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In vivo Antihypertensive and *In vitro* Vasodilatory Effect of the Crude Extracts and Fractions of *Moringa stenopetala* Leaves in Rodents.

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This is to certify that the thesis prepared by Bekesho Geleta, entitled: *In vivo* Anti-hypertensive and *In vitro* Vasodilatory Effect of Crude Extracts and Fractions of *Moringa stenopetala* Leaves in Rodents and submitted in partial fulfillment of the requirements for Degree of Master of Science in Pharmacology complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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ABSTRACT

Moringa stenopetala is an edible plant that has been medicinally claimed as a remedy for treatment of hypertension, diabetes, malaria and stomach pain mainly in southern parts of Ethiopia.

The aim of this study was to evaluate *in vivo* antihypertensive effect in fructose induced hypertensive male Wistar rats and *in vitro* vasodilatory effect in pre-contracted isolated guinea pigs thoracic aorta strip.

Rats were randomly divided into groups; three controls (positive, negative and normal control group) and 12 treatment groups of six male Wistar rats (n=6). Treatment groups were given daily crude extract, fraction and residue (250, 500, and 1000 mg/kg) of *M. stenopetala* orally with D-Fructose (66% w/v *ad libitum*). Whereas, positive, negative and normal control groups received Captopril (20 mg/kg/day with 66% w/v D-Fructose *ad libitum*), only D-Fructose (66% w/v *ad libitum*) and distilled water *ad libitum*, respectively for 15 days. On the other hand, for the *in vitro* vasodilatory study guinea pig was sacrificed by gentle cervical dislocation and the thoracic aorta ring was removed, cut spirally and mounted in an organ bath containing 37^{o}C maintained Kreb's Henseleit physiological solution aerated with carbogen (95% O_2 and 5% CO_2) for experiment.
The study showed that aqueous and 70% ethanol extracts a significant prevention in increment in systolic, mean and diastolic blood pressure in a dose dependent manner compared with negative and normal controls. The extracts also showed suppression in increment in a total cholestrerol, glucose and triglycerides plasma level compared with normal control. In addition, the oral daily administration of extracts produced a rise in liver enzymes but no effect on kidney metabolites compared with normal control group. All extracts showed a relaxation effect in pre-contracted isolated thoracic aorta of guinea pigs in dose dependent manner.
The *in vivo* antihypertensive, antidyslipidemic and *in vitro* vasodilatory effect was significantly shown with aqueous extract followed by 70% ethanol extract. But the effect was low in experimental groups that took ethyl acetate fraction and aqueous residue of aqueous extract.

Keywords: antihypertensive, vasodilatory, *in vivo*, *in vitro*, *Moringa stenopetala*, antidyslipidemic

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LISTS OF ABBREVIATIONS AND ACRONYMS

- ACEI: Angiotensin Converting Enzyme Inhibitor
- ALP: Alkaline Phosphatase
- ALT: Alanine Aminotransferase
- AQ: Aqueous
- ARB: Angiotensin Receptor Blocker
- AST: Aspartate Transaminase
- AU: African Union
- BB: Beta Blocker
- BG: Blood Glucose
- BRA: Baro Reflex Activity
- BUN: Blood Urea Nitrogen
- cAMP: Cyclic Adenosine Monophosphate
- CCB: Calcium Channel Blocker
- CI: Confidence Interval
- CO: Cardiac Output
- CVS: Cardiovascular System
- DBP: Diastolic Blood Pressure
- E^-: without endothelium
- E^+: with endothelium
- EC_{50}: Effective Concentration-50
- EDRF: Endothelium Derived Relaxing Factor
- eNOS: Endothelial Nitric Oxide Synthase
- EPHI: Ethiopian Public Health Institute
- EPI: Epinephrine
- EtAc: Ethyl acetate
- ETC: Electron Transport Chain
- EtOH: Ethanol
- GGT: Gamma Glutamyl Transferase

- GIT: Gastrointestinal Tract
- GLIB: Glibenclamide
- GLUT: Glutathione
- $HEPG_2$: Human Liver Hepatocellular Carcinoma Cell Line
- LD: Lactate Dehydrogenase
- LD_{50}: Lethal Dose-50
- LDL: Low Density Lipoprotein
- MAP: Mean Arterial Pressure
- MB: Methylene Blue
- MoFED: Ministry of Finance and Economic Development
- NADPH-OX: Nicotinamide Adenine Dinucleotide Phosphate Oxidase
- NAFLD: Non-Alcoholic Fatty Liver Disease
- NHE_3: Sodium Hydrogen Exchanger-3
- NO: Nitric Oxide
- OECD: Organization for Economic Cooperation and Development
- PAT_1: Putative Anion Transporter-1
- PPAR: Peroxisome Proliferator-Activated Receptor
- PVR: Peripheral Vascular Resistance
- RAAS: Renin Angiotensin Aldosterone System
- RAS: Renin Angiotensin System
- ROS: Reactive Oxygen Species
- rpm: revolution per minute
- SBP: Systolic Blood Pressure
- SNS: Sympathetic Nervous System
- STZ: Streptozocin
- TC: Total Cholesterol
- TG: Triglycerides
- TMMRD: Traditional and Modern Medicine Research Directorate
- WHO STEPS: World Health Organization Instrument for Stepwise Surveillance

1. INTRODUCTION

1.1 Background

Blood is carried from the heart to all parts of the body through blood vessels. Each time the heart beats it pumps blood into the vessels. Blood pressure (BP) is created by the force of blood pushing against the walls of blood vessels (arteries) as it is pumped by the heart. Hypertension, also known as high or raised BP, is a condition in which the blood vessels have persistently raised pressure exceeding 140 over 90 mmHg (WHO, 2013; Samardeep, 2013).

BP is majorly the result of interactions of nervous, endocrine and renal system function. Major pathophysiologic mechanisms of hypertension include activation of the SNS and RAAS. Endothelial dysfunction, increased vascular reactivity, and vascular remodeling may be caused, rather than consequences, of BP elevation; increased vascular stiffness contributes to isolated systolic hypertension in the elderly (Oparil *et al*, 2003).

Hypertension leads to complications with considerable morbidity and mortality. In general, hypertension can cause damage to the arteries, to the brain (cerebrovascular accident or stroke), to the heart (congestive heart failure, coronary artery disease, myocardial infarction and heart attack), retina (retinopathy or visual impairment) and to the kidneys (renal failure). The objective of management of hypertension is primarily directed at preventing these complications rather than just control of BP itself (AU, 2013; Samardeep, 2013; WHO, 2013).

1.2 Literature review

Cardiovascular disease (CVD) is the leading cause of death in developing countries where it causes nearly as many deaths as HIV, malaria and TB (Lopez *et al*, 2006). Prevalence and incidence of both hypertension and pre-hypertension are high. Population, epidemiologic changes and the hypertension epidemic is due to epidemiological transition, urbanization and population aging (AU, 2013).

Hypertension is a leading cause of CVDs such as MI and stroke worldwide. The proportion of the global burden of disease attributable to hypertension has significantly increased from about 4.5% in 2000, to 7% in 2010 (AU, 2013; WHO 2008). Moreover, the number of people with uncontrolled hypertension has increased to around 1 billion worldwide in the past three decades (Danaei *et al*, 2011). CVDs account for a death of approximately 17 million per year, nearly one third of the total (AU, 2013; WHO 2008).

Of these, complications of hypertension account for 9.4 million deaths worldwide every year. Hypertension is responsible for at least 45% of deaths due to heart disease and 51% of deaths due to stroke (WHO, 2008). At the beginning of the 20th century, CVD was responsible for less than 10% of all deaths worldwide, but by 2008 that figure had risen to 30%. In 2008, worldwide, approximately 40% of adults aged 25 and above had been diagnosed with hypertension; the number of people with the condition rose from 600 million in 1980 to 1 billion in 2008 (WHO, 2011). This makes hypertension the single most important cause of morbidity and mortality globally and highlights the urgent need of action to address the problem (AU, 2013). About 80% of the global burden of CVD death occurs in low- and middle-income countries (Gaziano *et al*, 2006).

Traditionally in Africa, communicable diseases and maternal nutritional deficiency induced disorder have accounted for the greatest burden of morbidity and mortality. This burden is fast shifting towards chronic non-communicable diseases, and by extension CVDs. Hypertension has become a significant problem in many African countries experiencing the epidemiological transition from communicable to non-communicable diseases. This phenomenon is what is being termed as a "double burden of disease" (AU, 2013; Kebede *et al*, 2013). Presently, the age specific mortality rates from CVDs are much higher in younger age groups in both men and women in Africa than in the developed world (Unwin, 2001).

CVD is the second leading overall cause of death in Africa, after HIV/AIDS, and is the leading cause of mortality among individuals over the age of thirty (Bertrand, 1999). Whereas high BP was almost non-existent in African societies in the first half of the 20thcentury, estimates now show that in some settings in Africa more than 40 % of adults have hypertension. The prevalence of hypertension has increased significantly over the past two to three decades. There were approximately 80 million adults with hypertension in Africa South of the Sahara in 2000 and projections based on current epidemiological data suggest that this figure will rise to 150 million by 2025 (AU, 2013).

WHO projects that over the next 10 years Africa will experience the largest increase in death rates from CVD and therefore the negative economic impact of CVD will be more felt on the continent (Alwan, 2011). The WHO STEPS survey conducted between 2003 and 2009 in 20 African countries reported high rates of hypertension in most countries. The prevalence ranges from 19.3% in Eritrea to 39.6% in Seychelles (AU, 2013).

Hypertension is the driver of the CVD epidemic in Africa where it is a major, independent risk factor for heart failure, and stroke and kidney failure (Brundtland, 2002). Even though some regional studies have been conducted in different parts of Ethiopia, there is no national survey that has been performed yet. Approximately 30% of adults in Addis Ababa have hypertension (above 140/90 mmHg) or reported use anti-hypertensive medication (Tesfaye *et al*, 2009).

According to WHO report, the annual hypertension deaths in Ethiopia reached 9,743 or 1.19% of total deaths which shows top 12 and ranks Ethiopia 72th in the world (WHO, 2011). The WHO STEPS survey conducted between 2003 and 2009 in 20 African countries reported high rates of hypertension in Ethiopia, which is about 31% prevalence (AU, 2013). The study done in Northern Ethiopia shows that the overall prevalence of hypertension and pre-hypertension was 18.1% and 37.2%, respectively (Mengistu, 2014).

The study done in Addis Ababa shows that the prevalence (95% CI) of isolated systolic hypertension was 8.9% (7.4, 10.3) in males and 8.3% (7.0, 9.5) in females. Isolated diastolic hypertension was 5.8% (4.5, 7.0) in males and 6.0% (4.9, 7.0) in females. Combined systolic diastolic hypertension was 16.0% (14.0, 17.9) in males and 13.6% (12.0, 15.1) in females (Tesfaye *et al*, 2009). In Ethiopia ischemic heart disease mortality rates (age standardized per, per 100, 0000) is 109-151; cerebrovascular mortality rates (age standardized per, per 100, 0000) is 132-240 (WHO, 2008).

Risk factors and management of hypertension

Hypertension is mainly associated with environmental and lifestyle factors rather than with genetics and has a stronger association and causal link with five particular beha-

viors: tobacco use, excessive use of alcohol, physical inactivity, unhealthy diet (high salt intake and, insufficient fruit and vegetable consumption) and obesity. Risk factors leading to hypertension can be reversible (modifiable), irreversible (non-modifiable), or associated with other predisposing disorders (AU, 2013).

Evidence from large clinical trials has shown a 40% reduction in stroke and a reduction of at least 25% in MI associated with treatment and control of hypertension (Neah *et al*, 2000). In developed countries, the improved control of hypertension has led to considerable reduction in overall morbidity and mortality over the last fifty years (Gu *et al*, 2010).

Treatment of hypertension with modern drugs

Controlling hypertension without producing unacceptable side effects is an important clinical need, which is, in general, well catered by modern drugs. Treatment involves non-pharmacological measures (e.g. increased exercise, reduced dietary salt and saturated fat, increased fruit and fiber diet, body weight and alcohol reduction) followed by the staged introduction of drugs, starting with those of proven benefit and least likely to produce side effects (Rang *et al*, 2007). Treatment is usually lifelong. Therefore drugs must be effective and safe over a long period use (Williams *et al*, 1996).

Some of the drugs that were used to lower blood pressure in the early days of antihypertensive therapy, including ganglion blockers; and adrenergic neuron blockers like reserpine, produced a fearsome array of adverse effects and are now obsolete (Rang *et al*, 2007). Moreover, the efficacy of these drugs are only 40%-60%, and usually two or more anti-hypertensive drugs from different categories need be combined to achieve optimal results.

However, side effects from these medications are important concerns (Du and Chen, 2005). To find a safe and effective way to manage hypertension has challenged medical professionals for centuries. Traditional medicine extracted from the natural plants may be one of the important future directions in the future.

Treating hypertension with medicinal plants

Even though several allopathic antihypertensive medications are available, most people living in developing countries rely on traditional medicine (TM) (Chatora, 2003). Various herbal preparations have been used and claimed to have benefit for treatment of hypertension. The hypotensive and antihypertensive effects of some of these plants have been validated and others disproved. Some of the plants used traditionally for treatment of hypertension include: *Moringa stenopetala* (Mengistu *et al*, 2012), *Moringa Oleifera* (Kajihara *et al*, 2008), *Allium sativum* (Ashraf *et al*, 2013), Syzygium guineense (Ayele *et al*, 2010), *Astragalus complanatus* (Li *et al*, 2005), *Hibiscus Sabdariffa* (Herrera-Arallano *et al*, 2007*), Carissa edulis* (Nedi *et al*, 2004) and *Thymus serrulatus* (Geleta *et al*, 2015).

Among these plants *Moringa stenopetala, Thymus serrulatus* and *Syzygium guineense* were reported to have vasodilatation effect (Ayele *et al*, 2010; Mengistu *et al*, 2012; Geleta *et al*, 2015) whereas *Astragalus complanatus, Hibiscus Sabdariffa, and Salvia miltiorrhiza* to act by inhibiting ACE (Herrera-Arellano *et al*, 2007; Li *et al*, 2005).

1.2.1 *Moringa stenopetala*

Several approaches are presently available to reduce BP in hypertensive patients. Among these, Ethiopians use different plants traditionally for the treatment of hypertension.

M. stenopetala is one of these medicinal plants widely used for treatment of variety of diseases including hypertension. *M.stenopetala* belongs to the family Moringacae represented only by a single genus *Moringa*. The genus is represented by 14 species to which *M. stenopetala* belongs. It is a branched tree that grows 6 to 10 m tall, thick at base bark with white to pale gray or silvery coloration (Abuye *et al*, 2003).

It grows abundantly in south western Ethiopia where the leaves are eaten as vegetable. The species is known by different vernacular names such as "Shiferaw" in Amharic, "Aleko" in Wollaytegna and Gamugna (Mekonnen Y. and Gessesse, 1998) and "Cabbage tree" in English. It grows widely at an altitude range of 1000 to 1800 m. The indigenous knowledge and use of *Moringa* is referenced in more than 80 countries and it's known in over 200 local languages. The history of *Moringa* dates back to 150 B.C. The family Moringaceae is a monotypic family of single genera with around 33 species of which 4 is accepted, 4 are synonym and 25 are not investigated. Out of these, 13 species, native of old world tropics are documented (Arora *et al*, 2013).

Among various types of Moringa species, *M. stenopetala* is native to Ethiopia, Northern Kenya and Eastern Somali and is the most economically important species after *Moringa oleifera* (Olson, 2001). *M. stenopetala* is a multipurpose plant (Abuye *et al*, 2003).

It has been reported that, *M. stenopetala* has hypotensive (Mengistu *et al*, 2012), antihyperglycemic and hypoglycemic (Mussa *et al*, 2008; Nardos *et al*, 2011; Toma *et al*, 2012; Sileshi *et al*, 2014; Toma *et al*, 2015), antileishmenial and antifertility (Mekonnen Y. and Gessesse, 1998), treatment of stomach pain, antispasmodic and to expel retained placenta following birth (Mekonnen Y., 1999), antimicrobial activity (Biffa, 2005) and also has a nutritional value (Abuye *et al*, 2003). The *in vitro* study demonstrated the beneficial bio-

chemical effects of *M. stenopetala* by inhibiting intestinal α-glucosidase, pancreatic cholesterol esterase and pancreatic lipase activities (Toma *et al*, 2014).

2. Induction of hypertension in rats

There are different models of inducing hypertension in animals. Such as, renovascular hypertension, dietary hypertension, endocrine hypertension, neurogenic hypertension, psychogenic hypertension, genetic hypertension and other models (Kaur *et al*, 2011). For the present study, the dietary induction of hypertension in male normal Wistar rats was employed using 66% w/v D-Fructose according to methods described by Jena *et al.* (2013), and Hwang *et al* (1987) with a slight modification.

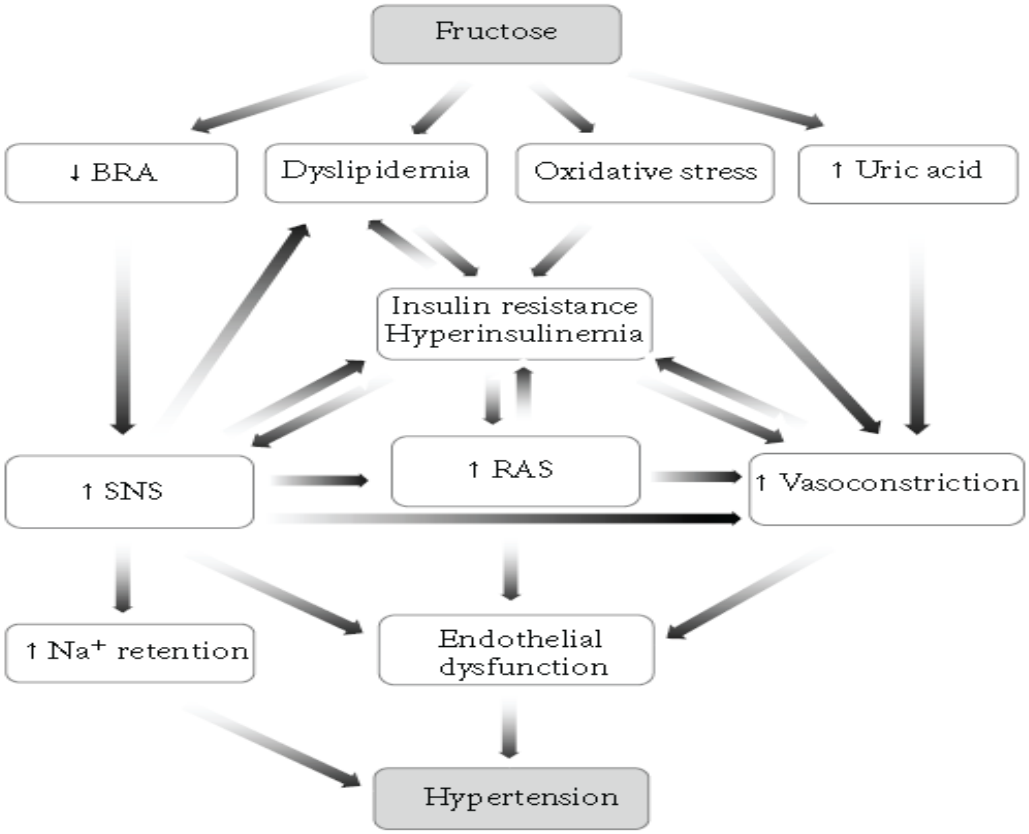

Figure 1: The proposed mechanisms by which fructose feeding results in hypertension (Abdulla *et al*, 2011).

2. OBJECTIVES

2.1 General objective

- To evaluate *in vivo* antihypertensive and *in vitro* vasodilatory effect of the crude extracts and fractions of *M. stenopetala* leaves in rodents.

2.2 Specific objectives

- To carryout phytochemical screening of the crude extracts and fractions of *M. stenopetala* leaves
- To evaluate *in vivo* antihypertensive activity of crude extracts and fractions of *M. stenopetala* leaves
- To evaluate effect of the crude extracts and fractions of *M. stenopetala* leaves on serum lipid profile (TC, BGL and TG), liver parameters (AST, ALT, ALP and GGT) and kidney paameters (BUN and creatinine).
- To evaluate the *in vitro* vasodilatory activity of the crude extracts and fractions of *M. stenopetala* leaves
- To carryout acute toxicity of the crude extracts and fractions of *M. stenopetala* leaves

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Drugs, chemicals and reagents

Ethyl Acetate (lot no: 8114/4, Park Scientific Limited, Northampton, UK), Absolute Ethanol (lot no: E35070/2, WINLAB, UK), Petroleum Ether (lot no: V2B668282B, Scientific limited, UK), Potassium Ferric Hexacynate (lot no: 0143290, The British Drug Houses, UK), Lead Acetate (lot no: V9H4049, Celtic Chemicals, South Wales, UK),

Ammonia Solution (lot no: 9457, Scientific limited, UK), Tween-80 (lot no: 4075, BDH laboratories, UK), Dinitro-2-4- Phenylhydrazine (lot no: 231523, VWR prolabo chemicals, USA), Sulfuric Acid (lot no: 8114/1, Scientific limited, UK), Glacial Acetic Acid (lot no: 2018/10209/03/L, Labort fire chemicals, India), Chloroform (lot no: 8114/1, Scientific limited, UK), Ferric acid (lot no: 4840, PVS Chemicals, USA), Hydrochloric Acid (lot no: 2571, Parchem fine and specialty chemicals, UK), Vanillin (lot no: 129F0635, Sigma chemical co., USA), D-Fructose (lot no: SL54161301, Loba Chemie, India), Captopril (lot no: 48794, EPSITRON Limited, Nicosia, Cyprus), Epinephrine (lot no: 111K1610, Sigma Aldrich Chemie Gmbh, Steinheim, Germany), Methylene Blue (lot no: 073K3413, Sigma Aldrich Chemie Gmbh, Steinheim, Germany), Acetylcholine Chloride (lot no: 12134/1, Sigma Aldrich Chemie Gmbh, Steinhein, Swizerland), Glibenclamide (lot no: 53917, Remedica, Cyprus), D-glucose Anhydrous (lot no: GL2863, Eurostar Scientific Limited, Liverpool, UK), Potassium Phosphate Monobasic (lot no: 46F-0522, Sigma Chemical Companyy, St. Louis, USA), Potassium Chloride (lot no: 8114/86, Park Scientific Limited, Northampton, UK), Sodium Hydrogen Carbonate (lot no: 205-633-8, Eurostar Scientific Limited, Liverpool, UK), Sodium Phosphate Monobasic (45H02675, Sigma Chemical Co., St Louis, USA), Calcium Chloride (lot no: 1501, Allied Chemical, General Chemical Division, New Jersey, USA), Magnesium Chloride (lot no: 86F-3524, Sigma Chemical Company, St Louis, USA), Magnesium Sulphate (lot no: 400290, The British Drug Houses Limited, B.D.H Laboratory Chemicals Division, UK) and Sodium Chloride (lot no: 108278, Riedel-de Haen, Germany) were used in the study. All the drugs and reagents used complied with the required standard and were of analytical grade.

3.1.2 Instruments and apparatus

Balance (Mettler Toledo, Korea), Whatman filter paper No.1 (Whatman International ltd, Maidstone, England), Orbital shaker (VWR DS-500, USA), Rota vapor (Buchi, Rotavapor R-210/215 B-490, Swizerland), Water bath (DVE-Kottermann, D-3162, Uetze-Hanigsen/W, Germany), Lyophilizer/ Freeze dry system (Labconco, 12 L Console Freeze Dry 230v-60 (7754040), Freeze Dry System, USA), Heparinized capillary tube with plastic sealing (Hawksley & Sous Limited, Sussex, UK), Centrifuge (Rotant 98, Hettich, Zentrifugen, UK), Clinical chemistry analyzer (Cobas-e-411, HITACHI, ROCHE, Germany), Grass polygraph (Model 7E, USA), BP analyzer (Model 179, USA), UV lamp (LA-MAG).

3.1.3 Plant material

The fresh *M. stenopetala* leaves were collected from Southern Ethiopia around Arbaminch, a town 502 km far south of Addis Ababa in September 2014. The plant material was authenticated by a taxonomist in the TMMRD, EPHI and a sample with voucher number AL-001 was deposited.

Figure 2: The leaves and tree of *M.stenopetala*

3.1.4 Experimental animals

The experiments were performed on adult, healthy male Wistar rats (*Rattus norvegicus)* and male guinea pigs (*Cavia porcellus*) with a weight range of 150-200 g and 350-400 g, respectively. The rodents were bred and obtained from the EPHI. All the animals used for this study were kept in standard animal cages and maintained under laboratory conditions of temperature (22 ± 3°C), relative humidity (40-70%) and 12 hour day-12 hour night and had free access to food (standard pellet diet and cabbage) and water *ad libitum* for acclimatization and experimentation. The animals were treated humanely throughout the study period and were kept in a well controlled area according to the guideline for use and care of animals (National Research Council, 2011).

3.2 Methods

3.2.1 Plant material preparation and extraction

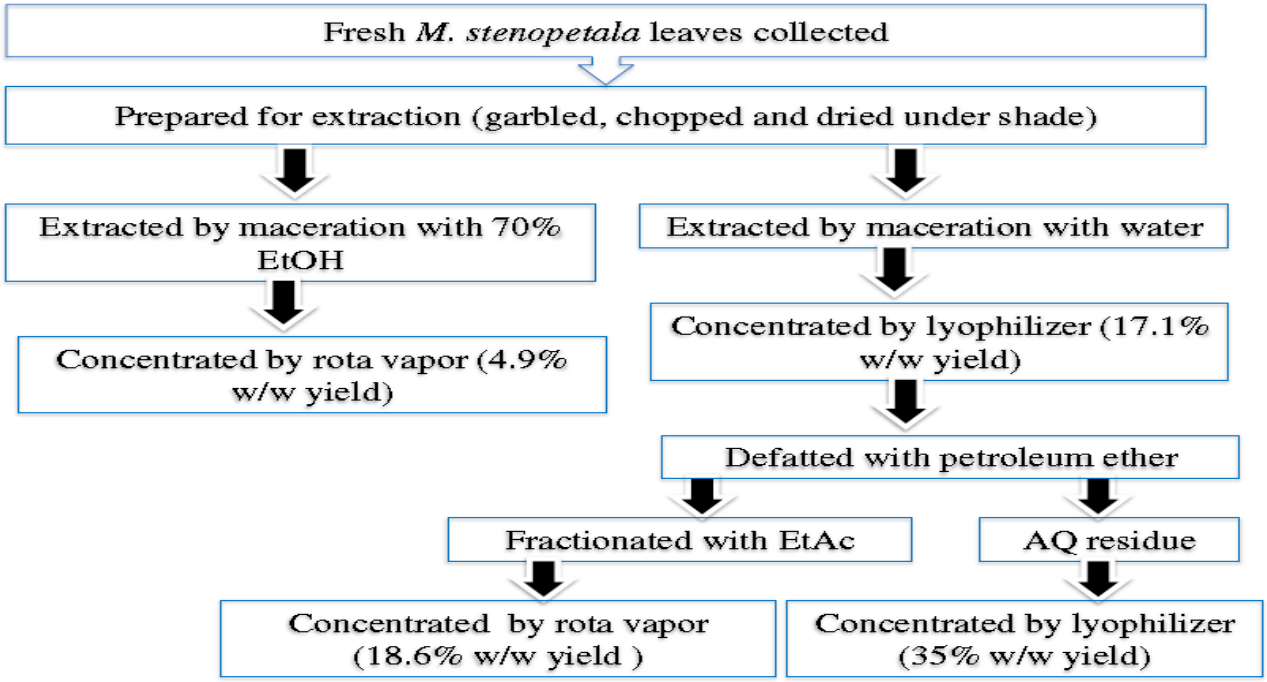

Figure 3: Flow chart showing plant material preparation and extraction process

3.2.2 Phytochemical screening

All extracts (AQ, 70% EtOH, EtAC and AQ residue) used for *in vivo* and *in vitro* antihypertensive activity study were subjected to phytochemical screening following methods described in Debella (2002). The extracts along with negative controls were tested for the presence of the following alkaloids, saponins, polyphenols, flavonoids, coumarins, terpenoids, anthraquinones, phytosterols, tannins and glycosides (see Annex 4).

3.2.3 Evaluation of *in vivo* antihypertensive activity

Rat preparation, handling and temperature maintenance

Each rat was trained and acclimatized to the restrainer and transducer, for about 15 minutes each day before beginning the experiment for 5 days. The rat was restrained in a low-stress environment and allowed to enter the holder freely at least 10 to 15 minutes prior to obtaining pressure measurements. The animal's nose was made to protrude through the front of the nose cone allowing for comfortable breathing and the tail of the animal was fully extended to exit through the rear hatch opening of the holder.

The rat was warmed but not heated using restrainer, the room temperature was maintained about 32–35.4°C, reduce stress and the blood flow to the tail was enhanced to acquire a BP signal. The rat never had its head bent sideways or its body compressed against the back hatch. The animal's temperature was monitored throughout the experiment (Malkoff, 2012).

Experimental course

Male Wistar rats were randomly divided into fifteen groups with 6 animals per group (n=6). Normal control rats (Group 1) received distilled water *ad libitum* only, negative control rats (Group 2) received 66% w/v D-Fructose *ad libitum* only, positive control rats (Group 3) received Captopril (20 mg/kg/day) with 66% w/v D-Fructose *ad libitum* and treatment rats (Group 4-15) received different extracts (250, 500, 1000 mg/kg/day) with 66% w/v D-Fructose *ad libitum* for 15 days.

The non-invasive (indirect) tail cuff method was widely used to evaluate the influence of antihypertensive drugs in experimentally hypertensive rats (Feng *et al*, 2008; Malkoff, 2012). The method was analogous to sphygmomanometry in human and applied at the tail of awake rats by quickly inflating well above suspected SBP and slowly released during which the pulse was measured and recorded by the non-invasive tail cuff BP analyzer (Ayele *et al*, 2010; Malkoff, 2012).

SBP, MAP and DBP measured on 1st day of experiment before being induced using (66% w/v) D-Fructose was stated as a basal BP (D_0). If $SBP_0 \leq 120$ mmHg, $MAP_0 \leq 100$ mmHg and $DBP_0 \leq 91$ mmHg or normotensive rats were a candidate for experiment and given (66% w/v) D-Fructose *ad libitum* except normal control group. Then BP was measured again every 5 days for 15 days. The SBP and MAP were read from the pulse tracings and DBP was calculated using formula-1.

$$DBP = (3MAP - SBP)/2 \quad \ldots\ldots\ldots\ldots\ldots\ldots 1$$

Every measurement was made in triplicate and the average value was reported (Ayele *et al*, 2010).

At the end of experiment, on 16th day the blood was collected in vacutainer tube by cardiac puncture from through-night fasted cervical dislocated rats. The serum was separated after centrifugation at 3000 rpm for 10 minutes. The serum lipid profile (TC, BG and TG), liver parameters (ALT, AST, ALP and GGT) and kidney parameters (BUN and creatinine) were assayed using methods described by the manufacturer (Roche diagnostics, Germany) using clinical chemistry analyzer instrument.

Evaluation

Mean values in SBP, MAP and DBP measured was determined and calculated as mean ± S.E.M and compared with positive, negative and normal control.

In addition mean ± S.E.M was calculated for serum lipid profile (TC, BG and TG), liver parameters (ALT, AST, ALP and GGT) and kidney parameters (BUN and creatinine) and was compared with normal, negative and positive control.

3.2.4. Evaluation of *in vitro* vasodilatory activity

The *in vitro* vasodilatory study was conducted on isolated guinea pig thoracic aorta according to the methods described by Vogel *et al.* (2008). The guinea pig was sacrificed by gentle cervical dislocation and the thoracic cavity was opened, and the aorta was identified. The descending thoracic aorta was then immediately removed and placed in Krebs-Henseleit physiological solution maintained at 37^{0}C. Excess adherent fat and connective tissue was trimmed off and cleaned; each aorta was cut spirally using heparinized capillary tube with plastic sealing to a strip of about 2 mm wide and 4 cm long.

The strip was immediately mounted in an organ bath containing 20ml Krebs-Henseleit physiological solution (118.4 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4.7H_2O$, 2.2 mM

KH_2PO_4, 1.3 mM $CaCl_2$, 24.9 mM $NaHCO_3$, 11.1 mM glucose, pH 7.4). The aortic strip was attached to isometric transducers connected to a polygraph and a resting tension of 1 g was applied to strip. Aorta strip was mounted under this resting tension onto two 0.2 millimeters L shaped stainless steel wire hooks gently inserted into the lumen to avoid damage to the endothelium in 20 ml organ baths containing Krebs-Henseleit physiological solution and allowed to stabilize for about 1 hour before commencing an experiment during which period it was washed by overflowing every 15 minutes.

The physiological solution was allowed to pass through a warm water jacket to maintain its temperature and was continuously aerated with carbogen (95% O_2 + 5% CO_2 gas mixture) at a pH of 7.4. The pH of buffer was checked in every 60 minutes of aeration with carbogen. Experiments were performed on aortic strip with intact and denuded endothelium. For the experiment on aortic strip with intact endothelium, the functional integrity of the endothelium was tested before extract and contracting agent administration by 1 μM acetylcholine in the organ bath should result in a transient relaxation.

On the other hand, the experiment was done on denuded endothelium by removing endothelial cells from strip by gently rubbing the intimal surface with a moist wooden stick for 30-60 seconds. The effectiveness of this procedure was subsequently investigated using acetylcholine (1mM), which normally relaxed aortic strips but had no such effect in rubbed strip pre-contracted with (80 mM) KCl.

After aortic strip equilibration period of 1 hour under a resting tension of 1 g, tissue viability was confirmed by adding (80 mM) KCl. After equilibration and tissue viability was checked, contraction of aortic strip was induced by addition of one of the following con-

tracting agents (80 mM) Potassium Chloride (KCl), (1 µM) Epinephrine (EPI), (10µM) Methylene Blue (MB) and (10µM) Glibenclamide (GLIB) to organ bath.

Once a contraction plateau was achieved, extracts of *M. stenopetala* was administered cumulatively every 15 minutes for their capacity of reducing aortic strip contraction induced and tension responses of the tissue was detected with transducers and recorded isometrically and was displayed on a Grass model 7E polygraph.

At the end the experiment, relaxation, which is a measure of inhibition of contraction in aorta ring pre-contracted with contracting agent, was determined using a measurement before and after extract administration and calculated using formula-2.

$$\%\text{Relaxation} = \frac{(Tc-Tt)}{Tc} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 2$$

Where, Tc stands for tension due to contracting agents, while Tt stands for tension after adding extract. In addition, EC_{50} for highly active extracts were determined.

3.2.5 Acute toxicity study

The extracts of *M. stenopetala* leaves were evaluated for their toxicity in female Wistar rats at a dose of 5000 mg/kg body weight according to OECD guidelines No. 425 (OECD, 2008). The animals (5 control and 5 experimental) were deprived of food for 18 hours with free access to water. Immediately after administration of the extract, the animals were carefully observed continuously for the first 4 hours for any overt signs of toxicity and death and then for the next 24 hours. Thereafter, they were kept under close observation up to 14 days to monitor the presence of any signs of morbidity or mortality. The weight of each animal was recorded at the 1^{st}, 7^{th}, and 14^{th} day of administration to

verify any unexpected weight change that might have occurred. Finally, after cervical dislocation, the rats were dissected at the 14^{th} day to observe gross pathology of the vital organs such as liver, kidney, spleen and pancreas.

4. STATISTICAL ANALYSIS

All experimental data's were expressed as mean values (measurement of BP or % relaxation) ± S.E.M and were subjected to bio-statistical interpretation by SPSS windows version 16 statistical packages all the way through a one-way ANOVA followed by post-hoc test (Tukey Test) for multiple comparisons of the mean differences and responses of different drugs and extracts. Statistical significance of P-value < 0.05 were considered as level of significance.

5. RESULTS

5.1 Phytochemical screening

Basic investigations of the extracts for their major phytocompounds is vital as the active principles of many drugs are these secondary metabolites found in plants. The various phytochemical screening tests performed on the crude extracts and fractions *M. stenopetala* leaves revealed the presence of different secondary metabolites (Table1).

Table 1: Phytochemical screening of the crude extracts and solvent fractions of *M.stenopetala* leaves

Type of extract	Alkaloids	Saponins	Polyphenols	Flavonoids	Coumarins	Terpenoids	Anthraquinones	Tannins	Phytosterols	Glycosides
AQ	+	+	+	+	+	+	+	+	+	+
AQ residue of AQ	-	+	-	+	+	+	+	+	+	-
EtAc fraction of AQ	-	+	+	-	-	+	-	+	+	-
70% EtOH	+	-	+	+	+	+	+	+	+	+

Key: + = present; - = absent

5.2. *In vivo* antihypertensive activity

5.2.1. Effect on body weight

All groups showed an increase in body weight. The negative control showed significant difference ($P<0.01$) in percent weight gain in 10^{th} day (D_{10}) and 15^{th} day (D_{15}) but not in 5^{th} day (D_5) compared with normal control (Fig. 4).

All groups did not show significant difference in percent weight gain in 5^{th} day and 10^{th} day except the group that took 500 mg/kg/day AQ residue of AQ extract ($P<0.05$), whereas, in the 15^{th} day the percent weight gain is high in groups that took 500 mg/kg/day followed by 250 mg/kg/day and 1000 mg/kg/day of extracts in similar manner in succession (Fig. 4).

All treatment groups that administered the highest dose (1000 mg/kg/day) of extracts, Captopril (20 mg/kg) and D-Fructose (66% w/v *ad libitum*) showed significant difference ($P<0.001$) in percent weight gain in 15^{th} day of the study compared with normal control group (Fig 4).

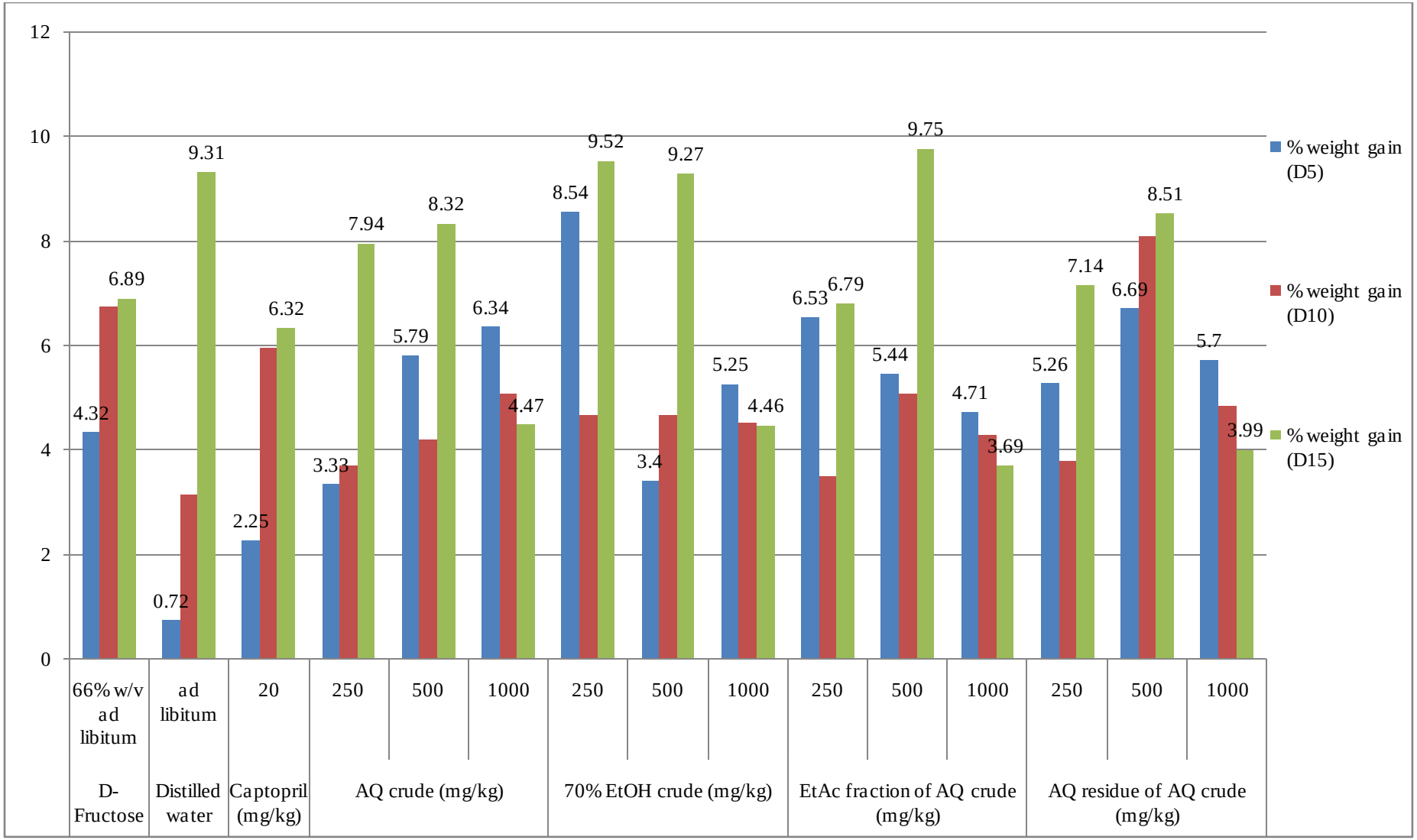

Figure 4: Effect of crude extracts and solvent fractions of *M. stenopetala* on body weight in D-Fructose (66% w/v *ad libitum)* induced hypertensive rats.

5.2.2. Effect on BP

The negative control showed significant increase in SBP, MAP and DBP compared with normal control ($P<0.001$) and positive control ($P<0.001$) in the D_5, D_{10} and D_{15} of the experiment (Table 3, 4 and 5). Those groups that received daily oral administration of 1000 mg/kg of AQ crude, 70% EtOH crude and AQ residue of AQ extract prevented a rise in SBP and did not show significant difference, whereas, EtAc fraction of AQ extract showed significant increase ($P<0.05$) in SBP compared with normal control in the D_5 of the experiment. Daily oral administration of 250 and 500 mg/kg of all crude extracts and solvent fractions showed significant increase ($P<0.001$) in SBP compared with normal and positive control in the D_5 of the experiment (Table 2).

In the D_{10} of experiment, the groups that received daily oral administration of 250 and 500 mg/kg of all extracts and 1000 mg/kg of solvent fractions showed significant increase in SBP compared with normal control ($P<0.001$) and compared with positive control ($P<0.001$), whereas, 1000 mg/kg/day oral administration of the crude extracts prevented a rise in SBP, did not show significant difference in SBP compared with normal and positive control (Table 2).

After consecutive oral daily administration for 15 days, all treatment groups showed significant increase in SBP compared with normal control ($P<0.01$) and positive control ($P<0.05$) (Table 2).

Table 2: Effect of crude extracts and solvent fractions of *M. stenopetala* leaves on SBP in D-Fructose (66% w/v *ad libitum*) induced hypertensive rats.

Substance administered	Dose (mg/kg)	SBP			
		D_1 (mmHg)	D_5 (mmHg)	D_{10} (mmHg)	D_{15} (mmHg)
D-Fructose	66% w/v *ad libitum*	113.50 ± 1.52	132.67 ± 0.71***^{3,} *^{3}	144.33 ± 1.33***^{3,} *^{3}	163.67 ± 1.12***^{3,} *^{3}
Captopril	20	113.83 ± 2.12	124.83 ± 1.19**^{3,} *^{3}	120.67 ± 0.88**^{3,} *^{1}	120.50 ±0.76**^{3}
AQ crude	250	114.17 ± 1.40	129.50 ± 1.38*^{3}	138.67 ± 0.88**^{3,} ***^{3,} *^{3}	149.67 ± 1.28**^{3,} ***^{3,} *^{3}
	500	112..50 ± 1.20	126.83 ± 0.95**^{2,} *^{3}	132.17 ± 0.70**^{3,} ***^{3,} *^{3}	136.50 ± 0.76**^{3,} ***^{3,} *^{3}
	1000	116.83 ± 1.78	121.83 ± 0.60**^{3}	123.00 ± 0.58**^{3}	124.67 ± 0.88**^{3,} ***^{1,} *^{2}
70% EtOH crude	250	116.17 ±1.01	138.67 ± 0.88**^{2,} ***^{3,} *^{3}	146.83 ± 0.60***^{3,} *^{3}	157.17 ± 0.95**^{3,} ***^{3,} *^{3}
	500	114.83 ± 1.96	130.50 ± 0.76***^{1,} *^{3}	137.50 ± 0.76**^{3,} ***^{3,} *^{3}	142.50 ± 0.76**^{3,} ***^{3,} *^{3}
	1000	115.17 ± 1.97	119.00 ± 1.39**^{3,}***^{2}	124.33 ± 0.49**^{3,} *^{3}	133.50 ± 0.76**^{3,} ***^{3,} *^{3}
EtAc fraction of AQ crude	250	118.33 ± 0.88	141.33 ± 0.80**^{3,} ***^{3,} *^{3}	151.17 ± 0.60**^{3,} ***^{3,} *^{3}	161.17 ± 0.60***^{3,} *^{3}
	500	119.67 ± 0.88	134.17 ± 0.70***^{3,} *^{3}	141.17 ±0.48***^{3,} *^{3}	151.50 ± 0.76**^{3,} ***^{3,} *^{3}
	1000	116.50 ±1.48	124.33 ± 1.33**^{3,} *^{1}	134.50 ± 0.76**^{3,} ***^{3,} *^{3}	145.33 ± 0.88**^{3,} ***^{3,} *^{3}
AQ residue of AQ crude	250	116.83 ± 1.78	137.83 ± 0.60**^{1,} ***^{3,} *^{3}	142.17 ± 0.70***^{3,} *^{3}	156.00 ± 0.58**^{3,} ***^{3,} *^{3}
	500	116.83 ± 1.54	132.67 ± 0.88***^{3,} *^{3}	139.50 ± 0.42**^{2,} ***^{3,} *^{3}	146.17 ± 0.60**^{3,} ***^{3,} *^{3}
	1000	117.33 ± 1.63	124.17 ± 1.01**^{3}	131.17 ± 0.60**^{3,} ***^{3,} *^{3}	139.50 ± 0.43**^{3,} ***^{3,} *^{3}

Results are expressed as X ± S.E.M (n=6), *=compared with normal control (baseline or basal value); **= compared with negative control; ***=compared with positive control; 1= P<0.05; 2= P<0.01; 3= P<0.001

In the D_1 of the experiment, daily oral administration of 250 and 500 mg/kg of extracts showed significant increase ($P<0.05$) in MAP compared with normal control. In addition, groups that received, 1000 mg/kg/day oral administration of all extracts except the one that received AQ crude extract showed significant increase ($P<0.001$) in MAP compared with normal control in the D_1 of the experiment (Table 3).

Daily oral administration of 250 and 500 mg/kg of all extracts and 1000 mg/kg of solvent fractions showed significant increase in MAP compared with normal control ($P<0.001$) and compared with positive control ($P<0.001$) in the D_{10} of the experiment, whereas, 1000 mg/kg/day oral administration of crude extracts prevented a rise in MAP in a similar manner with positive control (Table 3).

After consecutive oral daily administration for 15 days, all treatment groups except the one that received the highest dose (1000 mg/kg) of AQ crude extract showed significant increase in MAP compared with normal control ($P<0.001$) and compared with positive control ($P<0.001$) (Table 3).

Table 3: Effect of crude extracts and solvent fractions of *M. stenopetala* leaves on MAP in D-Fructose (66% w/v *ad libitum*) induced hypertensive rats.

Substance administered	Dose (mg/kg)	MAP			
		D1 (mmHg)	D_5 (mmHg)	D_{10} (mmHg)	D_{15} (mmHg)
D-Fructose	66% w/v *ad libitum*	96.95 ± 1.00	108.02 ± 0.80***3,*3	118.45 ±0.68***3, *3	130.88 ±0.50***3, *3
Captopril	20	97.40 ± 0.81	100.83 ± 0.99**3	99.55 ±0.74**3	99.38 ± 065**3
AQ crude	250	97.07 ± 0.75	106.83 ± 0.99***3,*3	112.88 ± 0.76**3, ***3, *3	121.35 ±0.74**3, ***3, *3
	500	97.17 ± 0.59	103.15 ± 0.50**3, *3	105.05 ± 0.43**3, ***3, *3	107.27 ± 0.84**3, ***3, *3
	1000	99.38 ± 0.68	101.27 ±0.58**3	100.23 ± 0.37**3	98.57 ± 0.75**3
70% EtOH crude	250	98.27 ± 0.44	110.45 ±0.58***3, *3	117.73 ± 0.56, ***3, *3	125.40 ± 0.48**3, ***3, *3
	500	97.28 ± 0.71	106.15 ± 0.48***3, *3	111.72 ± 0.63**3, ***3, *3	113.62 ± 0.34**3, ***3, *3
	1000	98.72 ± 0.79	100.68 ±0.81**3, *3	101.68 ± 0.53**3	106.18 ± 0.55**3, ***3, *3
EtAc fraction of AQ crude	250	99.77 ±0.43	111.70 ± 0.37**1,***3, *3	119.15 ± 0.85***3, *3	128.15 ± 0.51***3, *3
	500	99.55 ± 0.75	108.17 ± 0.49***3, *3	114.50 ± 0.52**2, ***3, *3	123.27 ± 0.32**3, ***3, *3
	1000	99.15 ± 0.70	105.00 ± 0.76***2, *3	112.50 ± 0.61**3, ***3, *3	119.67 ± 0.44**3, ***3, *3
AQ residue of AQ crude	250	99.17 ± 1.64	109.62 ± 0.56***3, *3	115.83 ± 0.69***3, *3	125.33 ± 0.47**3, ***3, *3
	500	97.95 ±0.83	108.12 ± 0.57*3	113.27 ±0.48**3, ***3, *3	120.28 ±0.42**3, ***3, *3
	1000	98.80 ± 0.89	103..38 ±0.57**2, *1	108.03 ± 0.49**3, ***3, *3	113.48 ±0.47**3, ***3, *3

Results are expressed as X ± S.E.M (n=6), *=compared with normal control; **= compared with negative control; ***=compared with positive control; 1= P<0.05; 2= P<0.01; 3= P<0.001

Daily oral administration of 250 mg/kg of all extracts ($P<0.01$), 500 mg/kg of all extracts except AQ crude ($P<0.01$) and 1000 mg/kg of EtAc fraction of AQ crude ($P<0.05$) showed significant increase in DBP compared with normal control in the D_1 of the experiment. However, those groups that received 500 and 1000 mg/kg/day of AQ crude, 70% EtOH crude and AQ residue of AQ crude extracts prevented a rise in DBP compared with normal control in the D_1 of the experiment (Table 4).

Groups that received daily oral administration of AQ crude (250 mg/kg), 70% EtOH crude (250 and 500 mg/kg), EtAc fraction (all tested dose levels) and AQ residue (all tested dose levels) showed significant increase in DBP compared with normal control in the D_{10} ($P<0.001$) and D_{15} ($P<0.001$) of the experiment, whereas, groups that received AQ crude extract (500 and 1000 mg/kg/day) and 70% EtOH crude extract (1000 mg/kg/day) prevented a rise in DBP when compared with normal control in the D_{10} and D_{15} of the experiment (Table 4).

After consecutive oral daily administration for 15 days, groups that received 1000 mg/kg AQ crude extract showed significant decrease ($P<0.05$) in DBP compared with normal control (Table 4).

Table 4: Effect of crude extracts and solvent fractions of *M. stenopetala* leaves on DBP in D-Fructose (66% w/v *ad libitum*) induced hypertensive rats.

Substance Administered	Dose (mg/kg)	DBP			
		D1 (mmHg)	D_5 (mmHg)	D_{10} (mmHg)	D_{15} (mmHg)
D-Fructose	66% w/v *ad libitum*	88.67 ±1.36	95.67 ± 0.88***^{3,*3}	105.50 ± 0.76***^{3,*3}	114.50 ± 0.76***^{3,*3}
Captopril	20	89.17 ± 0.87	88.83 ± 1.01**^{3}	89.00 ± 0.97**^{3}	88.83 ±0.95**^{3}
AQ crude	250	88.50 ±0.76	95.50 ± 0.99***^{3,*3}	100.00 ± 0.97**^{3,} ***^{3,*3}	107.17 ± 0.60**^{3,} ***^{3,*3}
	500	89.50 ± 0.76	91.33 ± 0.67**^{1}	91.50 ±0.76**^{3}	92.67 ± 1.45**^{3,} ***^{1}
	1000	90.67 ±0.67	91.00 ± 0.58**^{2}	88.83 ±0.31**^{3}	85.50 ± 0.76**^{3,} *^{1}
70% EtOH crude	250	89.33 ± 1.05	96.33 ± 0.88***^{3,*3}	103.17 ± 0.60***^{3,} *^{3}	109.50 ± 0.43**^{3,} ***^{3,*3}
	500	88.50 ± 0.76	94.00 ±0.58***^{2,} *^{2}	98.83 ± 0.60**^{3,} ***^{3,*3}	99.17 ± 0.60**^{3,} ***^{3,*3}
	1000	90.50 ±0.76	91.50 ± 0.76**^{1}	90.33 ± 0.88**^{3}	92.5 ±0.76**^{3}
EtAc fraction of AQ crude	250	90.50 ±0.76	96. 83 ± 0.60***^{3,*3}	103.17 ± 1.08***^{3,} *^{3}	111.67 ± 0.67***^{3,} *^{3}
	500	89.50 ±0.76	95.17 ± 0.60***^{3,} *^{2}	101.17 ± 0.60**^{1,} ***^{3,*3}	109.17 ± 0.60**^{3,} ***^{3,*3}
	1000	90.50 ± 0.76	95.33 ± 0.88***^{3,} *^{1}	101.50 ± 0.76**^{1,} ***^{3,*3}	106.83 ± 0.60**^{3,} ***^{3,*3}
AQ residue of AQ crude	250	90.33 ± 1.80	95.50 ± 0.76**^{3,} ***^{3,*2}	102.67 ± 0.88***^{3,} *^{3}	110.00 ± 0.58**^{2,} ***^{3,*3}
	500	88.50 ± 0.76	95.83 ± 0.79***^{3,} *^{3}	100.17 ± 0.79**^{2,} ***^{3,*3}	107.33 ± 0.49**^{3,} ***^{3,*3}
	1000	89.50 ± 0.76	93.00 ± 0.82***^{1}	96.50 ±0.76**^{3,} ***^{3,*3}	100.50 ±0.76**^{3,} ***^{3,*3}

Results are expressed as X ± S.E.M (n=6), *=compared to normal control; **= compared to negative control; ***=compared to positive control; 1= P<0.05; 2= P<0.01; 3= P<0.001

5.2.2. Effect on blood chemistry

5.2.2.1. Effect on total cholesterol, glucose and triglyceride plasma levels

The negative control showed significant increase in serum TC ($P<0.01$), BG ($P<0.001$) and TG ($P<0.001$) level compared with normal control. The positive control also showed significant increase in TG level ($P<0.001$) but not in TC and BG level compared with normal control. Although all extracts prevented a rise in TG level in dose dependent manner, there was significant increase ($P<0.001$) compared with normal control. However, groups that received 500 and 1000 mg/kg/day of all extracts showed significant difference ($P<0.001$) in TG level compared with negative control (Fig. 5).

Groups that received AQ crude extract (500 and 1000 mg/kg/day), AQ residue of AQ crude extract (1000 mg/kg/day) and normal control showed significant difference ($P<0.05$) in BG level compared with negative control. Daily oral administration of 250 mg/kg of all extracts and 500 mg/kg (70% EtOH crude and EtAc fraction of AQ crude extract) showed significant difference ($P<0.05$) in BG level compared with normal control, whereas, daily oral administration of AQ crude extract (500 and 1000 mg/kg), 1000 mg/kg AQ residue of AQ crude extract and normal control showed significant difference ($P<0.01$) in BG level compared with negative control (Fig. 5).

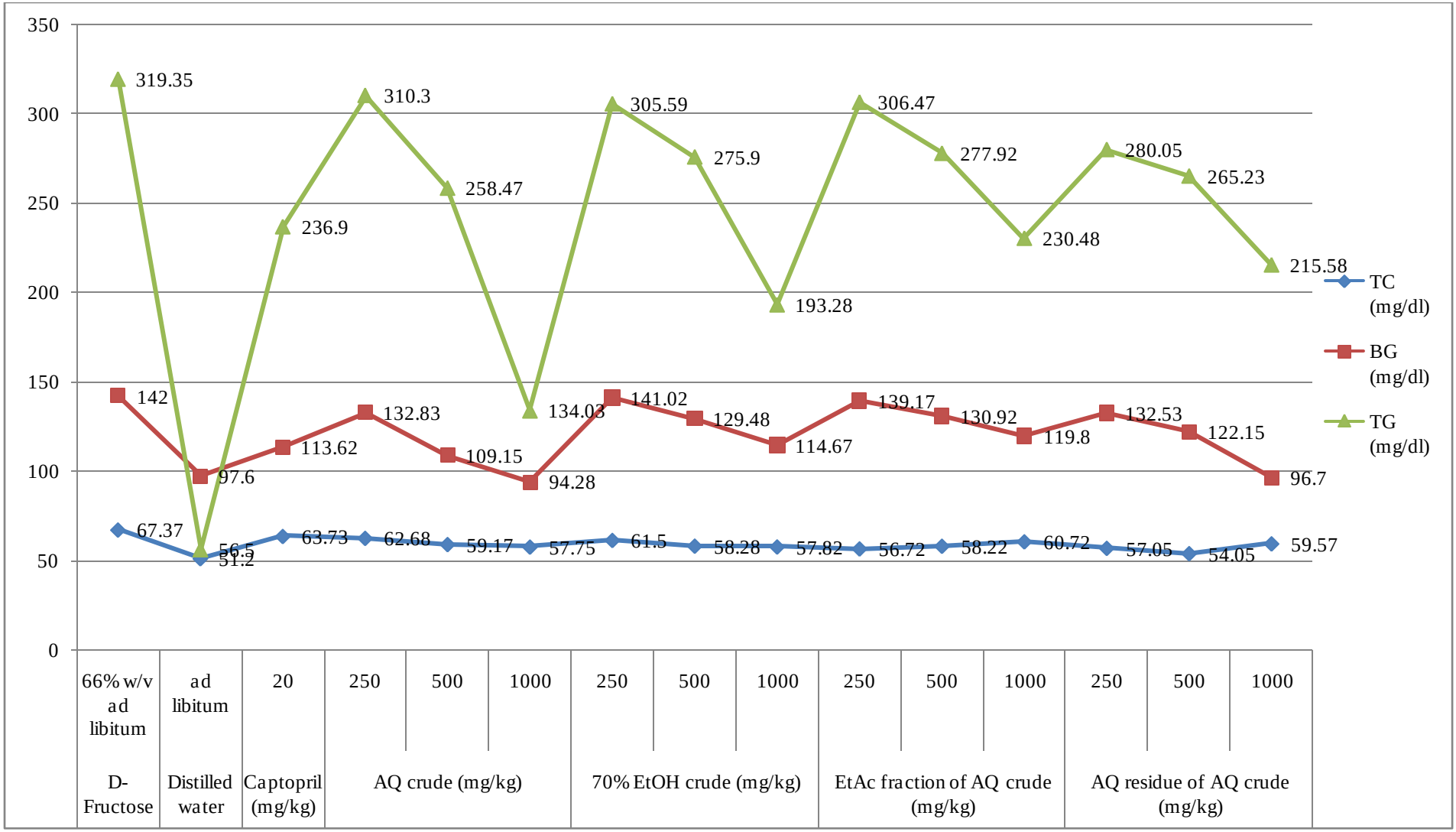

Figure 5: Effect of crude extracts and solvent fractions of *M. stenopetala* on TC, BG and TG in D-Fructose (66% w/v *ad libitum*) induced hypertensive rats

5.2.2.2 Effect on liver enzymes and kidney metabolites

All treatment groups elicited an increase in hepatocellular evaluation (ALT and AST) and hepatobiliary evaluation (ALP and GGT) in a dose dependent manner compared with normal control, whereas, groups that received 500 mg/kg/day 70% EtOH crude extract and 1000 mg/kg/day of all extracts showed significant difference ($P<0.05$) in ALP compared with positive control (Table 5).

The negative and positive controls showed significant increase ($P<0.05$) in creatinine, whereas, all treatment groups did not show significant difference in creatinine compared with normal control. Daily oral administration of 250 mg/kg 70% EtOH crude extract, 500 mg/kg (AQ crude extract, 70% EtOH crude extract and AQ residue of AQ residue of AQ crude), 1000 mg/kg of all extracts and normal control showed significant difference in creatinine compared with the negative ($P<0.05$) and positive ($P<0.01$) control (Table 5).

However, all groups showed significant decrease ($P<0.01$) in BUN compared with normal control (Table 5).

Table 5: Effect of crude extracts and fractions of *M. stenopetala* on liver enzymes and kidney metabolites.

Extract Administered	Dose (mg/kg)	Liver parameters				Kidney parameters	
		ALP(U/L)	ALT (U/L)	AST U/L)	GGT (U/L)	Creatinine (mg/dl)	BUN (mg/dl)
D-Fructose	66% w/v *ad libitum*	198.72 ± 14.72	66.47 ± 7.18	195.60 ± 26.02	2.83 ± 0.37	0.42 ± 0.03*[1]	21.95 ± 1.08*[3]
Captopril	20	149.98 ± 10.53	70.27 ± 7.83	218.43 ± 38.03	3.15 ± 0.19	0.44 ± 0.03*[2]	22.43 ± 1.39*[2]
AQ crude	250	168.03 ± 10.79	62.88 ± 3.65	162.57 ± 10.85	2.80 ± 0.21	0.43 ± 0.02*[2]	19.60 ± 1.01*[3]
	500	211.57 ± 10.85	77.87 ± 6.01	238.58 ± 42.96	3.06 ± 0.43	0.34 ± 0.01**[1, ***2]	22.03 ± 1.44*[3]
	1000	239.33 ± 8.43***[2]	80.50 ± 0.99	246.78 ± 14.08	3.58 ± 0.15	0.32 ± 0.00**[2, ***3]	23.33 ± 0.42*[2]
70% EtOH crude	250	179.18 ± 7.92	63.75 ± 3.55	176.15 ± 12.81	2.55 ± 0.18	0.30 ± 0.01**[1, ***3]	24.00 ± 0.81*[1]
	500	219.57 ± 15.24***[1]	64.10 ± 2.39	193.73 ± 27.64	2.71 ± 0.35	0.32 ± 0.01**[2, ***3]	22.55 ± 1.76*[2]
	1000	227.08 ± 15.62***[1]	66.67 ± 1.54	214.15 ± 9.84	3.31 ± 0.18	0.34 ± 0.02**[1, ***2]	21.15 ± 0.73*[3]
EtAc fraction of AQ crude	250	195.47 ± 11.59	65.03 ± 3.25	199.47 ± 35.39	2.8500 ± 0.56	0.39 ± 0.01	21.81 ± 1.39*[3]
	500	204.93 ± 25.91	65.93 ± 2.38	244.32 ± 20.88	3.5500 ± 0.49	0.35 ± 0.01***[2]	18.56 ± 0.73*[3]
	1000	222.02 ± 11.19***[1]	68.13 ± 1.17	251.33 ± 7.37	4.3000 ± 0.61	0.32 ± 0.00**[2, ***3]	24.48 ± 0.44*[1]
AQ residue of AQ crude	250	193.23 ± 13.29	57.18 ± 2.02	188.57 ± 19.75	3.1333 ± 0.17	0.40 ± 0.01	17.83 ± 0.89*[3]
	500	215.67 ± 4.11	64.20 ± 0.91	196.73 ± 5.55	3.5333 ± 0.39	0.30 ± 0.01**[3, ***3]	18.06 ± 1.21*[3]
	1000	237.53 ± 18.38***[2]	65.23 ± 3.02	200.27 ± 25.00	3.7500 ± 0.29	0.34 ± 0.01**[1, ***2]	22.33 ± 0.80*[3]
Distilled water	*ad libitum*	174.47 ± 8.64	66.63 ± 3.54	194.75 ± 18.18	3.9667 ±1.16	0.33 ± 0.00**[1, ***2]	29.80 ± 0.92**[3, ***2]

Results are expressed as X ± S.E.M (n=6), *=compared to normal control; **= compared to negative control; ***=compared to positive control; 1= P<0.05; 2= P<0.01; 3= P<0.001

5.3. *In vitro* vasodilatory activity

Cumulative dose administration of 2.5, 5 and 10 mg/ml of AQ residue and EtAc fraction of AQ crude showed significant difference ($P<0.001$) in percent relaxation compared with 10 mg/ml 70% EtOH crude extract (E^++KCl) and 10 mg/ml AQ crude extract (E^++KCl) in both intact and denuded endothelium aortic strip pre-contracted with KCl. And, administration of cumulative dose of all extracts showed a significant difference ($P<0.05$) in percent relaxation compared with 10 mg/ml 70% EtOH crude extract (E^++KCl) and 10 mg/ml AQ crude extract (E^++KCl) in intact endothelium aortic strip pre-contracted with MB (Fig. 6).

Administration of cumulative doses of AQ residue and EtAc fraction of AQ crude showed significant difference ($P<0.05$) in percent relaxation compared with 10 mg/ml 70% EtOH crude extract (E^++KCl) and 10 mg/ml AQ crude extract (E^++KCl) in intact endothelium aortic strip pre-contracted with EPI. A 70% EtOH crude extract (5.0 and 10 mg/ml) also showed significant difference ($P<0.05$) in percent relaxation compared with 10 mg/ml 70% EtOH crude extract (E^++KCl) and 10 mg/ml AQ crude extract (E^++KCl) in intact endothelium aortic strip pre-contracted with EPI (Fig. 6).

Cumulative dose administration of 1.25, 2.5, 5.0 and 10.0 mg/ml of all extracts except AQ crude extract showed significant difference ($P<0.001$) in percent relaxation compared with 10 mg/ml 70% EtOH crude extract (E^++KCl) and 10 mg/ml AQ crude extract (E^++KCl) in intact endothelium aortic strip pre-contracted with GLIB (Fig. 6).

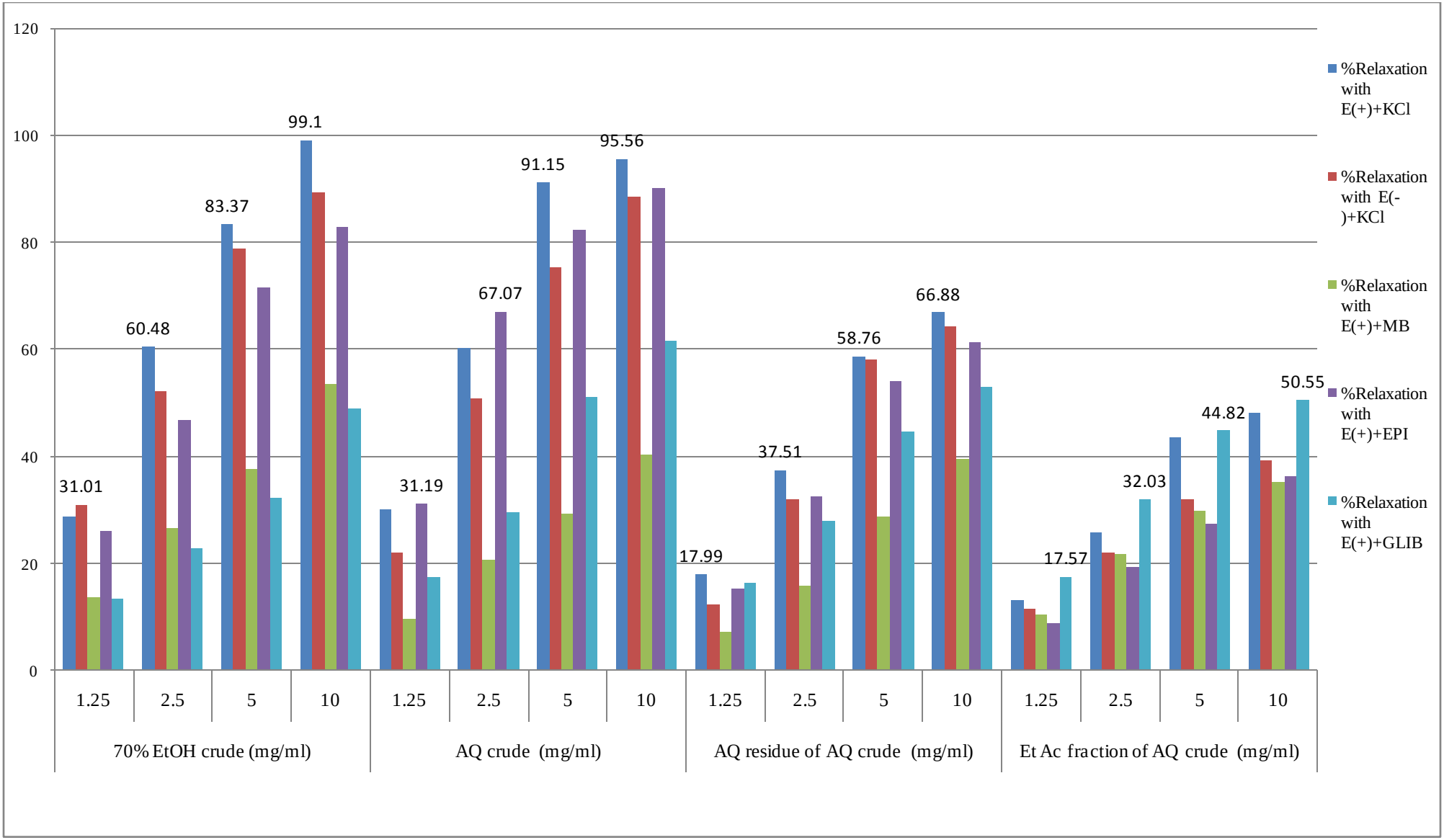

Figure 6: *In vitro* vasodilatory effect of crude extracts and solvent fractions of *M. Stenopetala* in pre-contracted isolated guinea pigs' aorta

5.4 Acute Oral Toxicity

Acute oral toxicity of AQ crude, 70% EtOH crude, EtAc fraction and AQ residue of AQ crude extracts of *M. stenopetala* leaves was evaluated in female Wistar rats according to OECD guideline No 425 (OECD, 2008). On the basis of oral reports of high consumption of the leaves for diet mixed with cultural foods, the chosen dose was highest (5000 mg/kg extract). The acute toxicity study indicated that the extract did not shown any signs of toxicity with close follow up for 4 hrs and cause no mortality within 24 hrs and 14 days at a dose of 5000 mg/kg body weight.

After 14 days of observation of the experimental rats, no body weight reduction as well as over increase was observed. Gross physical and behavioral observation also revealed no visible signs of toxicity. Additionally, there was no gross pathological alteration (color, size and texture) of the vital organs. Therefore, the LD_{50} of these extracts was greater than 5000 mg/kg.

6. DISCUSSION

6.1. Phytochemical Screening

The four solvent extracts (AQ crude, 70% EtOH crude, EtAc fraction and AQ residue of AQ crude) of the fresh *M. stenopetala* leaves were screened for the presence of different phytochemicals. The qualitative phytochemical screening of AQ crude extract showed the presence of all tested secondary metabolites. This finding is in agreement with the study done on AQ extract of *M.oleifera* (Brindha *et al*, 2014). And 70% EtOH crude extract showed presence of all tested phytochemicals except saponins. This finding is in line with the study done on EtOH extract of *M.oleifera* (Onyekaba *et al*, 2013). Tannin and phytosterol were present in all tested extracts. Saponin was present in all tested extracts but not in 70% EtOH crude extract. Only crude extracts showed a positive test result for alkaloids and glycosides.

The study done by Denen and coworkers showed the presence of alkaloids, tannins and glycosides but no saponins and anthraquinones in EtOH extract of *M.oleifera* (Denen *et al*, 2014). The other study done by Ojiako showed the presence of all tested metabolites (tannin, alkaloid, saponin and phenol) in both EtOH and EtAc crude extract of *M.oleifera* (Ojiako, 2014). On the other hand the study done by Nweze and Felix showed the presence of all tested metabolites (flavonoid, anthraquinone, alkaloid, saponin, terpenoid, glycoside, and tannin) in both EtOH and AQ crude extract of *M.oleifera* (Nweze and Felix, 2014).

The present study indicated that the fresh leaves extracts of *M. stenopetala* contain different classes of secondary metabolites. The yield obtained for secondary metabo-

lites of *M. stenopetala* leaves in the present study was recorded to be highest in the case of AQ crude extract followed by 70% EtOH crude, AQ residue and EtAc fraction of AQ crude extract in succession. The presence of these phytochemicals gave a great potential for extracts of *M. stenopetala* leaves in balancing abnormal rise in BP, BG level and metabolic disturbances that signifies the potential of the plant as a source of therapeutic agent.

6.2. *Invivo* antihypertensive activity

6.2.1. Effect on body weight

All groups of rats in this study showed increase in body weight which is in agreement with the study done on sub-acute administration of AQ crude extract of *M.stenopetala* leaves in mice (Ghebresellasie *et al*, 2011). However, the percentage of body weight gain was reduced in the highest doses of all extracts (1000 mg/kg/day). This reduction was significant compared to normal control in the D_{15} of experiment. This finding is in line with that of the previous study on sub-acute administration of AQ crude extracts of *M.oleifera* in rats (Adedapo *et al*, 2009).

6.2.2. Effect on blood pressure

The negative control which received 66% w/v D-Fructose were stated as a hypertension or diseased model with an average increase in SBP (50 mmHg), DBP (25 mmHg), and MAP (33 mmHg) from basal BP. Whereas, positive control which were given Captopril (20 mg/kg/day) with 66% w/v D-Fructose *ad libitum* showed the average change in SBP (7 mmHg), DBP (-1 mmHg), and MAP (2 mmHg) were considered as normotensive after 15 days of study period.

Daily oral administration of the crude extracts of *M.stenopetala* significantly prevented the increase in SBP, MAP and DBP in 66% w/v D-Fructose *ad libitum* consuming rats in a dose dependent manner. The highest daily oral dose of AQ crude extract (1000 mg/kg) significantly prevented the increase in SBP, MAP and DBP in a similar manner to positive and normal control, groups that received Captopril (20 mg/kg/day) and distilled water (*ad libitum*), respectively. The highest dose of 70% EtOH crude extract also elicited a significant suppression in SBP, MAP and DBP.

Whereas, the EtAc fraction and AQ residue of AQ crude extract did not show significant suppression effect in SBP, MAP and DBP, rather there was significant rise in SBP, MAP and DBP in a similar manner with negative control, groups that received only 66% w/v D-Fructose *ad libitum*. The AQ and 70% EtOH crude extract elicited the highest dose dependent suppression effect. This effect may be attributed to the presence of alkaloids and glycosides in crude extracts. This finding is in line with the study done on alkaloids of *M.oleifera* leaves on isolated frog heart (negative inotropic and chronotropic effect) (Dangi *et al*, 2002), on glycosides isolates of *M.oleifera* leaves (Faizi *et al*, 1995) and thiocarbamate glycosides *M.oleifera* pods and seeds in rats (Jansakul *et al*, 1997).

6.2.3. Blood Chemistry

6.2.3.1. Effect on total cholesterol, glucose and triglyceride plasma levels

Significant lowering of serum TC, BGL and TG could prevent atherosclerosis and ischemic conditions. In this study, it was observed that the extracts decreased TC, BG, and TG levels in a dose dependent manner indicating that they could prevent

atherosclerosis in animal models. The level of TG however, increased with the extracts.

This finding is in agreement with those of the previous studies carried out on antidiabetic and antihyperglycemic activity of different solvent extracts of *M. stenopetala* leaves using various models: *invitro* intestinal α-glucosidase and pancreatic enzyme inhibitory activity of 70% EtOH (Toma *et al*, 2014), 70% EtOH and its fractions in alloxan induced diabetic mice (Sileshi *et al*, 2014), n-butanol fraction of 70% EtOH in alloxan induced diabetic mice (Toma *et al*, 2012), AQ, 70% EtOH and butanol fractions in STZ induced diabetic rats (Toma *et al*, 2015), AQ and 70% EtOH extracts and fractions in alloxan induced mice (Nardos *et al*, 2011).

In addition, the finding is in line with those of the previous studies done on antidiabetic and antihyperlipidemic of different solvent extracts of *M. oleifera* leaves using various models: AQ extract in STZ and fructose induced rats (Divi *et al*, 2012), EtOH extract in STZ induced diabetic rats (Chinedu *et al*, 2014), EtOH extract of *M. oleifera* leaves in hypercholesterolemic rats (Denen *et al*, 2014), AQ extract of *M. oleifera* leaves in diabetic patients (Brindha *et al*, 2014), 98% EtOH extract in alloxan induced rats (Aja *et al*, 2015) and 100% methanol extract of *M.oleifera* pods in STZ induces rats (Gupta *et al*, 2012).

In the present study, AQ crude and residue extract of *M. stenopetala* showed the highest suppression in BG level increment in a dose dependent manner. The TC, BG and TG level suppression effect was high in groups that received AQ crude followed by 70% EtOH crude extract. The crude extracts showed a dose dependent suppression

in TC, BG and TG plasma level increment and a highest effect was observed at the maximum tested dose (1000 mg/kg/day).

6.2.3.2. Effect on liver enzymes and kidney metabolites

The liver and kidney are major organs of early screening efforts in the preclinical research and a major target organ in the repeated-dose non-clinical safety studies used to support clinical trials (Coolborn *et al*, 2012). If there is mild inflammation and tissue damage to these organs, the permeability of the cell membrane will increase and release cytoplasmic enzymes such as LD, ALP, and AST, while necrosis will release mitochondrial ALT as well as AST leaking into the blood and increase in levels (Hor *et al*, 2012).

Testing of AST and ALT are used for hepatocellular (damage to liver cells and liver disease) evaluation while ALP is used for hepatobiliary (cholestasis or hyperbilirubinemia) evaluation (Coolborn *et al*, 2012) which is used to determine potential target organ toxicity and associated time courses of the damage without the need of biopsy or necropsy samples. From the present study all treatment groups elicited an increase in AST, ALT, ALP and GGT level in a dose dependent manner though not significant. The largest increase in plasma ALP, AST, ALP and GGT level was observed for groups that received a maximum dose (1000 mg/kg/day) of AQ crude extract.

The results are in line with those of the studies done on extract of *M.stenopetala* leaves in $HEPG_2$ cells *in vitro* (Mekonnen N, *et al*, 2005), sub-chronic administration of 70% EtOH crude extract of *M.stenopetala* leaves in mice (Sileshi *et al*, 2014), sub-acute administration of AQ crude extract of *M.stenopetala* leaves in mice (Ghebresel-

lasie *et al*, 2011) and seven days administration of EtOH extract of *M. oleifera* leaves in rats (Aja *et al*, 2015).

Toxic agents may affect kidneys and impair their physiological functions. The effect on kidney can be investigated by cross checking the normally expected function, such as in excreting waste product like BUN and creatinine (Nafiu *et al*, 2011). In this study renal toxicity was assessed by measuring BUN and creatinine and the results showed that there was no significant increase in the plasma level of BUN and creatinine in comparison with normal control which indicated non toxic effect of the extract on the kidney.

This finding is in agreement with those of the studies done on sub-chronic administration of 70% EtOH crude extract of *M.stenopetala* leaves in mice (Sileshi *et al*, 2014), sub-acute administration of AQ crude extract of *M.stenopetala* leaves in mice (Ghebresellasie *et al*, 2011) and sub-acute administration of AQ crude extract of *M.oleifera* leaves in rats (Adedapo *et al*, 2009).

6.3. *Invitro* vasodilatory activity

From the present study, all extracts of *M.stenopetala* produced a relaxation of KCl (on intact and denuded endothelium), MB, EPI and GLIB induced contraction in isolated thoracic aortic strips of guinea pigs in a dose dependent manner. The greatest vasodilatory activity was observed at maximum tested doses of 10 mg/ml.

AQ and 70% EtOH crude extracts elicited a highest relaxation than the fraction and residue of AQ crude extract on pre-contracted isolated thoracic aortic rings. This finding is in line with those of the studies done on AQ extract of *M.stenopetala* leaves on

isolated thoracic aorta of guinea pigs (Mengistu *et al*, 2012) and on isolated compounds from crude extracts of *M. oleifera* leaves in isolated thoracic aorta of rabbit (Gilani *et al*, 1994).

This activity was attributed to the presence of alkaloids and glycosides in crude extracts which is in line with the study done on isolated guinea pigs ileum of alkaloids of leaves of *M.oleifera* (Dangi *et al*, 2002), and thiocarbamate glycosides isolated from *M.oleifera* pods and seeds (Jansakul *et al*, 1997). The percent relaxation of KCl induced contraction was found to be greater in intact than denuded endothelium of isolated thoracic aortic preparation of guinea pigs for all extracts. This showed that the presence of endothelium has a contribution for increment in the percent relaxation of pre-contracted isolated aortic strip which may be attributed to stimulation EDRF and release of NO by some phytoconstitutents present in the extracts.

As the extracts also produced significant relaxation in denuded endothelium in KCl pre-contracted isolated thoracic aortic strip, hence they have a potential to induce endothelium independent vasodilation. And, the largest dose dependent percent relaxation (% R_{max}) was achieved for KCl (intact endothelium) followed by KCl (denuded endothelium) and then by EPI (intact endothelium) pre-contracted isolated thoracic aortic strip than other agents.

Hence, the mechanism of the relaxation by *M. stenopetala* might be through Ca^{2+} channel blockade followed with a significant α_1-blocking effect which is indicative of the blockade of Ca^{2+} influx through both L-type voltage dependent and receptors op-

erated Ca^{2+} channels, respectively. This finding is in line with those of studies done on AQ extract of *M. stenopetala* leaves (Mengistu *et al*, 2012).

The presence of glycosides in the crude extracts might be contributed for vasodilatory effect by α_1-blocking which is in agreement with the study done on thiocarbamate glycosides isolated from *M.oleifera* pods and seeds (Jansakul *et al*, 1997). The AQ crude extract elicited the highest EC_{50} on intact endothelium isolated thoracic aorta strip pre-contracted with EPI and maximum smooth muscle relaxation. This was followed by AQ crude extract on intact endothelium isolated thoracic aorta strip pre-contracted with KCl and highest smooth muscle relaxation. Then followed by 70% EtOH and AQ crude extract on intact and denuded endothelium thoracic aorta strip pre-contracted with KCl, respectively.

6.4. Acute Toxicity Study

Aside from being highly revered for its medicinal value, *M. stenopetala* has also served the community as a source of food for centuries. With this prior information of safety, limit test was found to be the appropriate test for assessing the acute toxicity profile with high dose (5000 mg/kg) as per the OECD guideline (OECD, 2008). The acute toxicity study of oral administration of the limit dose of 5000 mg/kg of the AQ crude extract and its solvent fractions (EtAc fraction and AQ residue) and 70% EtOH crude extract of *M. stenopetala* leaves indicated that there was no mortality observed in all test animals during the course of the study period. Furthermore, no overt behavioral and physical signs of toxicity were discerned at this dose.

Consequently, the facts that there was no sign of morbidity as well as mortality at this specific high dose would in fact allow making suggestions that the oral LD_{50} of the extract could be greater than 5000 mg/kg. All experimental groups showed an increase in body weight and a reasonable percent weight gain in 1st and 2nd week of the experiment when compared with normal control.

This finding is in line with the study done on 70% EtOH extract of *M.stenopetala* leaves at 5000 mg/kg in mice (Nardos *et al*, 2011), n-butanol fraction of 70% EtOH extract *M.stenopetala* leaves at 5000 mg/kg in mice (Toma *et al*, 2012) and AQ extract of *M.oleifera* leaves at 2000 mg/kg in rats (Adedapo *et al*, 2009).

7. CONCLUSION

In general, this study demonstrated the *in vivo* and *in vitro* antihypertensive activity of the AQ and 70% EtOH crude extracts of *M.stenopetala* leaves in 66% w/v D-Fructose induced hypertensive male Wistar rats and pre-contracted isolated thoracic aorta strip of guinea pigs. The *in vivo* study also demonstrated the antihypreglycemic and anti-triglyceride activity. The effect decreased with the fraction and residue of crude extract, which gives indication that different active phytomolecules of the extract might be attributed for the *in vivo* and *in vitro* antihypertensive activity of the extracts synergistically. On the other hand, the safety result on biochemical parameters elicited a dose dependent increase in liver function indicators (AST, ALT, ALP and GGT) but no increase in kidney function indicators (BUN and creatinine).

8. RECOMMENDATIONS

Further studies are necessary to be performed for the antihypertensive activity of the extract against other hypertension models and effect of the extract on liver. Purification, isolation and characterization of the phytoconstituents responsible for the *in vivo* and *in vitro* antihypertensive effect and exploring the exact mechanism of action are needed.

DECLARATION OF INTEREST

I the undersigned Bekesho Geleta, declare that this thesis, "*In vivo* antihypertensive and *in vitro* vasodilatory effect of the crude extracts and fractions of *Moringa steno-petala* leaves in rodents" is my original work that has not been presented to any other university for a similar or any other degree award. It is fully sited and referenced with appropriate acknowledgment.

Name: Bekesho Geleta

Signature: _____

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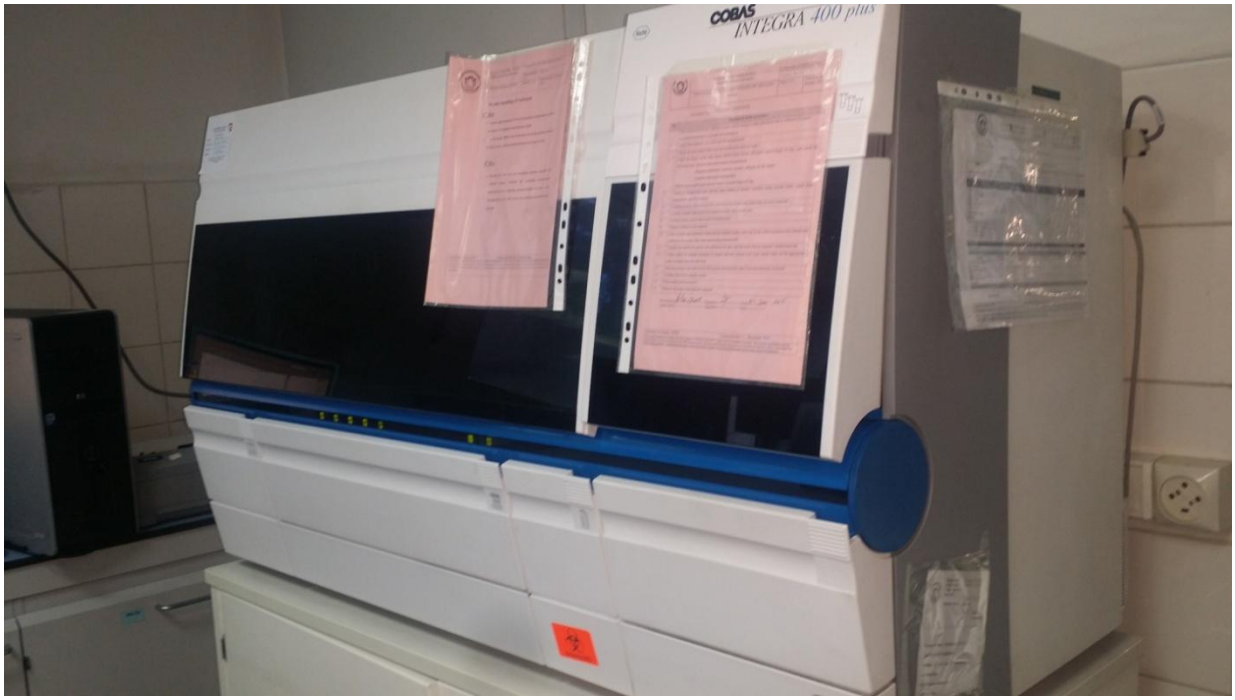
10. ANNEXES

Annex. 1: Model 179 tail cuff blood pressure analyzer instrument

Annex 2: Model 7E grass polygraph & organ bath with its accessories instrument



Annex 3: Model Cobas-e-411 clinical chemistry analyzer instrument

Annex 4: Protocol for phytochemical screening

a) Alkaloids

One and half milliliter of 10% HCl was added to 0.5 mg of the extracts in a test tube. The mixture was heated for 20 minutes. It was then cooled and filtered. To 1ml of the filtrate 5 drops Mayers and Draggendorff's reagents each were added. Formation of cream and orange colored precipitates respectively indicates the presence of alkaloids in the extracts.

b) Saponins

Froth test: An aqueous solution of 0.5 mg of the extract in a test tube was vigorously shaken for 2 minutes. Foam which persisted for 30 minutes and doesn't disappear upon warming was taken as an indication of the presence of saponin in the extract.

c) Polyphenols (Phenolic compounds)

Three drops of a mixture of 1 ml 1% FeCl3 and 1% $K_3Fe(CN)_6$ each were added to 2 ml of extracts. Formation of green or blue color was taken as an indication of the presence of polyphenols.

d) Flavonoids

To 2 ml of aqueous solution of the extract 4 drops of 2% lead acetate solution was added. Development of yellow or orange color confirms the presence of flavonoids.

e) Coumarins

Two milliliter of 10% ammonia solution was added to 5ml concentrated alcoholic solution of the extracts.The occurrence of an intensive fluorescence under UV light indicates the prescence of coumarin derivatives.

f) Terpenoids (Ketonic)

One milliliter of 2, 4-dinitrophenylhydrazine solutions (0.5g dissolved in 100ml of 2M HCl) was added to 2ml aqueous solution of the extract. Formation of yellow-orange coloration indicates the presence of a ketonic terpenoids.

g) Anthraquinones

Borntrager's test: Five milliletr of the extract was dried and shaken with 3ml petroleum ether. The filtrate was added to 2ml of a 25% ammonia solution. The mixture was shaken and formation of a red coloration was taken as an indication of the presence of free anthraquinones.

h) Tannins

Three drops of 5% ferric chloride solution was added to 1ml of the extract solution in water. A greenish or blue coloration or precipitation was taken as indication of the presence of tannins.

i) Phytosterols and Withanoids

Five drops of 3% vanillin in conc. H_2SO4 was added to a concentrated chloroform solution of extracts. Formation of a rose or reddish brown color indicates the presence of anoids or phytosterols.

j) Test for Glycosides (Keller-Killiani Test)

To 0.5 g of each extract suspended in 5 ml water, 2 ml of glacial acetic acid containing one drop of ferric chloride hexahydrate ($FeCl_3.6H_2O$) solution was added. This was mixed with 1 ml of concentrated sulfuric acid and observed for a brown ring at the interface or a violet ring below the brown ring; alternatively acetic acid was added and observed for a greenish ring above the brown ring which gradually spread throughout this layer.