Impact of phenolic compounds on ACE and CCK signaling to reduce blood pressure and food intake

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## Table of contents

List of abbreviations .................................................................................................................... 10
Aim of the study .......................................................................................................................... 13
Chapter 1 Literature review on phenolic compounds and their reported anti-hypertensive and anti-obesity effects ........................................................................................................... 19
  1.1 Phenolic compounds .......................................................................................................... 19
    1.1.1 Definition and classification ....................................................................................... 19
      Phenolic acids .................................................................................................................. 19
      Flavonoids ......................................................................................................................... 19
      Tannins ............................................................................................................................ 23
      Stilbenes ......................................................................................................................... 24
    1.1.2 Phenolic compounds content in food and dietary intake ........................................... 24
    1.1.3 Bioavailability and absorption of phenolic compounds .............................................. 27
    1.1.4 Metabolism of phenolic compounds .......................................................................... 30
  1.2 Hypertension ...................................................................................................................... 32
    1.2.1 Definition and status .................................................................................................. 32
    1.2.2 Blood pressure regulation ......................................................................................... 33
    1.2.3 Angiotensin converting enzyme (ACE) and its role in blood pressure ...................... 35
    1.2.4 ACE-inhibitors .......................................................................................................... 36
  1.3 Obesity .............................................................................................................................. 37
    1.3.1 Definition and status .................................................................................................. 37
    1.3.2 Causes, health consequences and treatment of obesity .............................................. 38
    1.3.3 Cholecystokinin and its role in food intake regulation ............................................... 39
  1.4. Overview of the anti-hypertensive and anti-obesity effects of phenolic compounds reported in animal and human studies ..................................................................................... 41
    1.4.1 Overview of the anti-hypertensive effects of phenolic compounds ......................... 41
      1.4.1.1 Anti-hypertensive effects of phenolic compounds reported in animal studies ...... 42
      1.4.1.2 Anti-hypertensive effects of phenolic compounds reported in human studies ...... 50
      1.4.1.3 Possible mechanisms of action ............................................................................ 57
        • 1.4.1.3.1 Phenolic compounds and oxidative stress ................................................... 57
        • 1.4.1.3.2 Phenolic compounds and endothelium ....................................................... 61
1.4.1.3.3 Phenolic compounds and RAAS ............................................................... 65

1.4.1.3.4 Other mechanisms.................................................................................. 67

1.4.2 Overview of the anti-obesity effects of phenolic compounds........................... 70

1.4.2.1 Anti-obesity effect of phenolic compounds and phenolic compounds-rich food reported in animals ................................................................. 70

1.4.2.2 Anti-obesity effect of phenolic compounds and phenolic compounds-rich food reported in human............................................................................. 75

1.4.2.3 Possible mechanisms of action................................................................. 77

1.5 Other bioactivities of phenolic compounds ..................................................... 82

Chapter 2 Angiotensin-Converting Enzyme Inhibitory Effects by Plant Phenolic Compounds: A Study on Structure Activity Relationships ......................................................... 85

2.1 Abstract ........................................................................................................... 85

2.2 Introduction ...................................................................................................... 85

2.3 Materials and Methods .................................................................................. 89

2.3.1 Products....................................................................................................... 89

2.3.2 In vitro ACE inhibitory activity assay ....................................................... 89

2.3.3 Molecular docking and quantitative structure-activity relationship analysis .... 90

2.4 Results ............................................................................................................ 91

2.4.1 Selection of solvent to dissolve phenolic compounds .................................... 91

2.4.2 ACE inhibitory activity by tannic acid, different phenolic acids, pyrogallol and catechol 92

2.4.3 ACE inhibitory activity of flavonoids and stilbenes ..................................... 94

2.4.4 Quantitative structure activity relationship for phenolic acids....................... 95

2.4.5 Molecular docking experiments .................................................................. 96

2.4.5.1 Phenolic acids, catechol and pyrogallol.................................................... 96

2.4.5.2 Flavonoids and stilbenes......................................................................... 100

2.5 Discussion......................................................................................................... 100

Chapter 3 Analysis of interaction of phenolic compounds with the cholecystokinin signaling pathway to explain effects on reducing food intake .................................................................. 111

3.1 Abstract ........................................................................................................... 111

3.2 Introduction ...................................................................................................... 111

3.3 Material and Methods.................................................................................... 113

3.3.1 Cell lines and products .............................................................................. 113

3.3.2 Cell-based bioassay to analyze activation of CCK1R .................................. 114
5.2.2 More animal and human trials on pure phenolic compounds are still important to confirm the claimed antihypertensive effects ................................................................. 149

5.2.3 In vivo experiments to investigate the effect of phenolic compounds on CCK-mediated food intake and body weight .................................................................................. 153
  • 5.2.3.1 Short term experiments .................................................................................. 153
  • 5.2.3.2 Long-term experiments .............................................................................. 154

References ................................................................................................................. 156

Summary .................................................................................................................... 184

Curriculum Vitae ....................................................................................................... 186
**List of abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
</tr>
<tr>
<td>ACE2</td>
<td>angiotensin-converting enzyme 2</td>
</tr>
<tr>
<td>AMPK</td>
<td>activated protein kinase</td>
</tr>
<tr>
<td>Ang I</td>
<td>angiotensin I</td>
</tr>
<tr>
<td>AngII</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>Ang-(1–7)</td>
<td>angiotensin (1–7)</td>
</tr>
<tr>
<td>AP-1</td>
<td>activating protein-1</td>
</tr>
<tr>
<td>ASH</td>
<td>American Society of Hypertension</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BP</td>
<td>blood pressure</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BW</td>
<td>body weight</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBG</td>
<td>cytosolic β-glucosidase</td>
</tr>
<tr>
<td>CCK</td>
<td>cholecystokinin</td>
</tr>
<tr>
<td>CCK-8S</td>
<td>sulfated cholecystokinin octapeptide</td>
</tr>
<tr>
<td>CCK1R</td>
<td>cholecystokinin type-1 receptor</td>
</tr>
<tr>
<td>CHO-CCK1R</td>
<td>Chinese hamster ovary cells expressing CCK1R</td>
</tr>
<tr>
<td>COMT</td>
<td>catechol-O-methyltransferase</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>DASH</td>
<td>dietary approach to stop hypertension</td>
</tr>
<tr>
<td>DBP</td>
<td>diastolic blood pressure</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>-------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>DOCA</td>
<td>deoxycorticosterone acetate</td>
</tr>
<tr>
<td>EDHF</td>
<td>endothelium derived hyperpolarizing factor</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ENaC</td>
<td>epithelial Na(^+) channel</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FMD</td>
<td>flow-mediated endothelium dependent vasodilatation</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GPx</td>
<td>glutathione peroxidase</td>
</tr>
<tr>
<td>HHL</td>
<td>hippuryl-histidyl-leucine</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>ISH</td>
<td>International Society of Hypertension</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>JNC 8</td>
<td>eighth Joint National Committee</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KKS</td>
<td>kallikrein-kinin system</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>LPH</td>
<td>lactase phloridizin hydrolase</td>
</tr>
<tr>
<td>MAP</td>
<td>mean arterial pressure</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MLR</td>
<td>Multiple linear regression</td>
</tr>
<tr>
<td>MMPs</td>
<td>matrix metalloproteinases</td>
</tr>
<tr>
<td>NAD(P)H</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
</tbody>
</table>
NOS  nitric oxide synthase
NPY  Neuropeptide Y
OPA  o-phthaldialdehyde
PAI-1 Plasminogen activator inhibitor-1
PL   pancreatic lipase
PYY  Peptide YY
QSAR quantitative structure–activity relationship
RAAS renin-angiotensin-aldosterone system
RIAI radioimmunoassay
ROS  reactive oxygen species
SAL  saccharic acid 1.4-lactone
SBP  systolic blood pressure
SHRs spontaneously hypertensive rats
SOD  superoxide dismutase
SREBPs sterol regulatory element-binding proteins
STC-1 secretin tumor cell line
SULT sulfation, sulfotransferases
tACE testicular ACE
TNF  tumor necrosis factor
UCPs uncoupling proteins
UGTs uridine-5’-diphosphate glucuronosyltransferases
WKY  Wistar Kyoto rats
**Aim of the study**

Globally, two important threats for public health are hypertension and obesity. It is well known that overweight or obese individuals are at more risk for the development of elevated blood pressure and cardiovascular diseases compared to normal individuals. As such, in the field of nutraceuticals and functional foods, focus is now given to the search for natural components that are able to reduce blood pressure and food intake targeting hypertension and obesity. Studies focusing on the potential role of phenolic compounds on these health issues increased over the last decades. Results from human and animal studies suggested the potential role of phenolic compounds in the battle against obesity and hypertension. However, mechanisms that are involved in the control and regulation of blood pressure, body weight and food intake, are many and complex. ACE (angiotensin-I-converting enzyme) plays a key role in the regulation of blood pressure. A few studies indicated that some flavonoids showed an ACE-inhibitory effect. For the regulation of body weight and food intake, it is known that cholecystokinin (CCK), a gut peptide hormone, plays an important regulating role in inducing satiety signals mediated by the activation of CCK receptors. Yet, the effects of phenolic compounds on the CCK signaling pathway and on the mechanistic insights related to satiety and body weight regulation are not available. Although a lot of studies demonstrated the effects of phenolic compounds in the regulation and inhibition of obesity and hypertension, more information is needed from mechanistic studies focusing on exact mechanisms that could be involved in the reported health claims. In addition, the reported claims in the reduction of risk disease by phenolic compounds are still needed to be discussed in more details in terms of structure-activity relationships. More information about these topics could lead
to a better insight and use of phenolic compounds.

Specifically, the objectives of this work were:

- To collect information from previous studies, in particular animal and human studies, on phenolic compounds with regard to their potential role in hypertensive and obesity management. A special attention was given to discuss possible physiological mechanisms responsible for these effects.
- To evaluate the potential of different pure phenolic compounds belonging to different (sub)-classes for their ACE-inhibitory activity in a cell-free system and to build a structure-activity relationship;
- To screen pure phenolic compounds for their effect on CCK and CCK-receptor signaling pathway using previously established Chinese Hamster Ovary (CHO) cells functionally expressing the rat CCK type-1 receptor (CCK1R) in a cell-based bioassay;
- To screen pure phenolic compounds for their effect on stimulation of CCK secretion. For this purpose, the intestinal secretin tumor cell line (STC-1) which possesses many features of native intestinal enteroendocrine STC-1 cells and are routinely used in screening platforms to identify compounds that modulate secretion of gut hormones and particularly CCK in vitro.

**Outline of the thesis**

The figure below shows a schematic overview of the outline of the study. **Chapter 1** focuses on phenolic compounds and food rich in phenolic compounds in relation to hypertension and obesity. Literature screening was done with the aim of collecting all information from human and animal studies on the potential role of phenolic compounds
and food rich in phenolic compounds on hypertension and obesity. In chapter 2, we studied the effect of phenolic compounds on ACE activity. A specific attention was given to investigate the key structural elements and differences that contribute to ACE inhibition and thereby blood pressure-reducing potency. Chapter 3 was performed to examine the effect of phenolic compounds on the CCK1R signaling pathway as an important pathway in food intake and satiety management. Further in chapter 4, we investigated pure flavonoids for their potential to stimulate CCK secretion from STC-1 cells. Finally, the general discussion and future perspectives from this work are presented in chapter 5.
Chapter 1

Literature review on phenolic compounds and their reported anti-hypertensive and anti-obesity effects

This chapter has been redrafted after:
Chapter 1 Literature review on phenolic compounds and their reported anti-hypertensive and anti-obesity effects

1.1 Phenolic compounds

1.1.1 Definition and classification

Phenolic compounds are a large group of phytochemicals characterized by having an aromatic ring, bearing one or more hydroxyl groups. They range from simple phenolic molecules to highly polymerized compounds (1). Structurally, phenolic compounds can be categorized into several classes: phenolic acids, flavonoids, tannins, and stilbenes (2). In the plant kingdom, they are commonly found conjugated to organic acids and sugars.

Phenolic acids

They are found in many plant species and consist of two subclasses, i.e. the hydroxybenzoic and hydroxycinnamic acids. Both subclasses share a backbone of an aromatic ring and differ in the substitution patterns of hydroxylation and methoxylation on the aromatic ring. The most common hydroxybenzoic acids are gallic, p-hydroxybenzoic, protocatechuic, vanillic and syringic acid. The most common hydroxycinnamic acids are caffeic, ferulic, p-coumaric and sinapic acid which share a three-carbon side chain. Phenolic acids are found in plants as conjugates and are quite seldom in the free form. Where the phenolic oxygen may be glycosylated with sugar, the carboxyl group can be esterified with sugar, tartaric acid, quinic acid or other organic acids (3, 4)

Flavonoids

Flavonoids are a group of phenolic compounds that form an important class of natural products present in fruits, vegetables, and beverages such as tea, coffee and red wine (5).
They share the basic C6–C3–C6 configuration and consist of 2 aromatic C6 rings: ring A and ring B connected by the heterocyclic C ring. The basic flavonoid skeleton can have different substituents such as hydroxyl groups, methyl groups, etc. with majority of flavonoids naturally exist as glycosides. Flavonoids are classified into six sub-groups namely flavonols, flavones, flavanols, anthocyanidins, flavanones and isoflavonoids. Table 1.1 represents the sixth sub-classes of flavonoids with their basic structures, the main dietary sources and the most common flavonoids within each sub-class.
<table>
<thead>
<tr>
<th>Sub-Classes</th>
<th>Food sources</th>
<th>The most Common flavonoids</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flavonols</strong></td>
<td>Onions, Leek Broccoli, Kale, apple</td>
<td><strong>Flavonols</strong></td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quercetin</td>
<td>OH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kaempferol</td>
<td>OH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rutin</td>
<td>Glycoside</td>
</tr>
<tr>
<td><strong>Flavones</strong></td>
<td>Peppers, Celery, Parsely</td>
<td><strong>Flavones</strong></td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Apigenin</td>
<td>H</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Luteolin</td>
<td>H</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Baicalein</td>
<td>OH</td>
</tr>
<tr>
<td><strong>Flavanols</strong></td>
<td>Cocoa, tea, Beans, chocolate</td>
<td><strong>Flavanols</strong></td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Epicatechin</td>
<td>OH</td>
</tr>
<tr>
<td><strong>Anthocyanidins</strong></td>
<td>Black, red, blue and purple berries, Aupergine</td>
<td><strong>Anthocyanidins</strong></td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cyanidin</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td><strong>Flavanones</strong></td>
<td>Orange, Grapefruit, Lemon</td>
<td><strong>Flavanones</strong></td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hesperidin</td>
<td>Glycoside</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Naringenin</td>
<td>OH</td>
</tr>
<tr>
<td><strong>Isoflavonoids</strong></td>
<td>Soyflour, Soybeans, Soymilk</td>
<td><strong>Isoflavonoids</strong></td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Genistein</td>
<td>OH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Daidzein</td>
<td>H</td>
</tr>
</tbody>
</table>
Flavonols are the most widespread of the flavonoids present in a wide variety of fruits, and vegetables. They are characterized by the presence of the double bond between C2 and C3 and the hydroxyl group at position 3. The most studies flavonols are kaempferol, quercetin and myricetin for which the presence in the aglycone form is limited (6). Flavonols are mainly found as O-glycosides preferentially at the 3-position of the C-ring but substitutions can also occur at the 7- and 4'-carbons, although rarely occurs in plant foods (7). Onions (8), broccoli (9), and lettuce (10) are good sources for quercetin, where kale is a significant source for both quercetin and kaempferol (11). Rutin was found in large amount in asparagus shoots (12). In addition to vegetables, beverages such as tea and red wine are considered good sources of flavonols (13).

Flavones are not widely distributed and less common than flavonols in fruits and vegetables. Apigenin, luteolin and baicalein are the most abundant flavones in plants. Celery and parsley are the main edible sources of flavones identified to date (14-16). Flavones have a wide range of substitutions including hydroxylation, methylation and O- and C-glycosylation. C-glycosides of flavones are reported to be present in cereals such as millet and wheat (17, 18).

Flavan-3-ols are the most structurally complex subclass of flavonoids. They range from simple monomers (catechins) to complex structures in which the monomer forms a structural unit in the oligomeric and polymeric proanthocyanidins. Catechins are found in fruits and red wines but tea and chocolate are the richest sources (6, 19, 20). The most common flavan-3-ols and generally found in the free form are catechin and its isomer epicatechin, the main flavanols in fruit, and gallocatechin and epigallocatechin, mainly found in tea (6, 20). Contrary to other classes of flavonoids, flavanols are not
glycosylated in foods. However, the presence of the glycosylated or esterified forms has been reported in some studies (21).

Anthocyanidins are widely spread throughout the plant kingdom. They present predominantly in the outer cell layers of different fruits such as cranberry, red grape, raspberry, strawberry, blueberry, and blackberry. Anthocyanidins are found as sugar conjugates in plant tissues and known as anthocyanins, which is responsible for their variable colors. Glycosylation occurs almost at the hydroxyl group on C3. The sugar residues can be D-glucose, D-galactose, L-rhamnose, L-arabinose, and D-xylose (22). The most widely spread anthocyanins are cyanidins which are found in the human diet from different sources such fruits, vegetables, and red wine (23).

Flavanones are characterized by the presence of a chiral center at C-2 and the absence of the double bond between C2 and C3 in the C ring which is attached to the B-ring at C2 in the α-configuration. Flavanones occur as glycosides in the plant kingdom (6) and are present only at high concentrations in citrus fruits such as lemon and orange (24, 25). The main aglycones are naringenin present in grapefruit and hesperetin in oranges (26). O-rutinoside, named hesperidin is the most common flavanone glycoside. Isoflavonoids such as daidzein and genistein have the B-ring attached at C3. They are classified as phytoestrogens because of the structural similarity with estradiol and their estrogenic activity in certain animal models (27). They occur mainly in leguminous plants, with the highest concentrations found in soybean. Non-fermented soya products contain the β-glycosides forms, while fermented products are rich in the aglycones as a result of hydrolysis of glycosides (28).

*Tannins*
Tannins are polyphenols widely distributed in fruits, vegetables, red wine, cocoa, and certain food grains, such as sorghum, finger millets, and legume. They are classified into two groups according to the polyphenol groups in their molecules: hydrolysable and condensed tannins. Hydrolysable tannins are esters of gallic acid (gallotannins and ellagitannins). Condensed tannins are polymers structurally more complex than hydrolysable tannins and built up from flavanoid precursors (29).

Stilbenes

Stilbenes have a C6–C2–C6 structure and are present in low quantities in the human diet (30). Resveratrol is the main stilbene which is present in red wine and peanuts (31, 32) and in lower amounts found in berries, red cabbage, spinach and certain herbs. Resveratrol occurs as cis and trans isomers, and conjugated derivatives, including trans-resveratrol-3-O-glucoside (33).

1.1.2 Phenolic compounds content in food and dietary intake

The structural diversity causes difficulty in the evaluation of phenolic compounds content in food. Together with lack of standardized analytical methods and variation of content in a particular foodstuff, caused the lack of available estimations on dietary intake of in the literature. Total phenolic compounds intake is calculated from the polyphenol contents in food and food consumption tables. Scalbert and Williamson (34) reported that the dietary intake of phenolic compounds to be a round 1g/day with approximately one third from phenolic acids. In the United States, a quite old study (35) reported that the daily intake of dietary flavonoids was determined to be between 1 and 1.1 g/day. In the Spanish diet, the daily intake of phneolic compounds was estimated to be between 2.55 g and 3.01 g (36). Flavonoids account for two thirds of the total dietary intake of phenolic compounds,
while phenolic acids make about one third.

Most foods contain a mixture of phenolic compounds but the distribution of these compounds at the tissue, cellular and sub cellular levels is not uniform (37). The outer layers of plants contain higher levels of phenolic compounds than inner parts (38). Also, some phenolic compounds like quercetin are found in all plant products; fruit, vegetables, cereals, fruit juices, tea, and wine, whereas flavanones and isoflavones are specific to particular foods. A lot of factors affect phenolic compounds content of plants like degree of ripeness at the time of harvest, environmental factors, processing and storage (39). Generally, it has been observed that ripening reduces the phenolic acids content. In contrast, anthocyanin concentrations increased during ripening. As a response to stress and infection, the concentrations of some phenolic compounds and especially phenolic acids may increase as they are directly involved in the healing process (40). Cooking also affects directly the concentration of phenolic compounds. For examples, onions and tomatoes lose between 75% and 80% of their initial quercetin content after boiling for 15 min, 65% after cooking in a microwave oven, and 30% after frying (14). Additionally, food processing could influence the content of some phenolic compounds. Phenolic compounds in wheat grain are mainly present in the outer layers and so the refined flour loses its content from phenolic compounds. Similarly, quercetin is found in the peel in apple (1 mg/g fresh weight) and thus it is lost during peeling. Storage has a major effect on the phenolic compounds content in food due to oxidation (39). Oxidation reactions result in the formation of more or less polymerized substances, which lead to changes in the quality of foods. Storage of wheat flour for six months results in a significant loss in the concentration of phenolic acids compared with fresh (38). In US diet, it was claimed
that vegetables (dry legumes included) provide 218 mg of total phenolic compounds/d based on Folin assay (41). In general, fruits are considered to have higher content of phenolic compounds than vegetables. The total phenolic content approximately is 1–2 g/100 g fresh weight for some fruits, such as plum and persimmon. Fruits such as apple, plum, and grape contain high amounts of proanthocyanidins. Cherries and other red fruits are rich in anthocyanins with contents vary from 0.15 to 4.5 mg/g in fresh fruits (42). Citrus fruits are the main food source of flavanones with hesperidin (from orange) being the most consumed flavanone. Oranges juice contains 125–250 mg/L (43). Chocolate is also very rich source in phenolic compounds and particularly contribute to catechin (44) and proanthocyanidin intake. Soy is the main source of isoflavones, which contains 1 mg of genistein and daidzein/g dry bean (45). Quercetin, which represents the main flavonol in the western diet, is present in many fruits and vegetables as well as in beverages. Mainly, it is abundant in onions (0.3 mg/g fresh weight) and tea (10–25 mg/L) (15).

Dietary habits and preferences affect to a large extent the dietary intake of phenolic compounds. For example, coffee is richer in phenolic acids than flavonoids and thereby heavy coffee drinkers probably consume more phenolic acids. Also, the proportion of particular classes or individual compounds depends on the consumed food stuffs. For instance, consumption of beverages like tea, red wine or beer will provide more flavanols and proanthocyanidins than other flavonoids (42). Catechins contents in green tea is 1 g/L (46). Red wine contains 270mg/L catechins and 26 mg/L anthocyanins (47). In western diets, the intake of flavonols, flavones and isoflavones is lower than that of phenolic acids and other flavonoids, such as proanthocyanidins, anthocyanins (48). The proportion of compounds such as quercetin and genistein does not exceed 2–4% of
the total phenolic compounds consumed in Western diets (34).

1.1.3 Bioavailability and absorption of phenolic compounds

Several definitions of the term “bioavailability” have been suggested. However, the most appropriate one could be as that fraction of an ingested nutrient or compound that reaches the systemic circulation and then distributes to the target tissues where it exerts its biological action various sites (49). Bioavailability of phenolic compounds is rather low and differs from one compound to another (50). After the consumption of 10–100 mg, the maximum concentration of a single phenolic compound in plasma rarely exceeds 1 μM. However, the total plasma concentration from phenolic compounds is probably higher due to the presence of metabolites formed in the body’s tissues or by the colonic microbiota (34). Singleton et al. (51) reported that the concentration of total polyphenols in plasma after the ingestion of 500 mg of polyphenols to be 50 μM. Another study showed an increase in the plasma polyphenol concentration of 15 μM after the consumption of one third of this quantity of red wine (100 ml) (52).

Several factors might influence the bioavailability of phenolic compounds directly or indirectly by affecting their content in food (53). As examples of factors affect the bioavailability are external factors such as the environmental factors (sun exposure and rainfall) and degree of ripeness. These may affect the content of phenolic compounds in plants and thereby the bioavailability in humans. Other factors that influence the bioavailability of phenolic compounds indirectly are food processing-related factors such as thermal treatment, cooking, culinary preparations, homogenization and storage that influence the content of phenolic compounds in food. Other factors affecting the bioavailability are food-related factors. Food components such as proteins, carbohydrates,
fiber and fat can interact directly with phenolic compounds and subsequently influence their bioavailability. Next, interaction with other compounds in the human body represents an important factor that affect the bioavailability of phenolic compounds. It was reported that quercetin metabolites and epigallocatechin-3-O-gallate possess a high affinity to blood proteins like albumin, which may influence the delivery of these compounds to cells and tissues. The chemical structure of the compound which depends on the degree of glycosylation, acylation, conjugation with other phenolics, molecular size, degree of polymerization, and solubility, is considered one of the main factors affecting its absorption (54). In food and apart from anthocyanins, most of phenolic compounds are present as polymers or in glycosylated forms and so they need to be hydrolysed by intestinal enzymes or by the colonic microflora to be absorbed. Moreover, the basic structure (i.e., benzene or flavone derivatives) of the compound as well as the type of sugar attached is the determinant of the rate and the extent of intestinal absorption.

Host-related factors like the intestinal factors are very important factors that can play a crucial role in the bioavailability of phenolic compounds. When dietary polyphenols are ingested, the absorption of some of these compounds happens in the small intestine. At the intestinal level, two possible mechanisms can be responsible for phenolic glycosides hydrolysis. The first one includes lactase phloridizin hydrolase (LPH), which is a cellular membrane-bound protein enzyme present on the luminal side of the brush-border of the small intestine epithelial cells. LPH deglycosylates phenolic compounds without transport of the glycosides into cells and then the produced aglycone with higher lipophilicity compared to its parent glycosides can enter the epithelial cell by
passive diffusion (55). A large number of flavonoid β-glucosides has been proved as substrates of LPH, such as daidzein-7-O-β-glucoside, genistein-7-O-β-glucoside, apigenin-7-O-β-glucoside, luteolin-7-O-β-glucoside, kaempferol-3-O-β-glucoside, quercetin-3-O-β-glucoside and quercetin-4’-O-β-glucoside. Therefore, the exclusive LPH expression in the small intestine suggests the important role of the intestinal mucosa in the deglycosylation of flavonoid glycosides and thereby a possible rapid intestinal absorption of flavonoids (56). The second mechanism that might attribute to phenolic glycosides hydrolysis, involves the transport of the polar glucosides into enterocytes through the active sodium-dependent glucose transporter SGLT1 where they undergo cleavage of the sugar moiety by cytosolic β-glucosidase (CBG) which is present within the epithelial cells (57).

Once they reach the colon, the non-absorbed phenolic compounds, and the fraction re-excreted in the bile undergo extensively structural modification by the microflora. The microbial conversion plays an important role in the biological activity of many phenolic compounds and in particular for those which are not absorbed in the small intestine and need this transformation to be absorbed (58, 59). For example rutin, a flavonoid glycoside, is not absorbed in the upper part of the intestinal tract, while the aglycone released by the action of colon microflora is reabsorbed (60). Most green tea catechins are mainly not absorbed in the small intestine and need the bacterial conversion in the colon which results in simpler compounds that could be absorbed (61).

Colonic microflora can hydrolyze glycosides into aglycone and further degrade them to more simple compounds like phenolic acids which might be well absorbed through the colonic barrier. Gut microflora can also hydrolyse flavonoid glucuronides
excreted in the bile and the produced aglycone can be reabsorbed and as a result entering into an enterohepatic cycle (7, 58).

Gut bacteria can not only hydrolyze glycosides, glucuronides, sulphates, amides, and esters (62) but also they carry out ring-cleavage, reduction, decarboxylation, demethylation, and dehydroxylation reactions (63). *Lactobacillus plantarum* is an example of the gut bacteria that can breakdown some hydroxybenzoic acids (protocatechuic acid and gallic and) and some hydroxycinnamic acids (caffeic, ferulic, p-coumaric, and m-coumaric acid) via decarboxylation and reduction of the phenolic acid (64). It is worthy to mention that there is a big inter-individual variability in producing these active metabolites which could be linked with the genetic characteristics of the subjects (65). For instance, the isoflavone daidzin is converted to daidzein in the intestine by β-glucosidase. Further, daidzein undergoes more transforming by dihydrodaidzein to produce O-desmethylangolensin by the intestinal bacteria to result in equol. However, it is estimated that only one-third of human individuals to have intestinal microorganisms have the capacity to convert daidzein into equol (59).

### 1.1.4 Metabolism of phenolic compounds

Phenolic compounds are metabolized in both tissues such as the small intestine, liver, and kidneys and by the colonic microflora. They undergo hydrolysis and conjugation by enzymes located in the small intestine and colon and in the liver. First, they are hydrolyzed to their free aglycones, and then are conjugated to form O-glucuronides, sulphate esters and O-methyl ether. Conjugation first occurs in the gut barrier (66) and then the conjugates reach the liver where they undergo further metabolization (67). When quercetin was perfused in the gut of living rats, quercetin glucuronides were formed in
the gut mucosa and secreted back either to the gut lumen or to the serosal side (68). Catechin is extensively metabolized in the liver. This has been shown when it was perfused in the gut of rats as 99% of the catechin excreted in the bile was O-methylated, while before reaching the liver only half of the catechin circulating in the mesenteric plasma was in the O-methylated form (67). Apart from green tea catechins, the free aglycones in the plasma are absent or present with low concentrations (69). However, some studies with pharmacological doses showed the presence of aglycones which might suggest possible saturation for the conjugation pathways (70).

The conjugation process occurs mainly in the liver. A methyl group from adenosyl-methionine transfer to phenolic compounds that contain a diphenolic moiety, such as quercetin, catechin, caffeic acid, and cyaniding. This is catalyzed by catechol-O-methyltransferase (COMT) enzyme which is highly active in tissue liver (53). Uridine-5’-diphosphate glucuronosyltransferases (UGTs) catalyzes the transfer of a glucuronic acid from UDP-glucuronic acid to polyphenols. UGTs are membrane-bound enzymes that are located in the endoplasmic reticulum in many tissues. Glucuronidation of polyphenols starts first in the enterocytes and then further continues in the liver (53). Regarding the sulfation, sulfotransferases (SULT) enzyme mediates the transfer of a sulfate moiety from phosphoadenosine-phosphosulfate to a hydroxyl group on various substrates such phenolic compounds (53).

In humans, the conjugation process is considered to be very efficient and this is evidenced by the predominant presence of the flavonoids in the conjugated forms in plasma and also in urine. Liquid chromatography analyses with and without hydrolysis of the samples with β-glucuronidases and sulfatases showed the appearance of flavonoid
conjugates in humans including $O$-methylated conjugates (69, 71, 72). The conjugation efficiency depends on the type of catechins as the catechin aglycone can be found in the plasma and it ranges from 10% to 80% (69). Also, the type of phenolic acids influences the conjugation percentage which is reported to range between 13% and 100% (72). With excluding anthocyanin glycosides (71, 73), the deglycosylation of flavonoids glycosides is also reported to be very efficient as evidenced by the absence of glycosides in the plasma of subjects supplemented with quercetin glucosides (74).

### 1.2 Hypertension

#### 1.2.1 Definition and status

Hypertension or high blood pressure is one of the most important risk factors that contribute to the development of cardiovascular disease (CVD). Hypertension is responsible for 9.4 million deaths worldwide every year and it affects one-quarter of the world’s adult population, resulting in a public health burden in both developed and developing countries (75). Blood pressure (BP) involves two measurements, systolic blood pressure (SBP) and diastolic blood pressure (DBP). Criteria to define high blood pressure or hypertension criteria have undergone changes although most guidelines recommend SBP/DBP $\geq 140/90$ mm Hg as a cutoff point. In 2013, the American Society of Hypertension (ASH) and the International Society of Hypertension (ISH) guideline recommended SBP/DBP $\geq 150/90$ as a criteria of high BP in 80 year old individuals or older. In 2014, the eighth Joint National Committee (JNC 8) guideline defined hypertension in 60 year old patients and older when BP $\geq 150/90$ mm Hg and $\geq 140/90$ in younger adults (76). The pharmacological therapies for high BP include the use of drugs from 6 different classes: Angiotensin converting enzyme (ACE)-inhibitors, calcium-
channel antagonists, diuretics, β-blockers, angiotensin II receptor blockers and direct vasodilators (77). Besides, non-pharmacological management or life style changes such as cessation of smoking, increased physical activity, reduced sodium intake, moderate alcohol intake and following a healthy diet are considered an important tool in the management and reduction of the risk of hypertension (78).

1.2.2 Blood pressure regulation

The regulation of blood pressure is a very complex process which involves different mechanisms. The diuretic or sodium system, the sympatho-adrenal system, and ACE regulated systems are the most important systems involved in the regulation of blood pressure. The diuretic system regulates indirectly the blood pressure by the release of atrial natriuretic peptide and vasopressin (79, 80). Vasopressin is a peptide hormone that plays a key role in homeostasis and the regulation of glucose, water, and salts in the blood. It affects the tissue's permeability, and thus controls the reabsorption of molecules in the tubules of the kidneys. Vasopressin increases peripheral vascular resistance, as well. This can result in an increase in arterial blood pressure (81). Two catecholamine transmitters are released in the sympatho-adrenal system, namely adrenaline and noradrenaline. The former is released from adrenal medulla and the latter is released from both nerve terminals and adrenal medulla. Catecholamine transmitters can induce an increase in the cardiac rate and contractile force and accelerate relaxation via β-adrenoreceptors. In addition, they can cause blood vessel contraction by interaction with the α-receptors (80).

ACE-regulated systems which are the renin-angiotensin-aldosterone system (RAAS) and the kallikrein-kinin system (KKS) have a major impact on cardiovascular
resistance and maintenance of blood pressure (figure 1.1). Renin-angiotensin-aldosterone system is a complex regulating system in which the decapeptide angiotensin I (Ang I) is released from angiotensinogen by the action of rennin. (ACE) metabolizes Ang I into the active octapeptide angiotensin II (AngII) which is known as a vasoconstrictor (82, 83). Ang II plays a crucial role in both acute and chronic blood pressure regulation. It functions as vasoconstrictive in blood vessels. In addition, AngII promotes the kidney to secrete aldosterone which causes sodium and water retention and potassium excretion. This altogether results in an increased BP. Ang II also has an important role in the growth and development of different cell types and thereby it can cause hypertrophy of the heart and blood vessels (77).

With regard to the kallikrein-kinin system (KKS), bradykinin and kallidin are bioactive kinins released from kininogen by kallikrein (82). Bradykinin and kallidin play a role in physiological functions as blood pressure regulation and heart function. They both and mainly bradykinin have vasodilatory functions. However, ACE inactivates both bradykinin and kallidin by hydrolysis into inactive fractions (84).
Figure 1.1: Role of angiotensin converting enzyme in blood pressure regulation (85).

### 1.2.3 Angiotensin converting enzyme (ACE) and its role in blood pressure

ACE is a metallopeptidase in which the presence of a zinc atom in the active site is essential for the catalytic activity of the enzyme. Two different isoforms of ACE are present in the human body, somatic ACE (sACE) of approximately 170 kDa (kilodalton), and testicular ACE of approximately 100 kDa (86, 87) (Figure 1.2). Somatic ACE is found in most cells in the body and it controls blood pressure and kidney function (88), whereas testicular ACE exists only in the testes and plays an important role in fertility.
Somatic ACE consists of two domains, called the N- and C-domains. Each domain has an active site with a typical zinc binding motif. On the other hand, the structure of testicular ACE is identical to the C-domain of somatic ACE (89). Somatic ACE has the predominant physiological role in blood pressure regulation. It acts as a dipeptidyl carboxypeptidase which releases a dipeptide from the C-terminus and thus converts Ang I to Ang II.

Figure 1.2: Structure of ACE derived from plasma, somatic cell and the testis. It shows the active catalytic sites and zinc dependency. Also, it shows the amino (long area)- and carboxyl (short)-terminus ends which are anchored to the plasma membrane through a single transmembrane domain (90).

1.2.4 ACE-inhibitors

The use of ACE inhibitors is one of the important approaches used in the medication of high BP. Synthetic ACE-inhibitors inhibit ACE in a competitive manner by binding its zinc atom in the active site. Three classes of synthetic ACE-inhibitors were recognized based on the group that binds the zinc atom, namely sulfhydryl-, carboxyl- and phosphinyl-containing inhibitors (77). Captopril and lisinopril are the most used synthetic ACE-inhibitors in the battle against hypertension. The use of synthetic ACE-inhibitors might be accompanied with side-effects such as skin rashes, dry cough, hypotension,
angioedema etc (91). Therefore, the need for alternative inhibitors from natural sources is of great interest.

So far, extensive research has been carried out to look for ACE inhibitors from natural products as the latter might have better drug profiles and less side-effect. Based on this research, some natural substances such as peptides (92, 93) and triterpenes (94) in the aim of ACE inhibition have been well described in literature. Moreover, recent studies also suggested the potential role of some phenolic compounds to inhibit ACE activity (95-98).

1.3 Obesity

1.3.1 Definition and status

Obesity is a complex disease considered as one of the major public health concerns in developed and developing countries. Each year, about 3.4 million adults die as a result of overweight or being obese (99). Obesity and overweight predispose individuals to serious chronic conditions as it is reported that 44% of diabetes, 23% of ischaemic heart disease and between 7% and 41% of certain cancer burdens are attributable to overweight and obesity (100-102).

Body mass index (BMI) is a simple tool used mostly to measure overweight and obesity in epidemiological studies. BMI is defined as the weight in kilograms divided by the square of the height in meters (kg/m²). A person with a BMI of more than 25 and 30 is considered to be overweight and obese, respectively. Measurements of skin fold thickness and waist circumference, waist to hip circumference are other methods used for evaluating body fat (99).
1.3.2 Causes, health consequences and treatment of obesity

The main cause of obesity is the impaired balance between energy uptake and physical activity which can be explained by increased consumption of energy-dense foods that are high in fat and sugar content and the sedentary nature of life nowadays according to WHO. Also, genetic predisposition, differences in hormone balances involving hunger and satiety and cultural/environmental situations could contribute in developing obesity (103). Additionally, the correlations between the composition and metabolic activity of the intestinal microbiota and obesity indicate the possible involvement of the intestinal microbiota in obesity (104).

Obesity is recognized as a chronic disease and the second leading cause of preventable death. Obesity is a major risk factor for hypertension, cardiovascular disease, type 2 diabetes mellitus, and some cancers. In addition, obesity could be involved in other pathological conditions including sleep apnea, osteoarthritis, idiopathic intracranial hypertension, gastroesophageal reflux, and urinary stress incontinence (105).

Medications used for the treatment of obesity and approved by FDA cause weight loss based on different mechanisms: 1) via reduction in calorie intake such as phentermine; 2) preventing the absorption of dietary fat through the inhibition of pancreatic lipase such as orlistat; 3) suppression of hunger and satiety induction such as lorcaserin (106-108). However, many of these drugs showed relative lack of efficacy. Besides, they are often responsible for cardiovascular and/or neurological side effects over a medication period of one-year or more. This limits their use to short term therapy with an exception of only orlistat to be approved for long term use (109, 110).
1.3.3 Cholecystokinin and its role in food intake regulation

Cholecystokinin (CCK) is a hormone peptide that is released from the entire endocrine cells (I cell) in the proximal small intestine in a response to the ingestion of nutrients mainly dietary fat and protein and their digestive products into the small intestine (111). The triangular shape with the apical surfaces oriented toward the intestinal lumen helps intestinal nutrients to stimulate I cells to release CCK from the basolateral site into the interstitial space where it can bind to CCK receptors located in the gastrointestinal tract. CCK presents in the gut and the brain and were found in different molecular forms that derive from the same precursor (single CCK gene ) and differ from each other as a result of posttranslational processing (112). A range of 4 to 83 amino acids were identified to be present in human tissue and blood (113, 114) with CCK-33 and CCK-8 are the most predominant forms in human tissue and blood (115, 116). CCK plays a role in diverse behaviors and states as anxiety, sexual behavior, learning, and memory and spontaneous activity (117). It could also be involved in the functional expression of other gut hormones such as ghrelin, insulin and leptin. (118-120). In the pancreas, CCK functions as a regulatory peptide that triggers secretion of digestive enzymes.

Figure 1.3 : Primary sequence of the most predominant mammalian forms of CCK: CCK-58, CCK-33, and CCK-8 (121).
CCK is one of the most important gut peptides involved in food intake regulation by both satiety and satiation induction (122-124). Its physiological action is mediated mainly via CCK type 1 receptor (CCK1R) activation. CCK1R is a G-protein coupled receptor (GPCR) which is mainly found in the alimentary tract. In human, it is expressed in chief cells, D-cells and mucus cells of the gastric mucosa, in glucagon secreting cells of the pancreas, in smooth muscle cells of the gall bladder, the pyloric sphincter, the intestines and the sphincter of Oddi. Additionally, CCK1R is present on vagal afferent fibers and nerve cells of the myenteric plexus. The activation of the receptor is responsible for controlling gastrointestinal functions which include delayed gastric emptying, inhibition of gastric acid secretion, stimulation of gall bladder contraction, slowing down gastrointestinal motility and stimulation of pancreatic exocrine secretion, and finally it leads to satiety regulation (125, 126). Although most reports from literature indicate to the role of CCK1R in the mediation of satiety, the few evidences that suggest the involvement of CCK2R in the control of food intake cannot be ignored when talking on CCK signaling [5, 26].

In vitro studies on second messengers involved in the signaling through G-protein coupled receptors (GPCRs) revealed the following cascade pathway presented in figure 1.4. In brief, binding of the agonist to GPCR, results in activation of phospholipase C (PLC). Phospholipase C (PLC) hydrolyzes phosphatidyl inositol 4, 5 biphosphate into inositol trisphosphate (IP$_3$) and diacylglycerol (DAG). Inositol trisphosphate (IP$_3$) stimulates intracellular Ca$^{+2}$ release which acts together with diacylglycerol (DAG) to
activate protein kinase (PKC). PKC mediates phosphorylation of other proteins in a cell type specific manner which leads to various cellular responses (127).

![Diagram of signaling pathway](image)

**Figure 1.4:** Simplified view of signaling pathway of the CCK1R via the Gq type of G-protein coupled receptor after binding with an agonist (128).

### 1.4. Overview of the anti-hypertensive and anti-obesity effects of phenolic compounds reported in animal and human studies

#### 1.4.1 Overview of the anti-hypertensive effects of phenolic compounds

Following a healthy diet is considered an important tool in the management and reduction of the risk of hypertension (78). Examples of a healthy diet are the Mediterranean diet or the Dietary Approach to Stop Hypertension (DASH) diet, which are both characterized by high amounts of fruit and vegetables. Several observational and intervention studies reported an inverse association between the risk of hypertension and the high intake of foods rich in phenolic compounds such as cocoa beverage, fruits and vegetables, tea and virgin olive oil. Indeed, phenolic compounds and food rich in phenolic compounds have been extensively investigated in terms of their potential effect on reduction hypertension. A lot of studies in different animal models as well quit good number of human
interventions proved the potential role of phenolic compounds and mainly flavonoids and food rich in phenolic compounds in reducing blood pressure.

1.4.1.1 Anti-hypertensive effects of phenolic compounds reported in animal studies

The effect of phenolic compounds on hypertension was reported in a lot of studies in animal models. In this context, many studies focused on flavonoids and food rich in flavonoids (table 1.2 and table 1.3). As such, evidence from many experiments carried out with spontaneously hypertensive rats (SHRs) suggest the potential role of pure or isolated flavonol compounds in lowering BP with quercetin being the most studied compound in terms of its effect on BP. The chronic effect of oral quercetin administration on reducing BP in hypertensive animals was reported in many studies in recent years. In this context, studies showed that long term oral administration of quercetin in SHRs induced a significant reduction in BP (129, 130). On the other hand, no effect of quercetin was observed in normotensive Wistar Kyoto rats (WKY) in the two studies. More recently, Galindo et al. (131) studied the acute effect of quercetin and its conjugated metabolites (quercetin-3-glucuronide and isorhamnetin-3-glucuronide) on BP in SHRs. They found that BP decreased after 2.5 h of oral administration of quercetin reaching a maximum effect 6 h after administration. Quercetin-3-glucuronide and isorhamnetin-3-glucuronide significantly reduced mean BP, after 1 and 2 h of metabolite injection (1 mg/kg i.v.), respectively. The maximum reduction induced by both compounds was reached after 3 h of injection. However, the inhibitory effect by quercetin and its two metabolites was prevented by D-saccharic acid 1,4-lactone (SAL), a β-glucuronidase inhibitor. Therefore, they suggested that deconjugation which is expected to occur intracellularly might be required for the effect of quercetin metabolites.
Some flavonol-rich extracts obtained from plants have been reported to reduce BP in animal models. After chronic treatment, Brasil et al. (132) showed that the oral administration of methanol extract of *C. papya* rich in quercetin resulted in a reduction in mean arterial pressure (MAP) and serum ACE activity in SHRs. Gasparotto Junior et al (133) showed that the acute administration of hydroethanolic extract of *Tropaeolum majus* (10 - 300 mg/kg), and its semi-purified fraction (12.5 - 100 mg/kg) was able to dose-dependently reduce MAP of normotensive and SHRs (maximal hypotensive effect around 15–20 mm Hg). Isoquercitrin (quercetin-3-O-glycoside) was found as the main compound in the hydroethanolic extract of *Tropaeolum majus* and its semi-purified fraction and when it was administered at a dose of 4 mg/kg, it induced a significant drop in MAP of the normotensive anesthetized rats (around 25 mm Hg).

With regard to flavan-3-ols, studies in animals have found that flavan-3-ols rich plants like grape seed (134, 135) and cocoa (136) are associated with improvement in endothelial function and with reduction of BP. The antihypertensive effect of dietary (-)-epicatechin was investigated in different animal models. However, results were not consistent as some reported an antihypertensive effect (137, 138) while some others did not (139, 140).

Concerning flavanones, the short and long-term effects of glucosyl hesperidin (a water-soluble derivative of hesperidin) and hesperetin were investigated in SHR as well as in normotensive WKY rats. Contrary to results in WKY, glucosyl hesperidin caused a significant reduction in blood pressure in short (141) and long term (142, 143) studies in SHR. The hypotensive effect of hesperetin was significantly inhibited when SHR were injected with L-NAME (141) before hesperetin administration. This indicates that the
antihypertensive effect of hesperetin might be mediated by the vascular nitric oxide synthase (NOS) pathway. On the other hand and in male Wistar rats, naringenin did not result in a significant reduction in blood pressure at a single dose (144), while naringin (flavanone-7-O-glycoside) caused a significant reduction in SBP when applied for long terms (145).

Some flavones have been reported for having synergistic effects in the reduction of BP in animal models. For instance, a mixture of luteolin and buddleoside resulted in acute and long term antihypertensive effects in SHRs. In contrast, a single dose of luteolin or buddleoside was reported not to induce a significant change in BP (146). In diabetic rats, both chrysin and luteolin alleviated diabetes-induced vascular complication and hypertension on the long term (147). Additionally, chrysin and luteolin caused impaired relaxation responses of aorta in diabetes and ameliorated dyslipidemia associated with diabetes.

The effects of lingonberry juice, rich in flavonoids with anthocyanins being the dominant compounds influencing BP, on vascular function and vascular inflammation was investigated in SHRs over a period of 8 weeks (148). At low concentrations of lingonberry juice, reduction in BP consistent with a decrease in inflammatory markers was observed while no change occurred in plasma ACE and aldosterone level. Additionally, lingonberry juice caused an increase in the total plasma calcium concentration which might have positive effect on BP since experimental and epidemiological studies reported a positive effect of dietary calcium on BP (149, 150).

Although quite good evidences are available for the flavonoids in terms of blood pressure lowering potency, few studies investigated the impact of phenolic acids. In this
context, Yeh et al reported that an oral daily dose (100 mg/kg) of caffeic acid for 6 weeks induced a reduction in blood pressure in SHR compared to the control group (151). Another study showed that a chronic treatment (8 weeks) with 50 mg/kg/day of ferulic acid significantly reduced SBP in both SHR and in young N (ω)-nitro-L-arginine methyl ester–treated Wistar rats (152). Suzuki et al. (153) showed that the single ingestion of (30-600 mg/kg) of chlorogenic acid reduced BP in SHR. Additionally, when the rats were fed with 300 mg/kg/day for 8 weeks, the development of hypertension was inhibited compared with the control diet group.

Tannic acid is a polymer that is hydrolyzed in to form gallic acid in the gastrointestinal tract. Thekkumkara et al (154) showed that a daily treatment with tannic acid (5mg/kg body weight) for 4 weeks of SHR induced a significant reduction in MAP in SHR in comparison with animals treated with vehicle control. In addition, withdrawal of tannic acid reversed its inhibitory effect on MAP.
<table>
<thead>
<tr>
<th>Sub-class</th>
<th>Pure or isolated flavonoids</th>
<th>Animals</th>
<th>Dose</th>
<th>Main Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonols</td>
<td>Quercetin</td>
<td>SHRs</td>
<td>10mg/kg BW/d for 13 weeks</td>
<td>Significant reduction in MAP. The chronic treatment with quercetin prevented the upregulated eNOS and p47 protein expression, the downregulated caveolin-1 expression, the increased NADPH-induced superoxide production in SHRs compared to nontreated SHRs</td>
<td>(129)</td>
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<tr>
<td></td>
<td>Quercetin</td>
<td>One-clip (2K1C) Gold blatt (GB) hypertensive rats</td>
<td>Oral administration of 10 mg/kg BW/d for 5 weeks</td>
<td>Significant reduction in SBP since the second week of the administration. Improve the decreased endothelium dependent relaxation to acetylcholine of aortic rings from GB rats. Increased endothelium-dependent vasoconstrictor response to acetylcholine and overproduction of TXB2 by aortic vessels of GB rats, being without effect in normotensive animals. Increased plasma NOx and TBARS, and decreased liver total glutathione (GSH) levels and glutathione peroxidase (G PX) activity in GB hypertensive rats compared to the control animals</td>
<td>(155)</td>
</tr>
<tr>
<td></td>
<td>Quercetin</td>
<td>Male Wistar rats</td>
<td>10mg/kg BW i/d intraperitoneally for 14 days</td>
<td>Quercetin treatment induced no significant changes in the hypertensive responses to angiotensin I and angiotensin II, as well as the hypertensive responses to bradykinin, compared to response to captopril. No significant differences were found in plasma ACE activity in rats treated with quercetin compared with those found in the control group</td>
<td>(156)</td>
</tr>
<tr>
<td></td>
<td>Quercetin</td>
<td>Male Dahl salt-sensitive (DSS) rats</td>
<td>10mg/kg BW/d for 4 weeks</td>
<td>Quercetin lowered SBP significantly in comparison to baseline. The reduction in SBP was in keeping with their increased urinary output, increased sodium output, decreased aldosterone and decreased AT1a mRNA.</td>
<td>(157)</td>
</tr>
<tr>
<td></td>
<td>Quercetin</td>
<td>Male SHRs</td>
<td>10 mg/kg BW/d for 4 weeks (oral administration)</td>
<td>A progressive decrease in mean BP reach maximum level at 6h of administration. Incubation of the mesenteric arteries with quercetin for 30 min caused a significant concentration dependent decrease in the vasoconstrictor response to phenylephrine.</td>
<td>(130)</td>
</tr>
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<td></td>
<td>Quercetin-3- glucuronide isolated from green bean pods isorhamnetin-3-glucuronide produced enzymatically from isorhamnetin</td>
<td>Male SHRs</td>
<td>1 mg/kg BW i.v (acute effect)</td>
<td>A progressive decrease in mean BP with maximum effects seen after 8h of administration. Incubation of the mesenteric arteries with quercetin-3-glucuronide for 1 and 2h and not for 30 min caused a significant reduction in the vasoconstriction induced by phenylephrine</td>
<td>(131)</td>
</tr>
<tr>
<td></td>
<td>Quercetin</td>
<td>Male Sprague Dawley rats</td>
<td>Intragastrically, 25 or 50mg/kg BW/d for 6 days</td>
<td>Quercetin restored the suppressed vascular responsiveness to bradykinin, acetylcholine, and phenylephrine in phenyl hydrazine control rats in a dose-dependent manner. Quercetin partially protected blood glutathione, suppressed plasma malondialdehyde levels, and largely suppressed nitric oxide metabolites and superoxide anion production</td>
<td>(158)</td>
</tr>
<tr>
<td>Enzymatically modified isoquercetin</td>
<td>Male SHRs</td>
<td>3, 25mg/kg BW/d for 50 Days</td>
<td>SBP was significantly lower than that in the control on d22, 36 and 50 of administration</td>
<td>(159)</td>
<td></td>
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<tr>
<td>Isoquercetin</td>
<td>Normotensive and SHRs</td>
<td>Intravenous administration of 0.5, 1, 2 and 4mg/kg BW</td>
<td>Dose dependent reduction in MAP with minor effect on heart rate. Reduced ACE activity in serum samples at 90 min after administration</td>
<td>(133)</td>
<td></td>
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<tr>
<td>Baicaline, flavone, or quercetin</td>
<td>SHRs</td>
<td>Oral administration10 mg/kg BW for 4 weeks. SBP was measured 24 hours after the last treatment</td>
<td>Flavone, quercetin but baicaline not reduced SBP compared to vehicle group. All the flavonoid-treated animals showed remarkably higher endothelium-dependent relaxations to acetylcholine. In contrast to other experimental groups, flavone pretreatment also enhanced the endothelium independent relaxations to sodium nitroprusside</td>
<td>(140)</td>
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</tr>
<tr>
<td>kaempferol</td>
<td>Normotensive and SHRs</td>
<td>Intravenous administration</td>
<td>No effect on MAP</td>
<td>(133)</td>
<td></td>
</tr>
<tr>
<td>Synthesized quercetin-39-sulfate</td>
<td>Male SHRs</td>
<td>1 mg/kg BW i.v (acute effect)</td>
<td>No effect on BP</td>
<td>(131)</td>
<td></td>
</tr>
<tr>
<td>Pentamethyloquercetin</td>
<td>Male Sprague-Dawley rats</td>
<td>2.5, 5, 10 mg/Kg BW</td>
<td>Treatment prevented the increment in SBP after abdominal aorta constriction</td>
<td>(161)</td>
<td></td>
</tr>
<tr>
<td>Flavan-3-ols</td>
<td>Rat model of HFD induced obesity</td>
<td>1mg/kg BW/d for 2 weeks</td>
<td>No change in SBP in both normal and HF diets groups, significantly decreased the rate of weight gain, glycemia and hypertriglyceridemi.</td>
<td>139</td>
<td></td>
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</tr>
<tr>
<td>Epicatechin</td>
<td>Obese diabetic (db/db) mice</td>
<td>0.25% in drinking water/d for 15 wk</td>
<td>Decreased BP, blood pressure, blood glucose, food intake, and body weight gain were not significantly altered</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>(-)-Epicatechin</td>
<td>SHR, Male wistar rats</td>
<td>3 g/kg diet corresponding to 250 mg/kg BW/d For 6 days</td>
<td>Prevented the increase in BP associated with the inhibition of NO production in a dose-dependent manner. Prevented oxidative stress and restored NO bioavailability</td>
<td>138</td>
<td></td>
</tr>
<tr>
<td>Epicatechin</td>
<td>Male Sprague–Dawley rats</td>
<td>0.2–4.0 g/kg diet for 4 days</td>
<td>No changes in blood pressure or heart rate were recorded (readings were taken immediately after compound administration at 0, 0.5, 1, 1.5, and 2 min post injection). Direct and immediate vasodilatory effect</td>
<td>163</td>
<td></td>
</tr>
<tr>
<td>Dihydrokaempferide</td>
<td>SHR</td>
<td>10 mg/kg BW/d (oral administration) for 28 days</td>
<td>Significant decrease in blood pressure, especially marked were the effects observed in the group that received isosakuranetin</td>
<td>164</td>
<td></td>
</tr>
<tr>
<td>Flavanones</td>
<td>Naringin</td>
<td>Male Wistar rats</td>
<td>Naringenin: 100 mg/kg BW/d mixed with diet For 8 weeks</td>
<td>Reduced SBP in High carbohydrate high fat diet fed group</td>
<td>145</td>
</tr>
<tr>
<td>Naringin, Hesperidin, glucosyl hesperidin</td>
<td>stroke-prone spontaneously hypertensive (SHRSP)</td>
<td>Naringin: 1000mg/kg diet Hesperidin: 825mg/kg diet glucosyl Hesperidin: 250, 500, 1000 and 2000 mg/kg diet for 4 weeks Mixed with the diet</td>
<td>Significant decrease in SBP, Improved endothelial function (enhanced endothelium-dependent relaxation induced by acetylcholine, increase in the metabolites of NO in urine samples after ingestion of naringin, hesperidin and glucosyl hesperidin)</td>
<td>143</td>
<td></td>
</tr>
<tr>
<td>Glucosyl hesperidin</td>
<td>SHR and normotensive Wistar-Kyoto rats (WKYs)</td>
<td>A single oral administration (10 to 50 mg/kg BW)</td>
<td>Significant dose-dependent reduction in SBP in SHR 9 and 12 h after administration in SHR</td>
<td>141</td>
<td></td>
</tr>
<tr>
<td>Glucosyl hesperidin</td>
<td>SHR and normotensive Wistar-Kyoto rats (WKYs)</td>
<td>50mg/kg diet (mixed with diet) for 8 weeks</td>
<td>G-Hesperidin had no effects in WKYs. On the other hand and in SHR, the ingestion of G-hesperidin inhibited the development of hypertension and enhanced endothelium-dependent vasodilation in response to acetylcholine, but had no effect on endothelium-independent vasodilation in response to sodium nitroprusside. In addition, G-hesperidin decreased mRNA expression of nicotinamide adenine dinucleotide phosphate oxidase subunits in aorta, which are the main source of superoxide anion in the vasculature</td>
<td>142</td>
<td></td>
</tr>
<tr>
<td>Naringenin</td>
<td>Male Wistar rats</td>
<td>100 mg/kg BW i.p. and BP was recorded for 2h (acute effect)</td>
<td>Naringenin caused a weak and not significant lowering of BP.</td>
<td>144</td>
<td></td>
</tr>
<tr>
<td>isosakuranetin, isolated from Brazilian green propolis</td>
<td>SHR</td>
<td>10 mg/kg BW/d (oral administration) for 28 days</td>
<td>Significant decrease in blood pressure, especially marked were the effects observed in the group that received isosakuranetin</td>
<td>164</td>
<td></td>
</tr>
<tr>
<td>Complex dioclein with β-cyclodextrin (1:1)</td>
<td>Mice</td>
<td>Single oral dose (10mg/kg BW) or 2.5mg/kg BW intraperitoneally</td>
<td>Induced a pronounced decrease in SBP. The onset of the hypotensive effect of complexed dioclein after oral administration is observed later and the maximal effect takes longer to be achieved compared to i.p. administration</td>
<td>165</td>
<td></td>
</tr>
<tr>
<td>Dioclein</td>
<td>Mice</td>
<td>Single oral dose (10mg/kg BW) or 2.5mg/kg BW intraperitoneally</td>
<td>Opposite to intraperitoneally, no significant change in SBP was seen when dioclein administered orally</td>
<td>165</td>
<td></td>
</tr>
<tr>
<td>Flavones</td>
<td>Purified luteolin from the outer cover of <em>Arachis hypogaea</em> (Fabaceae)</td>
<td>Male SHRs</td>
<td>Single dose (25 mg/kg BW, p.o.) (acute effect)</td>
<td>No remarkable decrease in SBP and DBP after 2h of administration</td>
<td>[146]</td>
</tr>
<tr>
<td>Budleloside from the dried flower of <em>Chrysanthemum morifolium</em> Ramat</td>
<td>Male SHRs</td>
<td>Single dose (25 mg/kg BW, p.o.) BP was measured after 2h of administration (acute effect)</td>
<td>No remarkable decrease in SBP and DBP after 2h of administration</td>
<td>[146]</td>
<td></td>
</tr>
<tr>
<td>Luteolin:Budleloside (1:1) mixture</td>
<td>Male SHRs</td>
<td>25 mg/kg BW or 50mg/kg BW of the mixture: Blood pressure was measured after 2h of administration 30, 60mg/kg for 30 days</td>
<td>Acute and long term antihypertensive effects. In addition, on the long term there were significant decrease in angiotensin II (Ang-II) levels and the plasma aldosterone. No significant influence in rennin levels in the SHR. Endothelin level decreased while nitric oxide level increased</td>
<td>[146]</td>
<td></td>
</tr>
<tr>
<td>Chrysos</td>
<td>Male wistar rats</td>
<td>Chrysin: 25mg/kg BW/day for 6 weeks (oral administration)</td>
<td>Attenuated DBP elevation in diabetic induce rats without affecting the developed hyperglycemia, prevented dyslipidemia (elevated levels of TGs, total and LDL cholesterol), attenuate diabetes-evoked impairment in endothelial-dependent relaxation</td>
<td>[147]</td>
<td></td>
</tr>
<tr>
<td>Luteolin</td>
<td>Male wistar rats</td>
<td>Luteolin: 100mg/kg BW/day for 6 weeks (oral administration)</td>
<td>Attenuated DBP elevation in diabetic induce rats without affecting the developed hyperglycemia, prevented dyslipidemia (elevated levels of TGs, total and LDL cholesterol), attenuate diabetes-evoked impairment in endothelial-dependent relaxation</td>
<td>[147]</td>
<td></td>
</tr>
<tr>
<td>Chrysos glucoside isolated from <em>Calycotome villosa</em></td>
<td>Anaesthetized Male Wistar rats</td>
<td>Intravenous injections of bolus doses (1–3 mg/kg BW) of the chrysos glucoside</td>
<td>Immediate and dose-dependent decrease in mean arterial blood pressure (MABP). Pretreatment of the rats with the nitric oxide synthase inhibitor reduced partially, but significantly the maximal decrease in MABP elicited by chrysos glucoside</td>
<td>[166]</td>
<td></td>
</tr>
<tr>
<td>Apigenin</td>
<td>SHRs</td>
<td>Oral administration 0, 0.007, 0.026, 0.104 and 0.417 g/kg BW/day</td>
<td>SBP was decreased. The transcription level of ACE2 mRNA in captopril positive control group and 0.417 g/kg bw apigenin group was significantly higher than the control group</td>
<td>[167]</td>
<td></td>
</tr>
<tr>
<td>Artemetin isolated from <em>Achillea millefolium</em> L. (Asteraceae)</td>
<td>Male Wistar rats</td>
<td>Oral administration (1.5 mg/kg BW) or intravenous (0.15–1.5 mg/kg BW)</td>
<td>Artemetin dose-dependently reduced the MAP at 3h of treatment. Intravenous injection of artemetin (0.75 mg/kg) significantly reduced the hypertensive response to angiotensin I while increased the average length of bradykinin-induced hypotension. Artemetin did not change angiotensin II-induced hypertension</td>
<td>[168]</td>
<td></td>
</tr>
<tr>
<td>Tilitin isolated from <em>Agastache mexicana</em></td>
<td>SHRs</td>
<td>Single oral administration 50 mg/kg BW (acute effect)</td>
<td>Significant decrease in systolic and diastolic blood pressures</td>
<td>[169]</td>
<td></td>
</tr>
<tr>
<td>Betuletol isolated from Brazilian green propolis</td>
<td>SHRs</td>
<td>Oral administration 10 mg/kg BW/d for 28 days</td>
<td>Significant decrease in blood pressure, especially marked were the effects observed in the group that received isosakuranetin</td>
<td>[184]</td>
<td></td>
</tr>
<tr>
<td>Chalcones</td>
<td>Dihydrosinocochalcone-A isolated from <em>Lonchocarpus xuel Lundell</em></td>
<td>SHRs</td>
<td>A single intragastric dose (50 mg/kg BW)</td>
<td>A significant decrease in SBP and DBP in SHR rats. No change in heart rate. Endothelium-dependent vasorelaxant effects.</td>
<td>[170]</td>
</tr>
</tbody>
</table>
methanolic extract of C. papaya (MCEP)

Hydroethanolic extract (HETM) obtained from Tropaeolum majus L.

Semi-purified fraction (TMLR) obtained from Tropaeolum majus L.

Carpobrotus rossii (CR) extract

Whole yellow passion fruit pulp (Passiflora edulis Sims f. flavicarpa Deg.)

Low molecular grape seed proanthocyanidin in extract (LM-GSPE)

Grape seed proanthocyanidin extract (GSPE)

Proanthocyanidins fraction (PAP) from Persimmon Leaf Tea

A natural flavonoid-enriched cocoa powder (CocoaOX)

Aqueous Extract (AE) of Cecropia glaziovii Sneth

Tea catechin extracts

**Table 1.3 The effect of flavonoids rich plant material on blood pressure reported in animals models**

<table>
<thead>
<tr>
<th>Plant or plant extracts</th>
<th>Identified flavonoids</th>
<th>Animals</th>
<th>Dose</th>
<th>Main results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanolic extract of C.papaya (MCEP)</td>
<td>Quercetin 47.1±1.0 µg/g extract, Epicatechin 36.1±0.3 mg/g</td>
<td>SHRs and wistar rats</td>
<td>100mg/kg BW</td>
<td>Serum ACE inhibition in both SHRs and in Wistar rats. Reduce MAP in SHRs. The chronic treatment with MCEP reversed both cardiac hypertrophy and the reduction of arterial baroreflex sensitivity.</td>
<td>(132)</td>
</tr>
<tr>
<td>Hydroethanolic extract (HETM) obtained from Tropaeolum majus L.</td>
<td>Isoquercitrin 38.6±4.0 mg/g, dry HETM</td>
<td>Normotensive and SHRs</td>
<td>Oral administration of HETM (10–300 mg/kg BW) MAP and heart rate were measured at 1.5, 3, 6 and 12 h of treatment</td>
<td>HETM significantly reduced, in a dose-dependent manner, the MAP in both normotensive and SHRs. In addition, these preparations significantly decreased the MAP for up to 3 h after the administration. Heart rate was not affected. Reduced ACE activity in serum samples at 90 min after administration</td>
<td>(133)</td>
</tr>
<tr>
<td>Semi-purified fraction (TMLR) obtained from Tropaeolum majus L.</td>
<td>Isoquercitrin 93.45±2.80 mg/g dry HETM</td>
<td>Normotensive and SHRs</td>
<td>Oral administration of TMLR (12.5–100 mg/kg BW) MAP and heart rate were measured at 1.5, 3, 6 and 12 h of treatment</td>
<td>TMLR significantly reduced, in a dose-dependent manner, the MAP in both normotensive and SHRs, MAP decreased for up to 3 h after the administration. Heart rate was not affected. Reduced ACE activity in serum samples at 90 min after administration</td>
<td>(133)</td>
</tr>
<tr>
<td>Carpobrotus rossii (CR) extract</td>
<td>3% w/v acylated spinacetin O-glucoside</td>
<td>Male Hooded-Wistar rats</td>
<td>35±0.32 mg/kg BW/d for 4 weeks</td>
<td>No change in SBP. Total cholesterol was lower but other blood chemistry parameters including HDL and triglycerides were similar.</td>
<td>(171)</td>
</tr>
<tr>
<td>Whole yellow passion fruit pulp (Passiflora edulis Sims f. flavicarpa Deg.)</td>
<td>Mg in 100 g in the yellow passion fruit pulp: Apigenin-3-mannoside (4.01±0.17), Luteolin-3-glucoside (3.63±0.15), Quercitin (2.21±0.32), Kaempferol (1.78±0.06)</td>
<td>SHRs</td>
<td>5,6 and 8g of Yellow passion fruit pulp/kg BW/d for 5 days</td>
<td>8g from the whole passion fruit significantly decreased SBP. Enhancement of the antioxidant status</td>
<td>(172)</td>
</tr>
<tr>
<td>Low molecular grape seed proanthocyanidin in extract (LM-GSPE)</td>
<td>Mg/g extract: Catechin 90.7±7.6, Epicatechin 55.0±8.8, Procyanidin dimer 144.2±32.2, Procyanidin trimmer 28.4±2.0, Procyanidin tetramer 2.0±0.2, Quercitin 0.3±0.0, Quercitin-3-O-gallate 0.2±0.0, Naringenin-7-glucoside 0.1±0.0, Kaempferol-3-glucoside 0.1±0.0</td>
<td>Wistar rats</td>
<td>375mg/kg BW (acute effect)</td>
<td>Decrease in both the SBP and DBP within 6 h from administration. Decrease in both the plasmatic and hepatic TC and TG levels</td>
<td>(134)</td>
</tr>
<tr>
<td>Grape seed proanthocyanidin extract (GSPE)</td>
<td>Epigallocatechin gallate 0.4±0.1, Procyanidin trimmer 28.4±2.0, Procyanidin tetramer 2.0±0.2, Epigallocatechin gallate 55.3±1.5, Quercitin 3-O-gallatoside 0.2±0.0, Naringenin-7-glucoside 0.1±0.0, Kaempferol-3-glucoside 0.1±0.0, Quercitin 0.3±0.0</td>
<td>SHRs and WKY</td>
<td>375mg/kg BW (acute effect)</td>
<td>GSPE caused a significant decrease in SBP of SHR (maximum decrease 6 h post-administration), while did not affect BP of Wistar–Kyoto rats. GSPE increased the activity of an antioxidant endogen system but did not affect plasma ACE value compared to non treated group. Improvement of oxidative stress (rapid increase in hepatic GSH, observed only 6 h post-administration in the GSPE group of SHR)</td>
<td>(135)</td>
</tr>
<tr>
<td>Proanthocyanidins fraction (PAP) from Persimmon Leaf Tea</td>
<td>Content of PAF (%)</td>
<td>Single dose 100, 300 mg/kg BW using stomach tube. BP was measured at 0, 2,4,6 8h</td>
<td>300 mg/Kg caused a gradual and significant decrease in SBP with minimum value obtained at 4h. 500mg/kg resulted in a maximum but not significant value of SBP at 2h</td>
<td>(173)</td>
<td></td>
</tr>
<tr>
<td>A natural flavonoid-enriched cocoa powder (CocoaOX)</td>
<td>(+)-catechin 5.18±0.09, (-)-epicatechin 19.36±0.03, procyanidin B2 16.8±0.06, procyanidin B1 1.25±0.07</td>
<td>SHRs</td>
<td>50, 100, 300, and 600mg/kg BW. BP was measured at 2, 4, 6, 8, 24, and 48 h Post administration</td>
<td>Does dependent antihypertensive effect up to the dose of 300mg. The maximum decrease in the SBP was achieved 4 h post-administration of 300mg/Kg</td>
<td>(136)</td>
</tr>
<tr>
<td>Aqueous Extract (AE) of Cecropia glaziovii Sneth</td>
<td>Catechins: 12%, Procyanidins: 19%, Flavones: 19%</td>
<td>Female normotensive Wistar rats</td>
<td>A single dose of AE (1 g/kg BW, p.o.) or repeatedly (0.5 g/kg/Bid, p.o.) for 60 days</td>
<td>The arterial blood pressure, heart rate and plasma ACE activity were not significantly modified within 24 h after a single dose administration of AE. Repeated treatment with AE reduced the mean SBP after 14 days. The plasma ACE activity was unchanged. Reduce BP not due to ACE</td>
<td>(174)</td>
</tr>
<tr>
<td>Tea catechin extracts</td>
<td>56.3% (+)-EGCG, 11.0% (+)-epigallocatechin (EGC), 9.9% (+)-epicatechin (EC), 5.1% ( )-gallocatechin-gallate</td>
<td>Malignant Stroke-prone spontaneous</td>
<td>0.5% tea catechin extract solution as drinking water for 3weeks</td>
<td>Tea catechin ingestion significantly delayed stroke onset by 10 days compared to the control group. Although there was no difference in blood pressure at 10 weeks, the rate of in blood pressure increase in the tea catechin group was significantly smaller than that in the</td>
<td>(175)</td>
</tr>
</tbody>
</table>
GCG), 3.2%
[-]epicatechin-gallate (ECG), 0.1%
[-]catechin-gallate (CG)

Hypertensive rats (BMSHRSP) control group.

<table>
<thead>
<tr>
<th>Green tea extract</th>
<th>Epicatechin (EC) 3.83 ± 0.15 [-]Epicatechin-gallate (ECG) 13.50 ± 0.92 [-]Epigallocatechin (EGC) 33.49 ± 1.31 [-]Epigallocatechin-gallate (EGCG) 28.54 ± 1.22</th>
<th>Diabets induced Male albino rats of Wistar strain</th>
<th>300mg/kg BW for 4 weeks</th>
<th>The increase in blood glucose, glycated hemoglobin and systolic blood pressure in diabetic rats were reduced upon green tea treatment.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tea catechin</td>
<td>31.7% of epigallocatechin gallate, 15.7% of epigallocatechin, 10.0% of epicatechin gallate, and 8.5% of epicatechin</td>
<td>Sprague-Dawley rats diabetic induced rats</td>
<td>5mg/day (in drinking water) for 12 weeks</td>
<td>No change in blood pressure</td>
</tr>
<tr>
<td>Lingonberries (Vaccinium vitis-idaea) juice</td>
<td>Content in juice mg/100g: Anthocyanins 5.9 ± 0.1 Flavonols 3.8 ± 0.1 Flavan-3-ols 3.0 ± 0.1</td>
<td>SHRds</td>
<td>Daily intake per rat: Anthocyanins 2.2 mg Flavonols 1.4 mg Flavan-3-ols 1.2mg Procyanidins 7 mg For 8 weeks</td>
<td>SBP decreased. Endothelium-dependent vascular relaxation was not improved. Slightly affected plasma inflammatory markers (reduction of NOx and sICAM-1) and clinical chemistry variables (decreased alkaline phosphatase and increased chloride and calcium levels). No change in circulating ACE between the different groups. Plasma aldosterone levels did not differ</td>
</tr>
</tbody>
</table>

14.1.2 Anti-hypertensive effects of phenolic compounds reported in human studies

The potential health benefits of phenolic compounds and phenolic compounds-rich food in humans has been addressed over the last decades. In this regard, human intervention trials provided indications that consumption of foods rich in phenolic compounds might be associated with a reduced risk of coronary heart disease and hypertension.

Cocoa products are the main sources of flavonoid-rich foods studied for their potential to lower BP. Some randomized controlled trials showed no significant effect for the daily ingestion of flavonoid-enriched chocolate on BP for 1 year (178, 179). However, evidence from other studies supports the health promising effects of consumption of flavonoid-rich cocoa products such as dark chocolate in the control of SBP and DBP (180-182). In this context, it is worthy to mention is that European Food Safety Authority (EFSA) has approved the health claims on consumption of cocoa flavanols in the high flavanol cocoa extract (i.e. in capsules or tablets) and maintenance of normal endothelium-dependent vasodilation. In order to obtain the claimed effect, 200 mg of cocoa flavanols should be consumed daily. This amount could be provided by less than one gram of high flavanol cocoa extract in capsules or tablets, and can be consumed
in the context of a balanced diet.

Fruits and vegetables are rich sources of anthocyanins and flavanols. Human intervention studies using fruits and vegetables for their potential antihypertensive effect have been less performed. However, there is a consistency in the results reported in terms of positive effects of fruits and vegetables rich in anthocyanins and/or flavanols in reducing BP and the potential role in the protection against CVD regardless of the study design and study period (183-186).

Grape and grape seed are rich sources of phenolic compounds. The involvement of these sources in promoting BP reduction was reported in many studies. In a placebo-controlled double-blind study, Park et al. (187) evaluated the effect of daily consumption of 5.5ml/kg body weight of grape juice (containing 472.8 mg total polyphenols per 240 mL) for 8 weeks in hypertensive Korean patients. At the end of the 8 weeks of intervention, both SBP and DBP were significantly reduced by 7.2 mm Hg and 6.2 mmHg, respectively. A meta-analysis of randomized controlled trials showed that a polyphenol rich grape seed extract significantly lowered SBP and heart rate (188). Very recently, a double-blind, placebo-controlled, randomized intervention study investigated the effect of a specific grape seed extract rich in low-molecular-weight polyphenolic compounds on ambulatory blood (ABP) in untreated subjects with pre- and stage I hypertension. This study showed that consumption of 300 mg/d grape seed extract during an intervention period of 8 weeks did not significantly lower the ABP in untreated subjects with pre- and stage I hypertension (189). Additionally, the consumption of raisins which are rich sources of a wide variety of polyphenols, catechins, resveratrol, and isoflavones (daidzein and genistein) was reported to be associated with lowering of blood
pressure (190, 191).

Until now, only few studies attempted to evaluate the impact of consuming isolated or pure phenolic compounds on BP. However, quercetin, which is one of the most abundant flavonoids in fruits and vegetables and probably the most studied molecule, has been investigated quite thoroughly for its antihypertensive potential. In a randomized, double-blind, crossover trial, 4 weeks of supplementation with quercetin (730 mg/day) resulted in a reduction in SBP (−7 ± 2 mmHg) and DBP (−5 ± 2 mmHg) in stage I hypertensive patients but not in those without hypertension (192). More recent studies in overweight–obese subjects with risk for metabolic syndrome showed that quercetin consumption (150 mg/day) for 5–6 weeks significantly reduces SBP and provides protection against CVD (193, 194). Hesperidin is another flavonoid that has been reported to have a beneficial effect on BP as stated in table 3. A randomized crossover study with healthy volunteers found that a 4-week consumption of orange juice (500 ml/day containing 292 mg of flavanon hesperidin) as well as a control drink plus hesperidin (146 mg) resulted in a significantly lower DBP (−3.2 ± 1.5 mmHg and -5.5 ± 1.8 mmHg, respectively) compared to a control drink plus placebo (195).

With regard to the impact of phenolic acids on blood pressure regulation in humans, a double-blind placebo-controlled study evaluated the effect of chlorogenic acid from green coffee bean extract on mild hypertensive subjects. It was found that a treatment with 140 mg/day of chlorogenic acid for 12 weeks caused a significant reduction in SBP and DBP by 10 and 7 mmHg, respectively (196).

In short, some promising effects on human blood pressure were reported with specific compounds and their food sources. However, most results are based on complex
food, extracts or beverages and thus the effect of compounds other than phenolic compounds cannot be excluded (178, 183, 197, 198). In addition, different designs, different intervention times and different doses might result in different results (194, 199, 200). Therefore, more human interventions using specific pure phenolic compounds are needed to confirm the role of these compounds in lowering blood pressure.
### Table. 1.4 Recent trials on the effect of flavonoids and flavonoids rich food on blood pressure in humans

<table>
<thead>
<tr>
<th>Authors</th>
<th>Participants</th>
<th>Study design</th>
<th>Intervention duration</th>
<th>Dose and treatment</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edwards et al. (192)</td>
<td>Men and women with prehypertension (n = 19) and stage 1 hypertension (n = 22)</td>
<td>Randomized, double-blind, placebo-controlled, crossover study</td>
<td>4 weeks</td>
<td>750 mg quercetin/d vs. placebo.</td>
<td>Blood pressure was not altered in prehypertensive patients after quercetin supplementation. In contrast, reductions in (P &lt; 0.01) systolic (-7 +/- 2 mm Hg), diastolic (-5 +/- 2 mm Hg), and mean arterial pressures (-5 +/- 2 mm Hg) were observed in stage 1 hypertensive patients after quercetin treatment.</td>
</tr>
<tr>
<td>Larson et al. (200)</td>
<td>Normotensive men (n = 5) and stage 1 hypertensive men (n = 11)</td>
<td>Randomized, double-blind, crossover placebo-controlled trial</td>
<td>Acute effect</td>
<td>1095 mg quercetin (single dose)</td>
<td>In stage 1 hypertensive individuals, mean BP decreased significantly 10 hours after administration. Plasma ACE activity, nitrites and brachial artery flow-mediated dilation were unaffected.</td>
</tr>
<tr>
<td>Dower et al. (201)</td>
<td>Healthy men and women (n=37)</td>
<td>a randomized, double-blind, placebo-controlled, crossover trial</td>
<td>4 Weeks</td>
<td>Participants received (-1)-epicatechin (100 mg/d), quercetin-3-glucoside (160 mg/d), or placebo capsules</td>
<td>Both epicatechin and quercetin-3-glucoside supplementation did not change flow-mediated dilation, blood pressure (office BP and 24 ambulatory BP), NO, endothelin 1, and blood lipid profile.</td>
</tr>
<tr>
<td>Perez et al. (199)</td>
<td>15 Healthy volunteers (6 female)</td>
<td>Randomized, double-blind, placebo-controlled trial</td>
<td>3 Weeks</td>
<td>Participants were given a capsule containing placebo or 200 or 400 mg of quercetin in daily</td>
<td>No changes were observed in BP.</td>
</tr>
<tr>
<td>Brüll et al. (202)</td>
<td>70 overweight-to-obese patients with prehypertension and stage I hypertension.</td>
<td>Randomized double-blind placebo-controlled cross-over trial</td>
<td>6-week treatment periods separated by a 6-week washout period</td>
<td>Patients were randomized to receive 396 mg of onion skin extract powder/d which contains 162 mg quercetin or placebo</td>
<td>In the total group, quercetin did not significantly affect 24-h ABP parameters and office BP. In the subgroup of hypertensives, quercetin significantly decreased 24-h, daytime, and night-time SBP when compared with placebo. In total group and also in the subgroup of hypertensives, vasoactive biomarkers including endothelin-1, soluble endothelial-derived adhesion molecules, asymmetric dimethylarginine, angiotensin-converting enzyme activity, endothelial function, parameters of oxidation, inflammation, lipid and glucose metabolism were not affected by quercetin supplementation.</td>
</tr>
<tr>
<td>Schroeter et al. (203)</td>
<td>Healthy Individuals n=6</td>
<td>Randomized cross-over study</td>
<td>Acute effects</td>
<td>(-1)-epicatechin (1 or 2 mg/kg BW)</td>
<td>Both doses resulted in similar and significant increase in FMD after 2h of ingestion compared with baseline and water controls.</td>
</tr>
<tr>
<td>Schroeter et al. (203)</td>
<td>Healthy Individuals n=10</td>
<td>Randomized, double-blind, cross over study</td>
<td>Acute effects</td>
<td>Cocoa powder with high (917 mg) or low flavanol content (37 mg) given on 2 separate days with &gt;days washout</td>
<td>The oral ingestion of the high-flavanol cocoa resulted in a significant increase in the FMD response at 1–4 h postconsumption compared with baseline of the same day. The high-flavanol cocoa -mediated increase in FMD was paralleled by an augmentation of plasma nitrate species (RNNO) concentrations at 1–3 h.</td>
</tr>
<tr>
<td>Blazer et al. (204)</td>
<td>Medicated diabetic patients (n=41)</td>
<td>Double-masked randomized, controlled trial</td>
<td>30 day</td>
<td>3 doses per day of Flavanol-rich cocoa (321 mg flavanol) per dose or a nutrient-matched control (25 mg flavanol) per dose</td>
<td>FMD was significantly increased compared to baseline. Endothelium-independent response, blood pressure, heart rate were not affected.</td>
</tr>
<tr>
<td>Curtis et al. (178)</td>
<td>118 Postmenopausal women with type 2 diabetes</td>
<td>Randomized double-blind, controlled trial</td>
<td>1 Year</td>
<td>27 g/day of flavonoid-enriched chocolate (850 mg flavan-3-ols, 90 mg epicatechin) and 100 mg isoflavones/ day</td>
<td>Significant reduction in estimated peripheral insulin resistance and improvement in insulin sensitivity. Significant reductions in total cholesterol. No effect on BP, HAa1c, or glucose was observed.</td>
</tr>
<tr>
<td>Davison et al. (205)</td>
<td>52 Subjects with untreated mild hypertension</td>
<td>Randomized double-blind, parallel study</td>
<td>6 Weeks</td>
<td>Reconstituted cocoa beverage containing 33, 72, 712 or 1052 mg/d day</td>
<td>There were significant reductions in 24-h systolic, diastolic and mean arterial BP only at the highest dose</td>
</tr>
<tr>
<td>Terauchi et al. (206)</td>
<td>96 Women</td>
<td>Randomized, double-blind, placebo-controlled pilot study</td>
<td>8 Weeks</td>
<td>Either low-dose (100 mg/d) or high-dose (200 mg/d) prostacyclin, or placebo</td>
<td>Cardiovascular parameters, were similar among the groups. SBP and DBP decreased in the low-dose and high-dose groups after 4 weeks.</td>
</tr>
<tr>
<td>Hassellund et. a. (207)</td>
<td>31 Men with screening blood pressure 4140/90mmHg without anti-hypertensive or lipid-lowering medication</td>
<td>Randomized double-blind, placebo-controlled crossover study</td>
<td>4 Weeks with 1 week washout</td>
<td>640mg Anthocyanins daily versus placebo</td>
<td>High-density lipoprotein (HDL)-cholesterol and blood glucose were significantly higher after anthocyanin versus placebo treatment. No effects were observed on inflammation or oxidative stress in vivo</td>
</tr>
<tr>
<td>Curtis et al. (208)</td>
<td>52 healthy postmenopausal women</td>
<td>Randomized, placebo-controlled study</td>
<td>12 Weeks</td>
<td>500 mg/d anthocyanins as cyanidin glycosides (from elderberry) or placebo (2 capsules twice/d</td>
<td>No effects on biomarkers of CVD risk as there was no change in plasma levels of inflammatory biomarkers (CRP, TNFα, IL-6, TNF R1 and RII, and RANTES), vascular activity (endothelin-1, platelet reactivity, blood pressure, pulse). In addition, plasma lipids and</td>
</tr>
<tr>
<td>Study</td>
<td>Participants</td>
<td>Study Design</td>
<td>Duration</td>
<td>Intervention</td>
<td>Outcomes</td>
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<tr>
<td>Barona et al. (184)</td>
<td>Men classified with metabolic syndrome (n = 24)</td>
<td>Randomized double-blind, cross-over study</td>
<td>30 days</td>
<td>Participants were randomized and allocated to 46 g/d of the grape polyphenols extract supplement</td>
<td>Systolic blood pressure (SBP) and plasma soluble intercellular adhesion molecule-1 (sICAM-1) concentrations were lower, whereas the response of the vascular endothelial function marker FMD was higher, during the GRAPE compared with the placebo period. Plasma NOx concentration did not differ after grape powder treatment. Changes in systolic BP were negatively correlated with changes in NOx concentrations</td>
</tr>
<tr>
<td>Habazui et al. (205)</td>
<td>Healthy postmenopausal women (n=48)</td>
<td>Randomized, double-blind, controlled clinical trial</td>
<td>6 weeks</td>
<td>Participants were randomly assigned to consume 340 mL grape fruit juice (GFJ)/d, providing 210 mg naringenin glycosides, or a matched control drink without flavonones with 2 months washout between beverage</td>
<td>Endothelial function in macro- and microcirculation, blood pressure, glucose metabolism, and biomarkers of inflammation and oxidative stress were not affected by the intervention. The mean carotid-femoral pulse wave velocity, which reflects central aortic stiffness, was statistically significantly lower after consumption of GFJ than after consumption of the matched control drink without flavonones</td>
</tr>
<tr>
<td>Johnson et al. (210)</td>
<td>Postmenopausal women with pre- and stage 1- hypertension (n=48)</td>
<td>Randomized, double-blind, placebo-controlled clinical trial</td>
<td>8 weeks</td>
<td>Subjects were given either 27 g flavonoid sweetie juice or 22 g macronutrient-matched control powder</td>
<td>SBP and DBP significantly lowered. Nitric oxide level increased in the blueberry group compared with the baseline level, whereas there were no changes in the control group.</td>
</tr>
<tr>
<td>Basu et al. (211)</td>
<td>60 volunteers (5 men and 55 women)</td>
<td>Randomized dose-response controlled trial</td>
<td>12 weeks</td>
<td>1 of the following 4 beverages: 1) low-dose freeze dried strawberries (LD-FDS); 25 g/d containing 78 mg anthocyanins/g; 2) low-dose control (LD-C); 3) high-dose freeze dried strawberries (HD-FDS); 50 g/d containing 155 mg anthocyanins/g; and 4) high-dose control (HD-C).</td>
<td>Significantly greater decrease in total and LDL cholesterol in the HD-FDS compared to LD-FDS. The decrease in LDL and total cholesterol remained significant over the 12 week treatment compared to control group. Both LD-FDS and HD-FDS treatments did not affect any measures of adiposity, blood pressure, glycemia, and serum concentrations of HDL cholesterol and triglycerides, C-reactive protein, and adhesion molecules.</td>
</tr>
<tr>
<td>Curtis et al. (179)</td>
<td>93 Postmenopausal women with diabetes</td>
<td>Randomized double-blind controlled study</td>
<td>1 year</td>
<td>Either 27 g flavonoid-enriched chocolate/d (850 mg flavan-3-ols [90 mg epicatechin] + 100 mg isoflavones [aglycone equivalents]/d) or matched placebo</td>
<td>no significant change in BP</td>
</tr>
<tr>
<td>Erlund et al. (183)</td>
<td>72 Subjects</td>
<td>Single-blind, randomized, placebo-controlled</td>
<td>8 weeks</td>
<td>160 g/day of different berries for 8 weeks, providing 837 mg/day of polyphenols of which 60% were anthocyanins</td>
<td>Berry consumption decreased BP and increased HDL concentration. Total cholesterol and triacylglycerol did not change</td>
</tr>
<tr>
<td>Naruszewicz et al. (186)</td>
<td>44 Coronary artery disease patients</td>
<td>A double-blind, placebo-controlled, parallel trial</td>
<td>6 weeks</td>
<td>Subjects were given either chokeberry flavonoid extract (255 mg/day; of which 25% anthocyanins, 50% monomeric and polymeric procyanidins) or a placebo</td>
<td>Flavonoids significantly reduced serum 8-iso-prostaglandins and Ox-LDL levels. Significant increase in adiponectin levels and reduction in SBP and DBP by a mean average of 11 and 7.2 mmHg, respectively.</td>
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<tr>
<td>Egert et al. (194)</td>
<td>93 Overweight or obese subjects with metabolic syndrome traits</td>
<td>A double-blind, placebo-controlled cross-over trial</td>
<td>6 weeks</td>
<td>Either 150 mg quercetin/day or placebo</td>
<td>In contrast to placebo, quercetin decreased SBP by 2-6 mmHg in the entire study group. Quercetin decreased serum HDL-cholesterol concentrations, while total cholesterol, TAG and the LDL:LDL-cholesterol ratio were unaltered. Quercetin significantly decreased plasma concentrations of athrogenic oxidised LDL, but did not affect TNF-α and C-reactive protein when compared with placebo.</td>
</tr>
<tr>
<td>Egert et al. (193)</td>
<td>93 Overweight-obese volunteers with metabolic syndrome traits in relation to apolipoprotein (apo) E genotype</td>
<td>A double-blind, placebo-controlled, crossover trial</td>
<td>6 weeks</td>
<td>Either 150 mg quercetin/day or placebo</td>
<td>Quercetin decreased SBP by 3.4 mm Hg in the apoE3 group, whereas no significant effect was observed in the apoE4 group. Quercetin decreased serum HDL cholesterol and apoA1 and increased the LDL:HDL cholesterol ratio in the apoE4 subgroup, whereas the apoE3 subgroup had no significant changes in these variables. Quercetin significantly decreased plasma oxidized LDL and tumor necrosis factor-α in the apoE3 and apoE4 groups.</td>
</tr>
<tr>
<td>Rechf et al. (212)</td>
<td>12 Patients with stage I hypertension,</td>
<td>Randomized Double-blind, cross-over study</td>
<td>12 weeks</td>
<td>Each patient received 0.5 L/day alternately high-flavonoid sweetie juice (677 mg/L of naringin and 212 mg/L of narirutin) and low-flavonoid</td>
<td>The high flavonoid sweetie juice was more effective than low flavonoid sweetie juice in reducing DBP. SBP declined with no significant difference between subjects in both groups.</td>
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<td>Study</td>
<td>Subjects/Design</td>
<td>Duration</td>
<td>Intervention</td>
<td>Results/Findings</td>
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<td>Naomi et al.</td>
<td>34 Healthy subjects divided in 2 groups (young and older groups)</td>
<td>4-6 Days</td>
<td>Sweetie juice (166 mg/L of naringin and 64 mg/L of narirutin), each for a 5-week period. The cocoa beverage contained the following flavanols/100 ml: epicatechin 9.2 mg, catechin 10.7 mg, and flavanol oligomers 69.3 mg. Cocoa alone induced no significant changes in BP at day 5 compared to baseline in both groups. Flow mediated vasodilation, measured by tonometry in the finger was enhanced with flavanol-rich cocoa in both groups, but significantly more among the old. Four to six days of flavanol-rich cocoa caused a rise in pulse wave amplitude in both groups.</td>
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<tr>
<td>Morand et al.</td>
<td>24 Healthy overweight men</td>
<td>4 Weeks</td>
<td>500 ml Orange juice (292 mg of hesperidin and 47 mg of narirutin) or 500 ml control drink plus capsules of pure hesperidin (2 × 146 mg) or 500 ml control drink daily capsules of pure hesperidin (2 × 146 mg). DBP was significantly lower and postprandial microvascular endothelial reactivity was significantly improved after 4 wk consumption of orange juice or control plus hesperidin compared with control.</td>
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<tr>
<td>Rizza et al.</td>
<td>24 individuals with metabolic syndrome</td>
<td>3 weeks</td>
<td>500 mg Hesperidin orally per day. Hesperidin treatment increased flow-mediated dilation and reduced concentrations of circulating inflammatory biomarkers (high-sensitivity C-reactive protein, serum amyloid A protein, soluble E-selectin).</td>
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<tr>
<td>Desideri et al.</td>
<td>90 Elderly men and women with mild cognitive impairment</td>
<td>8 weeks</td>
<td>Cocoa drink with flavanol-3-ols at high (990 mg flavanol-3-ols), intermediate (550 mg) or low level (45 mg). BP, insulin resistance, and lipid peroxidation decreased among subjects in the high-flavanol and intermediate-flavanol groups compared with the low flavonoid group.</td>
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<tr>
<td>Asgary et al.</td>
<td>21 hypertensive patients</td>
<td>2 weeks</td>
<td>Either 150 ml (8.7 mg anthocyanins/L/day of pomegranate juice or water. Both SBP and DBP decreased. A significant reduction in serum vascular cell adhesion molecule-1. Serum levels of E-selectin were elevated by the end of trial in the PJ group. Differentials for other parameters intracellular adhesion molecule-1 (ICAM-1), high-sensitivity C-reactive protein (hs-CRP) and interleukin-6 (IL-6) were not found to be significantly different between the study group.</td>
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</tbody>
</table>
1.4.1.3 Possible mechanisms of action

Figure 1.5 summarizes proposed actions of phenolic compounds underlying their potential antihypertensive activity.

- **Phenolic compounds**
  - ROS scavenging
  - Stimulation endogenous antioxidants enzymes: SOD, GPx
  - Inhibition ROS generating enzymes: NADPH oxidase, xanthine oxidase, lipoygenase
  - Activation eNOS and increase NO availability
  - Inhibition synthesis of ET
  - Activation EDHF
  - ACE inhibition
  - ACE2 activation
  - Renin inhibition
  - Other actions?

**Figure 1.5**: Proposed mechanisms involved in the potential role of phenolic compounds on blood pressure. ROS, reactive oxygen species; SOD, superoxide dismutase; GPx, glutathione peroxidase; NADPH, nicotinamide adenine dinucleotide phosphate; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; ET, endothelin-1; EDHF, endothelium derived hyperpolarizing factor; ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2.

- **1.4.1.3.1 Phenolic compounds and oxidative stress**

During the course of oxygen metabolism, significant amounts of reactive oxygen species (ROS) such as superoxide are produced under normal conditions. Superoxide, which is the primary ROS produced in the body, is a short-living, highly reactive, and cytotoxic molecule that can attack and modify adjacent molecules. The natural antioxidant system neutralizes ROS under normal conditions. However, the imbalance between the production of ROS and antioxidant capacity which can result from heightened ROS
generation, an impaired antioxidant system, or a combination of both, causes oxidative stress (214).

Although the connection between free radicals and hypertension has been known since the 1960’s, the role of oxidative stress in the development of hypertension began to be fully investigated in the 1990’s (215). Studies in animals suggests that the reduction in the bioavailability of nitric oxide (NO) can be one of the most important mechanisms by which oxidative stress can raise BP (216). When NO is released from endothelial cells, it causes smooth muscle relaxation, resulting in vasodilation, increased blood flow and a reduction in BP. NO is synthesized by various nitric oxide synthase (NOS) enzymes. Heightened ROS generation leads to a decrease in NO bioavailability by direct inactivation in the reaction that forms peroxynitrite (217, 218). Peroxynitrite oxidizes and destabilizes NOS which in turn results in more production of superoxide. The ROS such as superoxide can reduce NO production by uncoupling endothelial NOS by tetrahydrobiopterin oxidation (cofactor essential for NOS function) or by inhibition of dimethylarginine dimethylaminohydrolase (enzyme that degrades the NOS inhibitors, methylarginines) (215). Another mechanism that could be involved in the development of hypertension caused by oxidative stress is nonenzymatic oxidation of arachidonic acid and formation of isoprostanes, which have potent vasoconstrictor, proinflammatory and antinatriuretic activity (219). Additionally, ROS can increase vascular smooth muscle tone by increasing cytoplasmic ionized calcium concentration ([Ca^{2+}]_{i}) (214). Several studies reported that essential hypertensive patients and various animal models of hypertension to have excessive amounts of ROS and a diminished antioxidant status (215, 220, 221). Studies with mice with a genetically engineered deficiency in ROS-
generating enzymes showed that these models have lower blood pressure than wild type mice (222, 223). In addition, Ang II infusion in these mice did not cause hypertension (223). Therefore and due to the antihypertensive effect of inhibition of ROS-producing enzymes or the antioxidant therapy and ROS scavengers, it was proposed that ROS can be causative in the development of hypertension (224) and that oxidative stress can play a critical role in the pathogenesis of hypertension and its related complications (225-228). As a result, amelioration of oxidative stress was one of the important elements in the prevention and treatment of hypertension (215).

Phenolic compounds are well known as natural antioxidants. Thus, their reported effects in the prevention of cardiovascular disease might be partially attributed to their potential to reduce vascular oxidative stress through their direct superoxide anion scavenging activity or through interaction with other ROS such as hydroxyl radicals and peroxy radicals. Additionally, they have the potential to stimulate endogenous antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) (229, 230). On the other hand, it is reported that they inhibit the two major enzymes generating ROS namely, xanthine and NAD(P)H oxidases, which promote NO availability and thereby lower BP (231, 232). The antioxidant capacity of phenolic compounds could be attributed to scavenging ROS, chelating redox active transition-metal ions, inhibiting pro-oxidant enzymes, inhibiting redox sensitive transcription factors, and inducing antioxidant enzymes. Flavonoids are good electron donors and efficient scavengers of free radicals such as superoxide anions, NO, singlet oxygen, and peroxynitrite. The number of ortho-dihydroxyl and ortho-hydroxyketol groups, C2-C3 double bonds, concentration, solubility, and the accessibility of the active group to the oxidant are
important factors that determine the scavenging capacity of the molecules. The number of hydroxyl groups on the B ring contributes significantly to the hydroxyl scavenging activity and thereby to the antioxidant capacity. Moreover, flavonoids have a saturated heterocyclic ring, which might help ROS predominantly to attach to the ortho-dihydroxy site on the B ring. This results in electron delocalization and confers a higher stability to the resulting radical semiquinones. Next, the hydroxyl groups at C5 and C7 of the A ring; C3′ and C4′ of the B ring; and C3 of the C ring might enhance the inhibition of lipid peroxidation (233).

Cocoa extract rich in flavanols and procyanidins showed a positive effect on different oxidative stress parameters such as delaying low-density lipoprotein (LDL) oxidation and a reduction in the production of ROS in activated leukocytes and an inhibition of ultraviolet-induced DNA oxidation in vitro. However, it is worth mentioning that despite the huge number of studies in vitro that show the antioxidant activity of flavonoids, the evidence is still limited in vivo (234, 235). In hypercholesterolemic patients, flavanol-rich cocoa was reported to counteract lipid peroxidation and lower plasma levels of oxidized LDL and increases overall antioxidant capacity (236, 237). Green tea catechins were reported to decrease the biomarkers of oxidative stress and lipid peroxidation and to increase the plasma antioxidant capacity as well as protect human plasma against oxidation (233).

Generally, several factors influence the antioxidant activity of phenolic compounds in humans (238). First, absorption of phenolic compounds in the human gut, and uptake in the blood and brain is very low. After the consumption of flavonoid-rich foods, the maximum plasma level from these flavonoids is usually reached between 1 and 3 h.
These levels range between 0.06 and 7.6 µM for flavonols, flavanols and flavanones, and less than 0.15 µM for anthocyanidins. Also, the half-lives of flavonoids in human plasma are in the range of a few hours. In addition, they are extensively metabolized in the intestine and liver (232). Moreover, important to mention is that the majority of the in vitro studies are based on the use of phenolic compounds present in foods and not in the form they are present in the circulation. Therefore, it can be concluded that extrapolate the results to humans should be very critical. Next, the blood or brain levels of phenolic compounds are unlikely to be high enough to neutralize free radicals chemically. Accordingly, phenolic compounds may act as antioxidants in vivo in a more broad sense by interfering with prooxidant processes or by inhibition of prooxidant enzymes such as NADPH oxidases, lipoxygenases and myeloperoxidase-mediated modifications of LDL (232).

1.4.1.3.2 Phenolic compounds and endothelium

The endothelium plays a very important role in BP and blood flow regulation by synthesis and release of the vasorelaxing factor NO and the endothelium derived hyperpolarizing factor (EDHF) (231). Endothelial dysfunction which is a common feature in all forms of cardiovascular disease, including hypertension, is characterized by a decrease in vasodilator NO bioavailability which results in less endogenous opposition to circulating vasoconstrictor endothelin-1 and in an increase in levels of oxidized-low-density lipoprotein (239).

Flavonoids were reported to interact with pathways leading to generation of NO from vascular endothelium. Several in vitro studies were done using isolated vessels from animals to assess the endothelial function. Results from these studies showed that
flavonols, flavones, flavanols, flavanones, and anthocyanins can cause endothelium dependent vasorelaxation of isolated arteries via NO release from the endothelium (240, 241). Stangl et al. (242) reported that the endothelium dependent vasorelaxation induced by tea catechin occurs in response to significant dose dependent activation of endothelial nitric oxide synthase (eNOS) in the endothelial cells. Quercetin at physiologically relevant concentration also increases eNOS mRNA expression in HUVEC (243). In addition to the NO dependent pathway, the vascoprotective effect of flavonoids could be due to EDHF-mediated responses in porcine coronary arteries and aortic vessels (231, 244).

In animal models, studies reported that the BP reducing effect of quercetin was accompanied by improvements in the endothelial function induced by an increased NO bioavailability and NO production. In addition to the NO-dependent vascular function improvement pathway, other studies reported that quercetin decreases plasma ET-1 (vasoconstrictor) in rats (234). Gómez-Guzmán et al. (245) reported that the protective effect of epicatechin in deoxycorticosterone acetate (DOCA)-salt-induced hypertension was mediated by the improvement in endothelium function and the reduction in plasma ET-1 levels. Additionally, the study showed that the effects on aortic endothelial function were associated to an attenuation of vascular oxide content and an increased phosphorylation of endothelium NO synthase. Yamamoto et al. (141) suggested that the hypotensive effect of hesperetin in SHR involved NO-mediated vasodilation. This is because hesperetin enhanced endothelium-dependent relaxation had no effect on endothelium-independent relaxation. Moreover, in the presence of nitric oxide synthase inhibitor, the depressor effect of hesperetin was significantly inhibited.
In humans, accumulating data on tea, cocoa, dark chocolate and other related sources suggest that phenolic compounds can improve endothelial function (240, 246). Acute and chronic green and black tea consumption by healthy subjects resulted in a significant improvement in flow-mediated endothelium dependent vasodilatation (FMD) of the brachial artery (247, 248). Similarly, the short (hours) and long term (weeks) intake of flavonoids derived from chocolate or cocoa resulted in an increase in FMD of conduit arteries and microcirculation, the subcutaneous vessels and the coronary arteries (204, 249). Most studies suggest that catechins present in tea and chocolate, at least in part, attribute to the observed vascular effects and this effect is NO mediated. In accordance, Schroeter et al (203) showed that the oral administration of pure (−)-epicatechin showed similar acute vascular effects as flavanol-rich cocoa as both administration of catechin-rich cocoa or pure epicatechin enhanced FMD with acute elevation in the level of circulating NO species. The chronic consumption of flavanol-rich cocoa correlated with the increase in the urinary excretion of NO metabolites, suggesting a prolonged and augmented NO synthesis. In addition, the administration of L-NG-monomethyl arginine, a competitive NO synthase inhibitor, significantly decreased the increase in blood flow, supporting the concept of NO-dependent effects (203, 204). A randomized controlled study in healthy men showed that quercetin and (−)-epicatechin resulted in a significant increase in plasma S-nitrosothiols, plasma nitrite and in a significant reduction in plasma endothelin-1 concentration, a potent vasoconstrictor after 2h of oral administration (250). In contrast, a very recent study showed that consumption of quercetin or epicatechin for 4 weeks did not change FMD, NO and endothelin-1 (201). However, there was a difference in the dosages and duration of administration used in
both studies which might attribute to the difference in the results.

Flavonoid-rich vegetables and fruit diets were reported to increase endothelium dependent microvascular reactivity and plasma NO levels as well as to improve inflammatory status in people with an increased risk of CVD (251). A study on individuals classified with metabolic syndrome demonstrated that, compared with placebo, the daily consumption of freeze dried grape rich in flavans, anthocyanins and flavonols for 30 days significantly improved vascular endothelial function and decreased circulating inflammatory molecules in addition to lowering SBP (184). Very recently, daily intake of fresh pomegranate drink rich in anthocyanin by hypertensive patients for a period of 2 weeks resulted in a significant reduction in both SBP and DBP which was accompanied by a significant reduction in serum vascular cell adhesion molecule-1, which is a biomarker of endothelial dysfunction and vascular inflammation (185). Contrary to these results, consumption of onion extracts rich in quercetin for 6 weeks did not change endothelin-1, soluble endothelial-derived adhesion molecules, endothelial function and parameters of oxidation in hypertensive individuals (202).

Collectively, evidences from some studies might suggest that one potential mechanism by which phenolic compounds or food rich in phenolic compounds decrease BP could be through improvement of the NO dependent endothelial function. However, the contradiction seen between results from different studies using pure compounds or different food sources rich in phenolic compounds cannot be ignored. The reasons behind this inconsistency could be attributed to differences in doses used, study duration, and the characteristics of individuals participating in the studies at baseline. In addition, phenolic compounds rich extracts can also contain other compounds interfering with the results,
and partly explain differences compared to studies with pure flavonoids. Moreover, the possible synergistic effect that can be seen because of the presence of several phenolic compounds in phenolic compounds rich extracts. This highlights the importance of conducting more studies to investigate pure compounds individually and the relation between lowering BP and biomarkers of endothelial function.

1.4.1.3.3 Phenolic compounds and RAAS

RAAS has been recognized for decades for its major role in the regulation of BP and fluid balance in the body. Renin, which is released by the kidney in response to a decrease in blood volume and renal perfusion, cleaves angiotensinogen to form the inactive decapeptide Ang I. ACE metabolizes Ang I into the active octapeptide AngII which is known as a vasoconstrictor. AngII promotes the kidney to secrete aldosterone which causes sodium and water retention and potassium excretion. This altogether results in an increased BP. Overactivation of RAAS leads to hypertension and other cardiovascular diseases (252). It is already known that the use of ACE inhibitors or specific angiotensin receptor blockers that can interfere with RAAS is one of the approaches used to decrease BP (234). The detailed effects of phenolic compounds on RAAS are still not clear. There are some indications from in vitro studies which suggest that the inhibition of ACE enzyme activity could be one of the mechanisms in which flavonoids could reduce BP (97, 98, 253). Very few studies checked the role of phenolic compounds in RAAS in vivo. With the data available, no consistency in the results was obtained so far. In SHRs, the reduction in ACE activity was reported by the application of quercetin-rich extracts (132), the 3-O-glucoside of quercetin and plant extracts rich in this flavonoid glucoside (133) after 30 days, and 90 min of administration, respectively.
Mackraj et al. (157) indicated that the effect of quercetin in lowering BP in Dehal salt sensitive hypertensive rats is probably due to a modulation of renal function. On the other hand, Neto Neves et al. (156) suggested that other mechanisms than ACE inhibition are probably involved in the antihypertensive and protective cardiovascular effects associated with quercetin. In agreement, treatment with procyanidins and catechins-rich extract for 60 days did reduce BP but did not change plasma ACE activity in female normotensive Wistar rats (174). In stage1 hypertensive men, the decrease in BP caused by quercetin application was not accompanied by a change in plasma ACE activity (200). Worthy to mention is that rennin is the first enzyme involved in the cascade of RAAS activation and thus it plays a crucial role in hypertension. However, very few reports on renin inhibition by phenolic compounds are available. Recent results from in vitro studies provided evidence that some phenolic compounds might have an antihypertensive potential through renin inhibition. Deng and his colleagues (254) showed that baicalin inhibited the activity of ACE and rennin and the fluorescence emission of both renin and ACE were efficiently quenched by baicalin. In addition, they showed that baicalin–rennin complex formed through three-sites binding including the active site, but there was only one binding site for the baicalin–ACE complex with a much smaller binding constant. Another study reported that epigallocatechin gallate, (-)-epicatechin gallate and (-)-epigallocatechin inhibited rennin activity, with epigallocatechin gallate showing the strongest inhibitory activity (255).

Recently, RAAS has been extended by including new components, such as the angiotensin-converting enzyme 2 (ACE2), the heptapeptide angiotensin (1–7) (Ang-(1–7)), and Mas receptor. ACE2, a homologue of ACE, is mainly expressed in endothelial
cells and vascular smooth muscle cells of the vascular wall. It converts Ang II to Ang-(1-7), and thereby causing vascular protective effects (256). A very recent in vitro study showed that baicalin could protect endothelial cells from Ang II-induced endothelial dysfunction and oxidative stress via activating ACE2/Ang-(1-7)/Mas axis and modulating the expression of other factors (257).

To conclude, the detailed effects of phenolic compounds on RAAS are still not enough to make a firm conclusion. Some indications from in vitro studies suggest that ACE inhibitory activity could be one of the mechanisms involved in the reduction of BP caused by phenolic compounds. However, more studies are still important to confirm this effect.

- **1.4.1.3.4 Other mechanisms**

Phenolic compounds might influence other responses involved in BP regulation. For instance, increased alpha-adrenergic response in small mesenteric arteries has been involved in increased BP in SHR. Quercetin and isorhamnetin incubated with mesenteric vessels from SHR for 30 min, were reported to cause a significant reduction in the vasoconstrictor response induced by the alpha-adrenergic receptor agonist phenylephrine (131). The epithelial Na\(^+\) channel (ENaC) in the kidney plays a key role in the regulation of blood pressure by contributing to the Na\(^+\) reabsorption in renal tubules. In salt sensitive hypertensive animals, the BP-lowering effects of flavonoids might be mediated by a modulation of renal function. Quercetin was reported to cause reduction in the SBP elevated by high salt diet associated with diminishing of the alphaENaC mRNA expression in the kidney (258). There is also evidence that quercetin may decrease blood pressure through endothelium independent vasorelaxation by directly acting on the
vascular smooth muscle. However, it remains unclear how quercetin evokes endothelium-independent relaxation, but it has been suggested that it results from inhibition of protein kinases involved in the Ca\(^{2+}\)-sensitizing mechanisms responsible for smooth muscle contraction (259).

It is known from human and animal studies that inflammation might play an important role in the development of hypertension (260). Flavonoids have been reported to have anti-inflammatory activity *in vitro* through inhibition of different mediators of the inflammatory process such as reactive C protein or adhesion molecules and NOS. In addition, the molecular activities of flavonoids include inhibition of transcription factors such as NF-kappaB and activating protein-1 (AP-1), as well as activation of nuclear factor-erythroid 2-related factor 2 (Nrf2) (261-263). Droke et al. (264) showed that administration of soy isoflavone in C57BL/6J mice reduces the risk of cardiovascular disease associated with chronic inflammation, by down-regulating the expression of the proinflammatory mediator tumor necrosis factor (TNF)-alpha at endothelial level. In human, a study assessing associations between flavonoid intake and inflammatory biomarkers reported that total flavonoids, flavanones, and flavones were inversely associated with IL-18 concentrations and that flavonols were inversely associated with soluble vascular cell adhesion molecule concentrations (265). Another study reported that habitual intakes of anthocyanins are associated with inflammatory biomarkers involved in the regulation of glucose homeostasis, β-cell function, and insulin signaling (266). So far, more investigation is still important and needed to suggest that anti-inflammatory activity might be one of the mechanisms that could be involved in the antihypertensive potential of phenolic compounds.
Indeed, we have to be critical when we discuss the possible mechanisms of action that might be involved in the antihypertensive potential of phenolic compounds. At this moment, there is no much information on all possible mechanisms that can mediate the effect of phenolic compounds on hypertension. New drug classes eg, inhibitors of vasopeptidases, aldosterone synthase and soluble epoxide hydrolase, agonists of natriuretic peptide A and vasoactive intestinal peptide receptor 2, and a mineralocorticoid receptor antagonist are used in phase II/III of development. On the other hand, inhibitors of aminopeptidase A, dopamine β-hydroxylase, and the intestinal Na+/H+ exchanger 3, and agonists of components of the angiotensin-converting enzyme 2/angiotensin(1–7)/Mas receptor axis are used in phase I or preclinical development (267). Accordingly, it is important to investigate the effects of flavonoids on these therapeutic targets. To our knowledge and based on research we did, very little information is reported on the effects of phenolic compounds on these pathways (257, 268, 269).

1.4.1.4 Conclusion

Data from in vitro and in vivo studies suggest a potential role of phenolic compounds and food rich sources in a reduction of the blood pressure. Results from some randomized control trials in human confirmed the beneficial effects of flavonoid-rich food towards blood pressure control and influence on cardiovascular risk factors. However, further research with well-designed and long-term studies to optimize the doses of phenolic compounds in different food matrices and to confirm the observed clinical benefit, is still required. Plausible mechanisms supported by different studies are a decrease in oxidative stress, improving the endothelial function and interference with RAAS. However, the exact mechanism(s) of action by which phenolic compounds might
decrease the BP in humans still needs more investigation and confirmation. Lastly and in parallel to focus on phenolic compounds rich food, attention should be given to study pure and individual phenolic compounds through feeding studies in animal models and further in humans. This could help in better understanding of the possible beneficial effects of individual compounds. Moreover, it allows not only to evaluate exactly the role phenolic compounds in the treatment and management of hypertension but also to determine the exact mechanism(s) responsible for the action.

1.4.2 Overview of the anti-obesity effects of phenolic compounds

Because of the side-effects accompanied by the medication, the use of supplements and alternative forms from natural products became of medical interest and is considered as a promising tool in management and treatment of obesity (270-272). Within the plant metabolites, phenolic compounds are one of the important phenolic compounds that have been investigated for possible anti-obesity effects and that are still gaining considerable interest. Although not consistent in all studies, results from many animal and human studies suggest the positive role of phenolic compounds and phenolic compounds-rich food in the fight against obesity. Here in the following section we give an overview of data reported from previous studies on the role of phenolic compounds in obesity prevention and treatment.

1.4.2.1 Anti-obesity effect of phenolic compounds and phenolic compounds-rich food reported in animals

The potential anti-obesity effects of phenolic compounds and phenolic compounds-rich food in animals have been reported in several studies. Flavonoids such as catechins and anthocyanins have been considered to play an important role in the treatment of obesity.
Chan et al. (273) showed that the consumption of epicatechins isolated from jasmine tea (*Camellia sinensis*) leaves (5.7 g/kg diet) for 4 weeks caused a reduction in body weight, serum parameters (total cholesterol and triacylglycerols) and hepatic lipid levels in hamsters. The purity of the isolated tea epicatechins was 95% in which epigallocatechin gallate, epigallocatechin, epicatechin gallate and epicatechin made 62.3, 19.2, 8.3 and 4.6%, respectively. Murase et al. (274) reported that long-term consumption of tea catechins is beneficial for the suppression of diet-induced obesity. In this study, the composition of the isolated tea catechins was epigallocatechin gallate (74%), epicatechin gallate (18%), gallocatechin gallate (6%) and others (2%). They showed that male C57BL/6J mice fed a high fat diet supplemented with catechins isolated from green tea (*Camellia sinensis*) (5 g/kg diet) for 11 months, presented a significant reduction in body weight gain compared with male C57BL/6J mice fed a high fat diet without supplementation of catechin. Supplementation of epigallocatechin gallate purified from green tea (10 g/kg diet) for 4 weeks resulted in a dose-dependent attenuation of body fat accumulation in male New Zealand black mice (275). Similarly, another study showed that feeding male C57BL/6J mice with cyanidin 3-glucoside-rich purple corn color (PCC) at a cyanidin 3-O-β-D-glucoside concentration of 2 g/kg diet for 12 weeks significantly suppressed the high fat diet-induced increase in body weight gain in addition to white and brown adipose tissue weights (276). Anthocyanin-treated mice (1 g/kg of high-fat diet) showed a significant decrease in weight gain as reported by Jayaprakasam (277). Moreover, a decreased lipid accumulation in the liver, including a significant decrease in liver triacylglycerol concentration, was observed in these mice.

The beneficial effects of isoflavones in preventing the development of obesity
were reported in many studies. Kishida et al. (278) examined whether the estrogenic property of soy isoflavone, containing 155 mg/g genistein and 127 mg/g daidzein, can affect food intake and body weight in Sprague-Dawley rats. It was found that isoflavone supplementation at 100-300 mg/kg diet attenuated food intake and body weight gain in female rats, whether or not the animals had undergone ovariectomy. Therefore, they suggested that other mechanisms, next to the estrogenic effect, could be behind the effect on body weight reduction. In agreement with these results, another study showed that reduced food intake resulting from isoflavone supplementation was accompanied by reducing ghrelin and NPY levels and increased CCK and PYY levels (279). Kim et al. (280) showed that genistein supplementation (2 g/kg diet) for 12 weeks in male C57BL/6J mice resulted in low body weight gain as well as improved lipid profiles and hepatic steatosis. Similarly, female C57BL/6 mice that were fed genistein-supplemented (1.5 g/kg) diet for 3 weeks, showed a decrease in food intake of 14% and in body weight of 9% (281).

Quercetin is one of the most studied flavonoids. It might exhibit a wide range of biological functions from which the anti-obesity effect has been reported in many studies. Revera et al. (282) analyzed effects of chronic administration of quercetin (2 or 10 mg/kg of body weight for 10 weeks) on metabolic syndrome abnormalities in obese Zucker rats. It was found that the chronic oral administration of high doses of quercetin caused a reduction in the final body weight and body weight gain in both obese rats and their lean littermates. Kobori et al. (283) reported that administration of diets containing 0.1 and 0.5 % quercetin for 2 weeks significantly reduced body weight and visceral and hepatic fat in male C57/BL6J mice. Another study showed that quercetin supplementation (66 mg/kg
body weight/day) protected C57BL/6J mice against weight gain induced by a high fat diet (284). On the contrary, the effect of quercetin on body weight was not confirmed by some other studies. Steward et al. (285) evaluated the effect of dietary supplementation with quercetin (0.8 % of the diet) for 3 weeks and 8 weeks in C57BL/6J mice fed a high-fat diet. No effect on mean body weight was observed during the experiment. Dietary supplementation with quercetin resulted in transient increases in energy expenditure at 3 weeks and not at 8 weeks. However, quercetin at the levels provided was effective in reducing circulating markers of inflammation (TNF-alpha, IFN-gamma, IL1 and IL4) in animals on a high fat diet after 8 weeks of treatment. Mice that were fed a mild-high fat diet supplemented with quercetin (400 mg/ Kg BW) for 12 weeks did not show a significant difference in body weight and energy intake compared to the control group fed only a mild-high fat diet (286). A very recent study evaluated the effect of quercetin supplementation on weight gain, caloric intake and feed efficiency in exercised and sedentary rats (287). Rats were exercised and/or orally supplemented with quercetin (25 mg/ kg on alternate days) during six weeks. It was concluded that quercetin did not induce a reduction in weight gain as there were no differences between the placebo and quercetin condition either in the sedentary or in the training condition. In an experiment carried out with Wistar rats fed on a high fat diet, treatment with 25 mg of quercetin/kg body weight/day did not alter body weight gain and body composition as well as leptin level (288). Another experiment performed in db/db mice showed that treatment with quercetin (100 mg/kg of body weight/d) for 7 weeks caused a reduction in plasma glucose without changes in body weight (289).

Contrary to quercetin, less information is available on kaempferol and rutin. The
anti-obesity effect of 3-O-beta-D-glucosyl-(1-->6)-beta-D-glucosyl-kaempferol isolated from *Sauropus androgynus* on male Wistar rats was studied by Shih-Fing et al. (290). A dose of 60 mg/kg/day for 4 weeks resulted in 15% reduction in food intake. This corresponded to a decrease in body weight as well as serum-free triglyceride. An investigation of the anti-obesity effects of kaempferol in high fat diet-fed rats was carried out by Chang et al. (291). Oral administration of kaempferol (300 mg/kg/day) for eight weeks caused a reduction in body weight gain, visceral fat-pad weights and plasma lipid levels. Hence, kaempferol resulted in reductions in hepatic triglyceride and cholesterol content and lowered hepatic lipid accumulation in high fat diet-fed rats. Choi et al. (292) investigated the anti-adipogenic activity of rutin in C57BL/6 mice fed with high-fat diet (64.4% of total calories as fat). The oral administration (25 and 50 mg/kg body weight/daily) of rutin with high fat diet for 4 weeks resulted in less body weight gain and significantly lower total cholesterol contents in blood in comparison to the high-fat diet alone fed group.

With regard to phenolic acids, fewer studies were reported. Gallic acid (293) and p-coumaric acid (294) supplementation at a dose of 100 mg/kg BW for 10 and 8 weeks, respectively, caused a significant decrease in body weight, organ weight of the liver, and adipose tissue weights of peritoneal and epididymal in Wistar rats with high fat diet-induced obesity. In addition, dyslipidemia, hepatosteatosis, and oxidative stress reduced in the animal group with gallic acid and p-coumaric acid supplementation. Another study reported that addition of gallic acid (50 or 100 mg/kg BW) to the high fat-diet of male C57BL/6 mice OVWE during a period of 16 weeks caused as significant reduction in
body weight gain (295) and this could be attributed to gallic acid (296).

In summary, results obtained from animal studies provide indication on the positive role of phenolic compounds in obesity prevention and treatment in spite of the absence of effect from some studies. Additionally, many studies showed that application of phenolic compounds inhibited the high fat diet induced obesity. This might help further the mechanisms by which phenolic compounds could reduce obesity in human.

1.4.2.2 Anti-obesity effect of phenolic compounds and phenolic compounds-rich food reported in human

Although there is much evidence about the potential effect of phenolic compounds against obesity from in vitro experiments and in vivo studies using animal models, little data is available from human intervention studies. Green tea and green tea extracts rich in flavonoids have been studied extensively with respect to body weight regulation. Catechins are major polyphenols in green tea which accounts for about 35% of its total dry weight (297). Data from human studies suggest the positive effect of green tea and green tea extracts on body weight and energy expenditure (298-300). Caffeine content is not the only factor responsible for this effect on energy expenditure because the thermogenic effect of green tea extract containing caffeine and catechins was found to be greater than that of an equivalent amount of caffeine (301). A cross-sectional survey, where 1210 adults were enrolled, reported that habitual drinkers of tea for 10 years showed a 19.6% reduction in body fat percentage and a 2.1% reduction in the waist to hip ratio compared to non-habitual tea drinkers (302). A meta-analysis study evaluated the anti-obesity effects of green tea supplementation based on 11 selected long-term studies. From this analysis, it was concluded that catechins have a small positive effect on body
weight loss and body weight maintenance. However, caffeine intake seemed to be a moderator influencing the effects of catechins (303). This could be attributed to the increase and more sustained effect of norepinephrine on thermogenesis resulting from inhibition of catechol O-methyltransferase (COMT), the enzyme that degrades norepinephrine, by catechins and prolonging the life of cAMP in the cell by caffeine (301). Similarly, another meta-analysis study addressed that the administration of green tea catechins with caffeine is associated with reductions in BMI, body weight, and waist circumference (304). On the other hand, many studies with green tea and green tea catechins did not report positive results related to obesity measures. Diepvens’s study found that supplementation of a low-energy diet with green tea for 12-weeks, independent of habitual caffeine intake, had no effect on measures of body weight or body composition in overweight women at 4 weeks or 3 months (305). Similarly, Hill et al. observed that there was no significant difference in body weight, BMI, waist circumference, abdominal fat and intra-abdominal adipose tissue between the placebo group and the EGCG-supplemented group in overweight/obese postmenopausal women (306). In addition, a randomized, double-blinded, placebo-controlled trial in Taiwan reported that there was no statistical difference in % reduction in body weight, BMI and waist circumference in the green tea catechins-supplemented (491 mg catechins) group after 12 weeks of treatment compared to the placebo group (307). The association between 3 flavonoid subgroups and BMI over a 14-year period with 4280 men and women aged 55-69 years in a prospective cohort study was assessed in the Netherlands. It was found that women with the highest intake of total flavonols/flavones and total catechins experienced a significantly lower increase in BMI of 0.40 and 0.31,
respectively (308). Recently, a randomized, double-blind, placebo-controlled trial showed that 118.5 mg/day of anthocyanins for 4 weeks resulted in significant changes in body mass, body composition, appetite and dietary intake (309). In contrast, a study on subjects exhibiting a BMI between 25 and 35 kg/m\(^2\) showed no effects on the nutritional status (body weight, waist circumference, fat mass and fat-free mass) after 6 weeks of treatment with 150 mg/day of quercetin (193). Very recently, a randomized, double-blind, placebo-controlled study was performed on pre-menopausal obese women. They found that dietary supplementation with 300 mg/day of EGCG for 12 weeks did not enhance energy-restricted diet-induced adiposity reductions, and did not improve weight-loss-induced changes in cardiometabolic risk factors in obese Caucasian women (310).

To summarize, evidence on the beneficial effects of phenolic compounds and food rich in phenolic compounds in fighting obesity is increasing. However, more human studies are required to provide consistent data. These data can be used to establish the exact anti-obesity effect of phenolic compounds. Further, this will help in getting these health claims approved.

1.4. 2.3 Possible mechanisms of action

Energy surplus over time which is stored in the body as fat, is an important etiological factor for obesity. The beneficial or preventive effect of natural compounds against obesity is suggested in many studies (270-272, 311-313). The potential of phenolic compounds in weight loss and obesity management could be through influencing one or more of the following pathways as illustrated in figure 1.6: lipid absorption, food intake and energy expenditure, pre-adipocyte differentiation and proliferation, lipogenesis and
lipolysis and inflammatory response (270).

Figure 1. 6: Diagram illustrates the possible mechanisms of action behind the anti-obesity effects of phenolic compounds. HSL, hormone sensitive lipase; PL, pancreatic lipase; UCPs, uncoupling proteins.

At the cell biological level, obesity is characterized by an increase in the number and size of adipocytes differentiated from fibroblastic pre-adipocytes in adipose tissue. Therefore, regulation of the size and number of adipocytes is a valuable path in combating obesity. Phenolic compounds can inhibit adipogenesis. Studies indicated that catechins such as catechin gallate and epigallocatechin gallate (298, 314-317), quercetin (318), rutin (319), luteolin (320), genistein and naringenin (321) might prevent obesity by suppression of the adipocyte differentiation which could be attributed to the down-regulation of the expression of adipogenic transcription factors (C/EBPα, PPARγ) (322, 323) and
activation of the AMPK signal pathway (319, 324, 325). Phenolic acids were reported to inhibit the preadipocyte population growth as the treatment of 3T3-L1 preadipocytes with chlorogenic acid, o-coumaric acid, and m-coumaric acid caused cell cycle arrest in the G1 phase (326). This might attribute to their antiobesity effects in vivo. Phenolic compounds were reported to induce apoptosis of mature adipocytes (281, 327, 328) via modulation of the ERK and JNK pathways (327). Catechins suppress fat accumulation in differentiated 3T3-L1 cells via activating AMPK and inhibiting acetyl-CoA carboxylase (329). In addition, catechins can also decrease lipid digestion and absorption by the inhibition of digestive lipases (330). The effect on obesity prevention and management could be due to interferences with lipid metabolism. Catechins were reported to stimulate hepatic lipid metabolism (274). Kaempferol was shown to increase lipid metabolism through down regulation of SREBPs and promoting the hepatic expression of ACO and CYP4A1 (291). Cyanidin 3-glucoside-rich purple corn color (PCC) suppressed the mRNA levels of enzymes involved in fatty acid and triacylglycerol synthesis and lowered the sterol regulatory element binding protein-1 mRNA level in white adipose tissue (276). Since adipocyte dysfunction plays an important role in the development of obesity and insulin resistance, regulation of adipocytokine secretion or the adipocyte-specific gene expression can be one of the important targets for the prevention of obesity and amelioration of insulin sensitivity. Tsuda et al. (331) showed that treatment of isolated rat adipocytes with the anthocyanin cyanidin stimulates adiponectin and leptin (adipocytokine) secretion which is reported to activate AMPK that is associated with the enhancement of fatty acid oxidation and suppression of triacylglycerol accumulation. In addition, treatment with anthocyanin up-regulated the adipocytes-specific gene
expression. In another study, Tsuda et al. showed that both cyanidin 3-glucoside and its aglycone up-regulated the hormone-sensitive lipase which mediates the mobilization of stored triacylglycerol and enhanced the lipolytic activity (332). The pancreatic lipase (PL) is responsible for the hydrolysis of 50-70% of the triacylglycerols, monoacylglycerides and free fatty acids which are absorbable by enterocytes. Therefore, the inhibition of PL could result in a reduced fat absorption and in turn energy uptake which is one of the key targets in obesity management (333). Phenolic compounds-rich extracts from teas, herbal and fruit sources were reported as PL inhibitors during in vitro experiments (312, 334-338). Their high PL inhibitory activity was usually attributed to the proanthocyanidins such as galallocatechin gallate, epigallocatechin gallate and catechin gallate (312, 334-336) or to phenolic acids (296, 337). The significant decrease in PL activity caused by gallic acid and black tea extracts in vitro was accompanied by suppression of the elevation of blood triglyceride after oral administration of a corn oil emulsion in male mice (296). Recently, a study showed that epigallocatechin-3-gallate, kaempferol, quercetin, genistein and luteolin could be potent PL inhibitors, with epigallocatechin-3-gallate being the most active (339). Additionally, phenolic compounds might increase the energy expenditure via up-regulating different uncoupling proteins (UCPs). Many studies showed that catechins interfere with the gene expression of UCPs (275, 340, 341). Phenolic compounds might interfere with enterocyte glucose absorption. Studies indicated that epicatechin gallate and epigallocatechin gallate interact with glucose transporters as antagonist-like molecules, thereby playing a role in controlling the dietary glucose uptake in the intestinal tract (342, 343). Cyanidin was also reported by Sasaki et al. (344) to reduce the blood glucose levels in mice. Some studies provided
evidence on the protective effect of phenolic compounds against obesity-related inflammations. For example, quercetin was shown not only to reduce markers of inflammation, macrophages and insulin resistance in adipocytes but also it attenuated circulating markers of inflammation in animal models (345, 346). Anthocyanins were reported to down-regulate inflammatory protein cytokines such as monocyte chemoattractant protein-1 (MCP-1) in the adipose tissue of mice (344). Green tea catechins were also reported to influence the inflammatory response via inhibition of the expression and secretion of proinflammatory resistin, PAI-1, IL-6, TNF-α, MCP-1 and MMPs (340, 347). Phenolic compounds might influence obesity by decreasing food intake through satiety-inducing hormones. However, this pathway has not been well studied yet and only a few data are available. Zhang et al. reported that the effects of isoflavones on food intake were accompanied by a reduction of ghrelin and NPY levels, and an increase of CCK and PYY levels (279). An in vitro study proved that the flavonoid hesperetin stimulates CCK secretion in enteroendocrine STC-1 cells (348). Very recently, epigallocatechin-3-gallate (EGCG) was found to induce CCK, GLP-1 and PYY secretion from Caco-2 cells (349). The same study showed that EGCG also released CCK from the duodenum, and GLP-1 from the ileum using murine intestines.

1.4.2.4 Conclusion

The effect of phenolic compounds against obesity has been reported in several studies. However, results are still inconsistent and incomplete. Therefore, more data on the putative beneficial effects of phenolic compounds on body weight management and their efficacy in combating obesity are required. Most studies showed that anti-adipogenesis and influencing lipid metabolism are the main mechanisms underlying the anti-obesity
effect of phenolic compounds. Additionally, the involvement of other mechanisms like affecting glucose uptake and gastrointestinal hormones were suggested. Further studies are still needed to investigate and better characterize exact mechanisms that can possibly mediate the potential anti-obesity effects of phenolic compounds.

1.5 Other bioactivities of phenolic compounds

Except for the above extensively discussed bioactivities, phenolic compounds have shown several other health-beneficiary effects such as antimicrobial, antiageing, and anticancer effects (350). However, the aim of this work is only to focus on bioactivities of phenolic compounds with regard to hypertension and obesity prevention and treatment. As it was stated clearly above in previous sections based on reported studies in animal and humans models, phenolic compounds might play an important role in the prevention and treatment of hypertension and obesity. Several pathways are already included in BP and food intake control. Many were already discussed in previous studies. However, some pathways still demand more work to be fully understood.

In this work we focus on ACE as crucial enzyme involved in BP and CCK and CCK1R as important pathways influencing food intake. Data from this work could help in better understanding of mechanisms involved in the reported antihypertensive and antiobesity effects of phenolic compounds. Discussing the results obtained from the phenolic compounds in terms of their structures might give a deep insight to the structure-activity relationships of these compounds and further help in developing new drugs based on natural compounds.
Chapter 2

Angiotensin-Converting Enzyme Inhibitory Effects by Plant Phenolic Compounds: A Study of Structure Activity Relationships

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Chapter 2 Angiotensin-Converting Enzyme Inhibitory Effects by Plant Phenolic Compounds: A Study of Structure Activity Relationships

2.1 Abstract

In this study, 22 phenolic compounds were investigated to inhibit angiotensin-converting enzyme (ACE). Tannic acid showed the highest activity (IC₅₀=230 µM). The IC₅₀ values obtained for phenolic acids and flavonoids ranged between 0.41-9.3 mM. QSAR analysis confirmed that the numbers of hydroxyl groups on the benzene ring play an important role for activity of phenolic compounds, and that substitution of hydroxyl groups by methoxy groups decreased activity. Docking studies indicated that phenolic acids and flavonoids inhibit ACE via interaction with the zinc ion and this interaction is stabilized by other interactions with amino acids in the active site. Other compounds such as resveratrol and pyrogallol may inhibit ACE via interactions with amino acids at the active site and thereby blocking the catalytic activity of ACE. These structure-function relationships are useful to design new ACE inhibitors and potential blood pressure-lowering compounds based on phenolic compounds.

2.2 Introduction

Hypertension medication includes the use of drugs of which inhibitors of angiotensin converting enzyme (ACE) are considered as one of the most important classes (77). As we already mentioned in paragraph 1.2.2, ACE catalyzes the conversion of the precursor angiotensin I into angiotensin II, which is a peptide responsible in triggering vasoconstrictive effects, (82, 83). Therefore, inhibition of ACE has become a promising way for regulation and treatment of high blood pressure. Although several synthetic ACE inhibitors as lisinopril, captopril and enalapril are widely used for successful treatment of
hypertension, the chronic use of these synthetic inhibitors may be associated with many undesirable side-effects such as persistent cough, postural hypotension, renal failure and angioedema (351, 352). Extensive research has been carried out to look for ACE inhibitors from natural products as the latter might have better drug profiles and less side-effects. We already indicated in paragraph 1.2.4 to the natural substances that have been described in literature and targeting ACE inhibition such as peptides and triterpenes. Also, recent studies demonstrated the effect of some phenolic compounds as effective ACE inhibitors in vitro (95-97). Although these compounds have a poor solubility and subsequent restriction in bioavailability, studies in animal and human showed a potential effects on the reduction of blood pressure as already extensively discussed and stated in paragraph 1.4.1.1 and paragraph 1.4.1.2. These confirming data form a basis for our study with phenolic compounds and also underline the interest to investigate the structural differences in terms of ACE inhibition and thereby blood pressure-reducing potency, especially because only a few studies have been done to address the relationship between the activity of phenolic compounds and their structures. Recently, Guerrero et al.(98) reported on the key structural elements of flavonoids which contribute to their ACE inhibitory activity.

The objectives of this study were first to evaluate the ACE inhibitory activity of a wide range of 22 phenolic compounds belonging to different classes and subclasses (Figure 2.1). Second, results were used to identify possible mechanisms of action based on structure-activity relationships and molecular docking. We believe these new insights about the structure-function relationships may be useful for designing new ACE inhibitors based on phenolic compounds.
1 Tannic acid

2 Benzoic acid, $R_1 = COOH$, $R_3 = R_4 = R_5 = H$
3 $p$-Hydroxybenzoic acid, $R_1 = COOH$, $R_3 = R_4 = H$, $R_4 = OH$
4 Protocatechuic acid, $R_1 = COOH$, $R_3 = R_4 = OH$, $R_5 = H$
5 Gallic acid, $R_1 = COOH$, $R_3 = R_4 = R_5 = OH$
6 Vanillic acid, $R_1 = COOH$, $R_3 = OCH3$, $R_4 = OH$, $R_5 = H$
7 Syringic acid, $R_1 = COOH$, $R_3 = R_5 = OCH3$, $R_4 = OH$

8 Ellagic acid

9 trans-Cinnamic acid, $R_1 = CH_2=CHCO_2H$, $R_3 = R_4 = R_5 = H$
10 Ferulic acid, $R_1 = CH_2=CHCO_2H$, $R_3 = OCH3$, $R_4 = OH$, $R_5 = H$
11 $p$-Coumaric acid, $R_1 = CH_2=CHCO_2H$, $R_3 = H$, $R_4 = OH$, $R_5 = H$
12 Caffeic acid, $R_1 = CH_2=CHCO_2H$, $R_3 = R_4 = OH$, $R_5 = H$

87
13 Catechol, $R_1 = R_5 = H, R_3 = R_4 = OH$
14 Pyrogallol, $R_1 = H, R_3 = R_4 = R_5 = OH$

15 Quercetin, $R_1 = R_2 = R_3 = OH$
16 Kaempferol, $R_1 = R_3 = OH, R_2 = H$
17 Rutin, $R_1 = Glycoside, R_2 = R_3 = OH$

18 Apigenin, $R_1 = R_3 = OH, R_2 = H$
19 Epicatechin, $R_1 = R_2 = OH$
20 Phloretin
Figure 2.1: Structure of the 22 phenolic compounds tested in this study.

2.3 Materials and Methods

2.3.1 Products

ACE from rabbit lung, hippuryl-histidyl-leucine (HHL), o-phthalaldehyde (OPA), HCl, BSA, tannic acid, gallic acid, benzoic acid, p-hydroxybenzoic acid, syringic acid, vanillic acid, ellagic acid, protocatechuic acid, catechol, pyrogallol, caffeic acid, ferulic acid, p-coumaric acid, trans-cinnamic acid, quercetin, rutin, kaempferol, resveratrol, trans-stilbene, apigenin, epicatechin, phloretin and ninhydrin were purchased from Sigma-Aldrich (Bornem, Belgium; St. Louis, MO). Lisinopril was provided by Merck & Co. (Rahway, NJ, USA). Sodium tetraborate (Na₂B₄O₇·10H₂O) was purchased from Acros Organics (Geel, Belgium), while NaOH and NaCl from Chem Lab (Lichtervelde, Belgium).

2.3.2 In vitro ACE inhibitory activity assay

For the selection of 22 phenolic compounds, the ACE inhibitory activity was measured using the colorimetric method previously described (353) with slight modifications. In brief, the ACE catalyzed reaction was performed in cuvettes containing 100 µL of sample solution, 100 µL of ACE solution, and 100 µL of HHL solution. The phenolic compounds were dissolved in 100% ethanol and different concentrations tested with a
maximum of 10% ethanol in the final reaction volume; tannic acid was dissolved in water. Appropriate control and blank reactions were performed concurrently. The reaction mixtures were incubated for 2 h at 37 °C, and the reaction was stopped by adding 2 ml of o-phthalaldehyde reagent. After incubation for 20 min at 25 °C, the absorbance was measured at 390 nm.

The concentrations of the phenolic compounds (mM) were plotted versus the corresponding ACE inhibitory activity values (%) and the dose-response curves were obtained by the nonlinear sigmoid regression with Prism v4 (GraphPad Prism, La Jolla, CA). The IC₅₀ value, expressing the concentration of the phenolic compound inhibiting 50% of ACE activity, together with the corresponding 95% confidence interval and the R² of the curve fitting were determined as previously described (354). However, 50% inhibition was not always reached due to a lower solubility of some phenolic compounds at high concentrations. In that case, IC₅₀ values were extrapolated from the linear regression plot of the percentage of ACE inhibition versus the concentrations used (355).

2.3.3 Molecular docking and quantitative structure-activity relationship analysis

Molecular docking of the phenolic compounds 1-7, 9-15, 17, 19 and 21-22 into testis ACE (tACE) was performed with Discovery Studio (DS) 2.5 (Accelrys, San Diego, CA) using a CHARMm-based protocol, CDOCKER. The crystal structure of tACE (1UZF) was obtained from the Protein Databank and the ligands (phenolic compounds) were sourced from PubChem. The receptor protein and the ligands were subjected to protein and ligand preparations to simulate the conditions used in the in vitro analysis (pH 8.3) using the Prepare Protein and Prepare Ligand protocols. The best conformation based on docking score and literature reports were used in subsequent analyses. In an attempt to
establish a linear relationship between the IC$_{50}$ values and docking scores, the generated docking poses were ranked by each of the four individual scores (shape, hydrogen bound, protein desolvation and ligand desolvation). The sum of the normalized scoring functions generated the FRED Chemgauss4 score, using OEDocking suite for OSX (ver. 3.0.1) (OpenEye, Santa Fe, NM).

Multiple linear regression (MLR) analysis of the tested phenolic compounds was used to determine the molecular properties influencing the ACE inhibitory activity of the test phenolic compounds. However, only the structure-activity relationship of phenolic acids was analyzed due to the limited number of flavonoids tested in this study because of their low solubility. Molecular descriptors consisting of 2D molecular properties and functional group counts were obtained from DS and E-Dragon 1.1(356). MLR was performed using SPSS 20 (IBM, Armonk, NY).

2.4 Results

2.4.1 Selection of solvent to dissolve phenolic compounds

Since many phenolic compounds are poorly soluble in water, a suitable organic solvent to dissolve the phenolic compounds was needed. With the use of 10% methanol and 10% DMSO in the enzyme solution, there was an inhibition of the ACE enzyme activity by 25 and 16%, respectively. In contrast, no loss of ACE enzyme activity was recorded with 10% ethanol in the reaction mixture. Therefore, the phenolic compounds were dissolved in 100% ethanol and their effect on ACE inhibition was tested with an amount of 10% ethanol present in the final reaction mixtures. Tannic acid having higher water solubility was dissolved in water.
2.4.2 ACE inhibitory activity by tannic acid, different phenolic acids, pyrogallol and catechol

Before testing the phenolic compounds, we evaluated the ACE inhibitory activity of different concentrations of the synthetic ACE inhibitor lisinopril that were prepared in water. After sigmoid curve fitting (figure 2.2), the IC\textsubscript{50} of lisinopril was 1.00 nM (95% confidence interval=0.85-1.26 nM; R\textsuperscript{2}=0.99), confirming the reliability of the ACE inhibitory bioassay.

![Figure 2.2: Sigmoid dose-response curve of ACE inhibition by lisinopril as calculated in Prism.](image)

Table 2.1 demonstrates the ACE inhibitory effects by tannic acid, phenolic acids, pyrogallol and catechol. It was clear that among the 22 different phenolic compounds tested, tannic acid, 1, was the most active with the lowest IC\textsubscript{50} value (230 µM). Both subgroups of phenolic acids, i.e. hydroxybenzoic acids (Compounds 2-8) and hydroxycinnamic acid (Compounds 9-12), showed ACE inhibition in a range from 2 to 9.3 mM. Among all hydroxybenzoic acids, only ellagic and gallic acid showed some ACE inhibition at a concentration between 0.75 and 1.5 mM, while the other
Hydroxybenzoic acids at this concentration range did not affect the ACE activity. However, at a concentration in the range of 10-14 mM, all hydroxybenzoic acids, except syringic acid, resulted in ≥84% ACE inhibition. Among the hydroxycinnamic acids, caffeic acid caused the highest ACE inhibition at a concentration in the range of 0.85 and 1.5 mM. As the hydroxycinnamic acids have a poor solubility at high concentrations, even in a 10% ethanol solution, a concentration in the range of 10-14 mM could not be tested. Furthermore, catechol and pyrogallol, 13-14, were also tested. While these compounds both showed a concentration-dependent ACE inhibition, the IC\textsubscript{50} value obtained for pyrogallol was 7-fold lower than that of catechol.

Table 2.1: Percent Inhibition of ACE Activity at Different Concentrations Together with Calculated IC\textsubscript{50} Values Obtained for Tannic Acid, Phenolic acids, Catechol and Pyrogallol.

<table>
<thead>
<tr>
<th>Class</th>
<th>Subclass</th>
<th>Compound</th>
<th>% Inhibition (Conc., mM)</th>
<th>% Inhibition (Conc., mM)</th>
<th>IC\textsubscript{50} Value (mM)</th>
<th>95% CI (mM)</th>
<th>R\textsuperscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tannins</td>
<td>Tannins</td>
<td>Tannic acid</td>
<td>88±2 (0.90)</td>
<td>97±1 (1.9)</td>
<td>0.23</td>
<td>0.08 - 0.69</td>
<td>0.79</td>
</tr>
<tr>
<td>Hydroxybenzoic</td>
<td></td>
<td>Benzoic acid</td>
<td>1±4 (0.85)</td>
<td>90±8 (13.6)</td>
<td>6.20</td>
<td>5.78 - 6.66</td>
<td>0.99</td>
</tr>
<tr>
<td>Phenolic acids</td>
<td></td>
<td>p-Hydroxybenzoic acid</td>
<td>3±9 (0.75)</td>
<td>94±2 (12.0)</td>
<td>5.95</td>
<td>5.65 - 6.26</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protocatechuatic acid</td>
<td>9±1 (0.80)</td>
<td>91±2 (10.8)</td>
<td>5.07</td>
<td>2.70 - 9.24</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gallic acid</td>
<td>26±2 (0.80)</td>
<td>84±1 (10.0)</td>
<td>3.70</td>
<td>1.86 - 7.29</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vanillic acid</td>
<td>9±7 (1.50)</td>
<td>85±3 (11.8)</td>
<td>8.00</td>
<td>7.60 - 9.23</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Syringic acid</td>
<td>4±3 (1.50)</td>
<td>54±4 (10.0)</td>
<td>9.30</td>
<td>8.35-10.42</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ellagic acid</td>
<td>32±1 (1.00)</td>
<td>ND</td>
<td>2.00*</td>
<td>ND</td>
<td>0.78</td>
</tr>
<tr>
<td>Hydroxycinnamic</td>
<td></td>
<td>trans-Cinnamic acid</td>
<td>10±4 (1.50)</td>
<td>ND</td>
<td>8.50*</td>
<td>ND</td>
<td>0.54</td>
</tr>
<tr>
<td>acids</td>
<td></td>
<td>Ferulic acid</td>
<td>18±2 (0.85)</td>
<td>ND</td>
<td>4.40*</td>
<td>ND</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p-Coumaric acid</td>
<td>19±1 (1.00)</td>
<td>ND</td>
<td>2.80*</td>
<td>ND</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Caffeic acid</td>
<td>34±2 (0.93)</td>
<td>ND</td>
<td>2.10*</td>
<td>ND</td>
<td>0.56</td>
</tr>
<tr>
<td>Other polyphenols</td>
<td>Other polyphenols</td>
<td>Catechol</td>
<td>18±1 (1.50)</td>
<td>64±3 (12.0)</td>
<td>7.70</td>
<td>5.96 - 9.90</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pyrogallol</td>
<td>47±5 (1.20)</td>
<td>90±4 (10.0)</td>
<td>1.12</td>
<td>0.76 - 1.64</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD based on three biological replicates. * indicates that IC\textsubscript{50} values were extrapolated from the linear regression plot of the percentage of ACE inhibition versus the concentrations used. ND, not determined due to poor solubility of the sample at a concentration in the range of 10-14 mM.
2.4.3 ACE inhibitory activity of flavonoids and stilbenes

Six flavonoids representing 4 subclasses (flavonol, flavanol, flavones and dihydrochalcone) (Compounds 15-20) and two stilbenes (Compounds 21-22), were tested. As presented in Table 2.2, all tested flavonoids showed ACE inhibitory activity with IC\textsubscript{50} values ranging between 0.415 and 1.381 mM. At a concentration of 0.075 mM, quercetin and kaempferol showed the highest activity. Rutin and epicatechin appeared to have an intermediate ACE inhibitory activity, while the lowest ACE inhibitory activity was observed with apigenin and phloretin.

Regarding stilbenes, resveratrol showed a concentration-dependent ACE inhibition, giving an IC\textsubscript{50} value of 0.970 mM (Table 2.2). trans-Stilbene did not show any activity at the concentrations tested (0.05 to 0.1 mM).

Table 2.2. Effect of Different Flavonoids and Stilbenes on ACE Activity

<table>
<thead>
<tr>
<th>Class</th>
<th>Subclass</th>
<th>Phenolic compounds</th>
<th>% of ACE Inhibition at 0.075 mM \textsuperscript{1}</th>
<th>IC\textsubscript{50} value (mM) \textsuperscript{2}</th>
<th>95% CI (mM)</th>
<th>R\textsuperscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoids</td>
<td>Flavonols</td>
<td>Quercetin</td>
<td>37\textsuperscript{a} ± 2.1</td>
<td>0.415</td>
<td>0.18-0.97</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kaempferol</td>
<td>29\textsuperscript{b} ± 4.4</td>
<td>0.512</td>
<td>0.19-1.40</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rutin</td>
<td>21\textsuperscript{c} ± 3.7</td>
<td>0.472</td>
<td>0.14-1.55</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>Flavones</td>
<td>Apigenin</td>
<td>13\textsuperscript{d} ± 3.3</td>
<td>0.667*</td>
<td></td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>Flavanols</td>
<td>Epicatechin</td>
<td>19\textsuperscript{e} ± 2.8</td>
<td>1.381*</td>
<td></td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>Dihydrochalones</td>
<td>Phloretin</td>
<td>8\textsuperscript{f} ± 3.4</td>
<td>1.110</td>
<td>0.71-1.70</td>
<td>0.98</td>
</tr>
<tr>
<td>Stilbenes</td>
<td>Stilbenes</td>
<td>Resveratrol</td>
<td>15 ± 1.3</td>
<td>0.970</td>
<td>0.70-1.33</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>trans-Stilbene</td>
<td>no activity</td>
<td>no activity</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{1} The values are expressed as means ± SD based on three biological replicates. Means with different superscripts are significantly different at P value <0.05 using one-way ANOVA.
\textsuperscript{2} Activity to inhibit ACE is expressed as the concentration of phenolic compound (mM) to inhibit 50% of ACE enzyme activity; the IC\textsubscript{50} value is calculated from the sigmoid curve in Prism and is given together with the 95% confidence interval; the goodness of curve fitting is given with the R\textsuperscript{2} value. * this indicates that the IC\textsubscript{50} values were extrapolated.
2.4.4 Quantitative structure activity relationship for phenolic acids

The best multiple linear regression (MLR) analysis equation obtained for the logIC$_{50}$ values of the phenolic acids (Compounds 2-7 and 9-12) is as follows (figure 2.3):

$$\log\text{IC}_{50} = 4.618 \pm 0.54 - 4.042 \pm 0.54 \text{AlogP} + 0.07 \pm 0.01 \text{vol} - 1.63 \pm 0.21 \text{HBA}$$

with $n=10$, $F=18.83$, $p=0.002$, $R^2=0.904$

Figure 2.3: Linear regression correlation between the observed and the predicted IC$_{50}$ value for 10 different phenolic acids (Compounds 2-7 and 9-12).

where AlogP is a measure of hydrophobicity based on the octanol-water partition coefficient, vol is the calculated 3D volume of each molecule, and HBA is the numbers of hydrogen bond acceptors. A high correlation ($R^2=0.9$) between observed and predicted IC$_{50}$ values (from MLR analysis equation) of the phenolic acids was obtained. It should be noticed that tannic acid and ellagic acid were not included in the analysis since they have a different and more polymerized structure compared to the other phenolic acids.
2.4.5 Molecular docking experiments

Here tACE was used since it is the only available structure and it is highly identical to the C terminal half of the somatic ACE that has been shown to be sufficient for blood pressure regulation (357). In addition, the ligand-protein interaction was evaluated on the basis of the Chemgauss4 score. The docking results from lisinopril were shown in figure 2.4.

![Docking of lisinopril in the binding cavity of ACE, showing interactions with zinc and amino acids in the active site. Lisinopril in the cavity is shown in yellow and the zinc ion is shown in blue. Green dashed lines are used to show hydrogen bonds, while the dashed orange ones represent charge-charge interaction. Orange stacks were used to show the pi stacking.](image)

**Figure 2.4:** Docking of lisinopril in the binding cavity of ACE, showing interactions with zinc and amino acids in the active site. Lisinopril in the cavity is shown in yellow and the zinc ion is shown in blue. Green dashed lines are used to show hydrogen bonds, while the dashed orange ones represent charge-charge interaction. Orange stacks were used to show the pi stacking.

2.4.5.1 Phenolic acids, catechol and pyrogallol

Table 2.3 represents the intermolecular interactions between ACE binding site residues and predicted poses for most of the tested phenolic compounds (Compounds 2-7, 9-15, 17, 19 and 21-22).
Table 2.3: Intermolecular Interactions between ACE Inhibitors and the ACE Binding Site

<table>
<thead>
<tr>
<th>Ligands</th>
<th>SI</th>
<th>S2'</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc</td>
<td>Tyr523</td>
<td>Ala354</td>
<td>Glu384</td>
</tr>
<tr>
<td>2</td>
<td>C</td>
<td>H</td>
<td>Pi, C</td>
</tr>
<tr>
<td>3</td>
<td>C</td>
<td></td>
<td>Pi, C</td>
</tr>
<tr>
<td>4</td>
<td>C</td>
<td></td>
<td>Pi, C</td>
</tr>
<tr>
<td>5</td>
<td>C</td>
<td>H</td>
<td>C</td>
</tr>
<tr>
<td>6</td>
<td>C</td>
<td>H</td>
<td>H, C</td>
</tr>
<tr>
<td>7</td>
<td>C</td>
<td></td>
<td>Pi, C</td>
</tr>
<tr>
<td>9</td>
<td>C</td>
<td></td>
<td>Pi</td>
</tr>
<tr>
<td>10</td>
<td>C</td>
<td></td>
<td>Pi</td>
</tr>
<tr>
<td>11</td>
<td>C</td>
<td></td>
<td>Pi</td>
</tr>
<tr>
<td>12</td>
<td>C</td>
<td>H</td>
<td>Pi</td>
</tr>
<tr>
<td>13</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>14</td>
<td>H</td>
<td>H</td>
<td>Pi</td>
</tr>
<tr>
<td>15</td>
<td>C</td>
<td>H</td>
<td>Pi, C, H</td>
</tr>
<tr>
<td>17</td>
<td>H</td>
<td>H</td>
<td>Pi, H</td>
</tr>
<tr>
<td>19</td>
<td>C</td>
<td></td>
<td>C, Pi</td>
</tr>
<tr>
<td>21</td>
<td></td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>22</td>
<td></td>
<td></td>
<td>Pi</td>
</tr>
<tr>
<td>Lisinopril</td>
<td>C</td>
<td>H</td>
<td>H, C</td>
</tr>
</tbody>
</table>

C, charge-charge interactions; H, hydrogen bonding; Pi, pi (stacking) interaction.

It can be seen that all phenolic acids formed charge-charge interactions with the zinc ion in the active site of ACE. Additionally, different types of molecular interactions with the amino acids in the active site were found for all phenolic compounds tested. Figures 2.5A-D show the docking results from the predicted poses for gallic, protochatechuic, caffeic and syringic acids at the ACE binding site. From these results it could be clearly observed that gallic, protochatechuic and caffeic acids are able to make interaction with the zinc ion in the active site via their carboxylate group. On the other hand, syringic acid interacts with the zinc ion via its hydroxyl group. Caffeic acid interacts with its acrylic
acid group with amino acid residues at both the S2’ and S1 binding site, while for protocatechuic acid the carboxylate group only interacts with the S2’ binding site. It should also be noticed that tannic acid could not fit into the active site of ACE.

Concerning pyrogallol and catechol, neither of these compounds interacted with the zinc in the active site. However, these compounds formed interactions with amino acids in the active site of ACE (Table 2.3).
Figure 2.5: Docking of A. gallic acid; B. protocatechuic acid; C. caffeic acid; D. syringic acid; E. quercetin; F. epicatechin; G. resveratrol and H. trans-stilbene in the binding cavity of ACE, showing interactions with zinc and amino acids in the active site. The ligands in the cavity are shown in yellow and the zinc ion in blue. Green dashed lines are used to show hydrogen bonds, while the full green ones represent charge-charge interaction. Orange stacks were used to show the pi stacking. Chemical structures were drawn not to scale.
2.4.5.2 Flavonoids and stilbenes

As shown in Table 2.3, quercetin, rutin and epicatechin formed molecular interactions with the ACE binding site residues. The quercetin molecule docks into ACE, interacting with the zinc via the 3-hydroxyl group in the C-ring (Figure 2.5E). Epicatechin is able to form an interaction with the zinc ion via the A-ring hydroxyl on position 7 (Figure 2.5F). On the other hand, rutin did not show any interaction with the zinc in the active centre of ACE.

With regard to stilbenes, resveratrol did not show interaction with the zinc metal but it establishes some interactions via its hydroxyl groups with amino acids in the active site of ACE (Figure 2.5G). In contrast, with the exception of pi-stacking, no interactions were observed between trans-stilbene and both zinc and amino acids in the active site of ACE (Figure 2.5H).

2.5 Discussion

The data from in vivo studies about the potential role of phenolic compounds in reducing BP formed a strong basis for our study with phenolic compounds. Also, the available data raised the interest to investigate the structural differences of phenolic compounds in terms of ACE inhibition and thereby blood pressure-reducing potency. We therefore gave attention in this paper to pure phenolic compounds with regards to their potency to inhibit ACE activity. The ACE inhibitory properties of 22 pure phenolic compounds representing different classes and subclasses were evaluated. Together with the obtained results, structure-activity relationship analysis and molecular docking were used for a better understanding how these phenolic compounds interact with the ACE enzyme. To discuss and analyze the results obtained, the phenolic compounds as included in this
study were categorized into three groups.

The first group includes tannic acid that was proposed to inhibit ACE. Considering all the results reported in this study, tannic acid exhibited the highest ACE inhibitory activity. Tannic acid is available in high quantities and is well-known to bind and/or precipitate proteins (358-362). Indeed, some studies showed that the high ACE inhibitory activity found for gallotannin (363) and procyanidin hexamer (364) was reduced by 86% and 65% after addition of BSA and albumin, respectively. To a similar extent, our earlier unpublished data confirmed that tannic acid can cause 88 ± 2% precipitation of BSA protein at a concentration of 3.5 mM which is >10 times higher than the calculated IC$_{50}$ value (0.23 mM) for inhibition of ACE. Also, it should be mentioned that the precipitation of BSA by tannic acid was only seen at very high concentrations and that precipitation with the ACE protein has never been confirmed in our own experiments testing ACE inhibition. Additionally, our docking experiments demonstrated that tannic acid did not fit into the active site of ACE due to its too large polymer size. However, it has been reported for phlorotannin, belonging together with tannic acid to the group of tannins, that the hexameric phenol, dieckol, also inhibited the activity of ACE and this by binding on the outside of the ACE molecule (365) As such, although we cannot explain the exact mechanism(s) of ACE inhibition by tannic acid, this compound showed a high activity, which is in line with the blood pressure lowering activity found for tannic acid in spontaneously hypertensive rats (154).

The second group includes phenolic acids and flavonoids like quercetin that are expected to inhibit ACE activity via interaction with the zinc ion in the active site. All the tested phenolic acids exhibited ACE inhibition with IC$_{50}$ values ranging between 2 and
9.3 mM. Our structure-activity relationship analysis helped to understand the key structural elements influencing ACE inhibitory activity of the phenolic acids. It was observed that the hydroxybenzoic acids, differing only in the numbers of hydroxyl groups on the benzene ring, showed a significant increase in ACE inhibitory activity with increasing numbers of hydroxyl groups. A similar observation was seen with the hydroxycinnamic acids. Indeed, previous studies reported the significance of hydroxyl groups on phenolic compounds for zinc metalloproteinase inhibition (366, 367). In addition, the 2-fold lower IC50 value of caffeic acid compared to protocatechuic acid and the similar observation seen when comparing ferulic acid with vanillic acid confirm the importance of the acrylic group for ACE inhibition (368). Another functional group that seems to influence the ACE inhibitory activity of phenolic acids is a methoxy group. Methylation of 3 and 5-hydroxyl groups of gallic acid to generate syringic acid caused a 3-fold decrease in ACE inhibitory activity. A similar reduction in ACE inhibitory activity was observed when comparing caffeic acid and ferulic acid. The reduced ACE inhibitory activity seen in the presence of a methoxy group might be explained by the steric hindrance caused by this structure, which might hamper the binding to the active site of ACE.

To determine the molecular properties that influence the ACE inhibitory activity of the phenolic acids under consideration, MLR analysis was done using molecular descriptors. It was clear that the presence of certain functional groups such as hydroxyl, carboxyl and acrylic acid groups, which can act as hydrogen bond acceptors or donors, seems to increase the potency to inhibit ACE. On the other hand, the presence of methoxy groups negatively influences its ACE inhibitory activity. For instance, syringic
acid, that is the least active phenolic acid, contains two methoxy groups, which makes the number of hydrogen bond acceptors is low. In contrast, gallic acid possessing five hydrogen bond acceptor groups, showed a higher activity. This suggests that the overall contribution, rather than the presence or absence of certain functional groups (hydroxyl, carboxyl, etc), influences the potential of phenolic acids to inhibit ACE activity. Furthermore, our docking studies revealed that most of the phenolic acids included in this study formed charge-charge interactions with the zinc ion in the active site of ACE via the oxygen atom in the carboxylate moiety. Additionally, these compounds made different molecular interactions with the amino acids at the active site of ACE which gives rise to a stable complex between the phenolic acid molecule and ACE. This interaction with the zinc ion, stabilized by other interactions with amino acids in the active site, may have caused the ACE inhibitory activity seen by phenolic acids. It is well known that the synthetic inhibitor lisinopril complexes the zinc via its carboxylic moiety and subsequently different interactions with key amino acids in the active centre are established to stabilize this complex (357). Therefore, it can be suggested that the phenolic acids inhibit ACE activity in a manner similar to lisinopril. Additionally, to confirm the competition with the substrate on the active site, the ACE inhibition pattern of phenolic compounds on ACE can be investigated by Lineweaver–Burk plot in which the enzyme activity can be measured in the presence of different concentrations of the substrate (HHL). Then, from the kinetic study obtained, it can be distinguish between competitive, non-competitive and inhibitor.

With regard to the flavonoids included in this study, these compounds all inhibited ACE but with different potencies. It is known that flavonoids have a wide range
of structures differing in substitution patterns to the A-, B- and C-ring (369) which might influence their bioactivity. However, it was not possible to test a wider range of flavonoids for ACE inhibitory activity due to the poor water solubility of flavonoids. As a consequence, a larger structure-activity relationship analysis could not be established in this project. Recently, a study reported on the importance of the catechol group on the B-ring in flavonoids for ACE inhibition (98) In agreement, we believe that this might explain the higher activity seen with quercetin compared to kaempferol and apigenin in which the catechol group is lacking. With respect to the flavonol quercetin, the flavanol epicatechin exerted a significantly lower activity at 0.075 mM and a 3-fold increase in IC\textsubscript{50} value. Epicatechin lacks the keto group in C4 and the C2-C3 double bond, causing the flavonoid skeleton to lose its planar structure and subsequently changing the molecular electronic distribution. Previous studies reported that the planar structure of flavonoids (370) and cyanidins (97) is an important factor for ACE inhibition. Docking results of both quercetin and epicatechin showed that the absence of the keto group and the C2-C3 double bond shifts the zinc binding site to the 7-OH moiety. Tsutsumi et al.(371) reported on the importance of the hydroxyl group on position 7 in the structure of flavonoids for inhibiting ACE enzyme activity. However, according to studies pertaining to metal chelation of phenolic compounds in solution, zinc and other metal ions have a stronger binding affinity to the 3-hydroxy-keto group, which is absent in epicatechin (372, 373) This may justify the higher activity seen for quercetin than for epicatechin.

The third group includes other flavonoids (rutin), stilbenes (resveratrol) and the other phenolic compounds (catechol and pyrogallol) that may inhibit ACE activity via only interactions with amino acids in the active site. ACE is a zinc metallopeptidase, in
which the zinc ion at the active site is essential for ACE catalysis. Thus, the interaction with zinc in the active site of ACE is considered an important mechanism in ACE inhibition. However, based on a quantum mechanical and molecular mechanical study, the interaction with the zinc ion does not seem to be the only mechanism, since it has been shown that ACE may cleave a substrate without interaction between the substrate and zinc (374). The third group of our phenolic compounds did inhibit ACE activity but did not show any interaction with the zinc in the active site. Therefore, we believe that the ACE inhibitory activity seen by this group may be due to the interactions established via hydrogen bonds between the hydroxyl groups and amino acids in the active site that block the catalytic activity of ACE enzyme.

Finally, the results in this study need to be placed in a larger perspective. To exert an ACE inhibitory effect *in vivo*, phenolic compounds have to be transported through the intestinal wall to reach the blood stream. The IC$_{50}$ values for phenolic compounds in this study are higher than the concentrations reported in blood plasma (375). Nevertheless, in previous studies several phenolic compounds were shown to have antihypertensive effects *in vivo* (154). To explain these ACE inhibitory activities *in vivo*, we agree with the statement of Actis-Goreta et al (253) that a local enrichment of phenolic compounds near the membrane surface in the vascular endothelial cells is possible since ACE is a membrane-bound enzyme. As such, repeated exposure to phenolic compounds coming from plant-based diets (39) might result in an accumulation near the endothelial lining with concentrations higher than those circulating in the blood hereby enhancing the interaction with ACE. Additionally, the existence of ACE activity in the intestinal mucosa was demonstrated in some studies (376, 377). This might suggest that in case of
high concentrations of phenolic compounds present in the gastrointestinal tract, enzyme inhibition at the intestinal level may occur.

In conclusion, the present data indicate that phenolic compounds inhibit ACE activity \textit{in vitro} and that the activity against ACE and the mode of action depend on the class (subclass) and the structure of the phenolic compound. We believe that these structure-function relationships can be useful to design new ACE inhibitors based on phenolic compounds. Further strategies like encapsulation and chemical modification to increase the solubility and bioavailability, and subsequently formulate biologically active phenolic compounds that reach the target site need to be evaluated.
Chapter 3

Analysis of interaction of phenolic compounds with the cholecystokinin signaling pathway to explain effects on reducing food intake

This chapter has been published in:
Chapter 3 Analysis of interaction of phenolic compounds with the cholecystokinin signaling pathway to explain effects on reducing food intake

3.1 Abstract

CCK regulates food intake by inducing both satiation and satiety which are mediated by CCK1R activation. In this study, we investigated the possible interactions of 7 phenolic compounds of different classes (tannic acid, gallic acid, benzoic acid, hydroxybenzoic acid, protocatechuic acid, and quercetin) with the CCK1R signaling pathway.

As major results, the tested phenolic compounds could not activate the CCK1R in a specific cell-based bioassay. In contrast, we observed an anti-CCK1R activity. This antagonistic action might be explained by blocking of the functioning of the CCK1R receptor, although the exact mechanism of interaction remains unknown. For tannic acid, we also measured a sequestration activity of the CCK hormone in vitro.

In conclusion, the reported activity of phenolic compounds against food intake and weight is not based on an activation of the CCK1R. Taking into account the complex regulation of food intake, further work is necessary to unravel other essential mechanisms involved to explain the reported effects of phenolic compounds against food intake.

3.2 Introduction

Because of the high cost of the drugs used in the treatment of obesity and the potential associated side-effects like gastrointestinal and kidney problems (378), the search for alternatives based on natural products is needed.

Phenolic compounds are one class of important natural compounds, already extensively studied in terms of their potential use in the prevention and treatment of different diseases, of which obesity is receiving an increasing attention. The potential role
of phenolic compounds in obesity management and body weight reduction was suggested by animal (282-284, 290-292) and human (298-302) studies. *In vitro* studies provided evidence on the beneficial effect of phenolic compounds in fighting obesity, mainly by inhibiting adipogenesis and inducing apoptosis of adipocytes (379, 380). In parallel, studies in animal models confirmed that some phenolic compounds suppress food intake (381-383). However, the mechanism(s) by which phenolic compounds might reduce food intake and weight is still under investigation.

Food intake is a complex process partially regulated by gastrointestinal hormones that mediate sensing and signaling related to food intake in the central nervous system (384, 385). Cholecystokinin (CCK) is considered one of the most important gut hormones that induces both satiation and satiety and thereby food intake reduction mainly by activating CCK1R (125).

Based on the reported *in vivo* data related to a possible reduction in food intake by phenolic compounds, we investigated the mechanistic effects by different classes of phenolic compounds on the CCK1R signaling pathway by measuring the increase in the calcium flux as an indication of the receptor activation. This analysis is done for the first time and we believe it is useful as the CCK pathway plays an essential role in the regulation of food intake. We used a cell-based bioassay with Chinese Hamster Ovary (CHO) cells overexpressing the rat CCK1R, and tested whether the different compounds could activate this G-protein coupled receptor (GPCR). In previous work, the natural ligand CCK activated this receptor at 1 nM (386). In addition, we analyzed whether phenolic compounds could antagonize the CCK signaling by a blockage of the CCK1R. We included also an experiment with thrombin receptor-activating peptide (Trap-7) being
the ligand of another GPCR, to investigate whether the effect of phenolic compounds is CCK specific or not. In other words, in case we see a decrease in response by Trap-7, this would indicate that the phenolic compounds interact and block the function of the GPCR in a non-specific manner. Of course in these experiments where we scored an antagonistic activity, we also investigated that the viability of the used CHO cells was not affected. In case this happens then this may lead to a false antagonistic effect against CCK1R due to cell mortality. Finally, we tested whether tannic acid and its monomeric metabolite gallic acid may sequester the CCK peptide and as such reduce the amount of freely available CCK hormone, because a protein precipitating activity has been reported before for tannic acid (387, 388). From this series of experiments at different levels of the CCK1R signaling pathway, it was aimed to unravel the mechanism behind the reported effects of phenolic compounds reducing food intake. Subsequently, this information is useful to better understand the role of the peptide CCK and its receptor in the complex regulation of food intake, and to document with mechanistic data a potential development of phenolic compounds as natural products in the prevention and treatment of obesity and metabolic syndrome diseases.

3.3 Material and Methods
3.3.1 Cell lines and products

CHO cells overexpressing the rat CCK type 1 receptor (CHO-CCK1R) were prepared by Prof. Peter Willems [23], while the native non-CCK1R-expressing CHO-K1 cells were provided by Prof. Georges Leclercq (Ghent University Hospital, Department of Clinic Biology, Microbiology and Immunology, Ghent Belgium). Cells were cultured as described before (386). Ham’s F12 medium (1:1) (DMEM-F12), Advanced Dulbecco’s modified Eagle’s medium, fetal bovine serum (FBS), geneticin (G-418 antibiotic),
Penicillin-Streptomycin-L-Glutamine, Fluo-4AM, Pluronic F-127, Dulbecco's phosphate-buffered saline (DPBS), Hank’s buffered salt solution (HBSS) and PrestoBlue™ were purchased from Invitrogen (Paisley, UK). Bovine serum albumin (BSA), HEPES, probenecid, lorglumide ((±)-4-[(3, 4-dichlorobenzoyl) amino]-5-(dipentylamino)-5-oxopentanoic acid sodium salt; CR-1409), and phenolic compounds were purchased from Sigma-Aldrich (Bornem, Belgium; St. Louis, MO). Sulfated cholecystokinin octapeptide (CCK-8S) was purchased from Bachem (Weil am Rhein, Germany) and clear bottom black 96-well plates from Greiner (Frickenhausen, Germany).

3.3.2 Cell-based bioassay to analyze activation of CCK1R

As we already described in paragraph 1.3.3, CCK1R is a G-protein-coupled receptor, which upon activation, evokes an inositol trisphosphate (IP3)-induced calcium release from the endoplasmic reticulum. This intracellular calcium flux is a measure for the receptor activation and can be visualized with fluorescent sensor dyes. The change in the fluorescence signal was measured in a cell-based bioassay described previously (386).

Briefly, CHO-CCK1R and native CHO-K1 cells (as a negative control) were seeded in 96-well plates for 20-24 h before the assay. Then, medium was removed and cells were incubated in a fluorescence indicator dye (Fluo-4AM) for 1 h at 19 °C in the dark. Then, cells were washed with modified HBSS (HBSS supplemented with 20 mM HEPES, 2.5 mM probenecid and 10 mg/ml BSA). When the receptor is activated by CCK-8S or other ligands, an increase in the fluorescence signal is observed. A microtitreplate reader Infinite pro 200 (Tecan, Männedorf, Switzerland) multimode plate reader with automated injection system was used to measure the fluorescence signal. Tecan was handled using i-control™ software. Excitation and emission wavelengths were set to 480 nm and 520 nm.
respectively, using Quad4 monochromators™ technology. Before sample addition, the basal fluorescence in a well was recorded for 6 s. Afterwards, CCK-8S or phenolic compound solutions (tannic acid, gallic acid, benzoic acid, hydroxybenzoic acid, protocatechuic acid, kaempferol, and quercetin) were injected automatically and the fluorescence measurements were taken continuously for an additional 36 s. All samples were prepared in modified HBSS. The phenolic acids were dosed at 20 and 200 µM, while quercetin and kaempferol were dosed at 10 and 100 µM.

3.3.3 Cell-based bioassay to analyze antagonist action against CCK1R

To investigate for antagonist activity, cells were pre-incubated for 30 min with different concentrations of the phenolic compounds given in section 3.3.2. Subsequently, cells were stimulated with 1 nM of CCK-8S and fluorescence measurements were done as described above in section 3.3.2. Concentrations of 20 and 200 µM were used for phenolic acids, while 10, 30, 60 and 100 µM were used for quercetin and kaempferol.

3.3.4 Analysis of cell viability upon exposure to phenolic compounds

Viability of phenolic compounds-treated cells was evaluated using PrestoBlue™. PrestoBlue™ reagent is a resazurin-based solution in which resazurin, blue in color and non-fluorescent, is converted to resorufin under the reducing power of viable cells. Resorufin has a red color and is highly fluorescent, thus can be used to determine cell viability and cytotoxicity. Briefly, after measuring the change in the calcium signal under the effect of pre-incubation with 20 and 200 µM of tannic acid and gallic acid and with 10 and 100 µM of quercetin and kaempferol, cells were washed with buffer and re-suspended in a fresh culture medium. Afterwards, PrestoBlue™ was added at a
concentration of 10% and cell viability was determined according to the manufacturer’s instructions.

3.3.5 Analysis of the CCK-8S sequestering ability of tannic acid and gallic acid
In this series, 20, 100 and 200 µM of tannic acid or gallic acid were incubated with 1 nM of CCK-8S for 30 min to allow for possible sequestration of CCK-8S peptide by the phenolic compound. Then, cells were challenged with the mixture of tannic acid+CCK-8S or gallic acid+CCK-8S, and subsequently fluorescence was recorded as described above in section 3.3.2.

3.3.6 Data analysis and statistics
Response curves were generated using the following procedures described earlier (386). First, baseline fluorescence F0 was recorded during the first 6 s of the experiment before sample addition. Afterwards, samples were injected automatically and the fluorescence Fi was measured for an additional 36 s. Fi measurements were normalized to the basal fluorescence level F0 before sample addition. The kinetic response curves were generated by plotting the normalized fluorescence (Fi/F0) versus time (t). Net response was calculated as the normalized fluorescence values from CHO-CCK1R cells minus the normalized fluorescence values from CHO-K1 cells. Net responses were expressed as a percentage of the maximum net response as evoked by 1 nM of CCK-8S. The inhibiting effect of tannic acid and gallic acid was calculated as 100% minus the net response. Based on these results, sigmoid dose-response curves for the percentage of the maximum response/percentage of inhibition versus sample concentrations were derived with Prism v5 software (GraphPad Prism, La Jolla, CA) (354). Median 50%-response concentrations, i.e. effective EC$_{50}$ (for CCK-8S) and inhibitory IC$_{50}$ values (for tannic acid and gallic
acid), were calculated from the dose-response curves. The EC$_{50}$ value represents the concentration of CCK-8S at which 50% of its maximum response is reached. The IC$_{50}$ value is the concentration of tannic acid or gallic acid at which 50% of the maximum response as evoked by 1 nM CCK-8S, is inhibited. Statistical analyses were performed using a one-way ANOVA analysis followed by Tukey post-hoc tests and differences were considered significant for p-values less than 0.05.

### 3.4 Results

#### 3.4.1 Phenolic compounds do not activate CCK1R

The 7 phenolic compounds were tested for their potency to activate the CCK1R with the CHO-CCK1R cells in comparison with non-transformed CHO-K1 cells. As a positive control, the cells were exposed to 1 nM of CCK-8S. It was clear that there was no fluorescence increase in both cell types with any of the phenolic compounds, while there was a clear stimulation with 1 nM CCK-8S alone (figure 3.1).
Figure 3.1: No activation of the CCK1R upon incubation of CHO-CCK1R and CHO-K1 cells with 8 phenolic compounds: tannic acid (A) and gallic acid (B), benzoic acid (C), hydroxybenzoic acid (D), protocatechuic acid (E), quercetin (F), and kaempferol (G). The phenolic acids were dosed at 20 or 200 µM, and quercetin, and kaempferol at 10 or 100 µM. The response to 1 nM of CCK-8S in CHO-CCK1R cells was used as a positive control representing full activation of the CCK1R; the response of CHO-K1 cells to 1 nM of CCK-8S as a negative control. The data are expressed as normalized fluorescence kinetics (Fi/F0) based on 5 measurements per treatment.

3.4.2 Phenolic compounds pose antagonistic action against CCK1R

When CHO-CCK1R cells were pre-incubated with 20 or 200 µM of tannic acid and gallic acid and then challenged with CCK-8S, a significantly lower response (p-values < 0.05) was measured as compared with the response to CCK-8S alone (Fig 3.2A, B). In contrast, incubation of CHO-CCK1R cells with benzoic acid, p-hydroxybenzoic acid and protocatechuic acid did not show any change in the fluorescence signal (Figure 3.2C-E).
When CHO-CCK1R cells were pre-incubated with quercetin, and kaempferol, this resulted in some inhibition but less than 50% effect even at the highest concentration of 100 µM tested (Fig 3.2F-G).
Figure 3.2: The antagonistic effect of 7 phenolic compounds on the CCK-8S-evoked response in CHO-CCK1R cells. Cells were pre-incubated during 30 min with tannic acid (A), gallic acid (B), benzoic acid (C), hydroxybenzoic acid (D) and protocatechuic acid (E) at 20 and 200 µM, and during 30 min with quercetin (F) and kaempferol (G) at 10, 30, 60 and 100 µM, and then exposed to 1 nM of CCK-8S. The kinetic response was measured as described in M&M. The response to 1 nM of CCK-8S in CHO-CCK1R cells without pre-incubation with phenolic compound was used as a positive control; the response of CHO-K1 cells to 1 nM of CCK-8S as a negative control. The data are based on a minimum of 5 measurements per treatment.
Based on dose-response curves (Figure 3.3A, B) with increasing concentrations of either tannic acid or gallic acid, the respective IC$_{50}$ value was 35 and 397 µM (Table 3.1). Due to a low water solubility of quercetin, and kaempferol, higher concentrations than 100 µM could not be tested, and therefore, IC$_{50}$ values for these 3 compounds could not be calculated.

**Figure 3.3:** Dose-response curve for the antagonistic effect by pre-incubation for 30 min of CHO-CCK1R cells with different concentrations of tannic acid (A) and gallic acid (B). Data are presented as percentages of inhibition of the maximum response as evoked by 1 nM of CCK-8S, and the curves were drawn in Prism v5. Each curve is based on 3 independent experiments in which 5 measurements were performed per treatment.

**Table 3.1:** Inhibitory effect of tannic acid and gallic acid on the CCK-8S-evoked response, expressed as IC$_{50}$ value.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IC$_{50}$ (95% CI; R$^2$) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tannic acid</td>
<td>35 (26-47; 0.959)</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>397 (309-511; 0.996)</td>
</tr>
</tbody>
</table>

IC$_{50}$ values, as calculated in Prism v5, represent that concentration of tannic acid and gallic acid at which 50% of the maximum response, as evoked by 1 nM of CCK-8S, is inhibited. Data are based on 3 independent experiments in which 5 measurements were performed per treatment.
3.4.3 Phenolic compounds also pose antagonistic action against TRAP receptor

In addition to the antagonistic activity by phenolic compounds against the response of CCK1R as evoked by CCK-8S, we also tested the effect of tannic acid and gallic acid against the response as evoked by thrombin receptor-activating peptide (Trap-7). Upon pre-incubation with the phenolic acids, it was clear that the response by 250 µM of Trap-7 was inhibited and this was the case in the two cell lines CHO-CCK1R and CHO-K1 cells (Figure 3.4A, B).

![Figure 3.4:](image-url)

**Figure 3.4:** The antagonistic effect of two phenolic compounds on the thrombin receptor-stimulating peptide (Trap-7)-evoked response in CHO-CCK1R and CHO-K1 cells. Cells were pre-incubated during 30 min with tannic acid (A) and gallic acid (B) at 200 µM, and then exposed to 250 µM of Trap-7. The kinetic response was measured as described in M&M. The response to 250 µM of Trap-7 in CHO-CCK1R and CHO-K1 cells without pre-incubation with phenolic compound was used as a respective negative control. The data are based on 5 measurements per treatment.

3.4.4 Phenolic compounds do not affect viability of CHO-CCK1R cells

In this set of experiments we evaluated whether the incubation with phenolic compounds had a negative effect on the viability of the CHO-CCK1R cells. In case the cells would be affected, this may lead to a false antagonistic effect against CCK1R. As shown in Table
3.2, treatment with tannic acid, gallic acid, kaempferol, and quercetin did not cause a reduction in cell viability compared to non-treated cells, at the concentrations investigated.

**Table 3.2:** Viability of CHO-CCK1R cells upon exposure to different concentrations of phenolic compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc. (µM)</th>
<th>Cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tannic acid</td>
<td>20</td>
<td>102 ± 3</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>102 ± 4</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>20</td>
<td>104 ± 4</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>10</td>
<td>98 ± 3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>Quercetin</td>
<td>10</td>
<td>99 ± 2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>99 ± 3</td>
</tr>
</tbody>
</table>

Data are given as percentages of the control (non-treated) cells. Each value is expressed as mean ± SEM based on 3 independent experiments.

### 3.4.5 Tannic acid can sequester CCK-8S

CCK-8S is a peptide and tannic acid is well known to sequester proteins/peptides. Therefore, it was hypothesized that tannic acid might bind CCK-8S and so reduce the amount of CCK-8S hormone freely available for CCK1R activation. In case this happens it may explain, or partially, the antagonistic activity against CCK1R as observed for tannic acid. To test this hypothesis, tannic acid and its monomer gallic acid were incubated with CCK-8S for 30 min without cells, and then CHO-CCK1R cells were exposed to the mixture of tannic acid+CCK-8S or gallic acid+CCK-8S. Figure 3.5A and 3.5B shows that the mixture with 200 µM tannic acid caused a small, but significant, decrease compared to the response with CCK-8S alone. The other mixtures did not show significant differences. Indeed, when different concentrations of CCK-8S (0.01-1 nM)
were combined with tannic acid at 20, 100 and 200 µM, then the 50%-effective concentration of CCK-8S was significantly increased with increasing concentrations of tannic acid (Table 3.3). The 95% confidence intervals of the EC$_{50}$ value were overlapping for CCK-8S alone and in mixture with 20 µM tannic acid. With tannic acid at 100 µM there was a small shift, and with 200 µM the estimated EC$_{50}$ value was increased 17 times. These data confirm that the effect by tannic acid is concentration dependent, and that there was no effect by the monomer gallic acid.

![Figure 3.5](image_url)

**Figure 3.5:** The effect of pre-incubation of 1 nM CCK-8S with tannic acid (A) and gallic acid (B) on CCK-evoked kinetics in CHO-CCK1R cells. Cells were stimulated with 1 nM of CCK-8S alone as well as with the mixture of 1 nM of CCK-8S with 20, 100 and 200 µM phenolic compound. The data are based on 5 measurements per treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EC$_{50}$ (95% CI; $R^2$) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCK-8S alone</td>
<td>0.024 (0.017-0.035; 0.985)</td>
</tr>
<tr>
<td>CCK-8S + 20 µM tannic acid</td>
<td>0.051 (0.029-0.090; 0.971)</td>
</tr>
<tr>
<td>CCK-8S + 100 µM tannic acid</td>
<td>0.169 (0.138-0.206; 0.995)</td>
</tr>
<tr>
<td>CCK-8S + 200 µM tannic acid</td>
<td>0.427 (0.315-0.580; 0.990)</td>
</tr>
</tbody>
</table>

EC$_{50}$ values represent that concentration of CCK-8S at which 50% activity of the maximum response, as evoked by 1 nM CCK-8S alone, is realized. The dose-response curves were drawn in Prism v5, and each curve is estimated with at least 5 concentrations of CCK-8S with each point based on 5 measurements per treatment.
3.5 Discussion

Recently, there are indications from a few studies on the possible role phenolic compounds and food rich in phenolic compounds in reducing food intake by mediating the satiety signals like CCK (279, 348).

In this study, we provide for the first time mechanistic data to understand the effects of phenolic compounds that were reported to reduce food intake in animal models. Specifically, we investigated whether phenolic compounds can activate the CCK1R signaling pathway which might contribute to their effect on food intake suppression. The effect of different phenolic compounds on the CCK response at the level of the CCK1R by measuring the change in the calcium flux and on the amounts of CCK-8S available was tested. However, all tested phenolic compounds did not show any activation of the CCK1R. Thus, it can be concluded that the reported food intake reduction by phenolic compounds is not mediated via an activation of the CCK1R signaling pathway.

Although not expected, in contrast to an activation of CCK1R, we observed an antagonistic activity against CCK signaling. We investigated whether this can be provoked due to a blockage of the CCK1R by the phenolic compound and/or a sequestration of CCK peptide hormone. The first hypothesis for an antagonistic activity can be supported by the fact that, when CHO cells were incubated with tannic acid and its monomer gallic acid, there was an antagonistic activity against the response evoked by thrombin receptor-activating peptide (Trap-7), which is binding on another membrane GPCR. In agreement, a previous study demonstrated that quercetin, apigenin and genistein were able to inhibit the thrombin receptor-mediated response (389). However, it should be remarked here that although phenolic compounds might potentially block
different membrane GPCRs *in vitro* and so inhibiting peptide-evoked responses, further research is needed to unravel the exact interaction of the phenolic compound with the membrane GPCR. Secondly, based on the literature, phenolic compounds can bind proteins and peptides (387, 390, 391). Thus, binding of CCK-8S and thereby sequestration of the peptide leading to a reduced amount of freely available CCK hormone, may be the reason for the inhibition of the CCK-evoked response. Indeed pre-incubation of CCK-8S with tannic acid did reduce the CCK-evoked response and the effect was dose-dependent. On the other hand, pre-incubation of CCK-8S with gallic acid, the monomeric metabolite of tannic acid, did not affect the CCK-8S-evoked response. This is in agreement with Kawamoto et al [12] who reported that the numbers of the galloyl groups, which are responsible for the formation of hydrophobic associations and hydrogen bonds, determine the power to bind and precipitate protein.

Taking all together, we can postulate that tannic acid and gallic acid may provoke a non-specific blockage of the CCK1R and thus hinder or even prevent further activation by CCK-8S, and for tannic acid we expect also sequestration of CCK peptide thus reducing the amount of freely available hormone. This agrees with a recent study where quercetin was reported as a non-specific inhibitor of GPCRs by the effect of colloidal formulation with the agonist hormone and with the receptor (392). However, it should be remarked that this inhibition of CCK1R signaling by both tannic acid and gallic acid was seen in vitro at high concentrations which are expected to be irrelevant to the physiological concentrations in vivo post consumption. Therefore, such effects are not likely to be achieved *in vivo*.

To conclude, this is the first work that investigated the effect of different phenolic
compounds on the CCK1R signaling pathway. We could demonstrate that phenolic compounds do not activate the CCK1R. Thus, their reported effect in literature with a reduction in food intake and weight could involve CCK secretion or other mechanisms/signaling pathways like CCK2R pathway or influencing other satiety hormones pathway like glucagon-like peptide-1 pathway, peptide YY and ghrelin. On the other hand, tannic acid and its monomer gallic acid antagonized the CCK signaling in vitro as they inhibited the CCK-8S-evoked response; however, this was at high concentrations which are biologically not reached. Given the importance of the physiological and biological role of CCK hormone and its receptor CCK1R but also the complex regulation of food intake, we believe that further work is necessary to unravel other essential mechanisms involved to explain the reported effects of phenolic compounds against food intake.
Chapter 4

Flavonoids stimulate cholecystokinin secretion from the enteroendocrine STC-1 cells.

This chapter has been submitted to Fitoterapia as: Al Shukor, N., Ravallec, R., Van Camp, J., Raes, K., Smagghe, G. Flavonoids Stimulate Cholecystokinin Secretion from the Enteroendocrine STC-1 cells.
Chapter 4 Favonoids stimulate cholecystokinin secretion from the enteroendocrine STC-1 cells

4.1 Abstract

Animal experiments showed that flavonoids might have the potential for an anti-obesity effect by reducing weight and food intake. However, the exact mechanisms that could be involved in these proposed effects are still under investigation. In this study, we investigated the possible effects of flavonoids (quercetin, kaempferol, apigenin, rutin and baicalein) on stimulation of the CCK release in vitro using enteroendocrine STC-1 cells. In comparison with the control, quercetin, kaempferol and apigenin resulted in a significant increase in CCK secretion with quercetin showing the highest activity. On the other hand, no significant effect was seen by rutin and baicalein.

Based on the cell-based results in this work, it can be suggested that the reported activity of flavonoids against food intake and weight could be mediated by stimulation of the CCK signal which in turn is responsible for inducing satiety and food intake reduction. Nonetheless, future animal and human studies are needed to confirm this conclusion at organism level.

4.2 Introduction

Cholecystokinin (CCK) as key hormone in food intake regulation is released from the endocrine cells (I cell) in response to the ingestion of nutrients into the small intestine. The STC-1 cell line was derived from murine intestinal neuroendocrine tumor cells and has already been proven to act as a suitable model to study CCK secretion and production (393).

In the previous chapter, we showed that phenolic compounds did not stimulate
CCK1R activation. Thus, in this study we go further to investigate the possible effect of some selected flavonoids (figure 4.1A) on stimulation of CCK secretion in vitro using enteroendocrine STC-1 cells. The data should help in a better understanding of the mechanism(s) behind the reported activities of flavonoids and foods rich in flavonoids to lower food intake. Forskolin was used as a positive control (figure 4.1B).

![Figure 4.1: A represents the structure of flavonoids used in the study. Quercetin, R1 = R3 = R4 = OH, R2 = H; Kaempferol, R1 = R4 = OH, R2 = R3 = H; Rutin, R1 = Glycoside, R2 = H, R3 = R4 = OH; Apigenin, R1 = R2 = R3 = H, R4 = OH; Baicalein, R1 = R3 = R4 = H, R2 = OH. B is the structure of forskolin which was used as a positive control.](image)

### 4.3. Materials and methods

#### 4.3.1 Materials

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), Penicillin-Streptomycin, Dulbecco's phosphate-buffered saline (DPBS), Hank’s buffered salt solution (HBSS), Pierce™ IP Lysis Buffer, Halt™ Protease and Phosphatase Inhibitor Cocktail (100X), PrestoBlue™, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were purchased from Invitrogen (Paisley, UK). HEPES, forskolin, quercetin, kaempferol, rutin, apigenin, baicalein were purchased from Sigma-Aldrich (Bornem, Belgium; St. Louis, MO). For the RIA (radioimmunoassay), EURIA CCK kit for CCK
determination was purchased from Euro-Diagnostica, Malmo, Sweden.

4.3.2 Cell culture

The intestinal neuroendocrine tumor STC-1 cells were purchased from ATCC (USA) and cultured according to instructions from ATCC. Cells were grown in Dulbecco's modified Eagle's Medium (DMEM, 4.5 g/L glucose) supplemented with 10% FBS, 1% penicillin and streptomycin. Then, incubation was done at 37°C in the presence of 10% CO₂.

4. 3.3 Stimulation of CCK secretion

Forskolin was used as a positive control as it is already reported to stimulate CCK secretion from STC-1 cells (394). Cells were seeded in T25 flasks for 2 days. On the third day, when confluency reached almost 70%, the medium was removed and cells were washed twice with Hanks' Balanced Salt Solution buffer (HBSS) supplemented with 20 mM HEPES, pH 7.4. Samples (with forskolin, quercetin, kaempferol, apigenin, rutin and baicalein) were prepared in the same buffer used for washing in the presence of 0.1% DMSO. Cells were incubated with flavonoid samples for 2h at 37°C, under 10% CO₂ atmosphere. As a final concentration, 100 µM of forskolin and 20 µM of flavonoids were used. The concentration of forskolin was selected based on reported literature, while the flavonoid concentration was based on their solubility and the limitation to avoid possible complexation for CCK as already reported in previous work (395). Control flasks contained only buffer. Supernatants were collected and kept at -80°C until CCK determination.

After collecting supernatants, cells were washed once with ice cold phosphate-buffered saline (PBS). Afterwards, 1 ml of cell lysis buffer supplemented with 1% protease and phosphatase inhibitor was added and flasks were incubated on ice for 5 min
with periodic mixing. Next, cell lysate was collected with a scraper and transferred to eppendorf tubes and centrifuged at 13,000 g for 10 min. Supernatants were transferred to other tubes and kept in -80°C for determination of CCK concentration.

CCK measurement was done by RIA using a rabbit antiserum raised against CCK-8 sulphate conjugated to bovine serum albumin. The cross-reactivities of this antiserum were really low for the non sulfated member of the gastrin/CCK family that share the same C-terminal penta-peptide (Cholecystokinin 26-33 non-sulfated <0.01%, Cholecystokinin 30-33 <0.01%, Gastrin-17 sulphate 0.5 %, and Gastrin-17 non-sulfated <0.01%) but it should bind all biological active forms with equimolar potency (Cholecystokinin 26-33 sulphate 100.0%, Cholecystokinin-33 sulphate 134.0%). The sensitivity of the assay has been optimized at 0.3 pmol/L.

4.3.4 Western Blot

Extracted protein from STC-1 cells was combined with loading buffer, vortexed, heated at 95 °C for 5 min and then electrophoresed in 4-20% polyacrylamide gel, with each lane receiving equivalent amounts of protein (10 µg). Gels were then transferred to polyvinylidene fluoride membranes (Immun-Blot™ PVDF) (BioRad, Nazareth Eke, Belgium) for antibody probing. Membranes were incubated with blocking buffer (50 mL of 1x phosphate buffered saline containing 0.2% (v/v) Tween-20 and 5% (w/v) bovine serum albumin) for 60 min at room temperature. Further, the membrane was incubated with rabbit polyclonal anti-CCK primary antibody (ab83180, immunogen: synthetic peptide designed within residues IQQARKAPSGRMSIVKNLQNLDPSHRISDRDYMGWMDFGRRSAEEYEYPS, corresponding to internal sequence amino acids 66-115 of human CCK) at the
recommended dilution 1:1000 over night and at 4 °C. Horseradish peroxidase conjugated goat anti-rabbit IgG (Gentaur, Kampenhout, Belgium) was used as secondary antibody at dilution of 1:2500. Next, the membranes were then treated with enhanced chemiluminescence (ECL) reagent (GE Healthcare, UK) and subsequently the signal was detected by means of a ChemiDoc MP Imaging System (BioRad, Nazareth Eke, Belgium).

4.3.5 Analysis of cell viability upon exposure to phenolic compounds

Viability of STC-1 cells treated with forskolin and flavonoids was evaluated by the PrestoBlue™ and MTT assay. PrestoBlue™ reagent is a resazurin-based solution in which resazurin, blue in color and non-fluorescent, is converted to resorufin under the reducing power of viable cells. Resorufin has a red color and is highly fluorescent, thus can be used to determine cell viability and cytotoxicity. The MTT assay is a colorimetric assay for assessing mitochondrial activity of viable cells. Briefly for both assays, Cells were seeded for 2 days at 40,000 cells per well in 96 well plates. Afterwards, medium was removed and cells were treated with 100 µM forskolin, 20 µM quercetin, kaempferol, apigenin, rutin and baicalein. After incubation for 2h, cells were washed with buffer and re-suspended in fresh culture medium. Both assays were carried out as reported before (395, 396). Each treatment was done in 3 technical replicates and 3 biological replicates.

4.4 Results

4.4.1 Flavonoids do not affect cell viability

In this set of experiments, the effect of incubation of cells with forskolin and flavonoids
at the concentrations planned to be used for the secretion experiment was evaluated. The results from the two cell viability assays with Presto blue and MTT confirmed that neither forskolin nor flavonoids resulted in a reduction in the viability of the exposed STC-1 cells compared to the controls (Figure 4.2).

**Figure 4.2:** Viability of STC-1 cells upon exposure to 100 µM of forskolin and 20 µM of flavonoids. Two assays were used, Presto blue and MTT. Each value is based on three biological replicates and expressed as the mean ± SEM. Values are presented as a percentage of the control (non treated cells).

### 4.4.2 CCK detection

#### 4.4.2.1 CCK production in STC-1 cells

First, the production of the prepropeptide of CCK by STC-1 cells was confirmed by western blot (Figure 4.3A). All samples (extracts from control cells and cells treated with forskolin and the different flavonoids) gave an intense band that was seen at around 12 kDa. On the other hand, in all samples from the culture medium including the control, 2 faint bands were seen: one was around 37 kDa in addition to a very faint one at around 10 kDa (Figure 4.3B). However, when an equal volume of sample (extract) was loaded on
the gel (and not equal amount of protein), the bands were clearer. Thus, the low concentration of the protein in the culture medium (supernatants) could be the reason behind seeing very faint bands when loading based on equal amount of protein.

![Image](image.png)

**Figure 4.3:** The prepropeptide of CCK in STC-1 cells. A represents the prepropeptide of CCK in the extracts of STC-1 cells. B and C represent the prepropeptide of CCK in the culture medium of treated cells compared to control (non treated). In B, equal amount of protein was loaded on the gel for all samples. In C, an equal volume of sample (extract) was loaded for all samples.

4.3.2.2 Flavonoids stimulate CCK secretion

Further, CCK release from STC-1 cells was studies and quantified by RIA assay as it is already known as the most sensitive method for quantification of the active form of CCK. As it is shown from figure 4.4, forskolin which was used as a positive control resulted in a significant increase in CCK secretion as a percentage of the total CCK content (secreted+ intracellular) compared to control (no-treated) cells. Interestingly, all flavonoids caused an increase in the CCK secretion at the concentration used. Quercetin, kaempferol, apigenin and rutin resulted in a significantly higher CCK release compared
with the control. Rutin showed a lower activity compared to its aglycone quercetin. On the other hand, incubation the cells with the different flavonoids did not increase the cellular CCK content (Figure 4.5).

**Figure 4.4:** The effect of forskolin and flavonoids on stimulation of CCK secretion from STC-1 cells. Data are the mean of three biological replicates ± SEM. Secreted CCK was expressed as a percentage of the total CCK content (secreted+intracellular). Different superscripts on the columns mean values were significantly different at P value <0.05 using one-way ANOVA.
4.5 Discussion

In this study, we aimed to investigate the stimulation of CCK release by different flavonoids. First, the production of the prepropeptide of CCK in STC-1 cells was confirmed by western blot though the incubation time used was only 2h which is probably not enough to check the effect of different treatments on CCK gene expression level. Next, we showed that the different flavonoids were able to induce the release of CCK by STC-1 cells in vitro with some difference in the activity. Quercetin showed a significant and higher CCK releasing potential compared to its glycoside named rutin. This is in agreement with a previous study which showed that hesperetin, but not its glycoside hesperidin, was able to stimulate CCK secretion (348). Indeed, it is known that the beneficial effects of flavonoids on human health, including antioxidant, anti-inflammatory and anti-obesity effects, depend on both the position and number of hydroxyl groups (397). O-glycosylation of flavonoid aglycones was reported to reduce
different bioactivities such as antioxidant and anti-inflammatory effects (398). Suh et al. (399) also demonstrated that both antioxidant and pancreatic-lipase-inhibitory activities of soybean were increased during fermentation due to the increase in the levels of aglycones and the level of the pancreatic lipase activity in vitro was highly correlated with anti-obesity effects in vivo. Therefore and from results obtained with the different flavonoids, it can be suggested that the OH groups in position 3 and 4 of the B ring and an OH group on position 3 in the C ring are important elements to stimulate CCK secretion. In addition, the aglycone has more activity than its glycosides.

The CCK secretion may result from (a) activation of phospholipase C-β which is followed by the generation of IP3 and diacylglycerol (DAG) and subsequently increases in $[\text{Ca}^{2+}]_i$ and activation of PKC, (b) activation of adenyl cyclase with subsequent elevation in cAMP concentrations and activation of protein kinase A (PKA); and/or (c) other pathways that involve the activation of calcium channels that are important for sustained CCK secretion (400). Gaillard et al. (401) excluded the involvement of a seven-transmembrane domain receptor in the peptone-evoked CCK release because the accumulation of phosphoinositides in peptone-treated cells was moderate, and PKA or PKC inhibition failed to modify the peptone-CCK secretion. However, the same study showed that stimulation of CCK release by peptones involved pertussis toxin-sensitive G protein(s) and was dependent on Ca$^{2+}$ availability. Similarly, Kim et al. (402) showed that hesperetin evoked-CCK release is mediated by the extracellular Ca$^{2+}$ influx and TRP channels, mainly TRPA1 (a member of the TRP family of ion channels). Another study suggested that the epigallocateachingallate can activate TRPA1 on the STC-1 cell surface and subsequently cause an increase in Ca$^{2+}$ (403). Hence, further work to explore the
exact mechanisms behind the stimulation of CCK release by flavonoids is very crucial. To our knowledge, this is the first report on the effect of quercetin, kaempferol, rutin, apigenin and baicalein on CCK secretion from STC-1 cells. Results suggest that the reported effect of flavonoids towards food intake and weight could involve the stimulation of CCK release which in turns evokes a satiety signal responsible for food intake reduction. However, further work is still needed to explore the effect of the tested flavonoids on CCK release and appetite regulation in animal model which could attribute to their reported anti-obesity effects.
Chapter 5

General conclusion and future perspectives
Chapter 5 General conclusion and future perspectives

5.1 Status and main outcomes obtained from this thesis:

High blood pressure and obesity are both major health risk factors that threaten the life of humans worldwide. Although in most cases medication is needed, the search for alternatives from natural based products has been of great importance and research continues to investigate natural sources with the aim of finding new bioactive compounds that can help in the prevention and treatment of hypertension and obesity. Phenolic compounds, a group of compounds that are naturally found in fruits and vegetables, have been claimed for their health promoting effects. *In vitro, in vivo* and human studies indicated a positive role of phenolic compounds in the battle against hypertension and obesity. Many studies have already focused on investigating the possible mechanisms behind this activity. However, mechanisms involved in the control and regulation of blood pressure, body weight and food intake are many and complex. Therefore, more research in this field is needed to deduce firm conclusions.

The aim of this work was to screen different phenolic compounds from different classes for their antihypertensive activity as determined by ACE inhibitory activity *in vitro* and to test their possible effects on the CCK pathway (CCK1R activation and CCK release/production) with use of different cell-based bioassays. Results obtained are discussed on the basis of their structures.

Here below the main conclusions and achievements of this work are summarized in detail:
In **Chapter 1**, a literature screening was done with the aim of collecting most recent information from human and animal studies on the potential role of phenolic compounds and food rich in phenolic compounds on hypertension and obesity. Based on this screening, we concluded that some phenolic compounds and their food rich sources showed promising effects as antihypertensive and antiobesity agents. Nonetheless, most results are based on complex food extracts or beverages and thus the effect of compounds other than phenolic compounds can often not be excluded (178, 183, 197, 198). In addition, different designs, different intervention times and different doses might result in different observations (194, 199, 200). Therefore and in addition to focus on phenolic compounds rich food sources, focus on specific compounds is very important. Studying phenolic rich food sources will provide information on possible synergistic/antagonistic effects of food matrix and its interference with possible effects of phenolic compounds. On the other hand, focusing on specific individual compounds is needed to confirm and approve the claimed health beneficial effects of these compounds and food sources in hypertension and obesity management.

In **chapter 1** also possible mechanisms involved in the antihypertensive and antiobesity effects reported by phenolic compounds has been discussed. With regard to blood pressure-reducing effects, a decrease in oxidative stress, improving the endothelial function and interference with RAAS, were suggested. However, the exact mechanism(s) of action by which phenolic compounds might decrease BP in humans still needs further investigation and confirmation. Concerning the plausible mechanisms involved in the antiobesity effects, inhibition of adipogenesis, interferences with lipid metabolism and energy expenditure were supported by most of the studies. In addition, possible effects on
food intake mediated by satiation and satiety inducing hormones should not be ignored although this is not well studied and investigated for the moment.

In **chapter 2**, we studied the effect of phenolic compounds on ACE activity. Therefore, 22 phenolic compounds from different classes and sub(classes) (tannic acid, gallic acid, benzoic acid, \(p\)-hydroxybenzoic acid, syringic acid, vanillic acid, ellagic acid, protocatechuic acid, catecho, pyrogallol, caffeic acid, ferulic acid, \(p\)-coumaric acid, \(trans\)-cinnamic acid, quercetin, rutin, kaempferol, resveratrol, \(trans\) -stilbene, apigenin, epicatechin, and phloretin) were screened for *in vitro* ACE inhibitory activity. Tannic acid showed the highest activity (IC\(_{50}\)=230 \(\mu\)M). All tested flavonoids showed ACE inhibitory activity with IC\(_{50}\) values ranging between 0.415 and 1.381 mM with quercetin being the most active compound. Phenolic acids showed ACE inhibition in a range from 2 to 9.3 mM. QSAR analysis was performed for phenolic acids to investigate the key structural elements responsible for the difference in activity. It was elucidated that the number of hydroxyl groups on the benzene ring plays an important role for their activity. Substitution of hydroxyl groups by methoxy groups decreased their activity. Furthermore, docking studies were performed to investigate possible mechanisms involved in the ACE inhibitory activity. We concluded from this experiment that phenolic acids and flavonoids inhibit ACE via interaction with the zinc ion and this interaction is stabilized by other interactions with amino acids in the active site. On the other hand, other compounds such as resveratrol and pyrogallol may inhibit ACE via interactions with amino acids at the active site thereby blocking the catalytic activity of ACE.

Food ingredients influencing food intake by the CCK pathway can stimulate indirectly or/and directly this pathway. They might stimulate CCK secretion which in its turns
stimulates satiety by activating CCK1R and/or they can directly stimulate CCK1R. The reported antiobesity effect of phenolic compounds could involve influencing satiety signals (279, 348). Therefore, in this part (Chapter 3) we investigated the effect of a group of phenolic compounds (tannic acid, gallic acid, benzoic acid, hydroxybenzoic acid, protocatechuic acid, kaempferol and quercetin) on the CCK1R signaling pathway as an important pathway in food intake regulation. We used a cell-based bioassay with Chinese hamster ovary (CHO) cells overexpressing the rat CCK1R, and tested whether the different compounds could activate this CCK1R. From the results obtained in this piece of work, we could conclude that phenolic compounds do not activate the CCK1R. Thus, the proposed effect of phenolic compounds in literature on food intake and weight is not mediated by CCK1R activation. As such, phenolic compounds might stimulate CCK secretion which further acts on its receptor. In addition, we showed in this part that tannic acid and its monomer gallic acid antagonized the CCK signaling in vitro as they inhibited the CCK-8S-evoked response.

In chapter 4, we selected some compounds out of the group of flavonoids (quercetin, kaempferol, apigenin, rutin and baicalein) to investigate possible stimulation of CCK release from the enteroendocrine STC-1 cells. First, we confirmed the production of CCK in STC-1 cells without treatment using western blot. No effect on CCK production by the tested flavonoids was found. A RIA assay was used to measure CCK production/release. Interestingly, a significant stimulation of CCK release from the cells was caused by quercetin, kaempferol and apigenin. At the same time, no toxic effects were seen on cell viability for all flavonoids studied.
5.2 Future perspectives

5.2.1 *In vitro* screening of ACE inhibitory activity and other mechanisms involved in blood pressure regulation

Although we were able to build a structure-function relationship with a group of phenolic compounds, enough information that can help to fully understand all key structural elements behind the potential ACE inhibitory activity of phenolic compounds are still not available. Therefore, more *in vitro* screening for phenolic compounds with different structures are still important. Data obtained from the *in vitro* screening about the structure-function relationships can help in developing new ACE inhibitors based on phenolic compounds.

For the moment, not much information exists on all possible mechanisms that can mediate the effect of phenolic compounds on hypertension. New drug classes are now of importance, as well. For instance inhibitors of vasopeptidases, aldosterone synthase and soluble epoxide hydrolase, agonists of natriuretic peptide A and vasoactive intestinal peptide receptor 2 are targeted to phase II/III of hypertension development. As well, inhibitors of aminopeptidase A, dopamine β-hydroxylase, and agonists of components of the angiotensin-converting enzyme 2/angiotensin(1–7)/Mas receptor axis are used in phase I or under preclinical development (267). Positive effects of phenolic compounds on some of these routes were already reported (257, 268, 269). Accordingly, it is important to open the door on these therapeutic targets and to investigate possible effects of phenolic compounds on these pathways. This could be done *in vitro* as an initial screening tool using pure phenolic compounds and phenolic compounds rich sources to investigate structure-function relationships.
5.2.2 More animal and human trials on phenolic compounds are still important to confirm the claimed antihypertensive effects

Literature screening showed promising effects by phenolic compounds and phenolic compound-rich foods. Yet, more research on phenolic compounds rich foods and on pure compounds following well-designed and long-term studies is necessary in the process of developing functional foods and/or functional food ingredients or food supplements based on phenolic compounds. This is needed to confirm and approve the reported health claims and to optimize doses in different food matrices and to establish a tolerable upper limit of intake.

This first needs to be performed in animal models whereby special attention should be given to control possible side effects particularly in case of the use of pure compounds as supplementation before moving to human trials. Phenolic compounds can be both pro-oxidants and antioxidants and hence safety issues should be considered if high doses of phenolic compounds are consumed (404). Quercetin supplementation at a dose of 1000 mg/d for 1 month have been reported to cause adverse effects such as headache, nausea, and tingling of the extremities in humans (405). This addresses the importance of the in vivo evaluation for the safety potential of a tolerable upper dose of a compound that can show health promoting effects and that is considered as safe on the long term. SHR is the most frequently used model in animal studies (see literature review) as this is an accepted model for human essential or primary hypertension. Blood pressure of these rats can be measured with a direct (arterial) measurement or by using the indirect (tail-cuff) method. Direct measurement is more precise but the tail-cuff method is less complex and has a low impact on the animal (406). The choice between the two techniques should be based on the study objectives. A long-term study is the next
step to provide information on the effect of a compound on the long term and on the required dose to induce the required effect. In addition, this helps to evaluate mechanisms involved. This can be done within several weeks as we showed previously in the literature review. For instance, blood pressure could be recorded on a daily basis.

For human studies, participants involved should be chosen from the target group. We already discussed results from several human intervention studies in the literature review. A double-blind, randomized, placebo-controlled design is the most appropriate for this type of research. Our literature review showed that very few pure compounds (mainly quercetin) were studied in humans. Therefore, it would be interesting that also other compounds that show activity in animals in parallel with food sources rich in phenolic compounds to be investigated in humans taking into account that data on the minimum and maximum dose that can be applied in humans should be obtained first from animal studies. Before going to the main human intervention trial, pilot study might be useful to confirm activity in humans, and to determine the dose requirements. This human pilot study can also provide the necessary information (magnitude of the effect, standard deviation) for the sample size calculation for the human intervention trial.

In the EU, food claims have to be approved by the European Commission after evaluation by European Food Safety Authority (EFSA). To be approved by EFSA, food claims have to meet the following requirements: 1) characterization of the food constituent, 2) Relevance of the claimed effect to human health, 3) Scientific substantiation of the claimed effect. For example in case of developing phenolic compounds rich extract targeting ACE inhibition and blood pressure, the subject of the
health claim (particular compounds, class of phenolics) have to be sufficiently characterized in the extract. The health claim ‘maintenance of normal blood pressure’ is considered relevant to human health. To meet the third criterion and to establish a cause and effect relationship, evaluation of the effect in human in a representative study is essential. To be considered representative, many issues should be taken into account that will influence the results obtained from the clinical trial and further interpretation. First, the start from the target group which in the case of hypertension should be prehypertensive or stage I population. Characteristics of participants at base line (smoking, weight, age, sex, medicated and non-medicated individual, etc…) need to be described. Perhaps specific epigenetic parameters could be evaluated before including these in the study. The dose applied and the way of administration needs attention. In case of functional foods, dosage and administration form (food matrix) should be the same as the product intended to be launched on the market. Diet restriction to avoid interference from additional intake of polyphenols from food items consumed daily should be avoided. The placebo group needs to be defined, as well as what they will receive. It's important to match for caloric content and appearance. For example in case the treatment group receives polyphenols-rich extracts/ dark chocolate rich in flavonoids, the placebo product should be low or zero content in polyphenol (maybe white chocolate low in polyphenols could be an option). Another example: fruit juice rich in particular compound(s) versus placebo which could be only fruit juice.

Interestingly, EFSA has already approved some health claims on phenolic compounds rich food sources:
- Cocoa flavanol: EFSA concluded a cause and effect relationship has been established between the consumption of cocoa flavanols in the high flavanol cocoa extract (i.e. in capsules or tablets) and maintenance of normal endothelium-dependent vasodilation. In order to obtain the claimed effect, 200 mg of cocoa flavanols should be consumed daily. This amount could be provided by less than one gram of high flavanols cocoa extract in capsules or tablets, and can be consumed in the context of a balanced diet. The target population is the general population.

- Polyphenols in olive: EFSA concluded that a cause and effect relationship has been established between the consumption of olive oil polyphenols (standardised by the content of hydroxytyrosol and its derivatives) and protection of LDL particles from oxidative damage. The Panel considers that in order to bear the claim, 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol) in olive oil should be consumed daily. These amounts, if provided by moderate amounts of olive oil, can be easily consumed in the context of a balanced diet. The concentrations in some olive oils may be too low to allow the consumption of this amount of polyphenols in the context of a balanced diet. The target population is the general population.

- Water-soluble tomato concentrate developed in two variant forms named WSTC I (completely water-soluble syrup) and its low-sugar derivative, WSTC II, supplied in powder format: EFSA concluded that a cause and effect relationship has been established between the consumption of water-soluble tomato concentrate (i.e.,
WSTC I and II) and the reduction in platelet aggregation in humans. In order to achieve the claimed effect, 3 g WSTC I or 150 mg WSTC II in up to 250 mL of either fruit juices, flavoured drinks or yogurt drinks (unless heavily pasteurised) should be consumed daily.

**5.2.3 In vivo experiments to investigate the effect of phenolic compounds on CCK-mediated food intake and body weight**

This can be done in short and long term studies. Afterwards, human interventions are important for confirmation.

- **5.2.3.1 Short term experiments**

The short term trials give information about the effect of the tested compounds on appetite within hours. Investigating the effect of the tested compounds on food intake is done within one day. This should include measuring meal frequency, meal duration and the total daily food intake (407). When a reduction in food intake is seen, further investigation for the role of CCK can be done by blood sample analysis. Therefore, the CCK concentration in the blood should be measured within 10 to 30 min after administration of the tested compound and compared with the values obtained from the control animals. Additionally, other gut hormones involved in food intake regulation like GLP-1 and PYY could be included to test whether the effect is specific to CCK only or to gut hormones in general. In case only a change is seen in the concentration of CCK in the blood of treated rats compared to the controls, it can be assumed that the CCK release is at least partially involved. In case of positive effects seen on the CCK concentration after treatment, dose-response experiments are needed to be established for a determination of the minimum concentration for which an effect can be seen.
5.2.3.2 Long-term experiments

As discussed in the literature review, evidence on the effect of some phenolic compounds on body weight reduction is already available (282-284, 290-292, 382, 383). However, negative results from some other studies (285-289) did not allow to reach a clear conclusion. Further research is thus necessary. The aim of the long-term experiments is to see a decrease in weight or weight gain in the test animals compared to the control animals. Rodents with environmentally driven changes in weight are used in research that investigates the tendency for weight gain and weight loss. These models are often developed by giving the animals access to high fat diets, usually over a period of 3-4 months (408). This results in generating animals with a high body weight gain and predominantly in the form of fat, insulin resistance, glucose intolerance and elevated cholesterol and triglyceride level. The C57BL/6 mouse is a well-known model prone to study effects on weight gain (409). In addition, many commonly used Sprague-Dawley rats have been proposed as a rodent model for polygenetic obesity because they readily become obese following a standard diet (410). Administration of the test compounds can be done in 2 ways. First, it can be by adding the compound at the same time when introducing the obesity-induced diet. The aim of this design is to check the effect of these compounds on the prevention of body weight gain. Also, application of test compounds could be after inducing obesity. In both designs, animals should receive during a certain amount of time, every day a certain dose of the tested compound and subsequently in addition to food intake measurement, their body weight should be monitored on a day-to-day or week-to-week basis (411). It is important in long term experiments to monitor adverse effects by daily inspection for signs of toxicity by e.g. measuring body
temperature or abnormal behavior of the treated animal compared to control animals.
In short- and long-term studies, it would not be appropriate to mix pure phenolic compounds with animal food or water, which might cause positive false effects on food intake because of taste aversion or individual preferences for flavors. In addition, since phenolic compounds have a low bioavailability (50), we suggest that the use of a gavage which brings the treatment to the stomach could be a preferred choice for administration. In this way the administered compound still needs to pass the gastro-intestinal tract to be absorbed into the blood stream, and so the effect of gastro-intestinal digestion and absorption is not excluded as in the case of intravenous injection. This also ensures an accurate and precise dosing in the animals.
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Summary

In this PhD project, a detailed overview is given from all animal and human studies published on the effects of phenolic compounds and their suggested role in fighting hypertension and obesity. The ACE and CCK pathways play an important role in blood pressure and food intake as well as body weight maintenance. In the cell-free experiments on ACE, we could demonstrate that phenolic compounds from different classes showed ACE inhibitory activity. However, it should be remarked here that for some compounds the inhibitory activity was only at a high concentration that might most likely not be reached in vivo. Nonetheless, our data on modeling and SAR analysis helped to understand the key groups of the phenolic molecule that could influence the activity, and this in turn should help to design new ACE inhibitors based on phenolic compounds.

Regarding the CCK pathway, none of the tested compounds could stimulate the CCK1R in vitro. Interestingly, cell-based assays with the enteroendocrine STC-1 cells demonstrated for the first time that some phenolic compounds showed a significant stimulation of the release of the satiety peptide CCK. Thus, mediation of the CCK pathway could be, at least partially, involved in the antiobesity effects of phenolic compounds.
Samenvatting

In dit doctoraatsproject wordt een gedetailleerd overzicht gegeven van alle dierlijke en menselijke studies gepubliceerd omtrent de effecten van fenolische verbindingen en hun voorgestelde rol in de strijd tegen hoge bloeddruk en obesitas. De twee pathways van ACE en CCK spelen een belangrijke rol in de regulatie van de bloeddruk en voedselinname en het onderhoud van het lichaamsgewicht. In de celvrije-experimenten omtrent ACE, kunnen we zien dat fenolische verbindingen uit verschillende klassen een remmende activiteit hebben op ACE. Er moet echter worden opgemerkt dat voor sommige verbindingen de remmende activiteit pas werd gezien bij een hoge concentratie die in vivo waarschijnlijk niet kan worden bereikt. Desalniettemin de verkregen data omtrent de modellering en structuur-activiteitanalyse zullen helpen om de bijdrage in de ACE-inhibitorische activiteit van de belangrijkste groepen van de fenolische molecule te begrijpen, wat op zich zou moeten helpen om nieuwe ACE-remmers op basis van fenolverbindingen te ontwerpen. Wat betreft de CCK pathway, vertoonde geen enkele van de geteste verbindingen een stimulerende activiteit voor CCK1R in de in vitro proeven met CCK1R-CHO cellen. Daarnaast werd ook de activiteit van een aantal fenolische verbinden op de vrijstelling/productie van CCK onderzocht met enteroendocrine STC-I cellen in vitro. Voor de eerste keer kon worden aangetoond dat fenolen de vrijstelling van het CCK verzadigingspeptide verhogen en dit zou kunnen bijdragen in de beschreven anti-obesitas effecten van polyfenolen.
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Publications in international scientific peer-reviewed journals

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Submitted papers to international scientific peer-reviewed journals


- **AL SHUKOR, N., Ravallec, R., RAES, K. VAN CAMP, J. & SMAGGHE, G. Flavonoids stimulate cholecystokinin secretion from the enteroendocrine STC-1 cells.** Submitted to Fitoterapia.

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- **AL SHUKOR, N., RAES, K. VAN CAMP, J. & SMAGGHE, G. Interaction of phenolic compounds with the cholecystokinin signaling pathway in vitro to explain effects on reducing food intake (2014). 20th National Symposium on Applied Biological Sciences, Louvain-la-Neuve, Belgium, oral presentation.**

Chapter in books:

- **AL SHUKOR, N., RAES, K., SMAGGHE, G., VAN CAMP, J. Flavonoids: Evidence for Inhibitory Effects Against Obesity and Their Possible Mechanisms of
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Yours sincerely

Nadin