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# Valorization of olive tree leaves: phytochemical and pharmacological characterizations, selection of extracts and formulation of herbal cream

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**ABSTRACT/RESUME**

***Abstract:*** *Olive tree (Olea Europea L.) is one of the most important crops in the Mediterranean countries. The global olive oil industry annually generates many tons of olive leaves as waste. The present study aims at a valorisation of Algerian olive leaves harvested from the region of Bouira for therapeutic use. In order to evaluate the biological effects of the wild olive tree, chemical characterization tests of the leaves were carried out in the Natural Substances Laboratory of Saidal group according to their validated protocols drawn from the French pharmacopoeia. A phytochemical screening has been realized and whose purpose is to refer to the extraction, screening and identification of the medicinally active substances in the plant. Different extractions have been carried out in several solvents in order to extract bioactive molecules from the leaves using several solvents. Subsequently, a pharmacological characterization has been completed by determining the following: antioxidant activity of the extracts was evaluated in vitro by the DPPH method, anti-inflammatory activity was studied in vivo by induction of carrageenan oedema; the antibacterial activity was achieved by the agar medium diffusion method. Finally, we formulated an anti-inflammatory and antibacterial ointments based on the results obtained.*

## I. Introduction

The olive tree (*Olea Europea L.*) is a species widely cultivated in the Mediterranean region since ancient times [1]. The best-known use of the olive tree is certainly its oil which is used for popular medicine for its stomachic, sedative and anti-inflammatory virtues. However, the medicinal properties of the olive tree are mainly attributed to its leaves, which are currently under research in the broad field of medicine and pharmacology [2]. Olive leaves are a residue from the olive-oil industry and usually are discarded or used as feed for livestock. However, olive leaves are sources of added value bioactive compounds [3]. The global olive oil industry annually generates approximately 750,000–1,500,000 tons of *Olea europaea* leaves as waste that are typically burned for energy production [4]. Nowadays, bioactive compounds from olive leaves can be of great interest in pharmaceutical, cosmetic and food industries. Polyphenols of olive leaves, especially Oleuropein, have interesting effects on the human body such as antioxidant capability, antihypertensive,hypoglycemic, ypocholesterolemic factors [5]. Treatments with low dose and high dose of olive leaves extract in diabetic rats showed remarkable reducing and protecting influences of physiological and histopathological alterations. Moreover, the highly treatment efficiency was noted in diabetic rats treated with high dose followed by low dose of olive leaves extract [6]. Among the bioactive compounds of olive leaves, we found phenolic compounds, mainly oleuropein and hydroxytyrosol, flavonoids, and sugars (mannitol, oligosaccharides) [7]. Olive leaves contain

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compounds with potent antimicrobial activities against microorganism, fungi, and true bacteria [8]. Additionally, they have inhibitor and anti-inflammatory activities [9]. Also, it had been found that Olive leaves inhibits acute infection and cell-to-cell transmission of HIV-1 and additionally inhibits HIV-1 replication. [10]. The major active elements in olive leaf is oleuropein (see Fig.1 a) and its derivatives, like hydroxytyrosol and tyrosol, additionally as cafeic acid, p-coumaric acid, vanillic acid, vanillin, luteolin, diosmetin, rutin, luteolin-7-glucoside, apigenin- 7-glucoside, and diosmetin-7- [11]. A number of studies proved that triterpenic acids have potent antimicrobial, antitumor, anti-inflammatory, cytotoxic activities [12], [13], [14]. Oleanolic acid *(see Fig. 1 b),* in particular, is a key component of olive pomace, and a major contributor to the health promoting effect of the human Mediterranean diet.

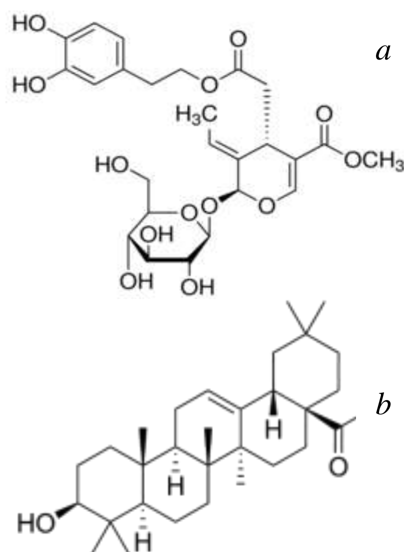


***Figure 1.*** *Chemical structure of Oleuropein (a) (PubChem CID: 5281544) and Oleanolic acid (b) (PubChem CID: 10494)*

Taking the total world production of olive oil in 2012 into account, 750,000–1500,000 tons of leaves are discarded on an annual base. Assuming a maximum content of up to 3.1 wt % of oleanolic acid, the main triterpenic acid present in the leaves of *O. europeae*, this corresponds to large amounts of this high-value compound that could be potentially extracted [4]. Triterpenic acids, such as oleanolic, betulinic and ursolicacids, are secondary plant metabolites typically found in barks, leaves or peels, with potential pharmaceutical and nutraceutical applications, they represent a typical example of such high-value compounds that can be extracted from agricultural by product [15]. Various methods have been used to isolate the bioactive molecules present in olive leaves, from the most common techniques to the more sophisticated including microwave-assisted extraction, pressurized liquid extraction and supercritical fluid extraction. It should be pointed out that most of these techniques suffer from high-energy costs as they operate under high pressure [12], [13]. To obtain natural extracts, methods using solvents are the most common, in particular for herbs solid-liquid extraction, with the simplest being maceration. The variables of the method are the solvent to be used, the extraction time and temperature, the grams of sample per solvent volume ratio, the agitation and the final separation method. For example, for the extraction of polyphenols, methanol, ethanol, acetone and water are commonly used as solvents [16]. Pharmaceutical semi-solid preparations are topical products intended for application to the skin or accessible mucous membrane to provide local or sometimes systemic effects at application sites. Topical agents like creams and ointments are more attractive than oral therapy because they reduce the potential for systemic adverse reactions. They are also suitable for self-administration of medication since no specialized appliances or personnel are required leading to improved patient compliance. Efficacy is also achieved by lower total daily dosage of drug since it is applied directly to the affected area [17]. Here, we investigate the extraction of oleanolic acid and other active components from Olive leaves, the influence of solvent type on the extraction yield, antioxidant, anti-inflammatory and antibacterial activities of olive leaves extracts were examined by various assays. Finally, extracts have been formulated into herbal creams for use as topical anti-inflammatory and anti-microbial agents. Thereby, the physicochemical and safety of the formulations were investigated.

## II. Materials and methods

### II.1. Chemicals

Thin-layer chromatography (TLC) silica gel 60 F254, chemicals, and solvents of analytical grade were purchased from Merck and Fisher Scientific**.**

### II.2. Plant material and preparation of the sample

The plant material consists of the leaves of olive tree, known by the botanical name *Olea europaea*. Is a species of small tree in the family Oleaceae, found in the Mediterranean Basin [18]. Collected in Ain lahjr at Bouira, Algeria, in the period of May – June 2017. The Specimen voucher was deposited in National Herbarium of the Research laboratory of Arid Zones LRZA Herbarium for authentication (N°10-2017 Boui; MB/LRZA/USTHB). The collected leaves were cleaned with water and shade dried. After drying, the leaves were crushed with traditional mortar to obtain a coarse powder, and then reduced to a fine powder using a propeller mill type Retsch SM 2000. We obtained a fine powder with a light greenish color. This powder was used for phytochemical and pharmacological investigations (*Figure 2).*

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***Figure 2.*** *Dried and powderd Olea europea leaves*

**II.3. Extraction and fractionation**

Extraction, as the term is used pharmaceutically, involves the separation of medicinally active portions of plant or animal tissues from the inactive or inert components by using selective solvents in standard extraction procedures [19]. Approximately 50 g of ground sample was extracted with 500 mL of methanol for 48 hours. Extraction was repeated and the extracts were pooled and filtered through Whatman number 1 filter paper. The filtrate was concentrated under reduced pressure in a rotary vacuum evaporator (RV10 Control, IKA, ProfiLab). The concentrated extract was air dried to a constant weight at room temperature. For the further studies, extractions using solvents is essential because the nature of active compounds depends on it. In a classic phytochemical study approach, a plant is successively subjected to several solvents of increasing polarity (e. g. Hexane > Ethyl acetate > Alcohol >Hydroalcoholic mixture) [20]. In this study, the objective was to cover all possible polarities of the compounds of the same extract and thus obtain all the characteristic compounds of the plant. Dried, powdered leaves samples of the plant under study were successively extracted with solvents (50g of drug's powder in 500ml of solvent) of different polarity: Water, Ethanol, Methanol, chloroform and Hexane. Solvent is preferably recovered under reduced pressure to save thermolabile constituents [19]. All leaves extracts were concentrated at 40-45°C by using a rotary evaporator (LABOROTA 4000-efficient) to 50 ml. [21]; and were been tested for antibacterial and antifungal activity. Water and ethanol extracts were being tested for antioxidant, and anti-inflammatory activities.

**II.4. Thin layer chromatography (TLC)**

The analysis of the crude extract by thin layer chromatography allows us to confirm the presence of the bioactive molecule in olive leaves which is Oleuropein. The TLC plates were prepared and heated for activation in an oven for 30 minutes at 110 °C. Approximately 10mg of Oleoropein and 1mg of rutine (standard solutions) in methanol were prepared for spotting on the prepared TLC plates. The plates were spotted with 10 uL of samples. For the developing system, the following solvent system was used: water, methanol, methylene chloride (1.5:15:85), the plates were air dried and visualized by spraying with vanillin reagent spray followed by heating for 5 minutes at 110°C and examine in the light of day [22]. Then, Rf value was calculated as the ratio of the distance travelled by the solute to the distance travelled by the solvent [23] and [24].

**II.5. Physicochemical analysis and phytochemical Screening**

Physicochemical parameters were done to examine appearance, loss on drying, total ash, sulfuric ash and hydrochloric acid insoluble ash, alcohol solubility and water solubility as per quality standards of European pharmacopoeia. So, for the phytochemical screening, major compounds in plants can belong to different classes of secondary metabolites. Phytochemical screening refers to the extraction, screening and identification of the medicinally active substances found in plants. Some of the bioactive substances that can be derived from plants are flavonoids, alkaloids, carotenoids, tannin, antioxidants and phenolic compounds.

The purpose of these tests is to know the composition of secondary metabolites. They are carried out either on the powder or on the infused. Phytochemical screening consists either of coloring or precipitation reactions [25]. The extract was subjected to standard phyto-chemical screening for alkaloids (Dragondroff and Mayer's test), flavonoids (sodium hydroxide, ferric chloride and lead acetate test), saponins (foam test), tannins (ferric chloride test) and phenols (Ferric chloride test) [26] and [27].

**II.6. Screening of antioxidant activity**

In the Free radical scavenging effect, antioxidants reduce the purple-colored radical DPPH (diphenylpicrylhydrayl) to a yellow compound (diphenylpicryl-hydrazine) whose color intensity is inversely proportional to the ability of antioxidants to give protons [28].

In practice, a DPPH solution is prepared by solubilizing 2.4 mg of DPPH in 100 ml of methanol. A volume of 25 µL of extract solutions and the reference antioxidant, BHT (hydroxytoluene-butyl) are added to 975 µL of DPPH. The mixture is left in the dark for 30 minutes and the discoloration with respect to the control containing only the DPPH solution is measured at 517 nm against a methanol

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blank. The control contains all the reagents except the test sample, which is replaced by an equal volume of methanol [20]. The antioxidant activity values of aqueous and alcoholic extracts were studied and compared with those of ascorbic acid. The scavenging ability of the plant extract was calculated using this equation;

DPPH Scavenging activity (%)= $[(Abs_{control} - Abs_{sample})]/(Abs_{control})] \times 100$

Where $Abs_{control}$ is the absorbance of DPPH + methanol; $Abs_{sample}$ is the absorbance of DPPH radical + sample (i.e.extract or standard).

## II.7.Anti-inflammatory activity

24 Albinos mice were divided into 3 groups (weight: Between 25g-30g, feeding: granules tap, water, housing condition: Temperature of 24°C, lighting 12/24 hours), each group consisting of 8 animals; Group1- control group treated with saline, Group 3 – standard group treated with diclofenac sodium at 100 mg/kg bwt and Group 2 – test group treated. The injection of carrageenan (to 25ml of distilled water, gradually add carrageenan (0.5g) and adjust the volume to 50 ml with distilled water) under the plantar fascia of the mouse leg (0.025ml per mouse) causes an inflammatory reaction that can be reduced by an anti-inflammatory product. [29]. This study compares the reduction of plantar edema after injection of equal doses (0.5ml) of the anti-inflammatory product (aqueous and alcoholic extracts) and a reference product (diclofenac sodium 75mg) to the mice after 30 mn from the injection of carrageenan [5]. The percentages of inhibition of oedema, induced in mice by the injection of carrageenan, reflect the presence of anti-inflammatory activity, the higher these percentages are, the greater the anti-inflammatory activity is [30].

## II.8. Antibacterial and antifungal screening by Agar disk-diffusion method

Generally, antimicrobial agent diffuses into the agar and inhibits germination and growth of the test microorganism and then the diameters of inhibition growth zones are measured [31]. In this well-known procedure, agar plates are inoculated with a standardized inoculum of the test microorganism strains used: *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 3521 and the fungi strain *Candida albicans* ATCC 10231 provided by the National Pharmaceutical Control Laboratory (LNCCP).

Then, filter paper discs (about 6 mm in diameter), containing the test compound at a desired concentration, are placed on the Mueller-Hinton agar surface. An additional negative control disk without any sample but impregnated with the equivalent amount of methanol solvent was also used in the assay. The antimicrobial activity was considered beyond a diameter of 9 mm or more.

The Petri dish was inoculated with 1 µL of bacterial suspension adjusted and diluted to the concentration of $10^7$ CFU (0.5 McFarland standard diluted to 10%), and the final inoculum required is $10^4$ CFU per spot. Gentamycin and ciprofloxacin were used as positive controls for bacteria.

The minimum inhibitory concentration (MIC) of *C. albicans* was performed as for bacteria but the culture medium used was supplemented with Mueller-Hinton, 2% glucose and 0.5 µg/ mL methylene blue with pH of 7.4. The fungal suspension was set at 0.12-0.15 (λ=530 nm) at final concentration of $1\times10^3$-$5\times10^3$ CFU. Amphotericin B was used as positive control. All the plates were inoculated at 37 °C for 24 h. The MIC (of bacteria and yeasts) was considered as the weakest concentration for which there was considered as the weakest concentration for which there was no visual growth. The data were the mean of three replicates.

## III. Formulation of a cream

The following components were used to prepare the formulation using established methods (% weight): paraffin 30, glycerol 2, propylene glycol 1, stearyl alcohol 7; glycerol stearate 1, lauric acid 0.5, beeswax 1, extract *Olea europea* leaves extract (1-10) and purified water (55-60) [32].

The different components of the formulation are weighted, the two phases of the emulsion are then formed, depending on the solubility of the different components of the formulation: the oily phase contains the different fatty substances (oils, waxes), lipophilic emulsifiers, the lipophilic viscosifying agent and lipophilic antioxidants. The aqueous phase contains purified water, humectants, water-soluble viscosifiers, water-soluble emulsifiers, antimicrobial preservatives and water-soluble antioxidants. Both phases are heated to a temperature of 60-80°C, under slow agitation to melt the oil phase and dissolve the various components. The increase in temperature also makes it easier to emulsify and obtain a finer emulsion. Emulsification is achieved when both phases are maintained at the same temperature. It is done by a slow addition (drip) of the internal phase to the dispersant phase, under a more or less violent and constant agitation. This reduces the internal phase to small cells. At the end of the emulsification process, the mixture is subjected to a progressive and slow cooling to room temperature, under an increasingly slow agitation avoiding air inclusions. When the cooling process is complete, the active ingredients are added (Olive leaves extracts). The mixtures were stirred properly to ensure that the sample was uniformly mixed with the molten stage of the cream.

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### III.1. Evaluation of physicochemical parameters of a cream

Three pH measurements were taken by dipping the pH meter electrode into the preparations to be examined and then reading, 5 ± 0.01g of the cream was weighed accurately in a 100ml beaker. 45ml of water was added and dispersed the cream in it. The pH of the suspension was determined at 27ºC using the pH meter [33]. In addition, the spreading test was conducted by checking the homogeneity of the cream by applying a thin layer (0,5g) on a flat surface with a spatula. The regular or irregular distribution of extract in the excipients was noted [34].

#### III.1.1. Rheological properties

The rheological measurements of the ice cream mixes were made using a rheometer (*Physica MCR 301 rheometer*) with a cone and plate geometry. All the steady, time-dependent and viscoelastic (dynamic) properties were determined. A rheogram representing shear stress as a function of shear rate is plotted by regularly increasing and decreasing the shear rate, in order to return to the initial value [35] and [36].

#### III.1.2. Dermal irritation test

The rabbit irritation test was carried out using established procedure [37]. Healthy, 6 adult New Zealand rabbits (weighting 2.5-3 kg, age 18 weeks, half of male) were obtained by Pasteur Institute of Algiers. They were placed in polypropylene cages, provided with standard laboratory diet and water ad libitum. The animal facility was maintained at 22°C-24°C, a relative humidity of 55% ± 10%, and a 12 h light/dark cycle at 160-290 lux throughout the experiment. Animals were kept under acclimatization for eight days before application and were clipped free of fur 24 h before the application of the formulations. The cream formulated (500 mg) was applied on the right and left hind limbs of the rabbit, the site of approximately 25 $mm^2$ and covered with a patch (semi-occlusive) using a non-occlusive bandage to wrap the test areas *(Fiure 3 a, b).* After 24 h, the patch and test materials were removed and the sites were examined for skin irritation. The reactions were assessed using the scoring system and response categories according to OECD test guideline 404 [37] and [38]. The score of primary irritation was then calculated for each rabbit.

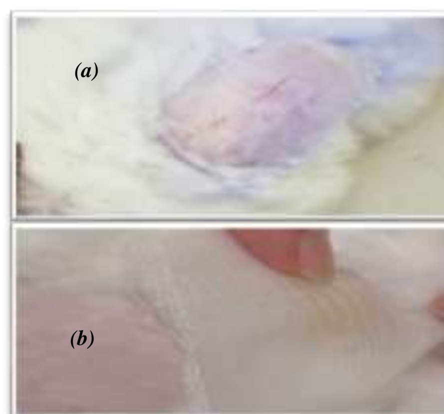


***Figure 3.*** *Photograph of a rabbit skin after scarification (a) and the application of the cream on the scarified skin (b).*

## IV. Results and discussion

### IV.1. Thin layer chromatography

The analysis of the crude extract by thin layer chromatography (TLC) allows us to confirm the presence of the bioactive molecule which is *Oleuropein* in in the plant. We notice the migration of the solution droplets deposited on the plate and the appearance of a green-brown band, which confirms the presence of the active ingredient (oleuropein) in our sample. In addition, a yellow-brown band representing Rutin appears. These results were explained in accordance with European Pharmacopoeia 6.0.

The retention factor ($R_f$) value of oleuropein is 0.6 with a green brown band that corresponds to oleuropein according to the European Pharmacopoeia. Oleuropein is a secoiridoid which is the most abundant phenolic compound in olive leaves and fruits and is responsible for the characteristic bitterness of olive fruit. Oleuropein concentrations of dry matter in the leaves can reach up to 60-90 mg $g^{-1}$. Several previous studies have reported oleuropein content of olive leaf, ethanolic and methanolic extracts [39].

### IV.2. Physicochemical and phytochemical analysis

The physical constant evaluation of the drugs is an important parameter in detecting adulteration or inproper handling of plants. The results of the physico-chemical analyses of the plant material (olive leaf powder) were presented in table 1.

The moisture content of olive tree leaves is found to be in acceptable range. The total ash and acid insoluble ash were performed to find the residue of the extraneous matter (e.g. sand and soil) [40].

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**Table 1.** *Comparative physicochemical analysis of olive leaves*

| Standards evaluated | Yields % | European Pharmacopoeia Standards (2008) % |
|---|---|---|
| Appearance | Green yellow | Green yellow |
| Loss on drying | 8.1 | NMT 10 |
| Total ash | 8.21 | NMT 9 |
| Sulfuric ash | 7,48 | NMT 8 |
| Ashes insoluble in hydrochloric acid | 0,957 | NMT 2 |

*NMT: not more than.*

The results obtained from physicochemical analysis for olives leaves were in accordance with all aspects and quality standards limit prescribed in European Pharmacopoeia, loss on drying result obtained (8.1%) is in accordance with standards (NMT 10% according to the European Pharmacopoeia, 2008), from this result we deduce the efficiency of the drying method carried out.
The percentage of total ash obtained is below the authorized limit, which means that the leaves were well treated before being processed into powder.
Ash values are used to determine quality and purity of crude drug. It indicates presence of various impurities like carbonate, oxalate and silicate. The acid insoluble ash consist mainly silica and indicate contamination with earthy material. Moisture content of drugs should be at minimal level to discourage the growth of bacteria, yeast or fungi during storage. [41].Several previous pharmacognostic studies were done for different parts of plants to evaluate pharmacological action as well as therapeutic efficacy and toxicity of the plant to establish as the drug, regulations on herbal medicinal products vary from country to country, and herbal preparations do occur not only in the form of medicinal products but also as less strictly regulated product groups like dietary supplements. [4].

### IV.3. Phytochemical Screening

Most of the drugs have definite specific chemical constituent's to which their biological or pharmacological activity was attributed. Qualitative and quantitative characterization of the active ingredient should be assayed using biomarkers. Defining of the biomarker has to be very specific and a lot of insight has to go into it before declaring any distinct molecule. Additionally, the mixture should be analyzed to develop finger printprofile [43]. Phytochemical screening has allowed us to highlight the presence of some secondary metabolites (Table 2) such as glucoside, anthocyanins, tannins, catechetical tannins, gallic tannins, lencoanthocyane, saponins, coumarins, flavonoids, free quinones, combined quinones, and mucilages on olive leaves. The detection of these phytoconstituentsis based on component solubility tests, precipitation reactions, and colour change.
The results obtained from phytochemical tests have revealed the richness of this plant in tannins, gallic tannins, saponins and flavonoids. The tests for lencoanthocyanins, alkaloids and combined quinones are marked negative; these results are identical to those of the previous work already carried out on olive leaves [44]. Knowing that olive leaves are rich in flavonoids, this richness could explain its antioxidant activity, linked to their polyphenolic structure [45]. On the other hand, it has also been found that olive leaves are rich in Saponins, which have a particularly strong anti-inflammatory and anti-edematous effect [46].

**Table 2.** *Comparative phytoconstituents of olive leave*

| *Phytoconstituents* | *Detected/ not Detected* | *Coloration* |
|---|---|---|
| *Glucosides* | + | *Purple* |
| *Anthocyanins* | *Rests* | *Red* |
| *Tannins* | +++ | *Blue to black* |
| *Catcheticaltanins* | ++ | *Red* |
| *Galictanins* | +++ | *Blue to black* |
| *Lencoanthocyanins* | - | *No red coloration* |
| *Saponins* | +++ | *white precipitate* |
| *Coumarins* | +++ | *Trouble formation* |
| *Flavonoids* | ++ | *Red to orange* |
| *Free Quinones* | +++ | *Red* |
| *Combine Quinones* | - | *No red coloration* |
| *Mucilages* | +++ | *white precipitate* |
| *Alkaloids* | - | *No red precipitate* |
| *Iridoid* | + | *Black precipitate* |

Similar results were reported by Malik [46]. Phytochemical screening is usually carried out to screen for and to characterized the constituents available in a given plant sample. Generally, in the phytochemical screening of any plant one normally identifies secondary metabolites that have accumulated to some extent at specific organ of the plant. These metabolites that are mainly used by the plant for protection against herbivores may have pharmacological activity when tested on animals. Result of phytochemical screening of *Olea europea*

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leaves us of the various extracts showed the presence of saponins, sterols, steroid, terpen and flavonoids. All these compounds were previously reported to occur in olive leaf. The compounds are found in various plant parts such as stems, roots, leaves, bark, flowers or fruits [47].

**IV.4. Extractions**

The percentages of the extractions carried out are represented in Table 3, Color and aspect of the different extracts are also represented. The choice of solvent was influenced by what was intended with the extract. Since the end product will contain traces of residual solvent, the solvent should be nontoxic and should not interfere with the bioassay. The choice will also depend on the targeted compounds to be extracted. [7]. So, table 3 shows the weight yields in percentage obtained in each of the different types of solvent, according to the procedure performed to obtain the extracts from Olive leaves. The influence of the type of solvent on the extraction performance was observed. The highest extraction yields were obtained by maceration with alcoholic and aqueous solutions, while the lowest yield corresponded to the extraction with hexane. For instance, many researchers obtained similar yields with extracts from maceration [16]. Also in the review performed by Melo et al., similar yields were reported for rosemary [48]. Therefore, for olive leaves, the extraction yield increased as the polarity of the solvent used to obtain the extracts increased. However, solvent composition should match the polarity of the target solutes. Chen et al. also reported low yields using non-polar solvents, and an increase in the yield with more polar solvent [49].

***Table 3.*** *Yields, aspect and color of olive leaves extracts in different solvents*

| *Extracts* | *Yields (%)* | *Aspect* | *Colour* |
|---|---|---|---|
| *Aqueous* | *36,64* | *Pastey* | *Brown* |
| *Ethanol* | *44,39* | *Friable* | *Black* |
| *Methanol* | *43,21* | *Friable* | *Green* |
| *Hexane* | *4.92* | *Tights* | *Yellow* |
| *Chloroform* | *7.25* | *Friable* | *Glossy black* |

**IV.5. Antioxidant activity results**

The antioxidant activity and radical scavenging properties of plants are related with its therapeutic properties. The antioxidant ability of the prepared olive leaf extracts (aqueous and ethanoic) was evaluated using DPPH assay. A freshly prepared DPPH solution has a dark purple colour with maximum absorption at 517 nm. This colour generally disappears when an antioxidant is present in contact with this radical. Subsequently, antioxidant particles can trap the free radical DPPH. What's more, convert it into a lacklustre item [50]. In this case, the antioxidant activity values of olive leaf crude extract were studied and compared with those of ascorbic acid. The results are represented in figure 4. Ethanoic extract has shown a higher antioxidant activity than that of aqueous extract, for a concentration of 20mg/ml, the ethanoic extract has reached an inhibition percentage of 94.22%, and for the same concentration, aqueous extract produces a lower inhibition percentage of 80.71%.

The activities of the two extracts were compared with that of ascorbic acid that has an important antioxidant activity [51]. The results indicated that ethanoic extract has a significantly higher activity than ascorbic acid (90.33%) and for aqueous extract; the activity was lower but close to ascorbic acid and it remains important. This result was not unexpected, since the extracts contain a mixture of compounds whose proportions depend on their solubility in the extraction solvent.

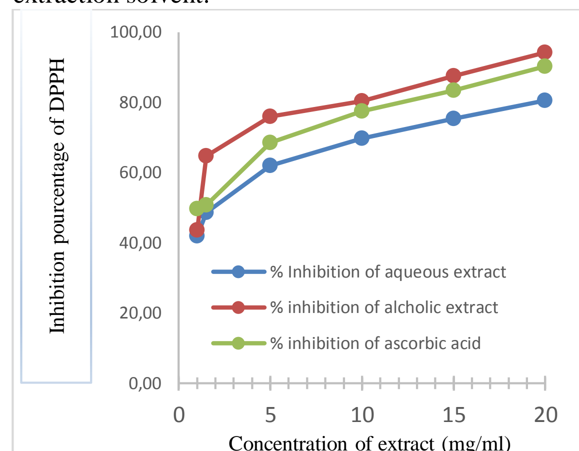


***Figure 4.*** *Percentage of inhibition of free radical DPPH vs. concentrations.*

For ethanoic extract, similar antioxidant activity was proved and reported by Vieiteza using different plant [16]. The antioxidant capacity of olive leaves proved in our study is due to Oleuropein, the most abundant biological active ingredient in olive leaves [52]. So, similar results were found in vitro by Jemai, in this study the lipid-lowering and the antioxidative

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activities of oleuropein, oleu- ropeinaglycone and hydroxytyrosol-rich extracts in rats fed a cholesterol-rich diet were tested. They highlighted olive tree by-products as a source of antioxidants able to reduce the frequency of cardiovas- cular diseases [53].
Olive leaf extract seems to have a very important antioxidant activity. This effect may be due to the phenolic compounds it contains. The content of phenolic compounds in olive leaves may exceed 250 mg/g of dry matter [54]. Thus, the antioxidant activity of each extract depended on the nature and potency of their phenolic compounds. In the extract from olive leaves, oleuropein was found to be the major phenolic compound. Rutin, vanillin and caffeic acid are minor compounds. In fact, oleuropein, itself (a powerful antioxidant), has given ethanoic extract an interesting antioxidant effect [55].

**IV.6. Anti-inflammatory activity results**

The current study interested on a new alternative source of phytochemicals with an anti-inflammatory activity. The percentages of inhibition of oedema, induced in mice by the injection of carrageenan, reflect the presence of anti-inflammatory activity, the higher these percentages are, the greater the anti-inflammatory activity is. The results are represented in figure 5:

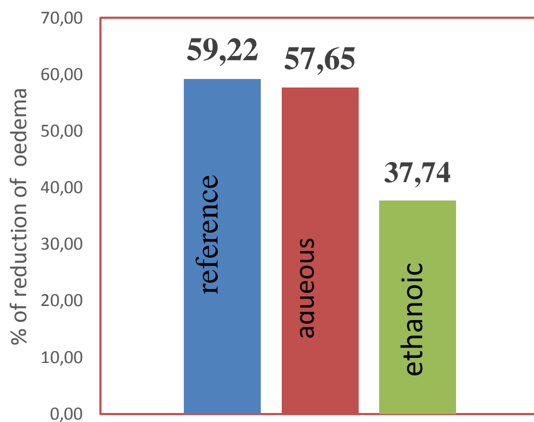


*Figure 5. Percentage of oedema inhibition of both extracts and reference (Diclofenac).*

We noted that the aqueous extract reduced the inflammation from the first test performed after 4 hours of injection of the irritant solution to 57.65%, and almost similar to Diclofenac that inhibited 59.22% of the inflammation. For alcoholic extract, we noted that the percentage of inhibition is lower compared to the aqueous extract and to the reference with a value of 37.74%. This value can also be considered as an important value. The anti-inflammatory activity of our extracts can be explained by the presence of flavonoids, according to Bruneton [56]. Flavonoid mainly known for their anti-inflammatory activity.

In general, the anti-inflammatory properties of flavonoids involve the inhibition of the activity of several proinflammatory biochemical mediators (cytokines, adhesion molecules, NO•) and enzymes (COX-2, LOX, iNOS). In addition, these compounds may also interact with important transcription factors and signaling pathways, including nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) thus regulating the proinflammatory genes' expression [57].

**IV.7. Results of antibacterial screening**

Plants and other natural sources can provide a huge range of complex and structurally diverse compounds. Recently, many researchers have focused on the investigation of plants and microbial extracts, essential oils, pure secondary metabolites and new synthetized molecules as potential antimicrobial agents [58]. The results of the antimicrobial properties of the different extracts are represented in table 4 and Fig.6.

***Figure 6.** Inhibitory effect of aquous extract against Staphylococcus aureus ATCC 6538.*

Aqueous and alcoholic extracts were found to be very active on *Staphylococcus aureus* with an inhibition diameter of 24.5 ± 0.1mm for aqueous extract and 20 ± 0.8mm for ethanoic extract, as well as on *Bacillus subtilis* (23.2 ± 0.1mm and 15.2 ± 0.3mm respectively). Hexane extract showed lower antibacterial activity. Additionally, chloroformic extract showed no antimicrobial effect except against *Bacillus subtilis*. Ruiz-Barba et al. [59] have proved similar results on olive leaves composition as antimicrobial agent.
The bactericidal effect of olives was largely owing to the presence of oleuropein [60]. According to Žugčić, the prebiotic capacity of olive leaves extracts could be related with the high amount of total phenolic compound. In this regard, the phenolic compounds may modulate microbiota in the intestine by selectively increasing the growth of potential beneficial bacteria as *Bifidobacterium spp.* and *Lactobacillus spp.* and decreasing the growth of harmful bacteria such as clostridia. On the other hand, ligstroside (a polyphenolic compounds present in olive leaves) showed a strong antimicrobial activity against a broad spectrum of microorganisms both Gram-positive and Gram-negative. Olive leaves

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also contain other important phenolic compounds with different biological activities. Some of these molecules exhibit a potent antimicrobial potency, which differs according to the extraction method as well as the targeted compounds. In fact, the inhibition of Gram-positive and Gram-negative bacteria growth varied according to the different molecules extracted from the leaves [61].

### IV.9. Results of control of cream formulation and Rheological propriety

According to the very interesting results found, olive leaf extracts can have a beneficial role for human health, in order to valorise and exploit its therapeutic effects, a cream formulation have been carried out. The pH of the cram ranged between 5.32 - 5.36 that is near skin pH. The absorption of creams into the skin through the stratum corneum has been shown to be affected by the pH partition hypothesis (figure 7). The stratum corneum is remarkably resistant to alterations in pH, tolerating range of 3-9 [17].

***Figure 7.*** *Photograph of the formulated cream based on olive leaves extract*

This result indicated that the cream formulation will be well tolerated on the skin and will not cause any form of irritation. According to the spreading test carried out, it was noticed that the cream spreads very well, and the absence of lumps and phase separation (figure 8), therefore the appearance of a conforming cream. The rabbit irritation test which was carried out showed that the irritation index in vivo was lower than 0.125. This value is in the negligible category for primary irritation. The skin sections of all the animals presented no visible lesions indicating that the formulation was well tolerated in the animals; the cream formulation can thus be regarded as safe for use. The stream properties affect each progression of the pharmaceutical improvement process; therefore, it is essential for pharmaceutical scientists to outline the flow properties. So, rheology is the study of the flow and deformation of materials under the effect of applied forces that are commonly measured using a rheometer.

**Table 4**. Inhibition diameters of olive leaves extracts

| Extract | Strains | Inhibition Diameter (mm) |
|---|---|---|
| **Aqueous extract** | *Staphylococcus aureus* ATCC 6538 | 31.5 ± 0.7 |
| | *Escherichia coli* ATCC 25922 | 0 |
| | *Bacillus subtilis* ATCC 3521 | 23.2 ± 0.1 |
| **Alcoholic extract** | *Staphylococcus aureus* ATCC 6538 | 20 ± 0.8 |
| | *Escherichia coli* ATCC 25922 | 24.5 ± 0.1 |
| | *Bacillus subtilis* ATCC 3521 | 15.2 ± 0.3 |
| **Hexane extract** | *Staphylococcus aureus* ATCC 6538 | 18.5 ± 0.7 |
| | *Escherichia coli* ATCC 25922 | 19.5 ± 0.3 |
| | *Bacillus subtilis* ATCC 3521 | 17 ± 0.3 |
| **Chloroformic extract** | *Staphylococcus aureus* ATCC 6538 | 0 |
| | *Escherichia coli* ATCC 25922 | 0 |
| | *Bacillus subtilis* ATCC 3521 | 14.6± 0.2 |

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***Figure 8.*** *Spreading test of the formulated cream*

Many commonly used materials and formulations have complex rheological properties, whose viscosity and viscoelasticity may vary according to external conditions, such as stress, deformation, duration and temperature. The rheological results of the cream are shown in the figure 9.

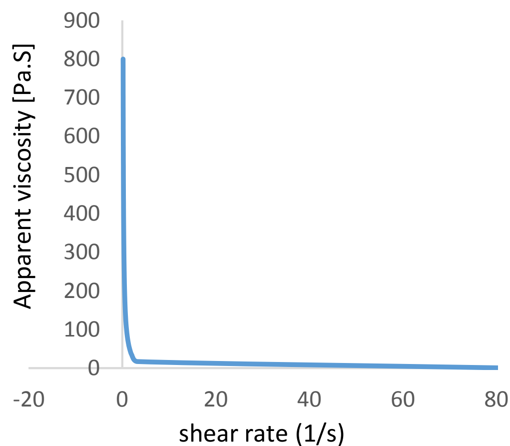


***Figure 9.*** *Apparent viscosity vs. shear rate of the formulated cream*

From the graph shown in figure. 10, it can be seen that with the increase in shear rate the viscosity decreased until it reached constant values, it can be concluded that the formulated cream is a non-Newtonian fluid. Often, the behavior of non-Newtonian fluids depends on time because changes in microscopic structures are not instantaneous. We thus have various manifestations of these phenomena in the rheogram plots.

This result is in accordance with Chi and Lee et al [61]. They found that the viscosity of the fluid varies with the shear stress and the consistency depends upon the duration and rate of shear.

The time-dependent change in viscosity is the desired property in the pharmaceutical formulations due to their requirement of the flexibility in drug delivery. If the rheological manifestation of viscosity induced structural changes is reversible and time-dependent, the effect is called thixotropy. The difference between thixotropical and shear-thinning behavior is only that of the time for the structure to regroup during shear or at rest. When a material is shear-thinning it changes the micro-structure instantly, whereas for a thixotropic material it takes some time. At present, shear-thinning materials also are considered as thixotropic since it always takes time, even though limited, to regroup the micro-structural elements [61]. A rheogram representing shear stress as a function of shear rate is plotted by regularly increasing and decreasing the shear rate to return to the initial value. The result is represented in figure 10.

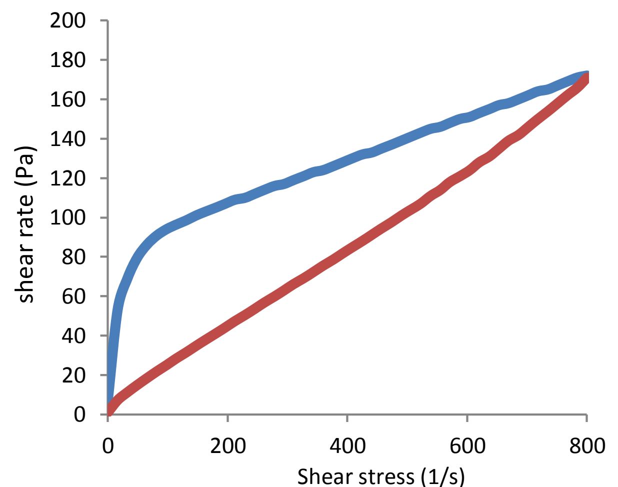


***Figure 10.*** *Shear stress vs. shear rate of the formulated cream*

The structure has been permanently modified during charging and the behaviour of the product is affected (different discharge rheogram, generally less viscous liquid), so it is said that the fluid is thixotropic. A fluid is thixotropic if its viscosity, at a constant shear rate, decreases over time if this phenomenon is reversible [35]. Thixotropy is the property exhibited by the pseudo plastic systems that exhibit the time-dependent change in the viscosity. Thixotropic systems demonstrate a decrease in viscosity with time under the constant shear. An enhancement of shear due to progressive break down of the structure of liquid and further rebuilding of the structure due to Brownian motion, which makes the particles move to their most favorable positions from a structure–entropy perspective and assumed the reason for pseudo plasticity [61].

## V. Conclusion

This work shows the importance of olive leaf extracts rich in oleuropein as an antioxidant, antibacterial and anti-inflammatory agent *in vitro*

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and *in vivo* tests. Phytochemical screening has shown the presence of several phenolic compounds such as tannins, flavonoids, glucoside, saponosides and anthocyanins that give the olive tree these various therapeutic activities. The antioxidant activity of the alcoholic extract was slightly higher than ascorbic acid; the antioxidant activity of the aqueous extract is lower but may remain high. The results obtained clearly show that polyphenols extracted from olive leaves are real competitors for ascorbic acid. In addition, they have the advantage of being of natural origin. The study of the anti-inflammatory activity of the two extracts of olive leaves extract on mice showed that the aqueous extract has a very important anti-inflammatory activity and almost similar to diclofenac followed by the alcoholic extract which gave a lower activity, but, considered as a good activity it should be also noted that olive leaf extracts can have a beneficial role in promoting probiotic bacteria and inhibiting harmful bacteria. Moreover, the herbal cream formulation from olive leaf extracts had acceptable physicochemical and safety profiles with significant anti-inflammatory and antibacterial activities, thus the cream formulation could be useful in the treatment of skin infections.

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