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Spring variation of total sugars and phenols on woody plants important for Red and Roe deer in Montesinho Natural Park (NE Portugal)

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Dissertation presented to the School of Agriculture of Bragança in partial Fulfillment of the requirements for the degree of Master of Science in Forest Resources Management

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Imane HADDOUCH

Abstract:

Red deer (*Cervus elaphus*) and Roe deer (*Capreolus capreolus*) are two ungulate species cohabite the same area in the Natural Park of Montesinho, this work aims to manage the study spring diet of both deer with exploring the importance of secondary metabolites on feeding selection by analysis phytochemical of three Mediterranean species *Quercus rotundifolia*, *Pterospartum tridentatum*, *Cistus ladanifer*. The collection of data was achieve during flowering period, from fives sites in the park.

Phytochemical analysis based on extraction of total sugar by methanol-aqueous (80,20) reagent using DNS method, and the extraction of total Phenolic compounds TPC by hot methanol and folin-ciocalteu reagent and using spectrophotometer to read the absorbance of each sample. The quantification of sugar was use as a calibration curve the "glucose", and for phenol we have used "acid Gallic".

The statistical analysis (Anova test, Tukey HSD) of all results obtained from the both extraction showed significative differences between plant species and between months and were used to compare between the choice of deer species on feeding during flowering period (March, April, June).*Quercus rotundifolia* showed highest phenol and sugar contents and seems to be avoided to consumption by deer. In contrast *Pterospartum tridentatum* showed an increase of sugar concentration and maintain phenolic contentsthat could explain the consumption during this period.

Keywords: *Capreolus capreolus*, *Cervus elaphus*, *Cistus ladanifer*, *Pterospartum tridentatum, Quercus rotundifolia*, total sugar, total phenol, diet composition

Resumo

O veado (*Cervus elaphus*) e o Corço (*Capreolus capreolus*) são duas espécies unguladas que coabitam a mesma área no parque natural de Montesinho, este trabalho tem como objetivo gerenciar a dieta de primavera do estudo de ambos os veados com a análise da importância dos metabolitos secundários na seleção de alimentação Por análise fitoquímica de três espécies mediterrâneas "*Quercus rotundifolia*", "*Pterospartum tridentatum*", "*Cistus ladanifer*". A coleta de dados foi alcançada durante o período de floração, a partir de cinco locais no parque.

Análise fitoquímica baseada na extração de açúcar total por reagente metanol-aquoso (80,20) usando o método DNS e extração de compostos fenólicos TPC por metanol quente e reagente folin-ciocalteu e utilizando espectrofotômetro para ler a absorvância de cada amostra. A quantificação de açúcar foi utilizada como curva de calibração, a "glicose", e para fenol utilizamos "ácido galo".

A análise estatística (teste Anova, Tukey HSD) de todos os resultados obtidos a partir das duas extrações mostrou diferenças significativas entre as espécies vegetais e entre meses e foram utilizadas para comparar a escolha dos cervídeos na alimentação, durante o período de floração (março, abril, junho). *Quercus rotundifolia* apresentou maior teor de fenol e açúcar e parece ser evitado para o consumo por veado. Em contraste, *Pterospartum tridentatum* mostrou um aumento da concentração de açúcar e manteve conteúdo fenólico, o que poderia explicar o consumo durante este período.

Palavras-chave: *Capreolus capreolus, Cervus elaphus, Cistus ladanifer, Pterospartum tridentatum, Quercus rotundifolia,* açúcares totais, fenóis totais, dieta

Résumé

Le cerf-élaphe(*Cervus elaphus*) et le chevreuil (*Capreolus capreolus*) sont deux espèces d'ongulés cohabitent dans la même région du parc naturel de Montesinho, ce travail vise à gérer l'étude du régime alimentaire des deux cerfs en explorant l'importance des métabolites secondaires lors de la sélection de l'alimentation Par analyse phytochimique de trois espèces méditerranéennes "*Quercus rotundifolia*", "*Pterospartum tridentatum*", "*Cistus ladanifer*".

La collecte des données a été réalisée pendant la période de floraison, à partir des cinq sites du parc. Analyse phytochimique basée sur l'extraction du sucre total par le réacteur méthanol-aqueux (80,20) utilisant la méthode DNS et l'extraction des composés phénoliques totaux TPC par le réacteur chaud au méthanol et au folin-ciocalteu et en utilisant un spectrophotomètre pour lire l'absorbance de chaque échantillon. La quantification du sucre a été utilisée comme courbe d'étalonnage du «glucose», et pour le phénol, nous avons utilisé «acide gallique». L'analyse statistique (test Anova, Tukey HSD) de tous les résultats obtenus à partir des deux extractions a montré des différences significatives entre les espèces végétales et entre les mois et ont été utilisées pour comparer le choix des espèces de cerf sur l'alimentation pendant la période de floraison (mars, avril et juin). *Quercus rotundifolia* a affiché des teneurs en phénol et en sucre les plus élevées et semble être évité par la consommation chez les cerfs. En revanche, *Pterospartum tridentatum* a montré une augmentation de la concentration de sucre et de maintenir un contenu phénolique qui pourrait expliquer la consommation pendant cette période.

Mots clés: *Capreolus capreolus, Cervus elaphus, Cistus ladanifer, Pterospartum tridentatum, Quercus rotundifolia*, sucres totaux, phénols totaux, régime alimentaire

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CHAPTER I: INTRODUCTION

Introduction:

All living beings-humans, birds, animals, insects are worthy of consideration and respect. Animals are not viewed as mere resources but as individuals and communities in their own right, they are as a special part of God's creation, so we need to treat them with compassion and not to abuse their life as community.

Several studies of large ungulate abundance have focused on animal abundance alone without linking it to habitat characteristics (Whipple et al,; 1994 Mayle et al., 2000;Smart et al., 2004). In addition, the relation between herbivores and plants has also been studied around the world, not only for wildlife (Hanley 1982, Holechek *et al.* 1982, Gordon e Iason 1989, Bryant *et al.* 1992, Bugalho *et al.* 2001, Illius *et al.* 2002) but also for domestic ungulates (Bourbouze e Guessous 1979, Rodriguez 1984, Launchbaugh *et al.* 2001).Habitat use is related to the manner that an animal uses a set of resources to fulfill its requirements (Block and Bernnan, 1993), namely food and cover. Habitat selection can be defined as the process involved in the choice of a resource (Johnson, 1980). Besides the land cover units, variables like ecotone proximity, vegetation productivity, water altitude, aspect and slope are identified as key factors influencing red deer and roe deer habitat use(e.g. San José etal.,1997;Garin,2000;Licoppe and de Crombrugghe, 2003). There are different methods for studying the resources available for deer diet. Direct methods is based on observation or tracking of animals, and provides measurements of habitat use/selection at individual or population level, through the assessment of specific parameters of the individuals (e.g. sex, age, productivity...). The indirect methods rely on the detection and counting of signs of animal presence, such as fecal pellets, or tracks, as that seems to be suitable for.

This document will present an analysis of habitat use by *Capreolus capreolus* and *Cervus elaphus*, adopting indirect method and specially fecal pellet method.(Alves et al, 2014). In fact, studies about habitat use/selection may provide the information needed to the management plans in several European countries (e.g. Italy: Lovari et al., 2007; Norway: Godvik et al., 2009; Poland: Borkowski, 2004; Borkowski and Ukalska, 2008; Spain: Carranza et al., 1991;Garin, 2000).

Animal preference for habitat characteristics are not uniform, and subsequently neither are the benefits of habitat management practices the same under all conditions (Pengelly 1972). Deer species may make considerable use of riparian areas, and clear cuts where forage is better quality or more abundant but avoid such areas if human disturbance exceeds certain threshold. In this document, we choose five sites on the natural park of “Montesinho” which are more occupied by roe deer and red deer, notwithstanding the fact that even these sites are located on hunting areas, where we can find also wolf as a primary predator to deer. The interaction among species modifies the ecosystem, particularly those interactions that control flows of energy and materials among trophic levels.

Understanding why deer select some foods and avoid others may enable us to manipulate their choices. Knowing how to reduce their interest in eating seedlings would greatly enhance our ability to manage their negative impacts. In general, herbivore diet selection is attributed to obtaining a balanced intake of nutrients (Westoby 1978) and minimizing toxins or plant secondary metabolites (Freeland and Janzen 1974) Proteins and energy are essential for survival (Parker et al. 1999). Secondary metabolites can be deleterious to an animal's health, limiting digestibility of other nutrients, or at least requiring additional resources to detoxify toxins (Cheeke and Shull 1985). An animal's ability to cope with toxins reflects not only the kind and amount of toxins, but available nutrients in all forages on offer (Villalba et al. 2002a). An ungulate's choice to consume a tree seedling therefore depends on the type and concentration of secondary metabolites and the animal's nutritional state. Deer tend to prefer foods with low concentrations of terpenes (Scholl et al. 1977, Connolly et al. 1980, Duncan et al. 1994) and tannins (Radwin et al. 1978, Alm et al. 2002). Deer given protein supplements, however, increase their intake of tannin-containing sagebrush (Artemisia spp.; Chris Peterson, Utah St. University, pers. comrn.). Similarly, domestic ungulates fed supplemental macro-nutrients increased their intake of foods that contain toxins as diverse as lithium chloride (Wang and Provenza 1996), terpenes (Banner et al. 2000, Villalba et al. 2002b), menthol (Illius and Jessop 1996), and tannins (Villalba et al. 2002~). Conversely, animals ingesting low-sodium diets restrict their intake of toxins, and the sodium-depleting effects of many toxins may deter herbivores from eating plants that are low in sodium (Provenza et al. 2003). Thus, manipulating nutrients available to an animal may offer the potential to increase their intake of plants habitually avoided or to decrease their intake of plants habitually eaten. This work study the variation of primary and secondary metabolites (with these parameters: total sugar and total phenol compounds), the effect of this variation on woody plants available for Roe deer and Red deer feeding in Montesinho Natural Park (NE Portugal).

2. Research aims:

This work aims to contribute to manage the habitat use of “red deer and roe deer” as two species of deer on the natural park of Montesinho, and also to select feeding strategy of this ungulate specie.

It includes these objectives:

- Explore the importance of Primary and secondary metabolites on feeding selection :

- quantify total sugar, total phenols content on woody plant collected at flowering period from different sites in natural park of Montesinho.

-Relate these results to changes in Deer diet in North-eastern Portugal, and explain the food choice’s by deer species.

3. Thesis outline:

This thesis was structured in five chapters that cover the research aims stated above:

Chapter 1: firstly we start by an introduction in which we will explain briefly the state of the topic and objectives that we hope to reach.

Chapter 2: Secondly we will give an overview of the study area; describing the different sites and factors affecting the preference of sites by deer and also influencing the feeding types and then the ecosystem process.

Chapter 3: in this chapter, we will describe all methods and materials used to reach thesis aims, analysis of sugar and phenol component.

Chapter4: from the results obtained, we will discuss outcomes/outputs and give a comparison between what we get and other experimentation on the same topic. Also, we discuss the relation between secondary metabolites and the change of habitat use by ungulate species and their change on feeding.

Chapter 5: finally, small conclusions where we cite some significantly result with real information about this work.

CHAPTER II: OVERVIEW OF THE STUDY AREA

1-Ecology of Roe and Red deer:

1.1 : Roe Deer (Capreolus capreolus):

The roe deer (*Capreolus capreolus*) is a unique and important animal: unique, because it is the only artiodactyls known to exhibit the phenomenon of delayed implantation, and important because by studying this process one can gain insight into the way in which the uterus is able to control the growth of the embryo.

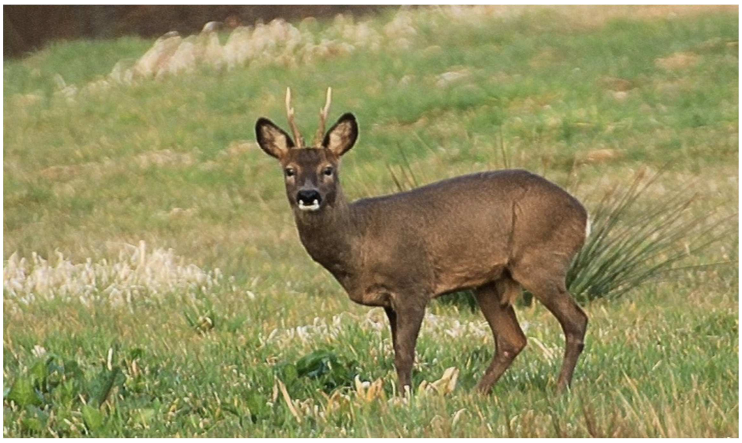


Figure1: Picture of *capreolus capreolus* from "Pixabay"

1-1-1.Taxonomy:

Table1 : Roe deer taxonomy (Linnaeus, 1758)

Kingdom	Animally
Phylum	Chordate
Class	Mammalian
Order	Cetartiodactyla
Family	Cervidae
Scientific Name	*Capreolus capreolus*

1-1-2: Physical appearance:

The roe deer is a relatively small deer, with a body length of 95–135 cm, a shoulder height of 65–75 cm and a weight of 15–35 kg. It has rather short, erect antlers and a reddish body with a grey face. Its hide is golden red in summer, darkening to brown or even black in winter, with lighter undersides and a white rump patch; the tail is very short (2–3 cm or 0.8–

1.2 in), and barely visible. Only the males have antlers. The first and second set of antlers are unbranched and short (5–12 cm or 2.0–4.7 in), while older bucks in good conditions develop antlers up to 20–25 cm (8–10 in) long with two or three, rarely even four points. When the male's antlers begin to re-grow, they are covered in a thin layer of velvet-like fur which disappears later on after the hair's blood supply is lost. Males may speed up the process by rubbing their antlers on trees, so that their antlers are hard and stiff for the duels during the mating season.(Wilson and Reeder 2005).

1-1-3: Range description:

The Roe Deer has a large range in the Palearctic region. It is found through most of Europe (with the exception of Ireland, Cyprus, Corsica, Sardinia, and most of the smaller islands), including western Russia (Stubbe 1999). Outside Europe, it occurs in Turkey, northern Syria, northern Iraq, northern Iran, and the Caucasus (Wilson and Reeder 2005). Along the Black Sea coast and in the northern Aegean region of Turkey, the Mediterranean sub-populations are close to extinction.

Figure2: Map of roe deer distribution by" IUCN (International Union for Conservation of Nature) 2016.*Capreolus capreolus*. The IUCN Red List of Threatened Species. Version 2017-1"

1-1-4: General Ecology:

Roe deer exist in solitary or in small groups, with larger groups typically feeding together during the winter. At exceptionally high densities, herds of 15 or more roe deer can be seen in open fields during the spring and summer. The reproductive cycle of the roe deer is summarized in fig. 3. The entire gestation period lasts about 10 months, beginning with the rut in late July or early August. Delayed implantation or embryonic diapause (Short & Hay, 1966) starts when the blastocyst has lost its zona pellucida, a few days after ovulation, and continues for 5 months until the end of December or the beginning of January. During this period the blastocyst only increases in diameter from 1 to 5 mm, but during the first 2 weeks of January a normal rate of embryonic growth is suddenly resumed and the blastocyst rapidly elongates. Embryonic elongation is followed by placental attachment and a further 5 months of normal gestation. Finally, one, two, or occasionally three, young are born in May. Their diet is varied and includes buds and leaves of deciduous trees and shrubs, bramble, rose, ivy, herbs, conifers, ferns, heather and grasses.

- The breeding season, known as the rut, is from mid-July to the end of August. During this time males become very aggressive in defending their territories. They fight other males by locking antlers and pushing and twisting. Fighting may cause injuries and occasionally one or both may die.

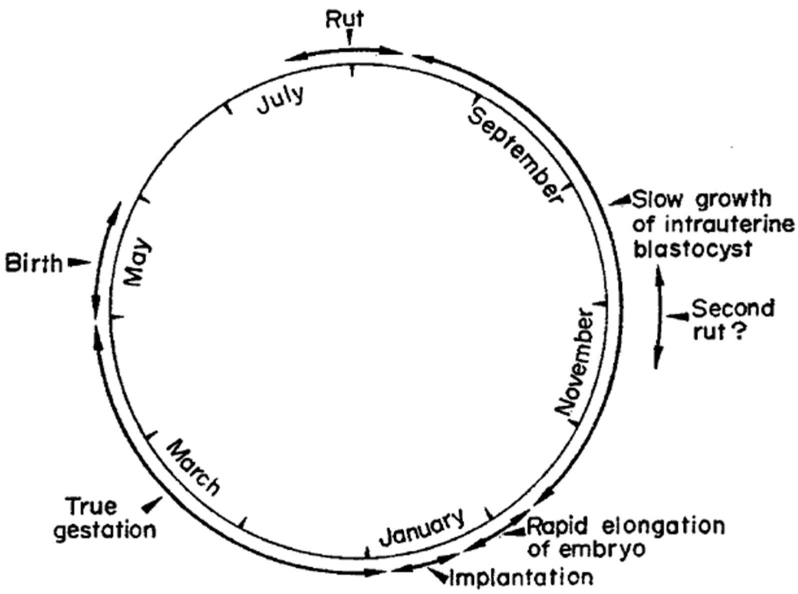


Figure3: The reproductive cycle of the roe deer (*Capreolus capreolus*)

1.2 :Red deer: Cervus elaphus. L

The Red deer has a long history in Britain, is one of only two native deer species in the UK, it's a beast highly prized by hunters, naturalists, artists, poets and photographers alike.

Figure4: Picture of *Cervus elaphus* From the British deer society "bds.org.uk"

1-2-1: Taxonomy:

Table2 : Red deer taxonomy (Linnaeus, 1758)

Kingdom	Animally
Phylum	Chordate
Class	Mammalian
Order	Cetartiodactyla
Family	Cervidae
Scientific Name	*Cervus elaphus*

1-2-2: Physical appearance:

Red deer male (stag) is typically 175 to 250 cm long and weighs 160 to 240 kg; the female (hind) is 160 to 210 cm long and weighs 120 to 170 kg. The tail adds another 12 to 19 cm and shoulder height is about 95 to 130 cm (37 to 51 in). In Scotland, stags average 201 cm in head-and-body length and 122 cm high at the shoulder and females average 180 cm (71 in) long and 114 cm (45 in) tall. Size varies in different subspecies with the largest, the huge but small-antlered deer of the Carpathian Mountains (C. e. elaphus), and weighing up to 500 kg. At the other end of the scale, the Corsican red deer (C. e. Corsicans) weighs about 80 to 100 kg (180 to 220 lb), although red deer in poor habitats can weigh as little as 53 to 112 kg (120 to 250 lb). European red deer tend to be reddish-brown in their summer coats.

The males of many subspecies also grow a short neck mane during the autumn. Red deer hinds (females) do not have neck manes. The European red deer is adapted to a Woodland environment.

1-2-3: Range description:

The Red Deer has a distribution extending from Europe into North Africa and the Middle East. It is widely but somewhat patchily distributed throughout most of continental Europe, although it is absent from northern Fennoscandia and largely from European Russia. It is present on a number of islands, including the British Isles and Sardinia. Whether the Red Deer is native to Ireland or introduced is still under debate (Carden et al.2011).

Red deer in Europe generally spend their winters at lower altitudes in more wooded terrain. During the summer, they migrate to higher elevations where food supplies are greater and better for the calving season.

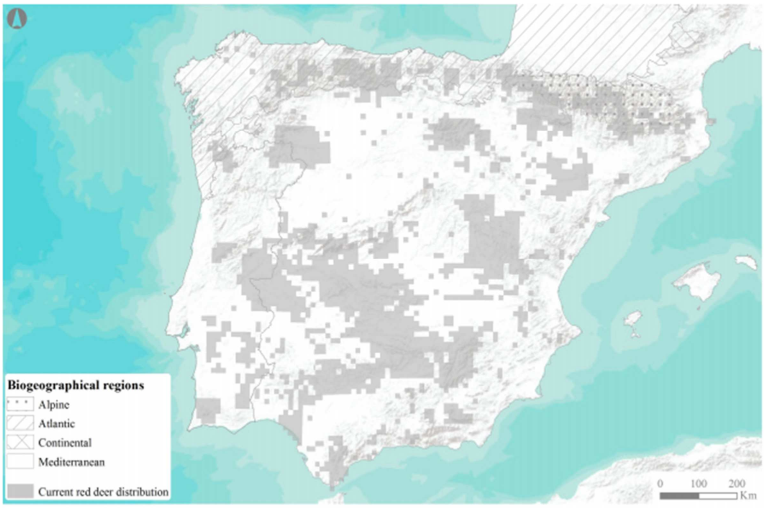


Figure 5: Current distribution of red deer (*Cervus elaphus*) in the Iberian Peninsula (depicted in grey), from Carranza (2007) and Salazar (2009). Biogeography regions are also shown (from European Environment Agency, 2011).

1-2-4: General Ecology:

- Red deer inhabits open deciduous woodland, mixed deciduous-coniferous and coniferous woodland, upland moors and open mountainous areas (sometimes above the tree line), Mediterranean maquis scrub, natural grasslands, pastures and meadows (Koubek and Zima 1999). It prefers broadleaved woodland interspersed by large meadows. In woodland, its diet consists mainly of shrub and tree shoots, but in other habitats it also consumes grasses, sedges and shrubs. Fruit and seeds are important in autumn.

- Females are sexually and socially mature at 1.5-2.5 years. Males attain sexual maturity as yearlings but they continue growing until at least 6 years of age and cannot compete for females with other males until then, by which time they reach social maturity.

2-Roe/ Red deer population:

2-1- In Europe:

During the past century, cervid populations throughout much of Europe have undergone substantial change. In Western Europe, native species such as red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), moose (*Alces alces*), and reindeer (*Rangifer tarandus*), experienced dramatic increases in numbers and have expanded their ranges as plantation forestry has increased and hunting has declined. The roe deer (*Capreolus capreolus*) has been particularly successful in utilizing lowland woodlands and agricultural habitat, and is today expanding throughout much of its range. It has recolonized areas from which it became extinct in recent historical times (e.g. Norway, and England and Wales). The red deer too has expanded its range considerably throughout most of Western Europe, particularly in the Alps and in parts of Scandinavia. Since the post-war period, both moose and reindeer have expanded their ranges and increased in numbers throughout much of Scandinavia. Declines in Western Europe have been few with only one subspecies of red deer (C. e. Corsicans) considered Endangered. The situation is far from clear in Central and Eastern Europe. In Central Europe, for example, the roe deer has been extremely successful, expanding its range and numbers by utilizing open agricultural fields. There is a paucity of information on the deer fauna in the former USSR, although several threatened species are reported. (IUCN, 1998)

2-2- In Portugal:

In Portugal, the species was extinct - in open areas - during the first 3/4 of the 20th century. The few rare observations of deer on free ground (so-called until very recently) occurred in areas bordering Spain (Bragança, Castelo Branco and Barrancos), while in some walled areas the existing and previously preserved specimens were depleted Management, food quality and over density. However, in neighboring Spain, the species was always present and the deer was the main catch of the largest game throughout the last century, whether it was in hunting or approaching or possibly waiting.

Likewise in Portugal all populations result from reintroduction or natural expansion from trans-border Spanish populations which in turn were reintroduced. It occurs from sea level to above the tree line (*c.* 2,500 m) in the Alps. The distribution is much more patchy and fragmented than the apparent continuity suggested by the distribution map.

It is a widespread and abundant species across much of its current range, although there is increasing fragmentation of populations in northern Africa and central Europe, and the species has been lost from some areas. In all Europe excluding Russia, the species numbered 1.25 million individuals in 1985 and 2.4 million in 2005. Densities are typically 1-5 individuals per km², sometimes up to 15 individuals per km² (Wilson and Mittermeier 2011).

3: Conservation status:

3-1- Roe deer:

Roe deer have been hunted from prehistoric times. They became extinct in England, Wales and southern Scotland during the 18th century and populations were re-introduced to southern England (Dorset) and East Anglia in the 19th century. As they have become more abundant, they have been treated as "vermin" because of damage to forestry, agriculture and horticulture, and consequently numbers are controlled. Roe deer may now number as many as 500,000, and are increasing. Since the 1970s there has been an increased interest in exploitation of roe as a game species and for meat. As a result they are now covered by various Acts of Parliament which impose close seasons (when deer may not be hunted), firearms restrictions and controls on poaching.

3-2-Red deer:

Red Deer in Europe have been affected to a large extent by translocations not only between far distant populations and different subspecies within the continent, but also by translocations of deer within the same regions with admixture of different 'park deer' of various mixed stock with wild ranging deer of 'pure' stock, and also by imported conspecifics from Central Asia and of *C. canadensis* from North America, and introduced Sika (Niedzialkowska *et al.* 2011, Smith *et al.* 2014, McDevitt *et al.* 2009, McDevitt and Zachos

2014, Zachos and Hartl 2011). As a result, most of the present deer populations of Europe areeither known hybrids on a subspecific or even specific level or their breeding background is insufficiently known for excluding such a possibility. Systematic investigation into the history and the genetics of all European Red Deer populations is therefore needed as a base for establishing a European Red Deer Management Plan. Part of this plan should be the identification of unpolluted autochthonous populations of this species and protection of their genetic integrity, thus preserving as much as possible of what is left of its natural variation. The development of stable meta-population networks by developing corridors and habitat connectivity will be important to ensure the viability of populations of Red Deer in the future (Zachos and Hartl 2011).

3-3- Alternatives to feeding:

Is a better way to help and to improve the wild ungulates, they benefit when we preserve and restore natural habitats and reduce human-caused disturbances, leaving them alone to conserve their energy to survive severe winter conditions.

- The best way to help wild ungulates survive in severe weather is to maintain high-quality habitat year-round. If animals enter the winter in good condition, most survive persistent deep snow and cold temperatures. Even in well-functioning natural ecosystems, however, some animals succumb during winter months. This is natural, winter mortality helps keep ungulates populations in balance with the available habitat.
- Another way to help wild ungulates in winter is to avoid disturbing them. Animals must conserve their energy to survive in winter conditions. Human-related causes of disturbance such as from recreation (e.g. snowmobile activity) and chasing by domestic dogs can result in wild ungulates expending valuable energy.

4- Study Area:

4-1- Natural park of Montesinho:

4-1-1- Physical environment:

The study area is located in the northeastern part of Portugal, included in the Natural Park of Montesinho (PNM, Parque Natural de Montesinho). This park is a protected area located in the municipalities of Vinhais and Bragança, in the administrative NUT Alto Tras-os-Montes, the mountainous region of northeast Portugal. It was created in 1979 and consists of 748 km2 of natural wooded landscape and traditional mountain agricultural landscape, with highly variable gradients. PNM lies in the vast northeast Tras-os-Montes plateau, with average altitude around 750- 900m, which is part of the Iberian Meseta northern block (Medeiros. 1987; Ribeiro etaI., 1987).However, in PNM elevation ranges more than 1,000 m, from the lowest point in the River Mente (436 m). It is western border, to the top of Montesinho, at 1,487 m. The main altitudinal belts correspond also to the main landforms found in the area.Most of the rivers in the area run below 700 m, and their valleys are the dominant landform in this area. Deep, with narrow bottom and steep slopes, (i.e. V-shaped, Tributaries of Sabor river in PNM: left) Onor crossing Rio de Onor.

- In the mountains, above 1,000 m elevation, human occupation is almost nonexistent and hence cultivated land almost disappears, giving way to highland pastures (Agroconsultores e Coba, 1991). This contour bounds the mountain domains and surrounds the three highest peaks of the area, the southern edges of the Spanish Galician-Leonese mountains (Pereira et al., 2003),Coraa (1,272 m), Nogueira (1,318 m, in fact outside but close to the southern border of PNM) and Montesinho (1,487 m).

- Hypsometry and hydrography have thus been coupled in forming the land, on which plant communities have installed themselves, soils have deepened, humans have settled and which is finally opening to tourism and the interest of entrepreneurs. Streams drain the whole area to the Douro River, more than 100 km to the south. The Douro is the Portuguese river with the largest drainage basin (though 4/5 of it is in Spanish territory), and it ranks second in total length (after the Tagus). The Douro drainage basin accounts for 1/3 of the Portuguese surface water resources and half of the energy generated in hydropower plants (Ferreira, 2005).

- Climate in Montesinho area is Mediterranean, although other influences, derived from the geographic position and the relief of this area, affect the general pattern (Gonçalves, 1985). Continental effects come from the inner Iberian Peninsula, as Atlantic influence is hampered by impressive mountain ranges (more than 1,500 m elevation in the west and more than 2.000 m in the north, in Sanabria, Spain, close to the border). They bring an increase in seasonal contrast (annual range is IS-20°C), in dryness, and in inter-annual variability

(Gonçalves, 1985). Continental effects are relevant in the eastern tract of PNM but they are drowned out to the centre and west and where the mountain effects take their place. Altitude is in fact the factor that best explains spatial variations in temperature (most of the area is below 12°C) and precipitation (mostly over 800 mm) (Gonçalves,1985;Figueiredo,1990).

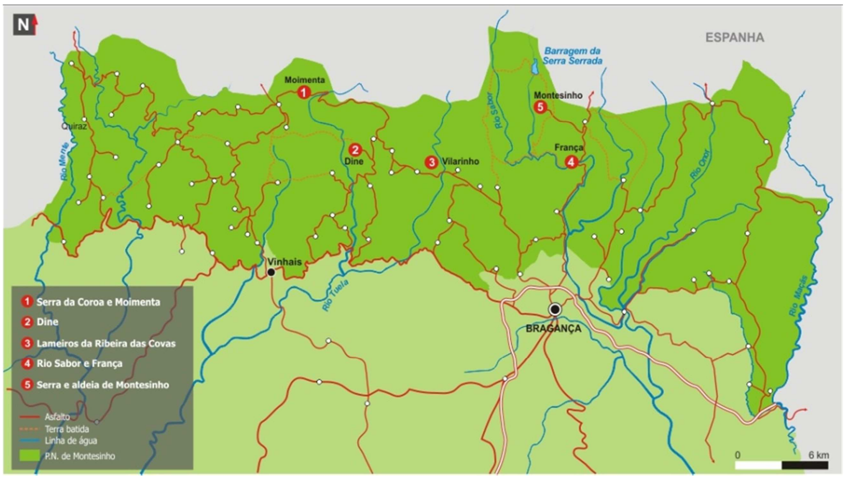


Figure6: Map of Natural park of Montesinho, published by natural.pt

4-1-2-Man and land use:

The PNM includes 92 small villages inhabited by less than 8,000 people. Demographically the western part of PNM, although less populated, has many villages, most of them with less than 200 inhabitants; the eastern part villages are fewer in number but more populated, frequently over 300 inhabitants. Economically the PNM still produces grains, chestnuts *Castanea sativa* and livestock, especially cattle and sheep. In recent years agriculture has declined, abandonment of the villages persists and the population is ageing. Most of the villages have too few children to keep the rural schools open. But it has been the traditional family livelihood of PNM inhabitants. This is based on small fanning and sheep herding that contributed definitely to the conservation of biodiversity in the region.(Castro., et all,.2009)

- The western part of the Montesinho Natural Park lies in the northern half of Vinhais municipality, where good examples of rural and agricultural lifestyles combined with wildlife preservation are found. At present, sweet chestnut plantations are the main driver or economic activity in Vinhais. Cattle husbandry of the local breed Mirandesa is also economically important but unfortunately it is declining.

Upland meadows, marked by a good network of hedgerows dominated by *Fraxinus angustifolia*, *Ulmus procera*, *Alnus glutinosa* and poplars *Populus* sp., together with heath lands and cropland, compose a very green landscape, typically associated with Mirandesa husbandry.(Castro., et all,.2009)

- The eastern part of the Montesinho Natural Park corresponds to the northern part Bragança municipality. Here, forestry, sheep herding and agriculture also combine with wildlife preservation. The Natural Park took its name from the most northern mountain of Bragança municipality that reaches an altitude of 1,487 m. The vegetation is composed of scrubs of *Erica* spp., *Genista* spp., *Pterospartum* sp. and *Salix* spp. Montesinho is the only village at such heights: agricultural activity has decayed over approximately 20 years but tourism and recreational activities have became more and more significant. For most of the year there are only about 200 sheep grazing(Castro., et all,.2009); however, grazing can be very intense from May to August when about 5,000 sheep are transported from the surrounding lowlands to graze in the highlands during summer months.

4-1-3-Major natural values:

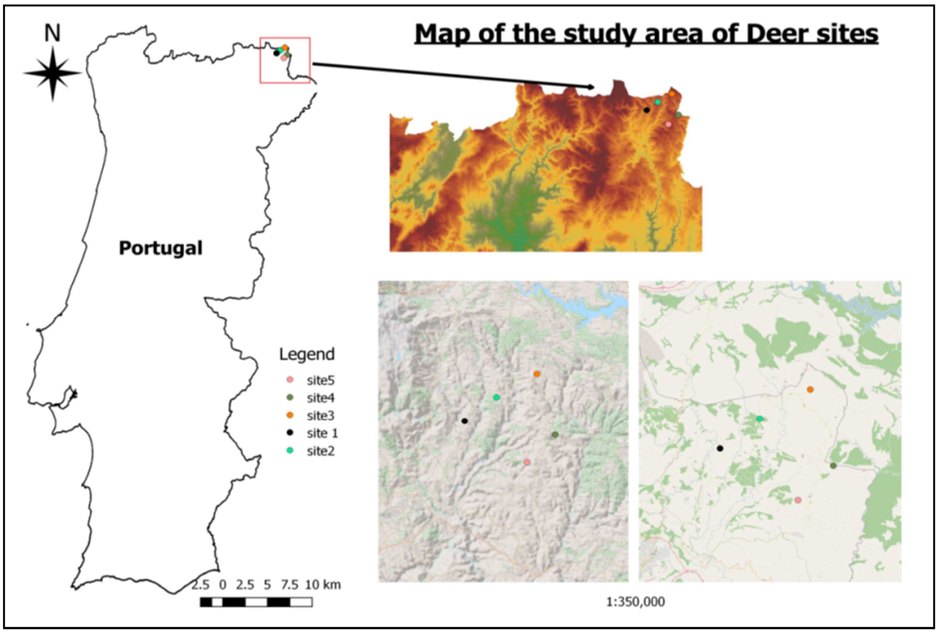
The main native forest cover was oak (*Quercus pyrenaica*) and chestnut trees (*Castanea sativa*). After centuries of destruction, wildlife populations are now recovering in the region in response to lessening of human pressure coupled with the expansion of scrub areas, better protection laws, the positive action of the Park management authority and a slow change in the attitude of individuals towards wildlife. Biodiversity is currently high but the densities of wild populations are lower than in the past (some, such as Brown-bear (*Ursusarctos*) and Iberean-lynx (*Lynx pardina*), reduced to extinction), mainly because man has destroyed most of the native forests. At the beginning of the twentieth Century, deer were exterminated, but by 1989, they came back via populations in Spain (Alves, 2004).

This Park has a high flora and fauna diversity and the latter expressively contributes to its emblematic biodiversity. All of the trophic levels are present and large carnivores and wild and domestic herbivores coexist in the region. A population or about 30 Iberian wolves (*Canis lupus signatus*) lives in the Park and it is linked to a larger Population of more than 120 wolves in neighboring regions in Portugal and Spain. Other carnivores include common genet (*Genetta genetta*) and red fox (*Vulpes vulpes*). Wild boar *(Suss crofa*), roe deer (*Capreolus*

capreolus), deer (*Cervus elaphus*) and, as mentioned above, domestic herbivores such as cattle, goat and sheep regional breeds, all inhabit the Natural Park. Main aquatic mammal species is the European otter (*Lutra lutra*) (lPB/ICN, 2006).

4-2- Sites selected:

The area selected was the whole Natural park of Montesinho, as we know that it occupied 74 229ha (ICNF.pt, classificação) and also it has a big diversity on fauna and Flora communities. For this study, we have chosen five sites with an importance deer population.



Coordinates (latitude; longitude)	Site1: Lat :41°53'45.04"N Lon :6°41'2.78"O Site2: 41°55'14.35"N 6°38'26.04"O Site3: 41°56'41.79"N 6°35'7.96"O	site4: 41°52'46.69"N 6°33'44.12"O site5: 41°51'3.66"N 6°36'3.89"O
	BRAGANCA	
District	Aveleda e Rio de Onor	SaoJuliao de palacios e deilao
Parish	10 635,21	8062,13
Area-ha		
Elevation(m) & Slopes(%) Site1 Site2 Site3 Site4 Site5	784 - 8-12 % 819 - 3-5% 872 - 0-3% 822 - 12-16% 905 - 16-25%	
Land Morphology	Hilltops	

Figure7: characteristics of study area

5- Primary and secondary metabolites:

Plant constituents comprise a wide variety of organic substances that are formed and accumulated by plants, they include:

5-1-Primary metabolites:

Primary metabolites are involved in growth, development, and reproduction of the organism. These compounds are a key component in maintaining normal physiological processes; thus, it is often referred to as a central metabolite. They are typically formed during the growth phase as a result of energy metabolism, and are deemed essential for proper growth. Examples of primary metabolites include carbohydrates, proteins, fats, and nucleic acids.

5-2-Secondary metabolites:

This kind of metabolites is typically organic compounds produced through the modification of primary metabolites by synthases. Secondary metabolites do not play a role in growth, development, and reproduction like primary metabolites do, and are typically formed during the end or near the stationary phase of growth. Many of the identified secondary metabolites have a role in ecological function, including defense mechanism(s), by serving as antibiotics and by producing pigments. Examples of secondary metabolites include: Phenolic compounds, terpenoids, organic acids, lipids, Nitrogen containing compounds, macromolecules.

5-3- the Role of primary and secondary metabolites on ungulate feeding:

Plant secondary metabolites (PMS) have many ecological functions, but have long been considered as defences against pathogens or herbivores, reducing the likelihood and extent of attack. However, mammalian herbivores ingest many foods containing PSM and use both behavioural methods and physiological strategies to limit their negative effects. Most physiological counter-adaptations are inducible in response to ingested PSM, providing efficient protection against toxic effects. Possible positive effects of PSM include antioxidant and anthelminthic properties and complex formation between protein and condensed tannins that protects dietary protein from degradation by the symbiotic micro flora of foregut fermenters, increasing its utilization by the animal. This protein effect is probably only beneficial to animals under a narrow range of nutrient-rich conditions found mainly in agricultural systems. There are many examples of PSM causing food avoidance or reducing food intake, but there is yet relatively little evidence for positive selection of them by herbivores. Although the feedback mechanisms relating the post-ingestive consequences of PSM to subsequent foraging behaviour are beginning to be understood, knowledge of the integration of behavioral and physiological strategies for regulating the effects of PSM is relatively poor. The opportunities for learned avoidance of PSM may be restricted in animals with complex diets that cannot associate a particular feedback signal with a given food type. (Iason et al. 2007)

6- Plant Species monograph.

Plant species were chosen by its abundance in the area and for deer diet. As an exploratory work, it was preferable to start this study with few species, instead of all plant species know to be eated by deer. Thus, for this work *Quercus rotundifolia*, *Pterospartum tridentatum*, *Cistus ladanifer* were selected.

6.1. Quercus rotundifolia Lam.

6.1.1. Geographical distribution and Habitat:

Holm oak (*Quercus rotundifolia)* is an evergreen oak in the Fagaceae family approximately 20-25 m in height. This tree is adapted to the Mediterranean climate, with a large population in the Iberian Peninsula. However, it can be found in any part of the Mediterranean region. Holm oak forests are dominant type of vegetation in a transition zone between temperate forests and scrublands. In this transition zone, plants have to cope with a selective pressure that result from double stress, winter cold and summer drought, which determine their morphological and eco-physiological evolutive response (Terradas, 1999). Holm oak can grow in heavy clay and acid, neutral or basic soils and it tolerates a wide range of soil textures.

The species has once been dominant in many landscapes of the Northeastern region of Portugal but it is today confined to steep slopes and unproductive soils where agriculture could not expand to (Azevedo et al., 2013). The species has also suffered from overexploitation for fuelwood. Holm oak woodlands are today in the region mainly facing East, West and North-West, and most of them are on slopes steeper than 6° and slopes steeper than 12° (Dias and Azevedo, 2008).

Figure 8 : Photo of holm oak, detail of the tree, the leaves and the fruit

6.1.2. Botanical description:

An evergreen tree of large size, growing 10–40 m (33–131 ft) tall, and the trunk is sometimes over 6 m in girth. The young shoots are clothed with a close grey felt. The leaves indoors, has a lower size and rounded shape , usually shorter and roundedas ovate-lanceolate, 4–8 cm long, 1.2–2.5 cm wide, with the back much more gray due to the presence of much more hairs. The petiole is 3–16 mm long. Fruits are produced one to three together on a short downy stalk, ripening the first season; the acorns usually 12–18 mm long in the UK, the cups with appressed, downy scales. (Royal Botanic Garden (2008)).

6.1.3. Plant Chemistry:

- **Phenolic compounds:**

Various works have assigned to identify and quantify the Phenolic compounds from *Quercus* species and especially *Quercus ilex*. Sub specie *rotundifolia*. There was intra-annual variability in these antioxidants: γ-tocopherol, total phenolics compounds (TPC), lipophilic and hydrophilic antioxidant activities (LAA, HAA) (acorn and grass) and condensed and hydrolysable tannins (CT, HT) except α-tocopherol, and inter-annual variability in all these antioxidants except the protein precipitating capacity.(Tejerina, et al.,2011). Both, acorns and grass, contain numerous substances with antioxidant properties. In particular, the acorns are rich in a- and c-tocopherol and tannins, and the grass in a-tocopherol and phenolic compounds (Cantos et al., 2003,.all, 2006).

❖ **Nutritional compounds: Total Sugar.**

Fermentable sugars production from three kind of steam-exploded oak wood was optimized by response surface methodology (RSM), using the severity factor (R_0), the pretreated total solids (TS%) and the enzyme loading (EL%) as variables of a central composite design result that a Oak wood species may be a good feedstock for the production of reducing sugars (Cotana et al., 2015).

6.2. Pterospartum Tridentatum:

6.2.1. Botanic description and habitats.

Pterospartum tridentatum (L.)Willk. Is a species of the Fabaceae family that grows spontaneously in Portugal under Mediterranean thermal conditions, it is endemic Portugal species where it is known as carqueja or carqueija (Carvalho, 2010).

Pterospartum tridentatum is a perennial shrub, which can reach up to one meter in height, with stems of woody and rigid consistency. The roots are straight and rather long. The stems are woody, erect or prostrate with winged branches laterally, false dark green leaves, trimmed and coriaceous in consistency or Sublenose. The branches thus have a flat shape with two or three expansions in Wing shape, with articulated appearance, ending with two or three teeth. The leaves, Persistent, alternate, unifoliolate and triangular, appear to be tridentate, by the leaflets are attached to the stipules. The flowers (Figure 1B, C and D) are a deep yellow and are arranged in Corymbiform inflorescences, in groups of 3 to 10, gathered in short bouquets and Tight. They have indumenta in the sepals that cover them. The fruit is a pod Oblong-linear with 10 to 12 mm in length (Pimenta, 2012).

Figure 9: ***Pterospartum tridentatum*** **(L.) Willk. (A, C and E) and flower detail (B and D) (https://www.flickr.com 2014).**

6.2.2. Phytochemistry.

Previous study identified as main constituents of the water extract, three derivatives of genistein. One new isoflavone glycosides showing a different oxygenated pattern was also isolated from the water extract and identified by spectroscopic methods. In addition, the flavonol glycoside isoquercirin was identified in a chromatographic fraction and in the water extract by different HPLC systems (Rute et al., 2004). Other study on Phenolic compounds from *Pterospartum tridentatum* detected twenty-one flavonoids (Roriz et al., 2014).

Pterospartum tridentatum is medicinal plant that requires a more detailed chemical characterization, given the importance of their consumption as infusions. Therefore, the individual profiles in tocopherols, free sugars, and organic acids were obtained by high performance liquid chromatography (HPLC) coupled to different detectors (fluorescence, refraction index, and photodiode array, resp.).This study result that *Pterospartum tridentatum* presented the highest fructose and total sugars content.

6.3. Cistus ladanifer L.

Figure 10: fruit and leaf of *Cistus ladanifer* detailed

6.3.1. Geographical distribution and Habitat.

It is a Mediterranean shrub, widely distributed in Portugal, being one of the most abundant species in the southern part of the country, occurring in large areas as pure dense stands (Teixeira et al., 2007). The wide distribution and morphological variation of *Cistus ladanifer* across northern Africa, the Iberian Peninsula, and southern France has resulted in the recognition of three sub-species: subspp. *Ladanifer*, *sulcatus*, and *africanus*.

6.3.2. Botanic description.

Cistus ladanifer L. is known as gum rockrose andbelongs to the Cistaceae family, having white flowers and viscid stems and leaves.It is a shrub growing 1–2.5 m (3 ft 3 in–8 ft 2 in) tall and wide. The leaves are evergreen, lanceolate, 3–10 cm long and 1–2 cm broad, dark green above and paler underneath. The flowers are 5–8 cm diameter, with 5 papery white petals, usually with a red to maroon spots at the base, surrounding the yellow stamens and pistils. The whole plant is covered with the sticky exudate of fragrant resin (Castroviejo, 1998).

6.3.3. Phytochemistry.

❖ **Phenolic compound.**

Recent research demonstrated that this aromatic plants is a good source of compounds with a positive impact in human health, such as Phenolic compounds (Vázquez et al., 2009), flavonoids (Danne et al., 1994) and terpenes (Adams et al., 1997)

The genus *Cistus ladanifer* is a characteristic species of the Mediterranean region (Warburg et al., 1968). A study on flavonoids glycosides from *Cistus* showed that the flavonoids patterns were rather uniform throughout the whole genus and did not allow a clear distinction between species or groups of related species based on qualitative differences (Poetsch et al., 1972). Several species of *Cistus* secrete large amounts of resin on the surfaces of leaves and stems, and the secreted flavonoids show very distinct patterns that might be useful in delineation of these species (Proksch, and Gulz, 1984).

❖ **Sugar total.**

Many studies result that *Cistus ladanifer* as an aromatic plant present a high values on reducing sugars, glucose and fructose and raffinose with 48.21mg/g for fructose. (Guimarães et al., 2009)

CHAPTER III: MATERIALS & METHODS

1. Sample collection

In this work, we have selected five sites from natural park of Montesinho which is characterized by a high value of fauna and flora species. The choice of flora species for this study was based on diet studies and previous knowledge about roe deer and red deer feeding behavior and plant species preferences. Three species were chosen, *Quercus rotundifolia* and *Cistus ladanifer* and *Pterospartum tridentatum* (Figure11) because of its importance in the area, as source of food (*Quercus* and *Pterospartum* species) or because of non-preference on diets. These species are also abundant in the area.

Plant samples were collected over the season of 2017 during spring at flowering period. From each species, we collect two samples on different months (Mars, April, June). In the total we have collected 86 samples which will be analyzed in laboratory. Only twigs, leaves and flowers were taken for the samples.

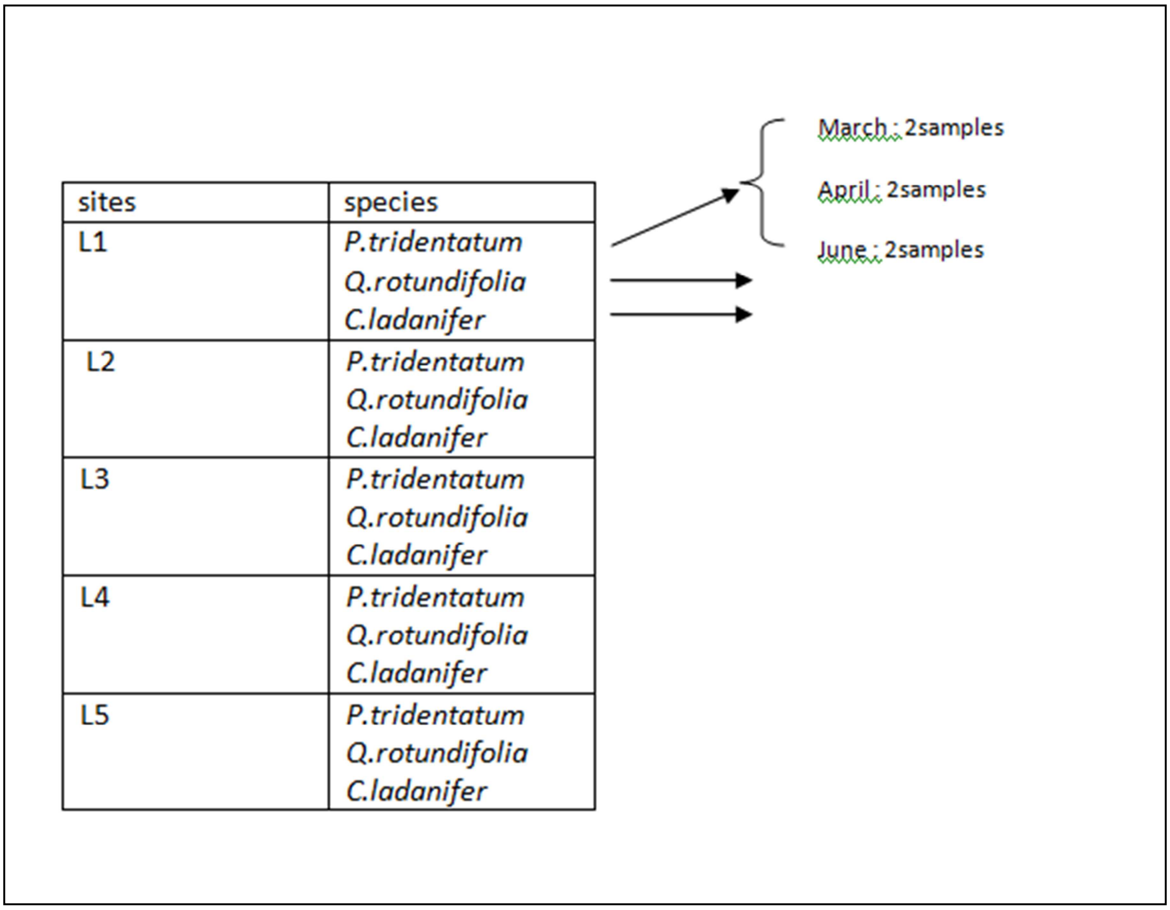


Figure 11: Schema shows the way how the collect of samples

Figure12: first picture shows a selected site for plant collection for this study; A is *Quercus rotudifolia*, B is *Pterospartum tridentatum* and C is *Cistus ladanifer*

The select of plants was randomly, harvested individually at about 5-10cm from the upper part of the species or from fresh part using a pair of scissor, placed in plastic bags and kept on ice. After getting all samples, they were dried in oven at 50°C for 2-3days, and later ground in a Wiley mill and stored in airtight containers at room temperature, in the dark, until analysis (Figure 13).

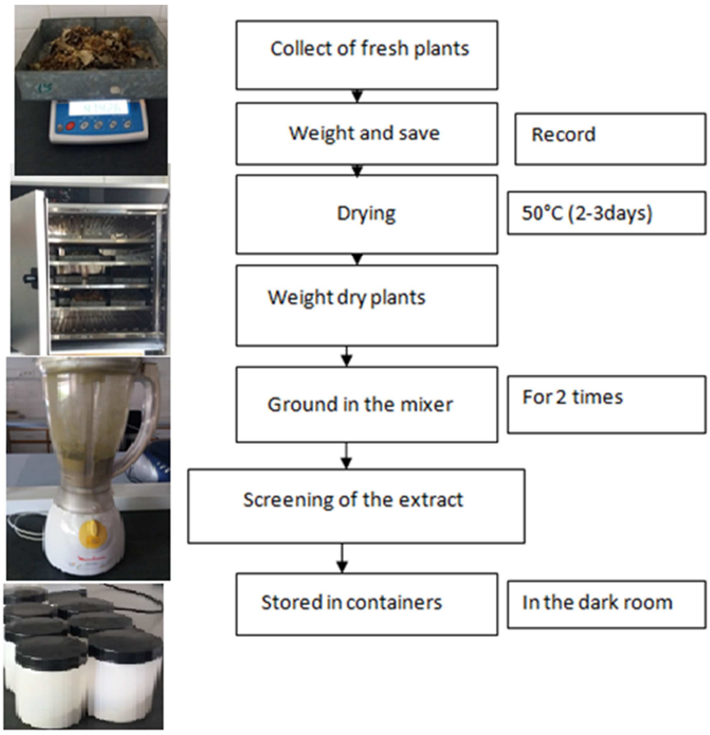


Figure 13: steps of sample's preparation for extraction

2. Equipments, standards and reagents.

2.1.Equipments required:

Spectrophotometer (AnalytikJena,, Germany), cuvettes for spectrophotometer, Water bath, magnetic stirrer, rotary evaporator (Büchi R-210), micropipette, centrifuge.

2.2.Required reagents and solvents:

DNS reagent, Folin-ciocalteu reagent, glucose, Gallic acid, methanol, Ether diethyl, carbonate of sodium.

3. Extraction procedure

The samples were analyzed for the chemical composition including the sugars and Phenolic compounds.

- **For the extraction of sugars.**

1. the dried and powdered aerial parts of each species (1g) was extracted with 40 mL of aqueous-methanol 80% (v/v) at 80 ºC for 90mn,
2. Later, The supernatant was concentrated at 60 ºC under reduced pressure and defatted three times with 10 mL of ethyl ether, successively,
3. After concentration at 40 ºC, the solid residues were dissolved in water to a final volume of 5 mL and filtered through 0.2 µm nylon filters from Whatman.
4. The resulting suspension (5ml) was centrifuged (Centurion K24OR refrigerated centrifuge, West Sussex, UK) at 4500rpm for 5min,

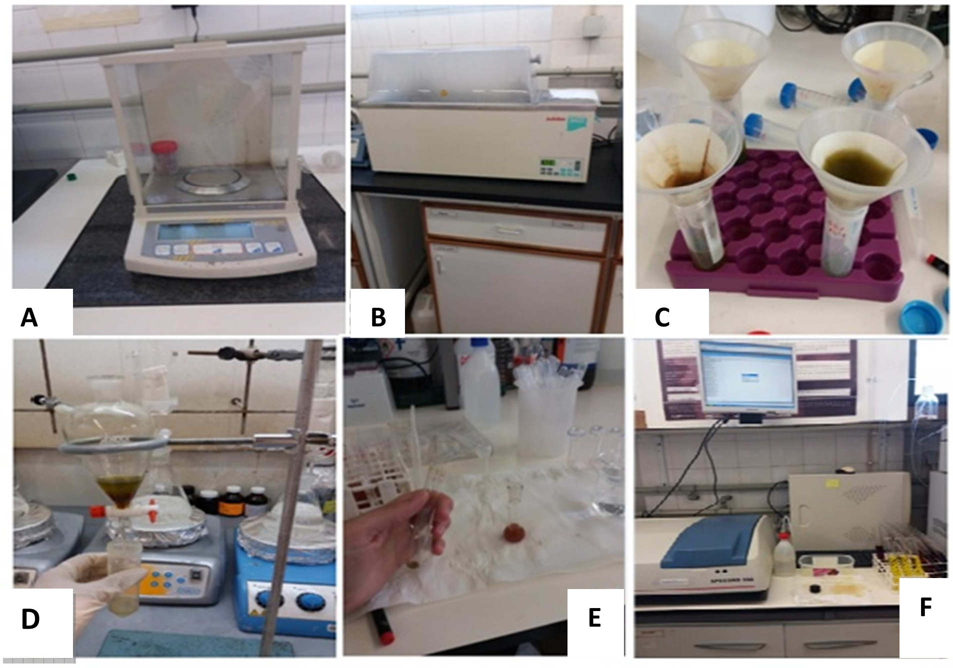


Figure 14: The processes of extraction de sugar until the measuring the absorbance. A: weight samples, B: water bath, C: Filtration of samples through Whatman No. 4 paper, D: cleaning; E: calibrate the tubes for centrifuge, F: Reading the results after performing the assay

- ❖ **For the extraction of Phenolic compounds:**

1. The extractions were performed using a fine dried powder (m= 500mg), stirring with 40-50 ml of methanol at 25°C at 150 rpm for 60mn and filtered through Whatman No. 4 paper.
2. The residue was then extracted with one additional 50 mL portion of methanol. The combined methanolic extracts were evaporated at 35ºC under reduced pressure (rotary evaporator Büchi R-210) and re-dissolved in methanol at a known concentration.

Figure 15: (A): stirring samples, (B): filtration using Wathman paper (C): Rotary Evaporation

4. Total Sugar.

4.1. Principal of reducing sugar.

For extraction of sugar and quantify the total sugar, we adopt the methodology of DNS (dinitrosalicylic acid spectrophotometric) described by Wolfe et al(2003).

This method tests for the presence of free carbonyl group (C=O), the so-called reducing sugars. This technic involves the oxidation of the aldehyde functional group present in glucose and the ketone functional group in fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions.

$$\text{aldehyde group} \xrightarrow{\text{Oxidation}} \text{carboxyl group}$$

$$\text{3,5-dinitrosalicylic acid} \xrightarrow{\text{Reduction}} \text{3-amino,5-nitrosalicylic acid}$$

Because dissolved oxygen can interfere with glucose oxidation, sulfite, which itself is not necessary for the colour reaction, is added in the reagent to absorb the dissolved oxygen. The above reaction scheme shows that one mole of sugar will react with one mole of 3,5-dinitrosalicylic acid. However, it is suspected that there are many side reactions, and the actual reaction stoichiometry is more complicated than that previously described. The type of reaction depends on the exact nature of the reducing sugars. Different reducing sugars generally yield different colour intensities; thus, it is necessary to calibrate for each sugar. In addition to the oxidation of the carbonyl groups in the sugar, other side reactions such as the decomposition of sugar also competes for the availability of 3,5-dinitrosalicylic acid. As a consequence, carboxy-methyl cellulose can affect the calibration curve by enhancing the intensity of the developed colour.

Although this is a convenient and relatively inexpensive method, due to the relatively low specificity, one must run blanks diligently if the colorimetric results are to be interpreted correctly and accurately. One can determine the background absorption on the original cellulose substrate solution by adding cellulase, immediately stopping the reaction, and measuring the absorbance, i.e. following exactly the same procedures for the actual samples. When the effects of extraneous compounds are not known, one can effectively include a so-called internal standard by first fully developing the colour for the unknown sample; then, a known amount of sugar is added to this sample. The increase in the absorbance upon the second colour development is equivalent to the incremental amount of sugar added.

DNS reagent:

- 10 g 3,4-dinitrosalicyclic acid
- 403 g potassium sodium tartrate tetrahydrate
- 16 g NaOH (anhydrous).

4.2. Methodology.

-For the colorimetric assay, the previously extracted sample (1mL) was mix with water (1mL) and with DNS (2 mL). The mixture was agitated and put in a bath at 100 °C for 5 min. Immediately, the tubes were put in an ice bath for 5 min to stop the reaction. Another batch of water was added (6mL), and then we dilute the extract by adding 40ml of water.

-Mixed and then measure the absorbance at 540 nm in a spectrophotometer (Analytik Jena, Germany).

- The results were calculated from equation obtained by calibration curve standard of glucose (y=0.594x-0.033, R2=0.999) established with different concentrations (0.125, 0.25, 0.5, 1, 2mg/ml), the contents of sugar were expressed on mg by g of dried weight of extract (Figure16).

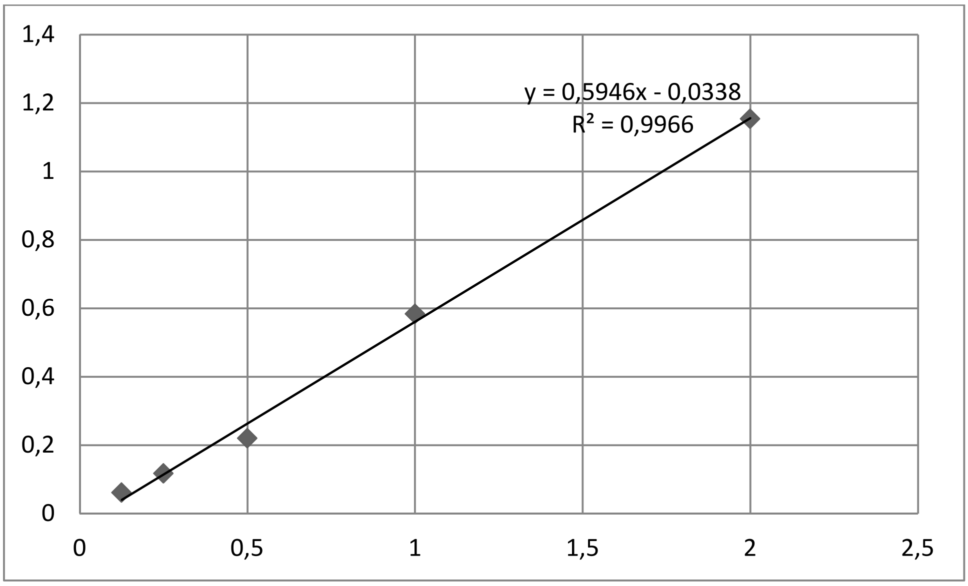


Figure16: The calibration curve was established by glucose at different concentrations

5. Total Phenols

5.1. Principle of methodology.

Polyphenols have become an intense focus of research interest because of their perceived health-beneficial effects. They occur in a variety of fruits, vegetables, nuts, seeds, flowers, bark, beverages, and even some manufactured food, as a component of the natural ingredients used. They have been reported to exhibit anti-carcinogenic, anti-atherogenic, antiulcer, anti-thrombotic, anti-inflammatory, immunemodulating, antimicrobial, vasodilatory and analgesic effects.

Phenolic compoundsQuantification Assay is based on Folin-Ciocalteu method. The FC reagent contains phosphomolybdic/ phosphotungstic acid complexes 3. The method relies on the transfer of electrons in alkaline medium from phenolic compounds to form a blue chromophore constituted by a phosphotungstic/ phosphomolybdenum complex where the maximum absorption depends on the concentration of phenolic compounds. The reduced Folin-Ciocalteu reagent is detectable with a spectrophotometer at 765 nm a (AnalytikJena, Jena, Germany).The reaction temperature has been used to reduce the time necessary to attain the maximum color (T= 37ºC). Generally, Gallic acid is used as the reference standard compound4and results are expressed as Gallic acid equivalents (mg/ml) (Wolfe at al., 2003).

Methodology:

An aliquot of the extract solution (0,5 mL) was mixed with *Folin-Ciocalteu* reagent (2,5 mL, previously diluted with water 1:10 (v/v) and sodium carbonate (75 g/L, 2 mL).

The tubes were vortexed for 15s and allowed to stand for 30 min at 40°C on water bath for colour development.

Absorbance was then measured at 765nm a spectrophotometer (AnalytikJena, Jena, Germany). Gallic acid was used to calculate the standard curve ($y = 0{,}985x + 0{,}010, R^2 = 0{,}997$) with using several concentrations (0,05; 0,1; 0,2; 0,4; 0,8 mg/ml)and the results were expressed as mg of Gallic acid equivalents (GAE) per g of extract.

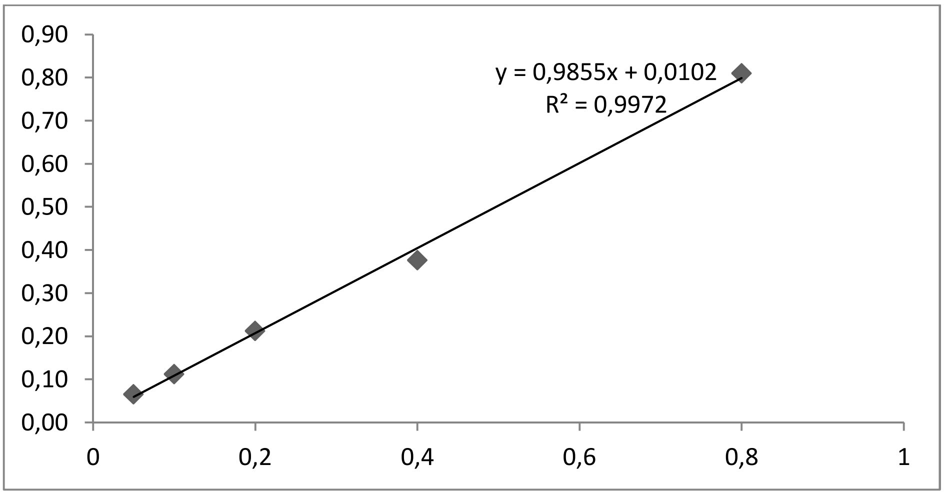


Figure17: curve of acid Gallic absorption

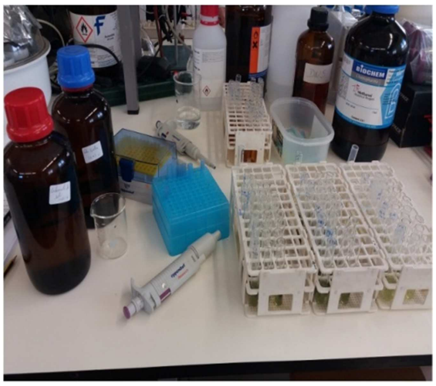


Figure 18: Addition of Folin-Ciocalteu and sodium carbonate

6-**Statistical analysis:**

For each one of the specimens, three samples were used and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD).

Data was log transformed, after verifying normal distribution with Kolmogorov-Smirnov test (Zar, 1996). The results were analyzed using two-way analysis of variance (ANOVA) followed by Tukey’s HSD Test with $\alpha = 0.05$. This analysis was carried out using SPSS v. 17.0 Program.

CHAPTER IV: RESULTS & DISCUSSION

1. Sugar results:

The results of total sugars estimated of three species analysed by spectrophotometer assay from a standard curve were shown in the table (3).

Table3: Quantity of total sugar (mg/g) in three tested species according to three successive months. (Mean ± standard deviation)

Month	*Cistus ladanifer*	*Pterospartum tridentatum*	*Quercus rotundifolia*
March	25.49±5.7	19.24±6.98	44.51±17.2
April	28.6±11.6	23.60±12.06	50.08±15.95
June	17.38±5.45	43.51±17.30	16.47±5

Standard calibration curves: glucose (y = 0,594x - 0,033, R^2 =0.9999)

The graph below (figure 19) shows the variation of total sugar during three months that include three phenological periods: before flowering (March), flowering period (April), after flowering (end of May - June).

Cistus ladanifer shows during all this period a slowly variation of sugar production. From the beginning of March until early April, the quantity of sugar produce doesn't change; it is stable around the mean 27.04 mg with a peak marked during this period in April with 28.6mg. Due to this, flower and floral reward availability (nectar and pollen) was high and visitation rates (pollinator visits per flower and unit time) low. Conversely, from mid April until June, the quantity of sugar decrease successively to reach a less quantity 17.38±5.45 mg, this decreasing of quantity due to availability of flower and floral reward which were much lower, and by result a much higher pollinator visitation rate.(Filella, et al, 2013/ PMC). Another study given that the flower duration observed in 1991 season was exceptionally extended, the prominent amount of sugar secreted was 18 ± 3 mg after 32hours (period age of flowers) and 6±1 mg after 50 hours (Talavera, 1992). A previous study explains that in the fruiting period (mid June until end of July) *C. ladanifer* as an aromatic plant present a high values on reducing sugars: glucose, fructose and raffinose with 48.21mg/g for fructose, when total sugar was around 77.51 ± 1.04mg/g (Guimarães et al., 2009).

Pterospartum tridentatum shows (figure19) a progressively increasing on the quantity of sugar produced before flowering, in the mid of flowering period, the plant start to increase the production to reach a peak at the end of flowering with 43.51±17.30 mg/g dw. In the same manner, previous study was produced at 2012 on spring period results that free sugar of *P. tridentatum* showed the highest levels of fructose and total sugars; nevertheless, the values obtained were much higher than the concentrations found in a wild sample previously studied (0.3 and 49.6 g/100 g dw for fructose and total sugars, resp.).(Rorizet al,2014)

For *Quercus rotundifolia*, the evolution of sugar production starts with high quantity (figure19,table3) during flowering period to reach a peak in the mid of April with 50.08±15.95 mg/g dw, then a rapid decreasing market with less quantity produced 16.47±5mg/g. Another study had act on data from February, to defend the relation between two species oh oak and sugar soluble and starch as a nonstructural carbohydrate. *Q. ilex* can assimilate more photosynthates during favourable days in winter than the semi-deciduous oak, as it retains a higher proportion of foliage in this season. However, early budbreak, thereby earlier leaf expansion, could be advantageous for *Q. faginea*, mainly growing in the understory, given that new leaves have higher photosynthetic potential than older leaves (Niinemets et al. 2005). High water availability in combination with high summer temperatures can account for the high photosynthetic activity in a potential water stress period. The replenishment of soil water reserves early in the growing season is critical to endure seasonal summer droughts in Mediterranean trees (Sperlich *et al.*, 2015).

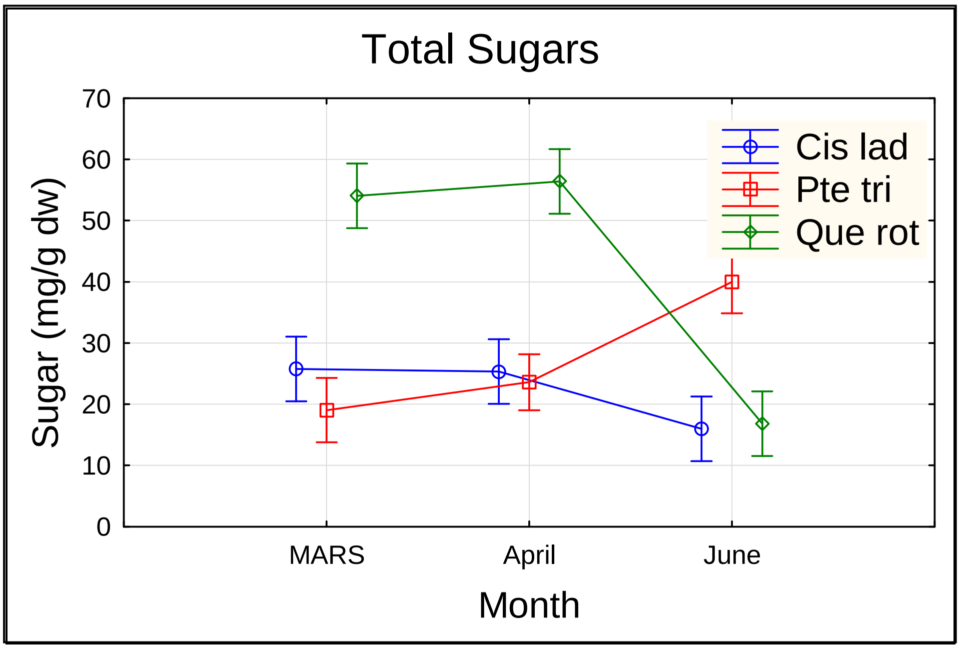


Figure19: Evolution of Total Sugar (mg/g dw) between species during three months

The results obtained by statistical analysis Anova, shows significant result between Months ($F_{2.160}$= 14,365, and P=0.000002) and also between species ($F_{2.160}$=45,826, and P=0,000000), which mean that there is an effect of month on sugar produced, as we can see in the Figure19, which also depend to the specie. The interaction between month and species indicate a significant effect ($F_{4,160}$=39.685 and P=0.000000).

Tukey HSD pos-hoc test indicates that June was different from other month and *Quercus rotundifolia* was responsible for the significant differences detected for species. For the interaction Month x Species, was possible to verify that the differences detected were for *Q. rotundifolia* in mars and April and *Pterospartum* in June.

2- Total phenol results:

The results of total phenol estimated of three species investigating by spectrophotometer assay from a standard curve were shown in the table 4.

Table4: Quantity of total Phenol (mg/g) in three tested species according to three successive months. (Mean ± standard deviation)

Month	*Cistus ladanifer*	*Pterospartum tridentatum*	*Quercus rotundifolia*
March	72.08±27.38	200.83±35.82	244.20 ± 48.79
April	78.66±15.56	161.79±38.45	302.98±27.65
June	317.28±102.06	195.42±64.62	376.99±95.79

Standard calibration curves: acid Gallic (y = 0,985x + 0,010, R^2 = 0,997)

The graph below (figure20) shows the fluctuation of total phenol during three months with three periods: before flowering (March), flowering period (April), after flowering (June). Before flowering, *Cistus ladanifer* starts to produce a stable quantity of phenol during this period until the mid of April, the flowering period when *Cistus ladanifer* mentions a rapid increasing of total phenol to reach at the end of this phenophase a high peak with 317.28±102.06 mg/g dw. This high quantity was explained by previous studies which they have demonstrated that phenol biosynthesis in *Cistus ladanifer* is induced by water stress and high temperatures, under natural conditions, the climatic parameters are those of spring and summer, which is when the plant secretes the greatest amounts of flavonoids (Chaves et al, 1997). In other studies, it has been shown that two possible functions of the flavonoids in *Cistus ladanifer* are as a filter of ultraviolet light (Chaves et al,2003) and as a potential defense against herbivory (Sosa et al, 2004). Thus, the synthesis of flavonoids is enhanced in summer, the season when ultraviolet radiation is the most intense and the plant is very sensitive to herbivore damage. Also, the greatest diterpene concentrations in the leaves correspond to winter, and the diterpenes pass into the soil during that period might interfere in the germination behavior of the seeds of *C. ladanifer*. (Alías,. et al. 2012)

Pterospartum tridentatum starts to reduce the quantity of phenol producing on flowering period until a minimum quantity= 161.79±38.45 mg/g dw in April, this point was the turning point for *Pterospartum tridentatum* to increase progressively the quantity produce of phenol until reach a peak = 195.42±64.62 mg/g dw at the end of flowering period. The total Phenolic content of *Pterospartum tridentatum* shows an appreciable levels at any time of harvest and are inferior to other previously studied showing a high levels of TPC in the same period of harvesting (flowering time), the total Phenolic content, ranged from 270.7 to 402.9 mg Gallic acid equivalents per gram of dry matter (Coelho et al,2011). Otherwise, twenty-one flavonoids were detected by using HPLC method at wavelength= 280nm and Methanol extraction (Roriz, et al,. 2014).

For *Quercus rotundifolia*, the figure 20 shows a gradually increasing continued of total phenol produced by *Quercus rotundifolia* over this flowering period to reach a quantity=376.99±95.79 mg/g dw. Anova test shows significant result between Months ($F_{2.477}$= 179,260, and P=0.00) and also between Species ($F_{2.477}$=355,855, and P=0,00), which mean that there is an effect of month on Phenol produced, as we can see in the Figure19, which also depend to the specie. The interaction between month and species indicate a significant effect ($F_{4.477}$=49.576 and P=0.00). This means that during spring, plants show important changes in phenolic contents, probably to protect new shoots and flowers from being eaten by herbivores, as a strategy to preserve flowers and plant reproduction period. Tukey HSD pos-hoc test indicates that June was different from other month and *Quercus rotundifolia* was responsible for the significant differences detected for species. For the interaction Month x Species, was possible to verify that the differences detected were for *Q. rotundifolia* in mars and April and *Pterospartum* in June (Figure20).

In fact, regarding this result, the average leaf Phenolic concentrations also varied significantly between study years. The variations during leaf development and growth are in accordance with the majority of hypotheses that explain investment in secondary metabolism compounds. Leaf phenolics concentrations decreased rapidly during leaf maturity and senescence, but this decrease depended on the time of leaf shedding, the concentration being substantially lower in the year when leaves had been attached longer to the tree. Variation of leaf phenolics concentration was greater in senescent leaves than in green leaves. Such high concentration variability represents a source of spatial and temporal heterogeneity not only for potential herbivores but also for the soil nitrogen cycle in terrestrial ecosystems. (Covelo and Gallardo, 2001).

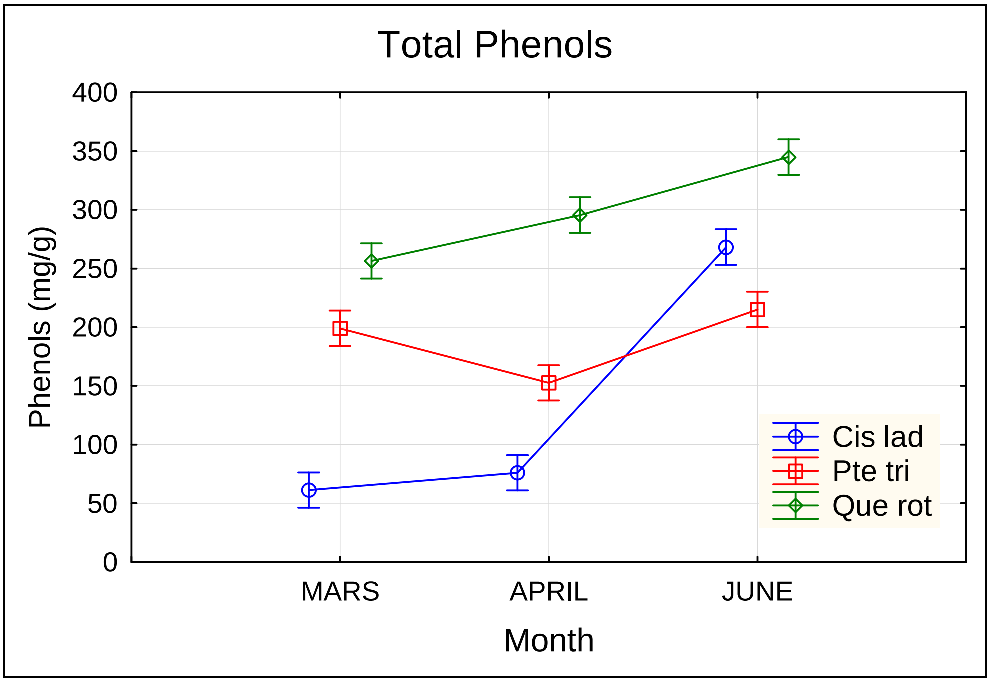


Figure20: Graph showing the evolution of Total Phenol (mg/g)

3- Diet and relation with phytochemical:

For this analysis, it was considered the results obtained by Cortez (2011), just to verify if the changes in consumption were related to those of we found for total sugar and total phenol content for the same physiological period.

The figures 21 & 22 show the degree of consumption by roe and red deer during two successive years, also with a preference degree of each species. We took just the species that we are studying in this dissertation to compare between the consumption and the evolution of secondary metabolites.

The micro-histological analysis of pellet fecal for roe and red deer give the result shown in the table4. As a consequence of this result, we conclude during the period of flowering (March, April, Mai, July) that the both deer species consumed a high quantity of

Pterospartum tridentatum, this could be related to the quantity of sugar produced and also the Phenolic compounds, in fact in this period *Pterospartum tridentatum* produce a gradually sugar to reach 43.51±17.30 mg/g (table 3) and a less quantity of phenol according to other species (table3). Otherwise, they don't consume *Cistus ladanifer* over these months due to the quantity of phenol produced in this period 317.28±102.06 mg/g dw (table4), in summer period *Cistus ladanifer* produce more Phenolic compounds as a potential defense against herbivory (Sosa .,et al.2004).

For *Quercus rotundifolia* is different, because as we can see in the table 9 that deer didn't consume the *Quercus rotundifolia* over two years in the same period this will be explained by 376.99±95.79 mg/g (table4) of phenol and a less quantity of sugar 16.47±5mg/g (table3), in contrast the Red deer pellet fecal indicate a less consumption in April-Mai-July of 2001, we can conclude that the requirements between red and roe deer are different. Previous study confirms that Red Deer can be classified among the intermediate feeders, with a mixed diet of grass & sedges (29%) and concentrate food items (63%). However, they also show Red Deer to be primarily a concentrate feeder (max. 75%) with no significant seasonal variation between the quantities of grass or sedges and concentrate food in the diet. (Gebert, et al, 2001)

On the Figure 21, is represented the percentage of consumption of the three plant species in the local diet of roe deer, indicating that during spring, this deer didn't consume the *Cistus ladanifer*, a short quantity of *Quercus* and a decrease in the consumption of *Pterospartum*. Taking in account the results for total sugar and phenol contents in these plants,it seems that higher concentrations of total phenols can explain this non consumption for *Cistus* and *Quercus*, during spring, which is produced for prevent the consumption by herbivory. In other hand, the consumption of *Pterospartum tridentatum* start to decrease during this period when in March the consumption was higher and also in February and during winter period. Roe deer seems to show ability to consume more *Quercus rotundifolia* during spring than red deer and that could be related to its capacity to select food with higher concentration of phenolic compounds...

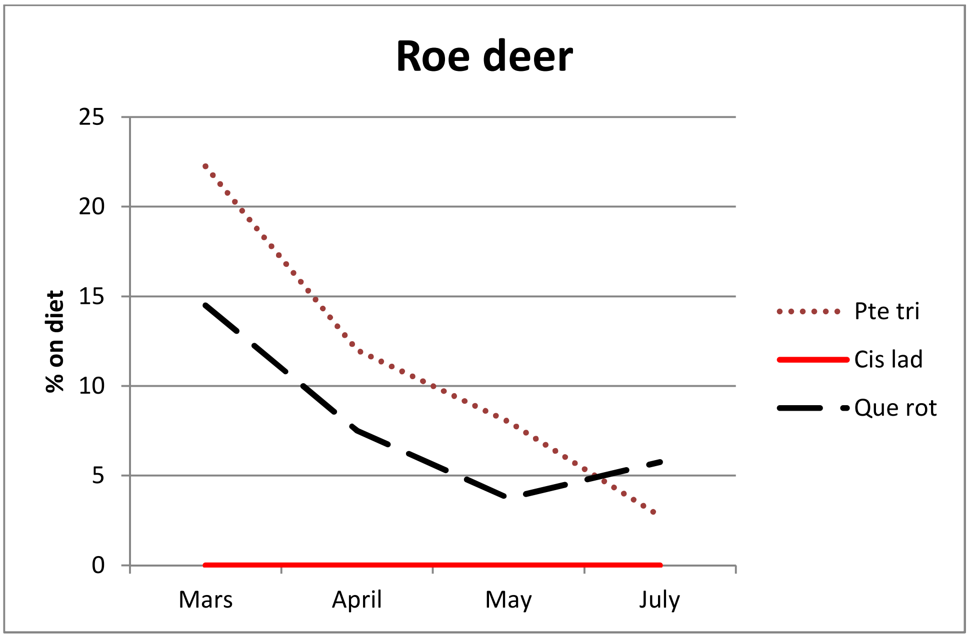


Figure21: Graph showing the evolution of spring consumption of the studied species by Roe deer.

The figure below represents the evolution of consumption in the diet of Red deer during two years successive, before flowering period we observe the high consumption of *Pterospartum tridentatum* and start to decrease during this period, in contrast of this, *Cistus ladanifer* show a lower consumption during mid-April to May. In spring period, the red deer didn’t show consumption of *Quercus rotundifolia*.

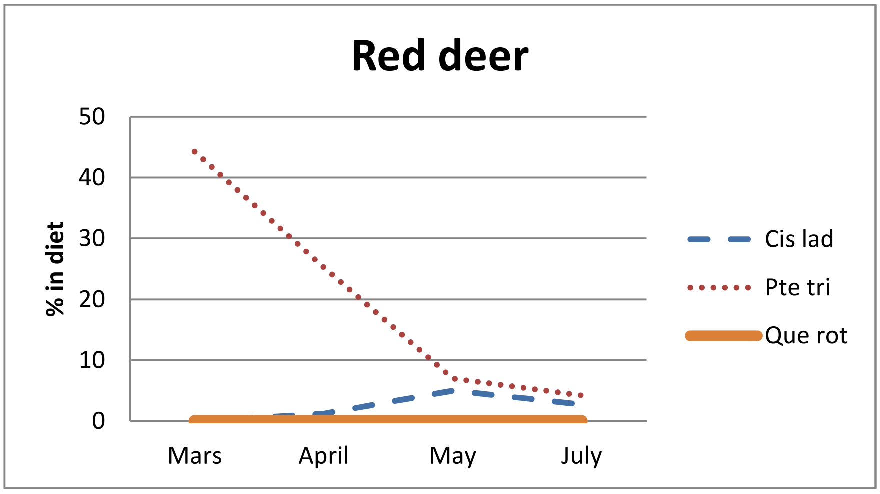


Figure 22: Graph showing the evolution of spring consumption of studied plants by Red deer

The result show that the main Portuguese deer species, *Capreolus capreolus* and *Cervus elaphus* have a difference preference of feeding and different reaction to season variation and part of plants eaten (leaves, fruits,..) also to the environmental variation. From this figures

(21, 22), we detect that the *Quercus rotundifolia* has a significant effect on consumption by deer, in flowering period it is consumed by Roe deer and not by Red deer. Red Deer, as a concentrate selector, particularly in winter, and more frugivorous and granivorous animal during oak mast years, is a potential competitor for European Roe Deer. Tixier& Duncan (1996) showed equivalent feeding behaviour for Red Deer when the abundance of seeds and fruits allows them to obtain enough of these kind of foods. Roe Deer is a generalist concentrate selector in forest habitats year-round (85.3%) and also consumes forbs, brambles and ivy instead of grass & sedges compared with Red Deer.

Conclusions and Final Considerations:

The phytochemical analysis of these Mediterranean plant species results that there is a relation between quantity of total phenol contain these species and consumption by ungulate species, as a result a high quantity of phenol prevent the consumption of the specie by herbivory during spring, also the Total sugar has an effect on choice of plants.

Our results may contribute to improve the understanding of the dynamics diet changes between woody plants and its relation to plant physiology during spring (start of flowering, flowering and post-flowering). From this work is also possible to verify that during spring, total phenols increase in the plant species we analyzed, but for total sugar concentration, plant species differ and only Pterospartum shows an increase of sugar concentration during this period. In relation to diet, phenols seem to explain better herbivore feeding behavior, but further studies are needed.

This study was focused on specific plants and during flowering period, the results obtain show a different between preference and avoidance against *Quercus rotundifolia* during spring. Actually, for better understanding of feeding behavior and strategies between roe deer and red deer, further investigation is needed and, based on the results presented here, should explore the variation in phenol and sugar contents during all year cycle for not only the plant species considered in this work but also many other species known to be consumed by red and roe deer, among trees, shrubs and herbs. It would be useful to include the availability of these key food resources in models of population dynamics, particularly during important changes in plants phenological stages. Moreover, other characteristics of the plants, like cellulose and lignin contents, needs to be studied to increase our knowledge on plants ecology and its importance as food for deer.

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