg/mL) significantly ($P < 0.05$) lower than fraction 3 (235 ± 33.05 µg/mL), fractions 1, 2, 4, 6 and 7 (>500 µg/mL), but not different from the standard drug, cisplatin (3.32 ± 0.09 µg/mL). The IC_{50} values of the standard drugs, doxorubicin (0.43 ± 0.02 µg/mL) and vincristine (0.05 ± 0.01 µg/mL) were significantly lower than that of R_f spot 5 (3.5 ± 0.21 µg/mL).

Table 4.12: Anticancer activity of fractions of *P. bicalyculata*

Fractions	**IC_{50} values (µg/ml)**
R_f spot 1	>500
R_f spot 2	>500
R_f spot 3	235 ± 33.05^{a}
R_f spot 4	>500
R_f spot 5	3.5 ± 0.21^{b}
R_f spot 6	>500
R_f spot 7	>500
Cisplatin	3.32 ± 0.09^{b}
Doxorubicin	0.43 ± 0.02^{d}
Vincristine	0.05 ± 0.01^{e}

Values are mean ± SEM

^{a,b,c,d,e} = Values with different superscript letters are significantly different ($P < 0.05$)

> 500 = IC_{50} values are higher than 500 µg/ml (inactive)

R_f = Retention factor

4.5.2 Acridine Orange/Ethidium Bromide Assay Staining of Human Mouth Epidermal Carcinoma (KB)

4.5.2.1 Apoptotic Effect of Partially Purified Extract of Peristrophe bicalyculata

There was a significant ($P < 0.05$) increase in percentage apoptotic cells 24 h and 48 h after treatment with the partially-purified anticancer fraction of *Peristrophe bicalyculata* (fraction 5) and cisplastin compared to control cells (Figure 4.16). The percentage apoptotic cells in cultures treated with 10 µg/mL (15.06 ± 2.31 %) cisplastin was not significantly different from cultures treated with fraction 5 at 250 (12.49 ± 1.96 %) and 500 µg/mL (16.25 ± 1.87 %); while the percentage apoptotic cells in cultures treated with 100 µg/mL cisplastin (22.74 ± 2.44 %) was not significantly different from that obtained in cells treated with 1000 µg/mL of fraction 5 (21.20 ± 2.17 %).

Forty-eight (48) hours after treatment, the percentage of apoptotic cells increased significantly ($P < 0.05$) compared to the percentage recorded after 24 h treatment, and in the control cultures. There was a significant increase ($P < 0.05$) in the percentage of apoptotic cells in cultures treated with 1000 µg/mL cisplastin (81.22 ± 3.62 %) compared to cultures treated with 100 µg/mL (63.35 ± 3.84 %), 10 µg/mL (49.73 ± 5.23 %) and those treated with fraction 5 (Figure 4.16).

4.5.2.2 Necrotic Effect of Partially-Purified Extract of Peristrophe bicalyculata

Treatment of cells with cisplastin and partially purified fraction (fraction 5) after 24 h resulted in a significant ($P < 0.05$) increase in necrotic cells, compared to control culture (figure 4.17). The percentage necrotic cells in cultures treated with the partially-purified fraction (fraction 5) at 1000 µg/mL was significantly higher than that of cells treated with 500 µg/mL (24.77 ± 3.68%) and 250 µg/mL (18.28 ± 2.12%) but was not different

from cultures treated with cisplastin at the same concentration at 1000 µg/mL (37.33 ± 3.22%).

After 48 h, the percentage necrotic cells increased significantly ($P < 0.05$) in cisplastin and extract-treated cultures at all concentrations compared to treatment after 24 hours. Cell cultures treated with 10 µg/ml (48.30 ± 7.10 %) cisplastin had significantly ($P < 0.05$) higher number of necrotic cells than those treated with 250 µg/mL fraction 5 (37.84 ± 5.68 %). However, there was no significant difference when cells were treated with 100 µg/mL (55.61 ± 6.98 %) and 1000 µg/mL (65.56 ± 3.07 %) cisplastin and those treated with fraction 5 at 1000 µg/ml (65.70 ± 6.93 %) (Figure 4.17).

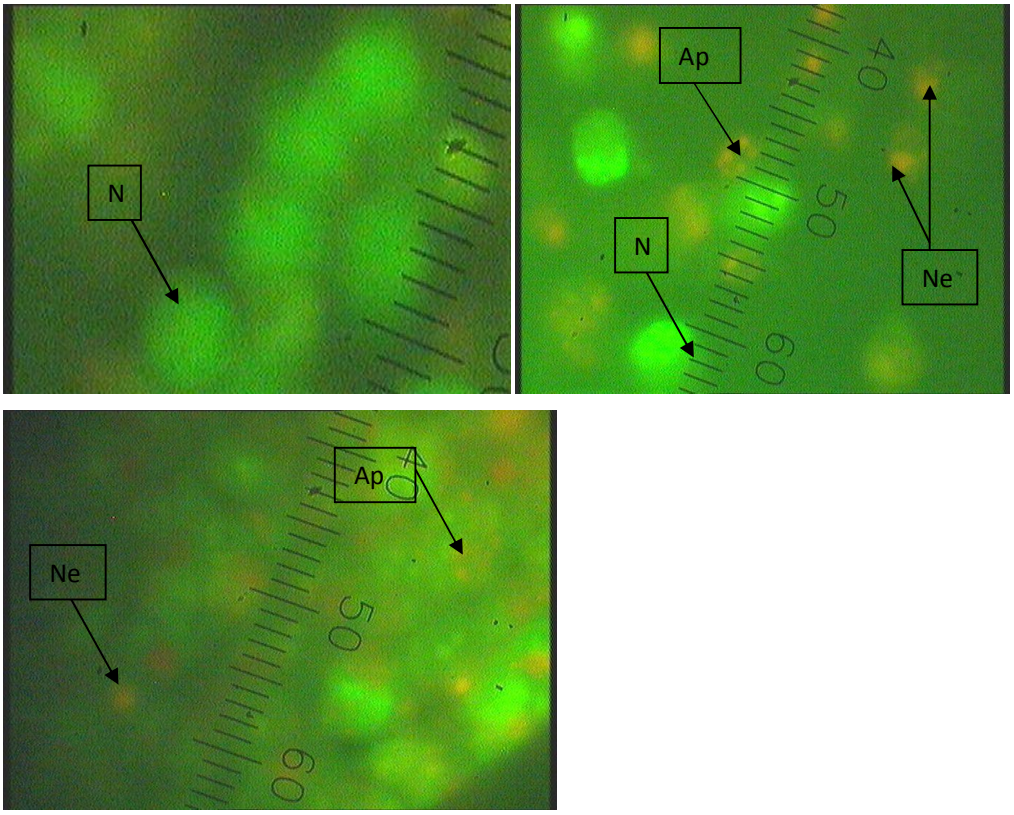


Plate XII: Effect of partially purified extract of *Peristrophe bicalyculata* on KB cells showing characteristic normal (N), necrotic (Ne) and apoptotic (A) morphology when stained with ethidium bromide and acridine orange stain (X 100 magnification). A: Control culture; B: cisplastin-treated culture; and C: extract-treated culture.

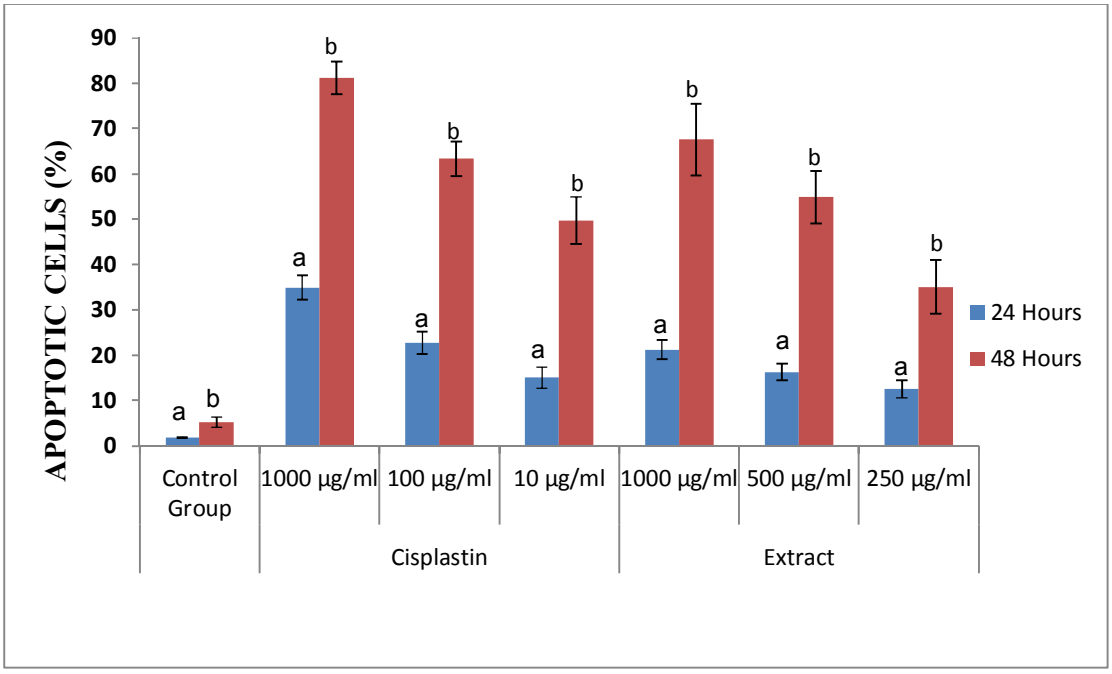


Figure 4.16: Percentage apoptotic human mouth epidermal carcinoma cells after 24 and 48 hours treatment with partially purified extract of *Peristrophe bicalyculata.*

[a,b] = Means with different superscript letters are significantly ($P < 0.05$) different

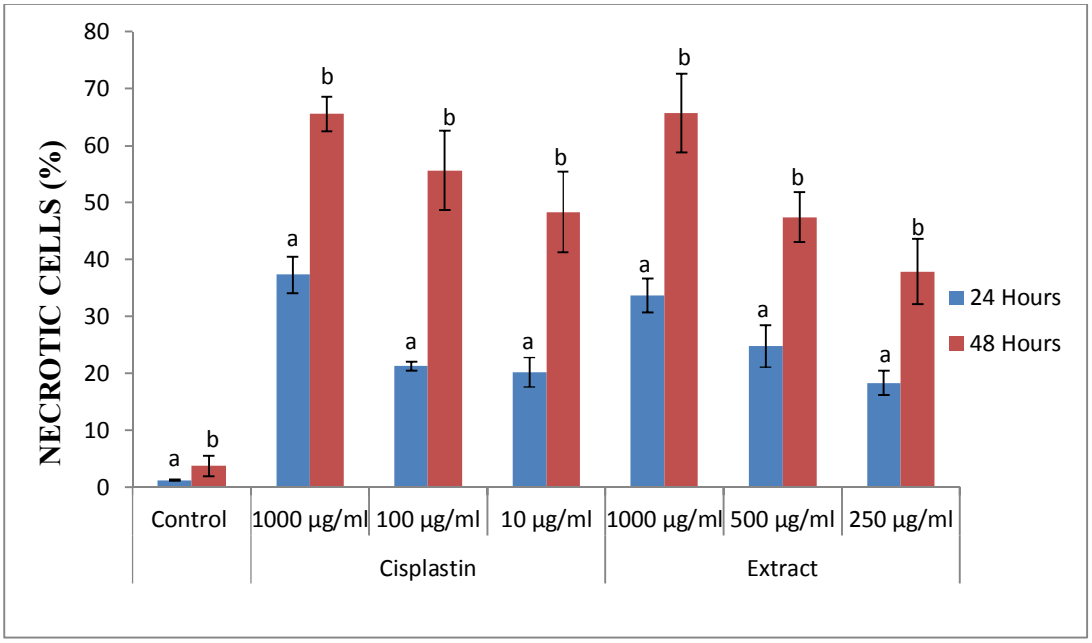


Figure 4.17: Percentage necrotic human mouth epidermal carcinoma cells after 24 and 48 hours treatment with partially purified extract of *Peristrophe bicalyculata*

[a,b] = Means with different superscript letters are significantly ($P < 0.05$) different

4.6 MELANOGENESIS INHIBITORY EFFECT of *Peristrophe bicalyculata*

To determine the effect of *Peristrophe bicalyculata* on the viability of $B_{16}F_{10}$ melanoma, the cells were treated with various concentrations of extracts of *Peristrophe bicalyculata* and results expressed as percentage viability relative to the control (Figure 4.18). The percentage viabilities of the cold water extract (99.58 ±7.01%), hot water extract (100.0 ± 4.52%), water fraction of methanol extract (103.0 ± 2.05%) and butanol fraction of methanol extract (96.0 ± 6.56%) were significantly higher ($P < 0.05$) than hexane extract (75.0 ± 12.23%), methanol extract (72.0 ± 11.53 %) and ethylacetate fraction of methanol extract (70.0 ± 10.26 %) at the lowest concentration (0.1 µg/mL). The percentage viability of the extracts decreased with increasing concentration of extract; but at the highest concentration of 1000 µg/mL, the cold water extract, water and butanol fractions of methanol extract were most viable at 113 ± 5.50, 101.23 ± 6.25 and 82.36 ± 10.56 percent respectively.

Table 4.13: Percentage viabilities of extracts and methanolic fractions of *P. bicalyculata* on $B_{16}F_{10}$ melanoma cells at varying concentrations

Extracts	Viability (%)				
	0.1 µg/ml	**1 µg/ml**	**10 µg/ml**	**100 µg/ml**	**1000 µg/ml**
Cold water	99.58 ± 7.01^{a}	103.0 ± 7.20^{c}	91.42 ± 6.90^{f}	88.80 ± 4.34^{h}	113 ± 5.50^{p}
Hot water	100.00 ± 4.52^{a}	88.52 ± 6.50^{e}	95.02 ± 7.60^{f}	70.82 ± 12.53^{j}	79.11 ± 8.90^{r}
Hexane	75.00 ± 12.23^{b}	72.39 ± 14.29^{d}	71.00 ± 9.63^{g}	70.00 ± 11.29	70 ± 9.02^{r}
Methanol	72.00 ± 11.53^{b}	66.10 ± 17.28^{d}	65.18 ± 10.26^{g}	76.72 ± 9.70^{j}	$63.31 \pm 11.45^{r,s}$
Methanolic ethylacetate fraction	70.00 ± 10.26^{b}	66.10 ± 12.57^{d}	66.52 ± 9.76^{g}	62.15 ± 13.04^{k}	55.01 ± 8.23^{s}
Methanolic butanol fraction	96.00 ± 6.56^{a}	100.0 ± 6.43^{c}	94 ± 5.52^{f}	78.83 ± 13.06^{j}	82.36 ± 10.56
Methanolic water fraction	103.00 ± 2.05^{a}	$99.69 \pm 4.02^{c,e}$	93.56 ± 5.67^{f}	93.71 ± 6.52^{h}	101.23 ± 6.25^{p}

Values are mean ± SEM

Values with different superscript in the same column are significantly different ($P<0.05$)

(PRINT FIGURE FROM LANDSCAPES)

The effects of the extracts on $B_{16}F_{10}$ cells at 1000 µg/ml were determined and their viability expressed as fold change with respect to control group. Results showed that the ethyl acetate fraction of methanol extract, hexane extract, hot water extract, water fraction of methanol extract and methanol extract were 1.87 ± 0.03, 1.80 ± 0.13, 1.74 ± 0.05, 1.71 ± 0.09 and 1.64 ± 0.13 folds of control (1.012 ± 0.01), respectively, and were significantly ($P < 0.05$) higher than the butanol fraction of methanol extract and cold water extract which were 1.18 ± 0.03 and 1.05 ± 0.03 folds of control respectively (Figure 4.18).

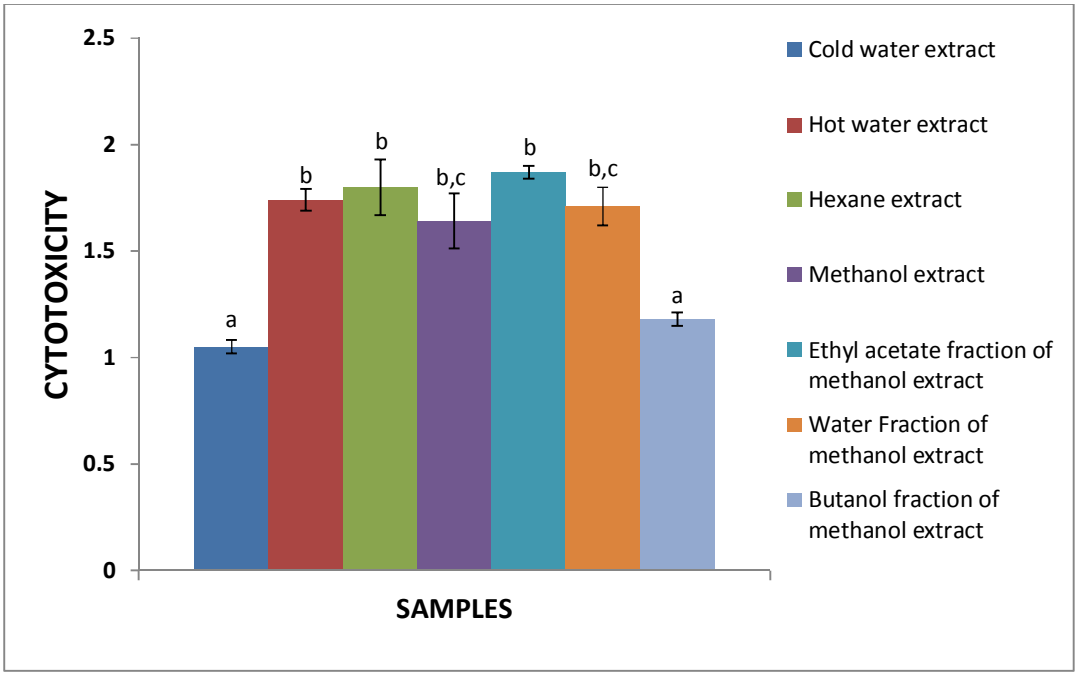


Figure 4.18: Cytotoxic effects of extracts and fractions of *Peristrophe bicalyculata* on $B_{16}F_{10}$ melanoma cells.

^{a,b,c} = Means with different superscript letters are significantly different ($P < 0.05$)

Figure 4.19 shows the melanin content of $B_{16}F_{10}$ melanoma cell cultures treated with Kojic acid, butanol fraction of methanol extract and cold water extract of *Peristrophe bicalyculata* compared to the control. From the results, the butanol fraction of methanol extract significantly ($P < 0.05$) reduced melanin content than the standard drug, Kojic acid and cold water extract at all concentrations. Also, the effect of all extracts on melanin formation was dose-dependent, with melanin content decreasing with increasing extract concentration.

The melanin content of $B_{16}F_{10}$ melanoma cultures treated with Kojic acid at 0.001, 0.01 and 0.1 mg/kg (1.52 ± 0.07, 1.35 ± 0.08 and 0.86 ± 0.1 mg/kg fold of control, respectively) was significantly ($P < 0.05$) higher than in cultures treated with butanol fraction of methanol extract (0.98 ± 0.04, 0.81 ± 0.04 and 0.59 ± 0.01 fold of control, respectively) and cold water extract (1.58 ± 0.06, 1.50 ± 0.08 and 1.20 ± 0.12 fold of control respectively) at similar concentrations. However, the melanin content in cell cultures treated with Kojic acid at 0.1 mg/kg (0.86 ± 0.1 fold of control) was not different from cells treated with butanol fraction of methanol extract at 0.001 and 0.01 mg/kg (0.98 ± 0.04 and 0.81 ± 0.04 folds of control, respectively).

The melanin content of cell cultures treated with cold water extract at 0.001 and 0.01 mg/mL (1.58 ± 0.06 and 1.50 ± 0.08 fold of control, respectively) was not significantly different from those obtained in cultures treated with kojic acid at the same concentrations (1.52 ± 0.07 and 1.35 ± 0.08 folds of control, respectively), but was significantly ($P < 0.05$) higher when compared at 0.1 mg/kg (1.20 ± 0.12 fold of control) with Kojic acid (0.86 ± 0.1 folds of control).

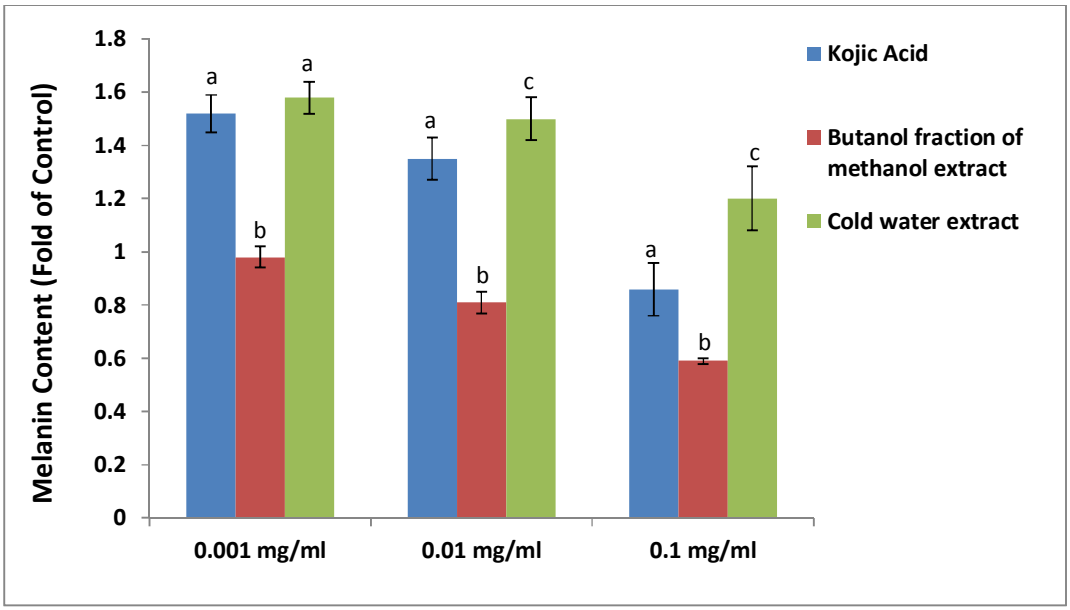


Figure 4.19: The melanin content of $B_{16}F_{10}$ melanoma cells after treatment with Kojic acid, butanol of methanol extract and cold water extracts of *Peristrophe bicalyculata* at various concentrations.

^{a,b,c} = Means with different superscript letters in the same column are significantly different ($P < 0.05$)

4.7 GAS CHROMATOGRAPY AND MASS SPECTOMETRY ANALYSIS OF PARTIALLY PURIFIED ANTIHYPERTENSIVE AND ANTICANCER FRACTIONS OF *Peristrophe bicalyculata*

4.7.1 Gas Chromatography and Mass Spectrometry Analysis of the Partially Purified Antihypertensive Fraction of *Peristrophe bicalyculata*

The results of the GC-MS analysis identified the various compounds present in the partially purified fraction. Figure 4.20 shows the gas chromatogram with five (5) distinct peaks identified by the GC-MS.

The major compound identified by GC-MS in the partially-purified antihypertensive fraction of *Peristrophe bicalyculata* was P,P,P-triphenyl-Imino(triphenyl)phosphorane with retention time (RT): 47.33 and molecular weight 278 g (Figure 4.21). Other compounds present are ethyl propanoic acid (RT: 3.41; molecular weight 102 g), 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one (RT: 11.71; molecular weight: 144 g), 1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy) tetrasiloxane (RT: 42.79; molecular weight: 444 g) and Andrographolide 2(3H)-furanone (RT: 57.39; molecular weight: 350 g).

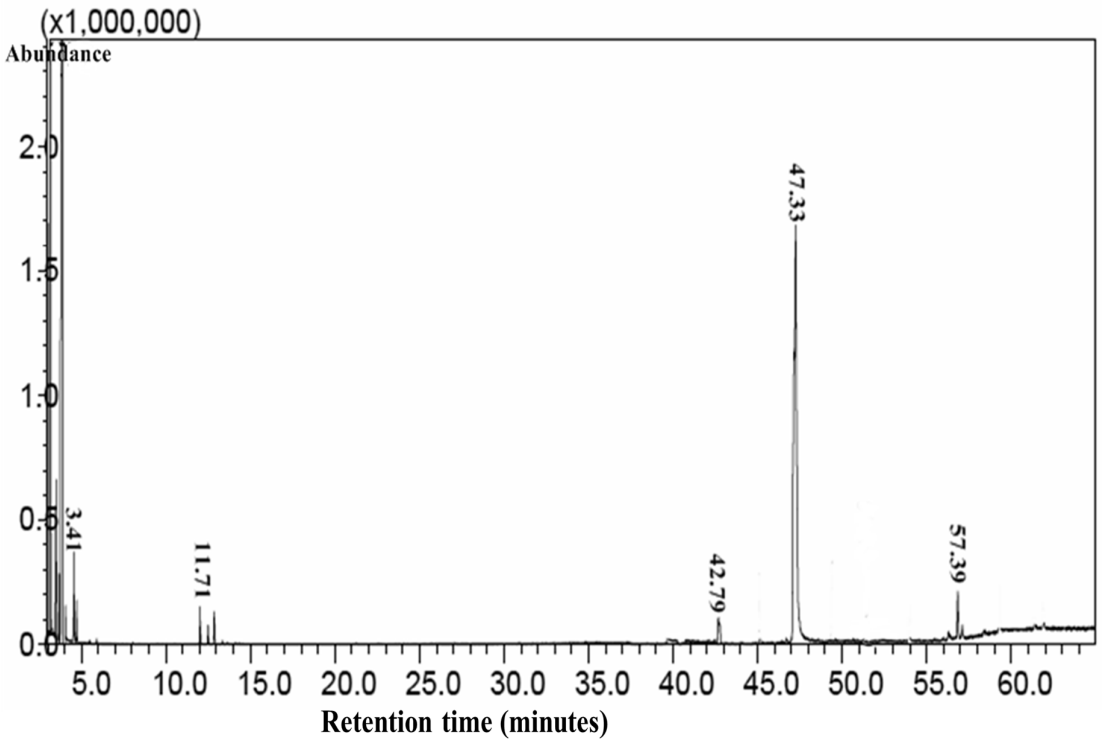


Figure 4.20: Gas chromatogram of partially-purified antihypertensive fraction of *Peristrophe bicalyculata*

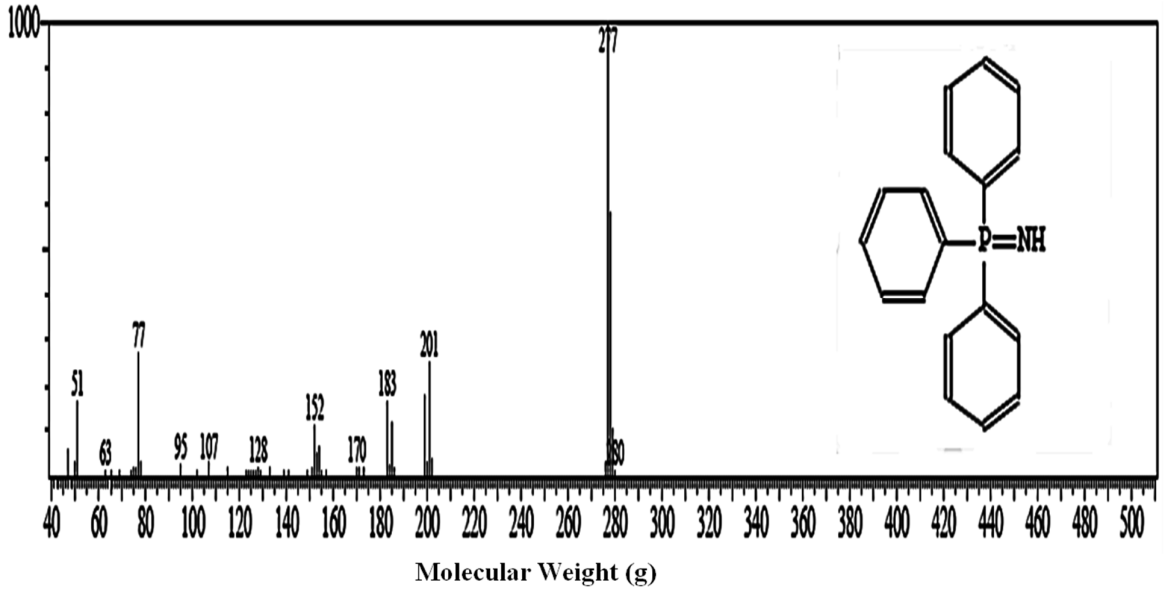


Figure 4.21: Mass spectrum of P,P,P-triphenyl-Imino(triphenyl)phosphorane Retention time: 47.33 minutes; $C_{18}H_{15}OP$; mol. Wt: 278 g)

4.7.2 Gas Chromatography and Mass Spectrometry Analysis of the Partially Purified Anticancer Fraction of *Peristrophe bicalyculata*

The gas chromatogram of the partially purified antihypertensive fraction of *Peristrophe bicalyculata* is shown in Figure 4.22. Six distinct peaks were obtained and the compounds identified were andrographolide 2(3H)-furanone (RT: 58.63 minutes; molecular weight: 350 g), 2,4,5-trimethoxybenzaldehyde (RT: 47.34 minutes; molecular weight: 196 g), diazoprogesterone (RT: 50.22 minutes; molecular weight: 338 g), cis-Z-.alpha.-bisabolene epoxide (RT: 52.04 minutes; molecular weight: 220 g), aromadendrene oxide (RT: 57.58 minutes; molecular weight 220 g) and 3,11-bis[(trimethylsilyl)oxy]androstan-17-one o-benzyloxime (RT: 56.33 minutes; molecular weight: 555 g). However, andrographolide 2(3H)-furanone was the most abundant of all the compounds identified.

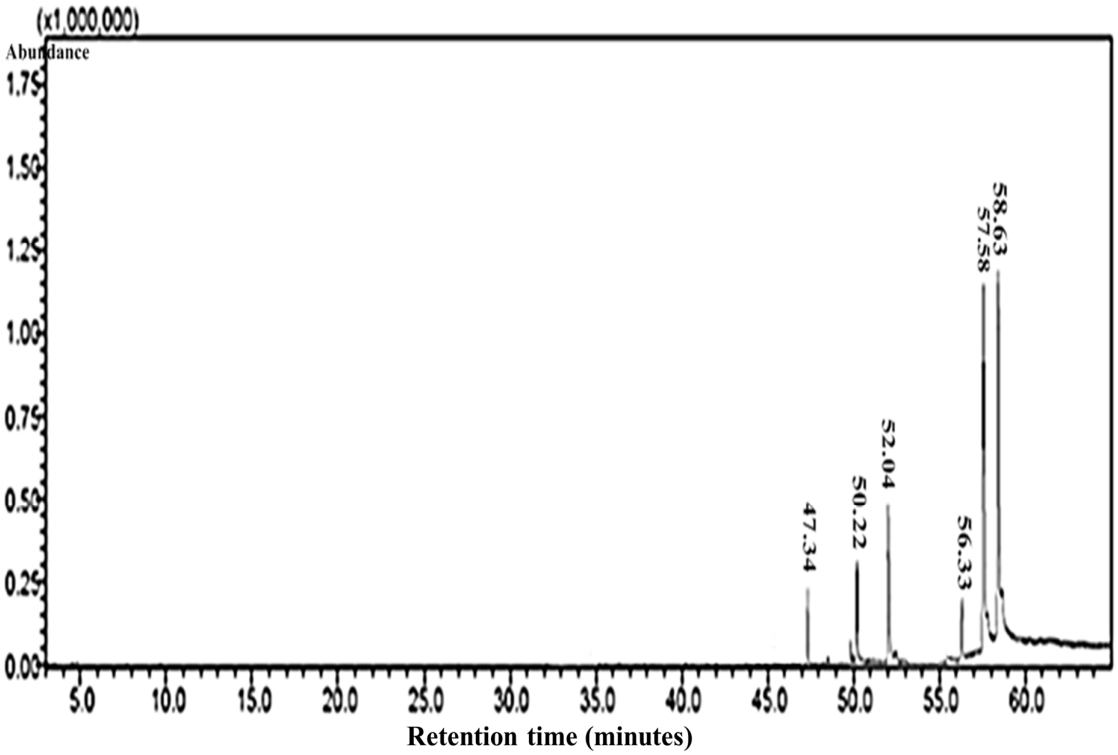


Figure 4.22: Gas chromatogram of partially-purified anticancer fraction of *Peristrophe bicalyculata*

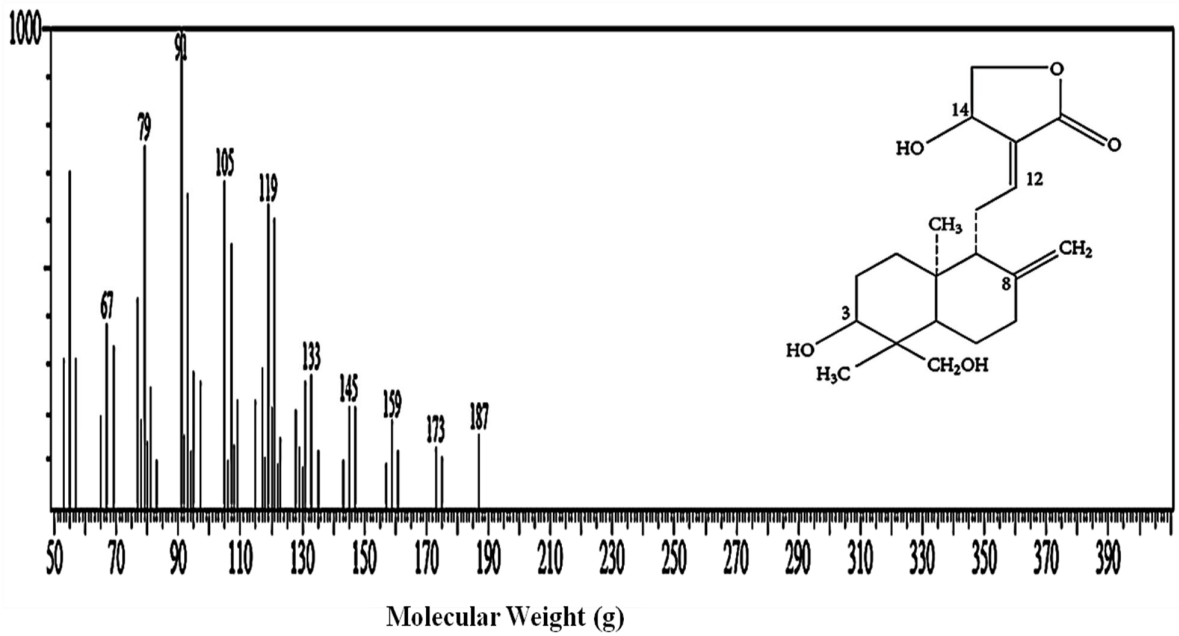


Figure 4.23: Mass spectrum of andrographolide 2(3H)-furanone (Retention time: 58.63 minutes; $C_{20}H_{30}O_5$; mol. Wt: 350 g)

CHAPTER 5

5.0 DISCUSSION

5.1 Antioxidant Activities of *Peristrophe bicalyculata*

Reactive oxygen species (ROS) including free and non-radical molecules play a significant role in the development of various diseases, including atherosclerosis, hypertension, ischaemia-reperfusion injury, inflammation, cystic fibrosis, diabetes mellitus, Parkinson's disease, Alzheimer's disease and cancer (Benz and Yau, 2008; Semchyshyn and Lushchak, 2012), hence the interest in identifying alternate, natural and safe sources of antioxidants from natural plant products (Zheng and Wang, 2002; Hajighasemi, 2011). Results of the radical scavenging, tyrosinase inhibition and metal ion chelating activities of extracts of *Peristrophe bicalyculata* (Table 4.4) demonstrated the antioxidant activity of the plant. The fact that radical-scavenging concentrations of the methanol extract (0.579 ± 0.02 mg/mL), butanol fraction of methanol extract (0.530 ± 0.01 mg/mL), and ethyl acetate fraction of methanol extract (0.510 ± 0.04 mg/mL) were not significantly ($P > 0.05$) different from one another, and that the values were significantly lower ($P < 0.05$) than those of the standard drug, vitamin C (0.705 ± 0.08 mg/mL) and all other extracts, showed that the methanol extract, butanol and ethyl acetate fractions of methanol extract may be considered better antioxidants than other extracts. This finding is in agreement with the results obtained by Sawadago *et al*. (2006) and Charoenchai *et al.* (2010) that extracts of plants belonging to the family Acanthaceae contain potent antioxidants, possessing these therapeutic values. Thus, the scavenging ability of the plant was apparently due to its ability to reduce DPPH radicals by donating hydrogen ions (Duan *et al*., 2007; Li *et. al*., 2009).

The results of the inhibitory effect of the extracts of *Peristrophe bicalyculata* on lipid peroxidation was compared with those of commercial antioxidants based on the amount of peroxides formed during seven days of incubation period. The high absorbance obtained was associated with high concentration of formed peroxides. The results of the present study showed that most extracts of the plant exhibited the same pattern of activity as vitamin E and BHA at different incubation times (Figure 4.1), demonstrating the ability of the extracts to inhibit lipid peroxidation. This finding is in agreement with the results obtained by Ebrahimzadeh *et al*. (2010) that the ability of some plant extracts to inhibit lipid peroxidation decreases with increasing incubation.

Extracts of *Peristrophe bicalyculata* demonstrated a considerable capacity for iron binding. Exceptions were the hot water extract (1.460 ± 0.18 mg/mL) and water fraction of methanol extract (1.380 ± 0.15 mg/mL), suggesting that the ability of the extracts to inhibit peroxidation was related to their ferrous-binding capacity (Table 4.4) (Ebrahimzadeh *et al*., 2010). Although there is paucity of information on the antioxidant properties of *Peristrophe bicalyculata*, the result of the phytochemical constituents of the plant (Table 4.2) showed that it is rich in flavones and phenols. This finding is in agreement with the results of Spitz *et al*., (2005) and Siriwardhana *et al*. (2003) that plants containing high levels of flavones, flavonoids and polyphenol possess very good antioxidant properties. It has also been reported that other bioactive components such as flavones and phenols found in proteins, polysaccharides and pigments present in plants, exhibit antioxidant activities (Duan *et al*., 2007; Li *et. al*., 2009). Thus, all the phytochemical constituents of the plant demonstrated that the extracts of *Peristrophe bicalyculata* possess strong antioxidant activity.

It has been reported that plants containing high levels of phenols are good antioxidants and are capable of inhibiting tyrosinase (Huang *et al.*, 2011); hence, the tyrosinase inhibitory activity of *Peristrophe bicalyculata* was determined in the present study. Results showed that the hexane (0.560 ± 0.06 mg/mL) and cold water (0.550 ± 0.050 mg/mL) extracts, butanol (0.557 + 0.04 mg/mL) and ethyl acetate (0.520 ± 0.080 mg/mL) fractions of methanol extract inhibited the tyrosinase enzyme at very low concentrations and so may be useful in preventing fruit browning and skin pigment formation (Chang, 2009).

5.2 Antihypertensive Effects of *Peristrophe bicalyculata*

The cold water extract and water fraction of methanol extract were investigated for antihypertensive activities based on the result of a previous study (Abdulazeez *et al.*, 2010). The water (aqueous) extract was found to be the most effective, while the angiotensin-converting enzyme inhibitory activity of the water fraction of methanol extract (15.16 ± 3.82 μg/mL) was not significantly different from that of the cold water extract (11.84 ± 0.88 μg/mL). This finding validates the claim that the aqueous extract of the plant possess antihypertensive activity, and demonstrated that the plant exert its effect via a mechanism involving inhibition of angiotensin-converting enzyme.

The consistent and normal systolic blood pressure in rats within the control group is evidence that the rats were healthy and not hypertensive. The significant ($P < 0.05$) increase in blood pressure observed when the rats were given L-NAME both on acute and chronic administration may be attributed to the inhibition of endothelial nitric oxide synthase by L-NAME, known to inhibit the synthesis of nitric oxide from L-arginine,

thereby causing severe and progressive arterial hypertension and renal injury (Garcia-Estan *et al*., 2006; Pechanova *et al*., 2006). The inhibition, apparently, occured as a result of vasoconstriction, associated with enhanced sodium and water renal reabsorption, increased oxidative stress and enhanced calcium signalling in smooth muscle cells (Zatz and Baylis, 1998). The inhibition of eNOS also explains the significant ($P < 0.05$) decrease in eNOS activity in serum and tissues of L-NAME hypertensive rats. Furthermore, the finding of the present study is in agreement with an increasingly large body of literature suggesting that alteration in the nitric oxide system plays an important role in the development or maintenance of clinical hypertension. The finding supports that of Augustyniak *et al*. (2005) who showed that certain forms of secondary hypertension are usually accompanied by accumulation of endogenous eNOS inhibitors, which may contribute to the development of hypertension.

Studies by Salazar *et al*. (1992) demonstrated that administration of L-arginine competitively reversed eNOS inhibition and, therefore, obliterate L-NAME–induced hypertension, only partially reduced blood pressure after a week; and the effect is reduced further after 4-6 weeks administration of L-arginine (Qiu *et al*., 1995). This fact demonstrated that once nitric oxide has been inhibited; the hypertension induced was no longer dependent exclusively on inactivation of the L-arginine/nitric oxide pathway. Indeed, the RAAS plays an important role because arterial hypertension and renal alterations, which occur on inhibiting nitric oxide are prevented, or almost completely corrected, by inhibiting ACE or type 1 angiotensin II receptors (Garcia-Estan *et al*., 2006). Therefore, the reduction in blood pressure and ACE activity in serum and tissues of L-NAME-induced hypertensive rats given captopril was evidence that the RAAS plays an important role in alleviating hypertension caused by eNOS-induced inhibition

(Fortepiani *et al*., 1999). Mombouli and Vanhoutte (1996) demonstrated that ACE inhibitors, such as captopril, prevent the conversion of angiotensin I to angiotensin II and, thus prevent degradation of bradykinin, causing the synthesis of vasodilator substances such as endothelium-derived hyperpolarizing factor (EDHF), prostacyclin and nitric oxide (Wiemer *et al*., 1991). Thus, the mechanism underlying the action of the cold water extract and partially-purified antihypertensive fraction (fraction 5) of *Peristrophe bicalyculata*, which in the present study were found to be potent ACE inhibitors may be linked to the prevention of conversion of angiotensin I to angiotensin II and synthesis of vasodilatory substances.

The significant increase in ACE activity observed in the serum and tissues of L-NAME hypertensive rats was due to increased expression of ACE mRNA (Figure 4.11). The ACE mRNA expression in the kidneys decreased when the rats were given captopril and the partially-purified antihypertensive fraction (fraction 5) of *Peristrophe bicalyculata,* demonstrating that the decrease in ACE activity was caused by the treatment at the level of gene expression. However, there was no significant difference in the expression of ACE mRNA in aorta of rats given the partially-purified fraction; even though the activity significantly ($P < 0.05$) decreased, showing that the fraction may decrease the activity of ACE in aorta by other mechanism. This mechanism may, however, not be at the level of gene expression of the enzyme.

The eNOS mRNA expression decreased significantly in the kidneys and aorta of hypertensive rats; apparently because chronic eNOS inhibition not only causes a decrease in eNOS activity, but also the expression of eNOS mRNA. This is because the

physiological bioavailabilty of nitric oxide is dependent on steady state levels of eNOS expression and a functional eNOS activity (Linz *et al*., 1995). Michel *et al.* (1996) also reported that chronic inhibition of eNOS by L-NAME upregulates the activity and expression of ACE and downregulates eNOS activity and mRNA levels, whereas treatment with nitric oxide or inhibitors of ACE inhibit ACE activity in a concentration-dependent and competitive manner. This inhibition reduces conversion of angiotensin I to II. Although not yet understood, it is believed that the effects of nitric oxide are mediated by guanylyl cyclase activation to synthesise cGMP, which acts by a feedback mechanism; such that when nitric oxide is inhibited, low levels of cGMP causes increase in ACE activity and decrease eNOS activity. The mechanism by which ACE inhibitors upregulate eNOS mRNA expression has not been elucidated, but studies have demonstrated that ACE inhibitors induce accumulation of endogenous kinins to mediate this effect. Another mechanism is believed to be through cyclic AMP. This is because ACE inhibitors and bradykinin enhance the turnover of endothelial prostacyclin via increased intracellular calcium ions. The increased intracellular calcium ions in turn stimulate the formation of cAMP, known to activate gene transcription via binding to nuclear-factor-1(NF1), which is present in the promoter region of eNOS. Therefore, the regulation of eNOS is involved in the development of hypertension, atherosclerosis and heart failure, in cases involving impairement of endothelium-derived nitric oxide (Augustyniak *et.al*, 2005).

Hypertension and other cardiovascular diseases, believed to be related to endothelial dysfunction are known to cause an imbalance in the endothelial production of NO and O^{2-}. This imbalance results in oxidative stress, which is associated with peroxidation of cellular lipids (Semchyshyn and Lushchak, 2012). Measurement of lipid peroxidation

end products such as TBARS is a good index of cell destruction, because cells and tissues damaged by any mechanism tend to peroxidize more rapidly than normal (Yokozawa *et al.*, 2000). Therefore, the concentration of lipid peroxidation products reflects the degree of oxidative stress (Halliwell, 1997). This explains the high level of TBARS in serum and tissues of L-NAME hypertensive rats. The administration of captopril and the partially-purified fraction of *Peristrophe bicalyculata* significantly reduced the levels of TBARS; probably because ACE inhibitors accumulate endogenous kinins, which activate eNOS and increase the synthesis of nitric oxide, known to scavenge reactive oxygen species (Gohlke *et al*., 1993). Also, the antioxidant activity of the partially purified fraction of *Peristrophe bicalyculata* may explain the ability of the fraction to scavenge reactive oxygen species.

5.3 Anticancer Activity of *Peristrophe bicalyculata*

The anticancer activity of *Peristrophe bicalyculata* (Table 4.10) shows that the hexane extract, methanol extract and butanol fraction of methanol extract were active against some of the cell lines, but they may only be classified as potentially harmful or moderately toxic (Boyd, 1989). The ethyl acetate fraction of methanol extract, with an IC_{50} of 15.6 ± 0.52 µg/mL may be classified to be potentially very cytotoxic against KB. Further purification of this fraction gave seven fractions; five (TLC spots 1, 2, 4, 6 and 7), which were inactive, with IC_{50} values above 500 µg/ml, and one (TLC spot 3) moderately toxic fraction (235 ± 33.05 µg/ml). TLC spot 5 was most active with an IC_{50} value of 3.5 ± 0.21 µg/mL. This result supports the findings of Ogunwande *et al.* (2010) and Ashokan and Muthuraman (2011), demonstrating the anticancer activity of extracts and essential oils of *Peristrophe bicalyculata* in different cancer cell lines. Several

studies have also reported potent anticancer properties of extracts of plants belonging to the same family as *Peristrophe bicalyculata,* Acanthaceae (Khajure and Rathod, 2011; Ashour, 2012; Ismail *et al.,* 2012).

It is a conceivable fact that one essential strategy for cancer therapy is to target the lesions that suppress apoptosis in the tumour cells, as can be found in most cancer chemotherapy drugs which exert cytotoxic effects on malignant cells by inducing apoptosis (Tu *et al*., 1996; Kaufmann and Earnshaw, 2000; Evan and Vousden, 2001). Thus, it is suggestive that the partially-purified anticancer fraction (TLC spot 5) of *Peristrophe bicalyculata* may be a possible anticancer drug candidate. This is because the morphological changes observed on treating KB cells with this fraction demonstrated its ability to induce apoptosis, although very little has been done to determine its mechanism of action. Besides, the anticancer activity of *Peristrophe bicalyculata* may be attributed to its high antioxidant activity and its phenol, flavone and alkaloid contents. These compounds are known to inhibit cancer cells by metabolizing xenobiotic enzymes that alter metabolic activation of potential carcinogens, alteration of hormone production, inhibition of aromatase to prevent the development of cancer cells and by disturbing the cellular division during mitosis at the telophase stage (Zhao *et al*., 2007). It has also been reported that phenols reduce the amount of cellular protein and mitotic index, and the colony formation during cell proliferation of cancer cells (Gawron and Kruk, 1992).

5.4 Melanogenesis Inhibitory Effect of *Peristrophe bicalyculata*

In the present study, the tyrosinase inhibition concentration at 50% activity (IC_{50}) of the butanol fraction of methanol extract (0.557 ± 0.04 mg/mL), ethyl acetate fraction of

methanol extract (0.520 ± 0.08 mg/mL), hexane (0.560 ± 0.06 mg/mL) and cold water (0.550 ± 0.05 mg/mL) extracts were significantly lower than Kojic acid (1.070 ± 0.05 mg/mL). On assessing the effects of these extracts on cell viability, only the butanol fraction of methanol extract and cold water extracts were found not to exert significant effect on cell proliferation at 1000 µg/ml. Thus, the effects of hexane extract, hot water extract, ethyl acetate and water fractions of methanol extract on melanin production were not studied due to the high cytotoxic effects, although the extracts exhibited active antityrosinase activities. Also, the methanol extract was not considered due to its low antityrosinase activity (0.650 + 0.07 mg/ml) and high cytotoxicity (63.31 ± 11.45%). This was done to ensure that the ability of an extract to inhibit melanin formation in $B_{16}F_{10}$ cells was not as a reult of its cytotoxic effects on the cells (Yu and Kim, 2010; Arunga *et al*., 2007).

The butanol fraction of methanol extract significantly ($P < 0.05$) reduced melanin content more than either the cold water extract or Kojic acid. This finding demonstrated the potential use of *Peristrophe bicalyculata* as a source of a skin whitening agent, or a new source for melanoma treatment. It has been documented that most antimelanogenesis agents function specifically to reduce the activity of tyrosinase (Yu and Kim, 2010). Consequently, the anti-melanogenesis activity of the butanol fraction of methanol extract of *Peristrophe bicalyculata* may be attributed to its ability to inhibit the tyrosinase enzyme, although further studies are required to elucidate its mechanism of action.

5.5 Gas Chromatography and Mass Spectrometry Analysis of the Partially Purified Antihypertensive and Anticancer Fractions of *Peristrophe bicalyculata*

P,P,P-triphenyl-Imino(triphenyl)phosphorane is the most abundant compound present in the partially-purified antihypertensive fraction of *Peristrophe bicalyculata.* This compound, which is a derivative of immunophosphorane has been demonstrated, for the first time, in the present study to possess antihypertensive activity. Also, the presence of a naturally-occurring immunophosphorane may be novel as the compound is usually synthesized via the Staudinger reaction (Shaik *et. al*., 2009; Ramzani *et al.,* 2011).

Other compounds present in the fraction include ethyl propanoic acid, used in the production of perfumes and fragrances, while 1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy) tetrasiloxane, like many other low molecular weight siloxanes, are used as vaporisable carriers in cosmetic preparations due to their high rate of vaporisation and pleasant feel on the skin (Eversheim *et al*., 2008). 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one is a malliard reaction product formed from carbohydrates (Marko, 2007).

The presence of compounds known to be cytotoxic against cancer cells in the partially purified anticancer fraction obtained in the present study may play major roles in the anticancer activity of the plant. Andrographolide 2(3H)-furanone, the most abundant compound present in the partially-purified anticancer fraction of *Peristrophe bicalyculata* is a derivative of andrographolide, the principal bioactive chemical constituent isolated from *Andrographis paniculata* Nees. Andrographoide has shown credible anticancer potential in various investigations (Chun *et al*., 2010; Varma *et al*., 2011). *In vitro* studies demonstrated its ability to induce cell-cycle arrest and apoptosis

in a variety of cancer cells at different concentrations (Kim *et al*., 2005). It has also shown potent immunomodulatory and anti-angiogenic activities in tumorous tissues. Synthetic analogues of the compound have also exhibited similar activities (Varma *et al*., 2011). Both *Peristrophe bicalyculata* and *Andrographis paniculata* Nees belong to the same family and, hence, the finding of the present study is in agreement with the report of Geethangili *et al.* (2008) and Chun *et al.* (2010), who demonstrated the presence of similar compound and action in plants belonging to the same family.

Aromadendrene oxide has been isolated and characterized from the stems of *Sandorium koetjape*, and demonstrated to be significantly cytotoxic against many cultured human cancer cells, especially P-388 cells (Kaneda *et al*., 1992). 2,4,5-Trimethoxybenzaldehyde has been isolated from *Antrodia camphorata* and shown to be chemoprotective against colon and breast cancer (Koki *et al*., 1999), and antiimflamatory by inhibitng cyclooxygenase 2 in MDA-MB-231 cells (Tsai *et al.,* 2005).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 SUMMARY

This study has demonstrated that the leaves of *Peristrophe bicalyculata* reduced blood pressure by acting via the renin angiotensin system as the partially-purified extract decreased the activity and expression of ACE mRNA in serum and tissues of hypertensive rats. The mechanism of action of the extract was exhibited apparently by inhibiting the activity of ACE, so that angiotensin I converted to angiotensin 1-7 by prolyl endopeptidases (PEP) activates B_2 kinin receptors to release vasodilatory substances such as nitric oxide.

The anticancer activity of *Peristrophe bicalyculata* showed that extracts of the plant was cytotoxic against KB cells. This study also demonstrated that *Peristrophe bicalyculata* inhibited cancer cells by inducing apoptosis, although, the exact mechanism by which apoptosis was induced was not investigated.

The butanol fraction of the plant had the highest antityrosinase activity and significantly reduced melanin formation, demonstrating the potential use of *Peristrophe bicalyculata* as a skin whitening agent and a new source for melanoma treatment.

P,P,P-triphenyl-Imino(triphenyl)phosphorane identified in the antihypertensive fraction of *Peristrophe bicalyculata* may be responsible for its antihypertensive activity, or it may enhance the biological function of other components present in the plant to exert

this effect. The anticancer activity of the plant may be attributed to andrographolide 2(3H)-furanone, aromadendrene oxide and 2,4,5-Trimethoxybenzaldehyde, known to possess such property.

6.2 CONCLUSIONS

1. The leaf extracts of *Peristrophe bicalyculata* contain potent antioxidants, inhibitors of lipid peroxidation and tyrosinase.

2. *Peristrophe bicalyculata* may play an important role in alleviating hypertension caused by NOS inhibition via the RAS, by decreasing the activity and expression of ACE mRNA in serum and tissues of hypertensive rats.

3. The extract increases NOS activity and expression probably by accumulation of endogenous kinins and endothelial cGMP, which is an index of NO synthesis and release.

4. The extracts of *Peristrophe bicalyculata* significantly reduced levels of thiobarbituric acid-reactive substances; probably because ACE inhibitors accumulate endogenous kinins, which activate eNOS and increase the synthesis of nitric oxide, known to scavenge reactive oxygen species.

5. The partially-purified anticancer fraction of *Peristrophe bicalyculata* promoted apoptosis, which made the fraction a potential source of valuable anticancer therapy.

6.3 RECOMMENDATIONS

1. Further purification on the extract should be conducted and investigation on the anticancer and antihypertensive compounds be performed.

2. The mechanism of apoptotic action of the anticancer fraction needs to be elucidated.

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APPENDICES

Appendix 1

Lipid peroxidation inhibitory activity of different extracts of *P. bicalyculata* at different incubation times

EXTRACTS	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7
Control	0	0.16 ± 0.02	0.25 ± 0.03	0.36 ± 0.01	0.44 ± 0.01	0.53 ± 0.03	0.65 ± 0.03
Vit E	0	0.04 ± 0.01	0.07 ± 0.01	0.13 ± 0.05	0.18 ± 0.02	0.28 ± 0.04	0.33 ± 0.02
BHA	0	0.04 ± 0.03	0.14 ± 0.02	0.17 ± 0.03	0.22 ± 0.03	0.28 ± 0.01	0.39 ± 0.01
Methanolic water fraction	0	0.08 ± 0.01	0.16 ± 0.04	0.3 ± 0.02	0.39 ± 0.02	0.42 ± 0.02	0.56 ± 0.04
Hexane extract	0	0.13 ± 0.01	0.05 ± 0.01	0.28 ± 0.02	0.44 ± 0.02	0.48 ± 0.02	0.53 ± 0.04
Hot water extract	0	0.08 ± 0.01	0.12 ± 0.05	0.28 ± 0.01	0.35 ± 0.06	0.45 ± 0.03	0.54 ± 0.02
Methanol extract	0	0.13 ± 0.02	0.06 ± 0.01	0.23 ± 0.01	0.3 ± 0.02	0.36 ± 0.04	0.46 ± 0.04
Methanolic butanol fraction	0	0.11 ± 0.01	0.14 ± 0.02	0.15 ± 0.02	0.16 ± 0.03	0.2 ± 0.02	0.25 ± 0.03
Methanolic ethyl acetate fraction	0	0.14 ± 0.02	0.11 ± 0.02	0.06 ± 0.01	0.15 ± 01	0.28 ± 0.02	0.33 ± 0.01
Cold water extract	0	0.13 ± 0.03	0.04 ± 0.04	0.12 ± 0.04	0.11 ± 0.02	0.14 ± 0.04	0.19 ± 0.03

Appendix 2

Effect of partially-purified extract of *Peristrophe bicalyculata* on body weight of L-NAME-induced hypertensive rats

	Control group	**Hypertensive group**	**Captopril (50 mg/kg)**	**Extract (2.5 mg/kg)**	**Extract (25 mg/kg)**
Week 1	9.66 ± 1.02	14.13 ± 1.55	12.59 ± 1.92	13.17 ± 1.15	11.54 ± 1.55
Week 2	15.54 ±1.36	20.15 ± 2.47	18.38 ± 2.43	19.47 ± 1.75	19.53 ± 1.11
Week 3	18.23 ± 2.49	23.19 ± 2.81	21.51 ± 2.10	21.58 ± 2.98	22.26 ± 2.47
Week 4	21.02 ± 1.09	26.08 ± 1.24	23.11 ± 1.05	23.52 ± 2.62	24.72 ± 1.51

Appendix 3

Effect of partially-purified extract of *Peristrophe bicalyculata* on systolic blood pressure of L-NAME-induced hypertensive rats

	Control group	**Hypertensive group**	**Captopril (50 mg)**	**Extract (2.5 mg/kg)**	**Extract (25 mg/kg)**
Basal	109.00 ± 2.65	113.35 ± 3.71[a]	110.67 ± 2.33[a]	110.00 ± 4.04[a]	108.67 ± 3.18[a]
Week 1	111.33 ± 1.20	169.30 ± 3.48[b]	172.00 ± 5.85[b]	176.00 ± 5.70[b]	180.00 ± 10.12[b]
Week 2	112.00 ± 2.65	189.00 ± 4.81[c]	142.00 ± 4.16[c]	172.33 ± 4.33[b]	144.33 ± 12.73[c]
Week 3	113.33 ± 2.91	195.00 ± 3.88[c]	118.67 ± 4.67[a]	138.33 ± 4.18[c]	107.33 ± 8.19[a]
Week 4	110.30 ± 2.60	212.00 ± 2.08[d]	74.00 ± 4.36[d]	103.33 ± 8.11[a]	94.00 ± 1.00[a]

Appendix 4

Effect of partially-purified extract of *Peristrophe bicalyculata* on activity of angiotensin-converting enzyme in the serum, kidney and aorta of L-NAME hypertensive rats

	Control group	**Hypertensive control**	**Captopril (50 mg/kg)**	**Extract (2.5 mg/kg)**	**Extract (25 mg/kg)**
Serum (µmol/min/mL)	0.05 ± 0.01^{e}	0.44 ± 0.04^{f}	$0.11 \pm 0.01^{e,h}$	0.28 ± 0.03^{g}	$0.19 \pm 0.05^{h,g}$
Kidney (µmol/min/mg)	1.38 ± 0.07^{a}	2.3 ± 0.17^{c}	$1.45 \pm 0.04^{a,d}$	1.66 ± 0.07^{d}	$1.56 \pm 0.02^{a,d}$
Aorta (µmol/min/mg)	1.08 ± 0.05^{x}	1.87 ± 0.11^{w}	1.07 ± 0.11^{x}	1.35 ± 0.05^{y}	$1.25 \pm 0.09^{x,y}$

Appendix 5

Effect of partially-purified extract of *Peristrophe bicalyculata* on activity of endothelial nitric oxide synthase (eNOS) in the serum, kidney and aorta of L-NAME-induced hypertensive rats

	Control group	**Hypertensive group**	**Captopril (50 mg/kg)**	**Extract (2.5 mg/kg)**	**Extract (25 mg/kg)**
Kidney (µmol/min/mg)	6.27 ± 1.25^{e}	5.43 ± 0.23^{f}	$7.53 \pm 1.15^{e,g}$	$4.69 \pm 0.50^{e,f}$	$6.44 \pm 1.13^{e,f,g}$
Aorta (µmol/min/mg)	11.97 ± 0.81^{h}	5.4 ± 0.03^{i}	9.84 ± 0.67^{j}	7.9 ± 1.65^{k}	$9.42 \pm 0.75^{j,k}$
Serum (µmol/min/mL)	9.50 ± 1.22^{a}	3.99 ± 0.61^{b}	$8.33 \pm 0.53^{a,c}$	5.92 ± 0.67^{d}	7.44 ± 0.74^{c}

Appendix 6

Effect of partially purified extract of *P. bicalyculata* on thiobarbituric acid reactive substances (TBARS) in serum, kidneys and aorta of L-NAME-induced hypertensive rats

	Control group	Hypertensive control	Captopril (50 mg/kg)	Extract (2.5 mg/kg)	Extract (25 mg/kg)
Serum (mmol/dl)	8.82 ± 0.16^{a}	35.43 ± 1.98^{b}	13.56 ± 0.79^{c}	23.83 ± 1.65^{d}	17.75 ± 1.08^{e}
Kidney (mmol/100g)	79.06 ± 3.59^{y}	152.85 ± 6.20^{w}	89.20 ± 4.11^{y}	129.36 ± 5.18^{p}	92.94 ± 4.18^{y}
Aorta (mmol/100g)	68.12 ± 2.16^{h}	128.51 ± 4.86^{i}	83.05 ± 1.74j	99.56 ± 3.42^{k}	93.28 ± 3.01^{k}

Appendix 7

Effect of *Peristrophe bicalyculata* on the expression of angiotensin-converting enzyme in the kidneys and aorta of L-NAME-induced hypertensive rats

	Control group	**Hypertensive control**	**Captopril (50 mg/kg)**	**Extract (2.5 mg/kg)**	**Extract (25 mg/kg)**
Kidney	0.35 ± 0.09^{a}	1.14 ± 0.04^{b}	$0.46 \pm 0.03^{a,d}$	0.84 ± 0.04^{c}	0.53 ± 0.02^{d}
Aorta	0.69 ± 0.06^{e}	$1.04 \pm 0.17^{g,f}$	0.73 ± 0.06^{e}	1.21 ± 0.03^{f}	$0.90 \pm 0.06^{e,g}$

Appendix 8

Effect of *Peristrophe bicalyculata* on the expression of endothelial nitric oxide synthase (eNOS) in the kidneys and aorta of L-NAME-induced hypertensive rats

	Control group	**Hypertensive control**	**Captopril (50 mg/kg)**	**Extract (2.5 mg/kg)**	**Extract (25 mg/kg)**
Kidneys	0.94 ± 0.06^{a}	0.76 ± 0.02^{b}	$0.84 \pm 0.01^{a,b}$	$0.86 \pm 0.04^{a,b}$	0.74 ± 0.07^{b}
Aorta	0.88 ± 0.01^{d}	0.49 ± 0.08^{e}	0.83 ± 0.05^{d}	0.91 ± 0.03^{d}	0.77 ± 0.03^{d}

Appendix 9

Effect of *Peristrophe bicalyculata* on the expression of angiotensin-converting enzyme and endothelial nitric oxide synthase in rat aortic endothelial cells

	Control group	**L-NAME (2mmol/L)**	**Captopril (1 mmol/L)**	**Extract (10 ug/ml)**	**Extract (100 ug/ml)**
ACE	0.55 ± 0.03^{a}	0.79 ± 0.05^{b}	0.52 ± 0.03^{a}	0.76 ± 0.03^{b}	0.64 ± 0.08^{c}
eNOS	1.71 ± 0.15^{c}	0.48 ± 0.03^{d}	1.01 ± 0.10^{e}	1.05 ± 0.09^{e}	0.95 ± 0.03^{e}

Appendix 10

Percentage apoptotic human mouth epidermal carcinoma cells after 24 and 48 hours treatment with *Peristrophe bicalyculata*

		24 Hours	**48 Hours**
Control Group	-	1.84 ± 0.13	5.23 ± 1.15
Cisplastin	1000 µg/ml	34.88 ± 2.74	81.22 ± 3.62
	100 µg/ml	22.74 ± 2.44	63.35 ± 3.84
	10 µg/ml	15.06 ± 2.31	49.73 ± 5.23
Extract	1000 µg/ml	21.20 ± 2.17	67.6 ± 7.90
	500 µg/ml	16.25 ± 1.87	54.88 ± 5.77
	250 µg/ml	12.49 ± 1.96	35.05 ± 5.68

Appendix 11

Percentage necrotic human mouth epidermal carcinoma cells after 24 and 48 hours treatment with *Peristrophe bicalyculata*

		24 Hours	48 Hours
Control	-	1.22 ± 0.11	3.71 ± 1.77
Cisplastin	1000 µg/ml	37.33 ± 3.22	65.56 ± 3.07
	100 µg/ml	21.24 ± 0.79	55.61 ± 6.98
	10 µg/ml	20.17 ± 2.63	48.30 ± 7.10
Extract	1000 µg/ml	33.62 ± 2.94	65.70 ± 6.93
	500 µg/ml	24.77 ± 3.68	47.42 ± 4.41
	250 µg/ml	18.28 ± 2.12	37.84 ± 5.68

Appendix 12

Cytotoxic effects of extracts and methanolic fractions of *Peristrophe bicalyculata* on $B_{16}F_{10}$ melanoma cells

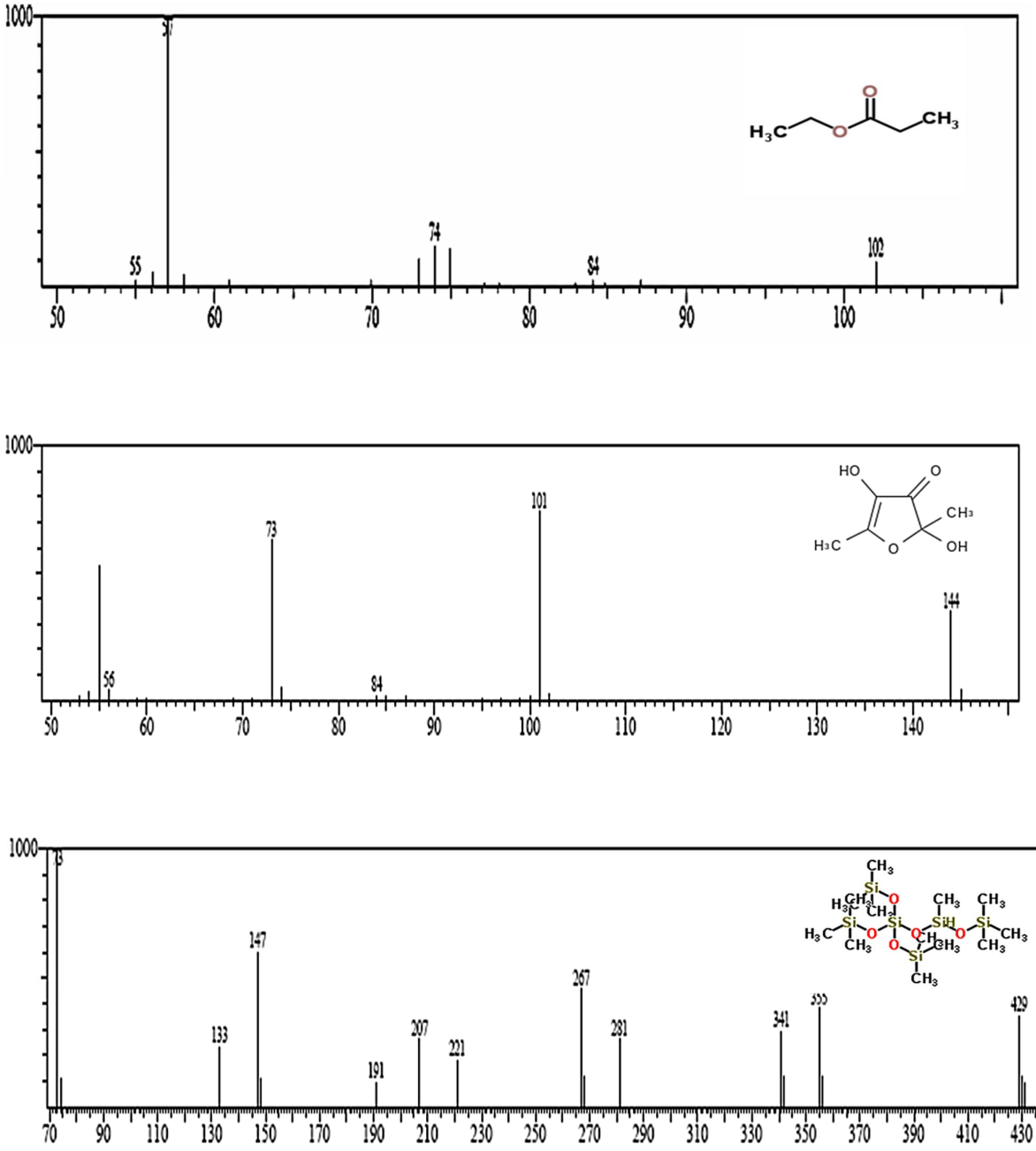
Extracts	Fold of Control
Cold water extract	1.05 ± 0.03^{a}
Hot water extract	1.74 ± 0.05^{b}
Hexane extract	1.80 ± 0.13^{b}
Methanol extract	$1.64 \pm 0.13^{b,c}$
Methanolic ethylacetate fraction	1.87 ± 0.03^{b}
Methanolic water Fraction	$1.71 \pm 0.09^{b,c}$
Methanolic butanol fraction	1.18 ± 0.03^{a}

Appendix 13

The melanin content of $B_{16}F_{10}$ melanoma cells after treatment with Kojic acid, butanol and cold water extracts of *Peristrophe bicalyculata* at various concentrations

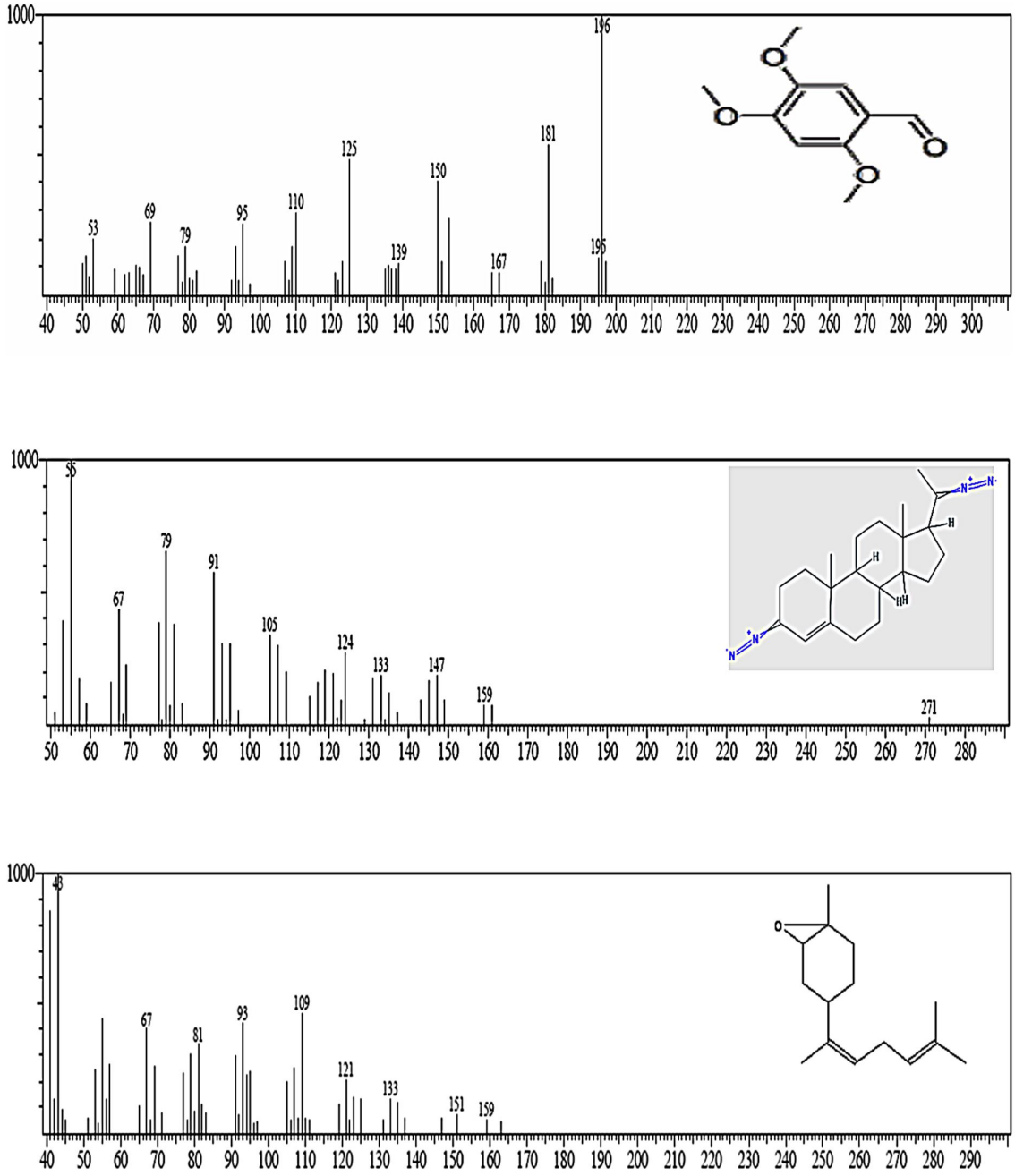
	0.001 mg/ml	0.01 mg/ml	0.1 mg/ml
Kojic Acid	1.52 ± 0.07^{a}	1.35 ± 0.08^{a}	0.86 ± 0.1^{a}
Methanolic butanol fraction	0.98 ± 0.04^{b}	0.81 ± 0.04^{b}	0.59 ± 0.01^{b}
Cold water extract	1.58 ± 0.06^{a}	1.50 ± 0.08^{c}	1.20 ± 0.12^{c}

Appendix 14



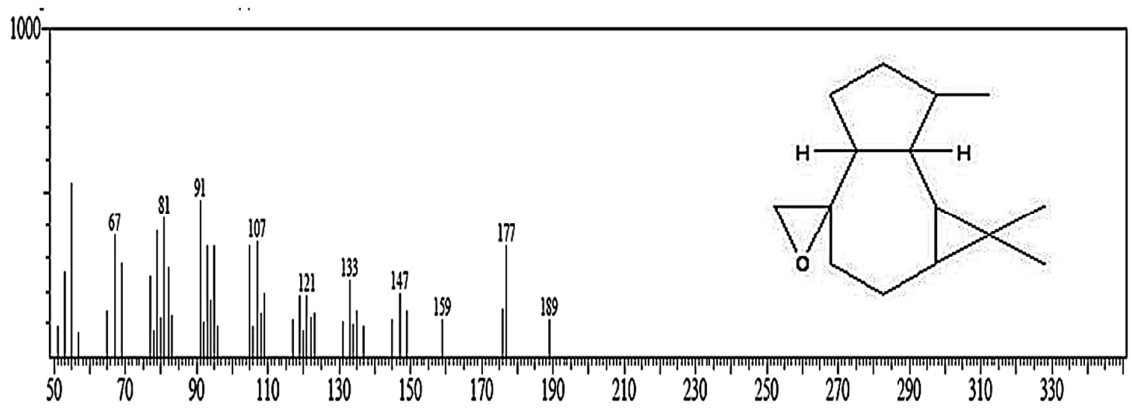
Mass spectrum of Ethyl Propanoic acid (Retention time: 3.41 minutes; $C_5H_{10}O_2$; mol. wt: 102 g), 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one (Retention time: 11.71 minutes; $C_6H_8O_4$; mol. wt: 144 g) and 1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy) tetrasiloxane (Retention time: 42.79 minutes; $C_{13}H_{40}O_5Si_6$; mol. wt: 444 g).

Appendix 15



Mass spectrum of 2,4,5-trimethoxybenzaldehyde (Retention time: 47.34 minutes; $C_{10}H_{12}O_4$; mol wt. 196 g), diazoprogesterone (Retention time: 50.22 minutes; $C_{21}H_{30}N_4$; mol wt. 338 g) and cis-Z-.alpha.-bisabolene epoxide (Retention time: 52.04 minutes; $C_{15}H_{24}O$; mol wt. 220 g).

Appendix 16



Mass spectrum of aromadendrene oxide (Retention time: 57.58 minutes; $C_{15}H_{24}O$; mol wt. 220 g)