BIOACTIVE COMPOUNDS INTO EDIBLE SYRIAN PLANTS: POMEGRANATE AND CAPPER

Settore Scientifico Disciplinare CHIM/10

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Ai miei professori,
genitori e alla mia
famiglia, Ai miei amici.
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Chapter 1: STRUCTURE AND AIMS OF THE THESIS

1. AIMS OF THE STUDY

The research developed within this PhD thesis has been developed according to different specific objectives depending on the selected plant. Overall the choice of these two matrices, the fruit of pomegranate and the root of capper, is related to the importance of these plant in Syria as better underlined in the next paragraphs.

POMEGRANATE FRUIT.

Due to the growing awareness of the contribution of diet to the health by consumers, the potential health benefits of pomegranate have led to a substantive increase of the popularity as well as worldwide marketing of such fruit and its juice. Its biological properties have been mainly attributed to polyphenolic compounds, such as anthocyanins and ellagittannins which greatly have contributed to the development of pomegranate-derived products present on the market. The comprehensive analysis of phenolic fingerprinting of the different parts of this fruit is a crucial starting point for assessing their biological and nutritional properties and to enhance the use of the whole fruit.

The principal objective of this first part of the work was to investigate on the discharge parts, mesocarp and exocarp of pomegranate fruits from the two varieties Laffan and Wonderful. To this aim all samples derived by water extractions have been treated to precipitate the crude polysaccharide. Overall, for the extraction only water and ethanol have been selected. A quick and efficient HPLC/DAD method was then applied to quantify and compare the different extracts in terms of HTs content. The quantitative data have been targeted to evaluate the ellagittannins, particularly punicalagins and ellagic acid derivatives. The main results regarding the tannin content are summarized in the draft of the work reported in Chapter 4 of this thesis.

Furthermore, another class of metabolites, really little studied to date, is a target of this research: the polysaccharides of this fruit. The efforts were targeted to: determine the amount of crude polysaccharides (CPS) in mesocarp and exocarp of Laffan and Wonderful cvs, optimize the process in terms of shorter time and higher yield of CPS, determine the sugar composition. Finally an in vitro evaluation of the prebiotic properties of these CPS on bifidobacteria and lactobacillus genera was carried out. The principal findings on CPS of pomegranate are illustrated and discussed in the Chapter 5 of this thesis.
It can be affirmed that our results allowing to improve the knowledge on the composition of these fruit-wastes contribute to valorise these by-products. Furthermore, the ability of these CPS to positively interact with well known probiotic agents, can open new perspectives for their potential use as food components able to positively interact with the human microbiota.

CAPPARIS SPINOSA ROOT.

Caper (Capparis spinosa L.) is one of the underutilized plant species in Syria. It is a wild spiny shrub, well adapted to harsh environments, dry heat and intense sunlight. In Syria the plants are collected to be traded by Syrian rural communities, in particular can be a resource for poor nomadic families living in the desert, and contribute to generate an important additional income. Products derived by caper are sold in herbal shops in Syrian towns and their use is well known by herbalists. Thanks to its economic importance and medical value but also to low cultivation requirements and good tolerance to adverse environmental conditions, the caper can contribute to livelihood of many small farmers.

Aim of this work was to improve the knowledge on the alkaloid content of the root of a Syrian sample of C. spinosa. Decoct and hydro-alcoholic and dichloromethane extracts have been prepared and analyzed by HPLC-DAD-MS. The different extracts obtained from the whole powdered root and from root cortex and the internal part separately have been evaluated in terms of alkaloids amounts. Moreover, preliminary findings from in vivo test on rats have pointed out the capacity to reduce the osteoarticular pain in rats of different root extracts of caper partially confirming the traditional use of this plant. Overall this thesis highlights the importance to improve the knowledge of the chemical compositions of the plant to support the medical and traditional uses of Capparis spinosa L roots.
Chapter 2: PUNICA GRANATUM L.

2.1. THE VALUE OF POMEGRANATE

The value of pomegranate is its large content in polyphenol compounds, which are present in the edible part as well as in the rest of the fruit, Pomegranate has a long history of nutritional (when consumed as raw fruit or juice) value. Apart from being eaten fresh, pomegranates are used to make juice, which is consumed around the world. About 100 g arils provides 72 kcal of energy, 1.0 g protein, 16.6 g carbohydrate, 1 mg sodium, 379 mg potassium, 13 mg calcium, 12 mg magnesium, 0.7 mg iron, 0.17 mg copper, 0.3 mg niacin and 7 mg vitamin C (Grove and Grove, 2008). And reported as an interesting source of essential vitamins as folate and vitamin K (USDA, 2010). In addition to the preferred consumption of pomegranate juice (PJ), extracts are used to prepare numerous products to exploit the specific nutritional and health-related properties of pomegranate phytochemicals. Pomegranate peel extracts have been found to be suitable for applications in the food industry as they are an important source of phenolics, flavonoids and tannins occurring as natural ingredients and co-products of PJ-related preparations (Viuda-Martos et al., 2010, 2013). It is well known that pomegranate is a good source of antioxidants. However, as Salgado et al. (2012) have shown, the antioxidant content of the other juices such as tomato and orange juice with strawberries can also be also be improved by the addition of as much as 0.5% dried pomegranate peel extracts to the juice. Pomegranate juice and seeds are used to make toppings, sauces and dips for many types of food. There are about 153 phytochemicals, including their derivatives, in pomegranate. Polyphenols are the major class of phytochemicals extracted from almost all parts of pomegranate tree, but are most abundant in fruits, primarily the peels. Phyto-nutrients derived from pomegranate fruits offer the best protection against many diseases (Jyotsana and Maity, 2010). Pomegranate peel extract might be useful as multi-functional preservative in foods (Ibrahim, 2010). The seeds of pomegranate fruits from Nigeria and Saudi Arabia showed significant differences in the percentage of ash, moisture, crude lipid, crude protein, crude fibre, available carbohydrate and energy value. Elemental analyses show that magnesium is the most abundant elements in the seeds of both countries (5650 mg/100 g and 1140.1 mg/100 g in Nigeria and Saudi Arabia, respectively) (Dangoggo et al., 2012). Human consumption of anthocyanins is increasing because of the growing awareness and interest in their potential health benefits. Pomegranate is one of the major sources of anthocyanins (Fischer et al., 2011a),
Pomegranate fruit parts also find use as nutrient feeds for ruminants. Taher-Maddah et al. (2012) recently developed the use of pomegranate seed and peel, which are by-products of the PJ industry in Iran, as a possible nutritive supplement there by reducing the dependence on cereal supplements of the ruminant diet. They showed that ensiled and dried seeds are a good nutritive source. However, it is a point worth mentioning the increasing role of seed oil in establishing the pharmacological mechanisms of pomegranate.

Recent scientific findings corroborate traditional usage of the pomegranate as a medical remedy and indicate that pomegranate tissues of the fruit, flowers, bark, and leaves contain bioactive phytochemicals that are antimicrobial (Hazeleger et al 2012), reduce blood pressure, and act against serious diseases such as diabetes and cancer. These findings have led to a higher awareness of the public to the benefits of the pomegranate fruit, particularly in the western world, and consequently to a prominent increase in the consumption of its fruit and juice. The development of industrial methods to separate the arils from the fruit and improvement of growing techniques resulted in an impressive enlargement of the extent of pomegranate orchards (Holland et al., 2009).

2.2. TAXONOMY AND MORPHOLOGY

Botanical Classification
The pomegranate family has a single genus Punica with two species viz., *P. granatum* and *P. protopunica*. The latter is considered to be the ancestor of the genus Punica which might have contributed to the evolutionary process of the cultivated form of pomegranate. *P. protopunica* is endemic to Socotra Islands (Yemen) and is the only relative of the cultivated pomegranate (Zukhovskij, 1950; Moriguchi et al., 1987; Guarino et al., 1990; Levin and Sokolova, 1979; Mars 1999; Levin 2006). *P. protopunica* differs in having pink unlike the red flowers of *P. granatum* and smaller, less sweet fruit. Over 1000 cultivars of *P. granatum* exist (Levin G.M., 1994). In India, the fruits of the wild pomegranate have thicker rinds and extremely high acidity compared with cultivated types (Bist et al., 1994). They are also reported to have much smaller arils (Kher, 1999). In Central Asia, the primary difference noted is the higher acidity in wild material (Kerimov, 1934).
Table 1. Systematic of pomegranate (*Punica granatum* L.)

<table>
<thead>
<tr>
<th>Botanical Name</th>
<th>Punica granatum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Division</td>
<td>Angiospermae</td>
</tr>
<tr>
<td>Class</td>
<td>Magnoliopsida</td>
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<tr>
<td>Subclass</td>
<td>Rosidae</td>
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<tr>
<td>Order</td>
<td>Myrtales</td>
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<tr>
<td>Family</td>
<td>Punicaceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Punica</td>
</tr>
<tr>
<td>Species</td>
<td>P. granatum</td>
</tr>
</tbody>
</table>

**Vegetative Growth**

Pomegranate is a shrub that naturally tends to develop multiple trunks and has a bushy appearance. When domesticated, it is grown as a small tree that grows up to 5m. Under natural conditions, it can sometimes grow up to more than 7m; at the other extreme, in severe natural environment, one can find creeping bush varieties (Levin 2006). In addition, there are dwarf cultivars that do not exceed 1.5m (Levin 1985, 2006; Liu 2003). Most of the pomegranate varieties are deciduous trees. However, there are several evergreen pomegranates in India. Singh et al. (2006)

**The Flower**

Flowering occurs about 1 month after bud break on newly developed branches of the same year, mostly on spurs or short branches. Flowers can appear solitary, pairs, or clusters. In most cases, the solitary flowers will appear on spurs along the branches while the clusters are terminal. In the northern hemisphere, flowering occurs in April-May. However, flowering may continue until end of summer, particularly in young trees. Such flowers are fertile, but the fruit will not properly mature because the trees enter the cooler season and the dormancy period in Mediterranean climatic conditions. Flowering and the consequent fruit set last about 1 month. During this period, there are three waves of flowering (Ben-Arie et al. 1984; Shulman et al. 1984; El Sese 1988; Assaf et al. 1991b; Hussein et al. 1994; Mars 2000). In evergreen cultivars in southern India, flowering season was observed in three
periods: June, October, and March (Nalwadi et al. 1973) or throughout the year (Hayes 1957).

The Fruit
Pomegranate fruits are globose or somewhat flattened, 5–12 cm in diameter, the fruit is originated from an infer ovary and contains the arils or seeds, which are the edible part, contributing to **55-60 %** of the whole fruit, The aril juice sac is composed of many epidermal cells and the colour of the arils range from deep red to virtually colourless according to different cultivars Figure 1, whereas the enclosed seed varies in content of sclerenchyma tissue, which affects seed softness (Levin, 2006).

![Figure 1 - Arils from different pomegranate cultivars](image)

while the skin supposes the **40-45 %** (Levin, 2006). There are varieties rich in mesocarp and the other poor Figure 2.

![Figure 2. Different varieties of pomegranate](image)

The husk is comprised of two parts: the pericarp, which provides a cuticle layer and fibrous mat; and the mesocarp (known also as the albedo), which is the spongy tissue and inner fruit wall where the arils attach. The multi-ovule chambers (locules) are separated by membranous walls (septum) and fleshy mesocarp. The chambers are organized in a non-symmetrical way. Usually the lower part of the fruit contains 2 to 3 chambers while its upper
part has 6 to 9 chambers (Fahan, 1976) divided in several chambers by a horizontal diaphragm and vertical septal membranes made of papery tissue, each chamber being filled by many seeds crowded on thick, spongy placentae; arils do not attach to septal membranes (Lawrence, 1951; Purseglove, 1968; Anonymous, 1969; Dahlgren and Thorne, 1984). There is interest in identifying or developing cultivars that have more locules to fill the fruit interior, fewer septal membranes for easier eating, and a thinner mesocarp. (Levin, 2006). Skin color ranges from light yellow to deep red as well as the color of the arils, depending on the cultivar, although, in general, (Kader, 2006). There are some exceptional cultivars, such as the black pomegranate which acquires its black skin very early and remains black until ripening time (Figure. 3 ). The skin (leathery exocarp) thickness varies among pomegranate cultivars and also no correlation exists between skin and arils color. Chemical composition of pomegranate arils changes as fruit ripens on tree, so it is important to know the most appropriated harvest date to have fully ripe fruits with high quality attributes, which last from 4.5 to 6 months after full bloom, depending on cultivar and agronomic and environmental conditions (Kader, 2006; Gil et al. 1995a).

Figure.3 - black pomegranate

In addition, the genus Punica L. is characterized by several easily distinguishable morphological features, such as the fruit with leathery pericarp and the pulpy seeds with edible sarcotesta (Dahlgren and Thorne, 1984). The ovules of Punica are thick with a multilayered outer integument and unicellular archesporium (Huang and Shi, 2002). The union of the ovary with the receptacle of the thalamus that forms a peculiar type of fruit, especially termed as “balusta” is also a very distinctive character of Punica (Nath and Randhawa, 1959). The fruit has a prominent calyx, which is maintained to maturity and is a distinctive feature of the pomegranate fruit.
Levin (2006) reports that occasionally metaxenia is observed such that there are several seeds of different color within an individual pomegranate. The arils vary in size and the seeds vary in hardness among different varieties. Varieties known as seedless actually contain seeds that are soft. There is no correlation between the outer skin color of the rind and the color of the arils. These colors could be very different or similar, depending on the variety. The external outer skin color does not indicate the extent of ripening degree of the fruit or its readiness for consumption because it can attain its final color long before the arils are fully ripened. The fruit ripens 5 to 8 months after fruit set, depending on the variety. The most pronounced difference in ripening time among cultivars is not derived from the differences in flowering dates but rather from the time required to ripening from anthesis.

The pomegranate tree requires a long, hot and dry season in order to produce good yield of high-quality fruit. Pomegranates are native to central Asia, but since the pomegranate tree is highly adaptive to a wide range of climates and soil conditions, it is grown in many different geographical regions including the Mediterranean basin, Asia, and California (Holland et al., 2009).

### 2.3. HORTICULTURAL ASPECTS

**Cultivars**

Owing to its wide distribution in many different world regions, pomegranate exhibits a broad array of diverse phenotypes and genotypes. Actually, more than 3000 pomegranate cultivars have been described. There are pink, red, green, and even almost black pomegranate varieties. In addition, some cultivars are well-adapted to the exigencies of the current global market, demanding high productivity.

Interesting pomegranate cultivars were reported from several locations all over the world, including Europe (Spain, France, Italy, Greece, and Cyprus), Asia (Turkey, Turkmenistan, Kirgizstan, Azerbaijan, Iran, India, China, Russia, Israel), and North Africa (Morocco, Tunisia, Egypt). The botanical differences between wild pomegranates and cultivated pomegranates are not obvious except for *P. protopunica*. Pomegranate cultivars were spread throughout different regions and continents, and it is probable that some of the pomegranate cultivars acquired different names in different countries and are in fact the same basic genotypes. (Levin, 1996).
Due to market request, it has become increasingly important to characterize the different cultivars and genotypes to identify those of high quality product that are economical to produce (Martínez et al., 2006). Cultivars and types exist across many countries, and several cultivars are grown commercially in the world. The names of most of these cultivars reflect the color or the shape of the fruit and one cultivar might have different names in different countries. The most important cultivars are:

- 'Ahmar', 'Aswad', and 'Halwa' in Iraq;
- 'Nab el-Jamal', 'Laffan', 'Sultani', and 'Wardi' in Syria;
- 'Red Laffani', 'Malissi', and 'Ras el-Baghel' in Palestine;
- 'Mangulati' in Saudi Arabia;
- 'Wonderful' in California;
- 'Mollar' and 'Tendral' in Spain;
- 'Schahvar' and 'Robab' in Iran;
- 'Hicaz' in Turkey;
- 'Zehri' and 'Gabsi' in Tunisia;
- 'Alandi' and 'Ganesh' in India. (Tubeileh, et al., 2004).

We observe that there are a lot of pomegranate varieties. Among these varieties, in respect of fruit taste, there are sweet, sour and sour-sweet. The last kind is called Loufani in Arabic, and some of them are extremely large and weigh an English pound. (Goor, 1967). And very little information is available about these cultivars. ‘Red luofani ’ ‘Malisi ’ and ‘Rasel baghl ’ in Syria (Yilmaz, 2007. Morton, 1987). and lefan are some of the most important varieties cultivated in Turkey in Hatay with yellow skin, large arils, a sweet-sour flavor, and very hard seeds (Ozguven and Yilmaz 2000; Ozguven et al. 2006). Lefan (or Lifani) the origin of this variety is Hatay the fruit color rind is yellow-red the arlis are pink the seed hardness intermediate( Ozguven, A.I., and C. Yilmaz. 2000 ). also, the Red Loufani, is a synonym of 'Wonderful'.(Goor and Liberman 1956). The two are very like one another in appearance and fruit. Both are late ripening, with a rather sour, and of excellent quality. And the Red Loufani ‘ and ‘Ras el Baghl’ are local strain from Israel and Palestine (Morton, 1987). And of The principal varieties in the Jewish sector (Goor, 1967).

Most of the cultivars known today are selections from an unknown origin, mostly chance seedlings or mutations collected from places where there are no records documenting their origin. However, some cultivars are the result of deliberate crosses. Such cultivars were
reported particularly from India (Keskar et al. 1993; Samadia and Pareek 2006), China (Zhao et al. 2006; Yang et al. 2007).

Pomegranates in the New World probably were imported by travelers from Europe. There is a relatively limited number of pomegranate cultivars in the United States ‘Wonderful’, the most important cultivar, originated in Florida and was discovered in Porterville, California, about 1896 (LaRue, 1980). This cultivar is the most widely planted commercial pomegranate cultivar in California. The fruit is a large with red arils, sweet-sour taste, and semihard seeds, and it ships well. The external appearance of the fruit is very appealing with red glossy color. The several Israeli landraces of ‘Wonderful’ are either ‘Wonderful’ seedlings (most likely) or sports. It is unclear whether the American ‘Wonderful’ is genetically distinguishable from any of the Israeli ‘Wonderful’ landraces. The American ‘Wonderful’ fruit is much harder and less prone to mechanical aril extraction than the Israeli landraces, but these differences could reflect variations in growth conditions. ‘Wonderful’ is also grown in western Europe and Chile (Stover and Mercure 2007).

In Sicily, Italy. Six Sicilian pomegranate selections were reported by Barone et al. (2001): ‘Dente di Cavallo’, ‘Neirana’, ‘Profeta’, ‘Racalmuto’, ‘Ragana’, and ‘Selinunte’. The local accessions were considered less attractive than the Spanish cultivars. Cultivars mentioned as important in the literature, but with no descriptions, include ‘Ahmar’, ‘Aswad’, and ‘Halwa’ from Iraq; ‘Mangulati’ from Saudi Arabia; and ‘Red Loufani’ and ‘Ras el Baghl’ from Israel and Palestine (Morton, 1987).

**Origin and cultivating regions**

Culture of pomegranate began in prehistoric times. It is estimated that pomegranate domestication began somewhere in the Neolithic era (Levin 2006; Still 2006). Pomegranates are thought to have been domesticated initially in the Transcaucasian-Caspian region and northern Turkey (Zohary and Spiegel-Roy 1975; Harlan 1992). The wild or semi-wild pomegranate still exists in the north of Syria, in Gilead, “Flora of Syria, Palestine and Sinai by G.E. Post” (Goor, 1967). Evidence for using pomegranates in the Middle East is dated at over 5,000 years ago. Pomegranate artifacts and relics dating to 3000 BCE and on were found in Egypt, Israel Armenia, and Mesopotamia (Goor and Liberman 1956; Still 2006; Stepanyan 2007) as seen on the walls of Karnak in the famous plates of the ‘Syrian Garden’ of that Pharaoh, where pomegranates figure among the plants imported from Canaan. (1408-1370 BC.) also’ 4 Bells were in themselves a symbol of the unfertilized pomegranate flower (male). 4. Stand decorated
with pomegranates (found at Ras-Shamra-Ugarib Syria, late Bronze Age) Figure 4 (Goor, 1967).

Carbonized fragments of pomegranate rinds dating from early Bronze Age were found in Jericho and Arad, Israel and even mentioned in the Bible and the Koran. (Still, 2006), in Nimrod, Lebanon, in Egypt (Still, 2006), and in Armenia (Stepanyan 2007). Pomegranates were introduced throughout the Mediterranean region to the rest of Asia to North Africa and to Europe. They traveled to the Indian peninsula from Iran about the first century CE and were reported growing in Indonesia in 1416. The Greeks and the successor empires distributed the pomegranate all over Europe. Spanish sailors brought pomegranates to the New World, and Spanish Jesuit missionaries introduced pomegranates into Mexico and California in the 1700s (Goor and Liberman 1956; Morton 1987).

Pomegranate is cultivated today throughout the world in subtropical and tropical areas in many different microclimatic zones. Commercial orchards of pomegranate trees are now grown in the Mediterranean basin (North Africa, Egypt, Israel, Syria, Lebanon, Turkey, Greece, Cyprus, Italy, France, Spain, Portugal) and in Asia (Iran, Iraq, India, China, Afghanistan, Bangladesh, Myanmar, Vietnam, Thailand; and in the former Soviet republics: Kazakhstan, Turkmenistan, Tajikistan, Kirgizstan, Armenia, and Georgia). In the New World, pomegranates are grown in the United States and Chile. New orchards are now established in South Africa, Australia, Argentine, and Brazil (Holland et al., 2009).
2.4. Pomegranate production

Current global production estimates for pomegranate are unavailable due to the rapid increase in the production and expansion, although it is estimated that around 1.5 million tonnes of pomegranate fruits are produced in the world annually (Holland and Bar-Ya’akov, 2008). However, it is widely grown in many countries where it is well adapted. In India more than 100,000 ha of pomegranate are produced. It is considered one of the most important fruits of the tropical and subtropical areas because of low maintenance cost, good yields, good keeping quality, and ability to thrive with limited moisture (Indian Council of Agricultural Research, 2005). In Iran, 65,000 ha of pomegranate produces 600,000 tons of fruit annually, with about 30% of yield exported (Mehrnews, 2006). Turkish production in 1997 was 56,000 tons/year (Gozlekci and Kaynak, 2000). Spain, with ≈3000 ha, is the largest western European producer of pomegranate, and production has been increasing as a result of high market prices (Costa and Melgarejo, 2000). Syria is the second largest producing country of pomegranates after Tunis in the Arab world (Anonymous, 2000). In the United States, there are 5600 ha of commercial pomegranate, mostly in the San Joaquin Valley. The ‘Wonderful’ cultivar dominates almost completely, but there is interest in earlier and later cultivars to extend the market season (Kotkin, 2006).

2.5. BIOACTIVE COMPOUNDS IN POMEGRANATE FRUIT

While detailed knowledge of relationships of the chemical content of pomegranates and their desirable pharmacologic endpoints has yet to be obtained, significant progress has been made over the past 8 years toward a much more comprehensive understanding of some of the important pharmacologic components of pomegranate. These are summarized, with their structures, in Table 2. In addition to the more common anthocyanins shown in the table, pentose glycosides of malvidine and pentunidin have been described in the pericarp and juice (Sharma and Seshadri, 1955).
<table>
<thead>
<tr>
<th>Chemical class</th>
<th>Compound name</th>
<th>Compound structure</th>
<th>Plant part: J: juice, P:peel S:seed</th>
<th>References</th>
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<tr>
<td>Hydroxy benzoic acids</td>
<td>Gallic acid</td>
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<td>J, P</td>
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<td>P</td>
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<td>P</td>
<td>Noda et al. (2002)</td>
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<tr>
<td>Ellagitannins</td>
<td>Corilagin</td>
<td>( P, L )</td>
<td>Satomi et al. (1993), Nawwar et al. (1994)</td>
<td></td>
</tr>
<tr>
<td>Ellagitannins</td>
<td>Pedunculagin</td>
<td>( P )</td>
<td>Satomi et al. (1993)</td>
<td></td>
</tr>
<tr>
<td>Ellagitannins</td>
<td>Granatin A</td>
<td>( P )</td>
<td>Tanaka et al. (1990)</td>
<td></td>
</tr>
<tr>
<td>Ellagitannins</td>
<td>Granatin B</td>
<td>( P )</td>
<td>Tanaka et al. (1990)</td>
<td></td>
</tr>
</tbody>
</table>
Polyphenol compounds have played a role in the successful evolutionary adaptation of plants. They are essential to the plant’s physiology, being involved in diverse functions such as structure, pigmentation, pollination, allelopathy, pathogen and predator resistance, growth and development (Croteau et al., 2000; Dewick, 2002). These phenolics are primarily derived from the phenylpropanoid and phenylpropanoid-acetate pathways. Phenolics range from simple, low molecular weight, single aromatic-ringed compounds to large and complex tannins and derived polyphenols. They can be classified into two main groups: flavonoids, a structurally diverse group of C15 compounds arranged in a C6-C3-C6 configuration and non-flavonoids which include condensed and hydrolysable tannins, stilbenes, phenolic acids and hydroxycinnamates (Del Rio et al., 2012). Among different kind of (poly)phenolic compounds, pomegranate contains mainly anthocyanins, ellagic acid and ellagitannins, gallic acid and gallotannins, flavan-3-ols, and proanthocyanidins.

**Anthocyanins**

Anthocyanins are a group of water-soluble natural pigments responsible for the attractive red-blue colour of flowers and many fruits, including pomegranate. They may play a role in the defence mechanisms of plants (De Pascual-Teresa & Sanchez-Ballesta, 2008). Likewise, anthocyanins therapeutic properties are wide-ranging and have been attributed to potential human health benefits of berries (de Pascual-Teresa et al., 2010; Seeram, 2008; Tsuda, 2012).

Pomegranate presents an anthocyanin profile characterised by six anthocyanins: cyanidin 3,5-di- and 3-O-glucoside, delphinidin 3,5-di- and 3-O-glucoside, pelargonidin 3,5-di- and 3-O-glucoside (Gil et al., 1995b). Three new cyanidin derivatives have been recently described in pomegranate juice: pentoside, pentoside-hexoside, and rutinoside (Fischer et al., 2011a). It is important to note that the amount of these coloured flavonoids is largely affected by the cultivar group; for instance, “Wonderful” variety is related to cyanidin

|------------------------|--------------|---|------------------------------------------|

*Table 2 Structures of polyphenolic compounds found in pomegranate peel and other parts (Punica granatum)*
3,5-diglucoside instead of cyanidin 3-glucoside, the main anthocyanin for Spanish “Mollar de Elche” cultivars (Gil et al., 2000; Pérez-Vicente et al., 2004).

**Ellagitannins**

Hydrolysable tannins are, together with condensed tannins, the main group of plant tannins. Hydrolysable tannins are polyesters of a sugar moiety and phenolic acids. These compounds are easily hydrolysed by diluted acids, bases, hot water, and enzymatic activity and because of this fact they are termed “hydrolysable tannins” (Khanbabaee & Van Ree, 2001). They are divided into two subclasses according to their structural characteristics: gallotannins and ellagitannins. If the phenolic acid is gallic acid, the compounds are called gallotannins and ellagitannins. On the other hand, ellagitannins are characterised by the presence of, at least, one hexahydroxydiphenoyl (HHDP) group, which spontaneously rearranges into ellagic acid when hydrolysed (Bakkalbasi et al., 2009).

With more than 500 structures hitherto identified, ellagitannins form the largest group of tannins. They are typical constituents of many plant families whilst the distribution of gallotannins in nature is rather limited (Niemetz & Gross, 2005). Nonetheless, despite hydrolysable tannins are widely distributed, their occurrence in foodstuffs is limited to a few fruits and nuts including raspberries (Rubus idaeus), strawberries (Fragaria ananassa), blackberries (Rubus spp.), and persimmon (Diospyros kaki), as well as walnuts (Juglans regia), hazelnuts (Corylus avellana), and oak-aged wines as they are leached from the oak barrels during wine aging (Landete, 2011). Pomegranate has also been pointed out as a rich source of ellagitannins (Gil et al., 2000).

Ellagitannins are characterised by one or more HHDP units esterified to a sugar core, usually glucose. HHDP groups are constituted by oxidative C-C bond formation between neighbouring galloyl residues. This complex class of polyphenolics can be categorized according to their structural characteristics into four major groups: monomeric ellagitannins, C-glycosidic ellagitannins with an open-chain glycoside core, oligomers, and complex tannins with flavan-3-ols(Yoshida et al., 2010). In addition, as noted earlier, HHDP moiety spontaneously undergoes lactonization to yield ellagic acid upon hydrolysis (Figure 13). Ellagic acid can be found in its free form or as ellagic acid derivatives through methylation, methoxylation, and glycosilation (Maas et al., 1991).

A broad array of ellagitannin structures have been found in pomegranate juice, doing of these hydrolysable tannins the main class of identified (poly)phenolics in pomegranate juice
(Borges et al., 2010; Fischer et al., 2011b). Ellagitannins are extensively found in pomegranate husk, mainly punicalagins (Gil et al., 2000). Punicalagin isomers (Figure 14) are ellagitannins in which gallagic and ellagic acids are linked to a glucose molecule \([2,3\text{-}(S)\text{-hexahydroxydiphenoyl}-4,6\text{-}(S,S)\text{-gallagyl-D-glucose}]\) and are usually extracted into juice during processing. Thus, the extraction process determines the amounts achievable in juice, displaying important differences between whole-fruit juices and arils-made ones (Gil et al., 2000). These compounds, when exposed to pH variations, are hydrolysed and the HHDP spontaneously rearranges into the water-insoluble ellagic acid. Likewise, this hydrolysis also renders punicalin (Figure 5), a gallagyl residue bounded to glucose (Clifford & Scalbert, 2000). Punicalin is other bioactive compound of pomegranate that has generated interest with regard to human health (Kasimsetty et al., 2010).

![Figure 5](https://example.com/figure5.png)

**Figure 5** - Proposed pathways for the conversion of ellagitannins to ellagic acid and urolithins in anaerobic faecal suspensions (González-Barrio et al., 2011)

**Other compounds**

Other phenolic compounds distinct from anthocyanins and ellagitannins have been described in pomegranate fruit. Catequin and gallocatechin have been identified as the major flavan-3-ols (De Pascual-Teresa et al., 2000). Likewise, prodelphinidins, a kind of condensed tannins derived from the polymerisation of gallocatechin, have also been found in pomegranate peel (Plumb et al., 2002). In addition, recently 35 flavanol-anthocyanin adducts have been detected in pressure extracted pomegranate juice (Sentandreu et al., 2010). Other phytochemicals such as phenolic acids and lignans have also been described in this juice (Bonzanini et al., 2009; Fischer et al., 2011a Fischer et al., 2012). Regarding potent antioxidant quercetin, it has also been recorded along with other flavonols, although quantities were not significant (Artik, 1998).
**Integral use and pomegranate-derived products**

Pomegranate fruits can be transformed into different food products: juices and beverages, wine, jams and jellies, dried arils, and fresh arils in modified atmosphere. Therefore, depending on the target product, flow diagram could be slightly turned over. Some of these products allow to extend the self-life of pomegranate fruits. On the other hand, the use of wastes such as peels and kernels to obtain extracts rich in polyphenol compounds and seed oil, respectively, entails the integral use of pomegranate fruits Figure 5.

![Proposed flow diagram for integral use of pomegranate fruits by producing pomegranate juice and revalorising by-products](image)

**Figure 5 - Proposed flow diagram for integral use of pomegranate fruits by producing pomegranate juice and revalorising by-products**

**Other pomegranate-derived products**

*Molasses pomegranate* The pomegranate molasses is commonly used in salad and many other dishes in Turkey and Syria (Marti et al., 2001; Poyrazoğlu et al., 2002; Altan and Maskan, 2006) Traditional methods are still being used to produce pomegranate molasses. It is concentrated simply by boiling, without the addition of further sugar or other additives (Tekeli, 1965; Gökçen et al., 1982; Uylaşer, and Başoğlu. 2000), to ensure long-term
storage. and the total polyphenol contents were found to be in between 551.61 and 9695.17 mg/kg. Karaali et al. (2006) identified this value to be 3100 mg/kg in pomegranate pulp and 1860 mg/L in juice. Total phenolic content of pomegranate molasses was very high in the research of Yılmaz et al. (2007) and reported as 52.6 mg gallic acid equivalent (GAE)/g.

*Extracts pomegranate peel* Polyphenol extracts: Pomegranate peel, which is a by-product of pomegranate juice processing, is a rich source of antioxidants compared to edible arils and can be used as a nutraceutical supplement due to its elevated content in ellagic acid, ellagitannins, gallotannins, and anthocyanins (Espín et al., 2007; Madrigal-Carballo et al., 2009). There are increasing numbers of pomegranate supplements in retail markets owing to the popularity of health benefits associated with pomegranate juice (Zhang et al., 2009). Commercially available pomegranate supplements are produced as three main different types of formulations: capsules, tablets, and soft gels when used for cosmeceutical industry.

On the other hand, pomegranate peel without extraction processes can also be used as a functional ingredient for juices (Navarro et al., 2011).

### 2.6 POMEGRANATE AND HUMAN HEALTH

**Therapeutic applications of pomegranate in medicine folk**

Phytotherapy is considered as a complementary approach for preventing and treating simple disease, although well grounded in medical tradition, it often lacks proper scientific validation (Cravatto et al., 2010). and in Ayurvedic medicine the pomegranate is considered “a pharmacy unto itself,” (Naovi et al., 1991). Fruits, peels and roots of pomegranate have been commonly used in herbal remedies by local healers in many countries. Pomegranate peels have been used in traditional medicine for treating diarrhea and dysentery (Ahmad and Beg, 2001; Braga et al., 2005; Voravuthikunchai et al., 2005; Reddy et al., 2007). In Yemen and other countries of the Arabian Peninsula, dried peels have been traditionally used for treating stomachache and for healing wounds. In this regard, astringency is a known pharmacological property of tannins (Fleurentin and Pelt, 1982; Cowan, 1999). the bark and roots believed to have anthelmintic and vermifuge properties (Naovi et al., 1991), today is documented in Italy and Bulgaria (Leporatti & Ivancheva, 2003), and the rind a traditional Chinese medicine used as antibacterial, anti-inflammatory and hemostasis agent, also for the treatment metrorrhagia, metrostaxis and bellyache. is rich of phenolic compounds (Lei et al., 2003 ; Panchavarnakili et al., 2012). the peels a powerful astringent and cure for diarrhea and oral aphthae, and the juice a “refrigerant” (
Arseculeratne et al., 1985) and “blood tonic” (Lad et al., 1986). Authors from India, Tunisia and Guatemala (Nagaraju et al., 1990; Boukef et al., 1982; Caceres et al., 1987) reported that the dried pomegranate peels are decocted in water and employed both internally and externally for numerous problems demanding astringents and/or germicides, especially for aphthae, diarrhea and ulcers. Mixtures of pomegranate seed, juice and peel products paradoxically have been reported to not only prevent abortion (Ramirez et al., 1988) but also conception (Gujral et al., 1960; Zhan et al., 1995). In Unani medicine, a Middle Eastern traditional medical system that later took root in India (Izhar et al., 1989) pomegranate flowers serve as a remedy for diabetes mellitus (Saxena et al., 2004). Finally, the flowers of P. granatum are used in folk medicine for the treatment of bronchitis, diarrhea, dysentery, ulcers, hepatic damage, sore eyes, and diabetes (Kirtikar and Basu, 2000).

**Therapeutic applications of pomegranate today**

The role of pomegranate on folk medicine has been largely established and in recent years a notable increase of scientific support has occurred. Evidence suggests that phenolic phytochemicals of pomegranate fruit, mainly anthocyanins and ellagitannins, could exert multiple therapeutic properties on health management as playing an essential role in oxidative stress balance, preventing important cardiovascular diseases, and fighting as chemoprotective agent against several kinds of cancer. In addition, pomegranate antioxidant bioactives could possess a role as neuroprotectors in some neurological disorders just as broad antimicrobial activities among other beneficial implications.

**Pomegranate and oxidative stress**

Pomegranate polyphenols have been suggested like antioxidant compounds and it has been confirmed on biological system. Research in animals and humans has demonstrated how pomegranate derivatives increase plasma antioxidant capacity and decrease oxidative stress, as lipid peroxidation as formation of protein carbonyl (Chidambara Murthy et al., 2002; Faria et al., 2007; Guo et al., 2008). The protective effect of pomegranate against hepatic oxidative stress after prolonged ingestion of pomegranate derivatives has also been pointed out in two different animal models (Chidambara Murthy et al., 2002; Faria et al., 2007). A point worth mentioning is how pomegranate antioxidants really act against oxidative stress. The protective effects of pomegranate bioactive compounds on oxidative stress have
been traditionally attributed to their ability as free radicals quenchers, diminishing the levels of ROS and, hence, lipid peroxidation and protein damage (Chidambara Murthy et al., 2002). Nevertheless, pomegranate health features should not be related to an antioxidant activity of polyphenols per se, as free radical scavengers, rather than the role of polyphenol metabolites in vivo, as signalling molecules able to exert modulatory actions in cell pathways (De Nigris et al., 2007a; Guo et al., 2008; Rosenblat et al., 2010). Actually, original pomegranate phytochemicals are usually absorbed and metabolised in other bioavailable compounds that can vary their biological properties, even losing their free-radical scavenging activity, as it occurs to the main ellagitannins metabolites registered in plasma, urolithins (Cerdá et al., 2004). However, this lack of direct antioxidant activity does not prevent these metabolites from exerting health benefits. Concerning this, in vivo metabolites, and not those phenolics present originally in fruits, seem to be the real responsible compounds for the protective effects linked to pomegranate consumption, and not necessarily due to a radical scavenging ability.

**Pomegranate and cardiovascular diseases**

The effects of pomegranate-derived products on prevention and attenuation of atherosclerosis have been largely tested, showing multiple anti-atherogenic effects (Aviram & Rosenblat, 2004; Aviram et al., 2008; de Nigris et al., 2007a). A study carried out with hypercholesterolemic mice, fed with high-fat diet, also exhibited the protective effects on atherosclerosis of a prolonged (6-months) pomegranate derivatives supplementation (de Nigris et al., 2007b). On the other hand, significant reductions in atherosclerotic lesion size were noted upon consumption of pomegranate by-products for 3 months by apolipoprotein E-deficient mice (which develop atherosclerotic injury similar to that displays in humans). The cardioprotective effect of pomegranate by-products on atherosclerotic lesion was attributed to macrophage oxidative stress attenuation along with a decrease in the extent of oxidised low-density lipoprotein uptake by macrophages since their lipids were oxidised in a lesser degree (Rosenblat et al., 2006). Likewise, in another trial carried out in hypertensive patients, a decrement in systolic blood pressure was also noted together with a reduction in serum angiotensin converting enzyme activity, which indicated pomegranate juice effects on attenuating the progression of atherosclerosis (Aviram & Dornfeld, 2001).
An approach on elucidating the responsible compound(s) for this anti-atherosclerotic activity by testing different pomegranate fruit parts has been reported. Aviram et al. (2008) pointed out that pomegranate juice and pomegranate arils extract, they both rich in anthocyanins, show a major influence in the drop of serum oxidative stress in contrast to pomegranate peel extracts, rich in hydrolysable tannins, which display a higher beneficial effect on the extent of oxidised low-density lipoprotein uptake by macrophages and on their oxidative status. Overall, pomegranate juice showed better anti-atherogenic properties than pomegranate peel extracts. Likewise, pomegranate juice was more potent than pomegranate peel purified phenolics like anti-atherogenic product.

On the other hand, pomegranate phenolics do not seem to modify serum cholesterol (both LDL- and HDL-cholesterol) levels, as well as other serum biochemical parameters (glucose, triacylglycerol, sodium, and potassium), in in vivo trials (Aviram et al., 2004; Aviram et al., 2008; de Nigris et al., 2007a; ), except for studies performed either in hyperlipidemic or hypercholesterolemic patients or animals, where LDL-cholesterol values were lowered (Bagri et al., 2009 ).

**Pomegranate and cancer**

Pomegranate, due to its phytochemical composition, has demonstrated to possess potential effects on multiple cancer such as colon, prostate, and breast, using cell lines and animal models assays.

Pomegranate juice and its constituents have been broadly studied for their antiproliferative and apoptotic activities in human cell cultures of colon cancer since they could exert their chemoprotective properties on the colon epithelium through a direct contact. Ellagitannin-derived compounds (punicalagins and ellagic acid) showed antiproliferative activity against all colon tumour cell lines tested (HT-29, HCT116, SW480, SW620), but in a lesser degree than pomegranate juice, which displayed the most prominent effect (Seeram et al., 2005). The superior bioactivity of pomegranate juice compared to its purified polyphenols was attributed to synergies in the way of action of multiple compounds presented in pomegranate juice. Likewise, ellagic acid and punicalagin induced apoptosis in human colon adenocarcinoma Caco-2 cells but interestingly not in normal colon CCD-112CoN cells (Larrosa et al., 2006). Ellagitannin-Derived metabolites, urolithin-A and –B, were also studied in Caco-2 cells. Novel gene expression profiles and deregulation of cellular functions related to cell cycle and proliferation were identified, suggesting that both ellagic
acid and urolithins-A and –B may exert a modulating role in the progression of colorectal cancer (González-Sarrías et al., 2009). The occurrence of urolithins in the human prostate gland upon consumption of pomegranate juice have positioned pomegranate as an ideal chemopreventive agent against prostate carcinoma in humans (González-Sarrías et al., 2010). Antiproliferative and proapoptotic activities of pomegranate derivatives have been evaluated in vitro in various cell lines of human prostate cancer. Antiproliferative properties of pomegranate ellagitannin metabolites, ellagic acid and urolithins, on androgen-dependent (LNCaP) and –independent prostate carcinoma cell lines (LNCaP-AR, DU145, and 22Rv1) in a dose-dependent manner have been confirmed (Seeram et al., 2007). It is also a point worth mentioning the fact that a combination of both different compounds and discrete fractions of pomegranate fruit have reflected possible synergies against cell proliferation (Lansky et al., 2005). In addition, a phase II clinical trial in men with recurrent prostate cancer and rising prostate-specific antigen (PSA) levels was conducted (Pantuck et al., 2006). Patients were supplemented with 8 ounces (~240 mL) of pomegranate juice daily until disease progression and results showed a significant prolongation of PSA doubling time. Consequently, prospects regarding chemopreventive properties of pomegranate juice and its constituents on prostate adenocarcinoma were substantially raised.

**Pomegranate and neurological disorders**

Unlike many other pathologies, protective effects of pomegranate on neurological disorders have been scarcely studied. Nonetheless, a few studies have been performed on animal models suggesting the promising potential of pomegranate bioactives in the prevention and treatment of some neurological disorders. Like that, transgenic mice with an Alzheimer’s disease-like pathology were supplied with pomegranate juice during their old age delaying the onset of cognitive impairment and enhancing the learning, as well as reducing significantly the accumulation of soluble amyloid-β and amyloid deposition in the hippocampus, a process related to disease progression (Hartman et al., 2006).

**Pomegranate and antimicrobial effects**

Pomegranate has been employed in folk medicine for the treatment of various microbial infections and, in fact, the potential antimicrobial properties of pomegranate are recently
being studied with promising results. Pomegranate extracts have displayed antagonist effects against all type of microorganisms causing urinary tract infections (El-Sherbini et al., 2010; Endo et al., 2010; ). Consequently, pomegranate-derived products represent an attractive prospect for the development of new management therapies for treatment of multi-drug resistant urinary tract infections.

On the other hand, pomegranate sun-dried rind is employed in some regions of India as an anti-malarial herbal preparation. The role of tannins-rich pomegranate rind methanolic extract on the treatment of cerebral malaria, a complication of the infection by Plasmodium falciparum, has been studied. Positive results were attributed to the anti-parasitic activity and the inhibition of pro-inflammatory mechanisms involved in the onset of malaria (Dell'Agli et al., 2010).

**Pomegranate and other diseases**

The most significant or, at least, more researched pomegranate therapeutic properties have been aforementioned, nevertheless, there are some other applications which have offered satisfactory results. In this sense, consumption of pomegranate derivatives has been related to possess anti-inflammatory activity and has been tested in various animal models. Pomegranate polyphenols extract strongly delayed the initiation, reduced the morbidity, and lowered the severity of collagen-induced arthritis in mice (Shukla et al., 2008). Likewise, another ellagitannins-rich pomegranate extract has shown to decrease oxidative stress in an inflammatory bowel model of rat, although it can not avoid colonic damage instead of urolithins-A, which reduced significantly colonic lesions. These differences regarding ellagitannins and their gut microbiota metabolites actions could be due to the inability for urolithins formation of colon-damaged rats (Larrosa et al., 2010).

**2.7. TOXICOLOGY STUDIES OF POMEGRANATE**

For thousands of years, Pomegranate has been widely consumed in many different cultures, by considering this fruit is generally safe. Studies of pomegranate constituents in animals at concentrations and levels commonly used in folk and traditional medicine did not indicate any toxic effects. Toxicity of the polyphenol antioxidant punicalagin, abundant in pomegranate juice, was evaluated in rats. No toxic effects or significant differences were observed in the treatment group compared to controls, which was confirmed via histopathological analysis of rat organs (Cerda et al., 2003).
Research in 86 overweight human volunteers demonstrated the safety of a tableted PFE in amounts up to 1,420 mg/day (870 mg gallic acid equivalents) for 28 days, with no adverse events reported or adverse changes in blood or urine laboratory values observed (Heber et al., 2007). Another study in 10 patients with carotid artery stenosis demonstrated PJ consumption (121 mg/L EA equivalents) for up to three years had no toxic effect on blood chemistry analysis for kidney, liver, and heart function. (Aviram et al., 2004). It would seem that hydroalcoholic extracts of Punica granatum fruit are innocuous when directly administered via the nasal cavity. It was shown that toxic effects of Punica granatum fruit extract occurred at higher doses from those antiviral or those used in Cuban traditional medicine (Vidal et al., 2003).

The toxic activity of a Punica granatum bark extract was related to its alkaloid content according to Tripathi and Singh (2000). The presence of alkaloids (e.g., pelletierine) in the peel is equivocal, positive by Dragendorff assay, but negative by Mayer assay (Vidal et al., 2003). According to Ferrara et al. (1989) the toxic effects of some medicinal preparations of Punica granatum may be explained by its pseudo-pelletierine content. Some people are allergic to Punica granatum. Several adverse reactions to pomegranate, including severe symptoms such as anaphylactic shock or laryngeal edema, have been described in recent years (Gaig et al., 1999).

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Capparis spinosa L. (family Capparidaceae) is a plant originating from dry regions in west or central Asia and spread particularly across the Mediterranean basin. (Baytop, 1984; Çalis, et al., 1999) The geographical location of Syria has provided an ideal environment for the growth of C. spinosa, which can be found growing wild everywhere in Syria around dry and rocky areas but are particularly abundant in the harsh environments of the Euphrates valley, Aleppo province, Idlib, Salamie area of the Hama province, and in Al Jazira (northeastern Syria). The caper is adapted to dry heat and intense sunlight. Capparis spinosa L. is a valuable commodity for the resource poor nomadic communities living in the Syrian desert. While caper is widely cultivated in other Mediterranean countries, in Syria caper is a wild species and is cultivated only on an experimental level in research nurseries. Unlike in other countries, the caper buds, collected before the flowers have formed, are used in Syria as trade items. (Giuliani and Padulosi, 2005).

Figure 1. The natural distribution of caper in the Eurasia and North Africa. Based on Inocencio et al. (2006) and Lin (2003).
3.1. PLANT DESCRIPTION:

The caper Capparis spinosa L (fig. 2 ) is a perennial lives 25 years spiny bush that bears rounded fleshy leaves and big (3-3.5 cm). polymorph shrub or climber armed with divaricate light yellow thorns, occurring in dry rocky. The flowers white to pinkish-white. The bush is native to the Mediterranean region, growing wild on walls or in rocky coastal areas throughout. The plant is best known for the edible bud and fruit (caper berry) which are usually consumed pickled. Other species of Capparis are also picked along with C. spinosa for their buds or fruits (Inocencio et al., 2002; Fici, 2001; Al-Safadi et al., 2014).

<table>
<thead>
<tr>
<th>Botanical Name</th>
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</tr>
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<tbody>
<tr>
<td>Kingdom:</td>
<td>Plantae</td>
</tr>
<tr>
<td>(unranked):</td>
<td>Angiosperms</td>
</tr>
<tr>
<td>(unranked):</td>
<td>Eudicots</td>
</tr>
<tr>
<td>(unranked):</td>
<td>Rosids</td>
</tr>
<tr>
<td>Order:</td>
<td>Brassicales</td>
</tr>
<tr>
<td>Family:</td>
<td>Capparaceae</td>
</tr>
<tr>
<td>Genus:</td>
<td>Capparis</td>
</tr>
<tr>
<td>Species</td>
<td>C. spinosa</td>
</tr>
</tbody>
</table>

Vernacular names:
- English: Caper, Caperberry, Caper bush.
- Finnish: Kapris.
- French: aprier, capres, fabagelle, tapana.
- German: Kapper, Kapernsrauch.
- Italian: Cappero, capperone (fruit).
- Marathi: Kabar.
- Spanish: Alcaparra, caparra,
- Arabic: l’Kabbar, Kabbar, Àssaf, Lasaf
- Shafalah (الكبر أوصاف الشفلح)

Fig. 2. the Capparis spinosa plant

Table 1 Capparis spinosa L. plant classification, Scientific Name and Common Name
Morphological features of root C. spinosa

This shrub has a high root/shoot ratio and the presence of mycorrhizae serves to maximize the uptake of minerals in poor soils. Different nitrogen-fixing bacterial strains have been isolated from the caper bush rhizosphere playing a role in maintaining high reserves of that growth-limiting element. (Lam, Han et al. 2009). Its roots are deep, Plants have been reported with 6-10 m long roots (Reche Mármol, 1967; González Soler, 1973; Luna Lorente and Pérez Vicente, 1985; Bounous and Barone, 1989). The root system may account for 65% of the total biomass (Singh et al. 1992).

Root pieces are up to 5.5 cm in thickness; bark rough to touch, thick showing longitudinal lenticels; freshly broken surface light yellowish; wood hard and compact; remnants of robots and slender rootless present on the bark; color varies from pale yellow to reddish-brown; odor of bud C. spinosa (e-Book government of india, 2008).

A transverse section of root characterized by outermost layer of slightly submersed corky zone of several layers showing irregular and broken outline; cork cambium make of 4 or 5 layers of thin walled, small, squares cells; cortex consisting of thin walled, irregular or somewhat tangentially elongated cells; angular sclereids in groups of 2 to 3 and up to 30 μ in size scattered in cortex; phloem in the form of multiple layers of cells forming a continuous cylinder around inner vascular zone, separated from the xylem by 4 to 5 layers of vascular cambium; wedges of vascular isolated or in groups of two, distributed uniformly among xylem parenchyma, which has granular contents; medullary rays of thin walled, mostly uniseriate, rectangular cells, often having granular contents; pith absent Powder - Powder shows vessel fragments with simple pitted thickness and tracheids with tapering or blunt ends. (Anonymousn, 2000; e-Book government of india, 2008)

3.2 Phytochemicals in caper

Alkaloids
The poly amine spermidine is analogue of diamine putrescine which is produced by reactive decarboxylation of lysin in microorganisms and animals (Cordell,1981). Several alkaloids contain spermidine joined with two cinnamic acid residue in any of the several ways for example lunarine, codonocarpine and pleurostyline (Cordell,1981). Structurally most interesting spermidine alkaloids are those having a macro cyclic ring. Typically, these compounds are formed by the condensation of one nitrogen with a carboxylic acid and the nucleophilic attack
of a second nitrogen on an electrophilic center with in the carboxylic containing unit (Cordell, 1981). Although the spermidine is found in microorganisms and animals, there are some reports of its occurrence in higher plants (Smith, 1970), mostly those used as food stuff including Brassica oleracea (cabbage), Cycopersicum esulentum (tonac) (Tabor H and Tabor C.W, 1968), chorella (Kanazawa et al., 1997) aid in Petunia pollen (Linskens et al., 1968). There are some evidences that spermidine is found in Triticum sativum (wheat) and in the embroys of other cereals (Bagni et al., 1968) Spermidine along with spermine also occur (12:1 ratio) in human semen which are formed primarily in prostate glands and may be present for stabilization of DNA (Cordell, 1981). The presence of spermidine alkaloids like capparidisine, capparisine or capparisinine and other in root bark of C. aphylla (Syn. Capparis decidua Edgew) and Capparis spinosa Table 02.

Capparisin (molecular formula C26 H 31 N 3 O 5 ) has same skeletal system but one methoxy group less than capparidisine, capparisinine; it is an isomer of capparidisidine with a methoxy group at a different position. (Arif,1986). Recently, Yang et al. (2010) found that fruits of Capparis spinosa contain a significant amount of compounds with many health benefits. Three new alkaloids, (1) Capparicone A, (2)Capparicone B, (3) Capparicone C and (4) two known alkaloids, (4) 2-(5-hydroxymethyl-2-formylpyrrol-1-yl) propionic acid lactone and (5) N-(3'-maleimidy1)-5-hydroxymethyl-2-pyrrole formaldehyde were isolated from the fruits of C. spinosa.

<table>
<thead>
<tr>
<th>Compound name and molecular weight</th>
<th>Compound structure</th>
<th>Cultivar</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Spermidine alkaloid</td>
<td><img src="image1.png" alt="Spermidine Structure" /></td>
<td>root bark of Capparis spinosa</td>
<td>Fu et al. 2008</td>
</tr>
<tr>
<td>Cadabicine 26-O-b-D-glucoside hydrochloride Mw 598</td>
<td><img src="image2.png" alt="Cadabicine Structure" /></td>
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<tr>
<td>Compound</td>
<td>Mw</td>
<td>Source</td>
<td></td>
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<tr>
<td>----------------------------------</td>
<td>----------</td>
<td>-------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Capparispine 26-O-b-D-glucoside</td>
<td>598.27</td>
<td>root bark of Capparis spinosa</td>
<td></td>
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<tr>
<td>Isocodonocarpine</td>
<td>465</td>
<td>root bark of Capparis Decidua</td>
<td></td>
</tr>
<tr>
<td>Cadabicine</td>
<td>435.21</td>
<td>root bark of Capparis Decidua, root bark of Capparis spinosa</td>
<td></td>
</tr>
<tr>
<td>codonocarpine</td>
<td>465.23</td>
<td>root bark of Capparis Decidua</td>
<td></td>
</tr>
</tbody>
</table>

References:
- Fu et al. 2008
- Ahmad et al. 1985
- Rathee et al., 2010; Chahlia, 2009
- Ahmad et al. 1987
- Rathee et al., 2010; Mishra et al., 2007;
<table>
<thead>
<tr>
<th>Name</th>
<th>Molecular Weight</th>
<th>Source</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capparidisine</td>
<td>Mw 495</td>
<td>root bark of Capparis Decidua</td>
<td>Ahmad et al. 1992</td>
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<tr>
<td>14-N-acetylcodonocarpine</td>
<td>C 28 H33 N3O6 Mw 507</td>
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<td>Ahmed et al. 1987</td>
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<tr>
<td>15- N-acetyl capparisine</td>
<td>Mw 507</td>
<td>root bark of Capparis Decidua</td>
<td>Ahmed et al. 1987</td>
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<td>Capparisinine</td>
<td>Mw 495.56</td>
<td>root bark of Capparis Decidua</td>
<td>Ahmed et al. 1987</td>
</tr>
<tr>
<td>Capparisine</td>
<td>Mw 465.22</td>
<td>root bark of Capparis Decidua</td>
<td>Gaind and Juneja, 1970</td>
</tr>
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### Table 2. The presence of spermidine alkaloids and other compound in capparis .sp

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mw</th>
<th>Source of Isolation</th>
<th>Reference</th>
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<tr>
<td>Stachydrine</td>
<td>144</td>
<td>root bark of Capparis Decidua</td>
<td>Arif, 1986</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>L-Stachydrine</td>
<td>143.1836</td>
<td>Capparis zeylanica leaves</td>
<td>Pradeep Singh et al.2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>root bark of Capparis Decidua</td>
<td>Windadri , 2001</td>
</tr>
<tr>
<td>3-hydroxy stachydrine</td>
<td>159.18</td>
<td>Capparis zeylanica leaves</td>
<td>Pradeep Singh et al.2011</td>
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<td></td>
<td>root bark of Capparis Decidua</td>
<td>Windadri, 2001</td>
</tr>
<tr>
<td>β-Sitosterol</td>
<td>846</td>
<td>root bark of Capparis Decidua</td>
<td>Rathee et al., 2010</td>
</tr>
</tbody>
</table>

#### 3.3 Pharmacological studies

**Antiviral and immunomodulatory effect of a lyophilized extract of Capparis spinosa L. buds.**

Herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) are common human pathogens that in particular cases can also cause severe problems especially in immunodeficient patients. The present paper reports the antiviral and immunomodulatory properties of a methanolic extract of C. spinosa buds (CAP), rich in flavonoids, including several quercetin and kaempferol glycosides. In particular we have investigated whether the in vitro exposure of human peripheral blood mononuclear cells (PBMCs) to CAP might inhibit
the replication of HSV-2 and modulate the induction kinetics of IL-12, TNF-alpha IFN-gamma. Our findings have shown that CAP treatment interferes with HSV-2 replication in PBMCs inhibiting the extra cellular virus release up regulating their production of IL-12, IFN-gamma and TNF-alpha. One could speculate that CAP may contribute in improving immune surveillance of PBMCs toward virus infection by up-regulating expression of peculiar proinflammator cytokines; it should thus be successfully employed for treatment of HSV-2 infections in immunocompromised hosts. (Arena et al., 2008)

Effect of Capparis spinosa on fibroblast proliferation and type I collagen production in progressive systemic sclerosis.

The effects of ethanolic extract from Capparis spinosa (ECS) on the fibroblast proliferation and type I collagen production in normal and progressive systemic sclerosis (PSS). METHOD: Cellular activity was determined by the MTT method. Apoptosis was detected by flow cytometry analysis of Annexin V-Stained cells. The expression levels of type I collagen messenger RNA and protein were analyzed by proliferation of fibroblast and reduced the expression of alpha2 (I) collagen mRNA and type I collagen protein in PSS in a dose-and time-dependent manner. ECS did not affect the proliferation of fibroblast and expression of type I collaged mRNA and protein in normal human. ECS could counteract the harmful effects on fibroblast by H202. conclusion: ECS can effectively inhibit the fibroblast proliferation and type I collagen production in PSS. (Cao et al., 2008).

Protective effect of Capparis spinosa on chondrocytes.

The in vitro chondroprotective effects of the lyophilized methanolic extract from flowering buds of Capparis Spinosa L (LECS). This plant, common to the Mediterranean basin, has been used by the traditional medicine for its diuretic and antihypertensive effects and also in certain pathological conditions related to uncontrolled lipid per oxidation. The extract contains many constituents, in particular some flavonoids (kaempferol and quercetin derivatives) and hydrocinammic acids with several known biological effects such as the anti-inflammatory and the antioxidant ones. In this study, we assayed the effect of LECS on human chondrocytes cultures stimulated by proinflammatory cytokine interleukin-1beta (IL-1beta) and we determined the production of key molecules released during chronic inflammatory events (nitric oxide, glycosaminoglycans, prostaglandins and reactive oxygen species). We observed that LECS was able to counteract the harmful effects induced by IL-1beta. This protection appeared to be greater than that elicited induced by IL-1beta. This protection appeared to be
greater than that elicited by indomethacin, which is usually employed in joint diseases. Since LECS possess a chondroprotective effect, it might be used in the management of cartilage damage during the inflammatory processes.(Panico et al, 2005).

Hypolipidemic activity of aqueous of Capparis spinosa L. in normal and diabetic rats
The effect of single and repeated oral administrations of the aqueous extract of Capparis spinosa L. (CS) at a dose of 20mg/kg on lipid metabolism in normal and streptozotocin – induced diabetic on plasma triglycerides concentrations 1 week (p<0.05).We conclude that the aqueous extract of CS (20 mg/kg) exhibits a potent oral administration of CS aqueous extract. (Eddouks et al. 2005).

Antiallergic and antihistaminic effect of two extracts of Capparis spinosa L. flowering buds.
The antiallergic properties of two lyophilized extracts obtained from Capparis spinosa L. flowering buds (capers) by methanol extraction, carried out at room temperature (CAP-C) or with heating at 60 degrees C (CAP-H), were investigated. The protective effects of CAP-H and CAP-C, orally administered (14.28 mg[sol] kg), were evaluated against Oleaceae antigen challenge-induced and histamine-induced bronchospasm in anaesthetized guinea-pigs. Furthermore, the histamine skin prick test was performed on humans, applying a gel formulation containing 2% CAP-C (the only extract able to protect against histamine-induced bronchospasm) on the sin for 1 h before histamine application and monitoring the erythema by reflectance spectrophotometer. The CAP-H showed a good protective effect against the bronchospasm induced by antigen challenge in sensitized guinea-pigs; conversely, a significant decrease in the responsiveness to histamine was seen only in CAP-C pretreated animals. Finally, the CAP-C gel formulation possessed a marked inhibitory effect (46.07%) against histamine – induced skin erythema. These two caper extracts displayed marked antiallergic effectiveness; however, the protective effect of CAP-H was very likely due to an indirect mechanism (for example, inhibition of mediator release from mast cells or production of arachidonic acid metabolites); conversely, CAP-C is endowed with direct antihistaminic properties. The different mechanisms of action of CAP-H and qualitative, quantitative chemical profile (Trombetta et al. 2005).

Antihepatotoxic activity
P-Methoxy benzoic acid isolated from the methanolic soluble fraction of the aqueous extract of Capparis spinosa was found to posses’ significant antihepatotoxic activity against carbon tetrachloride – and paracetamol-induced hepatotoxicity in vivo hepatocytes, using in vitro technique. (Gadgo li and Mishra ,1999).

**Anti-inflammatory activity**

The extract of Capparis spinosa contains many constituents, in particular some flavonoids (kaempfefol and quercetin derivatives) and hydrocinammic acids with several known biological effects such as anti-inflammatory and antioxidants effects.(Lam et al. 2009) Capparis spinosa was found to posses significant anti-inflammatory activity against carrageenan-induced oedema in rats (Ageel et al. 1986)

**Anti-hypoglycaemic activity in rats**

*Capparis spinosa* also possesses anti-hyperglycemic and hypolipidemic activities. The aqueous extract of powdered fruits C.s exhibits a potent anti-hyperglycemic activity without affecting basal plasma insulin concentrations. (Eddouks et al. 2004)It also exhibits a potent lipid lowering activity in both normal and severe hyperglycemic conditions after repeated oral administration of the extract.

*Capparis spinosa* is one of the constituents of the hepatoprotective formulation “Liv.52” administered to treat preliminary cases of acute viral hepatitis and cirrhosis of liver. It has also shown encouraging results against viral infection in man. The beneficial effects of *Capparis spinosa* on liver can be attributed to its potent antioxidant, antimicrobial, immunomodulating and anti-inflammatory activities. (Eddouks et al.2005; Arena et al., 2008)

**Antimicrobial properties of C. spinosa roots**

Ethanol, methanol and ethyl acetate root extracts exhibited higher antimicrobial activity than fruit extracts. Fruit ethanolic extract had higher activity against strain of Streptococcus sp. and Gram-negative bacteria. Thus, fruits and roots of C. spinosa can be traditionally used for treatment of bacterial infection in hemorrhoids and diarrhea. Antioxidant components other than phenolics play critical role in its antimicrobial activity (Mahboubi. M and Mahboubi. A 2014). A decoction from root of *Capparis spinosa* L. has shown a good antibacterial activity inhibiting the growth of *Deinococcus radiophilus* (Boga et al, 2011). The aerial parts of *Capparis spinosa* have shown a very good antimicrobial activity against gram-positive and gram-negative bacteria, and also moderate to good antifungal activity against *C. albicans* and *A. flavus*
(Mahasneh, 2002). A decoction from root of Capparis spinosa L. has a very good antibacterial activity on the growth of *Deinococcus radiophilus* (Boga et al., 2011).

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Mesocarp and Exocarp of Pomegranate as Source of Ellagitannins: a Comparison between Laffan and Wonderful Cultivars

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Abstract

Over the past decade, pomegranate (*Punica granatum*) is entitled as a wonder fruit because of its numerous pharmacological properties and the recent global trend of increased demand for pomegranates, whether as fresh fruit or as derived products, is growing. Well known countries producers of pomegranates are Iran, Turkey, Egypt, Italy and Syria. Pomegranate (*Punica granatum* L.) has been extensively studied and the fruit is well known in folk medicine of several countries. Last decade there has been a dramatic increase of interest in pomegranate as a medicinal and nutritional product due to its newly identified potential health effects, which include treatment and prevention of cancer and cardiovascular diseases. Pomegranate exocarp and mesocarp are usually discarded as waste during the juice extraction even could be good sources of bioactive compounds. In this study two cvs, Laffan from Syria and Wonderful from Italy have been selected with the aim to investigate on their composition in terms of ellagitannin content. For the extraction only water and ethanol have been selected as solvent suitable for food uses. A quick and efficient HPLC/DAD method was then applied to quantify and compare the different extracts in terms of ellagitannins (HTs) particularly punicalagins and ellagic acid derivates.

The results elucidated the differences in terms of different content of HTs between exocarp and mesocarp, highlighting higher amount of these molecules in mesocarp. The $\alpha+\beta$ punicalagins ranged from 66-80 % of the total content, ellagic acid and its derivatives (hexoside, rhamnoside and pentoside) ranged from 4.5 % to 9.6 for mesocarp and exocarp. Overall these wastes could be a suitable source of interesting amount of ellagitannins.

**Keywords:** tannins, punicalagin, ellagic acid, decoction, bioactive compounds
Introduction

Over the past decade, pomegranate (*Punica granatum*) is entitled as a wonder fruit because of its numerous pharmacological properties and the recent global trend of increased demand for pomegranates, whether as fresh fruit or as derived products, is growing. The global production and trade of pomegranates and the fruit's derivatives need to be improved mainly due to the garden character of production in the leading producing countries, India, China and Iran (Rymon, 2011). Pomegranate is cultivated around the world in subtropical and tropical regions with different microclimate. Well known countries producers of pomegranates are Iran, Turkey, Egypt, Italy and Syria. Iran contributed to at least half of the total world production (more then 700,000 tons/years; *FAOSTAT, FAO 2010*). Trading has grown steadily in last years, according to a greater awareness of consumers about potential health benefits.

The pomegranate has been widely investigated and several recent publications confirm this fruit as a powerful source of bioactive metabolites. According to literature demonstrating the health effects of this fruit, pomegranate represents a typical nutraceutical product to be included in human diet.

Punicalagins, the main ellagittannins of pomegranate have shown to possess remarkable anti-inflammatory and anti-genotoxic properties (Chen *et al*., 2003; Kulkarni *et al*., 2007). Ellagic acid, a minor constituent of pomegranate fruit, showed also antioxidant, anticancer and anti-atherosclerotic activities (Seeram *et al*., 2004).

Significant efforts have been made to define the individual active components and the pharmacological properties of peel (pericarp or rind); since 1830 in the United States Pharmacopeia and in the Philadelphia edition the use of the rind of pomegranate was reported. This part of the fruit is often discarded as waste even if it has shown potent biological activities (Hajimahmoodi *et al*., 2008; Gözlekçi *et al*., 2011). A recent review has provided an insight on the phytochemical components that contribute to antihyperglycemic, hepatoprotective, antihyperlipidemic effects of the pomegranate peel extracts (Middha *et al*., 2013).

The tannin-rich peels as byproducts of food industry are recently used in animal feeds in many developed countries such as USA. An interesting antimicrobial activity of pomegranate peel has been demonstrated against several pathogenic bacteria (Reddy *et al*., 2007; McCarrell *et al*., 2008).

According to the botanical classification, the husk of pomegranate fruit is constituted of two main parts: the exocarp (as cuticle layer) and the mesocarp known also as albedo, the spongy tissue and wall where the arils are attached. The multi-ovule chambers (locules) are separated
by a membranous walls (septum) from the mesocarp (Fahan, 1976). Nevertheless, in literature lacking data are available on the composition of the different parts of this fruit particularly of the mesocarp, usually discharged during the juice production. To date in the published works the botanical structure of this fruit is not so clearly defined; for example the peel has several synonymous as skin, rind, husk, and hull and it is often not distinguished by the mesocarp (Seeram et al., 2005; Zhou et al., 2008; McCarrell et al., 2008; Cuccioloni et al. 2009; Çağ and Hisıl 2010; Romani et al., 2012; Lu et al., 2008; Saad et al., 2012). Few years ago some authors reported that the peel or rind constitutes about 45-50% of the total weight of the fruit (Al-Said et al., 2009; Levin, 2006). This appears as an evident mistake from the botanical point of view because presumably the authors with the term peel have taken into account also the mesocarp. To the best of our knowledge, only in a recent work focused to study the constituents of the different parts of a Peruvian pomegranate is specifically cited the composition of the mesocarp (Fischer et al., 2011).

Even if the juice composition of pomegranate has been widely investigated, no data are available on the fruit of the Laffan variety, a typical cultivar cultivated in Syria. The Laffan variety (named also Red Loufani, or Lefan) has been described as a sour-sweet flavour fruit, large size with yellow skin, large arils, a sweet-sour flavor, and very hard seeds (Ozguven and Yılmaz 2000; Ozguven et al., 2006). It is a local strain from Syria particularly of Idlib (north of Syria), Palestine and Israel (Morton, 1987) and one of the main variety cultivated in Hatay, Turkey.

The Wonderful variety grows extensively in California, and it has become worldwide popular to produce from arils the commercial juice well known for its potential health benefits (Lansky and Newman, 2007; ). Several papers report data on juice composition of this variety (Mena et al., 2012), on findings related to its pleasant sensorial properties (Vázquez-Araújo et al., 2011), on HPLC/DAD/MS analysis of juice obtained by squeezing out of the whole fruits cultivated in Spain (Sentandreu et al., 2013).

Wonderful Pomegranate is used to obtain a standardized extracts named POMx obtained by the extraction of arils and peel and recently tested to reduce the prostate oxidative stress (Freedland et al., 2013). According to some authors (Goor, 1967), these two cvs, Laffan and Wonderful, seem to have several similarities with fruits very like one another in appearance, both are late ripening, with a rather sour and excellent organoleptic qualities.

Regarding chemical composition several data are available on the pomegranate peel where tannins are the main secondary metabolites, usually divided into two major groups the Hydrolysable Tannins (HTs) and the Condensed Tannins (CTs) (Hassanpour et al., 2011a,
the HTs are mainly located in the fruit peel and mesocarp (Gil et al., 2000; Fischer et al., 2011) with α and β punicalagins as predominant HTs (Haslam, 2007; Fischer et al., 2011; Romani et al., 2012; Çam and Hışıl, 2010; Lu et al., 2008).

Ellagic acid and some ellagic acid derivatives (ellagic acid hexoside, -pentoside, etc.) are also present, although in lesser amounts (Cerda et al., 2003b; Seeram et al., 2005; Fischer et al., 2012). In addition to ETs, pomegranate peel contains cyanidin, pelargonidin, and delphinidin (Noda et al., 2002) and flavonoids as kaempferol, luteolin, and quercetin (Van Elswijk et al., 2004). The presence of alkaloids (e.g., pelletierine) in the peel is equivocal positive by Dragendorff assay, but negative by Mayer assay (Vidal et al., 2003).

The aim of the work is to investigate on the discharge parts, mesocarp and exocarp of pomegranate fruits from the two varieties Laffan and Wonderful. To this aim all samples derived by water extractions have been treated to precipitate the crude polysaccharide. Overall, for the extraction only water and ethanol have been selected. A quick and efficient HPLC/DAD method was then applied to quantify and compare the different extracts in terms of HTs content. The quantitative data have been focused to ellagitannins as main constituents, particularly punicalagins and ellagic acid derivates. The work helps to clarify the composition in terms of HTs of the Laffan cv, a low investigated cultivar, compared to Wonderful cv, a well known commercial variety. To date, only scant data are specifically available on the tannin content of mesocarp and peel of these two varieties and our results can contribute to improve the value of these by-products.

**Experimental Part**

**Plant materials**

Fresh ripe fruits (about 5 kg of Laffan and 20 Kg of Wonderful) from two pomegranate cultivars were analysed. The Laffan cv (sour-sweet) was from Rif Idlib, Syria harvested in October 2011 and identified by a member of General Commission for Scientific Agricultural Research (Syria); the Wonderful cv was purchased from Ortofrutta Grosseto (Italy) in October 2013.

**Standard and solvents**

Ellagic acid purity ≥95%, α, and β punicalagins purity ≥98% were purchased from sigma Aldrich. All solvents (HPLC grade) and formic acid (ACS reagent) were purchased from Aldrich Company Inc. (Milwaukee, Wisconsin, USA).
Sample preparations
Arils, peels, and mesocarp were manually separated from fresh pomegranates, then washed with water separately. The fresh peel and mesocarp were freeze dried after cut into small pieces. Both vegetal parts were powdered in a grinder immediately before the extraction applying the methods described below. For all the extracts the applied drug/solvent ratio was 1:40 w/v starting always from the dried powdered material.

Extraction processes
Ethanol-water extraction. A hydro alcoholic treatment was applied using ethanol 70 % (v/v) stirring at room temperature for 24h. The extracts were then filtered through Whatman filter paper to remove solid residue.
Hot water extraction. The dried powdered material was boiled in distilled water stirring for 1 h obtaining Dec1. After cooling and centrifugation at 4500 rpm for 8’ at 4 °C to collect the supernatant the same procedure was applied on the solid residue (Dec2). The supernatants were then treated with 2 volumes of ethanol and kept for 3 h at 0 °C for precipitation of crude polysaccharides. Then the solutions were centrifuged at 4500 rpm for 12 min to remove polysaccharide fraction.
Water extraction at room temperature. The dried powdered material was soaked in distilled water stirring for 24 hours. To remove the polysaccharides was applied the same procedure of the hot water extraction.

Finally, all the extracts were dried under vacuum at 30°C, then redissolved in a mixture of EtOH:H2O (7:3 v/v) and centrifuged at 14.000 rpm, for 2’, before HPLC/DAD analysis.

HPLC-DAD analyses
The analyses were carried out using a HP 1100L liquid chromatography equipped with a DAD detector (Agilent Technologies, Palo Alto, CA, USA). A 150mm×2 mm i.d., 4μm, Fusion RP 80 A, column (Phenomenex, USA) equipped with a precolumn of the same phase was used. The mobile phases were (A) 0.1% formic acid/water and (B) CH3CN. The multi-step linear solvent gradient used was: 0-4 min 5–25% B; 4–8 min, 25–25% B; 8–14 min 25–35% B; 14–16 min 35–90% B with a final plateau of 2 min at 5%B; equilibration time 10 min; flow rate 0.4 mL min⁻¹ and oven temperature 26 °C. The UV-Vis spectra were recorded in the range 200–500 nm and the chromatograms were acquired at 240 nm, 350 nm, 370 nm, 380 nm.
Quanititative evaluation of tannins

The quantitative evaluation of the main constituents was performed using two external standards: the α+β punicalagins at 380 nm and ellagic acid at 370 nm. The first compound was used at 380 nm to quantify α, β punicalagins and derivatives, while the second one at 370 nm to determine ellagic acid and derivatives.

Stock solution of α+β punicalagins, 1mg/mL, was prepared in DMSO and then diluted with H₂O before the HPLC analyses; solution of ellagic acid 0.78 mg/mL was prepared in MeOH. The calibration curve of α, β punicalagins was in a linearity range between 0.5 μg and 8 μg with a R² = 0.9982; the calibration curve of ellagic acid was in the linearity range of 0.031–1.25 μg with R² = 0.9995.

Results and Discussion

Mesocarp and exocarp composition

First aim of the work was to perform a qualitative evaluation of ellagitannins content in discard materials from pomegranate fruit. These compounds have been chosen as target molecules of this study because recognized as the main phenols of pomegranate fruit (Fischer et al., 2012; Romani et al., 2012; Çam and Hışıl, 2010; Lu et al., 2008). Mesocarp and Exocarp, as by-products from pomegranate juice production, are very cheap material but can be interesting sources of polyphenols, mainly ellagitannins. Two different cultivars (Laffan from Syria and Wonderful from Tuscany) were compared in order to investigate on the ellagitannins content.

An hydro alcoholic mixture (70:30 EtOH:H₂O v/v) was firstly selected as common procedure for extraction of polar compound from different vegetal materials and then water was chosen as cheap and green solvent for the ellagitannin recovery. The decoction of fruit discards has also reported as domestic method to prepare pomegranate extracts used in the traditional Syrian medicine (Ibn al-Baytar, 1874). According to this tradition, decoction was applied but also a water extraction at room temperature was tested for both the exocarp and the mesocarp. Some authors reported the water as not effective solvent for the extraction of tannins from pomegranate peels compared to methanol (Çam and Hışıl, 2010; Elfalleh et al., 2012). These authors did not taken into account that the dried extracts obtained from water extraction certainly include the hygroscopic polysaccharides. This induces to apply further treatment (see experimental) to precipitate and separate this fraction and to have a cleaner extract mainly constituted by polyphenols. Again, the highest total phenolic amount was observed with methanol, followed by water then by ethanol, while the lowest value was with ethyl acetate.

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(Wang et al., 2011). On the opposite, other authors report that water extracts were the most efficient to recover the polyphenols from the dried peel obtaining up to 18% on dried weight, followed by ethanol 50%( v/v) with a 16% on dried material (Wissam et al., 2012).

First, ellagitannins identification was carried out working on the hydro alcholic sample from pomegranate peel by applying an HPLC/DAD method previously described (Romani et al., 2012). Successively a shorter analytical method performed using a new generation column (RP18 Synergi Fusion) was developed to reduce time of analysis and to improve resolution.

The retention times of α-punicalagin, β-punicalagin and ellagic acid at 5,76, 6,23, and 8,49 min respectively were in agreement with the polarity sequences but significantly shorter than those reported in previous procedures (Seeram et al., 2004; Lu et al. 2008; Romani et al., 2012). Overall the optimized HPLC/DAD method proposed requires half time of the analysis in comparison with other cited methods.

The rt values, the use of specific standards (alpha+beta punicalagins, ellagic acid) and the UV-Vis spectra allowed to characterize the main components of the various extracts (Figure 1).

**Figure 1:** UV-Vis spectra of α + β punicalagins (A) and ellagic acid hexoside, ellagic acid pentoside and ellagic acid(B)

This optimised analytical procedure was applied also for the sample from mesocarp. According to the previous works, five main molecules were identified: α+β punicalagins, ellagic acid hexoside, ellagic acid pentoside and ellagic acid, while other components were recognized as minor tannins (Figures 3a and 3b).

In Figure 2 are reported the chemical structure of the main bioactive compounds present in all the analysed samples.
**Figure 2:** Chemical structure of ellagic acid (A) $\alpha+$ $\beta$ punicalagins (B)

The Figure 3a and 3b compares the chromatographic profiles at 370 nm of three extracts from mesocarp and peel of Laffan cv; the corresponding profiles of Wonderful cv are not shown because very similar.
**Figure 3:** Chromatograms at 370 nm of three different (A, B, C) extracts from mesocarp (3a) and from exocarp (3b). 1) α-punicalagin, 2) β-punicalagin, 3) ellagic acid hexoside, 4) ellagic acid pentoside, 5) ellagic acid.

A comparison among the chromatographic profiles obtained from the three procedures (Figure 3a and 3b) did not show relevant qualitative differences. Both in mesocarp and exocarp the same components have been revealed, even if with different relative amounts as discussed in the next paragraph. All these solvents were able to extract the main polyphenols from both materials and most significant remarks are related to water extraction. Our results in agreement with previous work (Romani et al., 2012), demonstrate ellagitannins of pomegranate did not degrade even after a long boiling processes. Moreover the water at room temperature is able to efficiently recover the ellagic tannins from these by-products maintaining almost the same relative abundance (see next paragraphs).

**Quantitative Results**

The ellagitannins content in terms of α+ β punicalagins as most abundant compounds, ellagic acid and its glycosides as minor components, was evaluated in all the different extracts. Comparing the data related to dried material (mg/g DM) of mesocarp for the two cvs, (Figure 4A), it has been observed a comparable amounts both in terms of total tannins (close to 120
mg/g) and of each compounds. As expected, the α+β punicalagins resulted the main compounds reaching the 66- 80 % of the total tannins in all the extracts of mesocarp.

All data reported in histograms of Figure 4 are only related to the first step of decoction (Dec1) of both mesocarp and exocarp. Indeed the quantitative evaluation performed on Dec2 have highlighted a very low recovery of ellagitannins, about one order of magnitude lower than those obtained from only the Dec1 with values for mesocarp of 10,68 ± 2,28 mg/g DM for Laffan and 13,76 ± 0,33 mg/g DM for Wonderful. The exocarp showed lower recoveries: from 8,92 ± 0,48 for Wonderful to 11,65 ± 0,83 for Laffan. According to these results it can be assessed that a single step of decoction is able to extract almost all the ellagitannins from the two by-products.

It is known that water is a efficient solvent for polysaccharides extraction from herbal matrices, especially in high temperature treatments as the decoction. Nevertheless, to date only few data can be found on the polysaccharide content of pomegranate (Zhu and Liu, 2013). According to that consideration all aqueous extracts were processed (see experimental section) to remove the polysaccharides before the HPLC/DAD analyses. A detailed study on these components (see fifth chapter of this thesis) have pointed out the crude polysaccharides recovered from mesocarp were close to 10% of the dried weight while a lower amount (5 %) was in the dried peel.

The process was not required for the hydro-alcoholic extract because the high percentage of ethanol don’t allow their solubilization in the sample. Moreover, it is a common and widely applied procedure to add ethanol in a ratio of 2:1 v/v up to 4:1 v/v to precipitate the polysaccharides from water samples (Joseph et al., 2013; Jahfar et al., 2003). Comparing the decoction and the aqueous extract at room temperature from mesocarp (Figure 4A) it can be observed that both the cvs show a very similar content of total tannins, around 100 mg/g DM. The α+ β punicalagins, as already seen for the hydro alcoholic extracts, are the main compounds, reaching 66-67% of the total ellagitannins for the decoction and 74-78% for the aqueous extract at room temperature.

Regarding ellagic acid and its derivatives content in mesocarp, obtained amounts are shown in Table 1. Their content ranged between 4,4 -7.1 % of the total ellagitannin of mesocarp; due to the less polarity of these molecules if compared with the punicalagins, for both cvs a higher extractive efficiency was observed using the hydro alcoholic mixture compared with the water samples. A pomegranate acidified methanol extract (80% v/v) from mesocarp of a Peruvian fruit (Fischer et al., 2012 ) showed an amount of 40,59 mg /g of total ellagitannins, 0,23 mg/g of ellagic acid and 0,5 mg/g of ellagic acid hexoside plus pentoside. The higher amount
obtained in our extracts can be attributable to a longer extraction time and a more exhaustive recovery with respect to the Fisher’s work in which the extraction time was only of 30 min.

### Table 1: Content of ellagic acid and its derivatives (mg/g DM) in the three different extracts obtained from mesocarp; the data are a mean of triplicate of each extraction.

<table>
<thead>
<tr>
<th>Method</th>
<th>cultivar</th>
<th>ellagic ac hexoside</th>
<th>ellagic ac pentoside</th>
<th>ellagic ac</th>
<th>ellagic ac and der</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decoction</td>
<td>Laffan</td>
<td>2.71±0.22</td>
<td>1.49±0.07</td>
<td>3.10±0.33</td>
<td>7.30±0.62</td>
</tr>
<tr>
<td></td>
<td>Wonderful</td>
<td>2.28±0.08</td>
<td>1.28±0.04</td>
<td>2.08±0.26</td>
<td>5.63±0.34</td>
</tr>
<tr>
<td>Water (room temp)</td>
<td>Laffan</td>
<td>2.17±0.026</td>
<td>0.89±0.04</td>
<td>1.70±0.7</td>
<td>4.76±0.45</td>
</tr>
<tr>
<td></td>
<td>Wonderful</td>
<td>2.37±0.21</td>
<td>0.68±0.02</td>
<td>1.55±0.21</td>
<td>4.60±0.44</td>
</tr>
<tr>
<td>Ethanol 70 % v/v</td>
<td>Laffan</td>
<td>2.76±0.16</td>
<td>1.34±0.12</td>
<td>2.10±0.22</td>
<td>6.19±0.44</td>
</tr>
<tr>
<td></td>
<td>Wonderful</td>
<td>3.44±0.07</td>
<td>1.73±0.27</td>
<td>2.05±0.02</td>
<td>7.22±0.36</td>
</tr>
</tbody>
</table>

Regarding the exocarp, again a similar trend for the two cvs, was observed, although the total tannins content with respect to the DM was lower than in mesocarp with 77.50 mg/g for Laffan and 92.24 mg/g for Wonderful. Again, in this part of the fruit, α+ β punicalagins represent the major compounds reaching 61-76% of the total tannins. As shown in Figure 4B the peel contains the same tannins detected in the mesocarp but in lower concentration.

Analyzing more than 16 peel pomegranate varieties extracted with ethanol 40 % and determined by HPLC, the amounts of total punicalagins were from 45-121 mg/g DM (Lu et al., 2008); other authors reported up to 116.6 ± 12.2 mg/g α+ β-punicalagins on DM from pomegranate peels extract obtained with pressurized water (Çam and Hisıl, 2010).

A recent study (Gözlekçi et al., 2011) has evaluated the phenolic content in different parts of the fruit from four Turkish pomegranate cultivars and among these, also the Laffan cv was evaluated. Only a value of 3547 mg/L of total phenols has been reported in this work and the data was related not to dried matrix but to a ethanol/water extract (obtained with a final concentration of 1g dried peel /50 mL solution). Nevertheless these authors used the Folin–Ciocalteu colorimetric method, therefore a comparison with our results from HPLC-DAD is not possible.

Overall, from our data, ellagic acid and its derivatives in exocarp range between 3.4-8.9 % of the total ellagitannin; for both the cvs an higher extractive efficiency was reached using the hydro alcoholic mixture compared with the use of water alone (Table 2).
Table 2: Content of ellagic acid and its derivatives (mg/g DM) in the three different extracts obtained from exocarp; the data are a mean of triplicate of each extraction.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Sample</th>
<th>ellagic acid hexoside</th>
<th>ellagic acid pentoside</th>
<th>ellagic acid</th>
<th>ellagic acid + derivatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decoction</td>
<td>Laffan</td>
<td>1.64±0.10</td>
<td>1.54±0.06</td>
<td>2.73±0.16</td>
<td>5.63±0.20</td>
</tr>
<tr>
<td></td>
<td>Wonderful</td>
<td>2.32±0.03</td>
<td>2.07±0.03</td>
<td>2.80±0.07</td>
<td>7.19±0.17</td>
</tr>
<tr>
<td>Water (room temp.)</td>
<td>Laffan</td>
<td>1.11±0.01</td>
<td>1.04±0.02</td>
<td>2.36±0.18</td>
<td>4.51±0.14</td>
</tr>
<tr>
<td></td>
<td>Wonderful</td>
<td>1.10±0.0</td>
<td>0.72±0.04</td>
<td>1.61±0.03</td>
<td>3.43±0.02</td>
</tr>
<tr>
<td>Ethanol 70 % v/v</td>
<td>Laffan</td>
<td>2.03±0.09</td>
<td>1.78±0.12</td>
<td>1.92±0.14</td>
<td>5.72±0.29</td>
</tr>
<tr>
<td></td>
<td>Wonderful</td>
<td>2.86±0.23</td>
<td>2.89±0.16</td>
<td>3.14±0.29</td>
<td>8.89±0.67</td>
</tr>
</tbody>
</table>

A study carried out using water as solvent at elevated pressures (Çam and Hışıl, 2010) showed the ellagic acid content only of 1,25 ± 0.2 mg/g DM, and similar values for its derivatives (0.83-1,21 mg/g DM). These lower values were attributable to the low solubility of ellagic acid in this media compared to methanol which is used as extraction solvent in other studies. The maximum concentration of ellagic acid (10,8 mg/g DM) was for rind of Punica granatum Gansu Province (China) refluxed by methanol 50 % (Zhou et al., 2008). Recently, Fisher (2012) found similar content in peel of a Peruvian fruit with 0,64 mg/g ellagic acid and up to 0,97 mg/g or ellagic acid-hexoside.
Figure 4: Comparison among total ellagitannins, α+β punicalagines, ellagic acid and its derivatives (mg/g of DM) for different extracts of mesocarp (A) and exocarp (B) from Laffan and Wonderful pomegranate cvs. *Mean data carried out from extraction in triplicate.*

It has been reported that α-punicalagin and β-punicalagin interconvert each-other with an equilibrium constant ($K = [\beta]/[\alpha]$) of 1 in methanol and 4 in water (Doig et al., 1990). Nevertheless other authors reported a ratio of 1,60 in 40% of ethanol (Lu et al., 2008) and underlined this ratio will not change with changing of solvent. Our results summarized in Table 3, pointed out that this ratio is very similar for peel and mesocarp of these cvs, the values ranged between 1,21 to 1,40 and it is not influenced by the use of water or ethanol 70%.
<table>
<thead>
<tr>
<th>Extraction procedures</th>
<th>Part of the fruit</th>
<th>α-punical</th>
<th>β-punical</th>
<th>Ratio [β]/[α]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LM</td>
<td>28,50</td>
<td>36,13</td>
<td>1,27</td>
</tr>
<tr>
<td></td>
<td>WM</td>
<td>28,11</td>
<td>39,25</td>
<td>1,40</td>
</tr>
<tr>
<td></td>
<td>LE</td>
<td>19,73</td>
<td>27,36</td>
<td>1,39</td>
</tr>
<tr>
<td></td>
<td>LE</td>
<td>21,51</td>
<td>27,98</td>
<td>1,30</td>
</tr>
<tr>
<td>Decoction</td>
<td>LM</td>
<td>36,09</td>
<td>47,83</td>
<td>1,33</td>
</tr>
<tr>
<td></td>
<td>WM</td>
<td>27,94</td>
<td>39,55</td>
<td>1,42</td>
</tr>
<tr>
<td></td>
<td>LE</td>
<td>15,02</td>
<td>18,67</td>
<td>1,24</td>
</tr>
<tr>
<td></td>
<td>WE</td>
<td>13,59</td>
<td>16,46</td>
<td>1,21</td>
</tr>
<tr>
<td>Water (room temp)</td>
<td>LM</td>
<td>40,19</td>
<td>53,17</td>
<td>1,32</td>
</tr>
<tr>
<td></td>
<td>WM</td>
<td>37,89</td>
<td>51,12</td>
<td>1,35</td>
</tr>
<tr>
<td></td>
<td>LE</td>
<td>23,60</td>
<td>31,51</td>
<td>1,34</td>
</tr>
<tr>
<td></td>
<td>WE</td>
<td>26,27</td>
<td>35,41</td>
<td>1,35</td>
</tr>
</tbody>
</table>

**Table 3.** Ratios [β]/[α] punicalagins obtained by the yields values (mg/g of dried matter) for exocarp and mesocarp of the two cvs in water and ethanol 70% v/v. **LM: Laffan Mesocarp; WM: Wonderful Mesocarp; LE: Laffan Exocarp; WE: Wonderful Exocarp.**

As it can be easily observed by Figure 5 for the mesocarp, the different extracts for the two considered cvs, showed a very similar content of total tannins, around 140 mg/g DE. This data confirm that the use of hydro alcoholic mixture is not able to extract the polysaccharide fraction. Regarding the exocarp an higher variability of the quantitative data was evidenced, probably due to the non homogeneous lyophilized material Figure.5B
Figure 5: Quality of the extracts for different extracts of mesocarp and exocarp from Laffan and Wonderful pomegranate cvs. Mean data from extraction carried out in triplicate.
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Zhu, Caiping, and Xiaolin Liu.(2013)."Optimization of extraction process of crude polysaccharides from Pomegranate peel by response surface methodology." *Carbohydrate polymers* 92,(2) 1197-1202.
Recovery of polysaccharides from exocarp and mesocarp of pomegranate: the case of Wonderful and Laffan cultivars

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**Abstract**

Pomegranate (*Punica granatum* L.) has been used extensively in the folk medicine of many cultures. This fruit is well known for its ellagitannin content but it contain also polysaccharides that are not in deep investigated to date. It has been reported antioxidant, and antiglycation activities for a polysaccharide of pomegranate peel and anticancer activity in mice for a galactomannan recovered from the fruit rind. To date scant data have been published on the optimization of the extracting parameters of polysaccharides from mesocarp and exocarp, the main by-products of the juice production. Furthermore, no data are available on the prebiotic properties of these polymers recovered from pomegranate. Objectives of this study were to determine the amount of crude polysaccharides (CPS) in mesocarp and exocarp of pomegranate of Laffan and Wonderful cv, to optimize the process in terms of shorter time and higher yield of CPS, to determine the sugar composition and to evaluate the prebiotic properties of these CPS by *in vitro* test on bifidobacteria and lactobacillus genera.

The maximum extractive yields in terms of crude polysaccharides (10% w/w DM) have been achieved with a drug/solvent ratio of 1:40 (w/v) and applying a decoction for 60 min. The two varieties have been shown similar amounts, with the highest concentration in the mesocarp. Moreover these CPS were able to absorb up to 99.1% of water confirming a potential use as gelling agents in food chemistry.

Finally and to the best of author’s knowledge, this is the first report that allows to demonstrate that CPS from pomegranate mesocarp of Laffan and Wonderful can sustain growth of beneficial and potentially probiotic bacteria such as those used in this study and, therefore, possess prebiotic properties.

**Keywords:** Extraction; Sugar Composition; Gelling Capacity; Prebiotic
Introduction

Natural polysaccharides from plants are biocompatible and water soluble compounds with antioxidant, anti-bacterial and anti-virus activities making them suitable for pharmaceutical and biomedical uses. Even if the ellagitannins of pomegranate (*Punica granatum* L.) have been extensively studied and the fruit is well known in folk medicine of several countries, to date only few reports are available on the polysaccharides of this fruit.

Polysaccharides are a class of biological macromolecules that are relatively common in nature with tremendous structural diversity, and thus their biological properties have attracted substantial attention in medicine (Ooi & Liu, 2000; Sinha and Kumaria, 2001). Natural polysaccharides have been used extensively in the design of drug delivery systems due to their excellent biocompatibility, aqueous solubility, and stability (Bhardwaj et al., 2000; Liu et al., 2008). Some botanical polysaccharides have been commercially developed into important components of therapeutic drugs and skin care products (Deters et al., 2001; Wang and Fang, 2002) and water soluble and biodegradable polysaccharides have been extensively used as thickeners, gelling agents, carriers of hydrophobic drugs and base product to prepare nanoparticle (Marathe et al., 2002; Rubinstein, 2000). Polysaccharides have proven to be useful candidates in the search for effective, non-toxic substances with pharmacological effects and represent a relatively untapped source of new drugs, which may provide exciting new therapeutic opportunities (Beat and Magnani, 2009). Antioxidant, antitumor, immunomodulatory, antimicrobial, anti ulcer, hypoglycemic activity, and several other pharmacological activities from have been attributed to various natural polysaccharides (Franz, 1989; Liu, et al., 1997; Kardošová and Machová, 2006; Schepetkin and Quinn, 2006). Among the pharmacological properties of polysaccharides, have been cited the immunomodulatory and antitumor effects (Ooi and Liu, 2000). Numerous polysaccharides with in vitro antitumor effects have been isolated from plants, mushrooms, yeasts, algae, and lichens; of which the majority have been found to be non-toxic to normal cells and able to enhance the immune system of the host (Mahady, 2001, Leung, et al. 2006) or to act as biological response modifiers.

Pomegranate (*Punica granatum* L., family of Punicaceae) has been used extensively in the folk medicine of many cultures (Li et al., 2006) phytochemistry and pharmacological actions suggest a wide range of potential clinical applications as, antitumor, antibacterial, antidiarrhoeal, antifungal, antiulcer, and antioxidant properties (Valadares et al., 2010). Most of these authors have been pointed out the main role played by the ellagitannin as principal bioactive constituents of the different extracts obtained from the fruit of pomegranate.

Even if many reports have been focused on the extraction, chemical structure and biological activities of the antioxidants extracted from the pomegranate peels (often with this term the authors...
mean the sum of exocarp and mesocarp), which is reported to constitute about 45-50% of total weight of the fruit (Negi et al., 2003; Pan, et al., 2012; Saad et al., 2012; Al-Said et al., 2009; Levin, 2006), little attention has been devoted toward the extraction of the polysaccharides from this part of the fruit.

A polysaccharide from pomegranate peel have been emphasized marked antioxidant, antiglycation and tyrosinase inhibition properties (Rout and Banerjee 2007). A more recent study on the galactomannan, a polysaccharide recovered from the fruit rind of P. granatum has shown that this molecules exerts in vitro cytotoxicity, immunomodulatory, and free radical scavenging (Joseph et al., 2012). It shown, in vivo, anticancer activity in mice (Sreelekha et al., 2008) and also enhanced the survival and tumor reduction in mice bearing transplantable a scites tumors either alone and in combination with doxorubicin. Thus, this study provides evidence of the non-toxic nature of this plant-derived compounds, which could be used as an adjuvant or as single agent for the treatment of cancer (Joseph et al., 2013). Other authors (Li et al., 2014) reported that polysaccharides from pomegranate peels inhibits the proliferation of U-2 human osteosarcoma cancer cells by inducing apoptosis mainly through the mitochondrial signaling pathway (113).

Recent studies have suggested that polysaccharides introduced via the diet can have prebiotic activity, i.e. they can stimulate the growth of beneficial bacteria in the colon thereby contributing to the health status of the (Marotti et al. 2011; Di Gioia et al. 2014b). The intestinal tract harbors a complex bacterial community, referred to as gut microbiota, which has a great impact on the nutritional and health status of the host (Laparra and Sanz, 2010). A balanced gut microbial composition confers benefits to the host, due the modulation of host metabolic and immune functions, while microbial imbalances are associated with metabolic disorders and/or disease status (Tremaroli and Backhed, 2012; Di Gioia et al. 2014a). Therefore, the maintenance of a correct equilibrium between beneficial microorganisms, mainly belonging to the Bifidobacterium and Lactobacillus genera, and potentially pathogenic strains, is crucial for host health. The presence of abundant bifidobacteria and lactobacilli may provide some protection against incoming of enteric pathogens (Jankowska et al. 2008, Symonds et al. 2012). They are able to compete for nutrient with enteric pathogens, to strongly adhere to the intestinal mucosa thus preventing pathogen adhesion and to stimulate the development of the mucosal immune system.

To date scant data has been published on the optimization of the extracting parameters of polysaccharides from mesocarp and exocarp, the main by-products of the juice production. Furthermore, no data are available on the prebiotic properties of these polymers recovered from pomegranate. Objectives of this study were to determine the amount of crude polysaccharides (CPS) in mesocarp and exocarp of pomegranate of Laffan and Wonderful cv., to optimize the
process in terms of shorter time and higher yield of CPS, to determine the sugar composition and to evaluate the prebiotic properties of these CPS by *in vitro test* on bifidobacteria and lactobacillus genera.

**Experimental Part**

*Plant materials*

Fresh ripe fruits (about 5 kg of Laffan and 20 Kg of Wonderful) from two pomegranate cultivars were analysed. The Laffan cv (sour-sweet) was harvested from Rif Idlib-Syria in October 2011; the Wonderful cv was purchased from Ortofrutta Grosseto (Italy) in October 2013. The Syrian fruits were identified by General Commission for Scientific Agricultural Research, Damascus, Syria.

*Sample preparations and extraction processes*

The arils, the exocarp and the mesocarp were manually separated from fresh pomegranates, then washed with water separately. The fresh exocarp and mesocarp were freeze dried after cut into small. Both parts were powdered in a grinder immediately before the extraction applying the methods described below.

*Hot water extraction.* The dried powdered material was boiled in distilled water under stirring for 30, 60 or 120 min as reported in Table 1 (methods 1, 2) applying one or two successive extractive steps. After each boiling step, the sample was cooled and centrifuged at 4500 rpm for 8 min at 4 °C, to collect the supernatant containing the CPS. The Method 3 differs from Method 2 only for a pre-treatment of the sample: a stirring at 25 °C for 12 hrs. All the supernatants obtained applying the methods summarized in Table 1 were added with 2 volumes of ethanol and kept 3 h at 0 °C, to induce the precipitation of the CPS. Then the solutions were centrifuged at 4500 rpm for 12 min to recover the CPS.

*Water extraction at room temperature.* The dried powdered material was soaked in distilled water 1g DM/40 mL, under stirring from 8 to 24 hours, at room temperature, then to recover the CPS was applied the same procedure as described for methods 1-3 (Method 4). The solid residue was added water (ratio 1g DM/25 mL) and a single decoction of 60 min was applied. The recovery of CPS was done as previously described for the other methods. This procedure is named method 4 modified in Table 2.

*Ethanol-pretreatment and decoction.* The Method 5 consists of the addition of ethanol 70 % v/v, with a ratio of 1g DM/40 mL, then stirring at room temperature for 24h and a filtration to remove
the ethanol solution through Whatman filter paper. To the solid residue was added water (ratio 1g DM/25 mL) and a single decoction of 60 min was applied. The recovery of CPS was done as previously described for the other methods.

*Cleaning procedure of CPS.*

*Tannin removal.* All the crude polysaccharides CPS obtained applying the different methods were then washed with distilled water, and re-precipitated with 2 volumes of ethanol, then kept 3 h at 0 °C. The solutions were centrifuged at 4500 rpm for 12 min, the CPS were recovered as precipitate, then frozen and freeze-dried. The obtained recovery has been expressed as CPS yield (%) = (PS/DM) × 100; PS is the dried weight of polysaccharide in g and DM is the dried material weight (peel or mesocarp) in g. *Protein removal.* The proteins were removed with the Sevag method (Staub, 1965) with slight modification: the CPS were dissolved in water then added with chloroform and a liquid/liquid extraction by funnel was done to recover the aqueous phase containing the purified polysaccharide (PPS). The extractive steps were repeated several times until the water chloroform inter-phase became clear (Joseph et al., 2012).

*Filtration of PSP solution by cut-off filter.*

Some freeze dried PPS samples were redissolved in water then 500 µl of this solution was applied in the Amicon ultra filter device (cut off 10,000 Dalton) centrifuged at 14,000 x g for about 15 min; the precipitate was reconstituted with the original volume by adding distilled water and repeated the process up to 7 times to remove the 99.2% of fouling (low molecular weight materials and salts) from the sample as indicated by the Amicon supplier. After these cleaning steps the Amicon Ultra filter device with the unfiltered sample (PPS), was placed upside down in a clean micro centrifuge tube, for 2 minutes at 1,000 x g; then 500 uL of distilled water were added to redissolve the purified polysaccharide (PPS).

*Determination of the monosaccharide composition of PPS*

The PPS samples derived by purification steps of Laffan and Wonderful mesocarp and exocarp were hydrolyzed with a 2 M solution of trifluoroacetic acid at 120°C for 120 min, cooled on ice, then ultra filtered at 3500 x g for 20’ using centrifuge filter devices (Amicon ultra) having a cut off 3,000 Dalton and the supernatant was dried in a rotary evaporator. The samples were washed twice with MilliQ-grade water, re-dissolved in 1 ml deionized water and then analyzed by ion exchange chromatography using a Dionex ICS-2500 ion chromatograph (Sunnyvale, CA) with an ED50 pulsed amperometric detector using a gold working electrode (Dionex,Sunnyvale, CA). A Carbopac
PA1 4 mm by 250 mm column (Dionex, Sunnyvale, CA) was used. The eluents used were MilliQ-grade water (solution A), 0.185 M sodium hydroxide solution (solution B), and 0.488 M sodium acetate solution (solution C). A gradient elution was used consisting of a first stage (injection time to the 7th min) with an eluant constituted by 84% solution A, 15% solution B, and 1% solution C; a second stage (injection time from the 7th to 13th min) with 50% solution B and 50% solution C; and a final stage (injection time from the 13th to the 30th min) with 84% solution A, 15% solution B, and 1% solution C. The flow rate was 1 ml min\(^{-1}\). Unknown monosaccharides were detected according to retention time of standards purchased from Sigma.

**In vitro evaluation of the prebiotic properties**

The capability of CPS of stimulating the growth of beneficial bacteria (prebiotic activity) was assayed using as target strains *Bifidobacterium breve* B632, previously isolated from infant feces (Aloisio et al. 2012) and *L. plantarum* L12, previously isolated from adult feces (unpublished results) and available at the Bologna University Scardovi Collection of Bifidobacteria. The strains were stored as lyophilized strains. When necessary, they were re-vitalized in de Man Rogosa Sharpe (MRS) medium (Oxoid, Basingstone, UK) additioned with 0.05% cysteine and grown in anaerobic conditions at 37 °C for 24 h. Anaerobic conditions were created in a capped jar using an anaerobic atmosphere generation system (Anaerocult A, Merck, Darmstadt, Germany).

The MRS medium composition was modified to perform the growth experiment with the pomegranate polysaccharides. The modified medium (m-MRS) did not contain the carbon source (glucose), which was provided by the pomegranate polysaccharide, and had a halved amount of potential growth substrate, such as peptone, yeast extract and meat extract with respect to those present in the original medium (peptone, 5 g/L; yeast extract, 2 g/L, meat extract 5 g/L were the amount in m-MRS).

The prebiotic activity was evaluated using CPS at 0.5 % (w/v) in m-MRS. A positive growth control was performed using m-MRS with 0.05 % glucose and a negative control in m-MRS with no added carbon source. The medium containing CPS as the carbon source was prepared as follows: the m-MRS ingredients were weighted in a flask, dissolved in water and the medium was autoclaved at 120 °C for 15 min. 0.5% (w/v) fiber or glucose at the same concentration were added to the hot medium, stirred, and sterilized again at 102 °C for 10 min. This procedure allowed the fiber to dissolve in the medium and prevented risk of growth of undesired microorganisms. The *B. breve* B632 and *L. plantarum* M12 strains were grown overnight in the respective media, centrifuged, washed in phosphate buffered saline (PBS) and re-suspended PBS to obtain a solution having an Absorbance at 600 nm of 0.7. This suspension was used to inoculate at 2% (v/v) the flaks
containing the m-MRS medium plus the fiber, the m-MRS medium plus glucose (positive control) and the m-MRS medium with no additional carbon source (negative control). The flasks were incubated at 37 °C in anaerobic conditions for 48 hours and 1 mL culture was sampled from each flask for viable bacterial counts at pre-established times (0, 6, 24, 30 and 48 hours of incubation). The sampled amount was mixed with 9 mL of PBS, serially diluted in the same solution and plated on agarized MRS additioned with cysteine. Following incubation of the plates at 37 °C in anaerobic conditions for 24 h, the number of colonies, corresponding to the number of viable cells, was counted. The number of cells expressed as CFU/mL were transformed to Log<sub>10</sub> value (Log CFU/mL).

Results and Discussion

In recent decades, plant polysaccharides have attracted a great deal of attention in the biomedical field due to their broad spectra of therapeutic properties and relatively low toxicity. Nevertheless little attention has been devoted to the polysaccharides of pomegranate fruit. The most commonly method applied to recover these polymers is the use of hot water, followed by precipitation of interfering substances such as proteins, then collection of supernatant after centrifugation and a precipitation of polysaccharides by adding of ethanol (Huie and Di, 2004). In this study a similar procedure was applied to mesocarp and exocarp separately to evaluate their polysaccharide content.

CPS from Mesocarp

Three independent major variables, including extraction time (h), extraction temperature (°C) and ratio of raw material/ water (g/ml), were considered of significance for the extractive yields and were modified during this study. The first step was to test different methods working on mesocarp of Laffan pomegranate; in Table 1 were summarized the applied procedures.

The extraction time was firstly set at 30’ and then at 60’, in a single or two successive steps; the elective solvent was water for Methods 1-4, while also an hydro alcoholic solution was evaluated with Method 5, followed by a successive decoction. The extraction temperature was 100 °C for the hot treatments with water and room temperature only for Method 4. Finally the ratio raw material/extractive solvent was firstly 1:15 (Method 1) and then 1:40 for all the other methods. The choice of the best experimental parameters was done on the base of the percentage yields of the weight of crude polysaccharides (CPS) with respect to the weight of dried mesocarp (Table 1).

Applying a drug/solvent ratio of 1:15 (w/v), the yield of CPS increased from 5% to 8% when the extraction time was prolonged from 30’ to 60’, in two successive steps. This results are in agreement with previous data that pointed out the relevance of the extraction time to recover these
polymers from pomegranate (Zhongdong et al., 2006). Other authors underlined as the exposure of CPS to the extractive medium is a relevant aspect to favor the penetration of the liquid into the dried powdered material, dissolve the polysaccharide and allow the successive diffusion out of the matrix into the extraction solvent (Ye & Jiang, 2011).

Table 1: Different extractive methods applied to recover the crude polysaccharides from the Laffan mesocarp and corresponding extractive yields on DM. §The tests were carried out in triplicate from dried mesocarp; *sample under stirring.

<table>
<thead>
<tr>
<th>Method</th>
<th>Dried weight (g)/ solvent (mL)</th>
<th>Solvent</th>
<th>T (°C)</th>
<th>Time (min)</th>
<th>Steps</th>
<th>Yield (%) CPS/DW mesocarp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method 1</td>
<td>1/15</td>
<td>H₂O</td>
<td>100</td>
<td>30</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H₂O</td>
<td>100</td>
<td>30+30</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Method 2</td>
<td>1/40</td>
<td>H₂O</td>
<td>100</td>
<td>60</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>§Method 2</td>
<td>1/40</td>
<td>H₂O</td>
<td>100</td>
<td>60+60</td>
<td>2</td>
<td>9.8</td>
</tr>
<tr>
<td>§Method 3</td>
<td>1/40</td>
<td>H₂O</td>
<td>~25</td>
<td>60+60</td>
<td>2</td>
<td>9.1</td>
</tr>
<tr>
<td>§*Method 4</td>
<td>1/40</td>
<td>H₂O</td>
<td>~25</td>
<td>8-24 h</td>
<td>1</td>
<td>2-3.33</td>
</tr>
<tr>
<td>§*Method 4 modified</td>
<td>1/25</td>
<td>H₂O</td>
<td>100</td>
<td>60</td>
<td>1</td>
<td>7.8</td>
</tr>
<tr>
<td>§*Method 5</td>
<td>1/25</td>
<td>H₂O</td>
<td>100</td>
<td>60</td>
<td>1</td>
<td>7.15</td>
</tr>
</tbody>
</table>
According to Table 1 the CPS yields achieved a maximum value, close to 10% with a drug/solvent ratio of 1:40 (w/v) and applying an extraction time of 60’ (Method 2) in a single step. Applying a second step of 60’ and maintaining the same drug/solvent ratio, it was not observed a consistent increase of the yields, with only a recovered of very small amounts (0,36 – 0,5 %). In light on these results it was chosen one hour as extraction time because considered an enough time for recovering most of the CPS from mesocarp. Furthermore, it has been also observed that the stirring of the sample before the boiling process does not lead to an increase of the yields extractive.

After this pre-screening working only on the Laffan mesocarp some of these procedures have been also applied to the other matrices and in Table 2 is shown a comparison in terms of % extractive yields of CPS from mesocarp of Laffan and Wonderful. Again the recovery obtained working at room temperature (Method 4) was considerably lower and close to 2% DM after 8 hours of stirring, and up to 3,33 % after 24 hrs of stirring (Table 2). A successive boiling of the solid residue (Method 4 modified), allowed to increase the yield up to 7,80%±0,28 for Laffan cv and 5,67 % ±0,58 for Wonderful cv. These results support the use of an hot treatment for recovering CPS, due to the higher solubility of these polymers in hot water and agree with previous reported findings (Sun Li, et al., 2010; Sun Liu, et al., 2010; Yin and Dang, 2008).

Overall, for both the exocarp and mesocarp of Laffan and Wonderful the best procedure in terms of % CPS/DM was the method 2 with the following experimental conditions: dried material/water ratio of 1g /40 mL, extraction temperature 100 °C and 60 min in a single step as extraction time. Under these conditions the maximum yields were for mesocarp with values ranging from 8 ±0,01 % for Wonderful and 9,80±0,28 for Laffan (Table 2). The obtained yields are in agreement with previous data from peel of pomegranate (Zhu and Liu ,2013).

Finally, with the objective to avoid a co-precipitation of the ellagitannins together with the CPS, a different procedure (Method 5) was applied. This procedure was characterized by a first extraction of the ellagitannins from the mesocarp by an hydroalcoholic mixture at room temperature (see chapter 4 of this Thesis), then the solid residue was treated with hot water (decoction) to recover the CPS. It was observed that the boiling of the dried mesocarp without a pretreatment, involved a partially co-precipitation of ellagitannins, thus making necessary to wash the CPS to remove this fraction (Figure 2). Nevertheless with method 5 the yields of CPS were below (6,70±0,66 % for Wonderful and 7,15±0,21 for Laffan) if compared with those derived by method 2 (Table 2). Anyway this approach can be useful if the objective is to obtain a polysaccharide fraction free of tannins.
CPS from exocarp

Regarding the exocarp, a similar trend for the two cvs, was observed, although the polysaccharide content is lower than that of mesocarp; the maximum values are between 4,47±0,50 % for Laffan cv 4,7±1,15 % for Wonderful obtained again with method 2 (Table 2). As expected, the percentages of CPS from the exocarp show a higher variability (RSD from 11% to 25 %) mainly related to the non homogeneous thickness of the raw material that contain a variable residual part of mesocarp difficult to be removed. On the opposite, a very good reproducibility of the extractive method (RSD below 3%) was obtained for the mesocarp.

The decoction after extraction with EtOH: H₂O⁺ (7:3 v/v) allowed to obtain CPS amounts near 4 % for the two cvs and similar to those derived by method 2. On the opposite, for both the cvs a lower recovery (close to 1,93 % CPS) is derived by the extraction at room temperature; this latter result is also in agreement with literature data (Rout and Banerjee, 2007).

Table 2: Polysaccharides content (CPS) in mesocarp and exocarp of Wonderful and Laffan. The values are a mean of triplicates.

<table>
<thead>
<tr>
<th>Procedures</th>
<th>Samples</th>
<th>Yield (%) CPS/DW mesocarp</th>
<th>Yield (%) CPS/DW exocarp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method 2</td>
<td>Laffan</td>
<td>9,80±0,28</td>
<td>4,47±0,50</td>
</tr>
<tr>
<td></td>
<td>Wonderful</td>
<td>8±0,00</td>
<td>4,7±1,15</td>
</tr>
<tr>
<td>Method 4</td>
<td>Laffan</td>
<td>3,7±0,42</td>
<td>1,93±0,23</td>
</tr>
<tr>
<td></td>
<td>Wonderful</td>
<td>3,33±1,15</td>
<td>1,99±0,02</td>
</tr>
<tr>
<td>Method 4 modified</td>
<td>Laffan</td>
<td>7,80±0,28</td>
<td>4,20±0,20</td>
</tr>
<tr>
<td></td>
<td>Wonderful</td>
<td>5,67±0,58</td>
<td>4,13±0,31</td>
</tr>
<tr>
<td>Method 5</td>
<td>Laffan</td>
<td>7,15±0,21</td>
<td>3,93±0,12</td>
</tr>
<tr>
<td></td>
<td>Wonderful</td>
<td>6,70±0,66</td>
<td>4,07±0,31</td>
</tr>
</tbody>
</table>
Washing of crude polysaccharide

For impurities determination in mesocarp and exocarp, the crude polysaccharides were dissolved in distilled water and re-precipitated again with ethanol to evaluate the amount of these impurities by a gravimetric determination. Amounts of non-polysaccharide substances (impurities such as proteins, ellagitannins and other polar compounds) varies depending on the extraction method; applying an hot water extraction (Method 2) to the mesocarp the amounts of these impurities evaluated as dried weight range from 74 mg/g DM for Laffan, to 50 mg/g DM for Wonderful. Regarding the exocarp these residues are close to 30 mg/g DM for both these cultivars. From this control it emerges that only low amounts of impurities are entrapped in the CPS and the values range from 2-6 mg/g for both mesocarp and exocarp of the two cvs. In light of these data it can be concluded that the washing process of CPS is not necessary particularly when applying the extractive methods 4 and 5. The co-precipitation of ellagitannins together with the other impurities within the CPS leads contributes to confer a dark color of this crude fraction. In light of this observation the ellagitannins entrapped in the CPS have been evaluated by HPLC/DAD applying a method previously optimized (see chapter 4 of this Thesis).

**Figure 2.** Amounts of the ellagitannins co-precipitated in the CPS from Laffan and Wonderful mesocarp and exocarp. The data are a mean of triplicates.
Regarding the mesocarp the values ranged from 11.52 ± 1.09 mg/g DM for Laffan cv to 6.47 ± 0.82 mg/g DM for Wonderful cv, while the exocarp showed lower amounts ranging from 3.93 ± 0.49 mg/g DM for Laffan cv, to 3.87 ± 0.39 mg/g DM for Wonderful cv after washing the polysaccharides obtained by Method 2.

Sugar composition by hydrolysis

Samples were treated with trifluoroacetic acid to hydrolyze the polysaccharide strands and subsequently determine sugar composition by ion exchange chromatography according to previous methods (Erbing et al., 1995). The composition of four purified polysaccharide fractions is reported in Table 3. On the whole, diverse monosaccharides were found, and these samples showed a very similar composition for both the cultivars. Within the hexoses galactose, glucose/mannose, and fructose; then aldopentose arabinose, pentose xylose, rhamnose and galacturonic acid were found (Table 3). Glucose and mannose, having very close retention times, were co-eluted in one peak on the chromatograms. In the entire analyzed sample, glucose, xylose and glucuronic acid were the most abundant sugars (Table 3).

Normakhmatov, Rakhmnberdyeva, & Rakhimov (1999) pointed out that the main monosaccharides in the water-soluble polysaccharides from pomegranate peel varied with the growth site, and were constituted by glucose, galactose and xylose, with larger amounts of xylose, arabinose and galactose detected in the peel of other varieties of pomegranate harvested in different growth sites. In another study the composition of a polysaccharide from pomegranate peel showed glucose at 52.8% and glucuronic acids at 33.5%. Other monosaccharides included arabinose and galactose close to 5.0%, mannose and rhamnose close to 1.6% and only 0.5% for xylose. (Jahfar, Vijayan, & Azadi, 2003). From the few reports available to date on polysaccharides of pomegranate fruits, it emerges that there is a certain variability in the sugar composition that is mainly related to the variety and growth site but is also affected by the different purity grade of the polysaccharide itself, as analyzed by various authors. Notably, the two cultivars (one mainly localized in Syria and the other widely
diffused throughout the western world) showed a very similar compositional profile of the polysaccharides. However, this result is not completely unexpected, and is in agreement with a previous document that hypothesized that the Wonderful variety is derived from the more ancient Laffan cultivar (Goor, 1967). Glucofructan from the pectin of the peel, having a molecular weight 31,000, was also detected using a Sephadex G-100 column (Khodzhaeva et al., 1985). Further studies will be performed to define the molecular weight of these polysaccharides applying a gel permeation method and by the use of mass measurements with MALDI.

Table 3: Sugar composition of the crude polysaccharides obtained by chemical hydrolysis (Erbing et al., 1995). The name of the samples have been defined as follow: Ps for polysaccharides; M for mesocarp and E for exocarp; L1 for Laffan and W1 for Wonderful.

<table>
<thead>
<tr>
<th>Sugars</th>
<th>Molar %</th>
<th>PsMW1</th>
<th>PsML1</th>
<th>PsEW1</th>
<th>PsEL1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhamnose</td>
<td></td>
<td>13,25</td>
<td>11,33</td>
<td>3,20</td>
<td>3,18</td>
</tr>
<tr>
<td>Arabinose</td>
<td></td>
<td>27,36</td>
<td>28,02</td>
<td>35,63</td>
<td>26,67</td>
</tr>
<tr>
<td>Galactose</td>
<td></td>
<td>6,22</td>
<td>8,76</td>
<td>11,06</td>
<td>10,68</td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td>34,98</td>
<td>29,09</td>
<td>39,24</td>
<td>25,33</td>
</tr>
<tr>
<td>Xylose</td>
<td></td>
<td>8,63</td>
<td>6,93</td>
<td>5,26</td>
<td>1,47</td>
</tr>
<tr>
<td>Fructose</td>
<td></td>
<td>0,39</td>
<td>0,35</td>
<td>0,42</td>
<td>0</td>
</tr>
<tr>
<td>Galacturonic acid</td>
<td></td>
<td>33,18</td>
<td>43,91</td>
<td>28,76</td>
<td>32,94</td>
</tr>
</tbody>
</table>

A filtration step by the use of filter with 10,000 Dalton as cut off was applied to further purify the polysaccharide from mesocarp of Table 3 and then submitted to the chemical hydrolysis. It emerges that the two samples before and after cut off-filtration gave the same results in terms of molar % for all the sugars (data not shown) implying a lack of interference compounds (e.g., salts). These results suggest that the samples in Table 3 do not need to be further purified by the use of these filter device before the chemical hydrolysis.

Gelling capacity of CPS.
The water holding capacity of plant polysaccharides is determined by their chemical and structural properties and also by the pH and osmolality of the surrounding fluids (Eastwood et al., 1976). When expressed as water retained per gram of dried material, a range of 1.5 g water per gram of fiber for maize to 23.7 g water per gram of fiber for lettuce has been reported (Eastwood et al., 1976; Connell et al., 1974). It is well known as purified plant fibers such as carrageenan can form gels containing 99% of water/g dried material (Eastwood et al., 1973).

A preliminary test was carried out on some CPS extracted in this work to evaluate their gelling capacity by measuring the amount of adsorbed-entrapped water. The % of water adsorbed by CPS of pomegranate ranged from 98.6 to 99.1 % confirming these polymers as potential gelling agents to be used in food chemistry (Table 4). These preliminary data, helping to improve the knowledge on the physical properties of the polysaccharides recovered by a typical by-products of pomegranate fruit, add value to this waste and open future perspective for its optimal use.

**Table 4.** Gelling capacity evaluated by the % of the adsorbed water. CPS: crude polysaccharides

<table>
<thead>
<tr>
<th>Samples</th>
<th>Amount of CPS (g)</th>
<th>% H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPSML</td>
<td>0.32</td>
<td>99.2</td>
</tr>
<tr>
<td>CPSMW</td>
<td>0.3</td>
<td>99</td>
</tr>
<tr>
<td>CPSEW</td>
<td>0.3</td>
<td>98.6</td>
</tr>
</tbody>
</table>

*In vitro evaluation of prebiotic properties.*

In this study the ability of *B. breve* B632 and *L. plantarum* L12 strains to use crude polysaccharides from pomegranate exocarp and mesocarp as the carbon source was studied and compared to growth on glucose, i.e. an easily fermented carbon source.

Figure 2 shows that both strains could grow well on the CPS of Laffan and Wonderful mesocarp reaching the maximum growth on these substrate after 24 hours of incubation. Growth at 24 h on the Laffan variety was 0.6 and 0.1 Log CFU/mL lower with respect to that on glucose for *L. plantarum* L12 and *B. breve* B632, respectively. Growth at 24 h on the Wonderful variety was 1.0 and 0.2 Log CFU/ml lower with respect to that on glucose for the same strains. Growth on glucose was also maximum at 24 h of incubation but, differently from the Laffan CPS, the stationary phase
was longer, lasting at least until 30\textsuperscript{th} hour. A longer stationary phase was also observed for the Wonderful variety in the \textit{B. breve} strain. Growth on the medium with no added carbon source was 1 Log CFU/mL at 24 hours compared to the beginning of incubation.

On the whole, the results obtained allow to conclude that CPS from pomegranate mesocarp of Laffan and Wonderful can sustain growth of beneficial and potentially probiotic bacteria such as those used in this study and, therefore, possess prebiotic properties. Growth of the \textit{Lactobacillus} strain on the Laffan variety was higher with respect to the Wonderful one, whereas growth of the \textit{Bifidobacterium} strain was very similar on the CPS deriving from both varieties.

**Figure 2.** Evaluation of prebiotic properties of CPS from mesocarp of Laffan and Wonderful on the \textit{L. planctarum} PCS20 (a) and \textit{Bifidobacterium breve} B632.

\textit{C-}: growth obtained on m-MRS with no added carbon source
\textit{C+}: growth obtained on m-MRS with 0.5 % glucose
**Bifidobacterium breve B632**

![Bar chart showing the Log CFU/ml of Bifidobacterium breve B632 at different time points (T0, T6, T24, T30, T48). The chart includes four conditions: C-, C+, Laffan, and Wonderful.](image)

- **C-**
- **C+**
- **Laffan**
- **Wonderful**
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An insight on the alkaloid content of *Capparis spinosa* L. root by HPLC-DAD-MS and MS/MS

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Abstract

The *Capparis spinosa* L. has a wide distribution in the Old World from South Europe, North and East Africa, Madagascar, Southwest and Central Asia to Australia and Oceania. The relationship between capers and human beings can be traced back to the Stone Age. The traditional use of *Capparis spinosa* root as remedy against different pains in human is well known since the antiquity as highlighted by recent review. Various secondary metabolites have been found in caper plant, among them alkaloids, phenols, sterols, most of them in free or glycosylated forms. Nevertheless, few studies have been focused to the analysis of root constituents. To date, three main alkaloids (capparispine, capparispine 26-O-β-D-glucoside and cadabicine 26-O-β-D-glucoside) have been isolated from the root of *C. spinosa* and a more polar derivative, the stachydrine.

Aim of this work was to improve the knowledge on the alkaloid content of the root of a Syrian sample of *C. spinosa*. To this scope decoction, hydro-alcoholic and dichloromethane extracts have been prepared and analyzed by HPLC-DAD-MS. To the best of our knowledge, this is the first HPLC-DAD-MS profile that pointed out the copresence of stackydrine, several isobaric forms of capparispine and/or capparisine in free and glycosylated forms and some isobars of isocodonocarpine or codonocarpine all extracted from a sample of *C. spinosa* root. The quantitative evaluation was carried out by the use of cinnamic acid as external standard. The total alkaloids were almost doubled in hydro alcoholic extract (2,8 mg/g dried root) with respect to decoction. Coupling the use of ultrasounds even if of low potency, with the ethanol mixture, an increment close to 30% of the extractive yield was reached (3,9 mg/g dried root). Finally, the use of methylene chloride for a fractionation process allowed to increase the alkaloids amount almost of 7 times with respect to the native hydro alcoholic sample.

**Keywords:** capparisine; glycosilated alkaloids; stachydrine
Introduction

The *Capparis spinosa* L. has a wide distribution in the Old World from South Europe, North and East Africa, Madagascar, Southwest and Central Asia to Australia and Oceania (Jacobs, 1965; Fici, 2004). The caper is a perennial spiny shrub, which can be found growing wild everywhere in Syria around dry and rocky areas. It is particularly abundant in the harsh environments of Euphrates valley, Aleppo province, Idlib, Salamie area of the Hama province, and in Al Jazira (northeastern Syria). The caper is adapted to dry heat and intense sunlight. *Capparis spinosa* L. is a valuable commodity for the resource poor nomadic communities living in the Syrian desert. While caper is widely cultivated in other Mediterranean countries, in Syria the caper is a wild species and cultivations are only found in the research nurseries. Unlike to other countries, the caper buds collected before flowering are used in Syria as trade items (Giuliani and Padulosi, 2005). The caper buds are collected by private enterprises, then sell to foreign traders of the neighboring countries, mainly of Turkey. The foreign factories process the capers, and sell them to the European countries. The estimated yearly harvest is about 4000 tons that corresponds to only a quarter of the potential caper that could be harvested (Giuliani, 2005).

The value of caper in antiquity. The relationship between capers and human beings can be traced back to the Stone Age. Rests of *Capparis spinosa* were unearthed in archaeological sites in Syria as early as the lower Mesolithic (on the area of Tell Abu Hureyra, 9500–9000 B.C., Hansen, 1991). Carbonized flower buds and unripe fruits were unearthed in a jar at the site of Tell es-Sweyhat, Syria, dated to about 2400–1400 B.C., and were considered to be stored as a condiment (Van Zeist and Bakker-Heeres, 1985). The first recorded use of the caper bush for medicinal purposes was by the Sumerians in 2000 BC: the root bark of caper was used as analgesic and carminative agent Sher and Alyemeni, (2010). A high economic and medicinal value in different traditional medicines like Iranian, Unani, Chinese, Ayurvedic and Greco-Arabian contexts has been recognized for this plant. The plant is widely known with the common name caper in several countries (Azaizeh et al., 2003) and the different parts of the plant can be easily found at herbal markets in Syria (Giuliani et al. 2005). Several authors (Al-Said et al. 1988; Sher and Alyemeni, 2010) reported that in traditional medicines teas made with caper root and young shoots are considered to be beneficial for the treatment of rheumatism and stomach problems, against colicky pains, dyspepsia, dropsy, anemia and gout (Inocenico et al., 2002; Eddouks et al., 2004; Lemhadri, 2007). The moist pastes prepared from
the root bark are used for topical applications to treat swollen joints, skin rashes, burns, wounds and dry skin (Eddouks et al., 2004), and also as cataplasm for spleen troubles. The oral administration of the root bark has been described as appetizer, purgative, emmenagogue, and expectorant against chest diseases (Yadav, 2013), but also as diuretic and tonic (Batanouny et al., 1999). According to Calabrian popular medicine (southern Italy) the decoction of roots of C. spinosa L. is used to cure several diseases of the mucous membranes such as abscesses of the oral cavity (Boga et al. 2011).

Dr. A. Kattaa, from Aleppo University, has pointed out the medicinal value of caper plant in Syria reporting its use as good coadjutant for the immune system; the Syrian people use to drink the water where the roots have been previously soaked (Giuliani et al. 2005). The powdered root, mixed with olive and other oils, is used to treat rheumatism and diseases of the urinary tract (Giuliani, 2005).

The traditional use of Capparis spinosa root as remedy against different pains in human is well known since the antiquity as highlighted by a recent review (Jiang et al, 2007). The powdered root is widely used for oral administration in the Syrian tradition, alone or associated to local applications on the painful part of “dough” prepared mixing the powder with water. In Jordanian traditional medicine, the root bark are macerated and placed between gauze on aching area for 15-25 min in order to relieve the inflammation and muscle pain (Hudaib et al., 2008). This ability to reduce pain in humans is particularly interesting because few natural compounds have been recognized as efficacious to date.

**Root composition.** Various secondary metabolites have been found in caper plant, among them alkaloids, phenols, sterols, most of them in free or glycosylated forms. Nevertheless, few studies have been focused to the analysis of root constituents. Three main alkaloids, capparispine, capparispine 26-O-β-D-glucoside and cadabicine 26-O-β-D-glucoside, have been isolated from the root of C. spinosa (Fu et al., 2008). The spermidine alkaloids, isocodonocarpine, capparidisine and capparisinine, have been isolated from the dried root barks of C. deciduas, and their structures were elucidated by mass spectrometry, UV, IR and NMR spectroscopy (Ahmad et al., 1985; Ahmad et al., 1987; Ahmad et al., 1989). Successively, two new N-acetylated spermidine alkaloids, 14-N-acetylisocodonocarpine and 15-N-acetylcapararisine, have been isolated from the roots by the same research group (Ahmad et al., 1992). Other recently isolated compounds from C. deciduas root are capparisine, codonocarpine, cadabacine, rutin, 1-stachydrine and β-sitosterol (Rathee et al., 2010; Mishra et al., 2007). For the first time in 1969, the alkaloid stachydrine was isolated from the root of
Capparis spinosa (Multhamedova et al., 1969; Afsharypuor and Jazy, 1999). Both the root bark and the leaves contain stachydrine and 3-hydroxystachydrine, while glucobrassicin, neoglucobrassicin and 4-methoxy-glucobrassicin have been found only in the root (Batanouny and Lamnauer, 2005).

Pharmacological activities of the alkaloids of C. spinosa roots. The presence of spermidine alkaloids like capparidisine, capparisine or capparisinine in root bark of C. aphylla (Syn. Capparis decidua Edgew) reminds for careful use of Capparis plant in dietary or medicinal supplements (Rastogi RP & Mehrotra, 1995). Isocodonocarpine, present in roots was found to be responsible for antinflammatory and anti-asthmatic activity (Ahmad et al., 1992). The L-stachydrine exhibited anti-tuberculosis property in in vivo experiments (Tandon et al., 1961; Gaind and Juneja, 1970). This compound was able to increase the blood coagulation, thus shortening bleeding time and blood loss (Asolkar et al., 1992). The assumption of generous quantities of alkaloids, e.g., spermidine alkaloids and stachydrine, induced a depressant activity on central nervous system in rats (Aniszewski, 2007). Since the antiquity, the stachydrine containing species are widely used against rheumatism and other diseases in traditional medicines (Buckingham, 1994). Stachydrine was recognized as a potent anti-metastatic agent, which markedly inhibited invasive capacity of malignant cancer cells (Lam et al., 2009). Overall, in more recent literature, numerous studies about the biological properties of the C. spinosa L. are reported. However, the majority of these reports are related to the aerial parts of the plant, such as flowers and fruits. Other studies concerning the leaves have been published, but less attention has been devoted to the roots of C. spinosa L.

Aim of this work was to improve the knowledge on the alkaloid content of the root of a Syrian sample of C. spinosa. Decoction, hydro-alcoholic and dichloromethane extracts have been prepared and analyzed by HPLC-DAD-MS. The different extracts obtained from the whole powdered root and from external and internal parts have been evaluated in terms of alkaloids content.

Experimental section

Samples

The roots of Capparis spinosa L. (Capparaceae) were collected from Kafr Nabl, province of Idlib, in the north of Syria in 2011. The sample was authenticated by General Commission for Scientific Agricultural Research, Damascus, Syria. The roots were freeze-dried as whole and maintained at room temperature, in dark until the analyses.
Ethanol and water extraction. The extraction has been performed on the whole root, and on the root cortex and internal part separately. For all the extracts the applied drug/solvent ratio was 1:30 w/v starting always from the dried powdered material. The extraction with ethanol-water mixture 7:3 v/v was done in two successive steps (each of 12 h) on the powdered root stirred at room temperature. Exactly the same procedure was applied adding a sonication step of 30 min (35 MHz) for twice. The extracts were then filtered through Whatman filter paper to remove solid residue obtaining the sample EtH₂O. The decoction was done on dried powdered material boiled in distilled water under stirring for 1 hour; after cooling and filtration to collect the supernatant. This solution was treated with 2 volumes of ethanol and kept for 3 h at 0 °C for precipitation of crude polysaccharides and other impurities, then the solutions were centrifuged at 4500 rpm for 12 min to remove the precipitate obtaining the sample DEC. All the extracts were dried under vacuum at 30°C, then redissolved in a mixture of EtOH/H₂O (7:3 v/v) and centrifuged at 14,000 rpm, 5 min, before HPLC/DAD analysis.

Selective extraction of alkaloids. Briefly, an aliquot exactly measured of the hydro alcoholic extract was dried until water residue (below 30°C), added with NH₃ until pH 10, then CH₂Cl₂ was used for a liquid-liquid extraction (two steps, each with the same volume of the water residue). Then was used ethyl acetate in the same manner of methylene chloride (see Fig. 6). The organic phases were dried and dissolved in acidified mixture of ethanol/water/formic acid 70:30:1 v/v/v obtaining the CH₂Cl₂ and EtOAc samples for HPLC-DAD and HPLC-MS/MS analyses. The same procedure was applied to the water residue (H₂O-Res) to prepare the sample for the instrumental analysis.

HPLC/DAD analyses
The analyses were carried out using a HP 1200L liquid chromatograph equipped with a DAD detector (Agilent Technologies, Palo Alto, CA, USA). A 150 mm × 2 mm i.d., 4 μm Fusion, RP 80 A, column (Phenomenex, USA) equipped with a pre-column of the same phase was used. The mobile phases were (A) 0.1% formic acid/water and (B) CH3CN. The multi-step linear solvent gradient used was: 0-2 min 2 % B; 2–20 min, 2–25% B; 20–25 min 25–35% B; 25–28 min 35–95% B; 28–32 min 95–95% with a final plateau of 2 min at 2%B; equilibration time 10 min; flow rate 0.4 mL min⁻¹ and oven temperature 26 °C; injection volume 5 μL. The UV-Vis spectra were recorded in the range 220–500 nm and the chromatograms were acquired.
at 280, 350 nm. All solvents used were of HPLC grade; CH$_3$CN was from E. Merck (Darmstadt, Germany).

The MS and MS/MS experiments
The MS and MS/MS experiments were done in positive and negative mode using the same column and applying the elution method previously described for the HPLC/DAD analyses. The UHPLC was a Platin Blue (Knauer, Berlin, Germany), operating in the same conditions as described for HPLC-DAD analyses. The UHPLC was directly connected to a LTQ linear quadrupole ion trap mass spectrometer (Thermo Scientific, Bremen, Germany) through an ESI interface. The analyses were performed in scan mode in the range 134-800 m/z, alternating the two polarities. Instrumental parameters are listed below in Table 1.

<table>
<thead>
<tr>
<th>Positive Ions</th>
<th>Parameter</th>
<th>Negative Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>4700</td>
<td>Spray voltage (V)</td>
<td>-4400</td>
</tr>
<tr>
<td>20</td>
<td>Capillary voltage (V)</td>
<td>-40</td>
</tr>
<tr>
<td>90</td>
<td>Tube lens offset (V)</td>
<td>-55</td>
</tr>
</tbody>
</table>

Nitrogen was used as the sheath, sweep and auxiliary gas at the flow rate of 28, 6 and 4 (arbitrary units), respectively. The capillary temperature was 295°C.

The MS/MS and MS/MS/MS experiments were performed on some selected precursor ions in positive ion mode, at 144.1 m/z, 598.3 m/z and 436.2 m/z other ion 466. The precursor ions were isolated using a 3 mass unit width, collided with helium gas in the ion trap (Q 0.25, excitation time 30 msec) at 30% relative collision energy. The principal product ion of 598.3 m/z was at 436.2 m/z: this ion was also isolated and collided (30% relative collision energy) for MS/MS/MS experiment and its product ion spectrum recorded. The principal product ion of 436.2 m/z was also isolated and collided (27% relative collision energy) for MS/MS/MS experiment: the product ion spectrum of 348.1 m/z was recorded in the range m/z 95-460.

Quantitative evaluation of the alkaloid content
The quantitative evaluation of the main alkaloids was performed through the use of the p-coumaric acid as external standard (purity grade 98% by HPLC, purchased from Sigma-Aldrich, Germany). A five-point calibration curve was built at 280 nm, linearity range between 0.22 μg and 2.21 μg, \( R^2 = 0.998 \).
Results and discussion

Chemical characterization
Different extraction processes were performed on the powdered root trying to have a more complete picture of the secondary metabolites of the root, particularly of the alkaloids. To this aim, a decoction for 1 h, an extraction with ethanol at 70% v/v have been applied to the whole root but also on the external and internal parts, separately. The use of acetone at room temperature have been also applied but this solvent resulted in a poor extractive capacity compared with the hydro alcoholic and water extracts (data not shown), therefore not further used. The HPLC-DAD and HPLC-MS methods were optimized working on the hydro alcoholic extract from whole root as reference sample. The optimized elution procedure on the Fusion column was then applied to analyze all the other extracts; a profile at 280 nm of the main components of the reference extract is shown in Figure 1.

Figure 1. HPLC/DAD profiles at 280 nm of the hydro alcoholic extract (see also Table 3, sample 2-EtH$_2$O) of the whole root.

The same hydroalcoholic extract was then submitted to HPLC-MS and MS/MS analyses. The first full scan experiment was performed to check for the presence of molecular ions of the alkaloids cadabicine and capparispine in free or glycosylated forms, previously found in the root of Capparis spinosa (Fu et al., 2008; Khanfar et al., 2003) but also in other species as Capparis decidua (Arif, 1986).
Figure 2. (a) Base peak chromatogram of the hydro alcoholic extract of dried root of *C. spinosa* (black) with the associated extracted ion profiles at 436 m/z (red) and 598 m/z (green); the last two profiles are related to the main alkaloids in free and monoglycosylated forms as better highlighted by the zoom of the two areas (b).
Figure 2 shows the base peak chromatogram with a group of main signals in the range 10-13 min; and another series of peaks with a minor intensity related to more lipophylic compounds in the range 21-26 min.

The ion current profile of m/z 436 (Figure 2b) completely overlaps with the base peak profile showing also the same intensity of 3,4 E6. This profile highlights the presence of four isobaric forms but with strong differences in terms of relative ion intensity. Observing the profile in Figure 2c, it was possible to point out the presence of another group of peaks corresponding to at least four isobaric monoglycosides (mw 597 Dalton), attributable to cadabicine and/or capparispine derivatives. The intensity of the ions at 598 m/z [M+H]⁺ was one order of magnitude less intense compared to 436 m/z and taking into account that the glycosidic forms can be easily ionized in ESI-MS experiments, it can be assessed that the mono glycosilated alkaloids are in lower amount with respect to the corresponding free forms. Furthermore, the lower retention times of these glycosylated forms with respect to the peaks of the free alkaloids in the HPLC on reverse phase, are in agreement with the different polarity of these two groups of compounds. In Figure 3 are shown some reference structures of the main alkaloids detected in C. spinosa to date.

The next step was to carry out an HPLC-MS/MS experiment in positive ionization mode focused to induce the fragmentation of the main alkaloids by the selection of the corresponding protonated molecular ion at 436 Dalton. In Figure 4 the MS/MS spectra of the four isobars are compared, all showing the fragment ion at 419 m/z with 40-100 % of intensity. This key fragment can be explained by the cleavage of the bond between the N atom and the two adjacent carbons with a loss of ammonia residue from both the two possible isobaric opened ions. These two latter species can easily loss the neutral groups, 1,4-diaminobutane and 1,3-diaminopropane thus forming the ions at 348 m/z with higher intensity (50-100%) and the ion at 362 m/z, respectively. The ions at 291 m/z can originate by the loss of the 3-aminopropene group (57 Dalton) from the ion at 348 m/z. This fragmentation pattern is in agreement with that previously obtained by ESI-MS/MS experiments carried out on isolated cadabicine (Khanfar et al., 2003) and similar to that proposed for alkaloids extracted from root of Capparis decidua analyzed by FDMS (Arif,1986).
Figure 3. Some reference structures of the main alkaloids from *C. spinosa* root. 1, Cadabicine 26-O-b-D-glucoside; 2, Capparispine; 3, Capparispine 26-O-b-D-glucoside; 4, Isocodonocarpine; 5, Capparisine; 6, Stachydrine.
Figure 4. MS/MS spectra of the ions at 436 m/z related to four isobars corresponding to capparispine or capparispine alkaloids in free forms.

The structural hypothesis derived by the MS data agrees with the UV-Vis spectra of these molecules obtained by the HPLC/DAD analyses in water-acetonitrile and shown in Figure 5. The isolated capparispine, capparisine and capparisinine alkaloids in methanol solution showed maximum absorptions in the range of 280-283 nm with shoulders close to 308-310 nm (Arif, 1986). More recently, other authors (Fu et al., 2008) have reported almost the same maximum absorptions for free capparispine and capparispine 26-O-β-D glucoside. The spectra in Figure 5 have been grouped and overlapped taking into account the similarities among all the detected compounds and compared with the spectra of the p-coumaric acid selected as reference to measure the alkaloid content in all the extracts (see next paragraph).
Figure 5. Some UV-Vis spectra of the detected alkaloids and other unknown derivatives in the root of C. spinosa. a) standard of p-coumaric acid; b) 12.7 min, 13.2 min and 17.3 min; c) 11.7 min, 16.1 min and 16.3 min; d) 15.3 min, 18.1, 18.6 min and 19.1 min; e) 22.4 min, 22.8 min, 23.3 min, 23.9 min.

All these spectra are in agreement with the presence of cinnamoyl moieties within the molecular structures that are characteristic of the main alkaloids of Capparidaceae (Figure 3). The presence of another important alkaloid, stachydrine, has been confirmed by the MS and MS/MS experiments, but for this structure the UV spectra is not useful nor informative. This more polar compound typical of Capparidaceae, with the protonated molecular ion at m/z 144 [M+H]+, present an rt value of 1.5 min (Figure 1) almost coincident with the dead volume of the column. Our finding pointed out the interaction of this molecule with a C18 reverse phase (also starting the elution method with 98% of water) as negligible. In the same profile, according to the mass spectral data, it was possible to identify two monoglycosides at m/z 598 [M+H]+ corresponding to compounds 1 and 2, that can be related to capparispine 26-O- β-D-glucoside and/or cadabicine 26-O- β-D-glucoside, previously isolated from this root (Fu et al., 2008). The group of peaks in the range of 15-18 min corresponds to four isobars at m/z 436 [M+H]+ of capparispine and/or capparisine (compounds 3, 4, 5, 6 and 7 in Figure 1). Another
cluster of minor peaks between 18-21 min showed the presence of two main ions species at m/z 466 [M+H]⁺ and m/z at 435 detected for the compounds 8, 9, 10. The molecular weight of 466 Dalton suggests the presence of isocodonocarpine and/or codonocarpine, two isobaric alkaloids previously detected in *Capparis decidua* characterized by the presence of methoxyl groups on their structures that can explain the ion at m/z 435, due to a loss of 31 mu by the molecular ion species.

Further MS and MS/MS experiments on hydroalcoholic and water extracts have been carried out in flow injection, aiming to verify the ability to detect the main alkaloids in the extract without the need of a chromatographic separation. The obtained findings confirm this hypothesis, in fact the ion at 436 m/z corresponding to the protonated ion of capparisine and capparispine, was easily detected with a good intensity. Furthermore, the presence of the corresponding monoglucoside species, even if in consistently lower amount, have been detected. Due to the very similar structures and behavior in fragmentation, it was not possible to distinguish the contribution of each single isobaric molecule both in MS and MS/MS experiments.

To the best of our knowledge, this is the first HPLC-DAD-MS profile that pointed out the copresence of stackydrine, several isobaric forms of capparispine and/or capparisine in free and glycosylated forms and some isobars of isocodonocarpine or codonocarpine all extracted from a sample of *C. spinosa* root. Overall, the proposed HPLC/DAD/MS method result as a suitable tool to investigate on the alkaloid content of this root while the flow injection of these different extracts resulted able only to give a qualitative picture of the main alkaloids of this root.

*Extractive yields in terms of alkaloid content*

The alkaloid content in the root was measured by HPLC/DAD through the use of *p*-coumaric acid as external standard. This phenol was selected because a similar structure is always present in the main alkaloids of *C. spinosa* (Figure 3) and represent the determinant moiety to define the shape of the UV-Vis spectrum of these alkaloids (Figure 5). Naturally, selecting this standard the molar absorbitivity of all the alkaloids measured at 280 nm, was assumed as the same of that of *p*-coumaric acid. This approach is frequently applied in the analyses of complex vegetal matrices when more specific and suitable standards are not available.

The alkaloid content was determined in the extracts of whole root, but also on external and internal parts separately; Table 2 shows the recovery of some single alkaloids (compounds 1, 2, 3 and 7) and the total amount obtained with the different extracts. The choice to use a 70% hydro alcoholic mixture was done for recovering the larger amount of the different alkaloids.
only in one extractive step. At the same time also the decoction of root was applied because a similar procedure is described to prepare root extracts used in the traditional Arab and Greek popular medicine (Sher and Alyemeni, 2010; Moghaddasi et al., 2012).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Extract</th>
<th>Part of root</th>
<th>single alkaloid</th>
<th>Total Alkaloids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>S1</td>
<td>Dec</td>
<td>whole</td>
<td>0.09±0.01</td>
<td>0.30±0.06</td>
</tr>
<tr>
<td>S2</td>
<td>EtH₂O</td>
<td>whole</td>
<td>0.14±0.01</td>
<td>0.79±0.01</td>
</tr>
<tr>
<td>S3</td>
<td>EtH₂Os</td>
<td>Root cortex</td>
<td>0.20±0.05</td>
<td>1.25±0.22</td>
</tr>
<tr>
<td>S4</td>
<td>EtH₂Os</td>
<td>Inner part</td>
<td>0.33±0.02</td>
<td>1.71±0.20</td>
</tr>
<tr>
<td>S5</td>
<td>EtH₂Os</td>
<td>whole</td>
<td>0.31±0.03</td>
<td>1.52±0.12</td>
</tr>
</tbody>
</table>

Table 2. Amount of the alkaloids in different extracts; the data are a mean of triplicates.

DE = Dried Extract; DR = Dried Root; Dec= decoction; EtH₂O= hydro alcoholic extract; EtH₂Os hydro alcoholic extract with sonication

The main findings regarding the extractive yields obtained with the different procedures summarized in of Table 2 can suggest some general considerations. As expected the total alkaloids expressed as mg/g DR is almost doubled (2.75±0.02) in EtH₂O sample with respect to Dec sample. (1.36±0.07). Coupling the use of ultrasounds even if of low potency, with the ethanol mixture, an increment close to 30% of the extractive yield was reached (3.9 mg/g dried root). Applying the same process to external and internal parts of the root similar values in terms of total content were observed. This result suggests that the distribution of the alkaloids studied in this work is homogeneous within the root of *C. spinosa*. Furthermore it can be assessed the ultrasounds strongly help to raises the extractive yield, the results are in agreement with previous data (Ensminger and Leonard 2011), the extractive procedure shown a good reproducibility as confirmed by the RSD values in Table 2.

Composition of the extracts

The next step of this investigation was focused to apply a procedure to obtain extracts more concentrated in terms of alkaloids, suitable for MS investigations aimed to check the presence of minor alkaloids but mainly to evaluate the bioactivities of the alkaloids of root. To this latter
scope and according to the traditional uses, several extracts with different alkaloid composition have been selected for *in vivo* tests to evaluate their ability in reducing neuropathic pain in rats after oral administration (*see chapter 7 of this thesis*).

In literature a common procedure to recover different pools of alkaloids from complex vegetal matrices is to work at alkaline pH. In this medium, avoiding the positive charges on the nitrogen atoms, a liquid/liquid extraction by lipophilic solvents as dichloromethane and chloroform usually guarantees an efficient recovery of these molecules from aqueous residues. To this aim, the process summarized in Figure 6 applies this concept to fractionate the hydro alcoholic extract of the root for obtaining a partially purified fraction enriched in the non glycosilated alkaloids of capparisine and/or capparispine.

**Figure 6.** Scheme of the fractionation process applied to concentrate the alkaloid fraction
The alkaloid content of all the samples obtained by a direct extraction of the root or by the fractionation process of Figure 6 has been expressed with respect to the weight of the dried extract and compared in Table 3 and Figure 7.

**Table 3.** Alkaloid content in the extracts derived by the fractionation process of Figure 6 compared with the decoction and hydro alcoholic extract of whole root.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Extracts</th>
<th>Part root</th>
<th>main alkaloids (mg/g)</th>
<th>Total Alkaloids (mg/g DE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1  2  3   4</td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td>DEC</td>
<td>Whole</td>
<td>0,36 1,27 0,4 2,79</td>
<td>5,72</td>
</tr>
<tr>
<td>S2</td>
<td>EtH₂O</td>
<td>Whole</td>
<td>0,54 3,15 0,73 4,92</td>
<td>11,0</td>
</tr>
<tr>
<td>S6</td>
<td>CH₂Cl₂</td>
<td>Whole</td>
<td>0 1,67 7,37 57,39</td>
<td>76,53</td>
</tr>
<tr>
<td>S7</td>
<td>H₂O-Res</td>
<td>Whole</td>
<td>0,48 2,93 0,25 1,28</td>
<td>5,70</td>
</tr>
</tbody>
</table>

**Figure 7.** Distribution of the main alkaloids within root cortex (external) and inner root (internal); data as a mean of triplicates expressed on weight of dried extract (DE).
By the HPLC/DAD/MS analyses it was confirmed an increased amount of the glycosilated forms (alkaloids 1 and 2) in the aqueous residue (H₂O-Res) and an higher amount of free alkaloids in the organic phase (CH₂Cl₂). Overall this latter sample contain the largest amount of alkaloids up to 76.53 mg/g DE. The use of ethyl acetate (EtOAc) after methylene chloride does not give a more selective fractionation nor increased amounts of alkaloids (data not shown). Overall, the use of methylene chloride (Figure 6) allowed to increase the alkaloids amount almost of 7 times with respect to the native hydro alcoholic sample as evidenced comparing the data in table 3 with the; in light of this data the extract resulted eligible for biological tests focused on evaluating the activity of these pool of alkaloids (see chapter 7 of this thesis).
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Therapeutic Effects of *Capparis Spinosa* on Experimental Articular Pain in Rats

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Abstract

**Ethnopharmacological relevance.** *Capparis spinosa* L. originates from dry regions of Asia and Mediterranean basin. In traditional medicine of these areas, infusions from caper root are considered to be beneficial for the treatment of rheumatism, gout and against abdominal pains.

**Aim of the study.** To evaluate the pain reliever properties of a Syrian cultivar of *Capparis spinosa* roots in rat models of osteoarthritis and rheumatoid arthritis.

**Materials and Methods.** Decoction (DEC) and hydroalcoholic extract (EtH$_2$O) were obtained from powdered roots; the latter was further separated in CH$_2$Cl$_2$ and aqueous (H$_2$O-Res) fractions. The extracts were characterized in terms of spermidine alkaloids by HPLC/DAD/MS and stachydrine by NMR. Different amount of free and glycosilated forms of capparispine and analogues (from 0.5 % w/w for DEC up to 7.6 % w/w for CH$_2$Cl$_2$ fraction) were detected. Rat models of rheumatoid arthritis and osteoarthritis were induced by the intra-articular administration of Complete Freund’s Adjuvant (CFA) or monosodium iodoacetate (MIA), respectively.

**Results.** Fourteenth days after CFA or MIA injection, the different preparations of *Capparis spinosa* (3, 30, 100 and 300 mg kg$^{-1}$) were acutely administered *per os*. Powdered roots (300 mg kg$^{-1}$), DEC (100 mg kg$^{-1}$), and EtH$_2$O (300 mg kg$^{-1}$) significantly reduced hypersensitivity to mechanical noxious stimuli as well as spontaneous pain evaluated as hind limb bearing alterations in both models. The CH$_2$Cl$_2$ and the H$_2$O-Res (30 mg kg$^{-1}$) were the most potent in reverting pain threshold alterations despite the different content of free alkaloids.
Introduction

The traditional use of *Capparis spinosa* root as remedy against different pains in human is well known since the antiquity as highlighted by a recent review (Jiang *et al.*, 2007). The powdered root is widely used for oral administration in the Syrian tradition, alone or associated to local applications on the painful part of “dough” prepared mixing the powder with water. In Jordanian traditional medicine, the root bark are macerated and placed between gauze on aching area for 15-25 min in order to relieve the inflammation and muscle pain (Hudaib *et al.*, 2008). This ability to reduce pain in humans is particularly interesting because few natural compounds have been recognized as efficacious to date.

According to the traditional uses of this root overall in the medium-oriental area, aim of this study was to evaluate by *in vivo* models the ability of some extracts obtained by a Syrian sample of *Capparis spinosa* L. root to reduce the osteoarticular pain. The study was carried out with four different *Capparis spinosa* extracts tested in inflammatory and osteoarticular rat pain models. Pain was measured by paw pressure and hind limb weight bearing alterations tests measuring the effects over time from 15 to 60 min after the oral administration.

Material and Methods

*Complete Freund's Adjuvant-Induced Inflammatory Arthritis*

Articular damage was induced by injection of complete Freund’s adjuvant (CFA, Sigma-Aldrich) into the right knee joint. Briefly, the rats were slightly anesthetized by 2% isoflurane, the left leg skin was sterilized with 75% ethyl alcohol, and the lateral malleolus located by palpation; then, a 28-gauge needle was inserted vertically to penetrate the skin and turned distally for insertion into the articular cavity at the gap between the tibiofibular and tarsal bone until a distinct loss of resistance was felt. A volume of 50μL of CFA was then injected (day 0). The paw pressure and the incapacitance tests (see below) were performed 14 days after CFA injection.
Monoiodoacetate-Induced Osteoarthritis

Knee osteoarthritis was induced by injection of monoiodoacetate (MIA, Sigma-Aldrich) into the right knee joint. On day 0, rats were slightly anesthetized by 2% isoflurane, the left leg skin was sterilized with 75% ethyl alcohol, and the lateral malleolus located by palpation; then, a 28-gauge needle was inserted vertically to penetrate the skin and turned distally for insertion into the articular cavity at the gap between the tibiofibular and tarsal bone until a distinct loss of resistance was felt. 2 mg MIA in 25 μL saline was delivered into the left articular cavity. The paw pressure and the incapacitance tests (see below) were performed at day 14.

Drug treatments

All the extracts reported in Table 1 were suspended in 1% CMC and administered per os. The different doses were measured as dried amount of the corresponding extracts.

The preparation of the root samples has been reported in the chapter 6 of this Thesis; the following samples were evaluate: the hydroalcoholic extract (EtH₂Os), the decoction (DEC), the methylenchloride extract (CH₂Cl₂) and its correlated aqueous residue(H₂O-Res). All the dried extracts were suspended in 1% CMC and administered per os.

Paw pressure test

The nociceptive threshold in rats was determined with an analgesimeter (UgoBasile, Varese, Italy), according to the method described by Leighton et al. (1988). Briefly, a constantly increasing pressure was applied to a small area of the dorsal surface of the paw using a blunt conical probe by a mechanical device. Mechanical pressure was increased until vocalization or a withdrawal reflex occurred while rats were lightly restrained. Vocalization or withdrawal reflex thresholds were expressed in grams. Rats scoring below 40 g or over 75 g during the test before drug administration (25%) were rejected. An arbitrary cut-off value of 250 g was adopted. Fourteen days after the operation rats were tested twice in 30 min and mean was shown. The data were collected by an observer who was blinded to the protocol.

Incapacitance test

Weight bearing changes were measured using an incapacitance apparatus (Linton Instrumentation, UK) detecting changes in postural equilibrium after a hind limb injury. Rats
were trained to stand on their hind paws in a box with an inclined plane (65° from horizontal). This box was placed above the incapacitance apparatus. This allowed us to independently measure the weight that the animal applied on each hind limb. The value considered for each animal was the mean of 5 consecutive measurements. In the absence of hind limb injury, rats applied an equal weight on both hind limbs, indicating a postural equilibrium, whereas an unequal distribution of the weight on hind limbs indicated a monolateral decreased pain threshold. Data are expressed as the difference between the weight applied on the limb contralateral to the injury and the weight applied on the ipsilateral one (Δ Weight).

**Preliminary results**

*Effects of Capparis spinosa on CFA-Induced Inflammatory Arthritis*

With the aim of testing the pharmacological activity of *Capparis spinosa* root in articular inflammatory damage resembling human rheumatoid arthritis, the extracts were evaluated in the CFA-model. The pain threshold was measured 14 days after intra-articular CFA injection by paw pressure and incapacitance tests. The mechanical withdrawal threshold in ipsilateral- (CFA + saline) treated paw was significantly reduced as compared to the controlateral paw and control animals (saline + saline). The dried hydroalcoholic extract (at the doses of 300, 100 and 30 mg kg\(^{-1}\)), 15 minutes after administration, increased the withdrawal threshold and was still effective after 45 minutes. At the dose of 3 mg kg\(^{-1}\) was effective only 15 minutes after administration(Table 1).

CH\(_2\)Cl\(_2\) extract 10 mg kg\(^{-1}\) was also active for the first 45 minutes (Table 2),while the aqueous extract (100 and 30 mg kg\(^{-1}\)) increased the withdrawal threshold and was still effective after 60 minutes (P<0.05, Table 3). Decoction was effective at 100 and 30 mg kg\(^{-1}\) for the first 60 min (Table 4).

Incapacitance test confirmed these results. *Capparis spinosa* L. extracts significantly reduced hind paw unbalance in a time-dependent manner, being particularly effective 45 min after administration (data not shown). Decoction, at the dose of 30 mg kg\(^{-1}\), showed activity until 75 minutes (P<0.05) as reported in Table 5.
**Table 1. Effect of single administration of *Capparis spinosa hydro alcoholic extract* on CFA induced rheumatoid arthritis in the rat: Paw pressure test**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Paw Pretest</th>
<th>15 min</th>
<th>30 min</th>
<th>45 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sham + vehicle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>65.8 ± 0.8</td>
<td>64.1 ± 0.0.8</td>
<td>64.2 ± 0.1</td>
<td>64.1 ± 0.8</td>
<td>65.0 ± 1.4</td>
</tr>
<tr>
<td>contralateral</td>
<td>64.1 ± 0.8</td>
<td>63.3 ± 0.8</td>
<td>64.1 ± 0.8</td>
<td>64.1 ± 0.8</td>
<td>64.2 ± 0.8</td>
</tr>
<tr>
<td><strong>CFA + C. spinosa 300 mg kg⁻¹</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>44.0 ± 0.6**</td>
<td>66.0 ± 1.1^^</td>
<td>62.0 ± 1.0^^</td>
<td>52.5 ± 1.3^^</td>
<td>46.5 ± 0.6</td>
</tr>
<tr>
<td>contralateral</td>
<td>65.5 ± 1.0</td>
<td>66 ± 1.1</td>
<td>65.5 ± 0.5</td>
<td>64.5 ± 1.0</td>
<td>65.0 ± 1.2</td>
</tr>
<tr>
<td><strong>CFA + C. spinosa 100 mg kg⁻¹</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>42.5 ± 1.3**</td>
<td>56.5 ± 0.7^^</td>
<td>54.5 ± 1.0^^</td>
<td>49.0 ± 0.1^^</td>
<td>45.0 ± 0.8</td>
</tr>
<tr>
<td>contralateral</td>
<td>66.0 ± 1.9</td>
<td>65.0 ± 0.8</td>
<td>64.5 ± 1.0</td>
<td>64.5 ± 1.0</td>
<td>62.5 ± 0.8</td>
</tr>
<tr>
<td><strong>CFA + C. spinosa 30 mg kg⁻¹</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>44.5 ± 0.5**</td>
<td>55.5 ± 1.0^^</td>
<td>53.5 ± 0.6^^</td>
<td>49.0 ± 0.6^^</td>
<td>47.0 ± 0.6^^</td>
</tr>
<tr>
<td>contralateral</td>
<td>63.0 ± 1.0</td>
<td>65.0 ± 1.5</td>
<td>64.0 ± 0.6</td>
<td>65.5 ± 0.6</td>
<td>62.5 ± 0.8</td>
</tr>
<tr>
<td><strong>CFA + C. spinosa 3 mg kg⁻¹</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>46.0 ± 1.1**</td>
<td>52.0 ± 1.6^^</td>
<td>49.0 ± 1.1</td>
<td>48.0 ± 1.4</td>
<td>46.0 ± 1.4</td>
</tr>
<tr>
<td>contralateral</td>
<td>64.5 ± 1.3</td>
<td>65.5 ± 1.0</td>
<td>64.0 ± 1.1</td>
<td>63.5 ± 1.7</td>
<td>64.0 ± 0.7</td>
</tr>
</tbody>
</table>

*P<0.05 and **P<0.01 vs sham + vehicle treated animals; ^P<0.05 and ^^P<0.01 vs CFA + *Capparis spinosa* dose mg kg⁻¹ (pretest) treated animals. Each value represents the mean of 5 rats.
Table 2. Effect of single administration of *Capparis spinosa CH$_2$Cl$_2$ extract* on CFA induced rheumatoid arthritis in the rat: *Paw pressure test*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Paw</th>
<th>Pretest</th>
<th>15 min</th>
<th>30 min</th>
<th>45 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sham + vehicle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>65.8 ± 0.8</td>
<td>65.0 ± 1.4</td>
<td>65.8 ± 0.8</td>
<td>64.2 ± 0.8</td>
<td>64.2 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>contralateral</td>
<td>63.3 ± 0.8</td>
<td>65.0 ± 1.4</td>
<td>65.0 ± 0.8</td>
<td>64.2 ± 2.0</td>
<td>63.3 ± 0.8</td>
<td></td>
</tr>
<tr>
<td><strong>CFA + C. spinosa 30 mg kg$^{-1}$</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>41.2 ± 0.7**</td>
<td>64.2 ± 0.7^^</td>
<td>56.7 ± 1.4^^</td>
<td>50.0 ± 1.3</td>
<td>44.2 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>contralateral</td>
<td>65.8 ± 0.7</td>
<td>65.8 ± 0.7</td>
<td>65.0 ± 1.3</td>
<td>64.2 ± 0.7</td>
<td>64.2 ± 0.7</td>
<td></td>
</tr>
<tr>
<td><strong>CFA + C. spinosa 10 mg kg$^{-1}$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>43.3 ± 0.7**</td>
<td>60.8 ± 0.7^^</td>
<td>55.8 ± 0.7^^</td>
<td>55.0 ± 0.5^^</td>
<td>43.3 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>contralateral</td>
<td>64.2 ± 0.7</td>
<td>64.2 ± 0.7</td>
<td>65.0 ± 1.3</td>
<td>62.5 ± 1.3</td>
<td>62.5 ± 1.3</td>
<td></td>
</tr>
<tr>
<td><strong>CFA + C. spinosa 3 mg kg$^{-1}$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>44.2 ± 0.7**</td>
<td>50.8 ± 0.7^^</td>
<td>49.2 ± 0.7^</td>
<td>45.8 ± 0.7</td>
<td>44.2 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>contralateral</td>
<td>65.0 ± 1.3</td>
<td>63.3 ± 1.9</td>
<td>65.0 ± 1.3</td>
<td>62.5 ± 1.3</td>
<td>64.2 ± 1.9</td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05 and **P<0.01 vs sham + vehicle treated animals; ^P<0.05 and ^^P<0.01 vs CFA + *Capparis spinosa* dose mg kg$^{-1}$ (pretest) treated animals. Each value represents the mean of 5 rats.
### Table 3. Effect of single administration of *Capparis spinosa* aqueous extract on CFA induced rheumatoid arthritis in the rat: *Paw pressure test*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Paw</th>
<th>15 min</th>
<th>30 min</th>
<th>45 min</th>
<th>60 min</th>
<th>75 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham + vehicle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>65.8 ± 0.8</td>
<td>65.0 ± 1.4</td>
<td>65.8 ± 0.8</td>
<td>64.2 ± 0.8</td>
<td>64.2 ± 0.8</td>
<td>64.2 ± 0.8</td>
</tr>
<tr>
<td>contralateral</td>
<td>63.3 ± 0.8</td>
<td>65.0 ± 1.4</td>
<td>65.0 ± 0.8</td>
<td>64.2 ± 2.0</td>
<td>63.3 ± 0.8</td>
<td>64.2 ± 0.8</td>
</tr>
<tr>
<td>CFA + <em>C. spinosa</em> 100 mg kg⁻¹</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>43.3 ± 1.4**</td>
<td>65.0 ± 1.2^</td>
<td>64.1 ± 0.7^</td>
<td>56.7 ± 0.7^</td>
<td>51.7 ± 0.7^</td>
<td>46.7 ± 0.7^</td>
</tr>
<tr>
<td>contralateral</td>
<td>65.8 ± 0.7</td>
<td>66.7 ± 0.7</td>
<td>64.1 ± 0.7</td>
<td>65.8 ± 0.7</td>
<td>65.0 ± 1.3</td>
<td>65.0 ± 1.3</td>
</tr>
<tr>
<td>CFA + <em>C. spinosa</em> 30 mg kg⁻¹</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>45.0 ± 1.3**</td>
<td>65.8 ± 0.7^</td>
<td>60.0 ± 0.5^</td>
<td>54.2 ± 0.7^</td>
<td>47.5 ± 2.1^</td>
<td>44.2 ± 0.7</td>
</tr>
<tr>
<td>contralateral</td>
<td>65.0 ± 1.3</td>
<td>63.3 ± 1.4</td>
<td>62.5 ± 1.3</td>
<td>62.5 ± 1.3</td>
<td>63.3 ± 0.7</td>
<td>64.2 ± 0.7</td>
</tr>
<tr>
<td>CFA + <em>C. spinosa</em> 3 mg kg⁻¹</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>45.8 ± 0.7**</td>
<td>57.5 ± 0.3^</td>
<td>58.3 ± 0.7^</td>
<td>48.3 ± 0.7</td>
<td>45.8 ± 0.7</td>
<td>n.d.</td>
</tr>
<tr>
<td>contralateral</td>
<td>60.8 ± 0.4</td>
<td>63.3 ± 0.7</td>
<td>64.2 ± 0.7</td>
<td>63.3 ± 0.7</td>
<td>64.2 ± 0.7</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

*P<0.05 and **P<0.01 vs sham + vehicle treated animals; ^P<0.05 and ^^P<0.01 vs CFA + *Capparisspinosa* dose mg kg⁻¹ (pretest) treated animals. Each value represents the mean of 5 rats.

### Table 4. Effect of single administration of *Capparisspinosa decoction* on CFA induced rheumatoid arthritis in the rat: *Paw pressure test*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Paw</th>
<th>15 min</th>
<th>30 min</th>
<th>45 min</th>
<th>60 min</th>
<th>75 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham + vehicle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>65.8 ± 0.8</td>
<td>65.0 ± 1.4</td>
<td>65.8 ± 0.8</td>
<td>64.2 ± 0.8</td>
<td>64.2 ± 0.8</td>
<td>64.2 ± 0.8</td>
</tr>
<tr>
<td>contralateral</td>
<td>63.3 ± 0.8</td>
<td>65.0 ± 1.4</td>
<td>65.0 ± 0.8</td>
<td>64.2 ± 2.0</td>
<td>63.3 ± 0.8</td>
<td>64.2 ± 0.8</td>
</tr>
<tr>
<td>CFA + <em>C. spinosa</em> 100 mg kg⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>42.9 ± 0.9**</td>
<td>64.2 ± 0.9^</td>
<td>64.2 ± 0.3^</td>
<td>48.7 ± 1.0^</td>
<td>43.4 ± 0.6</td>
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</tr>
<tr>
<td>contralateral</td>
<td>64.2 ± 0.7</td>
<td>69.2 ± 1.4</td>
<td>66.7 ± 0.3</td>
<td>64.6 ± 0.9</td>
<td>64.6 ± 0.9</td>
<td>65.2 ± 0.5</td>
</tr>
<tr>
<td>CFA + <em>C. spinosa</em> 30 mg kg⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>44.2 ± 0.7**</td>
<td>58.3 ± 0.7^</td>
<td>53.3 ± 0.7^</td>
<td>49.2 ± 0.7</td>
<td>45.8 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>contralateral</td>
<td>65.0 ± 1.3</td>
<td>65.0 ± 1.3</td>
<td>64.2 ± 0.7</td>
<td>64.2 ± 0.7</td>
<td>63.3 ± 0.7</td>
<td>63.3 ± 0.7</td>
</tr>
<tr>
<td>CFA + <em>C. spinosa</em> 3 mg kg⁻¹</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>45.8 ± 1.4**</td>
<td>55.0 ± 0.5^</td>
<td>52.5 ± 1.3^</td>
<td>46.7 ± 0.7</td>
<td>45.0 ± 0.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>contralateral</td>
<td>65.0 ± 0.5</td>
<td>65.8 ± 0.7</td>
<td>65.0 ± 0.5</td>
<td>65.0 ± 0.5</td>
<td>64.2 ± 0.7</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

*P<0.05 and **P<0.01 vs sham + vehicle treated animals; ^P<0.05 and ^^P<0.01 vs CFA + *Capparisspinosa* dose mg kg⁻¹ (pretest) treated animals. Each value represents the mean of 5 rats.
Table 5. Effect of single administration of *Capparisspinosa* decoction on CFA induced rheumatoid arthritis in the rat: 
*Incapacitance test*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pretest (g)</th>
<th>15 min (g)</th>
<th>30 min (g)</th>
<th>45 min (g)</th>
<th>60 min (g)</th>
<th>75 min (g)</th>
<th>90 min (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham + vehicle</td>
<td>1.9 ± 2.7</td>
<td>-2.2 ± 3.2</td>
<td>2.0 ± 1.0</td>
<td>3.3 ± 3.7</td>
<td>2.1 ± 1.8</td>
<td>3.7 ± 0.5</td>
<td>2.0 ± 0.7</td>
</tr>
<tr>
<td>CFA + C. spinosa 100 mg kg⁻¹</td>
<td>62.4 ± 1.4**</td>
<td>13.5 ± 1.5^^</td>
<td>15.3 ± 2.8^^</td>
<td>25.2 ± 2.4^^</td>
<td>38.6 ± 0.9^^</td>
<td>57.9 ± 1.3</td>
<td>n.d.</td>
</tr>
<tr>
<td>CFA + C. spinosa 30 mg kg⁻¹</td>
<td>61.3 ± 0.2**</td>
<td>12.9 ± 0.9^</td>
<td>21.2 ± 1.0^^</td>
<td>31.1 ± 1.3^^</td>
<td>46.2 ± 2.4^^</td>
<td>56.5 ± 2.3^</td>
<td>60.1 ± 0.5</td>
</tr>
<tr>
<td>CFA + C. spinosa 3 mg kg⁻¹</td>
<td>49.6 ± 0.9**</td>
<td>39.0 ± 0.8^</td>
<td>47.1 ± 1.5</td>
<td>49.3 ± 4.2</td>
<td>56.0 ± 1.1</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

*P<0.05 and **P<0.01 vs sham + vehicle treated animals; ^P<0.05 and ^^P<0.01 vs CFA + *Capparisspinosa* dose mg kg⁻¹ (pretest) treated animals. Each value represents the mean of 5 rats.
Effect of Capparisspinosa on MIA-Induced Osteoarthritis

The effectiveness of capparis extracts was evaluated in the rat osteoarthritis induced by MIA. Fourteen days after MIA, the weight tolerated on the ipsilateral paw (MIA + saline) was significantly reduced as compared to the contralateral paw and control animals (saline + saline).

Hydroalcoholic extract (300 mg kg\(^{-1}\)), 15 minutes after p.o. administration, increased the withdrawal threshold and was still effective after 45 minutes. At the dose of 100 and 30 mg kg\(^{-1}\) it was effective until 45 minutes (Table 6).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Paw</th>
<th>Pretest</th>
<th>15 min</th>
<th>30 min</th>
<th>45 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham + vehicle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>62.5 ± 1.4</td>
<td>64.2 ± 0.8</td>
<td>64.2 ± 0.8</td>
<td>64.2 ± 0.8</td>
<td>64.2 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>contralateral</td>
<td>64.2 ± 0.8</td>
<td>62.5 ± 1.4</td>
<td>63.3 ± 0.8</td>
<td>65.0 ± 1.4</td>
<td>64.2 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>MIA + C. spinosa 300 mg kg(^{-1})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>40.6 ± 0.6**</td>
<td>59.3 ± 0.6^^</td>
<td>53.1 ± 0.6^^</td>
<td>48.1 ± 0.6^^</td>
<td>40.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>contralateral</td>
<td>64.2 ± 0.8</td>
<td>64.4 ± 0.6</td>
<td>64.4 ± 1.2</td>
<td>64.3 ± 0.6</td>
<td>63.7 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>MIA + C. spinosa 100 mg kg(^{-1})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>40.6 ± 0.6**</td>
<td>53.7 ± 1.3^^</td>
<td>49.4 ± 0.6^^</td>
<td>45.0 ± 1.0^</td>
<td>43.1 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>contralateral</td>
<td>66.8 ± 0.6</td>
<td>65.0 ± 1.8</td>
<td>65.6 ± 1.2</td>
<td>63.8 ± 0.7</td>
<td>64.4 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>MIA + C. spinosa 30 mg kg(^{-1})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>43.1 ± 1.2**</td>
<td>54.4 ± 0.6^^</td>
<td>50.6 ± 0.6^^</td>
<td>48.1 ± 0.6^</td>
<td>44.4 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>contralateral</td>
<td>63.4 ± 1.6</td>
<td>63.8 ± 1.6</td>
<td>63.4 ± 0.7</td>
<td>63.8 ± 1.6</td>
<td>63.8 ± 0.7</td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05 and **P<0.01 vs sham + vehicle treated animals; ^P<0.05 and ^^P<0.01 vs MIA + Capparisspinosa dose mg kg\(^{-1}\) (pretest) treated animals. Each value represents the mean of 5 rats.

CH\(_2\)Cl\(_2\) extract showed the same effectiveness than hydroalcoholic extract at the doses of 30 and 10 mg kg\(^{-1}\). Moreover, the aqueous extract significantly reduced hind limb weight bearing alterations, being effective until 60 minutes after administration at the dose of 100 mg kg\(^{-1}\) (data not shown). Regarding the decoction, at the doses of 100 and 30 mg kg\(^{-1}\) it was effective until 45 minutes (P<0.05); the dose of 3 mg kg\(^{-1}\) showed also effectiveness until 30 min (data not shown).

Incapacitance test confirmed the effect of Capparisspinosa extracts to reduce hind paw unbalance in rat; in particular the aqueous extract at the dose of 100 mg kg\(^{-1}\) showed
effectiveness until 75 minutes (Table 7). Decoction showed activity only for the first 45 minutes (P<0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pretest</th>
<th>15 min</th>
<th>30 min</th>
<th>45 min</th>
<th>60 min</th>
<th>75 min</th>
<th>90 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham + vehicle</td>
<td>1.9 ± 2.7</td>
<td>-2.2 ± 3.2</td>
<td>2.0 ± 1.0</td>
<td>3.3 ± 3.7</td>
<td>2.1 ± 1.8</td>
<td>3.7 ± 0.5</td>
<td>2.0 ± 0.7</td>
</tr>
<tr>
<td>CFA + C. spinosa 100 mg kg⁻¹</td>
<td>63.0 ± 2.1**</td>
<td>3.6 ± 1.3^^</td>
<td>18.0 ± 4.3^^</td>
<td>24.2 ± 0.8^^</td>
<td>33.5 ± 2.0^^</td>
<td>51.8 ± 0.2^^</td>
<td>60.1 ± 2.5</td>
</tr>
<tr>
<td>CFA + C. spinosa 30 mg kg⁻¹</td>
<td>66.6 ± 5.2**</td>
<td>13.5 ± 1.4^^</td>
<td>23.0 ± 0.6^^</td>
<td>33.5 ± 1.0^^</td>
<td>52.9 ± 2.7</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CFA + C. spinosa 3 mg kg⁻¹</td>
<td>53.6 ± 1.6**</td>
<td>30.9 ± 0.6^^</td>
<td>29.9 ± 0.5^^</td>
<td>41.3 ± 1.0^^</td>
<td>53.7 ± 0.1</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

*P<0.05 and **P<0.01 vs sham + vehicle treated animals; ^P<0.05 and ^^P<0.01 vs MIA + Capparisspinosa dose mg kg⁻¹ (pretest) treated animals. Each value represents the mean of 5 rats.

These preliminary results show that the aqueous extracts provided the best results, and the polar metabolites appear to be the most effective. On the contrary, the alkaloids (capparisina and its analogues) not seem to be the most active molecules.

However, other chemical characterization studies are needed.

In table 8 is shown the alkaloid content of the 4 tested samples expressed as total amount and as main alkaloids.
Table 8: Alkaloid content in the extracts derived by a fractionation process carried out at alkaline pH compared with the decoction and hydro alcoholic extract of whole root.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Extracts</th>
<th>Part of the root</th>
<th>main alkaloids (mg/g)</th>
<th>Total Alkaloids (mg/g DE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>S1</td>
<td>DEC</td>
<td>Whole</td>
<td>0,36</td>
<td>1,27</td>
</tr>
<tr>
<td>S2</td>
<td>EtH₂O</td>
<td>Whole</td>
<td>0,54</td>
<td>3,15</td>
</tr>
<tr>
<td>S6</td>
<td>CH₂Cl₂</td>
<td>Whole</td>
<td>0</td>
<td>1,67</td>
</tr>
<tr>
<td>S7</td>
<td>H₂O-Res</td>
<td>Whole</td>
<td>0,48</td>
<td>2,93</td>
</tr>
</tbody>
</table>

In light of the results shown in Tables 1--7 the aqueous extracts, DEC and H₂O-Res, have been resulted as more efficacious if compared with the EtH₂O and CH₂Cl₂ samples. These preliminary data suggest that the main alkaloids of *Capparis spinosa*, more concentrated in the CH₂Cl₂ extract and EtH₂O seem to be not the more active compounds in these in vivo test. Further studies are in progress to improve the knowledge on the composition of these aqueous extracts and to identify the main active metabolites of this root.