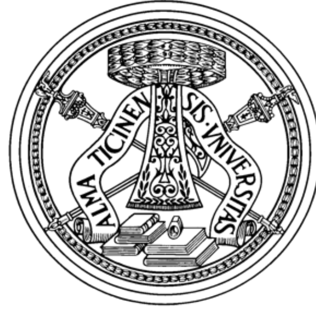

UNIVERSITÀ DEGLI STUDI DI PAVIA

DOTTORATO DI RICERCA IN SCIENZE CHIMICHE E FARMACEUTICHE

XXXI CICLO

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Study of traditional African foods useful for the preparation of functional foods and food supplements

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CHAPTER I

FUNCTIONAL FOODS, FOOD SUPPLEMENTS AND NOVEL FOODS

1 Functional food, food supplements and novel foods

1.1 Functional foods

The European Commission's Concerted Action on Functional Food Science in Europe (FUFOSE), a group which actively involved a large number of the most prominent European experts in nutrition and related sciences coordinated by the International Life Sciences Institute (ILSI) Europe, developed a European Consensus on 'Scientific Concepts of Functional Foods', in early 1996, publishing it in 1999 (Diplock et al., 1999). According to this consensus, a food can be regarded as functional if it has been satisfactorily demonstrated to beneficially affect one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either markedly improved health and well-being or reduction of risk of disease. A functional food remains a food, it is not a pill or a capsule but part of the normal food pattern, and it must demonstrate its effects in amounts that can normally be expected to be consumed in the diet:

In view of this definition of functional foods, to attribute "functional food" status to a specific food the following aspects should be considered:

1. The nature of the food i.e. the functional food must not be a pill, a capsule or any form of dietary supplement.

2. The demonstration of effects claimed by the food to the satisfaction of the scientific community.

3. The functional food must have beneficial effects on body functions, beyond adequate nutritional effects, relevant to an improved state of health and well-being and/or reduction of risk (not prevention) of disease.

4. Finally, the functional food must be able to be consumed as part of a normal food pattern.

From a practical point of view, a functional food can thus be a natural food or a food to which a component has been added, removed or modified, a food in which the bioavailability of one or more components has been modified, or any combination of these. The design and development of functional foods should rely on basic scientific knowledge of relevant target functions and their possible modulation by food components. This is because functional foods are not universal and the food based approach used in their development may thus be biased by local traditions or

beliefs, whereas the science based (function driven) approach is more universal.

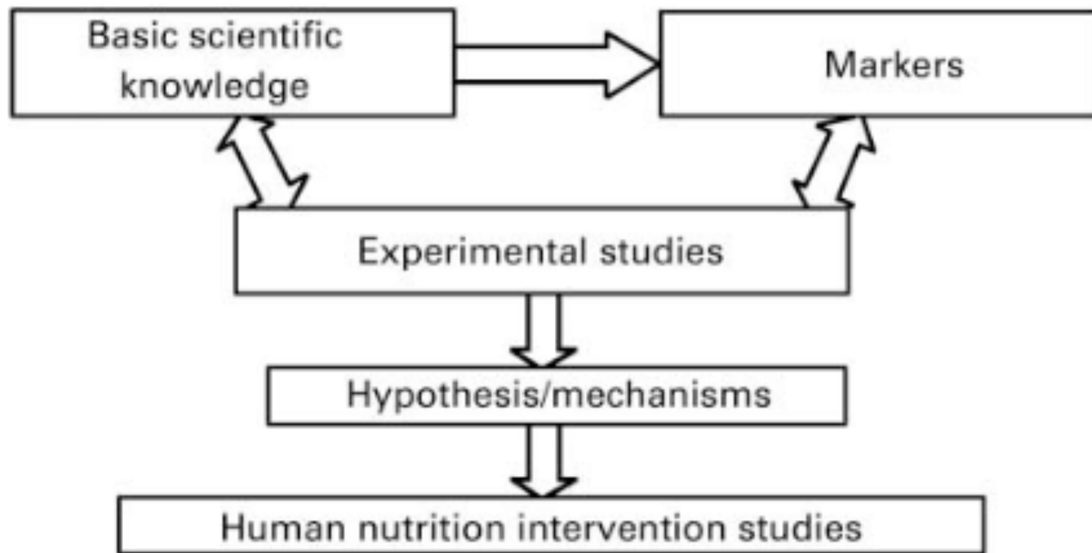


Figure 1.1: Schematic representation of the strategy for functional food discovery and development (Roberfroid, 2002).

From Figure 1.1, the basic knowledge of nutrition and related biological sciences serves in the understanding of the mechanisms by which a potential functional food or functional food component can modulate the target functions that have been proven to be relevant to the state of well-being and health, and/or the reduction of risk of adisease. A literature review is necessary to identify, define and characterize potential markers, given that the development of functional foods will, in most cases, rely on measurement of markers. The basic scientific knowledge underpinning these markers should also be evaluated and their relevance to physiological functions, well-being and health, and eventual disease risk should be assessed. Validation will then be necessary for both the methodology and the biological relevance. Experimental studies providing epidemiological data demonstrating a statistically validated and biologically relevant relationship between the intake of specific food components and particular health benefits will, if available, be very useful. All these should lead to the demonstration of a functional effect to be tested in a new generation of hypothesis driven human nutrition studies aimed to show that relevant (in terms of dose, frequency, duration, etc.) intake of the specified food will be associated with improvement in one or more target functions, either directly or indirectly, in terms of valid markers of an improved state of well-being and health and/or

reduced disease risk. Clinical studies conducted with a (double) blind design based on parallel groups, as opposed to crossing groups, will generally be appropriate. The long-term consequences of the interactions between functional foods and body functions will have to be monitored carefully.

As the market for functional foods grows, the question of how to communicate the specific advantages of these foods becomes increasingly important. The communication of health benefits to the public, through intermediaries such as health professionals, educators, the media, and the food industry, is an essential element in improving public health and in the development of functional foods. Communication remains a major challenge for functional foods, given that:

- science must remain the sole driving force, claims must be based on sound, objective and appropriate evidence, and
- evidence must be consistent, able to meet established scientific standards, and plausible.

Moreover, communication in nutrition generally comes from multiple sources that are sometimes contradictory, creating an impression of chaos, and chaotic information often generates ignorance and easily becomes misinformation. Thus there is the need for good communication to avoid problems associated with consumer confusion over health messages. With regards to functional foods, claims associated with specific food products are the preferable means of communicating to consumers. These remain an area of extensive discussion as they must be true and not misleading, must be scientifically valid, unambiguous and clear to the consumer. According to the Codex Alimentarius, 1991, a claim is any representation which states, suggests or implies that a food has certain characteristics relating to its origin, nutritional properties, or any other quality. Various types of claims exist, and the term ‘health claim’, traditionally used to communicate the benefits of foods, is defined in different ways in different part of the world. Codex Alimentarius, 1991, has classified and defined four different categories of claim excluding the term health claim, namely:

- relating to dietary guidelines,
- relating to nutrient content,

- being comparative (reduced, less, more) and
- describing nutrient function (contains ..., that contributes to the development of . . .).

These claims refer to known nutrients and their role in growth, development and normal function, as well as to the concept of adequate nutrition.They are based on widely accepted knowledge but do not refer to particular effects over and above those expected from consuming a balanced diet. Claims for functional foods should be scientifically based, and are usually classified into types A or B. Type A involves specific beneficial effects of the consumption of foods and their constituents on physiological or psychological functions or biological activities, but do not include nutrient function claims, while type B involve the reduction of risk of a disease, related to the consumption of a food or a food constituent in the context of the daily diet that might help reduce the risk of a specific disease or condition. Still, the main issue with these two claims concerns the ability to scientifically demonstrate the enhanced physiological function or the reduced disease risk, this can however be achieved using biological observations, epidemiological studies and intervention studies.

In Europe, regulation 1924/2006 (Regulation (EC) No 1924/2006 of the European parliament and of the council) entered into force in July 2007, and applies to any food or drink product produced for human consumption that is marketed on the EU market. It involves the EU conditions for the use of nutrition claims (such as low fat or high in vitamin C), and health claims (such as helps lower cholesterol). In 2012, a list of approved functional health claims, which comprises generic claims for substances other than botanicals which will be evaluated at a later date, went into effect. Disease risk reduction claims and claims referring to the health and development of children require authorization on a case-by-case basis, following the submission of a scientific dossier to EFSA (Regulation (EC) No 432/2012 of the European commission).

1.2 Food supplements

An adequate and varied diet could, under normal circumstances and in quantities which meet those established and recommended by generally acceptable scientific data, provide all the necessary nutrients for normal development and maintenance of a healthy life. However, surveys show that this ideal situation is not being achieved for all

nutrients and by all population groups across the European Community, so due to their particular lifestyles or for other reasons, consumers may choose to supplement their intake of certain nutrients through food supplements. There is an increasing number of products marketed in the European Community as foods containing concentrated sources of nutrients, presented to supplement the intake of those nutrients in the normal diet. These products are regulated in Member States by differing national rules that may impede their free movement, create unequal conditions of competition, and thus have a direct impact on the functioning of the internal market. It has then therefore been necessary to adopt Community rules on these products marketed as foodstuffs. So on the 10th of June 2002, the DIRECTIVE 2002/46/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL on the approximation of the laws of the Member States relating to food supplements, was adopted. According to this directive, food supplements are foodstuffs whose purpose is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities.

In this regulation, the term nutrients refers to vitamins and minerals, and these nutrients must be on annex I (list of vitamins and minerals admitted) or II (list of vitamin and mineral substances admitted) of the directive in order to be used in food supplements. Nevertheless, until 31 December 2009 a derogation from paragraph 1 of article 5 of the directive enabled nutrients not found in annex I or II to be used in making food supplements, provided said nutrient was in use in one or more food supplements marketed in the Community on the date of entrance in force of the directive, and also given that the European Food Safety Authority (EFSA) has not given an unfavorable opinion of the use of that nutrient, or its use in that form, in the manufacture of food supplements, on the basis of a dossier supporting the use of the substance in question to be submitted to the Commission by the Member State no later than 12 July 2005. Today nutrients not found on the list can be approved upon the submission of an appropriate scientific dossier to the EFSA for the evaluation of safety and bioavailability issues. Companies can submit dossiers to the European commission with the help of a guidance document available. This directive on food supplements also takes into account:

- Maximum and minimum levels of nutrients for the protection of the consumers

- Directives for labeling of food supplements
- Purity criteria of nutrients
- Notification to competent authorities

However, this regulation doesn't take into account other substances with physiological or nutritional effects. These substances usually include botanicals, fibres, amino acids, essential fatty acids etc… Nowadays, there is a great interest in botanicals and therefore many food supplements made from plants, algae, fungi or lichens are becoming widely available on the EU market. Despite this, there is no EU regulation specifically targeting botanicals. Food supplements made with botanicals are usually subject to the food legislation regulation 178/2002 (Regulation (EC) No 178/2001 of the European parliament and of the council) and the EFSA is currently discussing which types of botanical ingredients should be permitted, how their safety should be assessed, and also which health claims should be permitted and on which levels and types of evidence they should be based. Currently, specific national regulations are adopted and used at a member State level, and such products are marketed in EU countries other than those of origin on a mutual recognition basis.

To ease harmonisation, 3 (Italy, France and Belgium) of the top 10 countries by food supplement market size in the EU started the BELFRIT project to come up with common standards for botanicals. They came up with a common list that groups about 1000 herbal substances, which were assessed and approved by a scientific committee. Even though the BELFRIT is not legally binding to any EU country, it is likely to cause positive effects toward harmonization.

1.3 Novel foods

According to the regulation (EC) no. 258/97 of the European parliament and Council concerning novel foods and novel food ingredients, novel foods are defined as foods without a significant history of human consumption before the 15th of may 1997, the date in which the regulation came into force. According to article 1 of the same regulation, the foods concerned are:

- foods and food ingredients containing or consisting of genetically modified organisms within the meaning

of the Council Directive 90/220/EEC.

- foods and food ingredients produced from, but not containing, genetically modified organisms.
- foods and food ingredients with a new or intentionally modified primary molecular structure.
- foods and food ingredients consisting of or isolated from micro-organisms, fungi or algae.
- foods and food ingredients consisting of or isolated from plants and food ingredients isolated from animals, except for foods and food ingredients obtained by traditional propagating or breeding practices and having a history of safe food use.
- foods and food ingredients subjected to a production process not currently used, where that process gives rise to significant changes in the composition or structure of the foods or food ingredients which affect their nutritional value, metabolism or level of undesirable substances.

This regulation presents the procedure to follow to deposit a dossier for authorization of a novel food, with the aim to ensure that food approved as a novel food or novel food ingredient in the EU community doesn't present a danger for the consumer, mislead the consumer, or differ from foods or food ingredients which they are intended to replace to such an extent that their normal consumption would be nutritionally disadvantageous for the consumer.

On January 2018, the new regulation (EU) No. 2015/2283 came into force, it replaces the previous regulations (EC) No. 258/97 and (EU) No. 1852/2001 which were in force up to the 31th of December 2017. This new regulation facilitates procedures to bring new novel foods into the European market while maintaining a high level of safety for the consumers. This new regulation brings important points which include;

- A centralized and simplified procedure to request the authorization of novel foods managed by the European commission using an online process.
- A centralized safety evaluation of novel foods by the EFSA.
- A simplified assessment procedure for foods from third countries which are novel foods in Europe but

which have a history of significant human consumption in the third country, where there are no safety issues raised by the EFSA or member states.

- Promoting innovation by granting an individual authorisation for five years based on protected data.
- The improvement of efficiency and transparency by establishing deadlines for the safety evaluation and authorisation procedure in order to reduce the overall time spent on approvals.
- The establishment of a positive list of all approved novel foods
- The expansion of the categories of novel foods to foods originating from plants, animals, microorganisms, cell cultures, minerals, specific categories of foods like insects, vitamins, minerals, food supplements, to foods resulting from production processes and practices, and state of the art technologies (e.g. intentionally modified or new molecular structures, nanomaterials), which were not produced or used before 1997 and thus may be considered to be novel foods.
- Generic authorisations of Novel Foods, this means any food business operator can place an authorised Novel Food on the EU market, provided the authorised conditions of use, labelling requirements, and specifications are respected.

CHAPTER II

METABOLIC SYNDROME, DIABETES, OBESITY AND HYPERTENSION

2. Metabolic syndrome, diabetes, obesity and hypertension

2.1 The metabolic syndrome

The prevalence of the metabolic syndrome in the world ranges from less than 10% to as much as 84%, depending on the region, urban or rural area, the composition of the population (sex, age, race and ethnicity) and the definition employed (Desroches et al., 2007; Kolovou et al., 2007). In any case, the International Diabetes Federation estimates that one-quarter of the world adult population has the metabolic syndrome.

There have been several definitions of metabolic syndrome, but today the most commonly used criteria are given by the World Health Organization (Alberti et al., 1998), the European Group for the study of Insulin Resistance (Balkau et al., 1999), the National Cholesterol Education Programme Adult Treatment Panel III (Cleeman et al., 2001), the American Association of Clinical Endocrinologist (Einhorn et al., 2003) and the International Diabetes Federation (International Diabetes Federation). All these definitions agree on certain essential components used in the diagnosis of this syndrome which include obesity, dyslipidemia, hypertension and glucose intolerance, but differ in the details and criteria of each parameter (Eckel et al., 2005). The metabolic syndrome is a state of low grade inflammation as a consequence of the complex interplay between genetic and environmental factors which result in profound systemic effects like stroke, coronary heart disease, myocardial infarction, cancers and others, which may lead to death. (Kaur et al., 2014). The several factors that constitute the syndrome include; Insulin resistance, visceral adiposity, atherogenic dyslipidemia, endothelial dysfunction, genetic susceptibility, elevated blood pressure, hypercoagulable state, and chronic stress.

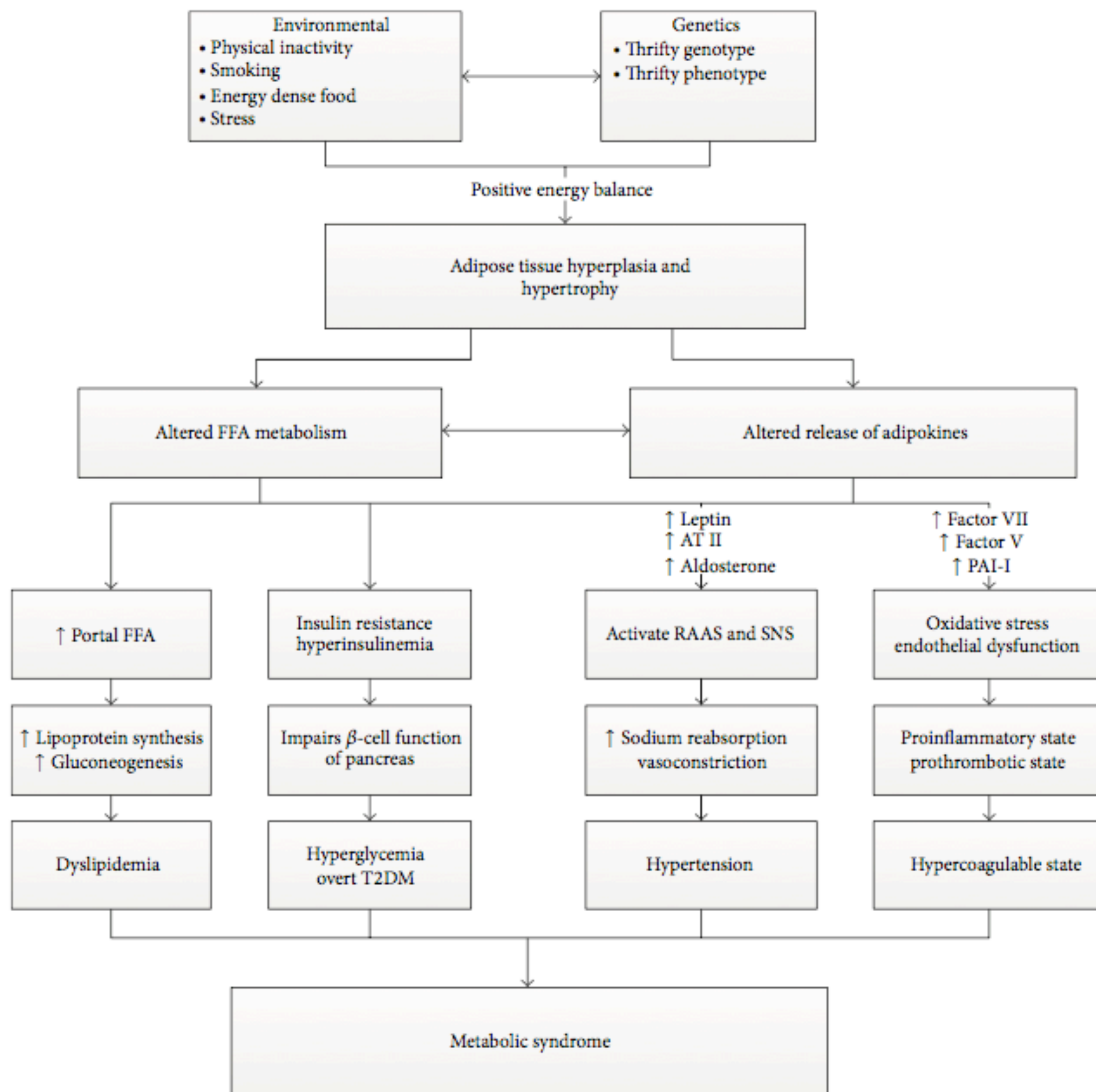


Figure 2.1: Schematic presentation of the metabolic syndrome. Abbreviations: FFA: free fatty acid, ATII: angiotensin II, PAI-1: plasminogen activator inhibitor-1, RAAS: renin angiotensin aldosterone system, SNS: sympathetic nervous system (Kaur et al., 2014).

On the figure above, the physiopathological mechanisms that lead to metabolic syndrome are described. Economic development and industrialization in recent decades has led to rapid growth of highly refined, transformed and energy dense foods, physical inactivity, smoking and consequent events such as stress. All these factors interact with the genetic background of individuals and trigger a cascade of metabolic pathways that lead to metabolic syndrome.

2.2 Obesity

Obesity is known to be a major public health problem that affects more than 1.9 billion adults, which means 39% of adults are considered obese and overweight (WHO, Geneva 2015). Estimations from the noncommunicable diseases (NCD) collaboration report that if these trends continue, by 2025 global obesity prevalence will reach 18% in men and surpass 21% in women; severe obesity will surpass 6% in men and 9% in women (NCD-RisC, 2016). Once considered a problem only in high income countries, overweight and obesity are now dramatically on the rise in low- and middle-income countries, particularly in urban areas. According to the World Health Organization (WHO), overweight and obesity are defined as abnormal or excessive fat accumulation that presents a risk to health. A person with a BMI of 30 or more is generally considered obese. A person with a BMI equal to or more than 25 is considered overweight. Overweight and obesity are major risk factors for a number of chronic diseases, including diabetes, cardiovascular diseases and cancer (Nabavi et al., 2015). Even though genetic factors contribute to this disease, overweight and obesity are usually a result of an impaired relationship between energy intake and energy consumption, this is a consequence of physical inactivity and the increased number of fast foods which render unhealthy food more available.

Adipose tissue, particularly abdominal adipose tissue, responds rapidly and dynamically to alterations in nutrient excess through adipocyte hypertrophy and hyperplasia (Ross et al., 2004). Adipocyte enlargement reduces blood supply to adipocytes, with consequent hypoxia (Cinti et al., 2005) triggering necrosis and macrophage infiltration into adipose tissue that leads to an overproduction of adipocytokines which include glycerol, free fatty acids (FFA), proinflammatory mediators like tumor necrosis factor alpha (TNFα) and interleukin-6 (IL-6), plasminogen activator inhibitor-1 (PAI-1), and C-reactive protein (CRP) (Lau et al., 2005). All these result in a local inflammation in adipose tissue that propagates to an overall systemic inflammation associated with the development of obesity related comorbidities (Trayhurn et al., 2004). Adipocytokines integrate the endocrine, autocrine, and paracrine signals to mediate multiple processes including insulin sensitivity (Saleem et al., 2009), oxidative stress (Tsimikas et al., 2009), energy metabolism, blood coagulation, and inflammatory responses (.Jacobs et al., 2009) which are thought to accelerate atherosclerosis, plaque rupture, and atherothrombosis.

FFA are often generated by upper body adipose tissue, An acute exposure of skeletal muscle to high levels of FFA induces insulin resistance by inhibiting insulin-mediated glucose uptake, while a chronic exposure of the pancreas to high FFA levels impairs pancreatic β-cell function (Boden et al., 2001). FFAs also increase fibrinogen and PAI-1 production (Kahn et al., 2001). Like FFA, TNF-alpha released by adipocytes also reduces their insulin sensitivity (Lau et al., 2005), it triggers apoptosis in adipocytes (Xydakis et al., 2004) and stimulates insulin resistance by inhibiting the insulin receptor substrate 1 signaling pathway (Hotamisligil et al 1996). TNFα is positively associated with body weight, WC (waist circumference), and triglycerides (TGs), whereas a negative association exists between plasma TNFα and High density lipoprotein–cholesterol (HDL-C) (Xydakis et al., 2004).

IL-6 is a systemic adipokine, which not only impairs insulin sensitivity but is also a major determinant of the hepatic production of CRP. IL-6 is capable of suppressing lipoprotein lipase activity. It has been shown to be positively associated with BMI, fasting insulin, and the development of T2DM and negatively associated HDL-C (Zuliani et al., 2007). Regardless of the presence or degree of metabolic syndrome in an individual, CRP levels independently predict the occurrence of future CVD events (Ridker et al., 2003). Because metabolic syndrome has been linked with a greater chance of future CVD events (Clearfield et al 2003), CRP levels may be an important independent predictor of unfavourable outcomes in metabolic syndrome. Elevated levels of CRP are associated with an increased WC (González et al., 2006), insulin resistance (Deepa et al., 2006), BMI (Guldiken et al 2007), and hyperglycemia (González et al., 2006) and increase with the number of the metabolic syndrome components. They are more likely to be elevated in obese insulin-resistant than in obese insulin-sensitive subjects (McLaughlin et al 2002). PAI-1 is a serine protease inhibitor secreted from intra-abdominal adipocytes, platelets, and the vascular endothelium (Lau et al., 2005). It inhibits the tissue plasminogen activator (Alessi et al., 2006) and thus is considered to be a marker of an impaired fibrinolysis and atherothrombosis. Plasma PAI-1 levels are increased in abdominally obese subjects and inflammatory states (Alessi et al., 2004), thus increasing the risk of an intravascular thrombus and adverse cardiovascular outcomes.

Adiponectin, also referred to as GBP-28, apM1, AdipoQ and Acrp30, is a protein hormone that regulates lipid and glucose metabolism, increases insulin sensitivity, regulates food intake and body weight, and protects against chronic inflammation. It inhibits the intrinsic production of glucose at the level of the liver, increases the transport of

glucose in muscles and promotes the oxidation of fatty acids (Eckel et al., 2005). Adiponectin also protects against atherosclerotic plaques by inhibiting endothelial activation, the transformation of macrophages to foam cells and arterial remodeling (Matsuzawa et al., 2004). Adiponectin is inversely associated with CVD risk factors such as blood pressure, low density lipoprotein cholesterol and triglycerides (Kazumi et al., 2002). Hypoadiponectinemia is associated with insulin resistance, hyperinsulinemia, and the possibility of developing type II diabetes mellitus (T2DM), independent of fat mass (Fumeron et al., 2004). Adiponectin is thus negatively associated with body weight, WC, TGs, fasting insulin, insulin resistance, body mass index (BMI), and blood pressure, whereas a positive association exists between adiponectin and HDL-C (Xykadis et al., 2004, Lee et al., 2007). The expression and secretion of adiponectin are reduced by TNFα (Maeda et al., 2001), possibly through stimulated production of IL-6 which also inhibits adiponectin secretion (Fasshauer et al., 2003). Adiponectin is however seen to be protective through its antagonism of TNFα action (Ouchi et al., 2000).

Leptin, the hormone of energy expenditure, is mainly secreted by adipose tissues. It helps to regulate energy balance by inhibiting hunger. Leptin is opposed by the action of the hormone ghrelin, the hunger hormone. Leptin levels in plasma increase during the development of obesity and decline during weight loss. Leptin receptors are mostly located in the hypothalamus and the brain stem and signals through these receptors control satiety, energy expenditure, and neuroendocrine function. Most overweight and obese individuals have an elevated level of leptin that does not suppress appetite, they are resistant to leptin and it is thought that leptin resistance is fundamental in the pathology of obesity (Hutley et al., 2005). The concentration of plasma leptin is correlated with adiposity, and hyperleptinemia is indeed considered to be an independent cardiovascular disease risk factor (Considine et al., 1996). In addition to its effects on appetite and metabolism, leptin acts in the hypothalamus to increase the blood pressure through activation of the sympathetic nervous system (Carlyle et al., 2002). Leptin is a nitric oxide (NO) dependent vasodilator, but also increases peripheral vascular resistance and sympathetic nerve activity (Shirasaka et al., 2003).

2.3 Diabetes mellitus

Diabetes mellitus is a group of metabolic diseases characterized by chronic hyperglycemia deriving from defects in the secretion of insulin, resistance to insulin or both. Some patients remain asymptomatic, particularly those with type II diabetes mellitus during the first years of the disease, others manifest marked hyperglycemia, particularly

children with absolute deficiency of insulin which can suffer from polyuria, polydipsia, polyphagia, lost of weight and blurred vision. Uncontrolled diabetes can lead to stupor, coma and, if untreated, to death due to ketoacidosis (American Diabetes Association 2014; Craig et al., 2009; Galtier et al., 2010). According to the International Diabetes Federation 2015, about 415 million of people in the world are affected by diabetes, 60 million in Europe and 3.5 million in Italy (IDF, 2015). According to the WHO, 1.5 million worldwide deaths are attributed to diabetes every year, with 90% of subjects with this pathology suffering from diabetes mellitus type II (http://www.who.int/diabetes/en/). The Middle East and North African regions have the highest prevalence of diabetes, however, the Western Pacific region has the highest number of adults diagnosed with diabetes and the countries with the highest prevalence. Low- and middle- income countries encompass 80% of cases "where the epidemic is gathering pace at alarming rates". Despite the fact that adult diabetes patients are mainly type 2 patients, it is not clear whether the million adults reported as being diagnosed with diabetes also include type 1 diabetes patients (IDF, 2015). The American Diabetes Association (ADA) classifies diabetes mellitus into type I, type II, others types and gestational diabetes (ADA, 1997). While classification of diabetes is important and has implications for treatment strategies, this is not an easy task and many patients do not easily fit into a single class, especially younger adults, and 10% of those initially classified may require revision.

Type 1 diabetes is characterized by an absolute insulin deficiency caused by the destruction of the pancreatic β cells, this destruction is usually linked to an auto-immune attack by auto-reactive T lymphocytes (T-cell mediated inflammatory response (insulitis) as well as a humoral B cell response). This kind of diabetes usually begins during childhood, manifests during puberty and progresses with age, for this reason it is also known as juvenile diabetes mellitus. In fact, 5 – 10 % of diabetic patients suffer from this typology (Kharroubi et al., 2015). Type 1 diabetes can manifest suddenly, over a period of time that goes from a few days to some weeks, the autoimmune attack on pancreatic β cells starts many years before the onset of the symptoms. In fact, the pancreatic autoantibodies which are characteristic of type 1 diabetes can be detected in the serum of these patients months or years before the onset of the disease. These auto-antibodies include islet cell autoantibodies and autoantibodies to insulin (IAA), glutamic acid decarboxylase (GAD, GAD65), protein tyrosine phosphatase (IA2 and IA2β) and zinc transporter protein (ZnT8A). Classical signs such as hyperglycemia and ketosis are usually late and manifest only after 90% of pancreatic β cells have been destroyed. Others symptoms include polyuria, polydipsia, and polyphagia. The basic

immunologic defect comes from the loss of immunologic tolerance of lymphocyte T, this can be caused by various factors which include; defects in the clonal deletion of auto-reactive T lymphocytes at the level of the thymus, functional defects of regulatory T lymphocytes, resistance of T lymphocytes to the suppressive effects of the regulatory cells. Autoimmune type 1 diabetes has strong HLA associations, with linkage to *DR* and *DQ* genes. HLA-DR/DQ alleles can be either predisposing or protective. In addition to a genetic predisposition, more and more studies are making an association between environmental factors such as viruses, vitamin D deficiency and others in the pathogenesis of this disease.

Diabetes mellitus type II is caused by a peripheral resistance to insulin linked to an inadequate compensatory insulin secretion response by pancreatic β cells. Diabetes II patients can present resistance to insulin or deficiency of β cells with a mild or severe symptomatology. In addition to insulin resistance, the increased demand for insulin is not met by the pancreatic β cells due to defects in the function of these cells. On the contrary, insulin secretion decreases with the increased demand for insulin over time due to the gradual destruction of β cells, which leads to certain type 2 diabetes patients progressing from being independent from insulin towards becoming dependent on insulin. Most type 2 diabetes patients are not dependent on insulin, where insulin secretion continues and insulin depletion rarely occurs. Dependence on insulin is one of the major differences from type 1 diabetes. Even if there were adequate insulin production by pancreatic cells, the quantity would still be inadequate to overcome insulin resistance and so glycaemia would rise regardless. Other differences include the absence of ketoacidosis in most patients of type 2 diabetes, and the lack of autoimmune destruction of β cells (Kharroubi et al., 2015). The reduced insulin activity also leads to the reduction of lipid metabolism, with a consequent increase in free fatty acids, triglycerides and a reduction of high density lipoproteins (Katzung et al., 2011). Diabetes mellitus type II is much more common than type I, in fact, 90% of diabetic patients are affected by this type (Kharroubi et al., 2015), it develops slowly, usually manifesting after the age of 40, most commonly between 50 and 60 years of age, and for this reason it is defined as adult diabetes (Kumar et al., 2013). Due to its mild symptomatology, diagnosis is usually delayed, this is mostly the case in low and middle income countries where check-ups without symptoms are not a part of the culture. This disease is complex and multifactorial, some of the factors involved in the pathogenesis of this disease include; environmental factors, physical inactivity, diet and genetic predisposition. Both type 1 and type 2 diabetes have genetic predisposition, this is stronger in type 2 but the genes are more characterized in type 1 (the *TCF7L2* gene is strongly associated with type 2 diabetes) (Kharroubi et al., 2015). In all cases abdominal obesity is commonly

involved in the development of this disease, because the risk of diabetes increases with increasing body mass index (Kumar et al., 2013).

The binding of insulin to its receptor results in a tyrosine phosphorylation of downstream substrates and activation of two parallel pathways which are: the phosphoinositide 3-kinase (PI3K) pathway and the mitogen activated protein (MAP) kinase pathway. The PI3K-Akt pathway is affected during insulin resistance, while the MAP kinase pathway functions normally. This leads to a change in the balance between these two parallel pathways. Inhibition of the PI3K-Akt pathway leads to a reduction in endothelial NO production, resulting in endothelial dysfunction and a reduction in GLUT4 translocation, leading to decreased skeletal muscle and fat glucose uptake. By contrast, the MAP kinase pathway is unaffected so there is continued endothelin-1 (ET- 1) production, an expression of vascular cell adhesion molecules, and a mitogenic stimulus to vascular smooth muscle cells. In these ways, insulin resistance leads to the vascular abnormalities that predispose atherosclerosis. While insulin-resistant individuals need not be clinically obese, they nevertheless commonly have an abnormal fat distribution that is characterized by predominant upper body fat. Regardless of the relative contributions of visceral fat and abdominal subcutaneous fat to insulin resistance, a pattern of abdominal (or upper body) obesity correlates more strongly with insulin resistance and the MetS than lower body obesity (Jensen et al., 1989).

Other types of diabetes which do not fall under the objectives of this thesis include monogenic diabetes, disease of the exocrine pancreas, diabetes caused by hormones and drugs, diabetes associated with genetic syndromes and gestational diabetes.

2.3.1 Complications of diabetes

Diabetes can give rise to acute or chronic complications. Acute complications are mostly common in diabetes type I and are due to an almost complete lack of insulin. In these cases the patient may undergo ketoacidosic coma due to the accumulation of products of the altered metabolism, namely ketones, which may cause loss of consciousness, dehydration and serious blood changes. In type II diabetes, acute complications are quite rare, while chronic complications affecting various organs and tissues, including the eyes, kidneys, heart, blood vessels and peripheral nerves are very common.

Long term complications of the different types of diabetes are similar. The pathogenesis of these complications are

multifactorial but the principal cause seems to be chronic hyperglycemia. Advanced Glycation End Products (AGE) result from non enzymatic reactions between intracellular precursors derived from glucose and amminic groups of intrecellular and extracellular proteins. The formation of AGE is accelerated by hyperglycemia, AGE bind to specific receptors known as RAGE expressed by inflammation cells (macrophages and lymphocytes), endothelium and smooth muscle cells of vessels, mediating the following effects; the release of pro-inflammatory cytokines and macrophage growth factors, generation of reactive oxygen species (ROS) in endothelial cells, stimulation of the pro-coagulant activity of endothelial cells and macrophages, and finally stimulation of the proliferation of vessel smooth muscle cells and the synthesis of the extracellular matrix. In addition to the receptor mediated activity of AGE, these also form direct cross linked bonding with proteins of the extracellular matrix, thus reducing the elimination of these proteins and promoting their accumulation. The complications of diabetes regard different organs including the eyes, kidneys, heart, blood vessels and peripheral nerves. The long term complications of these usually include diabetic microngiopathy and diabetic macroangiopathy.

In diabetic macroangiopathy, diabetes severely affects the circulatory system. This is typically indicated by early atherosclerosis of the aorta and medium-large vessels. In fact, proteins related to AGE can trap other plasma or interstitial proteins such as LDLs that remain trapped in the wall of large vessels damaged by AGE, accelerating atherosclerosis. Myocardial infarction secondary to this vascular degeneration, especially coronary infarction, is the leading cause of death in diabetics.

In diabetic microangiopathy, albumin, another plasma protein, remains trapped in the capillaries contributing to the thickening of the basal membrane which is typical of diabetic microangiopathy. This especially occurs in skin capillaries, skeletal muscles, retina, renal glomerulus, peripheral nerves and placenta. The various issues that microangiopathy can cause include;

- Retinopathy, where there is damage to the small blood vessels that irrigate the retina leading to reduced vision or even blindness, which are the most fearful complications. In this disorder, intracellular hyperglycemia activates PKC (protein kinase C), the effects of its activation are numerous and include the production of angiogenic factors, such as the endothelial growth factor (VEGF), involved in the angiogenesis of diabetic retininopathy (Kumar et al., 2013).
- Diabetic nephropathy, where the kidney is the main target of diabetes. Renal damage includes glomerular and nephrovascular lesions and pyelonephritis. Here there is a progressive reduction in the function of the kidney

filter which, if left untreated, can lead to renal failure potentially requiring dialysis and / or kidney transplantation (Kumar et al., 2013).

- In diabetic neuropathy, the nervous system is affected both centrally and peripherally. Hyperglycaemia appears to be the main cause of diabetic neuropathy (Kumar et al., 2013). It is one of the most frequent complications and, according to the World Health Organization, manifests itself at different levels in 50% of diabetics. It may cause loss of sensitivity, pain of different intensities and damage to the limbs, with the need for amputation in the most severe cases. It can lead to dysfunctions of the heart, eyes and stomach and is a major cause of male impotence.
- In the diabetic foot, changes in the structure of blood vessels and nerves can cause ulceration and problems in the lower limbs, with the foot being particularly affected due to the loads it bears. This may necessitate amputation of the limbs and statistically constitutes the first cause of amputation of the lower limbs of non-traumatic origin.
- Defects in the polyol pathway: in some tissues independent of insulin for glucose uptake (example; peripheral nerves, crystalline, kidneys and sangiovese vessels), hyperglycaemia leads to an accumulation of intracellular glucose which, is metabolized by aldose-reductase into sorbitol through an enzymatic reaction that uses NADPH as cofactor. This is also the cofactor of glutathione-reductase which regenerates reduced glutathione (GSH). GSH is one of the main cellular antioxidant mechanisms and its depletion exposes the cell to oxidative stress (Kumar et al., 2013).

Dyslipidemia can be a complication of insulin resistance, which occurs in several ways. An increase in lipolysis leads to the production of FFA, these FFA serve as a substrate for the synthesis of triglycerides and the stabilization of the production of apoB, the major lipoprotein of very low density lipoprotein (VLDL). Insulin also regulates the activity of lipoprotein lipase, the rate-limiting and major mediator of VLDL clearance. Thus, hypertriglyceridemia in insulin resistance is the result of both an increase in VLDL production and a decrease in VLDL clearance. VLDL is metabolized to remnant lipoproteins and small dense LDL, both of which can promote the formation of atheroma. The TGs (triglycerides) in VLDL (very low density lipoproteins) are transferred to HDL (high density lipoproteins) by the cholesterol ester transport protein (CETP) in exchange for cholesteryl esters, resulting in TG-enriched HDL and cholesteryl ester- enriched VLDL particles. Furthermore, the TG-enriched HDL is a better substrate for hepatic lipase, so it is cleared rapidly from the circulation, leaving fewer HDL particles to participate in reverse cholesterol

transport from the vasculature. Thus, FFA flux is high in the liver of insulin-resistant patients, TGs synthesis and storage are increased, and excess TG is secreted as VLDL (Lewis et al., 1996). It is believed that the dyslipidemia associated with insulin resistance is a direct consequence of increased VLDL secretion by the liver (Ginsberg et al., 2005).

2.4 Hypertension

According to the report “Global brief on hypertension” published by the world health organization in 2013, hypertension, also known as high or raised blood pressure, is a condition in which blood vessels have persistently raised pressure. Normal adult blood pressure is defined as a systolic blood pressure of 120 mm Hg and a diastolic blood pressure of 80 mm Hg. However, the cardiovascular benefits of normal blood pressure extend to a lower systolic of 105 mm Hg and a lower diastolic blood pressure level of 60 mm Hg. Hypertension is defined as a systolic blood pressure equal to or above 140 mm Hg and/or a diastolic blood pressure equal to or above 90 mm Hg. Normal levels of both systolic and diastolic blood pressure are particularly important for the efficient function of vital organs such as the heart, brain and kidneys and for overall health and wellbeing.

The higher the pressure in the blood vessels the harder the heart has to work to overcome said pressure in order to pump blood. If this pressure is left uncontrolled, hypertension can lead to a heart attack, an enlargement of the heart and eventually heart failure. Blood vessels may develop bulges (aneurysms) and weak spots due to high pressure, making them more likely to clog and burst. The pressure in the blood vessels can also cause blood to leak out into the brain and this can cause a stroke. Hypertension can also lead to kidney failure, blindness, rupture of blood vessels and cognitive impairment (WHO, 2013).

Globally cardiovascular disease accounts for approximately 17 million deaths a year, nearly one third of the total. Of these deaths, complications of hypertension account for 9.4 million worldwide every year. Hypertension is responsible for at least 45% of deaths due to heart complications and 51% of deaths due to stroke. In 2008, about 40% of the global population 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, 2013). Today hypertension affects over 1.2 billion individuals worldwide and has become the most critical and expensive public health problem (Rahimi et al., 2015).

The prevalence of hypertension is highest in the African Region with 46% of adults aged 25 and above affected, while the lowest prevalence of 35% is found in the Americas. Overall, high-income countries have a lower prevalence of hypertension - 35% - than other groups at 40% (WHO, 2013). Not only is hypertension more prevalent in low- and middle-income countries, there are also more people affected, because more people live in those countries than in high-income countries. Furthermore, because of weak health systems, the number of people with hypertension who are undiagnosed, untreated and uncontrolled are also likely to be higher in low- and middle income countries compared to high-income countries. The increasing prevalence of hypertension is attributed to population growth, ageing and behavioural risk factors, such as unhealthy diets, harmful use of alcohol, lack of physical activity, excess weight and exposure to persistent stress (WHO, 2013).

The cause of this disease is identified in 10% of the cases, these are secondary hypertensions, whereas in 90% of the cases no etiology is found, these are primary or essential hypertensions. In secondary hypertension, the disease arises as a consequence of another disease. Some examples include the hyper-secretion of the adrenal gland which results in high levels of aldosterone in the case of primary hyperaldosteronisms and high levels of catecholamine in the case of functional pheochromocytomas and paragangliomas. Another secondary cause is the stenosis of the renal artery, as a renal artery stenosis of more than 60% may induce renal ischemia that creates renin-dependent hypertension, reversible by removal of the obstruction (Steichen et al., 2014). In essential hypertension, the aetiology of the disease is usually unknown, however many risk factors are often involved. These risk factors include socioeconomic factors like globalization, urbanization, ageing, income, education and housing, behavioural risk factors like unhealthy diet, tobacco use, physical inactivity, harmful use of alcohol, and metabolic disorders like diabetes, obesity and high blood lipids (WHO, 2013).

Studies suggest that both hyperglycemia and hyperinsulinemia activate the Renin angiotensin system (RAS) by increasing the expression of angiotensinogen, Angiotensin II (AT II), and the AT1 receptor, which, in concert, may contribute to the development of hypertension in patients with insulin resistance. There is also evidence that insulin resistance and hyperinsulinemia lead to SNS activation, and as a result, the kidneys increase sodium reabsorption, the heart increases cardiac output, and the arteries respond with vasoconstriction resulting in hypertension. It has been recently discovered that adipocytes also produce aldosterone in response to ATII. In this regard, the adipocyte may be considered a miniature renin-angiotensin-aldosterone system (Kaur et al., 2014).

2.5 Others factors involved in metabolic syndrome

2.5.1 Endothelial function: various factors such as oxidative stress, hyperglycemia, advanced glycation products, FFAs, inflammatory cytokines, or adipokines cause an inability of the endothelium to serve its normal physiological and protective mechanisms. Furthermore, a reduction in NO, a key regulator of endothelial homeostasis, and an increase in reactive oxygen species result in endothelial dysfunction and a proatherogenic vascular bed (Kaur et al., 2014).

2.5.2 Chronic Stress and Glucocorticoid (GC) Action: Chronic hypersecretion of stress mediators, such as cortisol, in individuals with a genetic predisposition exposed to a permissive environment, may lead to visceral fat accumulation as a result of chronic hypercortisolism, low growth hormone secretion, and hypogonadism, while GCs increase the activities of enzymes involved in fatty acid synthesis and promote the secretion of lipoproteins, induce the hepatic gluconeogenic pathway, promote the differentiation of preadipocytes to adipocytes which could lead to an increased body fat mass, inhibit insulin-stimulated amino acid uptake by adipocytes and increase lipolysis or lipid oxidation which leads to peripheral insulin resistance (Kaur et al., 2014).

2.5.3 Genetics: Great variations in the susceptibility and age of onset in individuals with a very similar risk profile suggest a major interaction between genetic and environmental factors.

2.5.4 Diet: a high dietary fat intake is associated with oxidative stress and an activation of the proinflammatory transcription factor, namely nuclear factor kappa-beta, while a diet rich in fruits and fibres has no inflammation-inducing capacity compared with a high-fat diet even if it has the same calorific content (Kaur et al., 2014).

CHAPTER III

PREVENTION AND TREATMENT OF THE METABOLIC SYNDROME

3.0 Prevention and treatment of the metabolic syndrome

Given the large number of people affected by this disease, its high prevalence and the large sums of money required for the care of patients, it is clear that prevention becomes an ideal method to slow down the progression of these diseases. The first interventions able to prevent the onset of these diseases have their cornerstone in the application of an appropriate lifestyle: regular physical activity, healthy and balanced diet, functional foods and nutritional supplements. For example, some studies have shown that intervening on an individual's lifestyle with about 150 minutes of physical activity per week, in combination with with a low-calorie diet, reduces the risk of diabetes by 58% (Mul et al., 2015). In the last two decades, evidence from prospective observational studies and clinical trials has converged to support the importance of individual nutrients, foods, and other eating habits in the prevention and management of type II diabetes (Ley et al., 2014). In a prevention framework in which food policies prohibit certain foods and educational strategies impose preventive interventions, future functional foods, with their specific effects on health and well-being, could indicate a new way of considering the relationships between food and health in everyday life (Ballali et al., 2012).

3.1 physical activity

Physical activity is defined as any bodily movement produced by the contraction of skeletal muscles in which energy expenditure is greater than at rest. This means that physical activity is not limited to sports but also includes the activities of daily life associated with work, home, transport and non-competitive leisure time (Physical activity. Contexts and effects on health. INSERM 2008). Current physical activity guidelines recommend practical, regular, and moderate regimens for exercise. The standard exercise recommendation is a daily minimum of 30 minutes of moderate-intensity physical activity. However, a preference is given to 60 minutes of moderate-intensity brisk walking to be supplemented by other activities. The latter includes multiple short, 10 to 15 minute bouts of activity such as walking breaks at work, gardening or household work, using simple exercise equipment (like treadmills), jogging, swimming, biking, golfing, team sports, and engaging in resistance training, avoiding common sedentary activities in leisure time (television watching and computer games) is also advised (Kaur et al., 2014). Physical activity plays a fundamental role in the prevention of type II diabetes in subjects at risk and, in addition to being important in improving insulin-resistance and glycemic control, it is also involved in the control of lipids, blood

pressure, co-morbidity associated with type II diabetes, cardiovascular disease and mortality, while improving quality of life (Duclos et al., 2013).

Increasing physical activity in adult patients with type II diabetes has shown a complete or partial decrease in diabetes equal to 11.5% of patients in the first year of intervention, with an additional 7% after 4 years (Mul et al., 2015). Frequent physical activity leads to numerous adjustments in skeletal muscle that allow the muscle to be more effective in generating ATP and becoming more resistant to fatigue. There are two main mechanisms of the effects of physical activity on glucose metabolism:

➢ Increases glucose transport in skeletal muscle by translocation of GLUT4 from an intracellular compartment to sarcolemma (particularly in transverse tubules). It shares this effect with insulin.

➢ Increases insulin sensitivity for glucose transport in skeletal muscle (Mul et al. 2015).

Many factors modulate energy intake and energy expenditure with varying degrees of magnitude, and these contribute to the wide variability in adiposity levels observed between individuals. In this regard, body fat itself may modulate appetite, appetite-related hormones, and food intake after exercise. Many studies have examined the appetite-related responses during and after single bouts of continuous aerobic exercise (acute exercise), with the majority of these studies performed in lean, physically active males. Overall, these studies indicate that subjective feelings of appetite are transiently suppressed during exercise performed at or greater than 60% peak oxygen uptake (VO_2 peak), a phenomenon termed exercise-induced anorexia. This acute change has been reported in response to resistance exercise, but is less marked and is not observed consistently. Appetite perceptions typically return to resting control values within 30 to 60 min of cessation of exercise. Single sessions of exercise have consistently been shown to suppress circulating concentrations of the orexigenic hormone acylated ghrelin during aerobic exercise, at intensities above 60% VO_2 peak. The effect of resistance exercise on acylated ghrelin concentrations appears less definitive, with limited evidence reporting either a suppression or no change in concentrations, in response to exercise stimulus. Simultaneously, elevations in satiety hormone concentrations including peptide tyrosine tyrosine (PYY), glucagon like peptide-1 (GLP-1), and pancreatic polypeptide (PP) have been reported during aerobic exercise bouts (acute exercise), though again, these changes appear to be less profound during

resistance forms of exercise. The chronic effects of aerobic exercise (chronic exercise) on appetite parameters is largely conflicting. Some studies have shown that subjective appetite in a fasting state is increased after aerobic exercise training, whereas others have reported no change, or even a reduction in appetite. Many studies have examined changes in two tonic appetite suppressants, leptin and insulin, in response to exercise training. These generally report reductions in leptin after aerobic and resistance exercise training, whereas the findings for insulin are more variable, with some studies demonstrating a reduction and other studies demonstrating no change in concentrations after exercise training. A small collection of studies have shown an increase in concentrations of acylated ghrelin, PYY, GLP-1, and PP, but other evidence suggests that negligible differences exist after resistance exercise training (Dorling et al., 2018).

In line with evidence in individuals who are lean, exercise-induced anorexia has been reported in studies recruiting individuals who were overweight or with obesity. Studies with these participants reported no change in subjective appetite perceptions during acute aerobic exercise at moderate and high intensities, or acute resistance exercise. In addition, overweight and obese individuals do not demonstrate increased appetite after exercise. Similar to studies in individuals who are lean, reductions in acylated ghrelin and elevations in PYY, GLP-1, and PP have been reported concurrently with a suppression of appetite during aerobic exercise in overweight/obese individuals. Studies suggest that exercise training (chronic exercise) increases fasting hunger and postprandial satiety. This was shown by a study published by King et al., 2009, which demonstrated that 12 weeks of supervised aerobic training in overweight and obese individuals augmented fasting hunger but also increased the satiety response to a fixed meal. The effect of chronic exercise on hormone secretion in obese and overweight subjects is convulsionary and highly debated (Dorling et al., 2018).

Scientific investigations that analyse exercise-induced changes in appetite, appetite-related hormones and energy intake are less abundant in women than men. The preference to study men in exercise and appetite research is likely to be related to cyclical changes in sex hormones in women that engender fluctuations in appetite, appetite-related peptides, and energy intake during different phases of the menstrual cycle. Studies on appetitive changes after exercise have, however, been performed in women, suggesting that women may compensate for the exercise-induced energy deficit over the longer term in order to preserve higher body fat stores compared to men. Also, habitual physical activity and exercise is important, as evidence from cross-sectional research has indicated that

inactive individuals have impaired energy intake regulation, which may facilitate a positive energy balance and consequential weight gain (Dorling et al., 2018).

Several studies conducted in both normotensive and hypertensive subjects provided evidence of a decrease in total peripheral resistance by moderate and regular physical activities, whereas the mechanisms underlying the antihypertensive effects of exercise are yet to be fully clarified. In any case, data suggested the improvement of endothelium dependent relaxation, endothelial adaptation, mainly mediated by a significant increase in vascular NO production and/or decrease in NO scavenging by ROS as a product of exercise-induced changes in shear stress. These data suggest that the increase in NO bioavailability, mainly through the reduction of oxidative stress, remains an important contributor to the improvement of endothelial function associated with physical exercises. Moreover, exercise has also been demonstrated to normalize levels and/or expression of pro-inflammatory cytokines that decrease NO bioavailability by stimulation of ROS production. Furthermore, active muscles are associated with the release of several cytokines and various anti-inflammatory peptides, which in turn increase NO bioavailability via decreasing ROS production. The release of NO is associated with vasodilation. In line with these data, exercise-related vasodilation was also associated with the growth of new arterioles and the reduction of sympathetic vasoconstrictor tone in existing vessels. This represents direct evidence that in humans, physical training lowers sympathetic activity and supports the involvement of neuronal cardiovascular control in the lowering of blood pressure following training. Another cardiovascular disease risk factor, particularly critical for both hypertensive and diabetes type 2 patients, is left ventricular dysfunction, namely hypertrophy. In addition, this pathology involves endothelial dysfunction and chronic low-grade inflammation with raised levels of C-reactive protein. Data shows significant reductions in left ventricular mass index in hypertensive patients undergoing exercise training and increased endothelium-dependent vasodilatation, as well as anti-inflammatory effects, in type 2-diabetes. In addition, in hypertensive patients, physical exercise has clearly shown beneficial effects on various components of the plasma lipoprotein-lipid, including a decrease in total plasma cholesterol levels, a significant reduction in plasma low density lipoprotein cholesterol (LDL-C), and the reduction of plasma triglyceride (TG) levels (Lou M et al 2017).

In view of the important positive effects of exercise on the factors involved in metabolic syndrome, any type of physical activity should be encouraged. Lifestyle activity should be increased slowly in intensity and duration (by 5

min/session/week), starting from a low-intensity exercise (<3 metabolic equivalent) in sedentary subjects, to avoid excessive fatigue, muscle pain, strains, or injuries (Diabetes Prevention Program, 2002).

3.2 Some dietary interventions to reduce the metabolic syndrome

Energy restricted diets

This surely consists of the most commonly used and studied dietary strategy for combating excess weight and related comorbidities. These diets consist in personalized regimens that supply fewer calories than the total energy expended by a specific individual. These diets result in a negative energy balance and subsequently, in reduction of body weight (Bales et al., 2013). Weight loss is achieved via fat mobilization from different body compartments as a consequence of the lipolysis process necessary to provide energy substrate (Grams et al., 2015; Lazo et al., 2010). In obese individuals following a hypocaloric diet, a depletion of plasma inflammatory markers such as interleukin (IL)-6 has been observed, this is of particular importance given that the metabolic syndrome is associated with low grade inflammation (Rossmeislova et al., 2013). Also a hypocaloric diet will improve insulin resistance (Grams et al., 2015; Golay et al., 2013), reduce LDL-C, triglycerides and blood pressure (Wing et al., 2011; Fock et al., 2013).

Diets rich in omega-3-fatty acids

The very long-chain eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are omega-3 polyunsaturated fatty acids (n-3 PUFAs) essential for human physiology. Their main dietary sources are fish and algal oils and fatty fish, but they can also be synthesized by humans from α-linolenic acid (Fleming et al., 2014). Evidence suggests that EPA and DHA have a positive role in the prevention and treatment of the pathologies associated with metabolic syndrome. They have the ability to reduce the risk of developing cardiovascular diseases and cardiometabolic abnormalities as well as cardiovascular-related mortality (Wen et al., 2014), and these beneficial effects are thought to be mainly due to the ability of these essential fatty acids to reduce plasma TG levels (Lopez-Huertas et al., 2012).

Low glycemic index diets

The glycemic index of a food is a measure of the quality of its carbohydrates, this is based on the fact that foods

with the same amount of carbohydrates can give different glycemic indexes. The higher the index, the more promptly the postprandial serum glucose rises and the more rapid the insulin response. A rapid insulin response leads to rapid hypoglycemia, which is suggested to be associated with an increase in the feeling of hunger and to a subsequent higher caloric intake (Sun et al 2016). According to some authors, metabolic syndrome is a consequence of an elevated intake of high glycemic index foods over time, among other unhealthy dietary habits (Nakagawa et al., 2006). Under this approach, a diet rich in high glycemic index carbohydrates has been associated with hyperglycemia, insulin resistance, type 2 diabetes, hypertriglyceridemia, cardiovascular diseases, and obesity (Sun et al., 2016; Downs et al., 2004; Brand-Miller et al., 2009) abnormalities directly related to metabolic syndrome.

Diets with high total antioxidant capacity (TAC)

The metabolic syndrome is linked to low grade inflammation and oxidative stress, so diets with a high total antioxidant capacity are surely useful, either for protection against metabolic syndrome or for its prevention. In the Tehran Lipid and Glucose Study it was demonstrated that a high TAC has beneficial effects on metabolic disorders, especially preventing weight and abdominal fat gain (Bahadoran et al., 2012). Many others studies support the beneficial effects of diets rich in total antioxidant capacity in patients with metabolic syndrome. The World Health Organization recommendation for fruit and vegetable consumption (high TAC foods) for the general population is a minimum of 400 g a day (WHO: Geneva, Switzerland, 2000). Moreover, cooking with spices is recommended in order to increase TAC dietary intake and, at the same time, maintain flavor while reducing salt (Tapsell et al., 2006).

Moderate to high proteins diets

The distribution set of macronutrients in a weight loss dietary plan commonly consists of 50%–55% total caloric value derived from carbohydrates, 15% from proteins and 30% from lipids (Zulet et al., 2012; Abete et al., 2010). The mechanisms linking moderate to high protein diets and protection against metabolic syndrome include; increased diet-induced thermogenesis (Ray et al., 2012) and increased satiety (Giugliano et al., 2015). The increase in thermogenesis is explained by the synthesis of peptide bonds, production of urea and gluconeogenesis, which are processes with higher energy requirements than the metabolism of lipids or carbohydrates (Koppes., et al 2009). An increase in different appetite-control hormones such as insulin, cholecystokinin or glucagon-like peptide 1 may clarify the effect on satiety (Bendtsen et al., 2013). Other beneficial effects attributed to moderate-high protein diets

in the literature are an improvement in glucose homeostasis (Heer et al., 2015), the possibility of lower blood lipids (Layman et al., 2009), the reduction of blood pressure (Pedersen et al., 2013), the preservation of lean body mass (Daly et al., 2014) and decreased risk of cardiometabolic disease (Arciero et al., 2008; Gregory et al., 2011).

High meal frequency

The inverse association, between high meal frequency and metabolic syndrome, is yet to be consolidated. The first hypothesis proposed is an increment in energy expenditure, however studies carried out in this line have concluded that total energy expenditure does not differ with different frequency of meals (Smeets et al., 2009; Taylor et al., 2001). The second postulated hypothesis is that the greater the number of meals a day, the higher the fat oxidation, but again no consensus has been achieved on this (Smeets et al., 2009; Ohkawara et al., 2013). Another suggested hypothesis is that increasing meal frequency leads to plasma glucose levels with lower oscillations and reduced insulin secretion which is thought to contribute to a better appetite control. These results are still inconstant and need to be consolidated.

The Mediterranean diet

The traditional Mediterranean diet is characterized by a high intake of extra-virgin olive oil and plant foods such as fruits, vegetables, cereals, whole grains, legumes, tree nuts, seeds and olives, low intake of sweets and red meat and moderate consumption of dairy products, fish and red wine (Davis et al., 2015). The main contributors to the positive effects attributed to the Mediterranean diet are fiber, which helps in weight control by providing satiety, and nutrients such as n-3 fatty acids, oleic acid or phenolic compounds, which are high antioxidants and anti-inflammatories (Bertoli et al., 2015). Recent meta-analysis has shown that the Mediterranean diet is associated with decreased risk of developing type 2 diabetes and with better glycemic control in people affected by metabolic disorder (Esposito et al., 2015; Schwingshackl et al., 2015; Koloverou et al., 2014).

Also, it has been found a positive correlation between the adherence to a Mediterranean diet and reduced risk in the development of cardiovascular diseases. Many studies have found a positive association between following a Mediterranean diet and improvements in lipid profile by reduction of total cholesterol, LDL-c and TG, and an increase in HDL-c (Salas-Salvado et al., 2008; Martinez-Gonzalez et al., 2011; Fito et al., 2014; Estruch et al., 2013; Serra-Majem et al., 2006). Different studies also suggest that the Mediterranean diet may be a good strategy for the

treatment of obesity, as it has been associated with significant reductions in body weight and waist circumference (Kastorini et al., 2011; Esposito et al., 2011; Razquin et al., 2009).

This is not an exhaustive list of the different diets that may prevent or improve metabolic syndrome. Many others microelements also have a role in protection against metabolic syndrome, these include vitamin C, vitamin E and polyphenols, which are discussed in the next section.

3.3 Nutraceuticals and the metabolic syndrome

Nutraceutical science is the science that studies the healthy properties of foods. This usually includes functional foods, food supplements, and others foods with healthy properties. Some nutraceutical components which can be used against the metabolic syndrome are discussed in this section;

Plant proteins

Protein enriched diets have become a popular strategy for enhanced weight management and weight loss (Westerterp-Plantenga et al., 2009). Increasing protein intake may also have potential in the prevention of type II diabetes. Despite controversy over the optimal amount and quality of proteins involved, there are strong indications that for example, a high intake of red meat may be associated with an increased risk of type II diabetes, whereas plant proteins are generally associated with some improvement in diabetes risk and in the general management of hyperlipidemia. Two types of proteins have been extensively studied and their positive effects on the metabolic syndrome consolidated, namely lupin and soy proteins. Other plant proteins, in particular pea and wheat protein, have also shown some potential benefit (Sirtori et al., 2017).

Lupin proteins are well known for their reduction of cholesterol in plasma, largely attributable to an LDL-receptor activating mechanism (Sirtori et al 2004). A study also reported that lupin proteins lead to a 12.7% reduction of proprotein convertase subtilisin/kexin type 9 in humans, the key regulator of LDL-receptors. A direct modulation of the protein-protein interaction of PCSK9 (proprotein convertase subtilisin/kexin type 9) with the LDL-R has been found (Lammi et al 2016). In animal models, lupin proteins have displayed hypolipidemic and a remarkable antiatherosclerotic effect in a similar manner to soy proteins. A reduction in TG was noted in rodent studies,

apparently associated with reduced fatty acid synthesis and increased TG hydrolysis. Moreover, conglutin, a lupin protein with modulatory effects on the insulin signaling pathway, has been shown to reduce glycemia in both animal models and, moderately, in humans, particularly in the postprandial condition. The peptides obtained after the hydrolysis of lupin and soy proteins by pepsin and trypsin have been found to have inhibitory activities against dipeptidyl peptidase IV (DPP-IV), a new molecular target correlated to the development of diabetes mellitus type II (Lammi et al 2016).

Soy proteins, proteins from *Glycine max*, represent the prototype plant proteins. In addition to providing soy proteins, G*lycine max* also provide components such as isoflavones or phytoestrogens, with potential estrogenic activity and possible antiatherosclerotic effects (Anthony et al., 1997). Isoflavones may be of potential risk for growing children in particular, but, so far, there is no clear evidence of a significant activity on the major components of metabolic syndrome. This has been proven by an experiment in an animal model, where the careful extraction of isoflavones from soy did not change their lipid lowering and LDL-R stimulating activity (Fukui et al., 2002). There is clear evidence that soy proteins alone can raise the activity of LDL-R in different animal models and in familial hypercholesterolemic patients (Lovati et al., 1987). Decreased triglyceridemia and changes in other metabolic syndrome associated variables are less consolidated by studies. In a recent longitudinal EPIC-PANACE study (Vergnaud et al., 2013) increasing dietary plant source proteins by 5% at the expense of animal-source proteins in an isoenergetic diet, reduced weight gain by nearly 1 kg per year in men over a 5-year period, but not in women. A study specifically investigated patients with clinical features of metabolic syndrome; they received 30 g/days soy proteins in a parallel group vs animal food. A significant reduction of body weight was observed. The expected reductions of total and LDL cholesterol as well as of non-HDL-C vs milk proteins were found (Ruscica et al 2016). In post-menopausal women with metabolic syndrome, soy nuts were compared with soy proteins. The soy nut regimen reduced fasting glucose more significantly than the soy protein, in addition to an improved LDL-cholesterol reduction. A soy or lupin based beverage containing 50 g glucose significantly reduced glycemia for 4 hours post-beverage in diabetes type II patients, with no significant difference between lupin and soy. The insulin response was somewhat higher for lupin and soy compared to controls (Dove et al., 2011).

Probiotics

Studies have demonstrated that the administration of probiotics to obese subjects slightly reduced body weight,

waist circumference and body mass index (Park et al., 2015). The understanding of this complex issue comes from a meta-regression analysis reporting that the effects of probiotics on body mass index depend on the duration of intervention (greater than 8 weeks), number of species of probiotics and baseline body mass index greater than 25 Kg/m^2, thus highlighting the effectiveness of probiotics in reducing body mass index, especially in overweight or obese subjects (Zhang et al., 2015). A possible mechanism explaining these effects on weight loss is the improvement in the functioning of the intestinal barrier, which may reduce metabolic endotoxemia, thus ameliorating levels of lipopolysaccharides. In adipose tissue, endotoxins from LPS (lipopolysaccharides) trigger systemic and local inflammation with an increase in reactive oxygen species (ROS). As a possible associated event, bacterial fragments can increase the number of preadipocytes. Other proposed mechanisms linking probiotics and body weight reduction are the ability of the microbioma (i) to extract energy from the diet and (ii) to increase the secretion of the gut hormone glucagon-like peptide (GLP-1) enhancing satiety and reducing energy intake (Sartori et al 2017). Probiotics exert a hypocholesterolemic effect by reducing the intestinal absorption of cholesterol or by enhancing bile salt hydrolase activity. A lack of efficiency in modulating triglycerides and HDL-C has also been noticed (Ishimwe et al 2015). If only type 2 diabetic patients are considered, probiotic consumption reduced TG levels by -24.48 mg/dl with no HDL-C changes. HDL-C may be raised by 16 mg/dl if a longer duration of probiotic administration (>8 vs ≤ 8 weeks) is considered (Li et al., 2016).

Different studies showed that different strains of probiotics reduce both systolic and diastolic blood pressure. Interestingly blood pressure fell further when a sub-analysis was conducted taking into consideration duration of treatment ≥8 weeks, BMI ≥30 Kg/m^2, dairy source of probiotics, consumption of multiple probiotic species, daily dose consumption $\geq 10^{11}$ CFU and blood pressure at baseline ≥135/85 mmHg (Khalesi et al., 2014). The antihypertensive activity of probiotics has also been attributed to the reduction of inflammation mediated endothelial dysfunction, nitric oxide synthase down regulation, and release of peptides that inhibit the angiotensin converting enzyme (Robles-Vera et al., 2017).

Probiotics also slightly reduce fasting blood glucose even in diabetes mellitus type II patients, the extent to which they do this, as previously mentioned for blood pressure, depends on basal fasting blood glucose ≥126 mg/ml, length of intervention ≥ 8 weeks, baseline BMI ≥ $30Kg/m^2$, administration of multiple species, daily dose consumption

$\geq 10^{11}$ CFU and selected source (Nikbakht et al., 2016). The reduction of low grade inflammation promoted by gut derived LPS and metabolic endotoxemia has been proposed as a mechanism linking probiotics with metabolism of glucose (Akbari et al., 2016). Indeed, serum endotoxin levels correlate with fasting insulin (Creely et al., 2007) and are 2-fold higher in diabetes mellitus type II patients compared to non diabetics (Cani et al., 2007).

Prebiotics

Studies on the effects of prebiotic supplementation on metabolic traits such as body weight, glucose homeostasis and TG, indicates that dietary supplementation only impacts post-prandial glucose and insulin levels, leaving the other parameters unaffected. Interestingly, although self-reported, an improvement in satiety was found upon prebiotic intake. In fact, prebiotics have been related to a decrease in Ghrelin levels and to an increase in peptide YY and GLP-1 secretion, all involved in appetite regulation (Delzenne et al., 2005; Cani et al., 2009). In subjects with a BMI > 25 Kg/m^2 suffering from diabetes, evaluation of the effects of prebiotics compared to placebo showed an improvement in TG and HDL-C levels (Nikbakht E et al., 2016).

Curcumin

The multiple biological effects of curcumin include well consolidated antioxidant and anti-inflammatory properties, demonstrated by *in vitro* and *in vivo* studies. In fact, curcumin was found to increase serum total antioxidant capacity and superoxide dismutase, together with increasing glutathione concentrations and reducing lipid peroxides (Panahi et al., 2016; Panahi et al., 2017). Curcumin can also regulate cytokines, protein kinases, adhesion molecules and other enzymes (TNF-α, IL-1, IL-6, TGF-β, MCP-1, etc). Supplementation with curcuminoids has been associated with a statistically significant reduction in circulating high sensitivity (hs)-CRP levels, thus they reduce the state of inflammation (Sahebkar et al., 2014).

The main metabolic effect of curcumin seems to be related to activity as an insulin-sensitizer, demonstrated in animal and clinical studies. Some studies conducted on prediabetic patients for 9 months reported curcumin as preventing the onset of diabetes mellitus type II. These studies also showed that curcumin increases adiponectin levels while it reduces leptin levels. A shorter treatment period provided no comparable benefit on glucose homeostasis, with, however, a significant improvement of HDL-cholesterol and a reduction of TG and of non-HDL-

C.

The lipid-lowering mechanisms of curcuminoids seem to reside in their ability to increase cholesterol efflux via ABCA1 and APOA-I expression and to inhibit the expression of Niemann-Pick C1-Like 1 (NPC1L1) via the Sterol regulatory element-binding protein 2 (SREBP2) transcription factor. Curcumin also downregulates PCSK9 mRNA levels by up to 31-48% in different cell lines, thus promoting LDL-R expression on the cell surface and LDL-C uptake. The mechanism for down-regulation of PCSK9 appears to be associated with the inhibition of the HNF-1 transcription factor: this may lead to additional anti-inflammatory activity. Curcumin further demonstrated protection against atherosclerosis in LDL receptor deficient mice, by modifying peroxisome proliferator-activated receptors (PPAR)-α and γ, CETP and LPL expression, therefore affecting synthesis and catabolism of fatty acids (Sirtori et al., 2017).

Berberine

Berberine has a powerful cholesterol lowering activity in hypercholesterolemic patients. This activity is associated with an elevation in LDL-R expression, independent of the sterol regulatory element binding proteins (SRBPs), but consequent to the activation of an extra signal regulated kinase (ERK) (Kong et al., 2004). In endothelial cells, berberine has been reported to counteract pro- atherogenic and inflammatory stimuli induced by oxidized LDL and TNF-alpha, a mechanism involving the inhibition of lectin-like oxidized LDL receptor 1 and the modulation of AMPK and ERK1/2 pathways (Caliceti et al., 2017). In patients with newly diagnosed diabetes mellitus type II, berberine showed a reduction of glucose, hemoglobin A1c and TG; as well as remarkably, fasting insulin and the HOMA index (Yin et al., 2008). It also inhibited adipocyte differentiation, through the transcription factors PPAR and C/EBP, also inhibiting the full length PPAR, PPAR and their target genes involved in glucose homeostasis and lipid metabolism. In this way berberine may exert an effect on weight reduction, in addition to hypolipidemic and hypoglycemic activity (Ferri et al., 2017). Berberine has also been shown to reduce LDL-C and cholesterolemia. Findings clearly indicate that insulin sensitivity is improved by berberine by adjustment of adipokine secretion, both in primarily cultured adipocytes as well as in metabolic syndrome patients. A potential additional mechanism in improving insulin resistance is by way of inhibition of adipose tissue lipolysis. The antilipolytic activity is exerted by an increase in adipose tissue phosphodiesterase (PDE), leading to reduced cAMP and inhibited activation of the HSL (hormone sensitive lipase) (Zhou et al., 2011). Further findings have suggested that berberine can provide an

effective association with cholesterol lowering drugs. Berberine reduces the liver expression of PCSK9, a serine protease favoring degradation of the LDL-R, increased after statin therapy. The reduction of PCSK9 mRNA and protein levels are dependent on a down regulation of the hepatocyte nuclear factor (HNF-1), one of the essential cofactors for the transcriptional regulation of PCSK9 and responsible for the regulation of a variety of inflammatory conditions. Inhibited HNF1 transcription has also been reported after curcumin, as explained in its section above. Finally, berberine has a potentially significant activity in upregulating the reverse cholesterol transport when this is inhibited by the CETP inhibitor torcetrapib. In this condition, inhibited CETP in dyslipidemic hamsters does not lead to a stimulation of reverse cholesterol transport, whereas this is markedly stimulated in the presence of berberine (Sirtori et al., 2017).

Red yeast rice

Red yeast rice is produced by cultivating the *Monascus purpureus* strain of yeast on rice. The fermentation process enriches rice with bioactive components, including polyketides such as monacolins (compactin, monacolin K, M, L, J, X) that bear lipid-lowering effects as inhibitors of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase. The beneficial effects of monacolins are not limited to lowering cholesterol, they have also been found to promote endothelial protection, reduce fasting hsCRP, and improve post-prandial flow mediated dilation (FMD) in certain studies in humans (Zhao et al., 2004). These data could be possibly in line with a reduction in plasma concentrations of the matrix metalloproteinases MMP-2 and MMP-9, observed in previous clinical studies (Cicero et al., 2013). These effects could be due to an up-regulation of eNOS expression and inhibition of oxidative stress in vascular endothelia, as shown in rodents. Red yeast rice is usually used in combination, so to reduce the dose (sometimes there is the formation of a nephrotoxic mycotoxin named citrinin in the yeast, and red yeast rice also has myalgia and CK elevation as side effects) and to obtain effects beyond cholesterol reduction. For example, association with berberine, in addition to lowering total cholesterol, LDL, triglycerides, glucose, and raising HDL, also improves the leptin to adiponectin ratio, without changing adiponectin levels, and improves endothelial function and pulse wave velocity (PWV). Studies conducted on adults and the elderly demonstrated the efficacy of red yeast rice in preventing cardiovascular diseases by reducing the incidence of coronary events (Sirtori et al., 2017).

Polyphenols and the metabolic syndrome

Polyphenols are the most widely distributed secondary metabolites from plants in dietary sources. Although they are not considered essential micronutrients, a vast number of articles in the literature highlight their beneficial effects on human health, especially in diets associated with high consumption of fruits and vegetables. Polyphenols share a phenol carbon ring but have different structures, and more than 500 different polyphenol molecules have been found present in foods (Phenol-Explorer, 2017).

These present huge structural variability, which confers unique chemical, physical and biological properties, providing an explanation for their specific mechanisms of action and functional effects. Due to the presence of hydroxyl units in their structure, polyphenols are often present in nature as glycosylated, acetylated, methylated, or ester derivatives. Over 8000 polyphenols have been isolated and identified to date, and these are usually classified into different groups according to the number of phenolic rings in the structure, the binding elements between these rings and the substituent groups (Manach et al., 2004).

Polyphenols are commonly divided into two main groups: flavonoids and non-flavonoids. Flavonoids are the most abundant and most studied polyphenolic class and are characterized by a common skeleton made up by two aromatic rings, linked by three carbon atoms forming a pyran ring. Figure 3.1 below shows the common skeleton of flavonoid compounds. Depending on the degree of oxidation of the pyran ring, flavonoids are divided into six sub-classes: flavonols, flavones, flavanones, flavan-3-ols, anthocyanins and isoflavones (Daglia et al., 2012). Among the non-flavonoid components, phenolic acids are the most representative class, which includes both benzoic acids, such as gallic acid and protocatechuic acid, and cynnamic acids, such as ferulic, caffeic, and coumaric acids and their derivatives. Other important non-flavonoid components are stilbenes, of which resveratrol is the most representative, and lignans (Daglia et al., 2012).

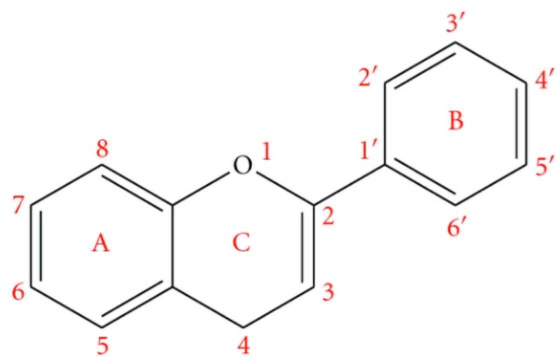


Figure 3.1: common structure of flavonoids

The consumption of polyphenols varies from one country to another and strongly depends on the type of diet consumed. The mean polyphenol intake of an adult subject is about 283–1000 mg of total polyphenols/day in France (Brat et al., 2006, Julia et al., 2016), 500–1100mg/day in Spain (Tresserra-Rimbau et al., 2016), about 700 mg/d in Italy (Godos et al., 2017, Vitale et al., 2016), 890 mg/day in Finland (Ovaskainen et al., 2008), 534 mg/day in Brazil (Nascimento-Souza et al., 2016), and around 1500 mg/day in Japan (Taguchi et al., 2015). The total flavonoid intake is about 190 mg/day in the UK and Ireland (Beking et al., 2011), about 240–350mg/day in the US (Goetz et al., 2016, Bai et al., 2014), around 450 mg/day in Australia (Johannot et al., 2006), between 50 and 500 mg/day in China (Zhang et al., 2014), and around 320 mg/day in Korea (Jun et al., 2016). These estimates are imperfect because there are a number of variables to take into account, such as the analytical method used for quantification and the approach used to determine the polyphenols. The estimation of the intake of polyphenols is performed by administering food frequency questionnaires to subjects in a study and translating this information to single or total polyphenols by using databases to calculate polyphenol intake from consumed food. The major public databases used for polyphenol content of foods and beverages are the United States Department of Agriculture (USDA) databases for flavonoids, proanthocyanidins, and isoflavones and the Phenol-Explorer database. USDA databases only presents values for flavonoids, but Phenol Explorer is a more complete database gathering information on all classes and types of polyphenols, including their metabolites (Pinto et al., 2017).

Even though polyphenol intake has the potential to alleviate metabolic syndrome components by decreasing body weight, blood pressure, and blood glucose and by improving lipid metabolism, they have a very low bioavailability of 5 to 10% in the small intestine (Chiva-Blanch et al., 2017). Many studies are now focused on improving this

bioavailability using various technologies. Polyphenols with their antioxidant and anti-inflammatory properties appear to be good dietary candidates to prevent the progression of metabolic syndrome, given that pro-oxidant status and low-grade chronic inflammation are hallmarks of metabolic syndrome (Upadhyay et al., 2015). Many studies report a negative association between polyphenol intake and metabolic syndrome, for example, in a cross-sectional study comprising of more than 8800 subjects, BMI, waist circumference, blood pressure, and triglycerides were found to be significantly lower among individuals in the higher quartiles of polyphenol intake, assessed by food frequency questionnaires. Furthermore, polyphenols have been shown to improve insulin resistance, to decrease blood pressure and body weight, and to improve lipid profile (Chiva-Blanch et al 2017).

In 2003, Wu found that regular tea consumers in a Chinese cohort showed a lower percentage of body fat and waist-to-hip ratio compared to subjects who did not consume tea on a regular basis (Wu et al., 2003) while a longitudinal analysis from a Netherlands Cohort Study has shown that an increased intake of flavones, flavonols, and catechins is associated with a lower increase in body mass index (BMI) associated with age in women but not in men (Hughes et al., 2008). While many others studies consolidate the intake of polyphenol and central obesity, weight loss induced by polyphenols is not clinically relevant in overweight and obese individuals (Farhat et al., 2017). Moreover, many of the interventional trials have had a duration of less than 3 months (Gemma Chiva-Blanch et al 2017).

As reported by Gemma Chiva-Blanch et al 2017, there are also many studies which consolidate the inverse association between polyphenol intake and insulin resistance. Some examples include the study conducted by Tresserra-Rimbau et al., in 2016, where in a Mediterranean cohort at high cardiovascular risk, total polyphenol, total flavonoid (flavanones and dihydroflavonols), and stilbene intake was associated with decreased risk of type 2 diabetes (Tresserra-Rimbau et al., 2016). Another one is the Nurses' Health Studies (NHS) I and II, where urinary excretion of flavanones (naringenin and hesperetin) and flavonols (quercetin and isorhamnetin), as well as caffeic acid, was associated with about 39%–48% decreased risk of type 2 diabetes in the middle term (5 years) but not at the long term (up to 11 years) (Sun et al., 2015).

There also exists an association between polyphenol intake and dyslipidemia. A meta-analysis has shown that green tea consumption, but not black tea (Wang et al., 2014), decreases total and LDL cholesterol (Onakpoya et al., 2014) with no effects on HDL cholesterol, though some studies have observed green tea consumption increasing HDL cholesterol levels (Imai et al., 1995). In the TOSCA.IT study with type 2 diabetic patients, high polyphenol intake

was associated with slightly lower levels of LDL and triglycerides and higher levels of HDL cholesterol (Vitale et al., 2016). In a subset cohort of the ATHENA study, total polyphenol and anthocyanin intakes were not associated with an improved lipid profile, but individuals with the serum paraoxonase/arylesterase 1 single- nucleotide polymorphisms rs854549 and rs854552 showed a positive association between HDL cholesterol levels and total polyphenol and anthocyanin intakes (Rizzi et al., 2016).

The same inverse relationship can be seen with hypertension, in fact, several observational studies have revealed a positive correlation between an increased intake of fruits and vegetables and a decreased prevalence of hypertension (Davinelli et al., 2016). In a Mediterranean population at high cardiovascular risk, the total polyphenol intake as measured by urinary polyphenol excretion was associated with lower blood pressure levels and lower prevalence of hypertension (Medina-Remón et al., 2011). Many others studies have made this association, so polyphenols, and specifically flavonoids, can be said to show potential antihypertensive effects, which may differ in relation to the status of the disease (healthy versus pre-hypertensive versus hypertensive individuals (Chiva-Blanch et al., 2017).

The epidemiological studies cited above illustrate the negative association between the polyphenol intake and metabolic syndrome or the different risk factors in involved in this syndrome. In any case, clinical trials are currently the best approach to demonstrate the effects of foods or food compounds such as polyphenols on human health.

3.4 Therapeutic approaches to the metabolic syndrome

Many pharmaceutical approaches targeting the various risk factors involved in the metabolic syndrome exist (Katzung et al 2011). These are beyond the scope of this PhD thesis.

CHAPTER IV

AIMS OF THE PhD RESEARCH

According to S. Desroches et al 2007 and G. D. Kolovou et al in 2007, as previously reported above, the prevalence of metabolic syndrome in the different regions of the world ranges from under 10% to as much as 84%, and according to the International Diabetes Federation estimates one-quarter of the world's adult population has metabolic syndrome. The best way to control and reduce the prevalence of such a highly dissipated condition whose aetiology is highly linked to life style is to improve prevention, also given that pharmacological therapy is costly and usually gives rise to many side effects. With this perspective, this research project aims to study foods consumed in Africa for which traditional uses indicate potential activity against metabolic diseases, and which not being commonly consumed in Europe fall under the category of novel foods. For this purpose, the different edible parts of *Adansonia digitata* L. (leaves, fruit pulp, raw seeds and toasted seeds), commonly known as baobab, and the red flowers of *Hibiscus sabdariffa* L. commonly known as karkadè were studied with a view to develop innovative functional foods which could have healthy properties against the risk factors involved in metabolic syndrome.

The baobab tree is indigenous to Africa and its edible parts have a significant and consolidated story of human consumption in this area, where it is used both as a food and in traditional medicine where it is used against various illness. Various parts of the baobab tree are used as a panacea, to treat almost any disease, with specific documented uses including the treatment of malaria, tuberculosis, fever, microbial infections, diarrhoea, anaemia, dysentery, and toothache among others (Rahul A et al 2015). Very limited studies report the activity of *Adansonia digitata* against metabolic syndrome in humans, with baobab fruit pulp reducing subjective hunger (Garvey et al 2017), improving glycemic response and satiety (Coe et al 2013), and reducing serum lipids (Gadou O. M et al 2017). Baobab fruit pulp and bark are also reported to help in maintaining glycemic control in animals (Savaranara et al 2017; Tanko et al 2008), the fruit pulp also reduces the degradation of starch *in vitro* (Coe et al 2013). The leaves have been reported to inhibit the enzymes alpha amylase and alpha glucosidase involved in the pathogenesis of diabetes (Irondi et al 2017). Metabolic syndrome is a state of chronic inflammation, and the baobab could also help in this as the leaf extract has been shown to inhibit pro-inflammatory iNOS, possibly through the inhibition of NF-kB (Ayele Y et al 2013), and the baobab fruit pulp has been found to have anti-inflammatory, analgesic, and antipyretic properties in mice (Ramadan A et al 1994). Baobab fruit pulp is very rich in vitamin C and polyphenols, with rich polyphenol content also being reported for leaves (Irondi et al 2017). Vitamin C and polyphenols are well known for their antioxidant and anti-inflammatory activities, these antioxidants thus present considerable advantages for patients

suffering from metabolic syndrome, as oxidative stress is one of the hallmarks of the syndrome.

Due to a lack of scientific data consolidating the beneficial effects of baobab on metabolic syndrome, the various edible parts of the baobab were tested in this PhD thesis against the hypothesis that they may prevent the risk factors involved in the metabolic syndrome or to improve the state of the disease. For this purpose, a set of 5 *in vitro* enzymatic assays that target the major risk factors involved in the metabolic syndrome have been developed and used to assay the inhibitory capacities of the extracts of baobab leaves, fruit pulp and seeds against the enzymes. The assays include the alpha amylase and alpha glucosidase inhibitory assays which target hyperglycaemia, which is a risk factor of diabetes mellitus, the HMG-CoA reductase and the pancreatic lipase inhibitory assays which target hypercholesterolemia and hypertriglyceridemia, and finally the angiotensin converting enzyme inhibitory assay which targets angiotensin II, a potent vasoconstrictor involved in the pathogenesis of hypertension. *In vitro* cellular assays are also being conducted on adipocytes, in these cellular assays the cytotoxicity of the extracts on the adipocytes were tested, the effects of the extracts on the differentiation of adipocytes to preadipocytes are being conducted and finally the effects of the extract on insulin resistance are also being assayed. In these assays, other than the positive controls, the extract from the flowers of *Hibiscus sabdariffa* was used as a positive control food plant, given that it is highly reported in the literature for its beneficial effects against metabolic syndrome and other troubles (Da-Costa-Rocha I et al 2014). Also, an animal study on wistar rat to determine the effects of the baobab and hibiscus extracts, and their corresponding functional pasta on the lipid metabolism of these animals is being conducted. Presently, only the results for the hibiscus extract and functional pasta are available and have been reported in this PhD thesis.

Also, because knowledge regarding the chemical composition of baobab is limited, the metabolite profiling of extracts from *Adansonia digitata* L. (baobab) fruit pulp and leaf, and *Hibiscus sabdariffa* L. (Karkadè) flowers, and the quantification of their major components, was conducted by means of reverse-phase high-performance liquid chromatography with photodiode array detection, coupled to electrospray ion-trap mass spectrometry (RP-HPLC-PDA-ESI-MS/ MS) and high field nuclear magnetic resonance (NMR) spectroscopy. This helps support and explain the growing amount of evidence on the nutritional and biological properties of baobab and karkadè, and provides suggestions about their possible uses by food, pharmaceutical and cosmetic industries.

CHAPTER V

IN-VITRO INHIBITORY ACTIVITIES OF THE BAOBAB FRUIT PULP, LEAVES, SEEDS AND OF HIBISCUS FLOWERS EXTRACTS AGAINST SOME ENZYMES INVOLVED IN THE METABOLIC SYNDROME.

5.1 Introduction

Adansonia digitata L. also known as baobab, is a tree very common in sub-saharan Africa and Madagascar (Gebauer et al., 2002; Diop et al., 2006). Almost all parts of this tree (fruit pulp, seeds, leaves, flowers, roots and bark) are used as food in the African tradition (Sugandha et al., 2017), whereas in Europe and in the USA, only the fruit pulp is consumed as a food since its authorization as novel food ingredient by the European parliament and council under the regulation (EC) No. 258/97 (Commission Decision 2008/575/EC), and in 2009 as a food ingredient by the Food and Drug Administration (FDA, U.S. Food and Drug Administration, 2009).Baobab fruit pulp has been reported to contain as much as 3 times the ascorbic acid content of oranges (Manfredini et al., 2002), can contain as high as 56% pectin (Nour et al., 1980) and is rich in linoleic acid, linolenic acid, oleic acid (Glew et al., 1997). It is also rich in vitamin B1, B2, B6 (Glew et al., 1997), in trace elements like P, Mg, Ca, K, Na and Fe (Glew et al., 1997; Kalenga et al., 1994) and polyphenols, with proanthocyanidins and hydroxycinnamic acids in particular (Li et al., 2017; Shahat et al., 2006). Baobab fruit pulp has been reported to reduce starch digestion, glycemic response (Coe et al., 2013) and subjective hunger in humans (Garvey et al., 2017). Also, baobab fruit pulp was shown to have anti inflammatory, analgesic, and antipyretic properties in mice (Ramadan et al., 1994), hypoglycemic properties in Alloxan induced diabetic rats (Gwarzo et al., 2013) and significant protective effects against acetaminophen-induced hepatoxicity in rats (Hanafy et al., 2016). Baobab leaves are traditionally used in sub Saharan Africa as food but are yet to be introduced into the European union as a novel food. These leaves have been reported to be rich in palmitic acid, oleic acid and linoleic acid. They represent an excellent source of Ca, K, Mg and are very rich in Fe. Their vitamin content is remarkable, they are rich in Vitamin C, B1, B2, B3, pro vitamin A and β carotene for the young leaves. Baobab leaves contain 9 – 12% of mucilage which explains their traditional use as a thickening agent in sub-saharan African culture. The leaves are also rich in phenolic compounds, particularly flavonols and tanins (Diop et al., 2006). Baobab leaf extract has been shown to inhibit pro-inflammatory iNOS, possibly through the inhibition of NF-kB (Ayele et al., 2013). Baobab leaves also present antibacterial activity (Djeussi et al., 2013), antioxidant capacity, 15-lipoxygenase inhibitory activity, antimycobacterial activity (Dzoyem et al 2014), antiviral activity (Hudson et al., 2000) and analgesic activity (Mumtaz et al., 2017). Furthermore, Irondi et al., in 2017 reported the potential of baobab leaves to inhibit enzymes involved in the digestion of carbohydrates such as alpha amylase, alpha glucosidase and aldose reductase. Baobab seeds are rich in calcium, phosphorus and magnesium. The seeds are also rich in tanins, and proteins, and they contain about 15% of lipids, comprising palmitic acid, oleic acid and

linoleic acid. They also contain cyclopropenic fatty acids (malvatic acid, sterculic acid and dihydrosterculic acid), these compounds are potentially carcinogenic and the traditional mode of preparation is made in such a way as to lower the amounts of these substances to acceptable levels for consumption. In sub-saharan culture, the seeds are eaten raw or toasted and used as a substitute of coffee (Diop et al 2006). Arabinogalactan proteins have been extracted from Baobab seeds by Zahid et al., 2017 and demonstrated to influence the innate immunity of human keratinocytes *in vitro*. Komane et al., in 2017 showed that the oil extracted from these seeds possesses hydrating, moisturising and occlusive properties when topically applied to the skin, and could thus be a valuable functional ingredient for cosmeceutical applications. Babiker et al., 2017 evaluated the antioxidant capacity of Baobab seed oil and showed that it contains a considerable total phenolic content (TPC) and worthy antioxidant activity. Shukla Y.N et al in 2003, showed that the ethyl acetate fraction of the methanolic extract of the combined pulp and seed portion of the Baobab fruit is active against Staphylococcus aureus, Staphylococcus epidermidis, Sterptococcus mutans and Pseudomonas aeruginosa.

Hibiscus sabdariffa flowers are traditionally used as a food in herbal drinks, in hot and cold beverages, as a flavouring agent in the food industry and as a herbal medicine. Early studies reported that *Hibiscus sabdariffa* flowers contain macronutrient-like proteins (1.9 g/100g), fat (0.1 g/100g), carbohydrates (12.3 g/100g) and fibres (2.3 g/100g). They are also rich in micronutrients such as vitamin C (14 mg/100g), B-carotene (300 μg/100g), calcium /1.72 mg/100g), iron (57 mg/100 g) and polyphenols including phenolic acids, organic acids, and flavonoids. The main constituents of these flowers relevant in the context of their pharmacological activity are organic acids, anthocyanins, polysaccharides and flavonoids. Various biological activities have been attributed to Hibiscus flower, including antioxidant, antibacterial, antifungal, antiparasitic, nephro and hepato-protective, diuretic, anti-cholesterol, anti-diabetic, anti-hypertensive, antispasmodic, antipyretic, antinoceptic, anti-inflammatory, cancer-preventive, anti-anemic activities as well as others (Da-Costa-Rocha et al., 2014).

As previously seen in section 2, the metabolic syndrome is a state of low grade inflammation as a consequence of a complex interplay between genetic and environmental factors which results in profound systemic effects including stroke, coronary heart disease, myocardial infarction, cancers and others, all which may lead to death. (Kaur et al., 2014). This syndrome usually englobes a cluster of risk factors that include hyperglycaemia, abdominal obesity, hyperlipidemia and hypertension. The International Diabetes Federation estimates that one-quarter of the world's

adult population has metabolic syndrome. Given the high prevalence of this syndrome, prevention should be the first solution to be considered. Preventive measures against metabolic syndrome, as previously seen in section 3, include lifestyle changes, primarily weight loss, diet, exercise and also the consumption of functional foods and food supplements. Due to the fact that many drugs present side effects, the pharmacological approach should be considered only if the changes in life style do not satisfactorily reduce risk factors. So in this context, the purpose of this study is to investigate if baobab fruit pulp, baobab leaves, baobab seeds and *Hibiscus sabdariffa* flower extract are able reduce the risk factors involved in the metabolic syndrome. These foods could be used to develop innovative functional foods or functional foods that could help in the prevention of the metabolic syndrome.

Enzymes can play a crucial role in the control of the risk factors involved in metabolic syndrome, so in this study, some of the enzymes involved in the pathogenesis of hyperglycaemia (alpha amylase, alpha glucosidase), obesity (HMG-CoAR and pancreatic lipase) and hypertension (ACE) were targeted and assayed in a series of *in vitro* enzymatic assays with baobab and hibiscus extracts, to evaluate their inhibitory capacity on these enzymes and hence improve health conditions. Baobab fruit powder and Hibiscus flowers were also used to prepare pasta, this has been done so as to verify if the pasta production and the cooking process could somehow affect their *in vitro* activity. Furthermore, the components present in the food extracts were identified and the major components quantified using a validated HPLC-PAD method, these components may be responsible for the enzymatic inhibitory activities of the food extracts.

5.2 Materials and reagents

HPLC-grade water was obtained from a LC-Pak™ Millex system (Millipore Coorporation, Billerica, MA). Formic acid, methanol, alpha-amylase, sodium chloride, sodium phosphate dibasic, sodium phosphate monobasic, starch, dinitrosalicylic acid, acarbose, alpha glucosidase, 4-Nitrophenyl-α-D-glucopyranoside, trichloroacetic acid, bistris HCl, bis tris, HMG-CoA reductase inhibition assay kit, angiotensin converting enzyme, N-[3-(2-Furyl)acryloyl]- L -phenylalanyl-glycyl-glycine, captopril, porcine pancreatic lipase, p-NPB, dimethyl formamide, orlistat, and morpholinepropanesulphonic, from Sigma-Aldrich, St. Louis, MO, USA. Semolina (*Triticum Durum*) was bought from a local chain supermarket Esselunga at Pavia, Italy.

5.3 Preparation of food materials

5.3.1 Food materials

In order to perform a more representative sampling, 5 samples of fresh baobab fruit pulp, baobab also known as *Adansonia digitata* var. congolensis (accepted name: *Adansonia digitata* L.), were collected, dried and mixed together, 20 samples of dried leaves were collected and milled together. 5 samples of karkadè dried flowers, also known as *Hibiscus sabdariffa* L. (accepted name: *Hibiscus sabdariffa*), were collected and milled together. These samples were collected during the harvest season of January–February 2016, in a local market in Ngaoundéré, Cameroon, and transported by airplane in plastic bags inside a box to laboratories in Italy, where they were stored in vacuum plastic bags away from sunlight in a cool room.

5.3.2 Production of pasta

Pasta with baobab was prepared by mixing 25% of dried baobab fruit pulp powder and 75% durum wheat (*Triticum durum*). The dough, obtained by mixing the powders with water, was dried in a ventilated electric oven for 48 hours at 40 °C. The pasta was cooked in boiling water for 10 minutes, freeze dried and then ground for the extraction process.

5.3.3 Preparation of the food extracts

Before extraction the various foods, including the pasta, were ground into a powder using a mortar and a pestle. The seeds were used both raw and toasted (electric oven for 10 min at 240°C). Each extraction was carried out separately by weighing 10 g of the food powder into a conical flask and then adding 100 mL of a 50:50 mixture of methanol to acidified bi-distilled water with 0.1% formic acid. The conical flask, immersed in ice, was left stirring for 24 hours in a nitrogen atmosphere. After 24 hours, the mixture was vacuum filtered with paper filters, and the methanol was evaporated with nitrogen. Finally, the obtained extract was frozen at -20 °C and then freeze-dried to obtain a powder. The different extracts were dissolved in the different buffers for the enzymatic assays.

5.4 *In vitro* enzymatic assays

5.4.1 Alpha amylase inhibition assay

The alpha amylase inhibition assay was conducted according to the protocol described in the Worthington enzyme manual, with some modifications (Worthington, 1993). In summary, 20 μL of each food extract solution and 20 μL of α-amylase solution (0.5 mg/mL) in a 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) were incubated at 25 °C for 10 min. After preincubation, 20 μL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube at timed intervals. The reaction mixtures were then incubated at 25 °C for 10 min. The reaction was stopped with 40 μL of dinitrosalicylic acid color reagent. The test tubes were then incubated in a boiling water bath for 10 min and cooled to room temperature. The reaction mixture was then diluted adding 600 μL of bidistilled water, and absorbance was measured at 540 nm using a microplate reader (BMG Fluostar Omega microplate reader). The absorbance of sample blanks (buffer instead of enzyme solution) and a control (buffer in place of sample extract) were recorded as well. The final extract absorbance (A540 extract) was obtained by subtracting its corresponding sample blank reading. The α-amylase inhibitory activity was calculated according to the equation below:

$$\% \text{ Inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}}$$

5.4.2 Alpha glucosidase inhibition assay

The alpha glucosidase inhibition assay was performed according to the protocol reported in the Worthington Enzyme Manual, with some modifications (Worthington, V 1993). The assay consisted of incubating in a 96-well plates at 25 °C for 10 min, 50 μL of aqueous food extracts and 100 μL of 0.1 M phosphate buffer (pH 6.9) containing alpha-glucosidase (1 unit/mL) solution. After preincubation, 50 μL of 5 mM p-nitrophenylα-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25 °C for 5 min. Before and after incubation, absorbance readings were recorded at 405 nm by microplate reader (BMG Fluostar Omega microplate reader). The absorbance of sample blanks (buffer instead of enzyme solution) and a control (buffer in place of sample extract) were recorded as well. The results were expressed as percentages of alpha-glucosidase inhibition and calculated according to the equation reported below.

$$\% \text{ Inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}}$$

5.4.3 Hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibition assay

The assay was conducted according to the protocol reported by Sigma-Aldrich, the manufacturer of the HMG-CoAR kit, and also according to Soares et al., 2015. The assay was conducted by placing 181 μL of phosphate buffer with 1 μL of food extract or positive control (pravastatin) into 96 well UV plate, 4 μL of NADPH (to obtain a final concentration of 400 μM) and 12 μL of HMG-CoA substrate (to obtain a final concentration of 0.3 mg·mL−1) were then added. The analyses were initiated (time 0) by the addition of 2 μL of the HMG-CoA reductase (concentration of the enzyme stock solution (0.50–0.70 mg protein/mL) and incubated at 37 °C. The rates of NADPH consumed were monitored every 20 s for up to 600 s by reading the decrease in absorbance at 340 nm using a microplate reader (BMG Fluostar Omega microplate reader). The absorbance of sample blanks (buffer instead of enzyme solution) and a control (buffer in place of sample extract) were recorded as well. The results were expressed as percent of HMG-CoAR inhibition and calculated according to the equation reported below.

$$\% \text{ Inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}}$$

5.4.4 Pancreatic lipase inhibition assay

The pancreatic lipase inhibition assay was performed according to Dechakhamphu et al., 2015 and it consisted of preparing an enzyme buffer by adding 30 μL of solution of porcine pancreatic lipase (2.5 mg/mL in 10 mM morpholinepropanesulphonic acid and 1 mM ethylenediamine tetraacetic acid, pH 6.8) to 850 μL of Tris buffer (100 mM Tris–HCl and 5 mM CaCl2, pH 7.0). Then, either 100 μL of the plant extracts at selected concentrations or Orlistat was added and incubated for 15 min at 37 °C. Ten microliters of substrate 10 mM p-NPB (para-nitrophenyl butyrate) in dimethyl formamide were then added and incubated for 30 min at 37 C. Lipase activity was determined by measuring the hydrolysis of p-NPB to p-nitrophenol at 405 nm using a microplate reader (BMG Fluostar Omega

microplate reader). The results were expressed as percentages of Lipase inhibition and calculated according to the equation reported below.

$$\% \text{ Inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}}$$

5.4.5 Angiotensin converting enzyme (ACE) inhibition assay

The assay was performed according to the protocol described by Holmquist et., al in 1979 with some modifications. The assay consisted of mixing 5 µL (corresponding to 20 mU) of ACE (1 U/mL) with 50 µL of different concentrations of food extracts dissolved in Tris-HCl buffer 50 mM, pH 7.5, containing 0.3 M NaCl, followed by the addition of 0.25 mL of 5 × 10-4 M FAPGG dissolved in Tris-HCl buffer 50 mM, pH 7.5, containing 0.3 M NaCl. The decrease in absorbance at 345 nm was recorded over 5 min at room temperature using a microplate reader. The absorbance of sample blanks (buffer instead of enzyme solution) and a control (buffer in place of sample extract) were recorded as well. Captopril was used as a positive control. The results were expressed as percentages of ACE inhibition and calculated according to the equation reported below.

$$\% \text{ Inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}}$$

5.4.5 Statistical analysis

The IC 50 were calculated using linear or polynomial regressions when necessary logarithmic transformations were used to facilitate the calculations. Results were expressed as means ± SE, and $p < 0.05$ was considered statistically significant. The statistical significance of the data was assessed through one-way variance analysis (ANOVA). When significant differences were found, Bonferroni post hoc testing was used to determine the difference between the groups involved.

5.5 Results of the *in vitro* enzymatic assays

5.5.1 Yield of the extraction procedure

The extraction process yield was calculated by applying the formula:

$$\text{Yield of extraction} = \frac{\text{dried mass obtained}}{10\ \text{g}} \times 100$$

Food samples	Dried mass (g)	Yield (%)
Adansonia digitata leaves	1,2178	12,18
Adansonia digitata fruit pulp	2,2324	22,32
Adansonia digitata raw seeds	0,5954	5,95
Adansonia digitata toasted seeds	0,4923	4,92
Hibiscus sabdariffa flowers	3,8545	38,55
Pasta made with baobab fruit pulp powder	0,7994	7,994

Table 5.1: Extraction yield for the baobab fruit, leaves, raw and toasted seeds, karkadè flowers and pasta made with baobab**.**

Hibiscus sabdariffa extract gave the highest yield of 38,54% while toasted *Adansonia digitata* seeds gave the lowest yield of 4,923%.

5.5.2 Alpha amylase inhibition assay

As shown on figure 5.1 below, the alpha amylase inhibition assay used is based on the fact that alpha amylase breaks down starch, releasing reducing sugars which reduce dinitrosalicylic acid to 3-amino-5-nitrosalysilic acid which absorbs strongly at 540 nm. Therefore extracts strongly inhibiting alpha amylase will produce a low absorption, whereas extracts with inhibition will present a high absorption at 540nm. As can be seen on figure 5.1F below, acarbose, a known inhibitor of alpha amylase, inhibits the enzyme in a concentration dependent mode with an IC 50 of 0.036 mg/ml. In the scientific literature, Mogale et al., 2011

report an IC 50 of 0.0165 mg/mL for acarbose. The experimental IC 50 obtained in this study is 2 times greater than that one reported in literature, but these are of the same order of magnitude and the slight difference may be due to different lots of enzymes used, different environmental conditions, the dissolution solvent, or even the concentration considered (the concentration of the solution of acarbose added or the final concentration of acarbose in the well). Authors such as Gondokesumo et al., 2017, and Ademiluyi et al., 2013 have demonstrated the inhibitory activity of *Hibiscus sabdariffa* flower extracts against alpha amylase. Their IC 50 values, which are lower than the ones obtained here, are not directly comparable with our results because of many factors including different extractions, different geographic harvest of hibiscus and further differences.

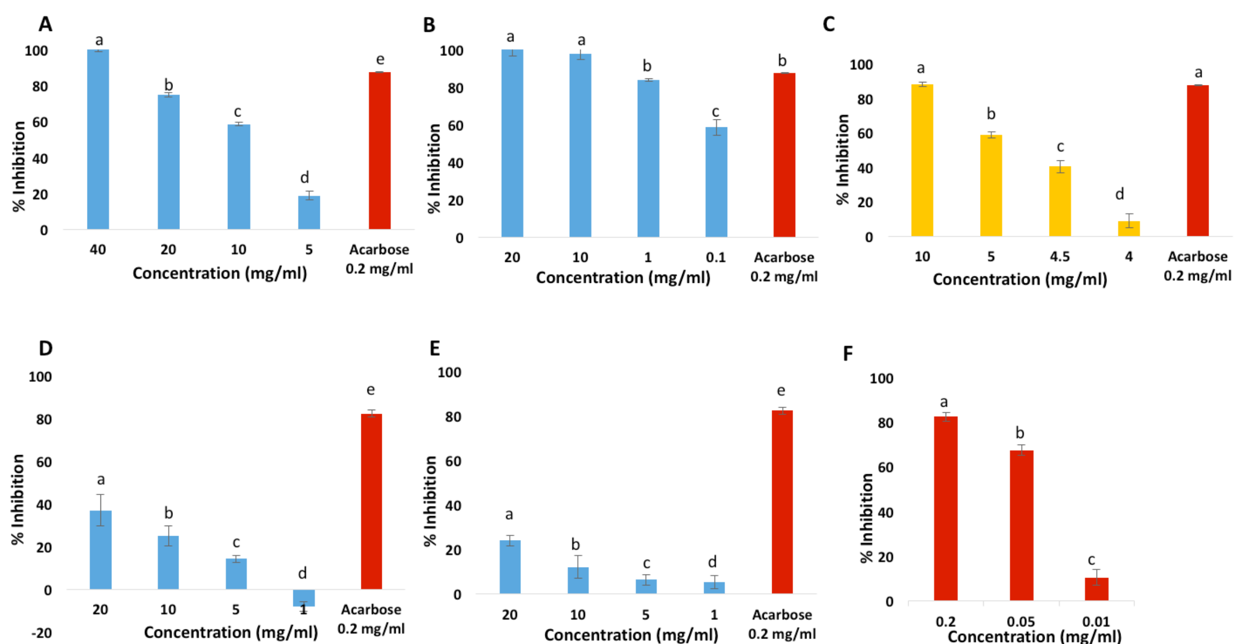


Figure 5.1: Inhibition of alpha amylase by A) *Adansonia digitata* fruit extract with IC50 = 9,97 mg/ml, B) *Adansonia digitata* leaf extract with IC50 < 0.1 mg/ml, C) *Hibiscus sabdariffa* flowers with IC 50 = 4,8 mg/ml D) *Adansonia digitata* raw seeds extract with IC50 > 20 mg/ml., E) *Adansonia digitata* toasted seeds extract with IC50 > 20 mg/ml, and by the positive control F) Acarbose with IC 50 = 0.036 mg/ml.

Hibiscus sabdariffa extract was thus used as a positive control food extract i.e. a food which is known in the scientific literature to inhibit the enzyme. From the data obtained in this study, *Hibiscus sabdariffa* inhibits

alpha amylase in a concentration dependent manner with an IC50 of 4.8 mg/ml. The baobab leaf and fruit pulp extracts also inhibit alpha amylase in a concentration dependent manner with IC50 <0.1 and = 9.97 mg/ml respectively. The baobab leaves show the most potent activity against alpha amylase, even though they show lower activity than the positive control acarbose. Both the raw and toasted baobab seeds weakly inhibit alpha amylase, though the raw seed extract seems to be more active.

5.5.3 Effect of cooking on the inhibition of alpha amylase

To test the effect of thermal treatment on the inhibition of alpha amylase, a functional pasta was made including baobab, boiled in water for 10 minutes, freeze dried, ground and then extracted by the extraction procedure reported in section 5.3.3. This process was adopted to ascertain that the baobab still inhibits the enzymes involved in the digestion of carbohydrates following the boiling process, as pasta is not eaten raw but once boiled. The pasta was freeze dried after cooking in order to better estimate the weight after the almost complete removal of water content during the freeze drying process.

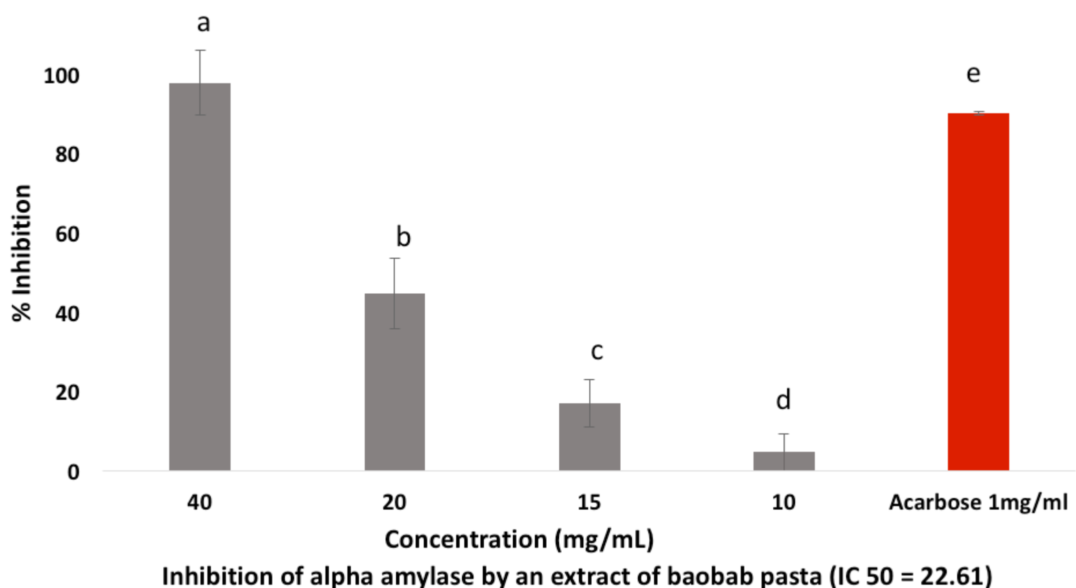


Figure 5.2: Inhibition of alpha amylase by a baobab pasta extract (25% of baobab powder)

The baobab pasta extract inhibited the alpha amylase with an IC 50 of 22.61 mg/ml. Considering that the pasta was made using only 25% baobab fruit pulp, and that the fruit pulp has an IC 50 of 9.97 mg/mL as reported above, it can be concluded that the pasta maintains its activity even after cooking, and the activity appears to be even higher.

5.5.4 Alpha glucosidase inhibition assay

Alpha-glucosidase is one of the glucosidases located in the brush-border membrane of the surface of intestinal cells, it breaks down oligosaccharides and disaccharides into monosaccharides. This assay is based on the ability of alpha glucosidase in breaking down the α 1-4 glycosidic bond of 4-Nitrophenyl α-D-glucopyranoside, releasing 4-nitrophenol which absorbs strongly at 405 nm. As can be seen on figure 5.3 below, acarbose, a known inhibitor of alpha glucosidase, inhibits the enzyme in a concentration dependent manner with an IC 50 of 0.66 mg/ml. In the scientific literature, Zhang et al., 2014, reported an IC 50 of 0,2 mg/mL for acarbose. The experimental IC 50 obtained in this study is 3 times greater than that reported in literature, but these remain in the same order of magnitude and the difference may be due to the various reasons listed above. As reported above, Gondokesumo et al., 2017, and Ademiluyi et al., 2013, also demonstrated the inhibitory activity of *Hibiscus sabdariffa* flower extracts against alpha glucosidase, but their lower IC 50 are not directly comparable with the ones obtained here because of the same factors as listed above.

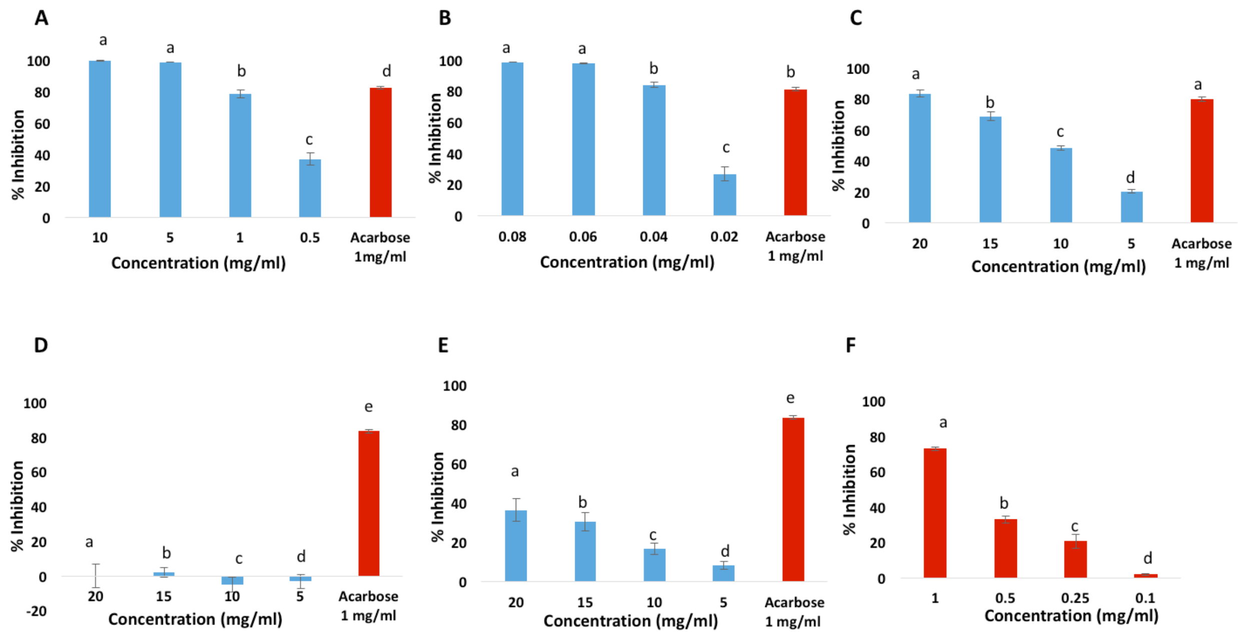


Figure 5.3: inhibition of alpha glucosidase by A) *Adansonia digitata* fruit extract with IC50 = 0.64 mg/ml, B) *Adansonia digitata* leaf extract with IC50 = 0.027 mg/ml, C) *Hibiscus sabdariffa* flowers with IC 50 = 9.86 mg/ml D) *Adansonia digitata* raw seed extract with IC50 > 20 mg/ml., E) *Adansonia digitata* toasted seed extract with IC50 > 20 mg/ml, and the positive control F) Acarbose with IC 50 = 0.66 mg/ml.

Here too, the *Hibiscus sabdariffa* extract was used as a positive control food extract. From the data obtained, *Hibiscus sabdariffa* inhibits alpha amylase in a concentration dependent manner with an IC50 of 9.86 mg/ml. The baobab leaf and fruit pulp extracts also inhibit alpha amylase in a concentration dependent manner with IC50 = 0.027 mg/ml and = 0.64 mg/ml respectively. The baobab leaf extract shows the most potent activity against alpha glucosidase with an IC 50 smaller than even that of acarbose. Both raw and toasted baobab seeds weakly inhibit alpha amylase, though in this case the toasted seeds seem to be more active, unlike in the alpha amylase assay.

5.5.5 Effect of cooking on the inhibition of alpha glucosidase

As previously reported for alpha amylase, the effect of cooking was also tested on alpha glucosidase to see if the thermal treatment could somehow affect the activity of the extract on this enzyme.

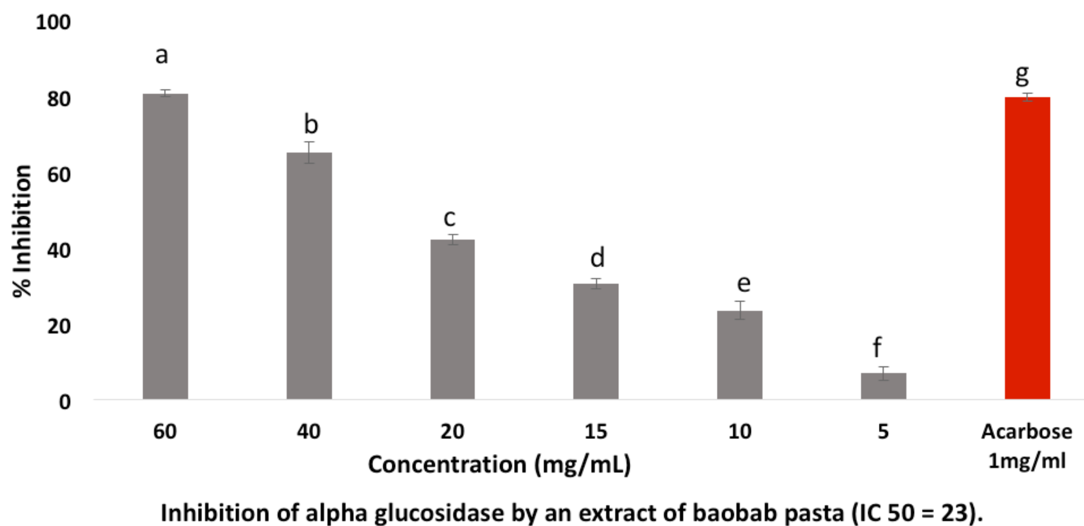


Figure 5.4: Inhibition of alpha glucosidase by an extract of baobab pasta.

As can be seen on figure 5.4, the baoabab maintains its activity even after cooking. The IC 50 is much greater, showing that there may be a certain lowering of the inhibitory activity.

5.5.4 HMG-CoA reductase inhibition assay

HMG-CoA reductase is the rate-controlling enzyme of the mevalonate pathway, the metabolic pathway that produces cholesterol. This enzyme converts HMG-CoA (3-hydroxy-3-methyl-glutaryl-coenzyme A), its substrate, to the product mevalonate using NADPH as cofactor. NADPH strongly absorbs at 340 nm and is converted by enzymatic catalysis to NADP+ which poorly absorbs at 340 nm, thus creating a decrease in absorbance at 340 nm. Pravastatin, the positive control, inhibits the enzyme in a concentration dependent manner, with an IC50 of 22.7 μM. Kurakata et al., 1996, report pravastatin sodium salt as inhibiting HMG-CoA reductase in human lymphocytes with an IC50 of 5.6 μM, and as previously stated for alpha amylase and alpha glucosidase, the slight difference that is observed may be for several reasons as listed above.

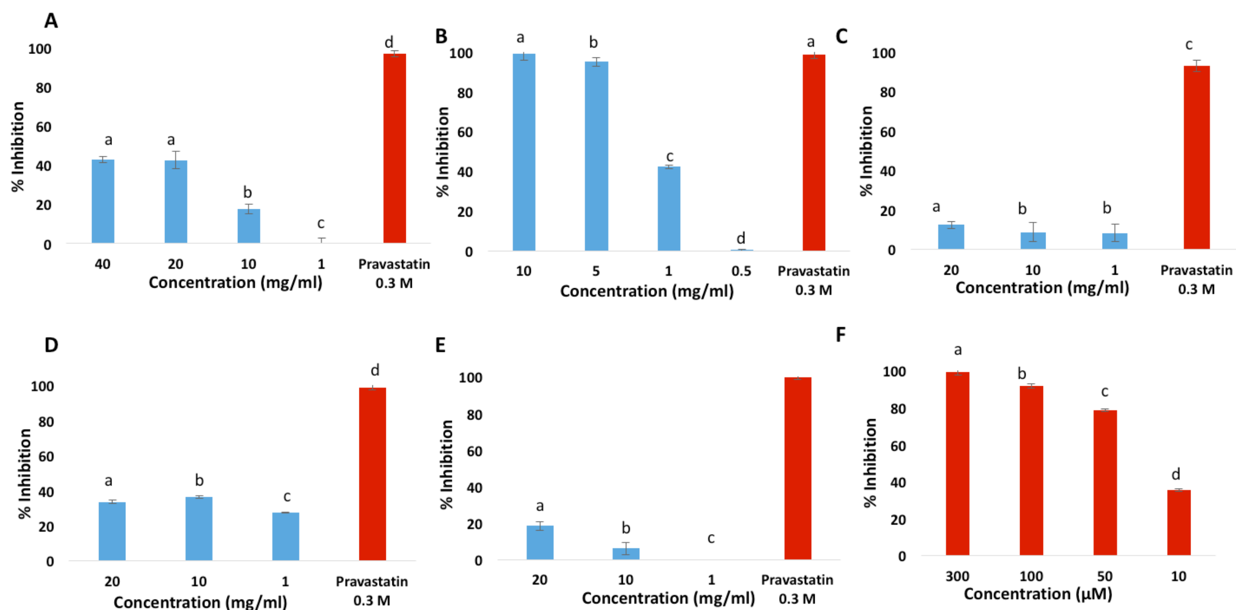


Figure 5.5: inhibition of HMG-CoA reductase by **A)** *Adansonia digitata* fruit extract with IC50 > 20 mg/ml, **B)** *Adansonia digitata* leaf extract with IC50 = 1.9 mg/ml, **C)** *Hibiscus sabdariffa* flowers with IC 50 > 20 mg/ml **D)** *Adansonia digitata* raw seed extract with IC50 > 20 mg/ml., **E)** *Adansonia digitata* toasted seed extract with IC50 > 20 mg/ml, and by the positive control **F)** pravastatin with IC 50 = 22.7 uM.

Baobab leaves show the highest inhibitory activity against HMG-CoA reductase with an IC50 of about 1.9 mg/mL, followed by the fruit pulp and raw seed extracts which show an IC50 of greater than 20 mg/mL. The baobab fresh seed and hibiscus flowers extracts show very low inhibition. Duangjai et al., 2011, showed that *Hibiscus sabdariffa* inhibits HMG-CoA reductase, but in the present experiment the inhibition obtained was low.

5.5.5 Pancreatic lipase inhibition assay

Pancreatic lipase, secreted from the pancreas, is one of the main digestive enzymes, converting triglyceride substrates found in ingested oils to monoglycerides and free fatty acids which are then absorbed into the lymphatic system. This assay is based on the fact that pancreatic lipase breaks down p-NPB to release p-nitrophenol that strongly absorbs at 405 nm. An increase in absorbance at 405 nm thus implies that the enzyme is functioning. In the presence of Orlistat, the positive control, there has been a low increase in absorbance

thus demonstrating that it effectively inhibits the enzyme.

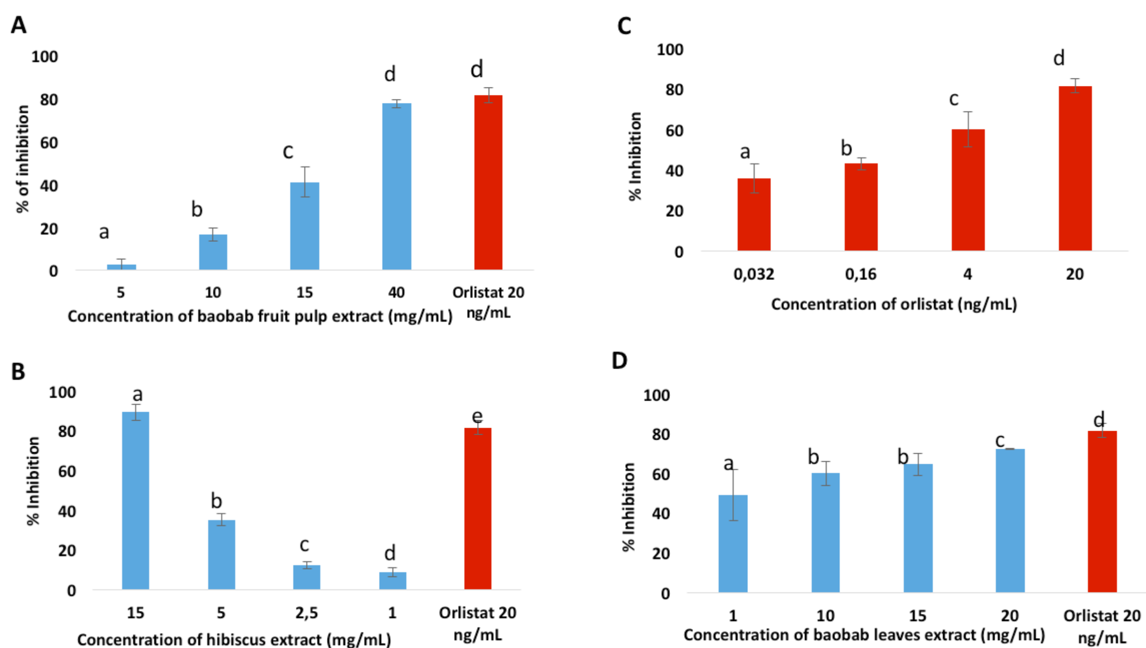


Figure 5.6: inhibition of pancreatic lipase by **A)** *Adansonia digitata* fruit extract with IC50 = 20.44 mg/ml, **B)** *Hibiscus sabdariffa* flowers with IC 50 = 8,2 mg/ml **C)** Orlistat with IC50 = 1.94 ng/ml, **D)** *Adansonia digitata* leaf extract with IC50 = 1.85 mg/ml.

Orlistat, the positive control, and hibiscus flower extract were found to inhibit pancreatic lipase in a concentration dependent manner. Gupta et al., 2015, reported orlistat to have an IC50 of 2.73 µg/mL, but the result obtained in this study is much lower (IC 50 = 1.94 ng/ml). This may be due to different solvents used in the solubilization of orlistat or different test procedures. Buchholz et al., 2016, report *Hibiscus sabdariffa* to have high inhibitory activities on pancreatic lipase with IC50 = 35.8 ± 0.8 µg/mL. The data obtained in this experiment shows a much greater IC 50 of 8,189 mg/mL, with the differences perhaps being due to the reasons previously mentioned. In any case, the leaves remain the most potent inhibitor with an IC 50 of 1.85 mg/ml followed by hibiscus and then

baobab fruit pulp, which has an IC 50 of 20.44 mg/mL. The raw and toasted seed extracts showed no inhibition at all of pancreatic lipase and so no histogram was reported.

5.5.6 Angiotensin converting enzyme (ACE) inhibiting assay

ACE is the enzyme that converts angiotensin I to angiotensin II a potent vasoconstrictor. The inhibition of this enzyme is used in pharmacotherapy to lower blood pressure. In this assay, ACE breaks down FAPGG (Furylacryloyl-Phe-Gly-Gly) which strongly absorbs at 340 nm, releasing FAP (Furylacryloyl-Phe) which absorbs less at 340 nm. So when the enzyme functions there should be a drop in initial absorbance. In this assay, the enzyme functioned correctly and the positive control captopril was able to prevent the fall in absorbance by inhibiting the enzyme. Captopril, the positive control, inhibits the enzyme in a concentration dependent manner with an IC50 of 0.03 uM. Ben et al., 2013, report the IC50 of captopril to be in the range of 1.79 – 15.1 nM, the value of 0.03 μM obtained in this experiment is a little bit greater but of the same order so it fits with literature.

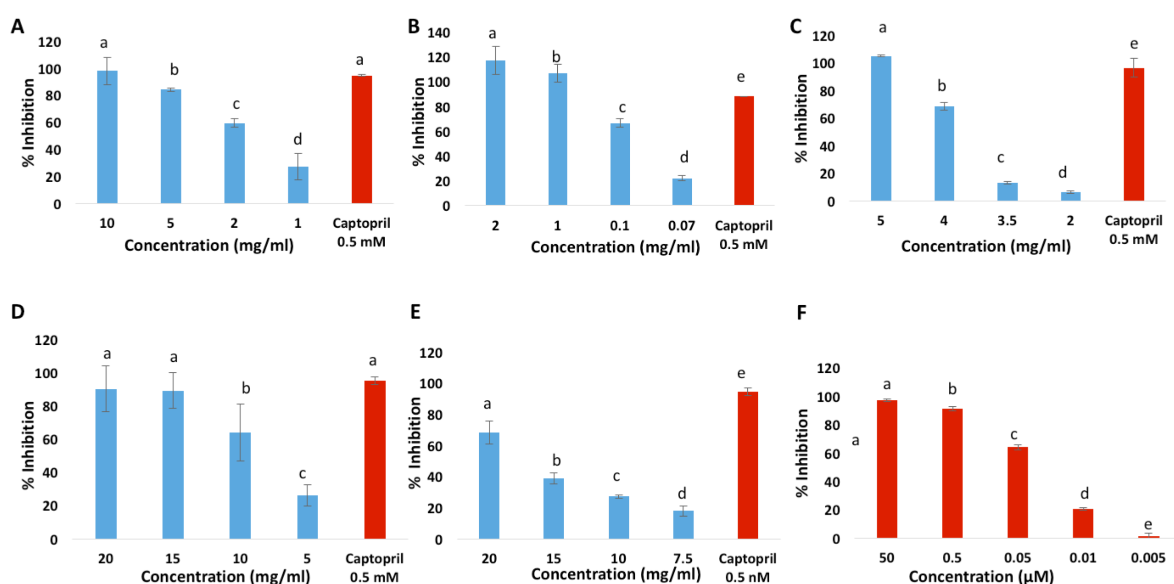


Figure 5.7: inhibition of ACE by A) *Adansonia digitata* fruit extract with IC50 = 1.7 mg/ml, B) *Adansonia digitata* leaf extract with IC50 = 0.08 mg/ml, C) *Hibiscus sabdariffa* flowers with IC 50 = 3.86 mg/ml D) *Adansonia digitata* raw seed extract with IC50 = 7.77 mg/ml., E) *Adansonia digitata* toasted seed extract with IC50 = 17 mg/ml, and the positive control F) captopril with IC 50 = 0.03 uM.

Infusion of *Hibiscus sabdariffa* flowers is traditionally recognized for its healthy properties against

hypertension. Ojeda et al., 2010, showed that *Hibiscus sabdariffa* flower extract and its components inhibit ACE, so this may be the mechanism by which it lowers blood pressure. In this assay, all the extracts were found to inhibit ACE in a concentration dependent mode, the baobab leaves extract show the highest inhibition followed by the fruit pulp, Hibiscus, then the raw seeds and the toasted seeds.

5.6 Conclusion

Currently many drugs exist for use in targeting many enzymes involved in the pathogenesis of the metabolic syndrome. Most of these drugs present many side effects and are expensive, and thus alternative options are required to solve these problems. Many centuries ago, Hippocrates came out with the famous phrase "let food be your first medicine", he already understood in these ancient times that food should underlie the prevention of disease given that it is a basic component of our everyday lives. Furthermore, food extracts including plant extracts are very complex and contain numerous active components, offering a higher probability of finding various components that can target different metabolic pathways, thus suggesting the utility of food extracts in complex pathogenic processes like the metabolic syndrome which involves many enzymes and metabolic pathways.

In this study, the inhibitory activities of the *Adansonia digitata* fruit, leaves and seed extracts were investigated against some of the enzymes involved in the metabolic syndrome, based on the comparison of their activities with those of positive controls and the positive control food sample. The positive control, according to Butterweck et al., 2012, is a standard reference compound that is known to possess the desired therapeutic property. One basic recommendation according to the same author is the selection of an appropriate model that has to be sensitive to the standard reference in a dose-dependent fashion. During the experiments in this study, acarbose was used as a positive control for the alpha amylase and alpha glucosidase inhibition assays, pravastatin for the HMG-CoAR inhibition assay, orlistat for the pancreatic lipase inhibition assay and captopril for the ACE inhibition assay. The positive controls all inhibited the enzymes in a concentration dependent manner hence validating the viability of the assays. In general, the baobab leaves were found to be the most active in all the enzymatic assays. All the extracts except the seed extract were then subjected to testing in more complex *in vitro* (*in vitro* cellular assays) and *in vivo* (animal studies) systems in order to confirm the activities obtained in the enzymatic assays, and their chemical profile was also determined. The seeds did not show satisfying activities against the enzymes, and for this reason were not considered for further investigations in the *in vitro* cellular assays and their chemical characterization was not executed.

CHAPTER VI

IN-VITRO CELLULAR ASSAYS ON HUMAN ADIPOCYTES

Effect of baobab and hibiscus extracts on cellular vitality, morphology and uptake of glucose

6.1 Introduction

In section 2.2 abdominal obesity has been established as one of the risk factors associated with the metabolic syndrome. This abdominal obesity is usually characterized by an accumulation of adipose tissues which is a result of an imbalance between energy intake and energy lost. In the case of apoxia, these adipocytes secrete adipokines (FFA, TNFα, IL-6, PAI-1, CRP). Adipocytokines integrate the endocrine, autocrine, and paracrine signals to mediate multiple processes including insulin sensitivity (Saleem et al., 2009), oxidant stress (Tsimikas et al., 2009), energy metabolism, blood coagulation, and inflammatory responses (Jacobs et al., 2009) which are thought to accelerate atherosclerosis, plaque rupture, and atherothrombosis.

In this study the aim is to determine whether the baobab and hibiscus extracts can inhibit the conversion of preadipocytes to adipocytes, hence reducing all the effects mediated by adipocytes. The other goal is to investigate the capacity of these extracts to increase the absorption of glucose into the cells and to monitor the upregulation of PPARγ and SREBP1 which are important factors involved in the metabolic syndrome. In previous studies the raw and toasted seeds showed very low activity and thus haven't been considered for further investigation.

In order to realize the above mentioned aims, the extracts need to be tested for their cytotoxicity on human adipocytes. In this PhD thesis only the part relating to the cytotoxicity (MTS and morphological studies) is reported, as the others studies are ongoing.

6.2 Materials and methods

6.2.1 Materials and food extracts

Human liposarcoma cell line (SW872, ATCC® HTB-92 ™) was obtained from the American Type Culture Collection (ATCC®, Manassas, VA, USA), DMEM/F-12 culture medium (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12), HEPES buffer (2- (4- (2-Hydroyethyl) -1 -piperazinyl) -ethansulfonsaur), sodium bicarbonate, L-glutamine, fetal bovine serum (FBS), penicillin, streptomycin, trypsin, EDTA, oleic acid, dimethylsulfoxide (DMSO), MTS reagent, and phenazine methosulfate were obtained from Sigma-Aldrich. The food extracts were obtained using the extraction process reported in section 5.5.3.

6.2.2 **Adipocyte cell culture**

The human liposarcoma cell line (SW872, ATCC® HTB-92 ™) was grown as recommended by the vendor. The cells were cultured in DMEM / F-12 culture medium, containing 15 mM of HEPES buffer and sodium bicarbonate to which 2 mM L-glutamine, 10 % fetal bovine serum (FBS) and 1 % penicillin (100 U/ml), streptomycin (100 μg/ml) were added. Then, these were kept in culture in a 75 cm^2 petri dish at 37 °C in an incubator set at 95% air and 5 % CO_2. The culture medium was renewed every two days (48 hours). After seven days (170 hours) of culture corresponding to a confluence percentage of approximately 90 – 100 % (Figure 6.1), 2 mL of a trypsin-EDTA solution was added to the plate to detach the cells and the plate was then incubated at 37 °C for 5 minutes. Subsequently, the trypsin-EDTA-cell suspension was centrifuged at 1200 rpm for 5 minutes to separate the cells. The cell pellet was resuspended in 10 mL of medium (DMEM) containing 10% FBS. For different densities, the cells were seeded in plates for further work.

6.2.3 Adipocyte differentiation

The SW 872 preadipocyte cells were cultured as described above in section 6.2.2 and after reaching a percentage of confluence close to 100%, a solution of oleic acid (500 μM) freshly prepared in the culture medium was added to initiate cellular differentiation of preadipocytes into mature adipocytes (Figure 6.1). The plant extracts were dissolved in sterilized dimethylsulfoxide (DMSO) and added to the cells for a final concentration of DMSO not exceeding 0.1%.

6.2.4 MTS Cell Proliferation Assay

The cell viability assay was conducted using the Cell Titer 96 aqueous non-radioactive cell proliferation assay (Promega, Madison, WI, USA) according to the method described by Kim et al., 2018 with little modifications. In brief, cells were seeded in a sterile, flat bottom, 96-well plate at a density of 2×10^4 cells/well and incubated at 37 ^oC for 24 h in a humidified incubator containing 5% CO_2 (Thermofisher Forma™ Steri-Cycle™, MI, USA). Different concentrations of extracts (1, 10, 50 and 100 μg/mL) were prepared directly in fresh serum-free MEM media and 100 μL of each treatment was added to each well and incubated for 24 and 48 h. Then, 20 μL of the MTS reagent in combination with the electron coupling agent phenazine methosulfate was added into each well and allowed to react for 1 h at 37 ^oC. After 2 minutes of shaking at minimal intensity, the absorbance was measured at 490 nm using the EnSpire PerkinElmer Multimode Plate Reader. In the same conditions, controls and blanks which consisted of cells

with media containing DMSO (≤0.1%) and wells containing media without cells respectively, were performed. The cell viability values were determined using the equations below:

Two separate experiments were run in triplicate.

6.2.4 Morphological analysis

The human liposarcoma (SW872, ATCC® HTB-92 ™) cells were cultured as reported above in sterile flat bottom 6 cm^2 dishes at a density of 5×10^5 cells/dish, then incubated at 37 ^oC for 24 h in a humidifed incubator containing 5% CO_2 (Thermofisher Forma™ Steri-Cycle™, MI, USA). Two different concentrations of extracts (1 and 100 µg/mL) were prepared directly in fresh serum-free media and 3 mL of each treatment was added to each dish and incubated for 24h and 48 h. After the treatment, cells were visualized on ZEISS microscope (ZEISS, VA, USA) using X10 and X32 magnifications.

6.2.5 Glucose uptake Assay

The insulin-resistant cell model and glucose uptake were set-up and measured using 2-NBDG (Abcam, ab146200) according to the method described by Bhakta et al., 2017, with some modifications. Briefly, SW872 cells were seeded in 96-well black plates at a density of 5x 10^4 cells/well, and incubated at 37°C for a duration not exceeding 48 hours (confluence cells in the range of 80-90%) in a humidified 5% CO2 incubator (Thermofisher FormaTM Steri-CycleTM, MI, USA). Two different schemes for inducing insulin resistance were tested:

- One included low-glucose DMEM as the medium for the first 24 h, the following 24 h (with the addition of insulin, see below) and the next 24 h (with treatments). No glucose DMEM was then used for the 30 min insulin shock + the 30 min glucose uptake period.
- The other included high-glucose DMEM as the medium for the first 24 h and the following 24 h (with the addition of insulin, see below). Then, no glucose DMEM was used for all the subsequent treatments.

Cells were treated with 10^{-6} M insulin for 24 hours to induce insulin-resistance. After that, different concentrations of plant extracts (1, 10 and 20 µg/mL) or 10 µM metformin (a non-cytotoxic concentration) were prepared in the different culture media containing no serum, then 100 µL of each treatment was added to each well and incubated

for 24 hours. The following day, 10^{-7} M insulin was added, and cells were incubated for 30 minutes. Then, to determine 2-NBDG uptake, 100 µL 2-NBDG (50 µM) freshly prepared in DMEM without glucose were introduced into each well and incubated at 37 °C in the dark for 30 minutes. To finish, cells were washed twice with cold PBS and the fluorescent light intensity was measured for excitation and emission wavelengths at 485 nm and 528 nm, respectively, using the EnSpire PerkinElmer multimode plate reader. Under the same conditions, the controls and the blanks, consisting of cells treated only with insulin or not, were tested. The relative percentage of glucose uptake produced were determined using the equation below:

% relative glucose uptake = ((Mean FI Samples -Mean FI Blank) / (Mean FI Control -Mean FI Blank)) x 100.

With FI = fluorescence

6.3 Results

6.3.1 Adipocyte culture and differentiation

Figure 6.1 below depicts Oil Red O staining of SW872, both undifferentiated (left panel) and differentiated (right panel)

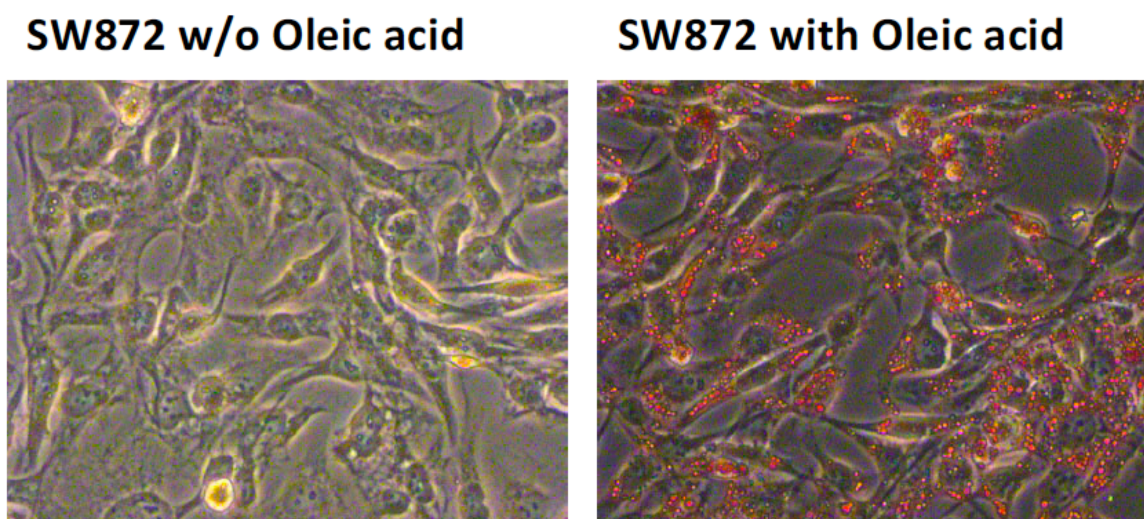


Figure 6.1: Differentiation of SW872 cells by the addition of oleic acid.

6.3.2 MTS Cell Proliferation Assay

The MTS Cell Proliferation Assay is a colorimetric method for sensitive quantification of viable cells in proliferation and cytotoxicity assay. The method is based on the reduction of a MTS tetrazolium compound by viable cells to generate a colored formazan product that is soluble in cell culture media. This conversion is thought to be carried out by NAD(P)H-dependent dehydrogenase enzymes in metabolically active cells. The formazan dye produced by viable cells can be quantified by measuring the absorbance at 490-500 nm. The assay can be used for the measurement of cell proliferation in response to growth factors, cytokines, mitogens, and nutrients, etc. It can also be used for the analysis of cytotoxic compounds such as anticancer drugs and many other toxic agents, pharmaceutical compounds and plants.

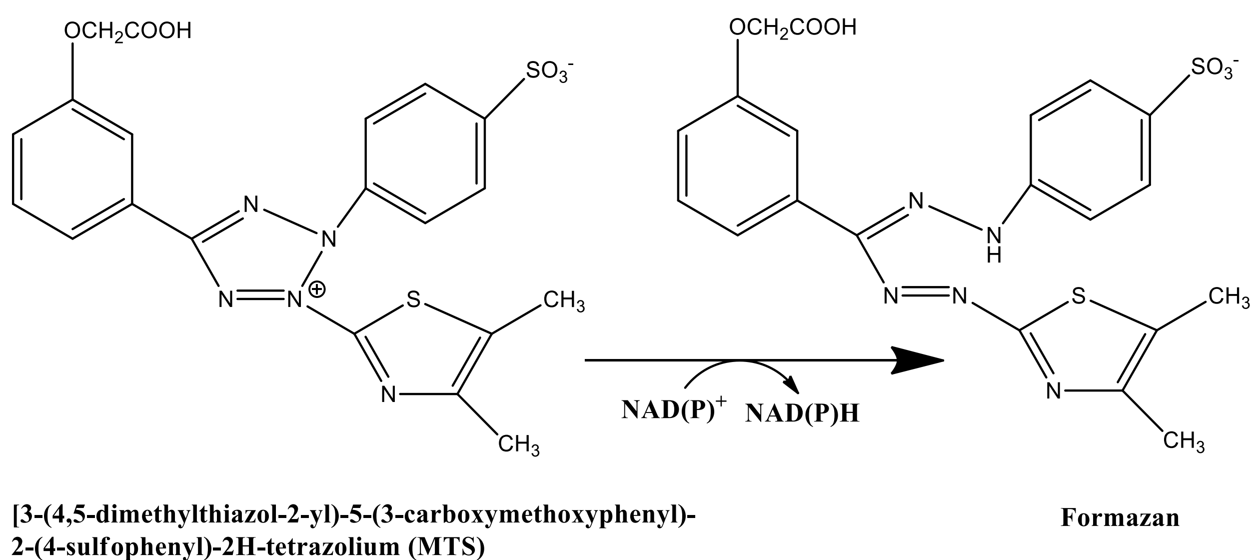


Figure 6.2: Conversion of MTS to formazan (that strongly absorbs in the range 490 – 500 nm) by metabolically viable cells by a NAD(P)H-dependent dehydrogenase.

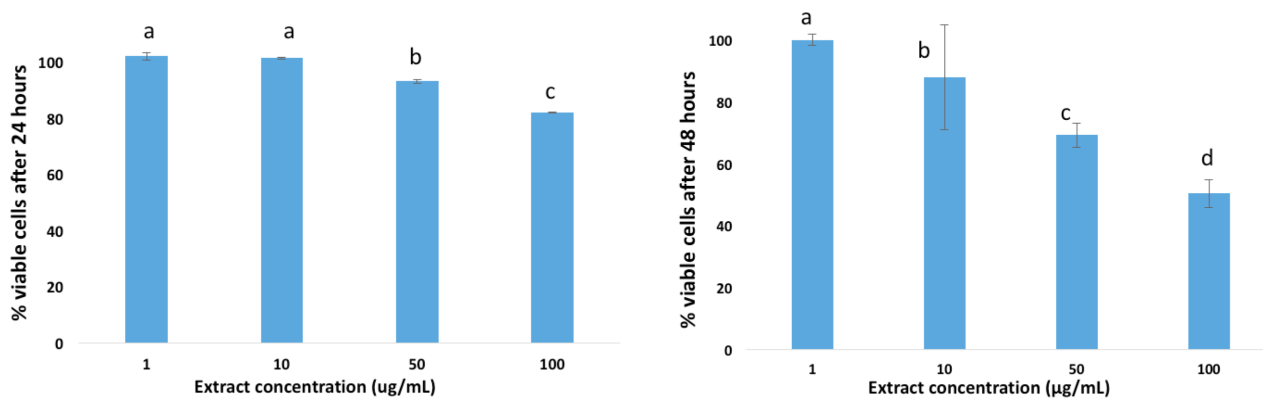


Figure 6.3: Cellular viability of human adipocytes after treatement with hibiscus flower extract for 24 and 48 hours**.**

The scope of this MTS study is to test the extracts for cellular viability so only extracts which are not toxic to the cells will be used in further experiments. If an extract is toxic to cells it may interfere with the normal functioning of the cells and thus the experimental data obtained may be biased.

Figure 6.3 shows that after 24 hours at concentrations of 1 and 10 μg/mL the hibiscus extract shows almost no toxicity, as the cells are 100% viable. The concentrations of 50 and 100 μg/mL show very negligible toxicity as the cellular viability is around 80%. Passing to 48 hours there is a slight increase in toxicity, but the cellular viability remains above 80% for the the concentrations of 1 and 10 μg/mL while this drops below 80% for the 50 and 100 μg/mL concentrations. Usually concentrations of extracts that give a viability above 80% are considered safe for cells and thus used in experimental studies.

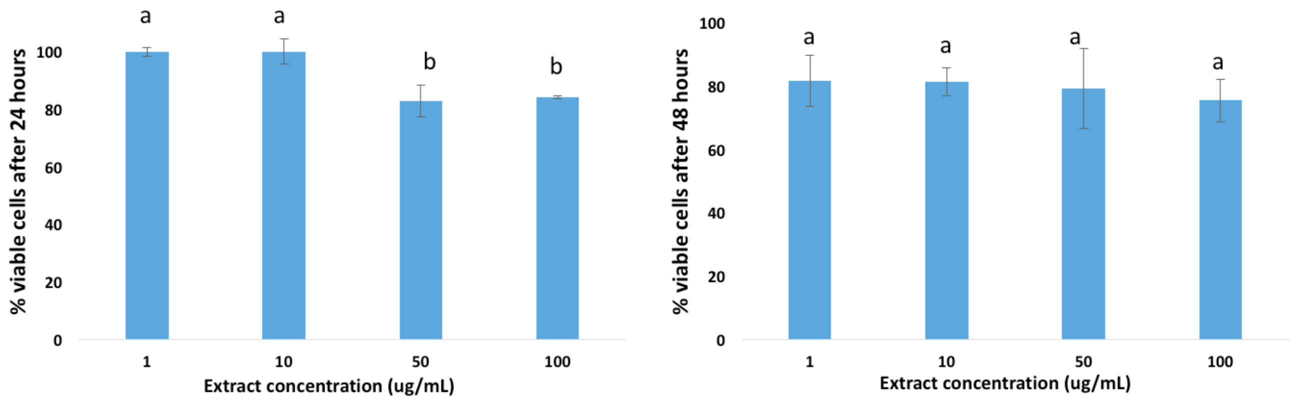


Figure 6.4: Cellular viability of human adipocytes after treatment with baobab fruit extract for 24 and 48 hours. After treatment with the baobab fruit pulp extract for 24 hours, the cellular viability revealed no toxicity for the concentrations of 1 and 10 µg/mL, whereas for 50 and 100 µg/mL there is slight toxicity but viability always remains above 80%. Progressing to 48 hours of treatment, all concentrations give a cellular viability of around 80%, and so all the concentrations tested with the baobab fruit pulp can be used for further experiments.

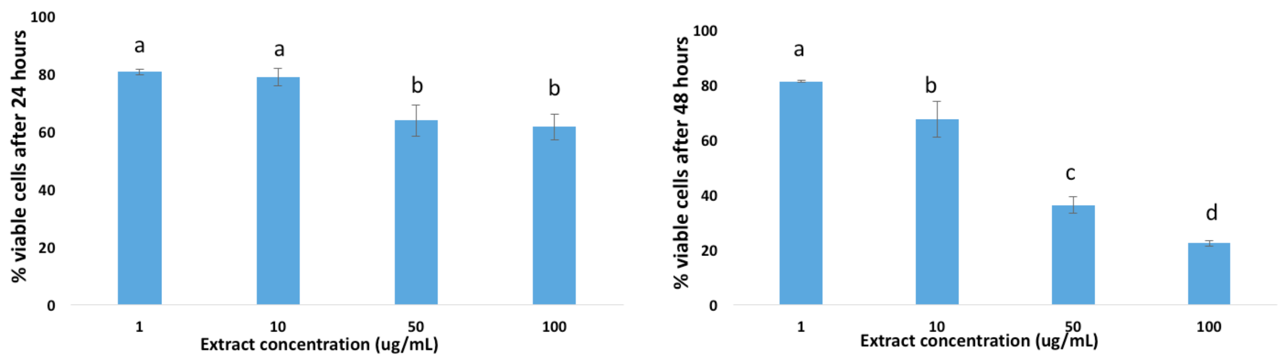


Figure 6.5: Cellular viability of human adipocytes after treatment with baobab leaf extract for 24 and 48 hours**.**

The baobab leaf extract was found to be the most toxic for cells, particularly after 48 hours of treatment. After 48 hours, only the concentration of 1 µg/mL reaches a cell viability of 80%, the others are below with the concentration of 100 µg/mL being the most toxic as it kills almost 80% of the cells. After 24 hours of treatment only the

concentrations 1 and 10 μg/mL reach 80% of viability, the others are around 60%. The toxicity after 24 hours of treatment is lower than the toxicity after 48 hours, but this is a bad indicator as future experiments may require more than one day of treatment. Compared to the baobab fruit and hibiscus extracts, the leaf extract is more toxic and caution must be taken when selecting concentrations for future studies.

6.3.3 Morphological studies

Studying the morphology of cells in culture (i.e., their shape and appearance) is essential for successful cell culture experiments. When coupled with cell counting (a method of counting or quantifying cells), the study can confirm cell health, inspecting cells by eye and microscope each time they are manipulated. This will quickly detect any signs of contamination or the presence of toxic agents. Morphological analysis has been conducted at the lowest and the highest doses tested (1 and 100 ug/mL) for 24 and 48 hours.

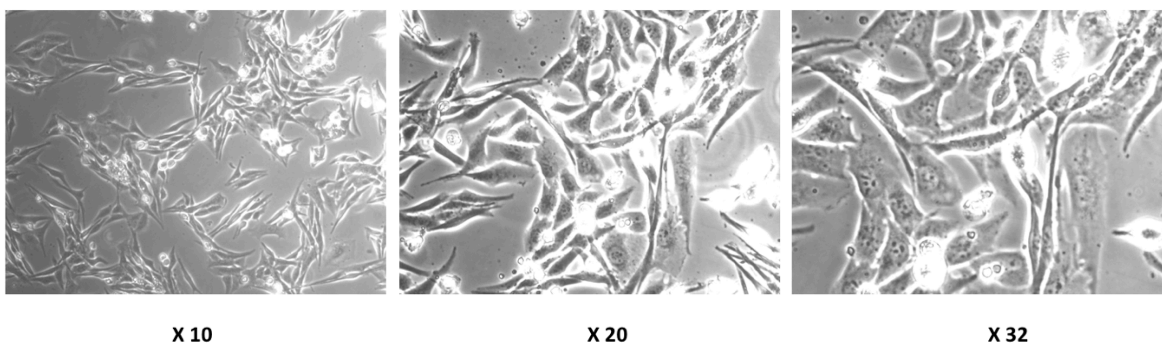


Figure 6.6: Morphology of human adipocytes after 48 hours without treatment.

In figure 6.6 above, control preadypocytes show a fibroblast-like morphology.

Treatment with baobab fruit extract (1 µg/mL) for 24 hours

Treatment with baobab fruit extract (1 µg/mL) for 48 hours

X 10 X 20 X 32

Figure 6.7: Morphology of human adipocytes after 24 and 48 hours of treatment with the baobab fruit pulp extract (1 µg/mL).

Treatment with baobab fruit extract (100 µg/mL) for 24 hours

Treatment with baobab fruit extract (100 µg/mL) for 48 hours

X 10 X 20 X 32

Figure 6.8: Morphology of human adipocytes after 24 and 48 hours of treatment with the baobab fruit pulp extract

(100 μg/mL).

Treatment with baobab leave extract (1 μg/mL) for 24 hours

Treatment with baobab leave extract (1 μg/mL) for 48 hours

X 10 X 20 X 32

Figure 6.9: Morphology of human adipocytes after 24 and 48 hours of treatment with the baobab leaf extract (1 μg/mL).

Treatment with baobab leave extract (100 μg/mL) for 24 hours

Treatment with baobab leave extract (100 μg/mL) for 48 hours

X 10 X 20 X 32

Figure 6.10: Morphology of human adipocytes after 24 and 48 hours of treatment with the baobab leaf extract (100 μg/mL)

Treatment with hibiscus flower extract (1 μg/mL) for 24 hours

Treatment with hibiscus flower extract (1 μg/mL) for 48 hours

X 10 X 20 X 32

Figure 6.11: Morphology of human adipocytes after 24 and 48 hours of treatment with the hibiscus flower extract (1 μg/mL).

Treatment with hibiscus flower extract (100 µg/mL) for 24 hours

Treatment with hibiscus flower extract (100 µg/mL) for 48 hours

X 10 X 20 X 32

Figure 6.11: Morphology of human adipocytes after 24 and 48 hours of treatment with the hibiscus flower extract (100 µg/mL).

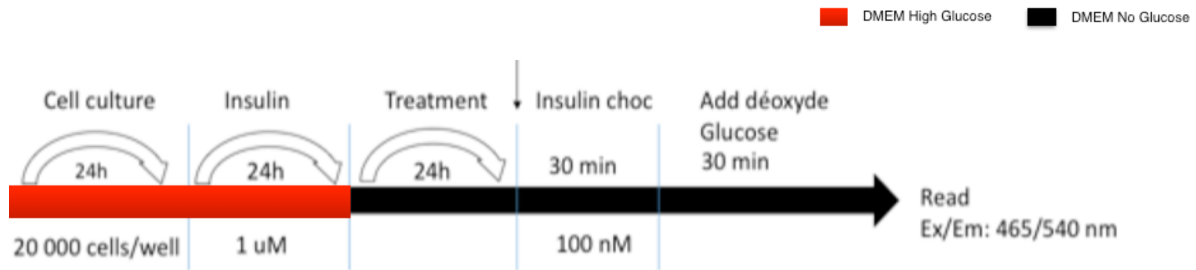
As can observed from the pictures above, no morphological changes were observed for the baobab fruit pulp and hibiscus flower extracts at all concentrations and exposure times (24 and 48 hours). In figure 6.10 we can see that there is a complete death of the adipocytes when they are treated with the concentration 100 µg/mL of the baobab leaf extract solution. This information goes in accordance with the data obtained in the MTS test as can be seen on figure 6.5, where treatment with baobab leaf extract can trigger the loss of up to 80 % of cellular viability.

6.3.4 Glucose uptake

Glucose is a ubiquitous energy source in most organisms and plays a pivotal role in cellular metabolism and homeostasis. 2-NBDG (2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino]-Dglucose) is a fluorescent deoxyglucose analog that can be taken up by cells through glucose transporters. 2-NBDG6P cannot be further metabolized and accumulates in cells, directly proportional to their glucose uptake. In this assay, 2-NBDG uptake is determined by a coupled enzymatic assay in which the 2-NBDG6P is oxidized, resulting in the generation of NADPH, which is then determined by a recycling amplification reaction in which the NADPH is utilized by glutathione reductase in a coupled enzymatic reaction that produces glutathione. Glutathione reacts with DTNB to

product TNB, with fluorescence which typically displays maximal excitation/emission at ~465/540 nm and can be visualized using optical filters designed for fluorescence (Jung et al., 2017; Bhakta et al., 2017).

After a series of preliminary experiments using low- and high-glucose culture media, it was found that adding insulin to the high glucose culture medium provided consistent results.



Studying culture in a high glucose medium allows for a better approximation of the conditions of insulin resistance, which was previously not the case with other culture media.

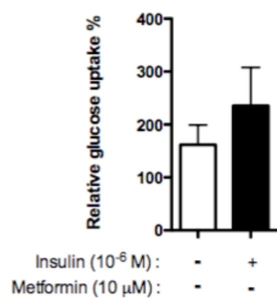


Figure 6.12: Explanatory schema of the final protocol. Use of High Glucose MEM culture medium for cell culture, induction of insulin resistance and use of no glucose DMEM for insulin shock, and addition of 2-NBDG.

In figure 6.12 above, the ability of 10 uM metformin (the insulin-sensitiser drug used here as a positive control) to increase glucose uptake is shown, thus counteracting insulin-resistance.

As can be ssen in figure 6.12 metformin increases the uptake of glucose in these cells.

6.4 Conclusions

The MTS and morphological studies have shown that the baobab fruit and hibiscus extracts were not toxic to adipocytes at all concentrations tested, while the baobab leaf extract was very toxic at a concentration of 100 μg/mL. These results will be taken into account while performing the tests on the inhibition of adipocyte differentiation by the extracts. Metformin, the positive control for glucose upatake, was found to be effective in increasing the uptake of glucose into the cells.

CHAPTER VII

IN-VIVO STUDY ON THE EFFECTS OF BAOBAB AND HIBISCUS ON THE LIPID METABOLISM OF WISTAR RATS

7.1 Introduction

As previously mentioned in chapter II, the metabolic syndrome is a cluster of disease risk factors which include hyperglycaemia, abdominal obesity, dyslipidaemia and hypertension which can lead to cardiovascular diseases like stroke and others. Hyperlipidaemia or dyslipidaemia, one of the disease risk factors for metabolic syndrome, is an abnormal increase in one or more plasmatic lipid components characterized by an increase in LDL-cholesterol (LDL-c), total cholesterol (CT), triglycerides (TG), and a reduction in HDL-cholesterol (HDL-c) (Vaessen et al., 2007; Subramanian et al., 2012). These lipids, especially LDL, can easily be oxidised and accumulate in the arteries causing atherosclerosis, which can lead to numerous pathologies like stroke, hypertension and well others (Simon et al., 2006).

In normal physiological conditions, the human body has various mechanisms for the regulation of plasmatic lipids, but when the lipid intake reaches an excess or when there is an uncontrolled hyper-production of cholesterol, hyperlipidaemia settles in and causes the various complications mentioned above (Raisonnier, 2004). Different types of blood lipid lowering drugs exist, including statins that inhibit HMG-CoA reductase, fibrates that reduce the hepatic synthesis of lipoproteins, ezetimibe that inhibits the intestinal absorption of bile cholesterol, among others (Katzung et al., 2011). These drugs have many side effects which can include hepatic toxicity, renal failure, gout, hyperglycaemia and myolysis (Ducobu, 2004; Bruckert et al., 2005). In such a situation, and as reported in chapter III, preventive measures can be taken to lower or maintain normal blood lipids levels, which include physical activity, diet and nutraceuticals. Food extracts are very complex and made up of different metabolites which may target different metabolic pathways thus avoiding some of the side effects drugs have due to over-inhibition of metabolic pathways.

For this purpose, the extracts of baobab fruit, leaves and Hibiscus flowers were investigated in an animal study to determine their effects on the blood lipids of hyperlipidemic rats. Baobab fruit powder and hibiscus flowers powder were also mixed with durum wheat (*Triticum Durum*) and used to make a functional pasta, with the aim to understand if the pasta making process and the cooking of the pasta could have any effects on the biological activity of these foods. It should be emphasised that the study is still ongoing and so the preliminary results obtained are based on a preliminary hibiscus extract and hibiscus pasta as used at the start of the study.

7.2 Materials and methods

7.2.1 Food materials

The food extract and the functional pasta were prepared as reported in section 5.3.3

7.2.2 Animal material

The animals consisted of adult male Wistar rats weighing between 200 to 250g. They were raised in the laboratory of the Laboratory of Biophysics & Biochemistry Food and Nutrition (LABBAN) where they had been fed on a normal diet (fish powder (20%), corn flour (59%), oil of soybeans (5%) (Table 7.1) and tap water. They were raised at room temperature and required humidity and subjected to a 12/12h light/dark cycle. The choice of rats for this study is justified by the sensitivity of their responses to nutritional conditions, and the fact that they are insensitive to environmental factors and have an omnivorous diet (Adrian et al., 1998).

		Normal diet		Lipid rich diet
	Ingredients	**Quantity (g/kg)**	**Energies (Kcal)**	**Quantity (g/kg)**
Proteins	Fish powder	200	800	140
Carbohydrates	Starch	590	2360	283
	Sugar	50	200	50
Lipids	Coco oil	-	-	250
	Egg yolk	-	-	300
	Soya oil	50	450	50
Others	Cellulose	50	-	-
	Minerals	50	-	50
	Vitamins	10	-	10
Total		1000	3810	1000

Table 7.1: Comparison between the normal and lipid rich diet

7.2.3 Induction of hyperlipidaemia

Hyperlipidaemia was induced by using the methods reported by Hamlat et al., 2008. Forty adult male rats were randomly divided in nine groups of five and tap water was used for the whole experiment, lasting 28 days. Seven groups (3, 4, 5, 6, 7, 8 and 9) were fed the lipid rich diet while the other two, groups 1 and 2 were fed with the normal diet so as to act as control groups. Body weight and remaining food in the different animal groups were evaluated every 3 and 1 days respectively. Table 7.1, above, shows the composition of the normal and the lipid rich diet. At the end of the experiment (after 28 days), one group of animals was randomly chosen amongst the groups

which received the lipid rich diet and another group was randomly chosen amongst the groups which received the normal diet, the chosen groups were culled and their lipid profiles determined to verify the onset of hyperlipidaemia.

7.2.4 Administration of the extracts to the hyperlipidemic animals

After the confirmation of hyperlipidaemia, the animals were treated with the food extracts and the functional pasta powder, with atorvastatin being administered to a group as a positive control. During the 14-day treatment period, the animals were fed with the normal diet reported in table 7.1 and the various treatments were administered every morning per os using an oesophageal probe. For this, the animals were randomly redistributed into the seven groups listed below;

- Normal control (N) made up of normal rats treated with 10 mL/kg of distilled water
- Negative control (HE) made up of hyperlipidemic rats treated with 10 mL/kg of distilled water
- Positive control (HA) made up of hyperlipidemic rats treated with 10 mg/kg of atorvastatin.
- Group 1 (HHNE1) made of hyperlipidemic rats treated with 250 mg/kg of Hibiscus flower extract.
- Group 2 (HHNE2) made of hyperlipidemic rats treated with 500 mg/kg of Hibiscus flower extract.
- Group 3 (HHE1) made of hyperlipidemic rats treated with 250 mg/kg of hibiscus functional pasta powder.
- Group 4 (HHE2) made of hyperlipidemic rats treated with 500 mg/kg of hibiscus functional pasta powder.

Parameters including body weight and food consumption were evaluated every 3 days for the whole length of the experiment (14 days). Animal faeces were collected at the beginning and at the end of the experiment. At the end of the experiment all the animals were starved for 12 hours, then anaesthetised by the inhalation of diethyl ether and blood was collected in heparinised tubes after a neck incision. The heart, liver, lungs, testicles, and the kidneys were collected, cleaned using glacial physiological water (0.9% sodium chloride solution) and then weighted for the evaluation of the relative body weight using the formula reported below:

$$\text{Relative weight} = \frac{Organ\ weight}{Animal\ weight} \text{ x } 100.$$

The collected blood was allowed to rest for 4 hours at room temperature (25°C) in its heparinised tubes, and then centrifuged for 10 minutes at 3000 rpm at 25°C. Plasma (the supernatant) was collected, aliquoted and conserved in

Eppendorf tubes at -20°C for further use in the quantification of lipid parameters such as total cholesterol, triglycerides and HDL-C.

Parts of the liver, heart, lungs testicles and kidneys were fixed in a solution of 10% formalin and contained in glass vases for histological sections. Finally, the remaining organs were crushed separately in a mortar, then 1 volume of crushed organ was homogenized with 9 volumes of phosphate buffer 0.1 M, pH 7.4. The organ homogenates obtained were conserved at -20°C for the quantification of transaminases and creatinine.

7.2.5 Evaluation of biochemical parameters

MONLAB test kits were used for the quantification of serum triglycerides (TG), with total cholesterol (TC) and HDL-cholesterol. LDL-cholesterol was determined by calculations.

7.2.5.1 Quantification of serum triglycerides

The triglycerides were quantified by the enzymatic method described by Kaplan et al. (1984). Quantification was accomplished by adding 10 μL of plasma, standard (R2), or distilled water to different test tubes and adding 1000 μL of enzymatic solution (R1). After homogenization and incubation at 37°C in a water bath for 5 minutes, absorbance was read at 500 nm after a further 60 minutes. The results were expressed by the formula reported below:

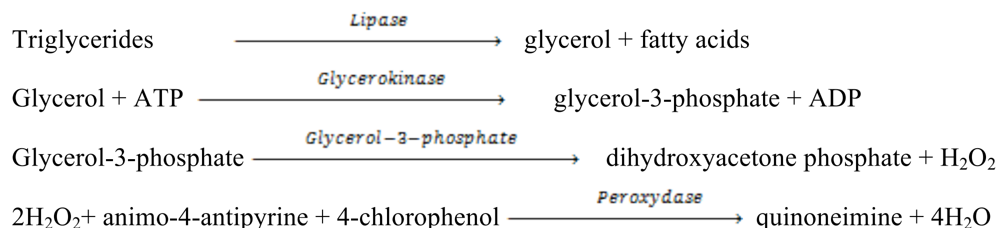
$$C_{sample} = 200 \times \frac{A\ sample}{A\ standard} \left(\frac{mg}{dL}\right)$$

Reagents	Composition	
R1	GOOD pH 6.3	(50mmmol/L)
	p-chlorophenol	(2 mmol/L)
	Lipoprotein lipase	(150000U/L)
	Glycerolkinase	(500U/L)
	Glycerol oxydase	(3500U/L)
	Peroxydase	(440U/L)
	4-aminophenazone	(0,1mmol/L)
R2	Standard	(200 mg/dL)

Table 7.2: Composition of kit used for total triglyceride determination.

This assay is based on the fact that under the action of lipases, the triglycerides (TG) are hydrolysed into glycerol and fatty acids. Glycerol is then transformed into hydrogen peroxide under the action of glycerol kinase and

glycerol-3-phosphate oxidase. The final step involves the formation of quinoneimine which absorbs strongly at 500 nm and thus serves as an indicator.



7.2.5.2 Quantification of total cholesterol (TC)

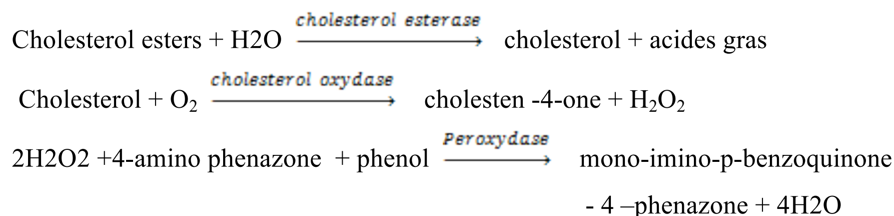
Quantification was accomplished by adding 10 µL of plasma, standard (R2) or distilled water to different test tubes and adding 1000 µL of enzymatic solution (R1). After homogenization and incubation at 37°C in a water bath for 5 minutes, absorbance was read at 500 nm after a further 60 minutes. The results were expressed by adopting the formula reported below:

$$C_{sample} = 200 \times \frac{A\ sample}{A\ standard} \left(\frac{mg}{dL}\right)$$

Reagents	Composition	
R1	PIPES pH 6,9	(90 mmol/L)
	Phenol	(26 mmol/L)
	Cholesterol esterase	(1000U/L)
	Cholesterol oxydase	(300U/L)
	Peroxydase	(650U/L)
	4-aminophenazone	(0,4mmol/L)
R2	Standard	(200mg/dL)

Table 7.3: Composition of kit for total cholesterol determination

Under the action of cholesterol esterase, esterified cholesterol is transformed into fatty acids. The oxidation of cholesterol in the presence of cholesterol oxidase produces cholesteren-4-one and hydrogen peroxide. Here too, quinineimine is formed from hydrogen peroxide, 4-aminoantipyrine and phenol under the action of peroxidase and serves as indicator.



7.2.5.3 Determination of HDL-cholesterol

Lipoproteins (chylomicrons, VLDL and LDL) were precipitated by adding phosphotungstic acid and magnesium chloride. After centrifugation, the clear supernatant containing the HDL fraction underwent the Monlab test for the determination of HDL-cholesterol, using the same procedure as the one reported for total cholesterol.

In practical terms, 200 μL of plasma and 500 μL of the precipitation reagent diluted 1-4 were added to a test tube. The tube was homogenised, allowed to rest for 10 minutes at room temperature, centrifuged at 4000 rpm for 10 minutes and the supernatant was collected. 100 μL of the supernatant, of the positive control (R2) or distilled water were added to different test tubes and 1000 μL of the cholesterol determination reagent were added (R1). After homogenisation and incubation at 37°C in a water bath, absorbance was read at 500 nm. The results were expressed according to the formula reported below:

$$\text{HDL-c} = 175 \times \frac{A.\ sample}{A.\ standard}\left(\frac{mg}{dL}\right)$$

Reagents	Composition
R1	Phosphotungstic acid (0,55 mmol/L)
	Magnesium chloride (25 mol/L)
R2	Standard (50 mg/dL)

Table 7.4: reagents used for the quantification of HDL-cholesterol

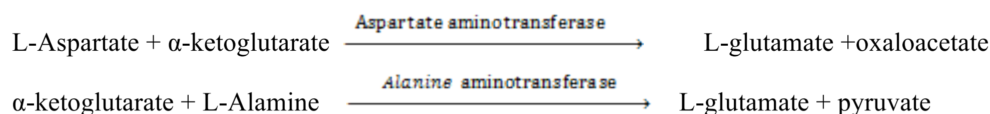
7.2.5.4 Determination of LDL-cholesterol

This was determined by direct calculation using the formula used by Friedwaldd et al., 1972. The concentration of LDL cholesterol is calculated from the difference between total cholesterol, HDL cholesterol and triglyceride concentration.

LDL-C (mg/dl) = Cholesterol total (mg/dl) - [HDL-C (mg/dl) +TG/5 (mg/dl)].

7.2.6 Quantification of serum transaminases

The levels of alanine and aspartate transferase (ALAT and ASAT) were determined by using the I MESCO kit according to the method used by Thomas, 1998. Alanine and aspartate transferase catalyse the reactions reported below:



ALAT and ASAT catalyse the transfer of an amino group respectively from aspartate to α-ketoglutarate to form the oxaloacetate, and from alanine to α-ketoglutarate to form pyruvate and L-glutamate. The oxaloacetate or pyruvate formed then reacts with 2,4-dinitrophenylhydrazyl (DNPH) to form a brown pyruvate hydrazone complex which absorbs at 546 nm in the presence of NaOH. The intensity of the absorbance is proportional to the quantity of pyruvate present and thus to the quantity of ASAT and ALAT.

7.2.6.1 Determination of ALAT

Test tubes were prepared with 0.1 mL of the samples and 0.5 mL of phosphate buffer, and the obtained solution was mixed and incubated at 37 °C for 30 minutes. After incubation, 0.5 mL of DNPH solution was added and the mixture incubated at 25 °C for 20 minutes. 5 mL of NaOH solution was then added and the absorbance read at 546 nm after 5 minutes.

7.2.6.2 Determination of ASAT

Substrate solution (0.1 mL) of ASAT was introduced into a test tube and preincubated for 5 minutes at 37°C. 0.02 mL of serum was added, the test tube incubated at 37°C for 1 hour, and 0.1 mL of coloration reagent was then added. The tubes were allowed to rest at room temperature for 20 minutes and the reaction stopped by the addition of 1 mL of NaOH 0.4 M. Absorbance was finally read at 505 nm.

7.2.7 Determination of creatinine

Creatinine was quantified according to the method using kit I MESCO. This quantification is based on the fact that in an alkaline medium, creatinine forms a yellow orange complex with picric acid. The intensity of the coloration formed is proportional to the amount of creatinine present in the medium. In practical terms, 1 mL of trichloroacetic acid and 1 mL of plasma were added to test tubes. The obtained mixture was mixed, centrifuged at 2500 rpm for 10 minutes at 37°C and the supernatant collected. 1 mL of the supernatant was then introduced into a test tube and 1 mL of the working solution, a mixture v/v of picric acid and NaOH, was added. The mixture obtained was allowed at rest for 20 minutes at 25 °C and the absorbance read at 520 nm. For the blank and positive control, instead of adding 1 mL of supernatants to the test tube, 0.5 mL of water and standard solution were added to the respective test tubes. The results were expressed using the formula reported below:

$$C_{sample} = 2 \times \frac{A\ sample}{A\ standard} \left(\frac{mg}{dL}\right)$$

7.2.8 Estimation of atherogenic index

The atherogenic index (IA) was determined by using the method from Solanki et al., 2010, in order to estimate the risk of cardiovascular events.

$$I_A = (VLDL_{-C} + LDL_{-C}) / HDL_{-C}$$

7.3 Statistical analysis

The obtained results were expressed as averages ± standard deviation. By using the software Statgraphics Centurion XV. II (Manugistics, Rockville, Maryland, USA, 1997), the analysis of variance (ANOVA) and the test by multiple comparisons DUNCAN were realized in order to appreciate the effects of different extracts on the measured parameters. The histograms were constructed with the software Sigmaplot. 11.0 (Systat software, C.A. USA). Significance was established at $p<0.05$.

7.4 Results and discussion

7.4.1 Effects of the lipid rich diet on animal body weight, lipid parameters and liver

The table 7.5 below shows the animal food consumption, gain in body weight, lipid parameters and hepatosomatic index of rats fed on the normal diet and the lipid rich diet. From the table it can be seen that the food consumption by both groups is not the same, the low food intake by animals fed on the lipid rich diet with respect to those fed with the normal diet could be a consequence of weight gain. Leptin, which is a hormone synthetized by adipocytic cells and crucial for the regulation of body weight, inhibits appetite through negative feedback. The more the stocks of lipids the more leptin is produced (Sherwood, 2004), leptin regulates weight gain at the level of the central nervous system through 3 targets. It decreases the release of dopamine (orexigenic effect) in the ventral tegmental area, decreases serotonin in the brainstem, increases the activity of the Pro-opiomelanocortin neurones (anorexigenic) and decreases the activity of Y neuropeptide (NPY) in the arcuate nucleus of the hypothalamus. All these actions lead to a decrease in appetite and consequently a decrease in food intake (Pénicaud et al., 2012; Sherwood, 2004). The weight gain in rats submitted to the lipid rich diet is higher than that of the rats fed with the normal diet, this means that the lipid rich diet provoked weight gain as a result of the accumulation of fats in the organism of the rats.

Parameters	Normal diet	Lipid rich diet
Food consumed (g/rat)	41,880,53[b]	23,91[a]
Weight gain (g/rat)	44,612,55[a]	85,132,05[b]
CT (mg/dL)	84,010,01[a]	144,583,80[b]
HDL-c (mg/dL)	49,690,60[b]	42,03±1,17[a]
LDL-c (mg/dL)	24,650,47[a]	116,362,85[b]
TG (mg/dL)	52,060,10[a]	83,155,88[b]
Hepato-somatic index	2,990,2[a]	3,960,6[b]

Table 7.5: CT: total cholesterol, C-HDL: HDL cholesterol, C-LDL: LDL cholesterol, TG: triglycerides, n=5. The data on the same line having different letters are statistically different with $p<0.05$.

The levels of triglycerides in rats fed on the lipid rich diet is higher than that of the rats fed on the normal diet, the increase in triglycerides and body weight are evident observations of a diet rich in fatty acids. The accumulated fatty acid can then be converted to cholesterol in the animal body (Nurjhan et al., 1992; Lewis et al., 2002), in fact, the total cholesterol and LDL-c are significantly higher ($p<0.05$) in the rats fed with the lipid rich diet than in the rats fed with the normal diet. Meanwhile the HDL-c levels are low in the rats fed with the high lipid diet compared to those fed with the normal diet. These results are in accordance with those obtained by Wansi et al., 2013, Ngacthic et al., 2013, and Aïssatou et al., 2017 who noted an increase in total cholesterol and LDL cholesterol in animals fed with lipid rich diet, of which the lipid source was coco oil.

The increase in cholesterol can also be due to the egg yolk, which is very rich in cholesterol, and of course coco oil which is mainly composed of long chain saturated fatty acids (Blackburn et al., 1988; Dayrit, 2003) known for their hypercholesterolemic potential (Hulshof et al., 2003). In fact, long chain fatty acids lower the activity of lecithin cholesterol-acyl-transferase which unbalances the inverse transport of cholesterol (Berard et al., 2004). Moreover, a diet rich in saturated fatty acids leads to a reduction in the activity of the LDL receptors, an increase in the hepatic synthesis of cholesterol LDL and of apoprotein B100 (Stad et al., 1996). The hyperlipidemic properties of this diet are further confirmed by the hepatosomatic index of the rats which is very high ($p<0.05$) (3.96±0.6) with respect to those fed with the normal diet (2.99±0.2).

7.4.2 Effect of the Hibiscus extract and pasta powder on the lipid parameters of hyperlipidemic rats

7.4.2.1 Effects on total cholesterol and LDL cholesterol

Cholesterol and lipids are fundamental for the organism, but an excess can lead to health issues. In fact, an increase in cholesterol is a risk factor for cardiovascular diseases, as mentioned in previous sections. The determination of blood cholesterol is a fundamental element in the diagnosis and classification of dyslipidaemia. Figure 7.1, reported below, shows the levels of cholesterol in the different groups of rats.

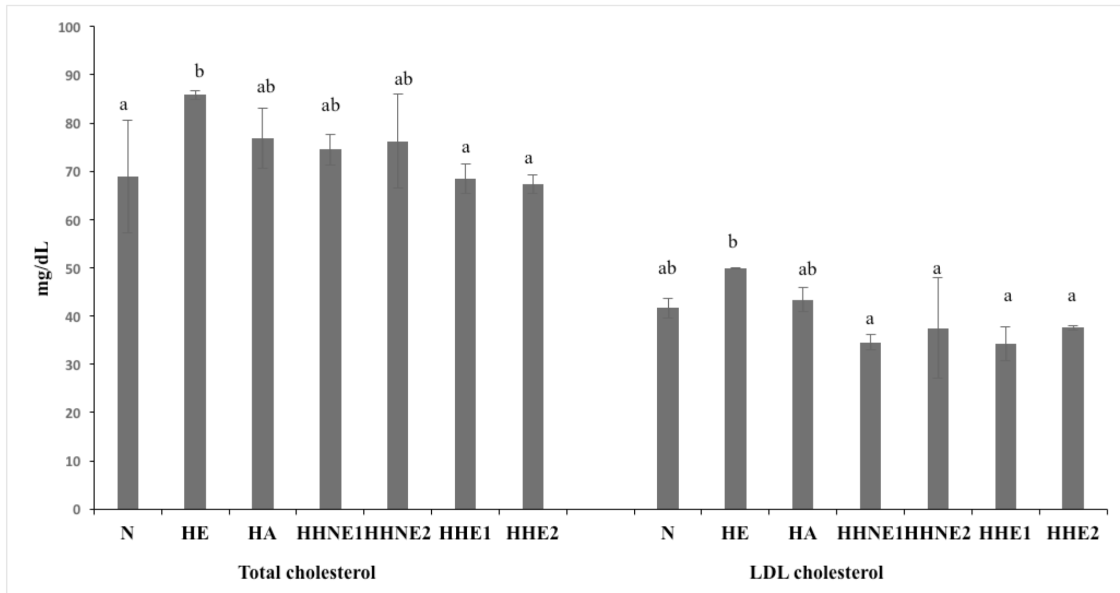


Figure 7.1: Levels of total cholesterol and LDL-c in hyperlipidemic rats treated with hibiscus extract and pasta powder. N: normal rats + distilled water, HE: hyperlipidemic rats + distilled water, HA: hyperlipidemic rats + atorvastatin (10 mg/kg), HHNE1: hyperlipidemic rats + hibiscus extract (250 mg/kg), HHNE2: hyperlipidemic rats + hibiscus extract (500 mg/kg), HHE1: hyperlipidemic rats + hibiscus pasta powder (250 mg/kg), HHE2: hyperlipidemic rats + hibiscus pasta powder (500 mg/kg), n=5. For each parameter the histogram bars with different letters are significantly different ($p<0.05$).

After two weeks of treatment, the levels of total cholesterol and LDL cholesterol were found to be lowered after the administration of hibiscus extract and pasta powder. In fact, the levels of total cholesterol and LDL cholesterol are significantly lower ($p<0.05$) in the treated groups HHNE1, HHNE2, HHE1 and HHE2 with respect to the negative control HE which was treated with distilled water. This effect is most marked in the groups treated with the hibiscus pasta powder HHE1 (250 mg/kg of hibiscus pasta powder) and HHE2 (500 mg/kg of hibiscus pasta powder) as they express the lowest levels of total cholesterol of 68.37±3.06 mg/dL and 67.31±1.88 mg/dL respectively for HHE1 and HHE2 which are almost equal to that of the normal control N (68.94±11.59 mg/dL) and even lower than that of

the positive control atorvastatin (76.88±6.29 mg/dL). The levels of LDL cholesterol are significantly lowered in all groups with values of 34.56±1.52, 37.47±10.45, 34.34±3.49 and 37.56±0.54 respectively for the groups HHNE1, HHNE2, HHE1 and HHE2. These values are even lower than those of the positive control treated with atorvastatin (43.41±2.44 mg/dL) and the normal control composed of healthy rats (41.63±1.98 mg/dL).

This lowering effect could be attributed to the components present in hibiscus flowers and flower extract, including fibres, flavonoids, phenolic acids, anthocyanins and tanins (Cissé et al., 2008; Ramirez-Rodriguez et al., 2011; Formagio et al., 2015). These compounds have the same hypolcholesterolemic effects as Ezetimibe and cholestyramine (Bansal et al 2009; Catapano et al 2014). So the mechanism of action of the biological effects of hibiscus could be similar, that is they may inhibit the intestinal absorption of cholesterol and/or complex bile acids thus favouring their faecal excretion. Bile acids are synthesised from cholesterol, so their complexation leads to the usage of plasma cholesterol to fulfil that lost in bile with the direct consequence of lowering blood cholesterol (Marfo et al 1990; Lasekan et al1995). Furthermore, as reported by Aïssatou et al., 2017, the mechanism could be the regulation of HMG-CoA reductase and LDL receptors, so further studies must be considered in order to understand the real mechanism behind this lowering of cholesterol.

The hibiscus pasta powder seems to have higher cholesterol lowering potential than the hibiscus extract, this may be due to the fact that the durum wheat somehow protects the bioactive components or the cooking process may enhance the activity of bioactive components. In any case, further studies have to be considered in order to fully understand this phenomenon.

7.4.2.2 Effect on the levels of HDL cholesterol

The lowering of HDL cholesterol, usually known as good cholesterol, increases cardiovascular risk. Figure 7.2 reported below shows the serum levels of HDL cholesterol in the different groups of treated hyperlipidemic animals.

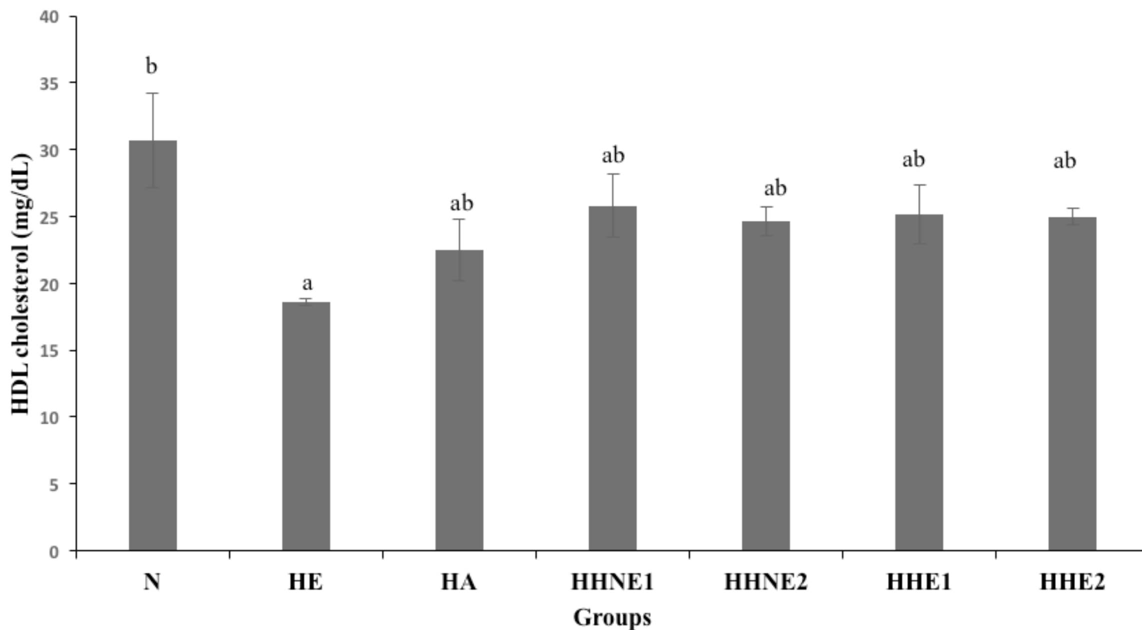


Figure 7.2: Levels of total HDL-c in hyperlipidemic rats treated with hibiscus extract and hibiscus pasta powder. N: normal rats + distilled water, HE: hyperlipidemic rats + distilled water, HA: hyperlipidemic rats + atorvastatin (10 mg/kg), HHNE1: hyperlipidemic rats + hibiscus extract (250 mg/kg), HHNE2: hyperlipidemic rats + hibiscus extract (500 mg/kg), HHE1: hyperlipidemic rats + hibiscus pasta powder (250 mg/kg), HHE2: hyperlipidemic rats + hibiscus pasta powder (500 mg/kg), n=5. For each parameter histogram bars with different letters are significantly different ($p<0.05$).

From figure 7.2 we can see that the levels of cholesterol with values of 25.79±2.35, 24.63±1.05, 25.16±2.16 and 24.98±0.64 mg/dL, respectively for the groups HHNE1, HHNE2, HHE1 and HHE2, are statistically higher than that of the negative control with the value of 18.61 ± 0.25 mg/dL. From the statistical analysis, there are no significant differences in the different treatment groups as they all have the same letters on the histogram bars. As previously reported, the noticed increase in HDL cholesterol may be due to the bioactive components present in hibiscus which may up-regulate the genes responsible for the synthesis of apo-A1 (Dievart et al., 2007).

7.4.2.3 Effects on the levels of triglycerides

Hypertriglyceridemia is a risk factor for cardiovascular diseases. The reduction in triglycerides leads to a decrease in atherogenicity, as the presence of triglycerides causes a delay in the catabolism of triglyceride rich lipoproteins and induces the appearance of dense and more oxydable LDL. The figure 7.3 reported below shows the levels of triglycerides in the different groups.

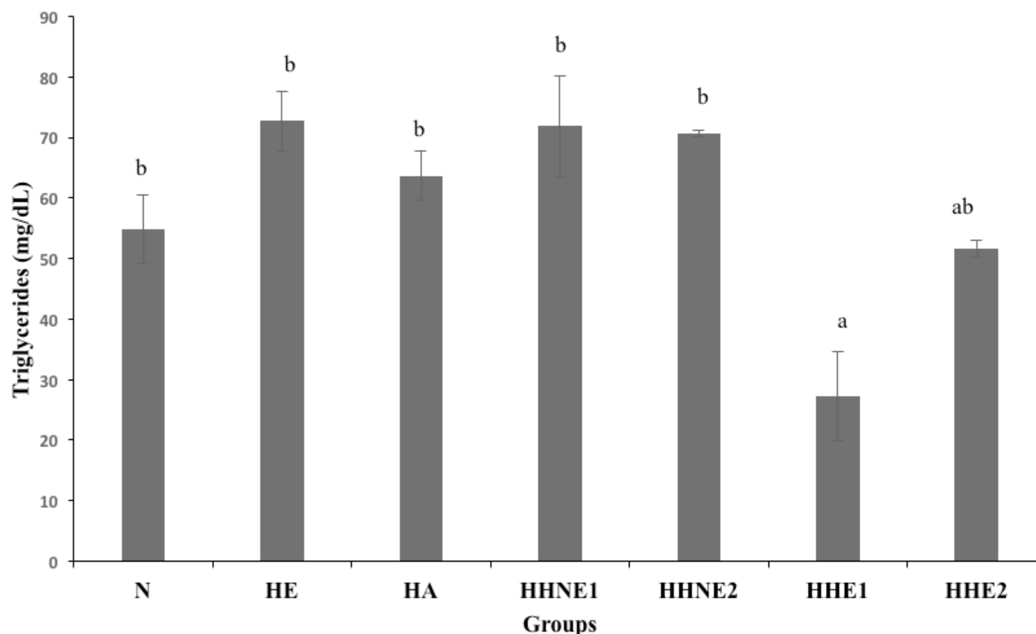


Figure 7.3: Effect of hibiscus extract and pasta powder on the levels of animal triglycerides. N: normal rats + distilled water, HE: hyperlipidemic rats + distilled water, HA: hyperlipidemic rats + atorvastatin (10 mg/kg), HHNE1: hyperlipidemic rats + hibiscus extract (250 mg/kg), HHNE2: hyperlipidemic rats + hibiscus extract (500 mg/kg), HHE1: hyperlipidemic rats + hibiscus pasta powder (250 mg/kg), HHE2: hyperlipidemic rats + hibiscus pasta powder (500 mg/kg), n=5. For each parameter the histogram bars with different letters are significantly different ($p<0.05$).

The hibiscus pasta powder lowers the level of hyperlipidemic rat serum triglycerides to 27.21 ± 7.42 mg/dL and 51.65 ± 1.34 mg/dL for HHE1 and HHE2 respectively, and these are statistically lower than those of the negative control (72.76 ± 4.96 mg/dL) and the positive control (63.65 ± 1.34 mg/dL). The groups treated with the hibiscus extract, HHNE1 and HHNE2, are not statistically different from the negative control and the positive control. Many studies have reported the efficacy of the extracts of medicinal plants on the activity of the enzymes involved in the

metabolism of triglycerides, for example Aïssatou et al., 2017 and Ntchapda et al., 2017, showed that the aqueous extract of the tubers of *Taca leontopetaloides* L. and the leaves of *Cassia occidentalis* reduce blood triglycerides levels. The hypotriglyceridemic effect could be attributed to a decrease in the activity of acylcoA synthetase, the inhibition of tessutal lipase and of acetylCoA carboxylase.

So we can say that the hibiscus pasta powder is more efficient in the reduction of blood triglycerides, but further studies need to be performed in order to fully understand this phenomenon.

7.4.3 Effects of the hibiscus extract and pasta powder on the food intake, weight gain, ratio total cholesterol/HDL-cholesterol and on the ratio LDL cholesterol/HDL cholesterol.

Groups	Food intake (g)	Change in body weight (%g)	LDL/HDL	CT/HDL
N	100,23±5,29[a]	2,87±0,71[b]	1,64±0,50[a]	3,89±1,92[a]
HE	101,11±5,37[a]	5,19±3,93[c]	1,62±0,42[a]	3,26±0,14[a]
HA	100,67±7,66[a]	-0,63±0,01[a]	1,98±0,34[a]	4,68±1,88[a]
HHNE1	99,34±2,56[a]	-0,10±0,30[a]	1,52±0,29[a]	3,14±0,41[a]
HHNE2	99,90±7,61[a]	-1,03±0,51[a]	1,46±0,38[a]	3,01±0,35[a]
HHE1	96,97±6,26[a]	-0,10±0,40[a]	1,83±0,30[a]	3,78±0,95[a]
HHE2	100,39±6,71[a]	-1,02±0,02[a]	1,99±0,23[a]	4,03±1,06[a]

Table 7.6: Change in body weight, food intake and the ratio CT/HDL and LDL/HDL of rats treated with hibiscus extract and pasta powder. N: normal rats + distilled water, HE: hyperlipidemic rats + distilled water, HA: hyperlipidemic rats + atorvastatin (10 mg/kg), HHNE1: hyperlipidemic rats + hibiscus extract (250 mg/kg), HHNE2: hyperlipidemic rats + hibiscus extract (500 mg/kg), HHE1: hyperlipidemic rats + hibiscus pasta powder (250 mg/kg), HHE2: hyperlipidemic rats + hibiscus pasta powder (500 mg/kg), n=5. For each parameter, different superscript letters represent significantly differences ($p<0.05$).

From table 7.6 it can be observed that the quantity of food consumed by the animals is almost the same in all the groups, as there is no statistically significant difference. This implies that extract and pasta powder affect the animal appetite similarly at both doses (250 and 500 mg/kg). The weight change was calculated by using the following formula:

Change in body weight = 100 × $\frac{Initial\ weight}{Final\ weight}$

The weight gain in the various groups is statistically less than that of the negative control. As previously reported, this difference may be due to bioactive compounds present in hibiscus that inhibit the accumulation of cholesterol and triglycerides.

The ratios of total cholesterol/HDL and LDL/HDL are the best indicators of cardiovascular diseases. The values should be strictly inferior or equal to 3.5 for the LDL/HDL ratio and 5 for the total cholesterol/HDL ratio. Also the LDL/HDL ratio or ratio atherogenicity helps to determine the predisposition of a subject to atherosclerosis. These ratios don't change across the different groups of animals. The fact that there is no lowering of this ratio with respect to the negative control may be justified by the fact that the experiment only lasted for two weeks.

7.4.4 Effects of the hibiscus extract and pasta powder on the levels of transaminase and creatinine of the kidney, liver and heart.

Creatinine is a product of the non enzymatic break down of creatine, a compound present in all muscular tissues and strictly eliminated by the kidneys. As such, creatinine is an important marker of the renal function, its increase or decrease reflect an impairment in the function of the kidneys. In this study, as can be seen on table 7.7, the levels of creatinine of the different tissues (kidneys, heart and liver) in the different groups of animals are not statistically different from those of the normal control, thus implying that the kidneys were functioning correctly and there hadn't been renal impairment.

ALAT and ASAT are enzymes with important metabolic activity inside the cells (Wallace et al., 2010). Their increase in organs reflects a cellular injury, especially in the liver (Kew et al 2000). The levels of ASAT and ALAT in heart tissue remained within the normal limits in the groups HHNE1, HHNE2, HHE1 and HHE2, compared to the normal control. These levels were statistically greater ($p<0.05$) than those of the normal control in the liver and kidney tissues. The high levels of ASAT and ALAT compared to the positive control in the liver and kidney tissue may be due to the provoked hyperlipidemia using the lipid rich diet (Gloria et al., 2010; Carip, 2014).

Groups	**Kidneys**			**Liver**			**Heart**	
	ALAT	ASAT	CREAT	ALAT	ASAT	CREAT	ALAT	ASAT
N	130±14[a]	305±1[a]	0,15±0,05[c]	1315±134	587±17[bc]	0,11±0,00[b]	750±56[de]	310±14[c]
HE	345±7[e]	426±5[e]	0,13±0,01[bc]	2670±552[bc]	805±7[e]	0,13±0,01[b]	800±113[e]	542±3[d]

HA	225±7^{d}	410±14^{d}	0,14±0,01^{c}	3225,00±167^{bc}	740±14^{d}	0,01±0,00^{a}	640±14^{abc}	272±3^{ab}
HHNE1	185±7^{b}	352±4^{b}	0,14±0 ,02^{c}	2700±240^{bc}	577±3^{b}	0,03±0,01^{ab}	680±0^{bcd}	240±14^{a}
HHNE2	195±7^{bc}	383±3^{c}	0,11±0,01^{bc}	2395±445^{ab}	607±9^{c}	0,10±0,11^{ab}	585±21^{ab}	239±29^{a}
HHE1	212±3^{d}	317±4^{a}	0,02±0,02^{a}	3650±395^{c}	542±11^{a}	0,09±0,01^{ab}	525±7^{a}	241±2^{a}
HHE2	245±7^{d}	341±2^{b}	0,07±0,01^{ab}	3400±212^{bc}	761±1^{d}	0,08±0,01^{ab}	715±49^{cd}	288±16^{bc}

Table 7.7: Levels of creatinine (mg/dL) and transaminases (U/L) in the heart, kidney and liver tissue of the animals. N: normal rats + distilled water, HE: hyperlipidemic rats + distilled water, HA: hyperlipidemic rats + atorvastatin (10 mg/kg), HHNE1: hyperlipidemic rats + hibiscus extract (250 mg/kg), HHNE2: hyperlipidemic rats + hibiscus extract (500 mg/kg), HHE1: hyperlipidemic rats + hibiscus pasta powder (250 mg/kg), HHE2: hyperlipidemic rats + hibiscus pasta powder (500 mg/kg), n=5. For each parameter the columns having different superscript letters are significantly different ($p<0.05$).

7.4.5 Effects of the hibiscus extract and pasta powder on the relative weight of the organs

The relative weight of an organ indicates the weight increase of the organ with respect to the body weight and this is calculated by applying the formula reported below:

Relative organ weight = $100 \times \frac{Organ\ weight}{Body\ weight}$

The change in the relative organ weight is an indication of toxicity, an increase of this ratio after the consumption of a substance implies that the substance is toxic.

Groups	**Relative weight of the organs (g)**				
	Kidneys	Liver	Heart	Lungs	Testicles
N	0,64±0,07^{a}	3,16±0,21^{b}	0,47±0,02^{b}	0,96±0,24^{b}	1,14±0,16^{d}
HE	0,52±0,09^{a}	2,81±0,28^{ab}	0,29 0,01^{a}	0,76±0,21^{ab}	0,91±0,18^{bcd}
HA	0,48±0,12^{a}	2,82±0,53^{ab}	0,28±0,06^{a}	0,65±0,05^{a}	0,70±0,03^{ab}
HHNE1	0,46±0,90^{a}	2,77±0,23^{ab}	0,29±0,03^{a}	0,56±0,20^{a}	0,70±0,08^{ab}
HHNE2	0,43±0,04^{a}	2,62±0,14^{a}	0,30±0,02^{a}	0,81±0,24^{a}	0,86±0,23^{a}
HHE1	0,59±0,12^{a}	2,94±0,09^{ab}	0,44±0,18^{b}	0,95±0,23^{b}	1,02±0,16^{cd}
HHE2	0,85±0,70^{a}	3,05±0,48^{ab}	0,33±0,05^{ab}	0,64±0,14^{a}	0,90±0,20^{bc}

Table 7.8: Relative weight of the organs in the different animal groups.N: normal rats + distilled water, HE: hyperlipidemic rats + distilled water, HA: hyperlipidemic rats + atorvastatin (10 mg/kg), HHNE1: hyperlipidemic rats + hibiscus extract (250 mg/kg), HHNE2: hyperlipidemic rats + hibiscus extract (500 mg/kg), HHE1:

hyperlipidemic rats + hibiscus pasta powder (250 mg/kg), HHE2: hyperlipidemic rats + hibiscus pasta powder (500 mg/kg), n=5. For each parameter the columns having different superscript letters are significantly different ($p<0.05$).

From the table 7.8 above the relative weight of the organs of the rats treated with hibiscus flower extract and pasta powder can be observed as lower than those of the normal control treated with distilled water. So we can say that the hibiscus extract and pasta creates no metabolic and clinical alteration in the Wistar rats.

7.5 Conclusion

This experiment, with the aim to study the hypolipidemic properties of hibiscus flower and pasta on hyperlipidemic Wistar rats, showed that the hibiscus extract and pasta powder significantly lower the levels of total cholesterol, LDL, triglycerides and increase the levels of HDL. The most significant effect was shown by the hibiscus pasta on the levels of triglycerides. Also, the treatment of the animals with the extracts and the pasta trigger a loss in weight but without altering metabolic and clinical parameters. We can conclude by saying that the hibiscus pasta seems to be more efficient, but the exact mechanisms by which these extracts and pasta mediate their effects remain unknown so future studies will have to focus on this in order to have a clear picture of the biological activity observed.

CHAPTER VIII

CHEMICAL CHARACTERIZATION OF BAOBAB FRUIT PULP, LEAVES AND *HIBISCUS SABDARIFFA* EXTRACT BY HPLC-MS AND NMR

8.1 Introduction

The scientific literature discussing the chemical composition of fresh baobab fruit is mainly focused on certain specific compounds. The ascorbic acid content has been found to be at least 3 times higher than that of other typical food sources of vitamin C (i.e. orange, kiwifruit, red fruits) (Vertuani, Braccioli, Buzzoni, & Manfredini, 2002). Furthermore, it has been reported that baobab fruit pulp is also rich in vitamins B_1, B_2, and B_6 (Diop et al., 2006) and in mineral salts (i.e. P, Mg, Ca, K, Na and Fe) (Glew et al., 1997). Baobab fruit pulp can contain as much as 56% pectin (Nour, Magboul, & Kheiri, 1980) and is rich in essential and nonessential fatty acids (i.e. linoleic acid, linolenic acid, oleic acid) (Glew et al., 1997). In 2017, Li et al., (2017) isolated and identified some polyphenolic compounds from dried baobab fruit pulp (i.e. four hydroxycinnamic acid glycosides, six iridoid glycosides, and three phenylethanoid glycosides).

Baobab leaves are traditionally used as a food in Africa, but are yet to be authorized as a novel food in Europe. Baobab leaves have been reported to be rich in palmitic acid, oleic acid and linoleic acid, and are an excellent source of Ca, K, Mg and Fe. Their vitamin content is also remarkable, with young leaves being rich in Vitamins C, B_1, B_2, B_3, and β-carotene. Baobab leaves contain mucilage (9 – 12%), which explains their traditional use as thickening agent in South-African culture. Phenolic acids (gallic and ellagic acids), hydroxycinnamic acids (chlorogenic and caffeic acid), flavonols (rutin, quercitrin, quercetin, and kaempferol), flavan-3-ols (catechin and epicatechin), and a flavone (luteolin) were identified in fresh baobab leaves through reverse-phase high-performance liquid chromatography with a diode-array detection (RP-HPLC-DAD) method (Irondi, Akintunde, Agboola, Boligon & Athayde, 2017).

Hibiscus sabdariffa flowers are also traditionally used as a food in herbal drinks, in hot and cold beverages, as a flavouring agent in the food industry and as a herbal medicine. Early studies reported that *Hibiscus sabdariffa* flowers contain macronutrients including proteins (1.9 g/100g), fat (0.1 g/100g), carbohydrates (12.3 g/100g) and fibres (2.3 g/100g). They are also rich in micronutrients like vitamin C (14 mg/100g), B-carotene (300 μg/100g), calcium /1.72 mg/100g), iron (57 mg/100 g) and polyphenols, including phenolic acids, organic acids, and flavonoids. The main constituents of these flowers relevant to their pharmacological activity are the organic acids, anthocyanins, polysaccharides and flavonoids (Da-Costa-Rocha I et al 2013).

However, despite the growing importance of baobab fruit and leaves due to their nutritional and biological properties, data on their primary and especially their secondary metabolites are extremely sparse in literature, and

little is known about their concentration of nutrients and bioactive components to date. On the other hand, *Hibiscus sabdariffa* has some available data regarding its chemical characterization but a deep insight into its chemical profile is still needed.

The analytical tools most often used to obtain a comprehensive picture of the composition of such foods are nuclear magnetic resonance (NMR) and mass spectrometry (MS) based metabolomic approaches, allowing for “high-throughput” spectroscopic/structural information on a wide range of compounds with high analytical precision (Sobolev et al., 2015). Metabolomics is a technology-driven approach advanced by recent developments in analytical tools, software, and statistical data analysis.

Here, we report the identification and quantification of primary and secondary metabolites occurring in extracts obtained from baobab fruit pulp, dried leaf and hibiscus dried flower samples using a multi-methodological approach, which consists of the combination of untargeted NMR spectroscopy and targeted reverse-phase high-performance liquid chromatography with photodiode array detection coupled to electrospray ion-trap mass spectrometry (RP-HPLC-PDA-ESI-MS/MS). The combination of these methodologies has been optimized in our previous investigations of plant foods and beverages (Mannina, Sobolev, Di Lorenzo, Vista, Tenore, & Daglia, 2015; Marchese, Coppo, Sobolev, Rossi, Mannina & Daglia, 2014, Sobolev et al., 2018). The choice of dried samples instead of fresh samples was made in view of the most common forms of baobab consumption worldwide, both in the countries of origin and in importing countries.

8.2 Materials and methods

HPLC-grade water was obtained from a LC-Pak™ Millex system (Millipore Coorporation, Billerica, MA, USA). Formic acid, MS grade methanol, citric acid, galacturonic acid, malic acid, glucose, sucrose, gallic acid, and caffeine were obtained from Sigma-Aldrich, St. Louis, MO, USA. Quinic acid, hydroxycitric acid, chlorogenic acid, delphinin sambubioside, cyanidin sambubioside, catechin, procyanidin B1, myricetin, procyanidin C1, kaempferol glucoside, kaempferol, rutin, and quercetin rhamnoside were obtained from PhytoLab, Vestenbergsgreuth, Germany.

8.2.1 Food materials

The food materials were obtained as reported in section 5.3.1.

8.2.2 Preparation of extracts from baobab fruit pulp, leaf and *Hibiscus sabdariffa* flowers

8.2.2.1 Extraction process for RP-HPLC-PDA-ESI-MS/MS analysis

Powdered baobab fruit pulp, dried leaves and hibiscus flowers were ground into fine powders prior to extraction. Each extraction was carried out separately by weighing 10 g of each food powder into a conical flask and adding 100 mL of a 50:50 (*v*/*v*) mixture of methanol and Millipore grade water acidified with formic acid (0.1%). The conical flask, immersed in ice, was left under agitation for 24 hours in a nitrogen atmosphere. Then, the extracts were filtered under vacuum through paper filters, and methanol was evaporated under nitrogen flux. Finally, the obtained extracts were freeze-dried. The various extracts were dissolved in Millipore grade water prior to chromatographic analysis.

8.2.2.2 Extraction process for NMR untargeted analyses - Bligh-Dyer extraction

Powdered baobab fruit pulp and dried leaves were submitted to extraction according to a modified Bligh–Dyer methodology (Bligh, & Dyer, 1959). A mixture of methanol/chloroform (2:1 *v*/*v*) was used to extract the greatest number of metabolites. The mixture (3 mL) was added to the sample (0.5 g) and agitated, followed by the addition of 1 mL of chloroform and 1.2 mL of Millipore grade water, and the emulsion was stored at 4 °C for 40 min after stirring. The sample was then centrifuged (800 *g* for 15 min at 4 °C). The upper (hydroalcoholic) and lower (organic) phases were carefully separated. The pellets were re-extracted using half of the solvent volumes (in the same conditions described above) and the separated fractions were pooled. Both fractions were dried under a nitrogen flow at room temperature until the solvent was completely evaporated. The dried phases were stored at -20 °C pending NMR analysis.

8.2.3 RP-HPLC-PDA-ESI-MS/MS analysis of the food extracts

Chromatographic analyses were performed using a Thermo Finnigan Surveyor Plus HPLC apparatus equipped with a quaternary pump, a Surveyor UV-Vis photodiode array detector (PDA), and a LCQ Advantage max ion trap mass spectrometer (all from Thermo Fisher Scientific, Waltham, MA, USA), coupled through an ESI source. RP-HPLC-PDA-ESI-MS/MS data were acquired under positive and negative ionization modes, using Xcalibur software. The ion trap operated in full scan (100-2000 *m/z*), data dependent scan and MSn modes; when greater discrimination was required, additional targeted MS^2 and MSn experiments were performed on selected pseudomolecular ions. To

optimize the MS operating conditions, a preliminary experiment was performed: 10 μg/mL caffeine (H_2O/MeOH: 50/50 with 0.1% formic acid) and 10 μg/mL gallic acid (H_2O/MeOH: 50/50 with 0.1% formic acid) solutions were directly infused through the ESI interface at a flow rate of 25 μL/min into the mass spectrometer. Optimized conditions were as follows: sheath gas 60, capillary temperature 220°C, auxiliary gas 25 and 20, spray voltage 4.5 and 5 kV, and capillary voltage at -26.13 V and 35 V respectively for negative and positive ionization modes.

8.2.3.1 HPLC separation of the baobab fruit pulp, leaves and hibiscus extracts

Separation of the baobab fruit pulp and dried leaf extracts was achieved on a Synergi Fusion RP18 80A (150 x 4.6 mm; 4 um) column, while the hibiscus extract was separated on a Luna Omega Fusion RP18 100A° (150 x 2.1 mm; 3 um) column, both operating at 25 °C and protected by their corresponding guard columns, both from Phenomenex, California, USA. A gradient elution was executed with acidified water (0.1% formic acid) as mobile phase A and methanol as mobile phase B, at a flow rate of 0.3 mL/min. The elution gradient for the separation of the baobab fruit pulp extract involved moving from 10% B to 70% B in 104 min, 70% B to 80% B in 5 min, 80% B to 100% B in 5 min, a 5 min isocratic of 100% B, 100% B to 10% B in 5 min, and finally an isocratic run of 10% B for 6 min. The elution gradient for the separation of the baobab leaf extract involved moving from: 10% B to 70% B in 84 min, 70%B to 80% B in 5 min, 80% B to 100% B in 5 min, 100% B for 5 min, 100% B to 10% B in 5 min, and finally 10% B for 6 min. The elution gradient for the separation of hibiscus extract consisted of moving from 2% of B to 70% of B in 5 min, 70% B to 80% B in 100 min, 80% to 100% B in 15 min, 100% B for 20 min, 100% B to 105 in 15 min, and finally 10% to 2% in 5 min. The temperature of the sample tray was set to 4 °C and the injection volume was 5 μL. Chromatograms for both baobab extracts were recorded at λ 254, 280, 330 nm while the chromatograms were recorded at λ 254, 280, 520 nm for the hibiscus extract. For all the extracts, spectral data were collected in the range of 200-800 nm for all peaks.

RP-HPLC-PDA-ESI-MS/MS analyses were performed in positive and negative ionization modes to obtain maximum information on the chemical composition of baobab fruit pulp, leaf and hibiscus extracts. Compounds were characterized on the basis of their UV-Vis and mass spectra, checking the molecular ion and fragment ions against fragmentation patterns of standard molecules, where possible, and with molecules described in the literature.

8.2.4 NMR analysis

NMR spectra were recorded at 27 °C on a Bruker AVANCE 600 spectrometer operating at a proton frequency of 600.13 MHz and equipped with a Bruker multinuclear z-gradient 5 mm probe head.

The dried hydroalcoholic phase of each sample was solubilized in 0.7 mL 400 mM phosphate buffer/deuterium oxide (D_2O), containing a 1 mM solution of (trimethylsilyl)-propionic–2,2,3,3-d_4 acid sodium salt (TSP) as an internal standard, and then transferred into a 5 mm NMR tube. The dried organic fraction of each sample was dissolved in 0.7 mL of a deuterated chloroform/deuterated methanol ($CDCl_3/CD_3OD$) mixture (2:1 *v*/*v*) and then placed into a 5 mm NMR tube. In order to avoid solvent evaporation, tubes were flame-sealed.

^{1}H NMR spectra of hydroalcoholic and organic extracts were referenced against the methyl group signal of TSP (δ= 0.00 ppm) in D_2O, and the residual deuterated methyl group (CHD_2) signal of methanol (set to 3.31 ppm) in a $CD_3OD/CDCl_3$ mixture, respectively. ^{1}H spectra of hydroalcoholic extracts were acquired using 256 transients and a recycle delay of 5 s. The residual deuterated water group (HDO) signal was suppressed using a pre-saturation pulse sequence. The experiment was carried out using a 45° pulse of 6.68 μs, and 32K data points. ^{1}H spectra of extracts in $CD_3OD/CDCl_3$ were acquired using 512 transients, a recycle delay of 5 s and a 90° pulse of 10 μs, with 32K data points. Two-dimensional (2D) NMR experiments, namely ^{1}H-^{1}H Total Correlation Spectroscopy (TOCSY), ^{1}H-^{13}C Heteronuclear Single Quantum Coherence (HSQC) and ^{1}H-^{13}C Heteronuclear Multiple-Bond Correlation (HMBC), were carried out under the same experimental conditions as previously reported (Mannina, Cristinzio, Sobolev, Ragni, & Segre, 2004; Capitani et al., 2013).

The mixing time for the ^{1}H–^{1}H TOCSY experiment was 80 ms, the ^{1}H–^{13}C HSQC experiments were performed using a coupling constant $^1J_{C–H}$ of 150 Hz, and the ^{1}H–^{13}C HMBC experiments were performed using a delay of 80 ms for the evolution of long-range couplings (Braun, & Kalinowski, 1998).

Pulsed field gradient spin echo (PGSE) experiments (Stilbs, 2010) were carried out with a pulsed field gradient unit, producing a magnetic field gradient in the *z*-direction with a strength of 55.25 G/cm. The sinusoidal gradient pulse was given a duration δ of 2.6 ms, with an intensity which was incremented in 32 steps, from 2% up to 95% of the maximum gradient strength, with a diffusion time Δ of 100 ms.

Signal area integration was used for quantitative analysis. Note that the quantitative analysis was performed on those metabolites not affected by overlapping peaks or deconvoluted peaks, using the program DIM2015 (Massiot et al., 2002). For water-soluble metabolites, the integrals of selected ^{1}H resonances were measured with respect to the integral of the TSP signal normalized to 1000 and used as an internal standard.

In the case of $CDCl_3/CD_3OD$ spectra, the integrals were labelled by numbers (I_1-I_{10}) for the calculations. First, integrals were normalized with respect to the integral of α-CH_2 groups for all fatty acid chains, I_4 (esterified fatty acids, α−CH_2, 2.35 ppm) + I_3 (free fatty acids, α−CH_2, 2.29 ppm) set to 100%. Then, the total content (% mol) of fatty acids of four different types (saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), linoleic fatty acid, and linolenic fatty acid) was calculated according to equations (1-4).

Lipid fractions (fatty acids, β-sitosterol, and squalene) were calculated according to equations (5-7).

Distribution of esterified fatty acids in pulp among phosphatidylethanolamine (PE), phosphatidylcholine (PC), monoacylglycerol (MAG) and other lipids was calculated according to equations (8-11)

(1) Linoleic = I_5; (I_5, CH_2, 2.78 ppm)

(2) Linolenic = $I_6/2$; (I_6, $2CH_2$, 2.82 ppm)

(3) MUFA=$I_7 - 2I_5 - I_6 \cdot 6/4$; ($I_7$, all unsaturated fatty acids, CH==CH, 5.36 ppm)

(4) SFA=100 – MUFA – Linoleic – Linolenic;

(5) Fatty acids = $\frac{I_3 + I_4}{I_3 + I_4 + I_2/3 + I_1 \cdot 2/3}$; ($I_1$, β-sitosterol, CH_3, 0.70 ppm; I_2, squalene, $2CH_3$, 1.68 ppm)

(6) β-Sitosterol = $\frac{I_1 \cdot 2/3}{I_3 + I_4 + I_2/3 + I_1 \cdot 2/3}$

(7) Squalene = $\frac{I_2/3}{I_3 + I_4 + I_2/3 + I_1 \cdot 2/3}$

(8) PE = $2 \cdot I_9/I_4$; (I_9, phosphatidylethanolamine, CH_2, 3.22 ppm)

(9) PC = $4 \cdot I_{10}/9 \cdot I_4$; ($I_{10}$, phosphatidylcholine, $N(CH_3)_3$, 3.26 ppm)

(10) MAG = $2 \cdot I_8/I_4$; (I_8, monoacylglycerol, CH, 3.62 ppm)

(11) Other lipids = 1 – PE – PC – MAG

8.2.5 Calculation of self-diffusion coefficients by NMR

The pulsed field gradient NMR experiment allows measurement of the translational diffusion of molecules. In this experiment, NMR signal intensity is attenuated depending on the diffusion time Δ and the selected gradient parameters (i.e. the gradient strength g and the gradient length δ). To obtain the self-diffusion coefficient, the intensity change of the NMR signal can be fit to the following equation (12):

$$\ln\left(\frac{I}{I_0}\right) = -D(2\pi)^2\gamma^2 g^2 \delta^2 \left(\Delta - \frac{\delta}{3}\right)$$

where I is the observed intensity, I_0 the reference intensity, γ the ^{1}H gyromagnetic ratio (4257 Hz/Gauss), Δ the diffusion time, and D is the self-diffusion coefficient.

8.3 Results and Discussion

Discussion of the results obtained from analysis of fruit pulp extracts, leaf extracts and hibiscus extract, using untargeted NMR and targeted RP-HPLC-PDA-ESI-MS/MS analyses, will be separated according to the class of compounds.

The chromatograms obtained at λ 330 nm following the separation of the baobab fruit pulp and leaf extracts, and the chromatographic and spectral properties of the compounds detected in fruit pulp and leaf extracts, are reported in Fig. 8.2, 8.3 and in Table 8.2, while the chromatogram obtained at 280 nm following the separation of hibiscus flower extract are reported in figure 8.8 and table 8.4. The assignment of ^{1}H spectra to the hydroalcoholic extracts of fruits and leaves solubilized in D_2O phosphate buffer was executed by 1D and 2D NMR experiments and literature data (Duarte, Goodfellow, Gill, & Delgadillo, 2005) on vegetable food matrices (Table 8.1). As an example, Fig 8.1 reports the ^{1}H spectrum of an aqueous extract of baobab pulp, the spectral ranges 5.5- 10 and 0 – 2 ppm have been vertically magnified, and are reported in inserts.

8.3.1. Analysis of the baobab fruit pulp extract

8.3.1.1. Sugars and derivatives

In the 3.2 – 5.5 ppm range, the ^{1}H NMR spectra of pulp extracts are dominated by the characteristic signals of glucose and fructose isomers, and sucrose (Fig. 8.1 and Table 8.1). These results have been confirmed by RP-HPLC-PDA-ESI-MS/MS analysis through the presence of peak 1fruit pulp which represents two different analytes that have been coeluted, showing MS spectra with parent ions at *m/z* 225 (hexose formate adduct), and *m/z* 387 (disaccharide formate adduct) (Fig. 8.2 and Table 8.2), with fragmentation patterns which correspond to those of the reference standard compounds (glucose and sucrose).

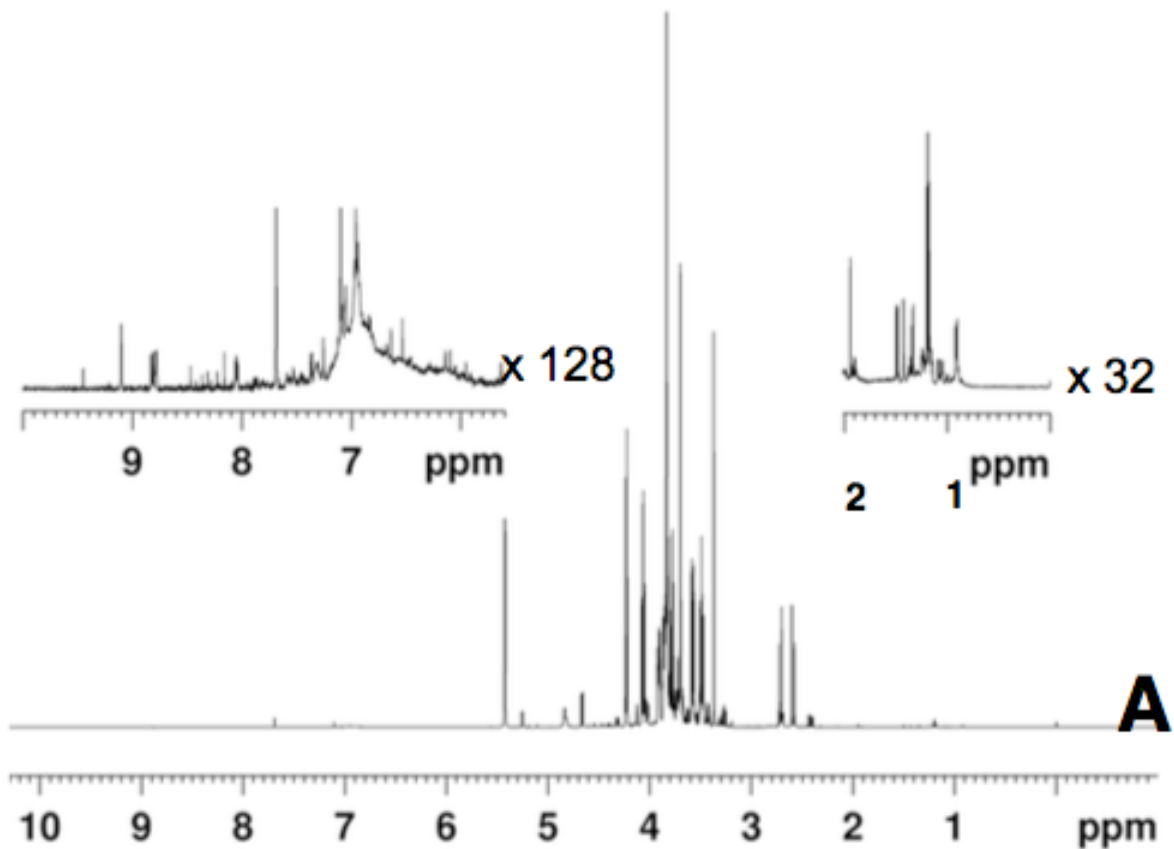


Figure 8.1: Region of the 600.13 MHz ^{1}H NMR spectrum of an aqueous extract of baobab pulp. The ^{1}H spectrum also showed characteristic signals of myo-inositol.

The ^{1}H signals of α and β-galacturonic acid, α-galacturonic acid pectic reducing end, and possibly α-galacturonic acid (pectic, non-reducing) /α-rhamnose, and β -galacturonic acid pectic reducing end (tentative assignment) were identified from literature data (Duarte et al., 2005).

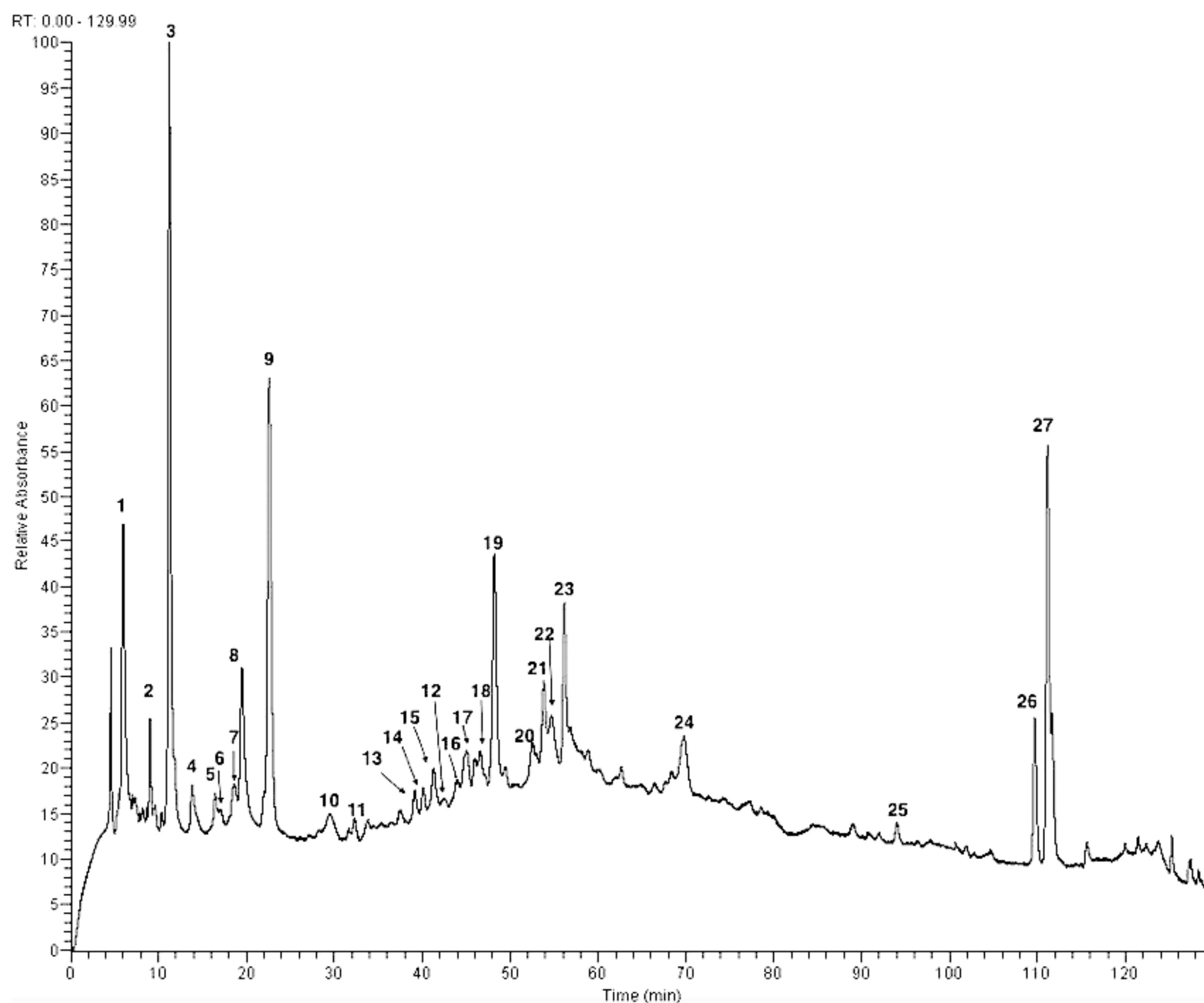


Figure 8.2: chromatogram of the baobab fruit pulp extract recorded at 330 nm.

This assignment was also confirmed by HPLC peak 3fruit pulp, with $[M-H]^-$ at *m/z* 193 corresponding to glucuronic or galacturonic acids, due to a fragmentation pattern which includes their typical fragments (131, 113, 103 and 89). These fragments are similar to those of the reference standard compound galacturonic acid. As baobab fruit is rich in pectins, and galacturonic acid is a pectin component, the proposed structure for peak 3fruit pulp was galacturonic acid (Nour et al., 1980; Brent, Reiner, Dickerson, & Sander, 2014).

Carbohydrates	Sample[a]	type	^{1}H	multiplicity	^{13}C
α-Glucose (α-GLC)	fruit pulp, leaves	CH-1	5.25	d [3.8]	93.1
		CH-2	3.55	dd [9.8;3.8]	72.3
		CH-3	3.72		73.8

		CH-4	3.43		70.6
		CH-5	3.84		72.6
		CH_2-6,6'	3.85;3.78		61.7
β-Glucose (β-GLC)	fruit pulp, leaves	CH-1	4.66	d [7.9]	97.2
		CH-2	3.27	dd [9.3;8.0]	75.3
		CH-3	3.50	t [9.1]	76.8
		CH-4	3.43		70.6
		CH-5	3.48		76.9
		CH_2-6,6'	3.91;3.74		61.8
β-D-Fructofuranose	fruit pulp, leaves	CH-1,1'	3.60;3.57		63.7
		CH-2			102.4
		CH-3	4.13		76.5
		CH-4	4.13		75.4
		CH-5	3.83		81.7
		CH_2-6,6'	3.81;3.68		63.5
α-D-Fructofuranose	fruit pulp, leaves	CH-3	4.13		83.0
		CH-5	4.07		82.4
β-D-Fructopyranose (FRU)	fruit pulp[b], leaves	CH-1,1'	3.57; 3.72		64.8
		CH-3	3.82		68.7
		CH-4	3.90		70.6
		CH-5	4.01		70.2
		CH_2-6,6'	3.72;4.03		64.4
Sucrose (SUCR)	fruit pulp, leaves	CH-1 (Glc)	5.42	d [3.8]	93.2
		CH-2	3.56		72.5
		CH-3	3.77		73.6
		CH-4	3.49		70.6
		CH-5	3.85		73.5
		CH_2-6	3.83		60.9
		CH_2-1' (Fru)	3.70		62.1
		C-2'			104.7
		CH-3'	4.23		77.4
		CH-4'	4.05		75.0
		CH-5'	3.90		82.3
		CH_2-6'	3.81		63.4
α-Galactose (α-GAL)	leaves	CH-1	5.29	d [3.9]	
		CH-2	3.83		
		CH-3	3.88		
		CH-4	4.01		
β-Galactose (β-GAL)	leaves	CH-1	4.61	d [8.0]	
		CH-2	3.51		
		CH-3	3.67		
		CH-4	3.96		

β-Galacturonic acid (β-GALU)	fruit pulp	CH-1	4.59	d(7.9)	
		CH-2	3.51		
		CH-3	3.71		
		CH-4	4.22		
β-Galacturonic acid (pectic, reducing end)[c]	fruit pulp	CH-1	4.63		
		CH-2	3.52		
		CH-3	3.78		
		CH-4	4.40		
α-Galacturonic acid (α-GALU)	fruit pulp	CH-1	5.31	d(3.9)	
		CH-2	3.84		
		CH-3	3.92		
		CH-4	4.28		
		CH-5	4.41		
α-Galacturonic acid (pectic, reducing end)	fruit pulp	CH-1	5.34		
		CH-2	3.86		
		CH-3	4.00		
		CH-4	4.31		
			4.43		
α-Galacturonic acid (pectic, non reducing)/ α-rhamnose	fruit pulp		5.11 [4.02. 4.14, 4.29, 3.75, 3.94]		
Myo-inositol (MI)	fruit pulp, leaves	CH-1	4.08		
		CH-2,5	3.55		
		CH-3,6	3.63		
		CH-4	3.30	t [9.38]	
Organic acids					
Citric acid (CA)	fruit pulp, leaves	α,γ-CH	2.53	d [15.0]	46.4
		α',γ'-CH	2.67	d [15.0]	46.4
Formic acid (FOA)	fruit pulp, leaves	HCOOC	8.46		
Quinic acid (QA)	leaves	CH_2-1,1’	1.88 2.08		41.8
		CH-2	4.31		68.1
		CH-3	3.56		76.2
		CH-4	4.16		71.5
		CH_2-5,5’	1.99 2.06		38.6
Malic acid (MA)	fruit pulp, leaves	α-CH	4.30	dd [9.9, 3.2]	71.4
		β-CH	2.68	dd [3.2, 5.4]	43.6
		β'-CH	2.38	dd [9.9, 5.4]	43.6
Succinic acid (SA)	fruit pulp, leaves	α,β-CH_2	2.41	s	35.2

Acetic acid	leaves	CH_3	1.94	s	24.6
Fumaric acid (FUA)	leaves	CH=CH	6.54	s	
D-threo-isocitric acid	fruit pulp	CH-3	4.00		
		CH_2-1,1’	2.51 2.44		
		CH-2	2.98		
Shikimic acid	leaves	CH_2-7	2.20 2.77		
		CH-6	4.00		
		CH-5	3.73		
		CH-4	4.42		
		CH-3	6.45		134.4
Ascorbic acid (AA)	fruit pulp	CH-5	4.55	d[2]	79.6
		CH-1’	4.04		64.5
		CH_2-2’	3.75		
Lactic acid	fruit pulp, leaves	CH_3	1.34	d	21.3
		α-CH	4.13		
Gallic acid (GA)	fruit pulp	CH-1	7.1	s	115.7
Amino acids					
Alanine (ALA)	fruit pulp, leaves	α-CH	3.79		51.5
		β-CH_3	1.49	d [7.3]	17.4
Asparagine (ASN)	leaves	α-CH	4.02		52.5
		β-CH	2.91	dd [16.9;7.2]	35.6
		β'-CH	2.95	dd [16.9;4.4]	35.6
Aspartate (ASP)	leaves	α-CH	3.92		
		β-CH	2.74		
		β'-CH	2.83	dd [3.9;17.4]	
Glutamate (GLU)	leaves	α-CH	3.78		
		β-CH	2.13		
		β’-CH	2.09		
		γ-CH_2	2.35		
Glutamine (GLN)	leaves	α-CH	3.78		55.1
		β-CH_2	2.15	m	27.3
		γ-CH	2.47	m	31.9
Isoleucine (ILE)	leaves	α-CH	3.63		
		β-CH	1.98		
		γ-CH	1.27		
		γ'-CH	1.48		
		γ-CH_3	1.02		
		δ-CH_3	0.95		
Leucine (LEU)	leaves	α-CH	3.75	d	
		β-CH_2	1.73		
		δ-CH_3	0.97		

		δ'-CH_3	0.95		
γ-Aminobutyrate (GABA)	fruit pulp, leaves	α-CH_2	2.30	t [7.4]	35.5
		β-CH_2	1.92		24.5
		γ-CH_2	3.02	t [7.5]	40.4
Proline (PRO)	leaves	α-CH	4.15		62.3
		β-CH	2.36		
		β'-CH	2.07		
		γ-CH_2	2.02		
		δ-CH	3.42		
		δ'-CH	3.35		
Phenylalanine (PHE)	leaves	CH-2,6, ring	7.34		130.5
		CH-3,5, ring	7.44		130.2
		CH-4, ring	7.39		128.8
Threonine (THR)	fruit pulp, leaves	α-CH	3.62		61.5
		β-CH	4.27		
		γ-CH_3	1.35		
Tryptophan (TRP)	leaves	CH-4	7.73		
		CH-7	7.54		
		CH-6	7.27		
		CH-5	7.20		
Valine (VAL)	leaves	α-CH	3.63		61.4
		β-CH	2.28	d [2.6]	
		γ-CH_3	1.01	d [7.0]	
		γ'-CH_3	1.05	d [7.0]	18.9
Miscellaneous metabolites					
Choline	fruit pulp, leaves	N-CH_3	3.19	s	55.0
		N-CH_2	3.52		68.6
		CH_2-OH	4.07		56.6
Ethanolamine	fruit pulp, leaves	N-CH_2	3.15		
		OH-CH_2	3.83		
Trigonelline	fruit pulp, leaves	CH-1	9.12	s	
		CH-3,5	8.83, 8.79	d [8], d [6]	
		CH-4	8.08	dd [6.0, 8]	
Uridine	fruit pulp, leaves	CH-5 ring	7.87	d [8]	
		CH-6 ring	5.91	d [8]	
		Rib CH-1	5.93	d [4.7]	
		CH-2	4.37		
		CH-3	4.24		
		CH-4	4.16		

Adenosine	fruit pulp	CH-1 ring	8.32	s
		CH-2 ring	8.23	s
		Rib CH-3	6.07	d [6]
		CH-4	4.78	
		CH-5	4.43	
		CH-6	4.29	

Table 8.1: Metabolites identified in the 600MHz ^{1}H NMR spectrum of aqueous extracts of baobab pulp and leaves. ([a] fruit pulp; leaves, [b] Traces, [c] Tentative assignment).

Peak 5fruit pulp with $[M-H]^-$ at *m/z* 209 was assigned to glucaric acid on the basis of its characteristic fragment ions at *m/z* 191 $[M-H-H_2O]^-$, 147, and 85, corresponding to subsequent decarboxylation, and de-hydratation (Spìnola, Pinto, & Castilho, 2015).

8.3.1.2. Organic acids

NMR signals of citric acid (CA), malic acid (MA), succinic acid (SA), D-threo-isocitric acid, lactic acid (LA), and ascorbic acid (AA), were identified in the 1.8 – 4.6 ppm region. Formic acid (FOA), fumaric acid (FUA), and gallic acid (GA) were identified in the low field region, along with a signal at 6.45 ppm belonging to shikimic acid.

The presence of citric and malic acids was confirmed by MS fragmentation in negative and positive ionization modes. Peaks 7fruit pulp, 8fruit pulp and 9fruit pulp correspond to citric acid and isocitric acid isomers with a molecular ion with *m/z* of 191 (in negative ionization mode). This breaks down into typical fragments at *m/z* 173, 155, 147, 131, 111, 87 corresponding to the loss of carboxyl and hydroxyl moieties (Brent et al., 2014), in accordance with the fragmentation of the reference standard compound (citric acid). The presence of malic acid was confirmed by peak 4fruit pulp showing a molecular ion at *m/z* 133, detected in negative ionization mode, and a fragmentation pattern comparable to that of the reference standard (malic acid), including fragment ions at *m/z* 115 $[M-H-H_2O]^-$, 97 $[M-H-H_2O- H_2O]^-$, and 89 $[M-H-COO]^-$ (Brent et al., 2014).

In addition to the organic acids determined by NMR, other organic acids were identified through RP-HPLC-PDA-ESI-MS/MS. The molecular ion at *m/z* 457 (peak 2fruit pulp) was tentatively identified as a malic acid hexoside derivative due to the presence of fragments corresponding to 341 $[M-H-116]^-$ where a loss of 116 could correspond to a malic acid or a deoxypentose residue (Llorent-Martínez, Spínola, Pinto, & Castilho, 2015). Peak 6fruit pulp, with a molecular ion at *m/z* 515, was only detected in negative ionization mode and was proposed to be dicaffeoylquinic acid. The different isomers of this molecule can usually be identified based on the collision energy

required. With the collision energy used in this experiment and the resulting fragmentation, 3,4- or 4,5-dicaffeoylquinic acid isomers were proposed, but we were unable to clearly distinguish between them (Alonso-Salches, & Berrueta, 2009). Peaks 15fruit pulp and 12fruit pulp, with a molecular ion at *m/z* 413, were proposed to be feruloylquinic acid isomers (formate adduct). These ions immediately lost formic acid, yielding fragment ions at *m/z* 367 which correspond to feruloylquinic acid isomers, which, in turn, broke down to give the fragment ion at *m/z* 191 $[M - H - 193]^-$ due to the loss of ferulic acid (Lin, & Harmly, 2008). Regarding the hydroxycinnamic acids, to the best of our knowledge this is the first time that dicaffeoylquinic acids and feruloylquinic acids have been identified in the powdered fruit pulp of baobab. These results are not in agreement with the results of Li et al., (2017), which identified different hydroxycinnamic acids (i.e. 1-O-(E)-feruloyl-β-D-glucose, 1-O-(E)-caffeoyl-β-D-glucose, 6-O-(E)-caffeoyl-β-D-glucose, 6-O-(E)-caffeoyl-α-D-glucose). These contradictory results could be due to the different origins of the Baobab fruit pulp and the extraction methods used.

8.3.1.3. Amino acids

Minor signals belonging to amino acids were observed (i.e. alanine, γ-aminobutyrate, and threonine) in the high field NMR spectral region between 0.50 and 3.60 ppm.

8.3.1.4. Phenolic compounds

Different classes of flavonoids (tannins, flavan-3-ols, flavonols, flavones) (Table 8.2) were detected by targeted RP-HPLC-PDA-ESI-MS/MS analysis.

Peak 19fruit pulp, with a molecular ion at *m/z* 577 and 579 in negative and positive ionization modes respectively, has tentatively been identified as a procyanidin dimer whose fragmentation pattern is reported in Table 8.2. It was not possible to differentiate between the various isomers of procyanidin dimer (A1, A2, B1, B2 etc..) with the information obtained. The fragmentation pattern of the molecular ion $[M- H]^-$ at *m/z* 577, consists of $[M – H -152]^-$ at *m/z* 425, and $[M - H - 289]^-$ at *m/z* 289, revealing the presence of two catechin units linked to either C4–C6 or C4–C8 (B-type procyanidin), which can be tentatively assigned to a procyanidin dimer.

Peaks 21fruit pulp and 22fruit pulp were assigned to procyanidin trimers. They were detected in both positive and negative ionization modes with respective molecular ions at *m/z* 867 and 865, giving characteristic fragmentation patterns. As reported for the dimer, it was not possible to differentiate between the different isomers.

Peak 23fruit pulp with its molecular ion at *m/z* 335 in negative ionization mode, was identified as catechin (formate adduct), losing formic acid to give the $[M-H]^-$ at *m/z* 289, yielding a fragmentation pattern which includes typical fragment ions at *m/z* 245, 203 and 187. The mass spectrum of catechin, procyanidin dimer and trimer were compared to those of the reference standard compounds and their identification was confirmed.

As far as flavonols are concerned, peaks 26fruit pulp and 27fruit pulp were assigned to kaempferol-3-O-(6-p-coumaroyl)-hexoside (tiliroside) based on the $[M+H]^+$ ion at *m/z* 595 and fragment ions at *m/z* 287 and at *m/z* 309, which are characteristic of kaempferol and the coumaroylglucoside moiety respectively (Felipe, Brambilla, Porto, Pilau, & Cortez, 2014).

Peak 25fruit pulp was assigned to kaempferol hexoside with a molecular ion at *m/z* 447, detected in negative ionization mode, a fragment ion at *m/z* 285 due to the loss of a hexosyl moiety (-162 amu), and a molecular ion at *m/z* 449 in positive ionization mode which gave the fragment ion at *m/z* 287 deriving from the loss of a hexosyl sugar moiety (Felipe et al., 2014; Lin, Chen, & Harnly, 2008). A standard compound for tiliroside is not yet available, and thus kaempferol and kaempferol glucoside were used to compare UV spectra and to identify common mass fragments, while kaempferol glucoside was used as a reference standard to compare the mass spectrum of kaempferol hexoside.

Of the flavones, peaks 17fruit pulp and 18fruit pulp were only detected in negative ionization mode with molecular ions at *m/z* 545. These were tentatively identified as pentosyl-hexosyl apigenin derivatives based on their fragment ions, as reported by Khoo, Azlan, Ismail & Abas (2012). Peak 20fruit pulp, showing a parent ion at m/z 447 detected in negative mode, was identified as apigenin-O-pentoside with sequential loss of formate adduct (*m/z* 401) and glycoside (*m/z* 269) moieties (Spínola, Pinto, & Castilho, 2015). To the best of our knowledge, this is the first time that apigenin derivatives have been isolated from baobab fruit pulp.

To summarise the secondary metabolites, both non flavonoids (hydroxycinnamic derivatives) and flavonoids (flavanols, flavones, flavonols, and proanthocyanidins) were identified in fruit pulp. While hydroxycinnamic acids (Li et al., 2017) and proanthocyanidins (Shahat, 2006) had already been found, this was the first time flavanol and flavone derivatives were isolated and identified in the powdered fruit pulp of baobab, to the best of our knowledge. The presence of these polyphenols, to which a large body of evidence ascribes antimicrobial properties (Daglia, 2012), explains and supports the use of baobab fruit pulp as part of traditional medicine against microbial diseases (Rahul et al., 2015)

8.3.1.5 Other compounds

NMR signals of choline, ethanolamine, trigonelline, uridine and adenosine were identified by means of their diagnostic signals (Table 8.1).

Peak No	(RT) (min)	λ Max (nm)	[M-H]⁻	Fragment ions	[M+H]⁺	Fragment ions	Proposed structure
				Sugars			
1fruit pulp	6.01	223, 265, 295	387	387 →341 (100) 341→ 179(100), 161(30), 135(10), 143(20), 119(20),	360	343(60), 325(100), 289(10), 307(5), 277(5), 259(10), 180(5), 163(5), 145(5), 127(5)	Disaccharide
1fruit pulp	6.01	223, 265, 295	225	225 → 179 (100) 179→161(100), 143(50), 99(20), 75(20), 135(10), 133(5), 125(5),	181	163 (100), 152(90), 148(70), 146(60), 135(30), 125(20), 166(80), 181(30), 184(10), 198(30), 86(10), 145(10)	Hexose
1leaves	6.00	217, 265	387	387 →341 (100) 341→179(100), 161(30), 135(, 143(20), 119(20),	/	/	Disaccharide
			225	225 → 179 (100) 179→161(100), 143(50), 99(20), 75(20), 135(10), 133(5), 125(5),			Hexose
				Aminoacids			
9leaves	27.3	219, 276, 298	/	/	205	188(100), 159(<5), 118(<5), 210(<5), 239(<5)	Tryptophan
				Organic and phenolic acids			
9fruit pulp	22.57	221, 282	191	111(100), 173(20), 87(<5), 131(<5), 154(<5), 129(<5), 101(<5), 155(<5), 147(<5)	210	210→193(100), 193→175(40), 147(10), 164(5)	Citric acid
8fruit pulp	19.48	221, 282	191	111(100), 173(20), 87(<5), 131(<5), 154(<5), 129(<5), 101(<5), 155(<5), 147(<5)	210	210→193(100), 193→175(40), 147(10), 164(5) ,	Citric acid

7fruit pulp	18.48	221, 282	191	111(100), 173(20), 87(<5), 131(<5), 154(<5), 129(<5), 101(<5), 155(<5), 147(<5)	210	210→193(100), 193→175(40), 147(10), 164(5)	Citric acid
			383	191(100)			Quinic acid dimer
			191	127(100), 173(70), 85(60), 111(40), 93(40), 171(10)			Quinic acid
5leaves	12.13	203, 216, 262			/	/	
			391	195(100)			Gluconic acid dimer
			195	129(100), 159(30), 177(30), 75(10), 99(10)			Gluconic acid
			391	195(100)			Gluconic acid dimer
4leaves	11.44	204, 223, 232, 280			/	/	
			195	151 (100) 159(30), 177(30), 75(10), 99(10), 85(5)			Gluconic acid
2fruit pulp	9.00	220, 294	457	341(100), 179(10), 149(10), 161(5), 143(5), 281(4), 439(<5), 310(<5), 325(<5), 217(<5), 411(<5)	/	/	Di- hexoside derivative of malic acid
4fruit pulp	13.86	221	133	115(100) 115→ 97 (10), 89 (40)	/	/	Malic acid
6leaves	15.81	203, 209, 232, 260	133	115(100), 71(10), 87(10)	/	/	Malic acid
7leaves	17.32	203, 209, 250, 263	133	115(100), 71(10), 87(10)	/	/	Malic acid
5fruit pulp	16.33	221	209	191(100), 147(20), 129(5),	/	/	Glucaric acid

				111(5), 85(5), 165(<5), 189			
3fruit pulp	11.18	227, 284	193	131(100), 113(70), 89(80), 103(40)	/	/	Galacturonic acid
6fruit pulp	16.94	220, 247, 312	515	353 (100), 173 (10)	/	/	3,4dicaffeoylquinic acid/ 4,5-dicaffeoylquinic acid
15fruit pulp	41.12	221, 280	413	367 (100), 191(5)	/	/	Feruloyl-quinic acid
12fruit pulp	42.36	221, 281	413	367 (100), 191(5)	/	/	Feruloyl-quinic acid
				Proanthocyanidins			
19fruit pulp	48.23	223, 280	577	425 (100), 407(40), 451 (30) 289(10), 559(5)	579	427(100), 409(60), 291(30), 247(10)	Procyanidin dimer
11leaves	43.03	224, 279	577	425(100), 407(40), 451(25), 289(10), 331(5) 245(5), 187(5)	579	427(100), 409(20), 291(25), 453(10), 247(10)	Procyanidin dimer
21fruit pulp	53.84	223, 280	865	695(100), 577(90), 713(60), 739(50), 587(30) , 425(35), 407(20), 449(10), 543(20), 525(10) 287, 363, 619, 847, 821)	867	579(100), 590(50), 409(20), 437(10), 255(10), 528(15), 697(20), 667(10), 715(10), 848(10)	Procyanidin trimer
22fruit pulp	53.84	223, 280	865	695(100), 577(90), 713(60), 739(50), 587(30) , 425(35), 407(20), 449(10), 543(20), 525(10) 287, 363, 619, 847, 821)	867	579(100), 590(50), 409(20), 437(10), 255(10), 528(15), 697(20), 667(10), 715(10), 848(10)	Procyanidin trimer
12leaves	47,31	224, 279	865	695(100), 577(60), 739(40), 587(40), 407(30), 451(20), 543(20), 713(30) 287(10), 395(<5), 847(15), 821(10)	867	579(100), 715(60), 409(30), 535(20), 697(50), 849(10), 517(40), 427(30),	Procyanidin trimer
13leaves	47,83	224, 279	865	695(100), 577(60), 739(40), 587(40), 407(30), 451(20), 543(20), 713(30) 287(10), 395(<5), 847(15), 821(10)	867	579(100), 715(60), 409(30), 535(20), 697(50), 849(10), 517(40), 427(30),	Procyanidin trimer
14leave	49,71	227, 277			/	/	Procyanidin

s			1008 1152 865 577 1440 1729 720 289	1008 → 736 (100) 1152→1000(80) 865→ 695(100), 577(60), 739(40), 407(20), 587(40), 577→ 425(100), 407(40), 451(25), 289(15) 1440→1152(100), 933, 701, 1264, 1421, 1368, 1173 1729→422(100)			oligomers
15leaves	51.99	223, 277	1008 1152 865 577 1440 1729 720 289	1008 → 736 (100) 1152→1000(80) 865→ 695(100), 577(60), 739(40), 407(20), 587(40), 577→ 425(100), 407(40), 451(25), 289(15) 1440→1152(100), 933, 701, 1264, 1421, 1368, 1173 1729→422(100	/	/	Procyanidin oligomers
			Flavan-3-ols				
23fruit pulp	56.16	226, 280	335	289 (100) 245 (5), 203(5), 187 (10)	291	139(100), 123(85), 165(70), 151(20), 273(10)	Catechin
			Flavonols				
26leaves	73.23	256, 355	609	301(100), 343 (20), 463 (20), 271(20), 179(5), 343(10)	611	303 (100), 465(60), 602(25), 593(20), 610(20), 447(15)	Rutin
23leaves	64,83	256, 355	609	301(100), 343 (20), 463 (20), 271(20), 179(5), 343(10)	611	303 (100), 465(60), 602(25), 593(20), 610(20), 447(15)	Rutin
			609	301(100), 343 (20), 463 (20), 271(20), 179(5), 343(10)	611	303 (100), 465(60), 602(25), 593(20), 610(20), 447(15	Rutin
27leaves	78.49	202, 223, 269, 319, 341					
			593	285(50), 300(20), 199(5), 257(5), 447(<5), 357(<5)	595	329(70), 309(30), 287(40), 395(60), 365(30), 431(40), 449(20), 626(50)	Kaempferol-3- O- coumaroylglucoside
27fruit pulp	111.14	227, 266 315	593	285(100), 447(30), 307(5), 257(5), 487(10)	595	329 (70), 309 (30), 287(40), 395 (60) 287(40), 365 (30), 431(40), 577 (100),	Kaempferol-3 -O-(6-p- coumaroyl)- hexoside

26fruit pulp	109.71	227, 266 315	593	285(100), 447(30), 307(5), 257(5), 487(10)	595	431 (40), 449(20), 626(50) 329 (70), 309 (30), 287(40), 395 (60) 287(40), 365 (30), 431(40), 577 (100), 431 (40), 449(20), 626(50)	Kaempferol-3 -O-(6-p- coumaroyl)-hexoside
25fruit pulp	94.04	220, 270	447	284(100), 285(50), 255(10), 227(<5), 151(5), 179(5), 327(20), 299(5)	449	287(100)	Kaempferol-3-O-hexoside
28leaves	80.5	202, 226, 266, 282 318	593	285(50), 300(20), 199(5), 257(5), 447(<5), 357(<5)	595	329(70), 309(30), 287(40), 395(60), 365(30), 431(40), 449(20), 626(50)	Kaempferol-3-O-coumaroylglucoside
24leaves	67.37	223, 266, 281, 345	739	285(100), 575(95), 593(30), 473(30), 393(30), 327(20), 255(20), 357(15), 429, 557, 668, 739	/	/	Kaempferol -3-O-(4´´-rhamnosyl) neohesperidoside
21leaves	62.68	228, 255, 355	755	301 (100), 591(45), 489(40), 609(15), 271 (25) , 255(10), 343(15), 409(10), 573(10), 737(15) , 755, 627	/	/	Quercetin 3-O-(2, 6-di-O-rhamnosyl -glucoside)
22leaves	63.33	228, 255, 355	755	301 (100), 591(45), 489(40), 609(15), 271 (25) ,255(10), 343(15), 409(10) , 573(10), 737(15), 627(5)	/	/	Quercetin-3-O-(2, 6-di-O-rhamnosyl) -glucoside
29leaves	82,66	227, 260, 346	607	463(100), 505(30), 545(10), 301 (10), 273(5), 353(5)	/	/	Quercetin 3-hydroxy-3-methylglutaryl-O-hexoside
				Flavones			
17fruit pulp	45.13	222, 274	545	443 (100), 473 (80), 309 (10), 351 (15), 272 (<5), 235 (<5), 175 (<5), 482 (<5), 499 (<5), 467 (<5)	/	/	Pentosyl-hexosyl apigenin
18fruit pulp	46.05	222, 280	545	443 (100), 473 (80), 309 (10), 351 (15), 272 (<5),	/	/	Pentosyl-hexosyl apigenin

				235 (<5), 175 (<5), 482 (<5), 499 (<5), 467 (<5)			
20fruit pulp	52.37	222, 282	447	401(100), 269(<5), 293(<5)	/	/	Apigenin-O-pentoside
25leaves	68.39	202, 226, 270, 331, 345	431	311(100), 341(25), 283, 353, 413, 153	433	415(40), 397(10), 367(5)	Isovitexin/Vitexin

Table 8.2: Chromatographic and spectral properties of compounds detected in baobab fruit pulp and leaf extracts.

8.3.2. Analysis of the baobab aqueous leaf extract

8.3.2.1. Sugars and derivatives

As in the case of pulp extract outlined above, leaf extract also contains glucose, fructose and sucrose, as again confirmed through RP-HPLC-PDA-ESI-MS/MS (1leaves). The ^{1}H spectrum also showed signals of α and β-galactose and *myo*-inositol.

8.3.2.2. Organic acids

Malic, citric, succinic, lactic, fumaric and formic acids, all detected in pulp, were also identified in leaf extracts, along with acetic acid, quinic acid, and shikimic acid. Some organic acids were also confirmed through RP-HPLC-PDA-ESI-MS/MS analysis.

The chromatogram obtained from leaf extract confirmed the presence of many organic acids. For instance, it showed the peak 5leaves due to a coelution of two substances, corresponding to molecular ions at *m/z* 191 and 383, which were only detected in negative ionization mode. The molecular ion at *m/z* 191 was assigned to quinic acid, and that at *m/z* 383, giving the fragment ion at *m/z* 191, was identified as quinic acid dimer due to a fragment ion at *m/z* 191 (Brent et al., 2014). The fragmentation pattern of quinic acid was compared to that of the reference standard and the identification was confirmed.

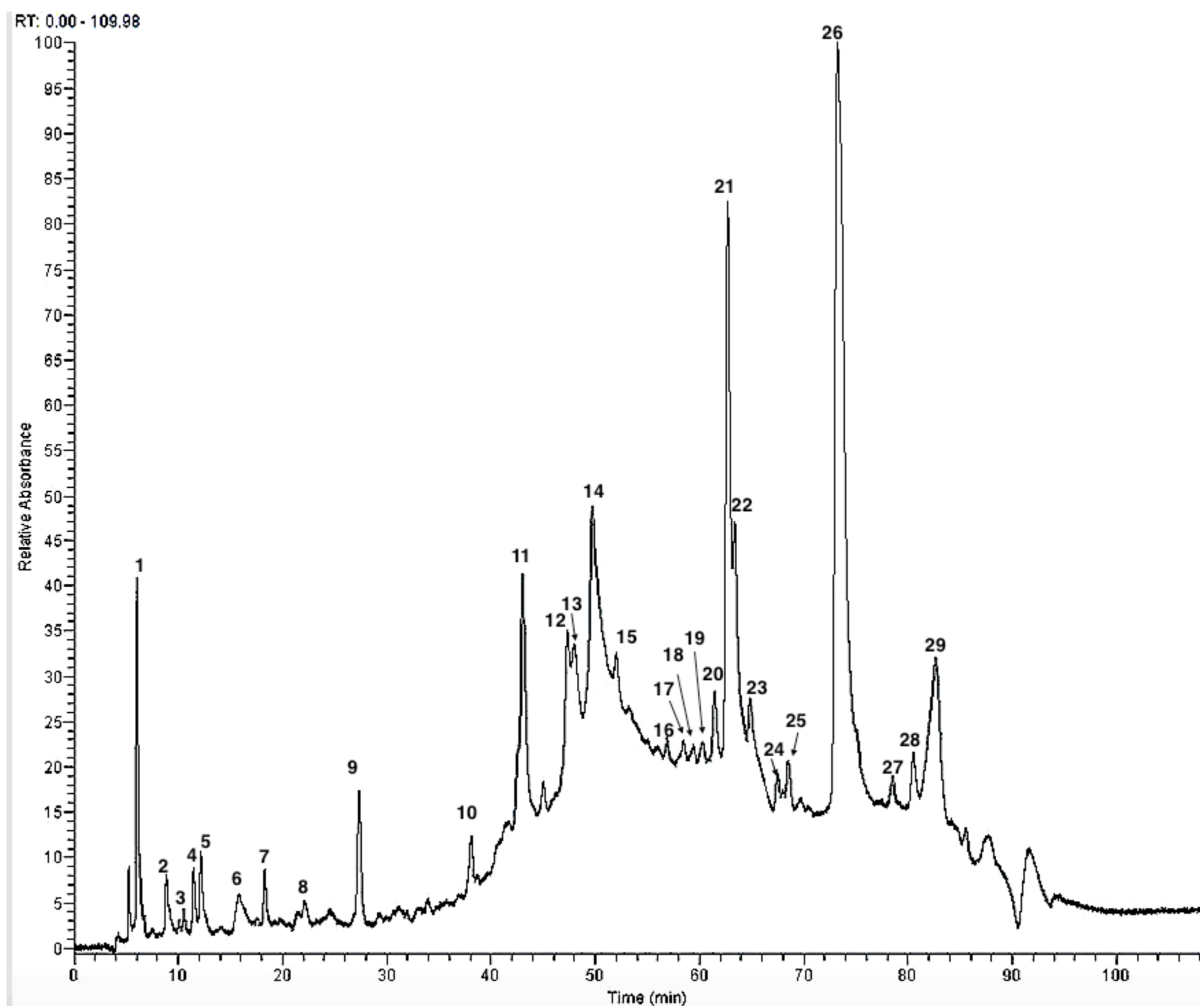


Fig 8.3: HPLC chromatogram of the baobab leaf extract recorded at 330 nm.

The chromatogram shows the peak 4leaves, a coelution of two compounds, both of which were only detected in negative ionization mode. The first was identified as gluconic acid with a molecular ion at *m/z* 195, whose fragmentation pattern consists of fragment ions at *m/z* 151 $[M\text{-}H\text{-}COO]^-$, 159 $[M\text{-}H\text{-}2H_2O]^-$, 177 $[M\text{-}H\text{-}H_2O]$ (Deng, & Yang, 2013). The second, with a molecular ion at *m/z* 391 giving the same fragmentation pattern as gluconic acid, was identified as gluconic acid dimer.

8.3.2.3. Amino acids

Alanine, γ-aminobutyrate, and threonine, previously identified in pulp, were also detected in leaf extracts. The ^1H spectrum of leaf extract also showed signals from asparagine, aspartate, glutamate, glutamine, isoleucine, leucine,

proline, and valine. Moreover, signals from aromatic rings of phenylalanine and tryptophan were detected in the low field region of the ^{1}H spectrum (Fig. 8.1). The presence of tryptophan was confirmed by the HPLC peak 9leaves, with the molecular ion at *m/z* 205 only detected in positive ionization mode according to the fragmentation pattern reported in Table 8.2 (Kosinska, Diering, Prim, Heritier, & Andlauera, 2013).

7.3.2.4. Phenolic compounds

As previously reported for baobab fruit pulp, and in accordance with the reference standards, peak 11leaves in the leaf extract with molecular ions at *m/z* 577 (in negative ionization mode) and 579 (in positive ionization mode), was tentatively identified as procyanidin dimer and its fragmentation pattern is reported in Table 8.2.

Peaks 12leaves (molecular ions at *m/z* 867 and 865, detected in positive and negative ionization modes respectively) were assigned to procyanidin trimers. Peaks 14leaves and 15leaves present a series of molecular ions at *m/z* 1152, 865, 577, 1140, 1729, 720, 289, detected only in negative ionization mode. On the basis of their fragmentations, these were tentatively identified as procyanidin oligomers (Hammerstone, Lazarus, Mitchell, Rucker, & Schmitz, 1999).

Peaks No 26leaves, 23leaves and 27leaves with molecular ions at *m/z* 609 and 611, detected in negative and positive ionization modes respectively, were identified as rutin, due to a fragmentation pattern which includes fragment ions typical of quercetin derivatives, such as that due to the loss of the disaccharide rutinose (fragment ion at *m/z* 302) corresponding to [M-H-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranose]. A comparison with the reference standard (rutin) confirmed this result.

Peak 27leaves represents two different analytes with different MS spectra that have been coeluted together, which allowed us to identify rutin (molecular ion at *m/z* 609) and kaempferol-3-O-coumaroylhexoside (molecular ion at *m/z* 593). kaempferol-3-O-coumaroylhexoside was also detected in peak 28leaves which showed a molecular ion at *m/z* 593 in negative ionization mode and at *m/z* 595 in positive ionization mode (Felipe et al., 2014).

The ion at *m/z* 739 (peak 24leaves) detected in negative ionization mode, was assigned to kaempferol-3-O-(4'-rhamnosyl)-neohesperidoside due to a fragmentation pattern which includes the characteristic aglycone fragment (*m/z* at 285) attributed to kaempferol, the fragment ion due to the loss of rhamnose $[M-H-146]^{-}$ (*m/z* at 593), and a neutral loss of 308 amu corresponding to the characteristic fragment mass of O-diglycoside (i.e., a neohesperidose disaccharide) (Ferreres, Pereira, Valentão, & Andrade, 2010). Here too, a standard reference for kaempferol-3-O-

coumaroylhexoside and kaempferol-3-O-(4'-rhamnosyl)-neohesperidoside was not found on the market, so kaempferol and kaempferol glucoside were used to compare UV spectra and to identify common mass fragments.

The molecular ions at *m/z* 755 (peak 21leaves and 22leaves), detected in negative ionization mode, had fragmentation patterns which led to a tentative identification as quercetin-3-O-(2,6-di-O-rhamnosyl)-glucoside, as these included the characteristic aglycone fragment (*m/z* at 301) attributed to quercetin, and the fragment ion $[M-H-146]^-$ (*m/z* 609) corresponding to the loss of rhamnose (Karar et al., 2016).

Peak 29leaves, which corresponds to a molecular ion at *m/z* 607 detected in negative ionization mode, was assigned to quercetin 3-hydroxy-3-methylglutaryl-O-hexoside on the basis of its fragmentation pattern, including the characteristic aglycone fragment (*m/z* at 301) attributed to quercetin and the loss of C4H6O3 (*m/z* 505, $[M-H-102]^-$) and C6H8O4 (*m/z* 463, [M-H-144]), both compatible with the presence of a 3-hydroxy-3-methylglutaryl substituent (Barreca, Gattuso, Laganà, Leuzzi, & Bellocco, 2016). Due to the difficulty in finding quercetin derivatives (quercetin-3-O-(2,6-di-O-rhamnosyl)-glucoside and quercetin 3-hydroxy-3-methylglutaryl-O-hexoside) on the market, quercetin and quercetin rhamnoside were used as reference compounds to compare UV spectra and to find common mass fragments.

The molecular ions at *m/z* 431 and 433, detected in negative and positive ionization modes respectively (peak 25leaves), were identified as vitexin/isovitexin, as it was not possible to differentiate between these two. The fragmentation pattern of the molecular ion $[M-H]^-$ included five basic fragment ions, three of which corresponded to the fracture of a glucosyl ring (i.e. *m/z* 353, 341 and 311) with the other two indicating a benzyl ion (*m/z* 283) and an aglycone ion (*m/z* 269) (Waridel, Wolfender, Ndjoko, Hobby, Major, & Hostettmann, 2001)

The results obtained for leaf extract show that the polar secondary metabolites consist of hydroxycinnamic acids and various classes of flavonoids, confirming data obtained by Irondi et al., (2017) on fresh baobab leaves. The presence of these antioxidant and anti-inflammatory polyphenolic compounds could at least partially explain the traditional use of baobab leaves in the treatment of diseases of the urinary tract, opthalmia and otitis, in which oxidative stress and inflammation are considered to be etiological factors (Rahul et al., 2015).

8.3.3. NMR analysis of pulp and leaf organic extracts

^{1}H NMR spectral regions of an organic extract of baobab pulp are shown in Fig 8.4 with the assignment of some selected resonances (I_1-I_{10}) which were used for quantitative analysis (Table 8.3).

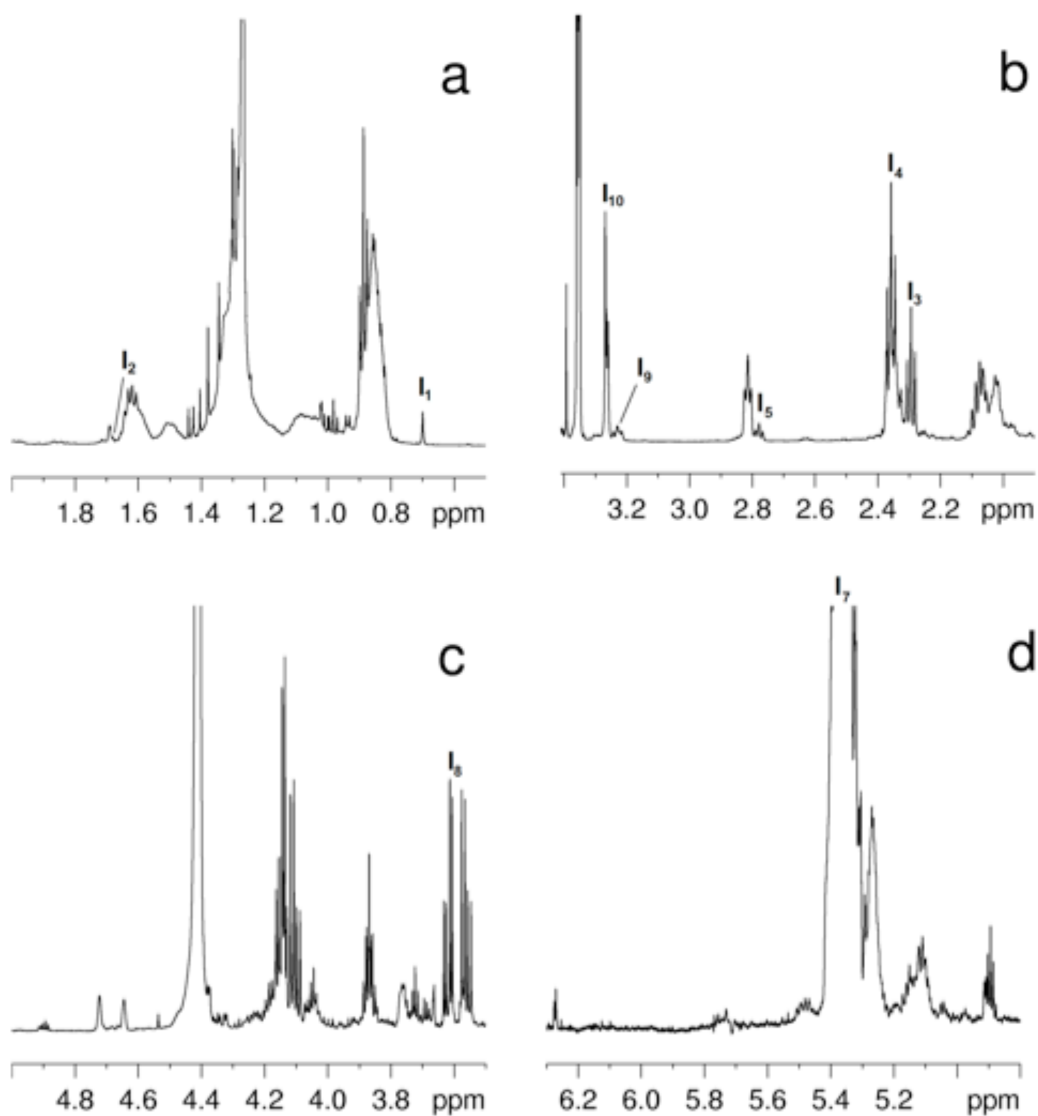


Fig 8.4: Regions of the 600.13 MHz ^{1}H NMR spectrum of a baobab pulp organic extract, signals used for quantitative analysis have been labelled with I_1–I_{10}. (a) 0.5–2.0ppm region, (b) 1.9–3.4ppm region, (c) 3.5–5.0ppm region, (d) 4.8–6.3 ppm region.

8.3.3.1. Sterols

The resonance at 0.70 ppm was ascribed to the CH_3-18 of β-sitosterol (I_1).

8.3.3.2. Phospholipids

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were the main diacylglycerophospholipids present in the baobab lipid fraction. The characteristic ^{1}H signals of these lipids belong to their head groups, specifically the singlet at 3.27 ppm of $N(CH_3)_3$ of PC (I_{10}) and the multiplet at 3.22 ppm of CH_2N of PE (I_9).

8.3.3.3. Fatty acid chains

The peak at 2.29 ppm was assigned to the α-CH_2 of esterified fatty acids (I_3), the peak at 2.78 was assigned to CH_2 - 11 of linoleic fatty acids (I_5), and the peak at 2.82 ppm was assigned to CH_2-11 and CH_2-14 of the linolenic fatty chain (I_6). The peak centred at 5.36 ppm was due to CH=CH protons in all unsaturated fatty acids (I_7).

8.3.3.4. Mono-, di- and triacylglycerols, and galctosyldiacylglicerols

The peak at 3.62 ppm was due to one of the two protons of monoacylglycerols (I_8). It was not possible to perform a quantitative analysis on di- and triacylglycerols due to the overlap of multiple strong peaks.

8.3.3.5. Squalene

The resonance of the very weak peak at 1.68 ppm was assigned to the two methyl protons of squalene (I_2). All resonances of squalene were assigned (Table 8.3), according to the labelling of the resonances reported in Figure 8.5.

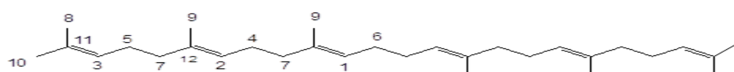


Fig 8.5: Sketch of squalene structure with nuclei labelling.

8.3.3.6. Isoprenoid compound.

Peaks at frequencies very similar to those of squalene appeared in the ^{1}H spectrum of baobab leaf organic extracts. Fig 8.6 compares selected ranges of frequencies of the ^{1}H spectra of pulp and leaf organic extracts.

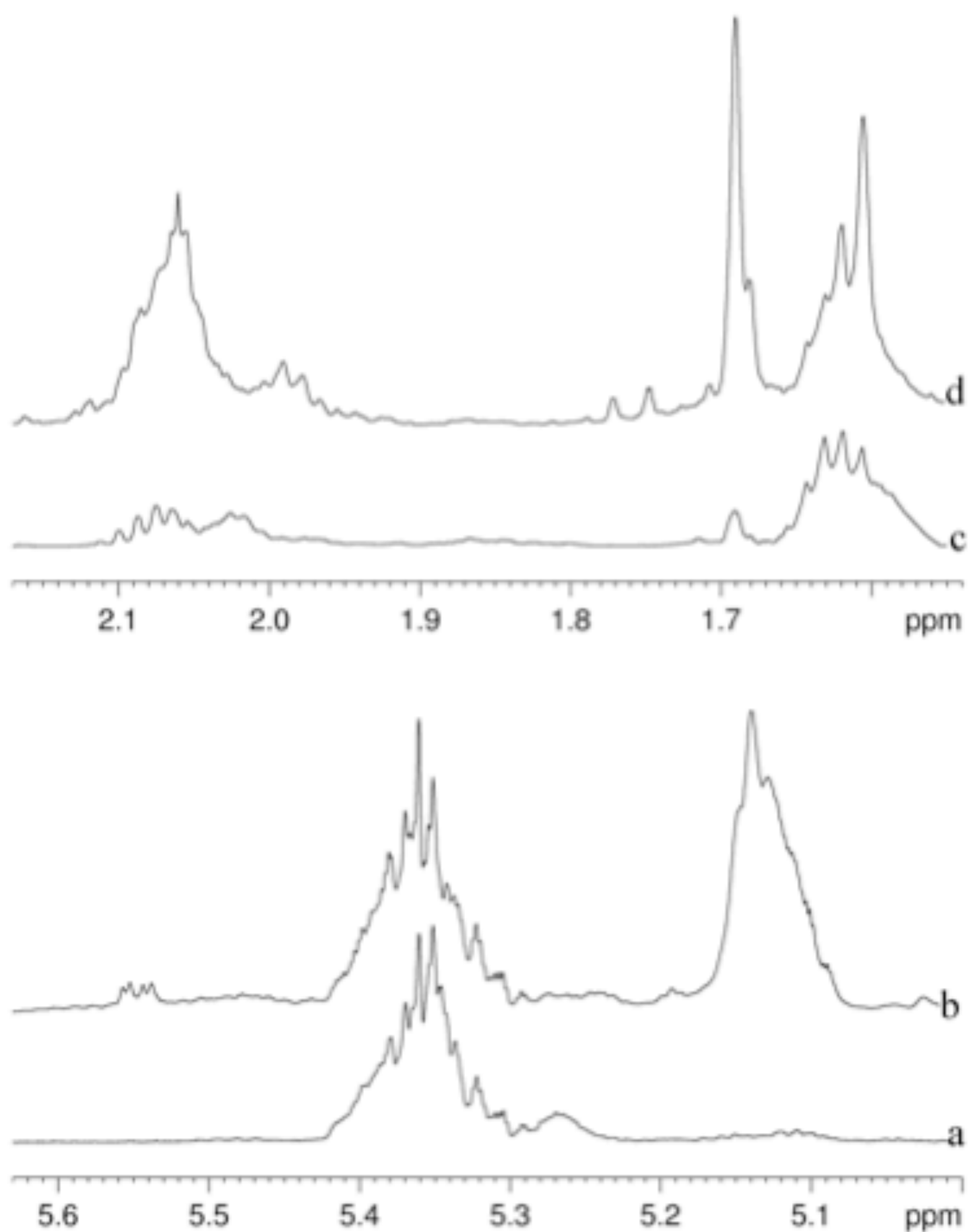


Figure 8.6: Comparison between two spectral ranges of the ^{1}H NMR spectrum of baobab pulp (bottom) and leaf (top) organic extracts. Intense peaks ascribed to the isoprenoic compounds are evident in the spectrum of leaf extracts.

An intense peak was found at 1.69 ppm, whereas peaks centred at 5.15, 2.08 and 2.06 ppm were stronger in leaf extract than in pulp extract. The TOCSY map revealed the presence of a spin system at 1.69, 2.06, 2.08, and 5.14 ppm. The HSQC map allowed the assignment of the corresponding carbon resonances at 25.5, 32.4, 26.8, and 125.3 ppm. The HMBC revealed long-range correlations between 5.14 ppm and 23.5 and 32.4 ppm, 1.69 ppm and 32.4, 125.3 and 135.5 ppm, 2.06 ppm and 26.8 ppm, 2.07 ppm and 32.4 ppm.

These data and data from literature (Maidunny, & Dulngali, 1985) allowed partial assignment of the unknown compound, see Table 8.3. Much like how the assignment of the repetitive unit of squalene perfectly matches that of 1,4-polyisoprene in trans conformation, the assignment of the repetitive unit of cis 1,4-polyisoprene matches that of the unknown compound which, for this reason, was labelled as an "isoprenoid" compound (Table 8.3 and Fig 8.7).

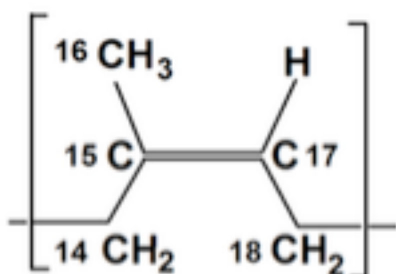


Figure 8.7: Sketch of the repetitive unit of the isoprenoid compound partially assigned, with the labelling. I_1, CH_3-18 β-sitosterol; I_2, $2CH_3$ squalene; I_3, α-CH_2 free fatty acids; I_4, α-CH_2 esterified fatty acids; I_5, CH_2 linoleic fatty chain; I_6, $2CH_2$ li- nolenic fatty chain; I_7, CH]CH all unsaturated fatty acids; I_8, CH MAG; I_9, CH_2 PE; I_{10}, $N(CH_3)_3$ PC.

This compound is probably an 1,4-polyisoprene oligomer with its chain in cis conformation, and if so the chemical structure of this isoprenoid compound closely resembles that of natural rubber, based on cis-1,4-polyisoprene chains. To identify other possible differences between squalene and the isoprenoid compound, the self-diffusion coefficients were calculated by fitting the collected NMR data to equation (12).

The self-diffusion coefficient of squalene was calculated as 3.79×10^{-10} m^2/s, with a coefficient of determination R^2 of 0.999, whereas the self-diffusion coefficient of the isoprenoid compound was found to be 4.62×10^{-10} m^2/s, with a

coefficient of determination R^2 of 0.998. As the self-diffusion coefficient is strictly related to translational molecular mobility, and thus to the molecular weight, these data indicate that the molecular weight of the isoprenoid compound should be slightly lower than that of squalene.

The molar composition of baobab pulp and leaf organic extracts is reported in Table 9.9. In the case of fatty acids, the amount of free FA in leaves was found to be about twice that of pulp, whereas the amount of esterified FA and monounsaturated fatty acids (MUFA), was found to be higher in pulp than in leaves. The amount of linoleic acid in leaves was about twice that of pulp. The amount of linolenic acid and saturated acids in pulp was comparable with that found in leaves. In both cases the lipid fraction was mainly comprised of about 90% fatty acids. The amount of β-sitosterol was comparable in pulp and leaves, whereas the amount of squalene was much higher in leaves than in pulp. Table 8.3 reports the distribution of esterified FA in pulp. Specifically, MAG was identified as the predominant component in the distribution of esterified FA in baobab pulp (39.7 %), followed by other lipids (33.6 %), PC (17.5 %), and PE (9.2 %).

Compounds	**Type**	**^{1}H (ppm)**	**Multiplicity (J Hz)**	**^{13}C (ppm)**
Saturated fatty chains	COO			175.0
	CH_2-2	2.35	t (*7.8*)	34.5
	CH_2-3	1.63		25.2
	CH_2 (n3)	1.32		29.5
	CH_2 (n2)	1.27		32.2
	CH_2 (n1)	1.31		22.9
	CH_3	0.88	t(*6.9*)	14.2
Oleic acid	COO-1			175.0
	CH_2-2	2.35		34.5
	CH_2-3	1.63		25.3
	CH_2-4,7	1.34		30.5
		1.32		29.5
	CH_2-8	2.03		27.5
	CH=CH-9,10	5.35		130.3
	CH_2-11	2.06		27.5
	CH_2-12,15	1.34		30.5
		1.32		29.5
	CH_2-16	1.28		32.2
	CH_2-17	1.30		23.0
	CH_3-18	0.88	t (*7.0*)	14.3
Linolenic fatty chain C18:3				
	COO-1(sn1,3)			175.6
	CH_2-2	2.33		34.5
	CH_2-3	1.63		25.2

	CH_2-4	1.33		29.50
	CH_2-5,7	1.34		30.4
	CH_2-8	2.07		27.4
	CH-9	5.35		130.5
	CH-10	5.35		128.5
	CH_2-11	2.81		25.9
	CH-12,13	5.35		128.5
	CH_2-14	2.81	t(*6.1*)	25.9
	CH-15	5.32		127.5
	CH-16	5.39		132.2
	CH_2-17	2.09		20.9
	CH_3-18	0.98	t(*7.5*)	14.4
Free fatty acids (FFA)				
	CH_2-2	2.29		34.5
	CH_2-3	1.62		25.2
	CH_2	1.32		24.6
Monoacylglycerol (MAG)				
	CH_2-sn1	3.56, 3.62	dd (*6.2, 11.4*) dd (*4.6, 11,4*)	63.6
	CH-sn2	3.87		70.3
	CH_2-sn3	4.10, 4.15	dd(*6.2, 11.4*) dd(*4.9,11.4*)	65.6
	COO			174.9
Diacylglycerol (DAG)				
	CH_2-sn1	4.19, 4.33		62.5
	CH sn2	5.09		72.6
	CH_2 sn3	3.69		61.0
Triacylglycerol (TAG)				
	CH_2 sn1,3	4.18, 4.34	dd (*6, 11.9*) dd (*4, 12*)	62.5
	CH sn2	5.27	m	70.1
Linoleic fatty chain C18:2				
	COO-1			175.6
	CH_2-2	2.33		34.4
	CH_2-3	1.63		25.3
	CH_2-4,7	1.32		29.5
	CH_2-8	2.06		27.6
	CH-9	5.36		130.3
	CH-10	5.36		128.5
	CH_2-11	2.78		25.9
Galactosyldiacylglycerols (DGDG)				
	CH-sn2	5.27		70.6
	CH_2-sn1	3.72, 3.96		68.2
	CH_2-sn3	4.24, 4.39		63.1
	CH-1’	4.23		104.2
	CH-2’	3.54		71.5
	CH-3’	3.51		73.6
	CH-4’	3.91		68.7

	CH-5’	------		
	CH2-6’	------		
	CH-1’’	4.91	d (*3.8*)	99.6
	CH-2’’	3.81		69.3
	CH-3’’	3.75		70.5
	CH-4’’	3.96		70.2
	CH-5’’	3.75		70.5
	CH_2-6’’	3.76, 3.86		62.2
Phosphatidylcholine (PC)	$(CH_3)_3N$	3.26		54.5
	CH_2OP	4.45		60.9
	CH_2N	3.77		66.2
	CH_2 sn3	4.17		63.4
	CH_2 sn1	4.38, 4.22		62.4
	CH sn2	5.27		70.1
Phosphatidylethanolamine (PE)	CH_2OP	4.24		63.4
	CH_2N	3.22		40.3
β-Sitosterol	CH_2-1	1.08, 1.87		37.6
	CH_2-2	---, 1.81		31.4
	CH-3	3.47		71.6
	CH_2-4	2.25		42.1
	CH-6	5.35		121.8
	CH_2-7	1.59, 2.06		32.9
	CH-8	1.55		32.8
	CH-9	0.94		50.6
	CH_2-11	1.51		21.5
	CH_2-12	1.19, 2.05		40.2
	CH-14	1.02		57.2
	CH_2-15	1.08, 1.59		24.6
	CH_2-16	1.30, 1.87		28.5
	CH-17	1.13		56.5
	CH_3-18 (I_1)	0.70		12.1
	CH_3-19	1.02		19.5
	CH-20	1.38		36.5
	CH_3-21	0.94		19.0
	CH_2-22	1.04, 1.34		34.4
	CH_2-23	1.11		26.2
	CH-24	0.94		46.3
	CH-25	1.64		28.9
	CH_3-26	0.86		20.7
	CH_3-27	0.85		19.9
	CH_2-28	1.30		23.0
	CH_3-29	0.86		12.1
Squalene	CH-1	5.12		124.7
	CH-2/CH-3	5.14		124.7
	CH_2-4	2.07		26.8
	CH_2-5	2.06		27.6
	CH_2-6	2.03		28.4
	CH_2-7	1.99		40.0
	CH_3-8	1.61		17.8
	CH_3-9	1.61		16.1
	CH_3-10	1.68		25.8
	C-11			131.6

	C-12/C-13		135.2
Isoprenoid compound (Only in leaves)	CH-17	5.14	125.3
	CH_3-16	1.69	23.5
	CH_2-14	2.06	32.4
	CH_2-18	2.08	26.8
	C-15		135.5

Table 8.3: Metabolites identified in the 600MHz ^{1}H NMR spectrum of organic extracts of baobab pulp and leaves.

8.3.4 HPLC-PDA-MS analysis of *Hibiscus sabdariffa* flowers

The results for the HPLC-PDA-MS analysis of Hibiscus flowers have again been separated by class of compounds for the purpose of the discussion. Figures 8.8, and 8.10 reported below show the chromatograms registered at 280 nm and 520 nm respectively, obtained after the separation of hibiscus extract, while table 8.4 shows the chromatographic and spectral properties of the compounds detected in the hibiscus extract. Figure 8.9 instead shows a magnification of the first 18 minutes of figure 8.8. The main classes of compounds identified include organic acids, hydroxycinnamic acids, anthocyanins and flavonols.

8.3.4.1Organic acids

As is expected, the first componds to elute from the C18 column are the most polar ones, represented here by organic acids. Compound 1 of table 8.4, with m/z 207 in the negative ionization mode, was assigned to hydroxycitric acid, this parent ion breaks into its basic fragment ions 127 $[M-H-H_2O-CO_2]^-$ (Rodríguez-Medina et al., 2009). Peak 2 at m/z 379 has been tentatively identified as hibiscus acid dimer, as it breaks down releasing hibiscus acid corresponding to the fragment at m/z 189 $[M-H-189]^-$ which further fragments to 127 $[M-H-189-H_2O-CO_2]$ which corresponds to a common fragmentation of hibiscus acid. Peaks 3, 4, 5 at m/z 351 were assigned as hibiscus acid hexoside, this molecular ion breaks down by first losing its hexoside moiety to form hibiscus acid 189 [M-H-162] (Ifie et al., 2016). Hibiscus acid then fragments giving rise to its basic fragment 127 $[M-H-H_2O-CO_2]$ as above. Peak 6 at m/z 531 corresponds to a dissacharide adduct of hibiscus acid, breaking down to release hibiscus acid and the fragment ion at m/z 189 [M-H-342]- which corresponds to the loss of a disaccharide adduct. Peak 7 at m/z 221 is a methanolic adduct of hibiscus acid instead, as it breaks losing methanol (molecular weight = 32) and forming hibiscus acid at m/z 189 $[M-H-32]^-$. Peaks 8 and 9 at m/z 203 were tentatively identified as hibiscus acid 6-methyl ester, which break down to give a basic fragment 141 $[M-H-H_2O-CO_2]$ while peak 14, identified as hibiscus

acid hydroxyethyl ester at m/z 235, breaks down to m/z 203 [M-H-CH_3OH] losing a methanol moiety and releasing hibiscus acid methyl ester which fragments as previously described. All the organic acids were only identified in negative ionization mode, no mass spectra have been obtained in the positive ionization mode.

8.3.4.2 Hydroxycinnamic acids

Peaks 20, 22, 23 and 24 at m/z 353 were identified as chlorogenic acids. They break up by the common fragmentation pattern of chlorogenic acids giving rise to fragments 179 [M-H-quinic acid]-, 191 [M-H-caffeic acid], 135 [M-H-quinic acid-CO_2] (Hofmann et al., 2016) and m/z 111 and 173 due to the lost hydroxyl and carboxyl moieties from quinic acid (Brent et al 2014). The identification of chlorogenic acid was confirmed by using the reference standard. Peak 25 at m/z 367 has been tentatively identified as feruloylquinic acid which breaks down to the fragment ion 191 $[M\text{-}H\text{-}193]^-$ due to the loss of ferulic acid, and 161 $[ferulic\ acid\text{-}CH_3OH]^-$ (Lin & Harmly, 2008) due to the loss of quinic acid and methanol. Peak 29 at m/z 335 has been identified as caffeoylshikimic acid, it fragments to 179 $[M\text{-}H\text{-}191]^-$ by the loss of a quinic acid moity, 161$[M\text{-}H\text{-}quinic\ acid\text{-}H2O]^-$ by the losst of a quinic acid and a water molecule and finally 135 [M-H-quinic acid-CO_2] by the loss of a carboxyl group and quinic acid. Peak 21 at m/z 337 was identified as coumaroyl quinic acid. It fragments to m/z 163 [M-H-191]- by the loss of a quinic acid moety, 119 [M-H-191-CO_2] corresponding to a loss of quinic acid and a carboxyl group, 191 $[M\text{-}H\text{-}146]^-$ due to the loss of coumaric acid, and 173 $[M\text{-}H\text{-}146\text{-}H2O]^-$ due to the loss of coumaric acid and a water molecule. Hydroxycinnamic acids were only detected in the negative ionization mode in this experiment, and all have a maximum peak absorption in UV-vis of around 310 nm.

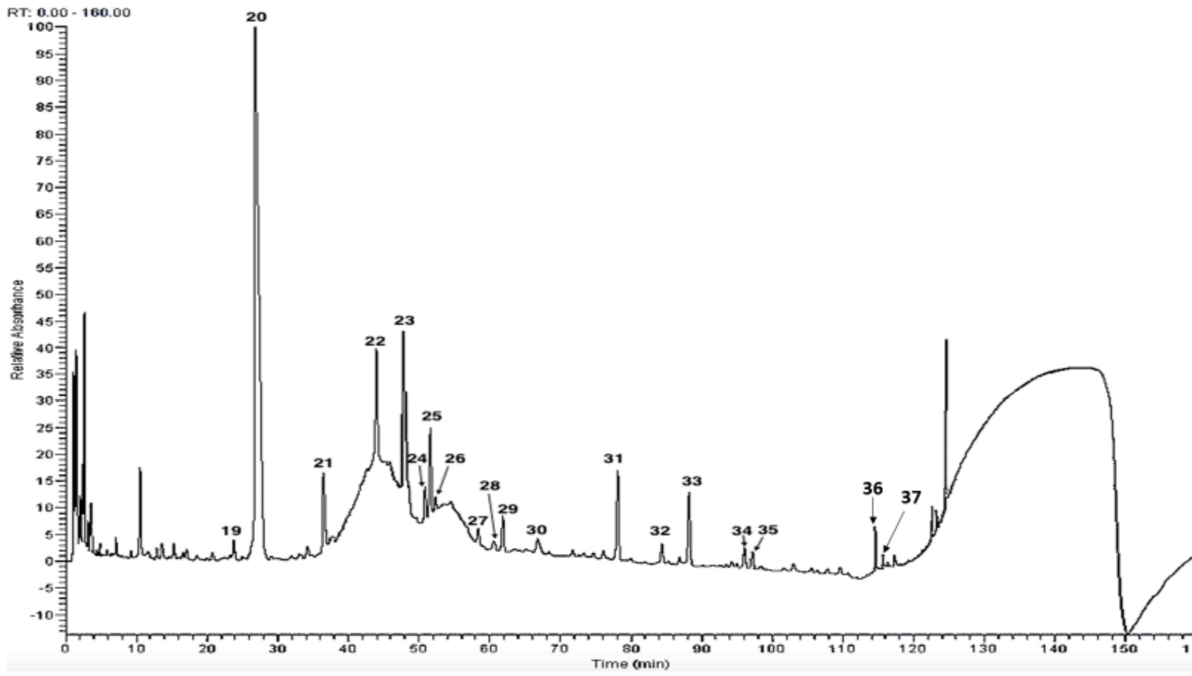


Figure 8.8: Chromatogram obtained at 280 nm after the separation of hibiscus extract.

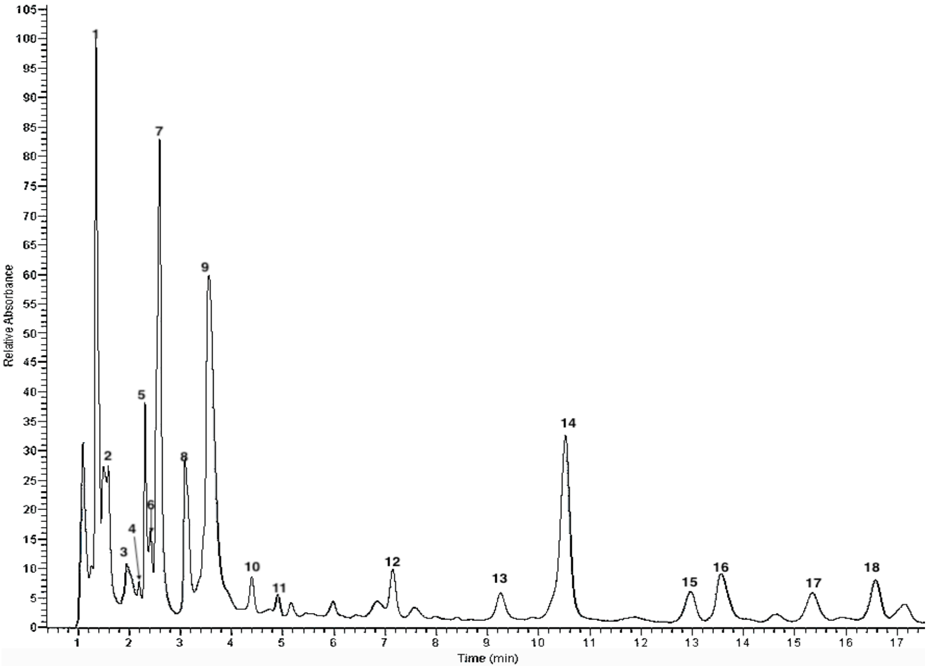


Figure 8.9: Initial part of the chromatogram obtained at 280 nm after the separation of hibiscus extract

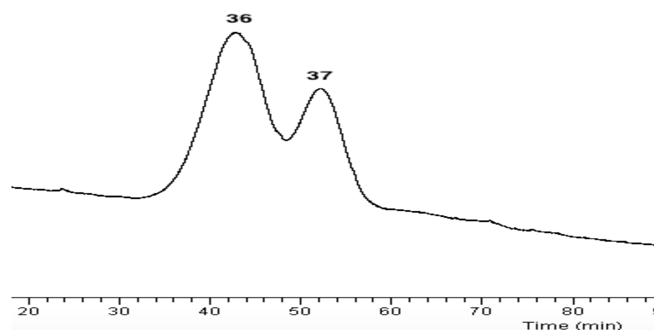


Figure 8.10: Chromatogram obtained at 520 nm after the separation of hibiscus extract, highlighting the anthocyanin peaks.

8.3.4.3 Anthocyanins

By the red colour of hibiscus flowers, one could suppose that they are very rich in the anthocyanins which typically give this strong red coloration to plants. In fact, peak 22 and 23, which had previously been identified as chlorogenic acid, in reality coelute with delphinidin sambubioside. This can be clearly clarified in figure 8.10 which shows peak 36 on the chromatogram, registered at 520 nm, which is a wavelength at which anthocyanins majorly absorb and hydrocynnamic acids don't absorb. So peaks 23, 24 and 36, correspond to delphinidin sambubioside with m/z 613 (aqueous adduct) in the negative ionization mode, which first fragments by losing the adduct (m/z 595 $[M\text{-}H\text{-}H_2O]^-$) then fragments to its principal fragment 301 $[M\text{-}H\text{-}glucose\text{-}xylose]^-$ due to the loss of a glucose and xylose moiety. This molecule was also detected in positive ionization mode at m/z 597, fragmenting to m/z 303 [M+H-glucose-xylose] $^+$ which correspond to the loss of glucose and hexose, forming delphinidin as previously reported (Du et al., 2004). Furthermore, peaks 24 and 25, previously identified as chlorogenic acid, and peak 25 as ferruloyl quinic acid, coelute with cyanidin sambubioside, this can be clarified in figure 8.10 on the chromatogram registered at 520 nm, which shows peak 37, corresponding to cyanidin as the other molecules don't abosrob at 520 nm. Peaks 24, 25, 26 and 27 have thus been identified as cyanidin sambubioside, and were detected both in positive (m/z 581) and negative ionization modes (m/z 579). In the positive ionization mode, this compound fragments to its principal fragment at m/z 287 [M+H-glucose-xylose] $^+$ losing glucose and xylose, thus having a similar fragmentation pattern to delphinidin sambubioside. In the negative ionization mode, it fragments to m/z 284 [M-H-glucose-xylose-H]- by losing glucose and xylose to give a deprototonated cyanidin, it also gives the fragment m/z 339 (Du et al., 2004). These results obtained with anthocyanins were confirmed using reference standards.

8.3.4.4 Flavonols

Flavonols were only detected in the negative ionization mode. Peak 31 at m/z 611 has been identified as myricetin arabino galactoside, which breaks losing an arabino galactose moety to give the fragment at m/z 316 $[M-H-295]^-$ which corresponds to myricetin (Rodríguez-Medina et al., 2009). Peak 33 at m/z 595 has been identified as quercetin sambubioside, it breaks down to quercetin with m/z 301 $[M-H-294]^-$ by losing a glucose and xylose moety, it also generates others fragments which include 463, 179, 271. Peak 34 at m/z 463 has instead been identified as quercetin glucoside, which breaks to its basic fragment at m/z 301$[M-H-162]^-$ by losing a glucose molecule corresponding to quercetin. Peak 35 at m/z 609 has been identified as rutin on the basis of its fragmentation pattern, which includes fragment ions typical of quercetin derivatives, such as the loss of the disaccharide rutinose fragment ion at 301 corresponding to [M-H-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranose]. This result was confirmed by comparison with the reference standard rutin (Tsetegho et al., 2019). Kaempferol-3-O-coumaroyl glucoside was detected at peak 37 with m/z 593, it breaks down to its principal fragment at m/z 285 $[M-H-308]^-$, which corresponds to the loss of a coumaroyl glucoside moety, and 447 [M-H-146]- corresponding to the loss of coumaric acid (Felipe et al., 2014).

8.3.4.5 Tannins

Ellagic tannin was attributed to peak 38 and has the fragmentation pattern reported on the table 7.4.

Peak No	(RT) min	λmax (nm)	$[M-H]^-$	MSn^-	$[M+H]^+$	MSn^+	Tentative identification
Organic acids							
1	1.35	203, 264	207	127(100)83(20), 73(<5), 99(<5), 117 (<5)	/	/	Isomer of hydroxycitric acid

2	1.57	221	379	189(100), 127(40)	/	/	Hibiscus acid dimer
3	2.00	202	351	189(100), 127(40), 291(50), 261(20), 231(5), 181(<5), 151(<5), 211(<5)	/	/	Hibiscus acid hexoside
4	2.19	202	351	189(100),127(40),291(50), 261(20), 231(5),181(<5), 151(<5), 211(<5)	/	/	Hibiscus acid hexoside
5	2.31	202	351	189(100),127(40) 291(50), 261(20), 231(5), 181(<5), 151(<5), 211(<5)	/	/	Hibiscus acid hexoside
6	2.42	202	531	189(100),291(5), 351(<5),413(<5), 445(<5), 513(<5)	/	/	Adduct of hibiscus acid disaccharide
7	2.59	223	221	189(100),127(40), 221(10),159(5), 171(<5), 83(<5)	/	/	Methanolic adduct of hibiscus acid
8	3.1	253	203	141(100),126(5), 185(<5),97(<5), 87(<5)	/	/	Hibiscus acid 6-methyl ester
9	3.55	245	203	141(100), 126(5), 185(<5), 97(<5), 87(<5)	/	/	Hibiscus acid 6-methyl ester

14	10.52	231, 283	235	203(100),141(60),131(10)	/	/	Hibiscus acid hydroxy ethyl ester
Hydroxycinnamic acids							
20	23.80	217, 300	353	191(100),179(65), 135(10),111(<5), 173(<5)	/	/	Chlorogenic acid
22	26.78	243, 324	353	191(100),179(65), 135(10),111(<5), 173(<5)	/	/	Chlorogenic acid
21	39.86	227, 310	337	163(100),119(10), 191(10),173(5)	/	/	Coumaroyl quinic acid
23	43.95	222, 294, 324	353	191(100),179(65), 135(10),111(<5), 173(<5)	/	/	Chlorogenic acid
24	47.79	219, 300, 326	353	191(100),179(65), 135(10),111(<5), 173(<5)	/	/	Chlorogenic acid
25	51.62	221, 295, 324	367	161(100),193(10), 133(10)	/	/	Feruloylquinic acid
29	61.82	234, 328	335	161(100),135(70), 179(10)	/	/	5-O-caffeoylshikimic acid
Anthocyanins							
	43.95	222, 293, 324	613	301(100),487(30), 461(10),257(10),	597	303(100)	Delphinidin sambubioside

22				569(10), 595(100)			
23	47.79	222, 293, 324	613	301(100),487(30), 461(10),257(10), 569(10), 595(100)	597	303(100)	Delphinidin sambubioside
24	51.62	221, 294, 325	579	339 (100), 284 (50)	581	581(100), 287(40)	Cyanidin sambubioside
25	55.76	227, 279	579	339 (100), 284 (50)	581	581(100), 287(40)	Cyanidin sambubioside
27	58.19	227, 285	579	339 (100), 284 (50)	581	581(100), 287(40)	Cyanidin sambubioside
26	57.44	227, 281	579	339 (100), 284 (50)	581	581(100), 287(40)	Cyanidin sambubioside
Flavonols							
31	78.11	222, 256, 353	611	316(100)	/	/	Myricetin arabino galactoside

33	88.19	254, 353	595	301(100),463(20),179(5),271(10), 475(5),577(5), 343(5),409(5)	/	/	Quercetin-sambubioside
34	96.02	254	463	301(100), 257(10),229(10),179(5), 373(<5),395(<5)	/	/	Quercetin 3-glucoside
35	97.10	254	609	301(100),343(15), 271(10), 179(10)	/	/	Rutin
37	114.52	230, 257, 313	593	447(10), 285(100)	/	/	Kaempferol-3-O-coumaroyl glucoside
Tannins							
38	115.92	233	609	463(100), 301(10)	/	/	Ellagic tannins

Table 8.4: Chromatographic and spectral properties of compounds detected in *Hibiscus sabdariffa* flower extract.

8.4 Conclusions

The primary and secondary metabolites determined in this study and their concentrations provide a comprehensive view of the chemical composition of baobab fruit and leaves. The data pertaining to primary metabolites confirms that powdered fruit pulp and dried leaves are rich in carbohydrates, amino acids, and lipids. These results, showing the high nutritional value of baobab fruit pulp and leaves, support the use of fruit pulp in importing countries as well as its sub-Saharan countries of origin, and suggest that leaves could be considered for evaluation as an authorized novel food in Europe and other Western Countries. In addition, as far as lipids are concerned, baobab fruit pulp and

leaves were found to be especially rich in omega-3 essential fatty acids and β-sitosterol, which may be useful in the food industry for preparation of functional foods assisting in the maintenance of normal blood cholesterol levels. In addition, the relatively high squalene content present in organic leaf extract could be useful in the cosmetics industry, especially in barrier products, protecting the skin from free radicals generated by exposure to solar UV radiation and maintaining moisture by lubricating the surface of the skin. The analysis of *H. sabdariffa* showed that it principally presents four classes of compounds, namely organic and phenolic acids, hydroxycinnamic acids, flavonols and anthocyanins. The organic and phenolic acids are represented by hydroxycitric acid, a hydroxycitric acid derivative, hibiscus acid hexoside, hibiscus acid disaccharide adduct, hibiscus acid-6-methyl ester, hibiscus acid hydroxyethyester and hibiscus acid. The hydroxycinnamic acids consist of chlorogenic acid, coumaroylquinic acid, feruloyl quinic acid and 5-O-caffeoylshikimic acid. The anthocyanins are represented by delphinidin sambubioside adduct, delphinidin-3-sambubioside and cyanidin-3-sambubioside. The flavonols are represented by myricetin arabinogalactoside, quercetin-3-O-sambubioside, quercetin-3-glucoside, rutin and kaempferol-3-p-coumarylglucoside. The tannins are represented by ellargic tannins.

In conclusion, the deeper understanding of the chemical composition of baobab and hibiscus provided by this investigation could enhance the value of baobab and hibiscus products and their industrial applications in various fields, both in the countries of origin and importing countries.

CHAPTER IX

QUANTIFICATION OF THE MAJOR COMPONENTS PRESENT IN THE BAOBAB FRUIT, LEAVES AND HIBISCUS FLOWERS EXTRCATS USING VALIDATED HPLC-MS METHODS AND NMR

9.1 Introduction

In the previous sections, the biological activity of baobab fruit pulp, leaves and hibiscus flowers have been explored, and their extracts have been chemical characterized so as to correlate the biological activities with the various components present. The major components present in the extracts have been quantified in order to better appreciate the correlation between these compounds and the noted biological effect, primarily through HPLC-PAD and NMR. The HPLC-PAD method was also validated, validation being a procedure that estimates the fitness of an analytical method to the realization of the goal for which it has been developped.

9.2 Materials and methods

9.2.1 Materials

The materials used here were reported in the previous section.

9.2.2 Methods

9.2.2.1 Quantification of the major components present in the baobab fruit pulp, leaves and hibiscus flowers by HPLC-PAD

The extracts used in this experiment were obtained using the same procedures as reported in section VIII. The 3 different HPLC-PAD-MS analytical methods developed in section VIII were validated and used to estimate the quantity of the major components present in the baobab fruit pulp extract, baobab leaf extract and Hibiscus sabdariffa flower extract. Due to the fact that some of the identified compounds are not yet available on the market, they were quantified in equivalent compounds which are mostly similar. For the fruit pulp extract, the major compounds identified included galacturonic acid, citric acid, catechin, procyanidin B1, procyanidin C1 and kaempferol-3-glucoside. Galacturonic acid and citric acid were quantified using their respective standards, procyanidin dimer was quantified in equivalent of procyanidin B1, procyanidin trimer in equivalent of procyanidin C1 and kaempferol 1-3-O-(6-p-coumaroyl)-hexoside in equivalent of kaempferol-3-glucoside. The major compounds identified in the baobab leaf extract include procynidin dimer, procyanidin trimer, rutin, procyanidin oligomers, Quercetin 3-O-(2,6 di O-rhamnosyl glucoside) and 3-Hydroxy-3-methylglutaroylof quercetin-7-O-hexoside. Procyanidin dimer was quantified in equivalent of procyanidin B1, procyanidin trimer and oligomers were quantified as equivalent of procyanidin C1, Quercetin 3-O-(2,6 di O-rhamnosyl glucoside) and 3-Hydroxy-3-methylglutaroylof quercetin-7-O-hexoside were quantified as equivalent of quercetin rhamnoside.

In the case of *Hibiscus sabdariffa*, the major components identified in extract include chlorogenic acid, cyaniding-3-sambubioside, delphinidin-3-sambubioside, myricetin-3-arabinogalactoside and quercetin 3-o-glucosyl pentoside. Chlorogenic acid, cyaniding-3-sambubioside and delphinidin-3-sambubioside were quantified by using their respective standards while myricetin-3-arabinogalactoside was quantified as an equivalent of myricetin and quercetin 3-o-glucosyl pentoside as an equivalent of quercetin. Calibration curves were generated to confirm linearity, based on a set of serial dilutions from a concentrated mixture of the various standards. The correlation coefficients (r) were then calculated as regression parameters by linear regression. As some of the compounds identified in the extracts are not yet available on the market, their accuracy was assessed by spiking a certain amount of analyte, not in the extract solution but in the solvent in which it is dissolved. Precision was evaluated using repeatability measurements (intraday on various concentrations) and intermediate precision measurements (interday). Reproducibility wasn't assessed and no interlaboratory trial was conducted, because the standardization of the analytical procedure wasn't required. The limits of detection (LOD) and limits of quantification (LOQ) were estimated using various calibration curves calculated as part of the validation procedure, LOD and LOQ were obtained as follows: LOD = 3,3δ/S, LOQ = 10δ/S.

9.2.2.2 Quantification of the major components present in the baobab fruit pulp and leaf extract by NMR

As previously reported in section 8.2.4, signal area integration was performed for quantitative analysis. Note that the quantitative analysis was not performed on those metabolites affected by overlapping peaks or on deconvoluted peaks, and was conducted using the program DIM2015 (Massiot et al., 2002). For water-soluble metabolites, the integrals of selected ^{1}H resonances were measured with respect to the integral of the TSP signal, normalized to 1000 and used as an internal standard. For the $CDCl_3/CD_3OD$ spectra, the integrals were labelled by numbers (I_1-I_{10}) for calculation. First, integrals were normalized with respect to the integral of α-CH_2 groups for all fatty acid chains, I_4 (esterified fatty acids, α−CH_2, 2.35 ppm) + I_3 (free fatty acids, α−CH_2, 2.29 ppm) being set to 100%. Then, the total contents (% mol) of fatty acids of four different types (saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), linoleic fatty acid, and linolenic fatty acid) were calculated according to equations (1-4).

Lipid fractions (fatty acids, β-sitosterol, and squalene) were calculated according to equations (5-7).

Distribution of esterified fatty acids in pulp, between phosphatidylethanolamine (PE), phosphatidylcholine (PC), monoacylglycerol (MAG) and other lipids, was calculated according to equations (8-11)

(1) Linoleic = I_5; (I_5, CH_2, 2.78 ppm)

(2) Linolenic = $I_6/2$; (I_6, $2CH_2$, 2.82 ppm)

(3) MUFA=$I_7 – 2I_5 – I_6 \cdot 6/4$; ($I_7$, all unsaturated fatty acids, CH==CH, 5.36 ppm)

(4) SFA=100 – MUFA – Linoleic – Linolenic;

(5) Fatty acids = $\frac{I_3 + I_4}{I_3 + I_4 + I_2/3 + I_1 \cdot 2/3}$; ($I_1$, β-sitosterol, CH_3, 0.70 ppm; I_2, squalene, $2CH_3$, 1.68 ppm)

(6) β-Sitosterol = $\frac{I_1 \cdot 2/3}{I_3 + I_4 + I_2/3 + I_1 \cdot 2/3}$

(7) Squalene = $\frac{I_2/3}{I_3 + I_4 + I_2/3 + I_1 \cdot 2/3}$

(8) PE = $2 \cdot I_9/I_4$; (I_9, phosphatidylethanolamine, CH_2, 3.22 ppm)

(9) PC = $4 \cdot I_{10}/9 \cdot I_4$; ($I_{10}$, phosphatidylcholine, $N(CH_3)_3$, 3.26 ppm)

(10) MAG = $2 \cdot I_8/I_4$; (I_8, monoacylglycerol, CH, 3.62 ppm)

(11) Other lipids = 1 – PE – PC – MAG

9.3 Results and discussion

9.3.1 Validation of the HPLC-PAD analytical method and quantification of the major components of *Adansonia digitata* fruit pulp extract

As can be seen on the table below, there is a variability of up to 20% in the accuracy and interday precision, while the intraday repeatability presents very low RSD.

Compound	Equation	Regression coefficient	Range (µg/ml)	LOD (µg/ml)	LOQ (µg/ml)
Catechin	y = 54439x - 34990	0,9992	10 - 50	0.25	0.77
Procyanidin B1	y = 52880x - 24166	0,99923	10 - 50	0.47	1.42
Procyanidin C1	y = 60038x - 246714	0,99809	10 - 50	0.21	0.64
Kaempferol-3-glucoside	y = 114297x - 391919	0,99848	10 - 50	0.04	0.13

Galacturonic acid	y = 22405x + 26027	0,99957	4800 – 38400	309	939
Citric acid	y = 131239x + 662524	0,99973	1200 - 28800	210	630

Table 9.1: Linearity, calibration curve, LOD and LOQ.

Compound	Recovery 10 μg/ml (%)	Recovery 20 μg/ml (%)	Recovery 30 μg/ml (%)
Catechin	96	85	80
Procyanidin B1	120	101	95
Procyanidin C1	119	112	94
Kaempferol-3-glucoside	117	112	104
Compound	**Recovery 9.6 mg/ml (%)**	**Recovery 19.2 mg/ml (%)**	**Recovery 28.8 mg/ml (%)**
Galacturonic acid	116	112	113
Citric acid	115	119	92

Table 9.2: Accuracy.

Compound	Repeatability 10 μg/ml		Repeatability 30 μg/ml		Repeatability 50 μg/ml
	STD	**RSD**	**STD**	**RSD**	**STD**
Catechin	4129,639573	0,816389223	27563,11107	1,71091267	20627,94132
Procyanidin B1	7619,03616	1,521996057	19782,9814	1,291713607	7711,929784
Procyanidin C1	3823,672624	0,954689788	24403,92479	1,562685619	16371,21994
Kaempferol-3-glucoside	1538,768339	0,187983417	87139,36141	2,884479662	85910,31842
Compound	**Repeatability 9.6 mg/ml**		**Repeatability 19.2 mg/ml**		**Repeatability 38.4 mg/ml**
	STD	**RSD**	**STD**	**RSD**	**STD**
Galacturonic acid	1666,0001	0,711812744	6335,101288	1,403450629	12633,49945
Citric acid	34978,30484	1,843160978	11759,71483	0,364716621	86950,65806

Table 9.3: Repeatability.

Compounds	Concentration prepared (μg/ml)	Mean measured concentration for 5 days (μg/ml)	RSD	Variation from concentration prepared (%)
Catechin	30	23,94993663	1,825749739	-20,17
Procyanidin B1	30	27,88356657	1,520741168	-7,05
Procyanidin C1	30	27,35577156	1,129988128	-8,814
Kaempferol-3-glucoside	30	33,17309991	0,766151211	10,58

Compounds	Concentration prepared (mg/ml)	Mean concentration (5 days)r	RSD	Variation from concentration prepared (%)
Galacturonic acid	28.8	35,17860299	0,989563401	22,14792705
Citric acid	28.8	29,97272762	1,463813807	4,0719709

Table 9.4: Inter-day precision.

HPLC-PAD quantification showed that the hydro-methanolic baobab fruit pulp extract primarily contains organic acids with galacturonic (or glucuronic acid) acid accounting for 44% and citric acid 7.5%. Polyphenols only represent 0.4% of the extract, with the major polyphenols (kaempferol-3-o-p-coumaroyl hexoside, procyanidin B1, procyanidin C1 and catechin) being present in similar quantities as no statistical difference was seen in their individual quantifications.

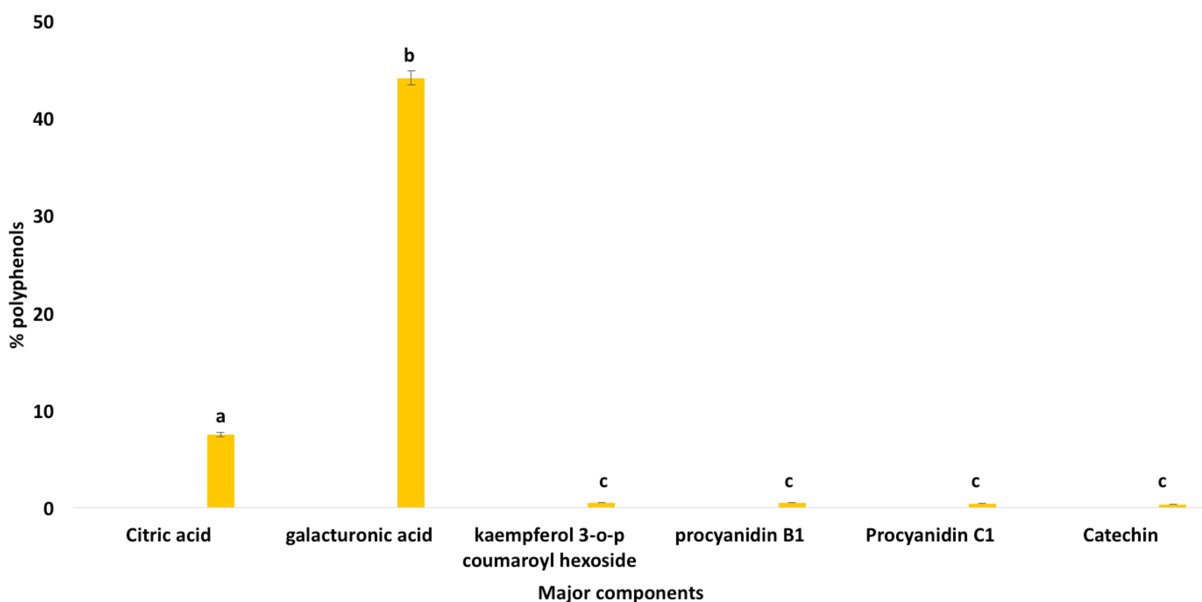


Figure 9.1: Quantification of the various components in the baobab fruit pulp extract.

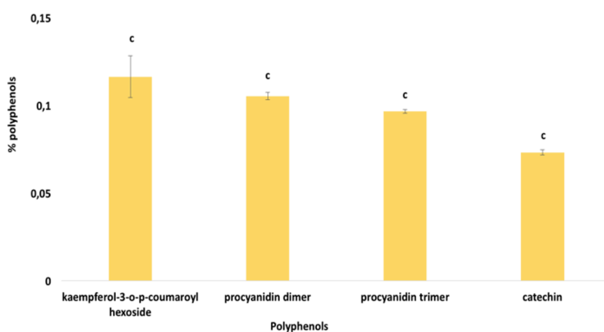


Figure 9.2: Quantification of polyphenols in the baobab fruit pulp extract.

9.3.2 NMR quantification of major components present in the baobab fruit pulp acqueous extract

Regarding the concentrations of sugars and their derivatives, sucrose accounts for about 86% of the total amount of sugars in baobab pulp extract, as indicated by the histograms reported in Fig. 9.3, with glucose in both its α and β isomers representing about 8%, and β-fructopyranose and myo-inositol forming 4% and 2%, respectively. Galacturonic acid represents less than 1% of the total amount of sugars and derivatives.

Histograms reported in Fig. 9.3 indicate that citric acid is present in high amounts in the baobab pulp extracts, at about 77.3%, followed by malic acid (17.6%), ascorbic acid (3.2 %), and gallic acid (1.3%), the other acids present are at amounts below 0.34 %.

Histograms reported in Fig. 9.3 indicate that alanine and γ-aminobutyrate are present in baobab pulp extracts at similar levels, about 54 and 46 %, respectively.

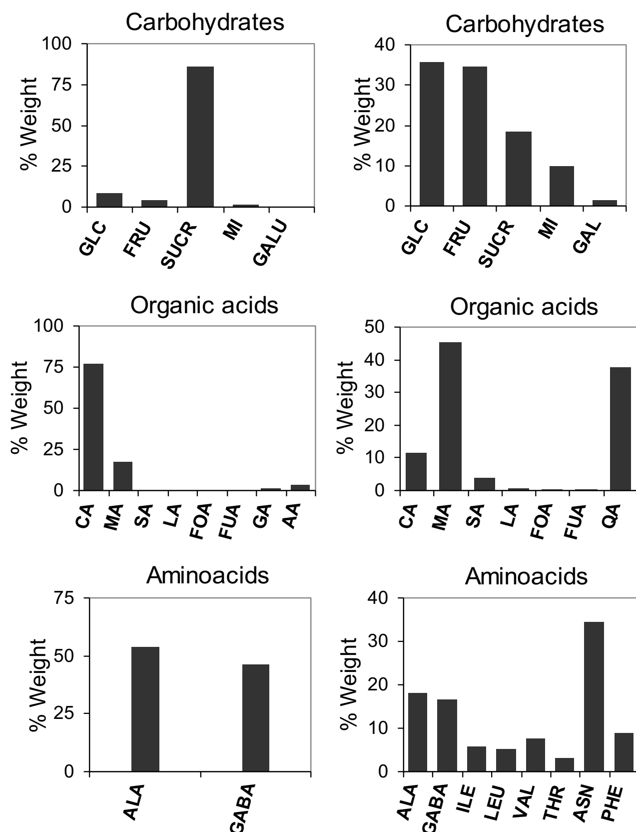


Figure 9.3: Histogram resulting from the quantitative NMR spectroscopic analysis of some metabolites present in aqueous extract of baobab: sugars and derivatives in fruit pulp (a) and leaves (b), organic acids in fruit pulp (c) and leaves (d), and amino acids in fruit pulp (e) and leaves (f).

9.3.3 Validation of the HPLC-PAD analytical method and quantification of the major components of *Adansonia digitata* leaves extract

As can be seen on the table below, aside from a variation in accuracy of up to 12%, the validation process gave good results.

Compound	Equation	Regression coefficient	Range (µg/ml)	LOD (µg/ml)	LOQ (µg/ml)
Procyanidin B1	y = 50608x - 62572	0,99907	10-100	0,51	1,55
Procyanidin C1	y = 54081x - 105942	0,99831	10-100	0,59	1,80
Rutin	y = 51812x - 152410	0,99934	20-200	0,62	1,87
Quercetin rhamnoside	y = 84139x -	0,99826	10-100	0,80	2,42

	651209				

Table 9.5: Linearity, calibration curve, LOD and LOQ.

Compound	Recovery 20 µg/ml (%)	Recovery 40 µg/ml (%)	Recovery 60 µg/ml (%)
Procyanidin B1	106	114	112
Procyanidin C1	102	104	106
Quercetin rhamnoside	94	95	91
	Recovery 40 µg/ml (%)	**Recovery 80 µg/ml (%)**	**Recovery 120 µg/ml (%)**
Rutin	95	105	104

Table 9.6: Accuracy

Compound	Repeatability 10 µg/ml		Repeatability 50 µg/ml		Repeatability 100 µg/ml
	STD	RSD	STD	RSD	STD
Procyanidin B1	7960,046545	1,777224542	124793,8119	5,154573067	83644,16601
Procyanidin C1	9862,808643	2,145262512	30742,84855	1,226598872	21838,12245
Quercetin rhamnoside	20205,2877	5,873135026	106505,6999	3,149253931	184181,2804
Compound	**Repeatability 20 µg/ml**		**Repeatability 100 µg/ml**		**Repeatability 200 µg/ml**
	STD	**RSD**	**STD**	**RSD**	**STD**
Rutin	9590,055839	1,100482516	180518,0056	3,596271838	149040,4839

Table 9.7: Repeatability.

Compounds	Concentration prepared (µg/ml)	Mean measured concentration for 5 days (µg/ml)	RSD	Variation from concentration prepared (%)
Procyanidin B1	40	46,00867122	1.799868933	13.05
Procyanidin C1	40	42,02164346	2.507079862	3.67
Quercetin rhamnoside	40	39.4	1.9	-1.5
Rutin	80	83,04637278	2.231409336	4.81

Table 9.8: Interday precision.

The HPLC-PAD quantification showed that the hydro-methanolic baobab leaf extract principally consisted of polyphenols, which account for about 7% of the dry extract. Of the polyphenols, the most representated is rutin at about 2.8%, followed by procyanidin oligomers which represent 1.7% and Quercetin-3-O-(2,6-di-O-rhamnosyl glucoside with 0.9%. The other major components are present in similar amounts of about 0.6%, and there was no significant difference between them in the statistical test.

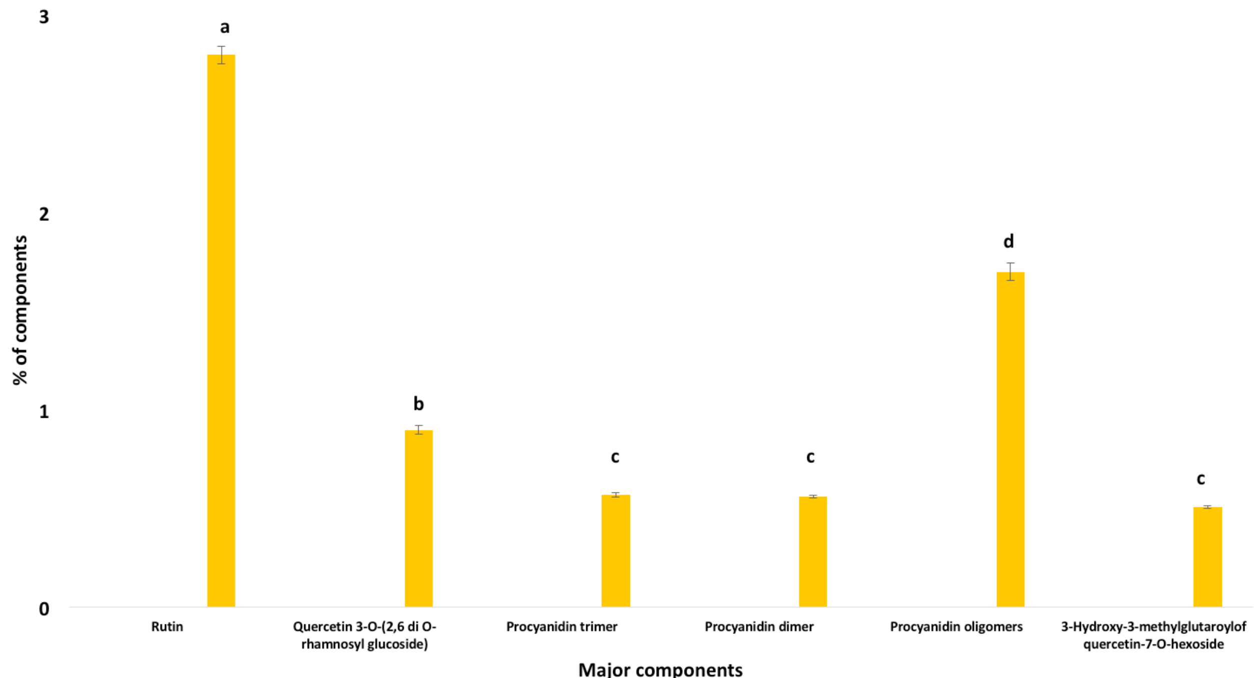


Figure 9.4: Quantification of the major components in the baobab leaves extract.

9.3.4 NMR quantification of major components present in the baobab leaves aqueous extract

The histograms reported in Fig. 9.3 compiled from the quantitative NMR spectroscopic analysis indicate that sucrose accounts for about 18% of the total amount of detected sugars in leaves, whereas glucose represents about 35% in both α and β isomers, and β-fructopyranose accounts for 34.5%. The amount of myo-inositol was found to be higher in leaves than in pulp, at about 10%, with both α and β galactose isomers accounting for 1.5% of the total amount of sugars detected in leaves.

As reported in the histogram in Fig. 9.3, malic acid (45.3%) was the most abundant acid, followed by quinic acid (37.9%), citric acid (11.6 %), and succinic acid (3.8%), other acids, namely lactic, formic, and fumaric were present in amounts of about 1.3%.

Asparagine is the most abundant amino acid (about 34.4 %) in baobab leaf extracts, followed by alanine (18%), γ-aminobutyrate (16.6%), phenylalanine (9 %), valine (7.7 %), isoleucine and leucine in very similar amounts, (5.8 and 5.2 % respectively), and threonine (3.2%).

9.3.5 NMR quantification of major components present in the baobab fruit pulp and leaf organic extracts

	% mol
	Pulp
Fatty acids (FA)	
Free FA	21.2
Esterified FA	78.8
Linoleic	3.2
Linolenic	12.7
Monounsaturated	16.5
Saturated	67.6
Lipid fractions	
Fatty acids	92.4
β-Sitosterol	7.4
Squalene	0.1
Distribution of esterified FA in pulp	
PE	9.2
PC	17.5
MAG	39.7
Other lipids	33.6

Table 9.9: Composition of baobab pulp and leaf organic extracts, and distribution of esterified FA in pulp.

The molar composition of baobab pulp and leaf organic extracts is reported in Table 9.9. With regards to their fatty acids, the amount of free FA in leaves was found to be about twice that of pulp, whereas the amount of esterified FA, and monounsaturated fatty acids (MUFA), was found to be higher in pulp than in leaves. The amount of linoleic acid in leaves was about twice that of pulp. The amount of linolenic acid and saturated acids in pulp was comparable with that found in leaves. In both cases the lipid fraction was mainly comprised of fatty acids, which account for about 90%. The amount of β-sitosterol was comparable in pulp and leaves, whereas the amount of squalene was

much higher in leaves than in pulp. Table 9.9 reports the distribution of esterified FA in pulp. Specifically, MAG was identified as the predominant component in the distribution of esterified FA in baobab pulp (39.7 %), followed by other lipids (33.6%), PC (17.5 %), and PE (9.2 %).

9.3.5 Validation of the HPLC-PAD analytical method and quantification of the major components of *Hibiscus sabdariffa* flowers extract

In validating the method used for the quantification of the major components present in *Hibiscus sabdariffa* flowers, cyanidin and delphinidin sambubiosides were revealed to have a low degree of detection accuracy. There are also quite high RSD in the determination of the interday precision, as can be seen in the figure below.

Compound	Equation	Regression coefficient	Range (µg/ml)	LOD (µg/ml)	LOQ (µg/ml)
Quercetin	y = 281774x - 3E+06	0,99487	10 - 125	0,05	0,15
Myricetin	y = 141667x - 540137	0,99727	10 - 125	0,06	0,20
Chlrogenic acid	y = 116965x + 191821	0,99605	20 - 100	1,99	6,03
Delphinidin-3-sambioside	y = 24346x - 345687	0,99859	100 - 500	2,79	8,46
Cyanidin 3 sambioside	y = 25389x - 76949	0,99855	100 - 500	2,04	6,17

Table 9.10: Linearity, calibration curve, LOD and LOQ.

Compound	**Recovery 50 µg/ml (%)**	**Recovery 75 µg/ml (%)**	**Recovery 100 µg/ml (%)**
Quercetin	90	97	88
Myricetin	90	91	97
	Recovery 20 µg/ml (%)	**Recovery 60 µg/ml (%)**	**Recovery 100 µg/ml (%)**
Chlorogenic acid	101	106	109
Compound	**Recovery 40 µg/ml (%)**	**Recovery 160 µg/ml (%)**	**Recovery 200 µg/ml (%)**
Cyanidin-3-sambubioside	87	88	83
	Recovery 100 µg/ml (%)	**Recovery 400 µg/ml (%)**	**Recovery 500 µg/ml (%)**
Delphinidin-3-sambubioside	81	81	98

Table 9.11: Accuracy.

Compound	Repeatability 20 µg/ml		Repeatability 40 µg/ml		Repeatability 100 µg/ml
	STD	RSD	STD	RSD	STD
Quercetin	110277,9487	3,527246429	163259,7851	1,929098413	299292,3697
Myricetin	69402,12058	3,442086299	98859,94329	1,852848983	279404,3764
Chlorogenic acid	72204,58054	2,915215823	498832,5134	1,987620222	38782,0205
Compound	**Repeatability 40 µg/ml**		**Repeatability 120 µg/ml**		**Repeatability 200 µg/ml**
	STD	**RSD**	**STD**	**RSD**	**STD**
Cyanidin-3-sambubioside	15834,14571	1,734037711	12358,72685	0,404830299	100579,2833
Compound	**Repeatability 100 µg/ml**		**Repeatability 300 µg/ml**		**Repeatability 500 µg/ml**
	STD	**RSD**	**STD**	**RSD**	**STD**
Delphinidin-3-sambubioside	20327,51773	0,98334389	133873,3526	1,875229497	231338,9542

Table 9.12: repeatability.

Compounds	Concentration prepared (µg/ml)	Mean measured concentration for 5 days (µg/ml)	RSD	Variation from concentration prepared (%)
Quercetin	75	74,01424014	2,500048762	98,68565351
Myricetin	75	60,34796624	6,610574637	80,46395499
Chlorogenic acid	100	110,7258411	1,698106394	110,7258411
Cyanidin-3-sambubioside	200	169,7639135	4,065146461	84,88195675
Delphinidin-3-sambubioside	500	491,4859594	9,670178364	98,29719187

Table 9.13: interday precision.

The major components present in *Hibiscus sabdariffa* are primarily polyphenols, with quantification showing a proportion of polyphenols as high as 4.6%. Delphinidin-3-sambubioside accounts for about 2.5%, followed by Cyanidin-3-sambubioside and chlorogenic acid which accounts for about 0.9% each. The least represented are the flavonols miricetin-3-arabinogalactoside and quercetin-3-O-glucosylpentoside.

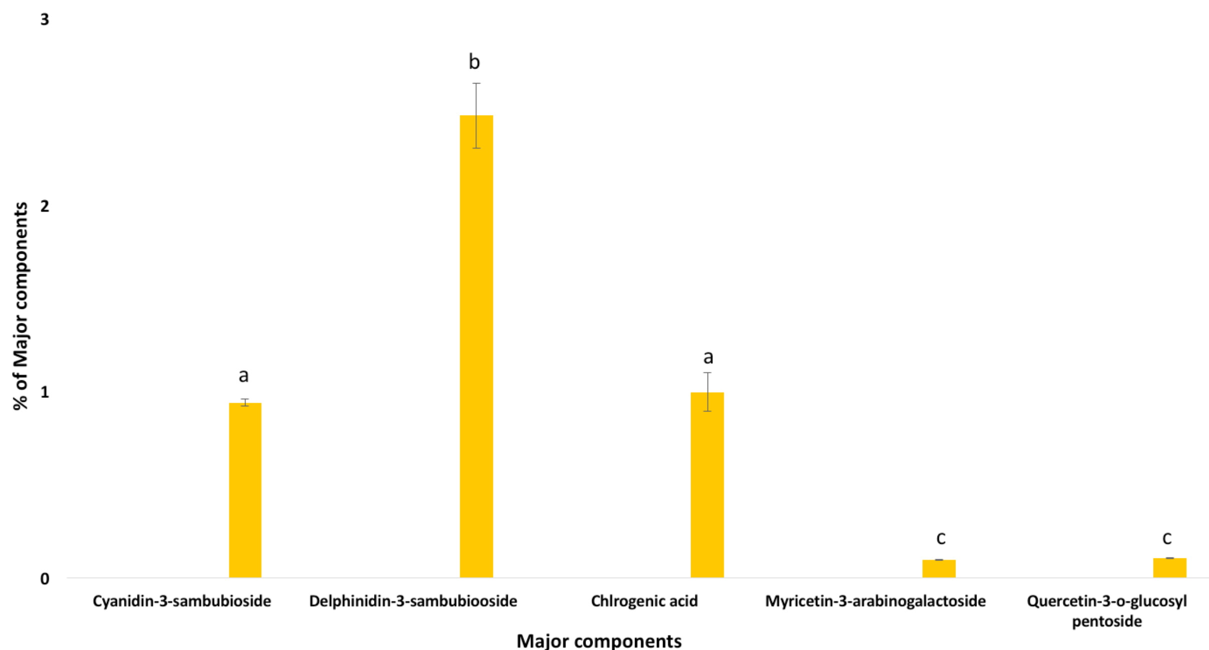


Figure 9.5: Quantification of the major components present in *Hibiscus sabdariffa* flowers extract

9.4 Conclusions

The quantifications showed that the baobab fruit pulp, leaves and hibiscus extracts are rich in polyphenols. As described in the previous section, polyphenols are associated with many beneficial effects on the metabolic syndrome. These results are very encouraging, but further studies must be performed in order to confirm the association between the polyphenols found and their biological activity. The extracts are also rich in omega 3, which is well known for its beneficial effects against the metabolic syndrome.

CHAPTER X

FINAL CONCLUSION

The biological activity of extracts of baobab fruit pulp, leaves and seeds, as well as hibiscus flower extract, have been investigated as part of this research program. Baobab and hibiscus extracts were found to inhibit some of the

enzymes involved in the metabolic syndrome. The extract of baobab leaves demonstrated the highest activity while the raw and toasted seeds showed very low activity. Furthermore, these extracts were tested on human adipocytes by effecting an MTS test and morphological studies, to evaluate if the extracts are toxic to the cells. This informs choice of concentration for use *in vitro* cellular assays, so that this remains below levels toxic to cells, thus not biasing the experiments. A glucose uptake assay has also been conducted, with metformin as a positive control, as this is known to increase the uptake of glucose in human adipocytes. Baobab and hibiscus extract were tested on the uptake of glucose by human adipocytes, and also their ability to stop the maturation of preadipocytes to adipocytes. Ongoing animal studies on rats and mice were established, with the aim of these experiments being to test the beneficial effects of baobab and hibiscus extracts *in vivo,* against important metabolic risk factors including hyperglycemia, hyperlipidemia, body weight, blood pressure and others. The hibiscus extract and pasta powder showed benefic effects on the lipid metabolism of wistar rats by lowering total cholesterol, LDL cholesterol and triglycerides.

The chemical characterization of the baobab and hibiscus extracts was conducted through HPLC-MS and NMR, identifying water-soluble metabolites belonging to different chemical classes such as sugars, amino acids, organic acids, and phenolic compounds, as well as metabolites soluble in organic solvent such as triacylglycerides, sterols, and fatty acids. The HPLC-PAD methods used for the metabolic profiling were then validated and used together with NMR to quantify the major components present in the extracts. This produces a clearer image of the possible compounds which may be responsible for the identified biological activities. In addition to being rich in many components that claim healthy properties, baobab and hibiscus extract also show a good biological activity, tested here against metabolic syndrome. This evidence sheds light on the nutritional and biological properties of these plant foods, and provides suggestions towards possible uses of baobab and hibiscus by food, pharmaceutical and cosmetic industries.

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