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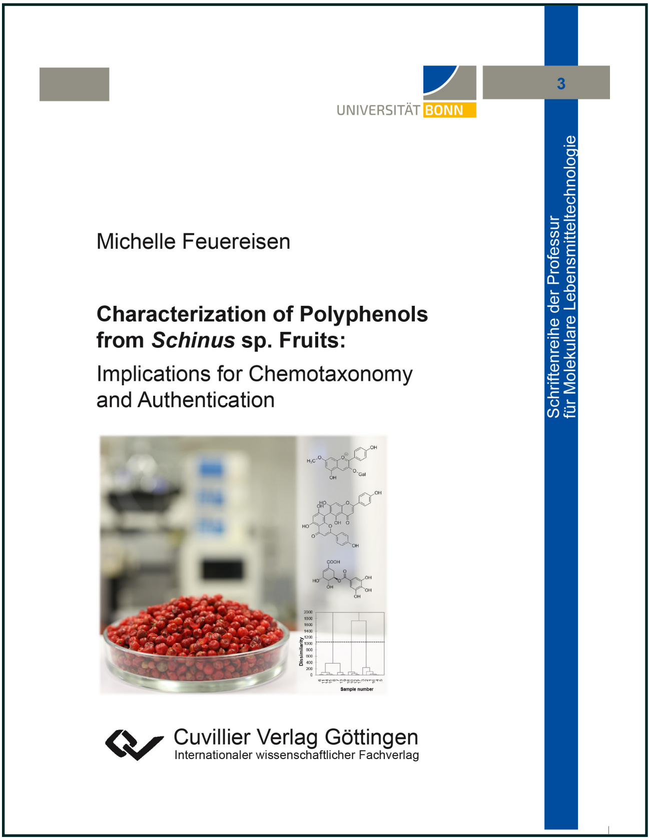
Characterization of Polyphenols from Schinus sp. Fruits

Implications for Chemotaxonomy and Authentication



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Chapter 1

General introduction

1 Need for new and natural bio-functional and techno-functional compounds

Human nutrition has always been a subject of constant change influenced by principal factors such as the way of life, current trends, academic research, and increasing global availability of eatables. In recent years, a major trend which partly stands in contrast to the convenience food trend has lead toward an increasingly health conscious and environment conscious lifestyle, most notably regarding the diet. This includes the recollection to more natural, less processed and sustainable groceries often under the label “all natural”. As a consequence, there is an increasing demand for natural products and natural food additives which, in contrast to synthetic additives like azo dyes or sodium nitrite, are valued as beneficial for health and fulfill the consumer expectation. For the industry, the so called “green processing” and “clean label” also implicate the challenge of ensuring the same food quality, shelf life and convenience while avoiding the use of synthetic additives and a reduction of food processing. (Daglia, 2012; Maqsood et al., 2013)

Besides the nutritional and the health value of groceries, food safety is an issue concerning consumers, regulatory agencies, and food industries. Contamination of food with bacteria and fungi causing spoilage and food intoxications are issues which have not yet been overcome. To ensure safe products, effective preservatives and detergents are some of the basic requirements. Unfortunately, there is an increase in microbial resistance against common preservatives and disinfectants. This can interfere with product shelf life and safety, elevating the need for novel antimicrobial agents. Progress in the development of novel antimicrobials is moreover essential due to the increasing prevalence of antibiotic-resistant bacteria and at the same time a decrease in the development of new antibiotics. Research is focusing on nature as a source of new agents, not only because of consumers' preferences and possible health benefits but also since nature has revealed distinguished bioactive and techno-functional compounds. (Brul & Coote, 1999; Chapman, 2003; Daglia, 2012; Negi, 2012) To enable pharmaceutical or food use, identification and characterization of promising natural sources as well as their compounds is a prerequisite. This is a challenging task, not only because of the overwhelming number of possible raw materials but also since chemical, bioactivity, and industrial aspects like synergetic effects, interactions, sensory properties, toxicity, and applicability of bioactive compounds should be taken into consideration. Therefore, interdisciplinary approaches, as conducted in this thesis, are recommended or even required for a selection and investigation of botanical sources and the establishment of a crucial basis for further research regarding their bioactivity.

2 Nature as a source of bioactive compounds

Ever since natural products have been a rich source of antimicrobial agents. For example, the first discovered and approved antibiotics like penicillin in 1940, tetracycline in 1948, and glycopeptides in 1955 are natural compounds. More precisely, they were yielded from fungi and bacteria. Spices have been used for the preservation for food since ancient days, and various plants have been employed in traditional medicine all the while. Although nowadays up to 25–50% of pharmaceutical products are of plant origin, humans have relied on these antibiotics of microbial origin. Due to the aforesaid reasons of consumers´ preferences and the increasing resistance of microorganisms against classic antimicrobials, there is a rising demand for alternative agents, also of natural origin. Plants are of increasing interest for pharmacology and food safety purposes because they have successfully been utilized in traditional medicine, including the treatment of infections, and since they represent a nearly inexhaustible source of novel molecules. At present, they are scanned for new antimicrobial or otherwise bioactive compounds. Secondary plant metabolites, especially phenolics, are a rather young research domain compared to other food related constitutes such as e.g. macronutrients. Thus, this class of compounds has not been evaluated entirely and further research is essential for the recovery and approval of effective novel and natural agents. Nevertheless, this field has already revealed promising results of which a few have been verified by *in vivo* studies. Encouraging examples are the neutralization of *Staphylococcus aureus* α-toxin by polymerized catechin, the disruption of *Helicobacter pylori* pathogenesis by the flavonol quercetin in guinea pigs, and the clinical efficacy of the chalcone derivative sofalcone on human *Helicobacter pylori* infection in a multidrug treatment (Cushnie & Lamb, 2011). In addition, techno-functional and bio-functional properties in respect of disease prevention, health benefits, and food application are of major interest. (Bourgaud et al., 2001; Cowan, 1999; Cushnie & Lamb, 2005; Sugathan et al., 2017)

2.1 Secondary plant metabolites

Secondary plant metabolites, also referred to as phytochemicals, occur ubiquitously in plants. They are products of the secondary metabolism, meaning that they have no anabolic, katabolic, or energetic function, and are, therefore, not part of the primary metabolism. Nevertheless, they possess a major role for the reproduction, proper growth, and survival of plants as well as their interaction with the environment and adaptation to environmental influences. Plant secondary compounds provide defense against abiotic and biotic stress caused by herbivores (anti-feeding properties), phytopathogens (phytoalexins), competing plants (allelopathy), UV radiation, and environmental pollution. Furthermore, they are attractants for pollinators or seed dispersing animals. As they are commonly classified according to their biosynthetic pathways, three major groups are considered: phenolic metabolites, terpenoids, and alkaloids including other nitrogen-containing metabolites. Since phytochemicals are part of the daily dietary intake, and thus likely also influence ingesting humans and animals, they have been focused by research in the last decades. Besides some toxic or anti-nutritive effects, various health benefits including prevention and treatment of diseases have been associated with the application or consumption of these plant constituents. (Bourgaud et al., 2001; Dillard & German, 2000; Molyneux et al., 2007) Due to the manifold of compounds, the further delineation in the present work is focused on the group of polyphenols, more precisely on some of their relevant subclasses which will be discussed in the following.

2.2 Polyphenols

With several thousands of identified structures, polyphenols including simple phenolics are the most abundant phytochemicals and widely distributed in higher plants. Basically, the polyphenol molecule consists of at least one aromatic phenyl ring linked to one or more hydroxyl groups, reaching from simple structures such as phenolic acids to highly polymerized tannins. Diversity and ubiquity of these natural plant compounds have led to different classification regarding their chemical structure, biological function, or source of origin. Categorization based on the chemical and structural characteristics usually results in the following subclasses: phenolic acids, flavonoids, stilbenes, and lignans. Frequently, further subgroups such as tannins or phenolic lipids are included. Polyphenols contribute to the organoleptic and nutritive properties of plant based food and several hundred structures can be found in edible plants. Besides fruits, vegetables, nuts, and whole grains, also processed products such as chocolate, wine, coffee, and tea contain high amounts that contribute to the daily intake. (Dai & Mumper, 2010; Dillard & German, 2000; Manach et al., 2004; Tsao, 2010)

The basic flavonoid structure C6-C3-C6 is composed of two aromatic rings (A, B) connected by a heterocyclic pyran ring (C). According to the different substitution degrees of the central C ring, flavonoids are further divided into six subgroups: flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanidins. The number and complexity of corresponding compounds is increased by the substitution patterns of rings A and B, for example, by hydroxylation, methoxylation, alkylation, or glycosylation. (Dai & Mumper, 2010; Manach et al., 2004; Tsao, 2010)

In nature, the less soluble free aglycones anthocyanidins rather occur glycosylated to one or more sugar moieties that are then called anthocyanins. Anthocyanins are water-soluble pigments imparting many flowers, leaves, fruits, vegetables, and some grains their red, pink, violet, purple, or blue coloration. Color, structure, and consequently stability are influenced, among other factors, by the pH value and metal ion complexation. From the basic structure of a 3,5,7,4'-tetrahydroxyflavylium cation more than 500 known anthocyanins can be derived by different methoxylation and hydroxylation patterns of ring B and glycosidic substitution. The predominantly occurring aglycones in nature are delphinidin, cyanidin, petunidin, peonidin, malvidin, and pelargonidin. (Dillard & German, 2000; Manach et al., 2004; Tsao, 2010)

Biflavonoids are dimeric flavonoids arising by oxidative coupling of two monomers. The heterocyclic ring (C) always has a single functional group, namely a carbonyl function leading to biflavones, flavanon-flavones, or biflavanones. Most naturally occurring dimers have a biphenyl linkage, but almost all possible positions of the interflavonoid bond exist. The variety is expanded by methoxylation, hydroxylation, and glycosylation as well as different oxidation states of the C ring. Nevertheless, there are only approximately 90 known natural biflavonoids, of which amentoflavone occurs most frequently. Further, biflavonoids are only found in a limited number of plants, for example, St. John's wort (*Hypericum perforatum* L.) or ginkgo (*Ginkgo biloba* L.), which display pharmacological activities. (Barnes et al., 2001; Hemingway, 1989; Hyun et al., 2005)

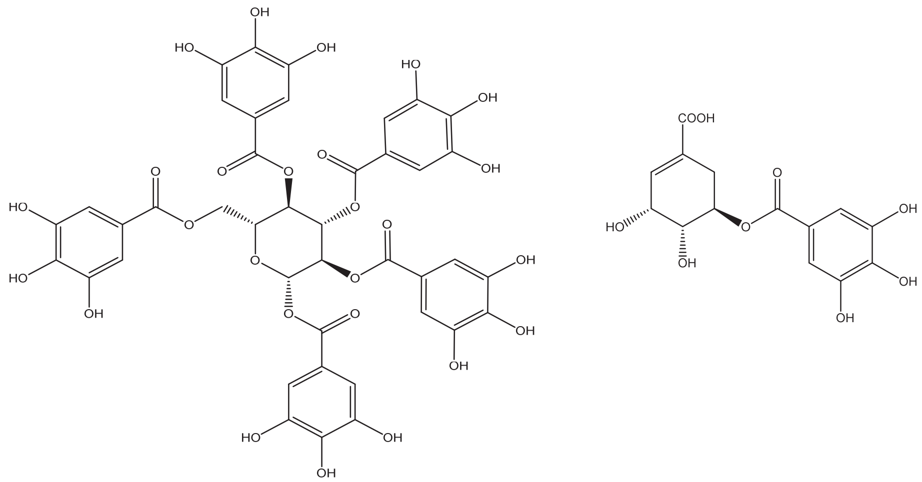

Figure 1: Chemical structure of penta-*O*-galloylglucose and of a mono-*O*-galloyl shikimic acid.

Tannins are commonly subdivided into condensed and hydrolyzable tannins and contribute to the overall sensory impression of many foods like vegetables, fruits, chocolate, and wine due to their astringent and bitter character. Condensed tannins are flavan-3-ol dimers to polymers also called proanthocyanidins. Hydrolyzable tannins, as exemplarily shown in Figure 1, consist of a central molecule (glucose or other polyol, e.g., shikimic acid) esterified with gallic acid (gallotannins) or hexahydroxydiphenic acid (ellagitannins) in different quantities and positions. (Dai & Mumper, 2010; Manach et al., 2004) An additional group also assigned to tannins are the oligomers of phloroglucinol, named phlorotannins. They are less common and have so far been reported only in brown algae (Okuda & Ito, 2011).

2.2.1 *Bioactivity of polyphenols*

Consumption of polyphenol rich food is associated with a decreased risk for acute and chronic diseases like cancer, cardiovascular indispositions (e.g. coronary heart disease, stroke), and neurodegenerative disorders (e.g., Parkinson's, Alzheimer's disease). For a long time, this was solely attributed to the antioxidative properties of polyphenols, but recent findings have shown that secondary compounds also seem to interact with cellular signaling pathways as well as cell receptors and thereby influence cell function. In addition, polyphenols have revealed further biochemical and pharmacological properties such as antiradical activity, chelation of metal ions, modulation of enzyme activity, anti-inflammatory, anti-proliferative, anti-diabetic, anti-allergic, spasmolytic, hepatoprotective, antiviral, antifungal, and antibacterial activity. (Daglia, 2012; Pietta et al., 2003; Vauzour et al., 2010) Due to its relevance for this work, the latter activity will further be discussed in the following.

Many previous studies dealing with plant extracts hamper insights into structure-activity relationships, modes of action, and the determination of active compounds. This is mainly because they lack an identification of polyphenols and since they apply crude extracts rather than purified fractions or isolated phenolics. Nevertheless, polyphenol rich plant extracts and some pure polyphenols have revealed convincing evidence for their potential as antimicrobial agents. Due to their higher antimicrobial activity and their wide activity spectrum in comparison to other classes of polyphenols, mainly flavonoids and tannins were in the focus of attention. Structure-activity relationships have been examined for several flavonoids and tannins. However, the results are partly ambiguous and to a certain extent contradictory. Therefore, more research is necessary since individual compounds likely have multiple cellular targets rather than one specific site of action and structural features might simply serve the uptake into bacterial cells. Reported modes of action are the inhibition of nucleic acid synthesis as well as cell wall and cell membrane synthesis, influence on cytoplasmic membrane function, inhibition of energy metabolism, and complexation of essential nutrients. Furthermore, polyphenols can suppress microbial virulence factors such as toxins or biofilm formation by influencing the crucial factors like enzymes, attachment to host ligands, or quorum-sensing and thereby attenuate the bacterial pathogenicity. (Cushnie & Lamb, 2005; Cushnie & Lamb, 2011; Daglia, 2012; Engels et al., 2011)

Various pathogenic and non-pathogenic bacterial strains have been included in a multitude of assays and some polyphenols have shown promising antimicrobial effects even against drug-resistant bacteria such as multi-antibiotic resistant *Staphylococcus aureus*. Thereby, some compounds showed the highest activity alone or in synergy with other phenolics or other antibacterial agents like antibiotics. Chemical modification of naturally occurring polyphenols can also enhance their antibacterial effects. Since polyphenols from edible plants have also revealed potential as antimicrobial agents and, among others, have an impact on food-borne bacteria, they could possibly be utilized as new plant based preservatives and prevent food spoilage as well as food intoxications. (Cushnie & Lamb, 2005; Cushnie & Lamb, 2011; Daglia, 2012; Taguri et al., 2004)

2.2.2 Polyphenols as chemotaxonomic and authenticity markers

Chemotaxonomy is a classification system for organisms based on the convergence and deviation of their chemical composition. Plant chemotaxonomy utilizes a broad spectrum of macromolecules (proteins, nucleic acids) and micromolecules (secondary plant products) for the elucidation of plant affiliations and can provide insights into plant systematics and evolution. Thus, the classification of plants has been constantly revised, considering the recent chemotaxonomic findings. (Crawford & Giannasi, 1982; Crockett & Robson, 2011; Feuereisen et al., 2017; 1989; Okuda et al., 1992)

Food fraud has a long and disreputable history reaching back to ancient times. Although statutory regulation und effective verification methods have reduced food adulteration, there still is a temptation to increase the rather low profit margin of the food sector in this way. Thus, the advancement of reliable methods for food authentication and species differentiation is relevant for participants of the supply chain as well as for food control authorities (Feuereisen et al., 2017; Schieber, 2008).

As the boundaries between chemotaxonomy and authentication are indistinct, species differentiation and phenolic profile determination, respectively, can be of use for both areas of application (see Chapter 3 and 5). Phenolic compounds and particularly the subgroup of flavonoids have been shown to be useful and suitable chemotaxonomic markers but also authentication markers. Their use is especially helpful when species differentiation based on morphological characteristics is challenging or when plant based food products are concerned. These compounds are suitable due to their ubiquity in higher plants, vast structural variety, and their comparatively straightforward extraction and analysis. (Crawford & Giannasi, 1982; Okuda et al., 1992; Schieber, 2008) Relevant techniques and methods of sample preparation, isolation and determination are discussed in Section 5.4.

2.3 Phenolic plant extracts as food ingredients

As mentioned, consumers prefer natural food and consequently additives of natural origin due to positive associations such as health benefits. The development of new preservatives is also necessary to overcome resistances (see section 1). Due to the great variety of bioactivities and structures, the application of suitable plant extracts or polyphenols as natural food additives is an encouraging approach. In this regard, phenolics may be, and in some cases have already been, utilized successfully as antioxidants, colorants, texturizers, and preservatives for food. Among other resources, agro- and food industry by-products are promising sources for the recovery of natural and sustainable food additives because they often contain high amounts of polyphenols and are accumulated as side streams in high quantities. As some polyphenols have been shown to prevent lipid oxidation and microbial growth, both principal causes for food spoilage, their application is conceivable and has partly been approved, even for perishable food like fish or meat. (Ayala-Zavala et al., 2011; Daglia, 2012; Maqsood et al., 2013; Negi, 2012; Pokorný, 1991; Schieber, 2017)

Although polyphenols from edible plants are regularly consumed by humans and, therefore, are considered safe, the toxicity of plant extracts, especially pure phenolics, and their reactions with food ingredients needs to be assessed. For example, essential oils from herbs and spices revealed distinguished antimicrobial activity but also strongly alter the sensory quality of products. Therefore, their application in foods is limited. This example demonstrates that the influence of the utilized phenolic source on the overall sensory impression of food should also be evaluated. (Negi, 2012; Pokorný, 1991)

3 Plant family Anacardiaceae

The Anacardiaceae, more commonly referred to as “cashew family” or “sumac family”, belongs to the Sapindales order and the immediate phylogenetic relatives are the Burseraceae (Kubitzki et al., 2011). For a long time, the classification of the Anacardiaceae into the five tribes Dobineeae, Mangifereae, Rhoideae, Semecarpeae, and Spondieae was common (Engler, 1892; Hegenauer, 1989). More recent publications prefer the denomination as Anacardieae, Rhoeae, Spondiadeae, Semecarpeae, and Dobineeae as characterized by Mitchell and Mori 1987 and shown in Figure 2 (Mitchell & Mori, 1987; Kubitzki et al., 2011; Pell, 2004). The tribes encompass approximately 72 genera with several hundreds of species (Hegenauer, 1989; Simpson, 2006; Kubitzki et al., 2011; Pell, 2004). It should be mentioned that the number of taxa and its order is conflicting as it can be based on different characteristics such as morphological or phylogenetic properties and is constantly revised (Barkley, 1957; Pell, 2004; Schulze-Kaysers et al., 2015).

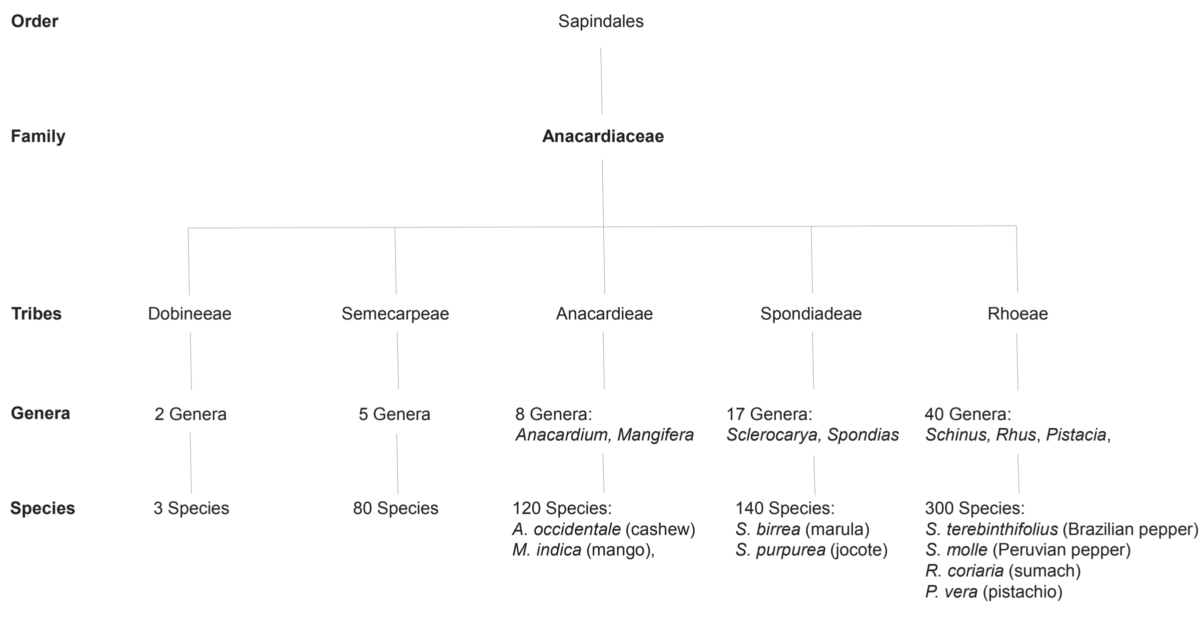

Figure 2: Taxonomic relationships of selected Anacardiaceae species (Tianlu & Barfod).

This plant family is of regional, general economic, and scientific interest due to a broad range of properties. On the one hand, some of the species of the Rhoeae tribe such as Eastern poison ivy (*Toxicodendron radicans* (L.) Kuntze), Western poison ivy (*Toxicodendron rydbergii* (Small ex Rydb.) Greene), Atlantic poison oak (*Toxicodendron pubescens* P. Mill.), Pacific poison oak (*Toxicodendron diversilobum* (Torr. & A. Gray) Greene), and poison sumac (*Toxicodendron vernix* (L. Kuntze)) are known to produce urushiol, which can cause contact dermatitis if oxidized. (Gladman, 2006; Senchina, 2006) On the other hand, many other species are used in traditional medicine or are of technological value. For example, *Spondias*, *Rhus* and *Schinus* representatives are exploited for diverse medicinal applications reaching from wound treatment over gastrointestinal diseases to various infections. *Schinopsis* and *Rhus* species are applied in the leather and textile industries for tanning and coloring purposes. (Kubitzki et al., 2011; Schulze-Kaysers et al., 2015; Shabbir, 2012; Venter et al., 2012) Furthermore, several edible fruits, some of which are of economic importance, can be found in this plant family. The most important food crops are cashew (*Anacardium occidentale* (L.)), mango (*Mangifera indica* (L.)), pistachio (*Pistacia vera* (L.)), but also pink pepper (*Schinus terebinthifolius* (Raddi), *Schinus molle* (L.)), and recently marula (*Sclerocarya birrea* (A. Rich.)) have gained attention. Many other species such as jocote (*Spondias purpurea* (L.)), sumac (*Rhus coriaria* (L.)), cajú (*Anacardium giganteum* (W. Hancock ex Engl.)) or blood plum (*Haematostaphis barteri* (Hook. F)) are of regional importance (Kubitzki et al., 2011; Schulze-Kaysers et al., 2015; Pell, 2004).

The Anacardiaceae exhibit additional unique features, e.g., they are known to extensively accumulate polyphenols (Hegenauer, 1989). This is one of the reasons why they have been increasingly focused by science. As a consequence, there are numerous studies demonstrating that this plant family, particularly the edible Anacardiaceae, harbor a high potential for the recovery of bioactive compounds (Schulze-Kaysers et al., 2015). Referring to mango and its varieties, a multitude of studies on the phenolic profiles of the fruit have revealed a broad spectrum covering alkylresorcinols, anthocyanins, tannins, gallic acid derivatives, flavonols, phenolic acids, and xanthones (Schulze-Kaysers et al., 2015). Furthermore, techno-functional properties as well as medicinally relevant bioactivities like effects on cancer cell lines could be shown (Noratto et al., 2010; Luo et al., 2014). For example, alkylresorcinols and gallotannins from mango peel and kernels exhibited antifungal (Cojocaru et al., 1986; Droby et al., 1987) or antibacterial activity (Engels et al., 2009; Engels et al., 2010; Engels et al., 2011; Engels et al., 2012). Although they have been investigated to a different extent, results regarding the bioactivity of their phenolics could be observed for several other edible species, such as cashew (Kubo et al., 1993a; Kubo et al., 1993b), marula (Street & Prinsloo, 2013), pistachio (Lim, 2012; Bisignano et al., 2013) or sumac (Shabbir, 2012; Rayne & Mazza, 2007). For further