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ANALYTICAL CHARACTERISATION OF SOME BUDS OF ETHERIC OILS USED IN COSMETICS

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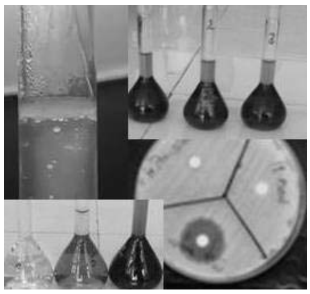
Etheric oils, or aromatic oils as they were once called, have been used by many cultures around the world for centuries; today, they are used successfully worldwide in many industries, particularly in perfumes, organic and dermato-cosmetics, homeopathic medicine and aromatherapy.
The aim of this paper is to pinpoint the potential of three etheric oils used in dermato cosmetic products, oils extracted from buds of *Populus nigra*, *Pinus sylvestris*, and *Abies alba*, in regenerating and nourishing the skin but also in treating many dermatological skin problems. Using some of the best analytical methods to evaluate the antioxidant activity: an adapted Folin Ciocâlteu assay, DPPH Radical Scavenging test and carrying out the analytical characterization, remarkable results have been reached.
To highlight further the antimicrobial activity, the procedure was directed towards determining the sensitivity of two bacterial strains, MRSA (Methicillin-resistant *Staphylococcus aureus* and *Klebsiella pneumonia*, on the etheric oils comparing with antibiotics. *Populus nigra* etheric oil has a high effectiveness on inhibiting the MRSA better than a synthetic drug and can be a top ingredient in dermato-cosmetics that treat *Staphylococcus aureus* and *Klebsiella*, in good accordance with the antioxidant activity. *Pinus sylvestris* and *Abies alba* recorded good results but lower than *Populus nigra.*



INTRODUCTION

Etheric oils are an important source of raw ingredients whose uses varied from religious purposes to healing the sick. They represent the *quinta essentia* of a plant and are best described by Paracelsus, the enormously influential sixteenth-century doctor and alchemist: "The quinta essentia is that which is extracted from a substance - from all plants and from everything which has life - then freed of all impurities and perishable parts, refined into highest purity and separated from all elements. The inherency of a thing, its nature, power, virtue, and curative efficacy, without any foreign admixture, that is the quinta essentia".[1] Depending on the plant species, etheric oils own a complex chemical composition, with low or high quantity of active principles rich in antioxidants.

In the last few years, the usage of organic cosmetics increased due to people's desire to maintain skin health with products free of chemicals, despite of the large number of products with synthetics, vigorously promoted.

Contrary to the synthetic drugs, antimicrobials of plant origin are not associated with side effects and have an enormous therapeutic potential to heal

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many infectious diseases.[2] The potential for developing antimicrobials from higher plants appears rewarding as it will lead to the development of phytomedicine to act against microbes.[3]

Although Romania offers more than 3400 medicinal plant species[4], according to the literature there are some species having etheric oils with high antioxidant and antimicrobial capacity: black poplar (*Populus nigra*), pine (*Pinus sylvestris*), and fir (*Abies alba*). The oils extracted from their buds are already used in different pharmaceuticals and organic cosmetic products to heal various dermatological issues. *Populus nigra* (poplar) is known to have various uses in medicine. Black poplar buds contain phenolic glycosides, volatile oil, fatty oil, tannins, resins, malic acid, gallic acid, saponins, mannitol and flavones. The etheric oil contains betulen, α, β and γ-betulenol, δ-humulen and α-caryophyllene.

The active principles contained by the black poplar buds act as an astringent, slightly anti-inflammatory and antimicrobial, weak analgesic, antiseptic and antipyretic. Propolis originating from black poplar buds has also many biological and pharmacological properties and his mechanism of action was studied in recent years, using different experimental models in vitro and in vivo.[5]

Etheric oil of pine buds contains many compounds such as α-pinene, β-pinene, α-tujene, tricilene, sabinene, borneol, thymol, limonene, β-felandren, E-caryophyllene, caryophyllene oxide, α-terpineol, α-terpinolen, α-terpinene, γ-terpinene, terpinene-4-ol, β-burbonen acid, gallic acid, p-coumaric acid, homovanillic acid vanilic, α, γ, δ-cadinene, α-humulen, myrcene, α, β-cubeben, catechin, epicatechin, taxiresinol.

The etheric oils of *Pinus* species demonstrate primarily antimicrobial, expectorant activities and promote blood circulation.[6] Antioxidative, free radical scavenging, insecticidal, phytotoxic, larvicidal, repellent, anti-inflammatory, antiviral, and antifungal properties of *Pinus* etheric oils have been reported as well.[7]

Abies alba (fir) is one of the most common species of conifers in Central Europe and therefore of particular importance. The etheric oil is rich in gallic acid and p-coumaric acid, p-hydroxybenzoic acid, homovanillic and vanillic acids, phenolic compounds such as catechin, epicatechin and taxiresinol.[8]

The etheric oil showed antioxidative and antibacterial activities[9] and the extract of silver fir and common mistletoe (*Viscum album*) mixture exhibited antiproliferative and anticancerogenic features. However, many other fir species have been recognized as rich sources of ligands, flavonoids and other phenols with antioxidant activity.[9,10]

Antioxidants are vital for our organism and they represent a wide class of chemicals compounds that fight against the oxidative processes.

Oxidative stress was defined as the organism`s status involving cell damage, by enhance release of radical or non-radical oxygenated species.[11] An antioxidant capacity is defined as the ability of compound (or mixture of compounds) to inhibit the oxidative degradation of various compounds like preventing lipid peroxidation.[12] Antioxidants are endogenous (uric acid, bilirubin, albumin, metallothioneins, enzymes superoxide reductase, glutathione-S-transferase) and exogenous - natural antioxidants (vitamin C, vitamin E, carotenoids, phenolic compounds such as flavonoids, phenolic acids, anthocyanin's, proteins such as transferrin, ceruloplasmin or albumin, some minerals like Se). The main compounds found in natural sources are phenolics (flavonoids or non-flavonoids), associated to health benefits resulted from the inhibition of low-density lipoprotein oxidation.[13-14] Among this various types of compounds, etheric oils from aromatic and medicinal plants are receiving particular attention due to their radical scavenging properties.[15,16]

Today, dermatological skin conditions like acne, dermatitis, irritations and skin allergies are healed using plant extracts under the recommendation of dermatologists and pharmacists. Acne is one of the most common skin disorders worldwide. It affects a very large number of people, both man and women, from 9 to 45 years old. There are multiple reasons why acne affects the epidermis: low immunity combined with a stressful life schedule, polycystic ovaries (at women), drugs for treating lung diseases, toxic working environments, genetic factors, MRSA (Methicillin-resistant *Staphylococcus aureus*), sebum excess etc.

The best methods to obtain a detailed characterization of the etheric oils are the Gas chromatography–Mass spectrometry (GC-MS), High Performance Liquid Chromatography (HPLC), Liquid chromatography–mass spectrometry (LC-MS). To analyse the antioxidant capacity of the etheric oils, the most common methods are Folin Ciocalteu Assay, 2,2-diphenyl-1-picrylhydrazyl Assay (DPPH), Cyclic Voltametry (CV) and Differential Pulse Voltammetry (DPV).[17-18]

The use of the DPPH assay provides an easy and rapid way to evaluate antioxidants by spectrophotometry, so it can be useful to assess various products at a time. The antioxidant capacity can be measured in pure substances as well as in mixtures of different samples of herbal and animal origin, such as plasma, blood, tissues homogenates of fruits and vegetables, juices and other foods. As we mentioned above, there are many methods for measuring of total antioxidant capacity (AC), but in literature the most often cited are the following: Folin Ciocâlteu assay, FRAP - Ferric Reducing Antioxidant Power,[19] ORAC - Oxygen Radical Absorbance Capacity,[20] and TEAC - Trolox Equivalent Antioxidant Capacity.[21]

The pH of the skin is a key factor in barrier homeostasis, stratum cornea integrity and antimicrobial defence. Maintaining its balance is very important. Skin pH is normally acidic, ranging in pH values of 4–6, while the body's internal environment maintains a near-neutral pH (7–9). This creates a steep pH gradient of 2–3 units between the stratum cornea and underlying epidermis and dermis. In spite of mounting evidence that skin pH plays a vital role in stratum cornea function, application of the "acid mantle - skin" concept in clinical care has lagged behind. The importance of preserving an acidic skin pH, especially in those affected by certain skin diseases, remains an under-recognized topic by practicing U.S dermatologists. This is evident by the scarcity of low pH soaps, cleansers, and moisturizers available in the US market.[22] On the other hand, the oxidation-reduction potential (ORP) is a measure of overall balance between oxidants and antioxidants, providing a comprehensive measure of oxidative stress.[23]

Given that cosmetology is closely related to dermatology and chemistry, the aim of this paper was to measure the pH, ORP, total phenols, antioxidant capacity and antimicrobial activity of three etheric oils used in dermato-cosmetic products, oils extracted from buds of *Populus nigra* (poplar), *Pinus sylvestris* (pine), and *Abies alba* (fir).

RESULTS AND DISCUSSION

Physico chemical parameters

In our opinion, cosmetics ingredients pH is important to be determined and it is of the same importance as the pH of the skin. We think that the oxidation reduction potential (ORP) values can be a starting point in evaluating the antioxidant capacity of the etheric oils on skin surface and furthermore, a criterion in evaluating medicinal oils benefits.

Although pH and ORP are not found in the EU Regulations as mandatory measurements for cosmetics products or quality parameters for cosmetic ingredients, we think it can be a starting point in product description. Table 1 presents the measured pH and oxidation reduction potential (ORP) of three etheric oils, all obtained from dried plant species. The pH values are low and this helps improving skin pH and skin metabolism.

Antioxidant activity

For antioxidant activity evaluation we followed the total phenols content (TP) determination using an adapted Folin Ciocalteu method and DPPH (2,2-diphenyl-1-picrylhydrazyl) Radical Scavenging test. All the results are presented in Table 2. TP in black poplar (*Populus nigra*), fir (*Abies alba*) and pine tree (*Pinus sylvestris*) was 21.37, 0, respectively 14.37 mg GAE/mL. Regarding the activity of free radicals, DPPH value for black poplar was 1.841, 0.923 for fir and 0,836 mg GAE/mL for pine tree. According to the results obtained, black poplar (*Populus nigra*) has the highest antioxidant activity due to its rich composition in bioactive compounds, followed by pine tree (*Pinus sylvestris*) and fir (*Abies alba*).

Table 1

Physical-chemical parameters of bud etheric oils used in cosmetics

Sample	pH, units	ORP, mV
Black poplar (*Populus nigra*) bud etheric oil	3.78	179.1
Fir (*Abies alba*) bud etheric oil	3.79	175.2
Pine tree (*Pinus sylvestris*) bud etheric oil	4.08	173.2

Table 2

Total phenols (TP) and antioxidant activity by DPPH test of buds etheric oils used in cosmetics

Sample	**TP, mg GAE/mL**	**DPPH, mg GAE/mL**
Black poplar (*Populus nigra*) bud etheric oil	21.37	1.841
Fir (*Abies alba*) bud etheric oil	0	0.923
Pine tree (*Pinus sylvestris*) bud etheric oil	14.37	0.836

Table 3

The sensitivity of two bacterial strains, MRSA (Methicillin-resistant *Staphylococcus aureus* and *Klebsiella pneumonia,* to investigated etheric oils comparing with antibiotics (mm diameters in complete inhibition)

MRSA				**Klebsiella pneumonia**			
pine buds (*P.sylvestris*) etheric oil	**11**	Biseptol Netilmicin Ciprofloxacin Ofloxacin Levofloxacin Moxifloxacin	20-28	pine buds (*P.sylvestris*) etheric oil	0	Levofloxacin	23
fir buds (*A.alba*) etheric oil	**11**			fir buds (*A.alba*) etheric oil	0	Netilmicin	0
poplar buds (*P.nigra*) etheric oil	**32**			poplar buds (*P.nigra*) etheric oil	**25**		

The Folin Ciocalteu method (F-C) is one of the simplest methods for the detection of a wide range of antioxidant compounds in a large variety of plant species. For many years now, the F-C method of assay has been in use as a measure of polyphenol in natural products, and the basic mechanism is an oxidation/ reduction reaction with the phenolic group being oxidized and the metal ion reduced. The major classes of antioxidant compounds: flavonols, flavones, flavanones, flavanols, proanthocyanidins, isoflavones, anthocyanins, phenolic acids are detected by the Folin methods.[24]

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is also a good method for characterizing a cosmetic ingredient. DPPH is an antioxidant assay based on electron-transfer that produces a violet solution in methanol or ethanol. This free radical, stable at room temperature, is reduced in the presence of an antioxidant molecule, giving rise to colorless solution.

Besides Folin Ciocalteu and DPPH assays, antioxidant capacity can be evaluated upon other methods as well. Recently, special interest has been bestowed on the application of electrochemical methods, as they have the advantage of fastness and relatively inexpensive instrumentation and small volumes of samples usage: voltammetric, amperometric, biamperometric, potentiometric and coulometric methods for total antioxidant capacity estimation.[25] Our group will take these methods into account in future scientific research.

Antimicrobial activity

Plants are known to have antimicrobial activity and this approach has started to gain the attention of many pharmaceutical scientists focused on finding less invasive treatments for acne vulgaris caused by bacteria.

Table 3 presents the results obtained regarding the antimicrobial activity of the investigated etheric oils.

Populus nigra (poplar) etheric oil had a high effectiveness on inhibiting the MRSA (32 mm), followed by *Pinus sylvestris* (pine) etheric oil (11mm) and *Abies alba* (fir) etheric oil (11mm). The results are similar with those obtained using antibiotics. *Populus nigra* (poplar) etheric oil was the only one with high effectiveness on inhibiting *Klebsiella* (25 mm), higher than antibiotics, while *Pinus sylvestris* (pine) and *Abies alba* (fir) etheric oils did not inhibit *Klebsiella* (0 mm).

There is significant in vitro evidence suggesting a possible pathogenetic role for *S. aureus* in acne vulgaris.[26] *Staphylococcus aureus* is one of the prominent medically important bacterial pathogen. Its potential to cause wide spectrum of pyogenic lesions involving several organs, hospital outbreaks and community acquired infections are well recognized. *S. aureus* infections are often fatal in nature and are associated resistance to several beta-lactam antibiotics used in hospitals.[27] Acne vulgaris is the most common disorder of human skin that affects up to 80% of adolescents and young adults in their lives.[28] The widespread use of antibiotics has unfortunately led to the emergence of resistant bacteria.[29]

The potential for developing antimicrobials from higher plants appears rewarding as it will lead to the development of phytomedicine to act as against microbes. Plant-based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials. In recent years, Klebsiella species have become important pathogens in nosocomial infections[30]. The genus Klebsiella belongs to the tribe Klebsiellae, a member of the family Enterobacteriaceae. Three species in the genus Klebsiella are associated with illness in humans: *Klebsiella pneumoniae*, *Klebsiella oxytoca,* and *Klebsiella granulomatis*.

EXPERIMENTAL

Chemicals

All used reagents were of analytical reagent grade. Gallic acid was purchased from Fluka (Buchs, Switzerland), Folin – Ciocalteau reagent from Merck (Darmstadt, Germany) and DPPH from Sigma Aldrich. Gallic acid (standard phenolic compound) 1×10^{-2} mol×L^{-1} was prepared by dissolving 0.1881g of gallic acid in 100 mL of methanol 50%. Folin – Ciocalteu reagent was diluted with distilled water 1:2 (V:V). DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) 1.268 mM was prepared by dissolving 0.1000 g in 200 mL methanol.

The sensitivity of two bacterial strains, MRSA (Methicillin-resistant *Staphylococcus aureus* and *Klebsiella pneumonia* to three etheric oils, poplar (*P. Nigra*), pine (*P. Sylvestris*) and fir (*A. Alba*), was performed at S.C. Laborator Biotest S.R.L., Constanta, ROMANIA by doctor Adelina Caizerliu, laboratory medicine physician. For this test we used the Mueller Hinton culture media and discs purchased from S.C. Mikrobiologie Labor Technik LLC. The Mueller Hinton culture media are the one that are used for performing pathogen susceptibility. The discs were Bioanalyse AST discs, in blank, impregnated with the solution and applied on Mueller Hinton plates that contained MRSA.

Sampling

The fresh buds of black poplar (*Populus nigra*), pine (*Pinus sylvestris*) and fir (*Abies alba*) were picked in 2016, at full bloom from Buzau county, Romania.

The plant materials were naturally dried on nets situated in an airy space for 2 weeks. For the oil extraction of each plant species only dried plants have been used. The method of extraction was hydrodistillation using 300 g dried and 1L distilled water in Neoclevenger device. The distillation was carried out for 3 to 4 h at moderate temperature.

Measurements

To characterise the investigated buds etheric oils there were determined their pH and oxidation reduction potential (ORP) using a pH – meter pH 300 from Oakton, USA with a combined electrode.

Alcoholic solutions of each sample there were prepared by dissolving a known amount of oil in ethanol in order to measure the total phenols concentration and to make DPPH radical scavenging test. Spectrometric measurements were carried out using a Camspec M330 UV VIS scanning spectrophotometer.

To plot the calibration curve for total phenols determination in the range of 0.68–4.76 mg GAE/L, 1 mL Folin Ciocalteu reagent 1:2 was added in 50mL calibrated flasks to different volumes of standard gallic acid solution, 1 mL sodium carbonate solution 20%, then the mixture was mixed and let standing 10 min at room temperature and fill up to the mark with distilled water. At the end the mixture was homogenized and let under room temperature 30 minutes for the color stabilization and after that the absorbance was read at 681 nm.

To perform the DPPH test, a calibration curve that follows the absorbance decreasing (not more than 50%) with the increase of the amount of gallic acid was drawn in the range of 0–2.72 mg GAE/L. To different volumes of standard gallic acid solution, 5 mL of DPPH solution were added in 25 mL calibrated flasks, filled up to the mark with methanol, then the mixture was homogenized and let to dark under room temperature for 45 minutes and the absorbance was read at 530 nm. For both spectrometric analyses, 1 mL of each oil alcoholic solution has been processed similar with samples for calibration curves.

To highlight further the antimicrobial activity the procedure was directed towards determining the sensitivity of MRSA (Methicillin-resistant *Staphylococcus aureus* and *Klebsiella pneumonia* to three etheric oils with antimicrobial activity: *Pinus sylvestris* (pine buds), *Abies alba* (fir buds) and *Populus nigra* (poplar buds). In our case, instead of using discs impregnated with antibiotics, we used discs impregnated with the etheric oils. In the same time, the sensitivity of MRSA and Klebsiella pneumonia was tested on antibiotics like Levofloxacin, Biseptol, Netilmicin, Ciprofloxacin, Ofloxacin, Levofloxacin and Moxifloxacin. Handling seeding and reading microbiology samples was done only inside the laminar flow hood. The working surface was decontaminated before and after with appropriate disinfectants. Media culture and discs were removed from the refrigerator and left to warm.

The media cultures were placed in thermostat at 37° C for one hour before using. A quantity of microbial substance was deposited on the surface of an agar culture medium pre-sown with the bacteria tested. Two phenomena occurred simultaneously: the dissemination of the etheric oils and bacteria growth. In areas where the antimicrobial achieves higher concentrations than MIC (Minimum Inhibitory Concentration), the bacteria does not grow. Once the culture enters in exponential phase 'critical moment', the bacteria divides faster than etheric oils diffuses and accumulates a canvas visible culture that is not influenced by subsequent changes of the etheric oils concentration. The bacteria tested, depending on the diameter of the inhibition area, was classified as sensitive or intermediate resistance.

The bacterial strains were isolated from biological samples (urine, exudate throat, nasal). The blank discs were left to impregnate in the etheric oils for 15-30 min. Impregnation was carried out in glass Petri dishes as the plastic dishes have been distorted, darkened and melted. We kept the disks for 2, 8 and 24 hours and read the results. Reading was done by measuring ruler graduated in complete inhibition mm diameters.

CONCLUSIONS

The usage of some etheric oils in cosmetics have a lot of advantages: they quickly penetrate the epidermis due to their rapid molecular recognition by skin metabolism, have a visible efficacy and applied as instructed, rejuvenate the epidermis, enhance the tissue tonicity and regenerate the epidermal cells. The *P. nigra* and *P. sylvestris* buds etheric oils have the highest ORP and polyphenolic compounds content, similar with those reported in the literature. The etheric oils taking into study are microbiologically pure and they can safely be used in cosmetics. All three etheric oils recorded considerable results in the microbiological study, inhibiting the MRSA (all three etheric oils) and *Klebsiella* (only *P.nigra* etheric oil) better than the antibiotics. This minor results are very important and will lead to deepening studies regarding antimicrobial activity of plant extracts, especially of the etheric oils. The experimental results are consistent with the practical results obtained in cosmetology by Pharm. Ioana Adina Oancea and Chem. Elena Oancea.

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