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HEALTH SCIENCES INSTITUTE

PHARMACOGNOSTICAL STUDIES ON *OLEA EUROPAEA L.*

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PHARMACOGNOSY

MASTER THESIS

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Özet

Oleacea familyasının bir üyesi olan zeytin ağaçları (Oleo europaea) Akdeniz bölgesinde yaygın olarak yetiştirilir. Ağaçların meyvesi ve meyvesinden elde edilen yağ Akdeniz mutfağının en önemli öğelerinden biridir. Zeytinin ana bileşenlerinden birisi oleuropeindir ve ham zeytine acı bir tat verir. Bu çalışmada, oleuropein yapraklardan hareketle hazırlanan etanolik ekstreden elde edildi. Saflaştırma işlemi için, ham ekstre, su-metanol karışımı ile karşıt faz silikajel üzerinde fraksiyonlandı. Son saflaştırma işlemi, oleuropein yönünden zengin olan fraksiyonların Sephadex LH-20 üzerinde su-metanol (1:1) karışımı kullanılarak gerçekleştirildi.

Oleuropeinin yapı tayini 1D (1H- and 13C-NMR, DEPT-135) ve 2D NMR (COSY, HSQC and HMBC) ölçümleriyle gerçekleştirildi. HL-60 hücreleri ile yapılan çalışmada, anlamlı antioksidan aktivite (IC50 10.02µg/ml) ile antienflamatuvar aktivite için bir indikatör olan iNOS inhibe edici (IC50 value 1µg/ml) etkisi gösterildi. Diğer taraftan, Hela, A549, MCF-7, U78MG, MPANC-69, MDA-MB-241, HEK-239, CaCo-2, RAW-264.7 ve PC-3 hücre panellerinde hücre gelişmesini arttıran bir rol oynadığı saptandı.

Ayrıca, Escherischia coli, Staphylococcus aureus, S. epidermidis, Enterecoccus faecalis, Salmonella typhimurium gibi hem Gram-pozitif hem de Gram-negatif organizmalara karşı 62.5 µg/ml dozda antimikrobiyal etkinlik gösterdiği belirlendi. Buna karşılık, E. faeciumas için bu etki 31.2 µg/ml dozda gözlendi. Oleuropeinin, aynı zamanda hidatise neden olan Echinococcus granulosus'a karşı aktiviteye sahip olduğu gösterildi. Oleuropeinin %2'lik çözeltisinin (0.1 mg/ml), parazitleri 5 dakika içinde öldürdüğü saptandı.

Sonuç olarak, bu çalışmada, zeytin ağacının ana bileşeni olan oleuropein, antimikrobiyal aktivite, hücre proliferasyonu, iNOS inhibisyon ve antioksidan aktiviteleri yönünden değerlendirildi. Bu sonuçlardan hareketle, oleuropein için yara iyileştirici, cilt koruyucu, enflamasyona bağlı barsak hastalıklarında koruyucu amaçlı farmakolojik ve biyolojik aktivite çalışmalarının yapılabileceği kanısına varıldı.

Anahtar Kelimeler: Olea europaea, oleuropein, Echinococcus granulosus, sitotoksisite, antioksidan, antimikrobiyal aktivite, iNOS.

Abstract

Olive trees (Olea europaea) from Oleaceae family are cultivated in Mediterranean area. Both the oil and the fruit are the main components of the Mediterranean diet. The major component is oleuropein which gives bitter taste to unripe olive fruits. In this study, oleuropein was isolated from the ethanolic extract (80%) of the leaves. For the isolation of oleuropein, crude extract was fractionated using vacuum liquid chromatography with a mixture of methanol-water on reversed-phase silica gel. Final purification was performed on the fractions rich in oleuropein using Sephadex LH-20 with a mixture of methanol-water (1:1). The structure elucidation of oleuropein was determined by 1D (1H- and 13C-NMR, DEPT-135) and 2D NMR (COSY, HSQC and HMBC) spectroscopic analysis. It has shown good results as antioxidant in HL-60 cells with IC50 value 10.02μg/ml and for iNOS inhibition as an indicator for anti-inflammatory with IC50 value 1μg/ml. On the other hand, oleuropein play a role as a cell growth inducer by increasing the viability Hela, A549, MCF-7, U78MG, MPANC-69, MDA-MB-241, HEK-239, CaCo-2, RAW-264.7 and PC-3cell lines. In addition, the ability of antimicrobial activity were screened against both Gram-positive and Gram-negative bacteria with a dose equal to 62.5 μg/ml for Escherischia coli, Staphylococcus aureus, S. epidermidis, Enterecoccus faecalis, Salmonella typhimurium, and 31.2 μg/ml for E. faeciumas. It showed most potent activity against Candida albicans with a dose equal to 31.2 μg/ml. Oleuropein has also shown activity against Echinococcus granulosus which causes hydatis. Oleuropein kills the parasite in 5 minutes with the concentration 2% which is equal to 0.1 mg/ml.

In conclusion, oleuropein as a main component of olive tree can exhibit antimicrobial, cell proliferation, iNOS inhibition and antioxidant activities as is shown in this study. Through these results we can make further pharmacological and biological studies such as wound healing, skin protection, supportive drug for inflammatory bowel disease, etc.

Keywords: Oleaeuropaea, oleuropein, Echinococcus granulosus, cytotoxicity, antioxidant, antimicrobial activity, iNOS.

PHARMACOGNOSTICAL STUDIES ON *OLEA EUROPAEA*

1. Introduction

Natural products have served as an important source of drugs since ancient times and a significant part of today drugs are somehow derived from natural sources. In recent years, a renewed interest in obtaining biologically active compounds from natural sources has been observed. Plants are a rich source of natural antioxidants and substances play major role in human physiology as well as in the food industry. The food industry has long been concerned with issues such as rancidity or oxidative spoilage of fruits, vegetables, oils, fat containing foods to prolong their shelf-life. The large scale availability of agricultural and industrial plant waste materials and their low cost makes them attractive sources of useful compounds (Perez-Bonilla et al., 2006).

The olive tree *(Olea europaea L., Oleaceae)* is one of the most important fruit trees in Mediterranean countries and virgin olive is appreciated throughout the world by consumers attentive to both health and nutritional aspects of food. So it is considered as one of the first domesticated agricultural tree crops in the family *Oleaceae.* The domestication of *Olea europaea* is supposed to be realized some 5700-5500 years ago in the Near East Mediterranean region such as Palestine, Syria, Spain, Italy, Greece, France, Turkey, Algeria, and Morocco (Nora et al., 2012).

In the Mediterranean area, there are nearly eight million hectares of cultivated olive trees. Leaves of the tree became important when olive leaf extract was reported to be potent in treating fever and malaria in 1854. Since then, several researchers demonstrated hypotensive, hypoglycemic, coronary dilatators, antiarrhythmic, antiuricaemic, antioxidant, anticomplementary, antimicrobial, thyroid stimulatory, and antiviral activities (Altinyay and Altun., 2006).

Oleuropein is the major phenolic secoiridoid in *Olea europaea,* which plays a very important role as an anti-inflammatory by decreasing the inflammatory cell recruitment and the release of inflammatory cytokines (Giner et al., 2013).The antibacterial effect of oleuropein against various Gram positive and Gram negative bacteria were observed on *Escherichia coli, Pseudomonas aerugiosa, Klebsiella pneumonia, and Enterobacter cloacae as Gram negative and Staphylococcus aureus, and Stearothermophilus as Gram positive* (Nora et al., 2012). It has important role as

an antioxidant by scavenging superoxide anions and inhibitory of the respiratory burst of neutrophils and hypochlorous acid derived radicals and many others effects (Jemai et al., 2009).

Olive tree, or in the other word *Olea europaea* from family Oleaceae, is characterized by having a gnarly trunk with a cracked bark, in-deciduous leaves, and small white tetramerous flowers grouped in racemes. Its fruit is notorious: the olive an ellipsoid drupe with a hard pit and a mesocarp rich in oil. The leaves are the opposite, sub sessile, entire and coriaceous, have a grayish-green upper side and a whitish underside with a sheen to it, a result of the presence of a fine down which can easily be scraped off. The drug tastes bitter. It can be identified by its microscopic characteristics, particularly the presence of many shield- shaped covering trichomes and sclerites clearly visible in the powder, those are long, have thick walls and are bent here and there, are highly refringent and end as if were truncated (Bruneton, 1993).

2. Secondary Metabolites of *Olea europaea*

Secondary metabolites such as phenolic acid compounds, flavonoids, iridoids, secoiridoids, lignans and others compounds play important roles in disease resistance, protection against pests and species dissemination. The interest on these compounds is related with their antioxidant activity and promotion of health benefits.

Phenolic compounds are a complex but important group of naturally occurring products in plants and are present in the Mediterranean diet, which include table olives and olive oil.

Olives contain high concentrations of phenolic compounds. The main classes of phenolic compounds present in olives are phenolic acids, phenolic alcohols, flavonoids, and secoiridoids (Silva et al, 2005).

2.1. Secoiridoids

Secoiridoids arise from the iridoid by cleavage of the 7, 8 bond of the cyclopentane ring. The most important secoiridoids in *Olea europaea* is oleuropein, which are abundant in Oleaceae, Gentianaceae, Cornaleae, as well as many other plants, and is responsible for the bitter taste of immature and unprocessed olive (Silva et al, 2005).

Iridoid and secoiridoids are compounds that are usually glycosidically bound and are produced from the secondary metabolism of terpenes as precursors of various indole alkaloids. Secoiridoids and its derivatives have characteristic absorbance at 240nm (Silva et al, 2010).The secoiridoids in Oleaceae are usually derived from the oleoside type of glucoside, which are characterized by an exocyclic 8, 9-olefinic functionality, a combination of elenolic acid and a glucosidic residue. Oleuropein is an ester of 2-(3, 4-dihydroxyphenyl) ethanol (hydroxytyrosol) and has the oleosidic skeleton that is common to the secoiridoid glucosides of Oleaceae mainly in its aglycone form, which makes the sugar moiety insoluble in oil (Omar, 2010).

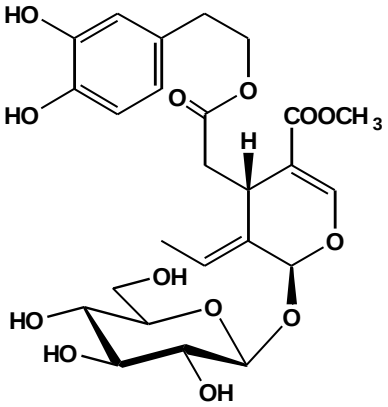
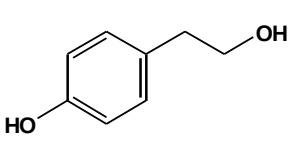
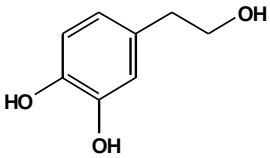


Fig.1. Structure of Oleuropein

Oleuropein during maturation, undergoes hydrolysis and yield several simple molecules like hydroxytyrosol, oleuropein aglycone and tyrosol (Silva et al, 2005).



Tyrosol



Hydroxytyrosol

Fig.2. Structures of Tyrosol and Hydroxytyrosol.

Oleuropein undergoes with many stages inside the olive fruits .Three phases are usually distinguished: a growth phase, during which accumulation of oleuropein occurs; a green maturation phase that coincides with a reduction in the levels of chlorophyll and oleuropein; and a black maturation phase that is characterized by the appearance of anthocyanin and during which the oleuropein levels continue to fall.

Elenolic acid glucoside and demethyloleuropein, glycosylated derivative of oleuropein appear at the beginning of green maturation as the oleuropein levels drop off. Later, they accumulate, reaching their maximum during black maturation, until demethyloleuropein becomes the major constituent of black olives. These two compounds are formed from oleuropein by the action of esterase because esterase activity is increased considerably during the first phase of maturation and reaches a maximum during black maturation.

Olea europaea also contains many types of secoiridoids. Some of them are related to oleuropein and others form different groups such as demethyloleuropein, 3, 4-DHPEA-EDA, elenoic acid glucoside, ligstroside, nuzhenide, oleoside, and oleuroside (Omar, 2010).

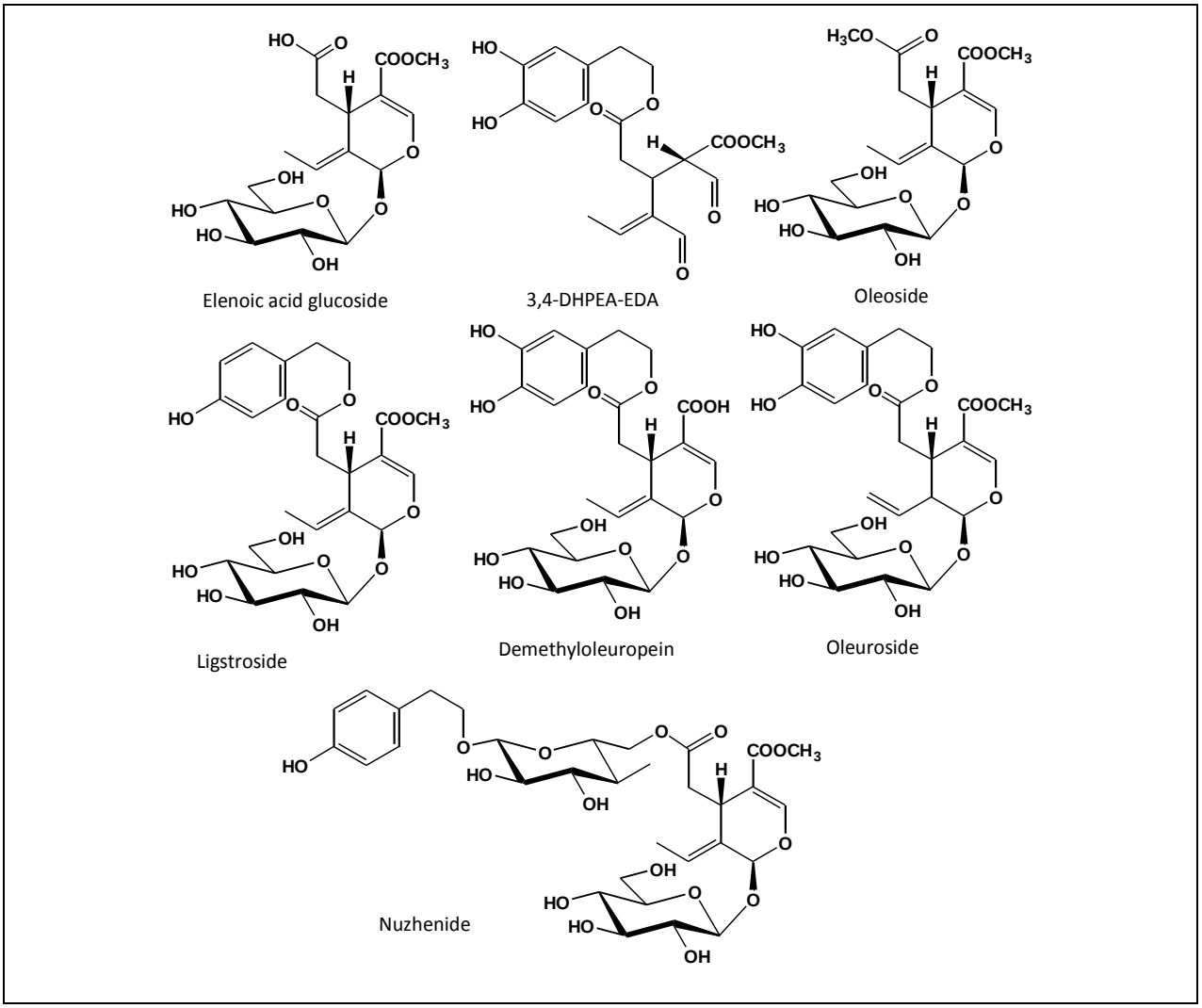


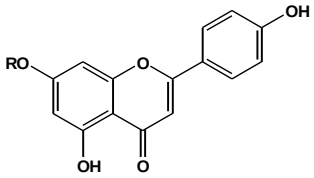
Fig.3. Structures of Demethyloleuropein, 3, 4-DHPEA-EDA, Elenoic acid glucoside, Ligstroside, Nuzhenide, Oleoside, and Oleuroside.

2.2. Other Metabolites

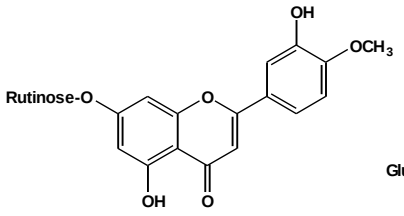
2.2.1. Flavonoids

The flavonoids, which occur both in the Free State and as glycosides, are the largest group of naturally occurring phenols, more than 2000 of these compounds are now known, with nearly 500 of them occurring in the free state. They are formed from three acetate units and phenylpropane unit and are typed according to the state of their oxygenation of the C_3 unit, so all flavonoids have γ-pyrone moiety (Evans, 2009).

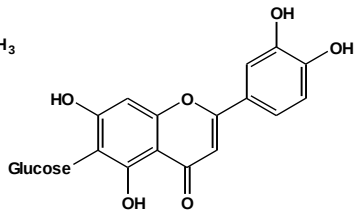
Olea europaea has significant amounts of flavonoids and its derivatives such as: flavonol glucosides, in particular rutin and luteolin-7-glucoside, anthocyanins like cyaniding and delphinidin glycosides (Cardoso, 2005).



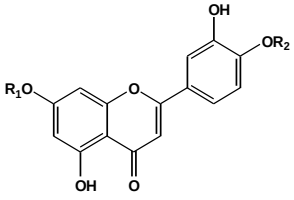
Flavones	R
Apigenin	**H**
Apigenin 7-*O*-glucoside	**Glucose**
Apigenin 7-*O*-rutinoside	**Rutinose [= Rhamnopyranosyl-(1→6)-glucopyranosyl]**



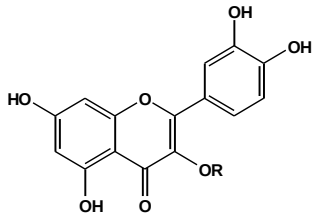
Hesperidin



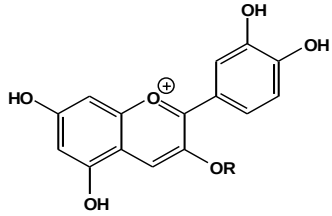
Homoorientin



Flavones	R_1	R_2
Luteolin	**H**	
Luteolin 7-*O*-glucoside	**Glucose**	**H**
Luteolin 4-*O*-glucoside	**H**	**Glucose**
Luteolin 7-*O*-rutinoside	**Rutinose**	**H**



Flavonols	R
Quercetin (Quercetol)	H
Quercetin 3-*O*-rhamnoside	Rhamnose
Quercetin 7-*O*-rutinoside	Rutinose [= Rhamnopyranosyl-(1→6)-glucopyranosyl]



Anthocyanins	R
Cyanidin	H
Cyanidin 3-O-glucosides	Glucose
Cyanidin 3-O-rutinoside	Rutinose [= Rhamnopyranosyl-(1→6)-glucopyranosyl]

Fig.4. Structure of flavonoids and anthocyanins in *Olea europaea*

2.2.2. Lignans

Lignans are dimeric compounds formed essentially by the union of two molecules of a phenylpropene derivative. Neolignan are also derived from the same units as lignans but the C_6-C_3 moieties are linked "head to tail" or "head to head" and not through the β-β' carbons. They occur in the heart-woods of trees (Evans, 2009). Lignans and neolignans, which are produced through a biosynthetic pathway starting from E-coniferyl alcohol, are a widely distributed and structurally diverse phytochemical class. Most of them and their intermediate products exhibit various biological activities. For example, 7-hydroxymatairesinol inhibits fungal growth, 5-demethoxyepixcelsin, anti-HIV activity, and trachelogemin, an antiviral property (Kadowaki et al., 2003).

In *Olea europaea* family, there are two types of lignin. Olivil which defined as 4,4',8',9-tetrahydroxy-3,3'-dimethoxy-7,9'-cyclolignan and 1-acetoxypinoresinol which is also a lignin like active (Kadowaki et al., 2003).

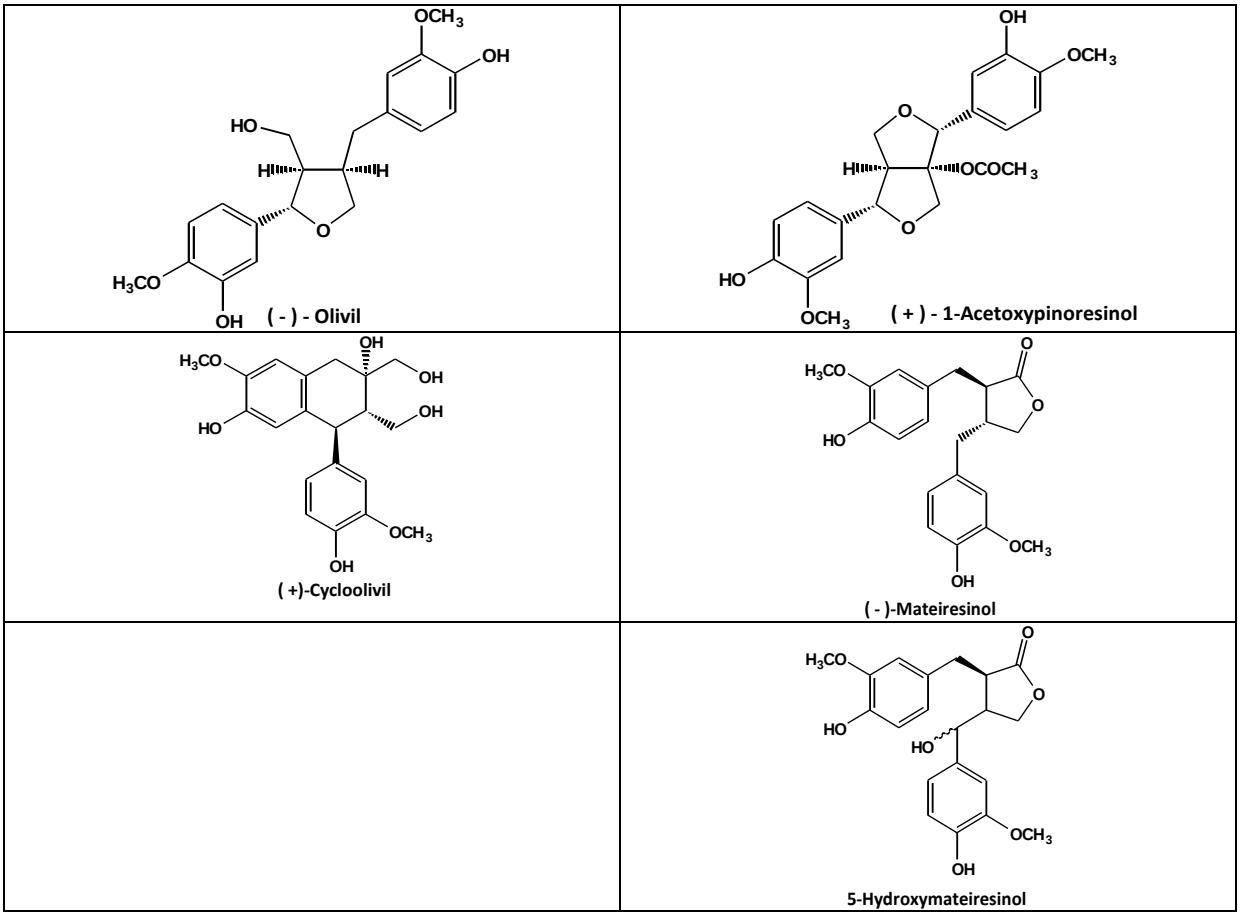


Fig. 5. Structure of Lignans in *Olea europaea*

2.2.3. Other Phenolic Compounds

Phenols probably constitute the largest group of plant secondary metabolites. Widespread in nature, and to be found in most classes of natural compounds having aromatic moieties, they range from simple structure with one aromatic ring to high complex polymeric substances. Phenols are important constituents of some medicinal plants and in the food industry they are utilized as coloring agents, flavorings, aromatizers and antioxidants (Evans, 2009).

Olea europaea provides variable amounts of phenolic compounds which have biological and pharmacological properties.

Some phenolic compounds which are found in *Olea europaea*:

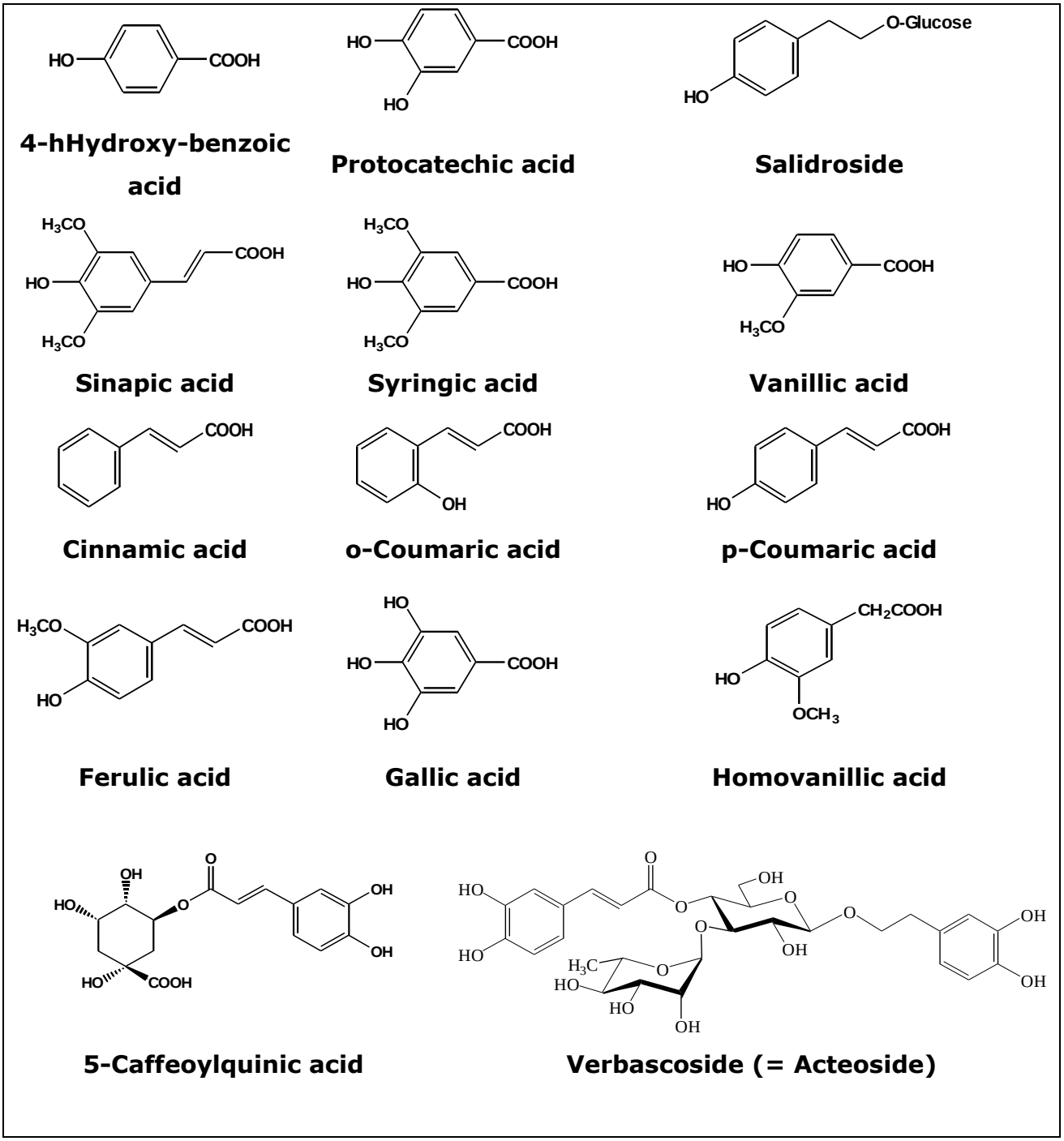


Fig. 6. Structures of phenolic compounds in *Olea europaea*

3. Pharmacological and Biological Properties Of *Olea europaea*

3.1. Antimicrobial and Antiprotozoal Activities

Pathogenic bacteria constitute a major cause of morbidity and mortality in humans. The emergence and spread of bacterial resistance made the treatment of infectious disease more problematic. The antimicrobial activity of a plant is highly related to secondary substances that are synthesized and produced by these plants.

Extract obtained from leaves of *Olea europaea* was studied for antibacterial activity. The antibacterial activity of aqueous leaves extract against various gram positive and gram negative bacteria were observed.

Gram negative: *E. coli ATCC25922, E. coli 2, Pseudomonas aeruginosa ATCC10145, Klebsuiella pneumonia, Enterobacter cloacae ATCC13047*.

Gram positive: *Staphylococcus aureus ATCC6538, S. aureus ATCC25923, Bacillus stearothermophilus ATCC11778.*

The results showed gram negative organisms have a slightly higher sensitivity to aqueous leaves extract of *Olea europaea* compared to the gram positive organisms.

The reason for different sensitivity between gram positive and gram negative bacteria could be ascribed to the morphological difference between these microorganisms membrane carrying. The structure of lipopolysaccharide components makes the cell wall impermeable to lipophilic solutes, where porins constitute a selective barrier to the hydrophilic solutes with an exclusion limit of about 600Da. The gram positive bacteria should be more susceptible since they have only outer peptidoglycan layer which is not an effective permeability barrier. Therefore, this aqueous extract has hydrophilic properties and it can penetrate inside the gram negative bacteria cell (Bouderba Nora et al., 2012).

Table 1. Antibacterial activity of the aqueous extract of *Olea europaea* leaves by disc diffusion method

Microorganisms	Inhibition Zone (mm)
Gram negative	
E. coli ATCC25922	***13.5***
E. coli	***15.3***
Pseudomonas aeruginosa ATCC10145	***13.3***
Klebsiella pneumonia	***11.7***
Enterobacter cloacae ATCC13047	***12.5***
Gram positive	
S. aureus ATCC6538	***9***
S. aureus ATCC25023	***7***
Bacillus stearothermophilus ATCC11778	***6.9***

Table 2. MIC values of aqueous extract of *Olea europaea* leaves by micro-dilution method

Microorganisms	MIC (µg/ml)
Gram negative	
E. coli ATCC25922	***9.8***
E. coli	***6.97***
Pseudomonas aeruginosa ATCC10145	***25.01***
Klebsiella pneumonia	***19.03***
Enterobacter cloacae ATCC13047	***21.29***
Gram positive	
S. aureus ATCC6538	***9.88***
S. aureus ATCC25023	***9.12***
Bacillus stearothermophilus ATCC11778	***26.36***

MIC: minimum Inhibition Concentration

Olive oil phenol compound shows in vitro activity against *Helicobacter pylori* which causes peptic ulcer and some types of gastric cancer and resistance of the microorganisms to antibiotic treatment. Virgin olive oil is an unrefined vegetable oil that contains a significant amount of phenolic compounds. These substances can diffuse from the oil into the gastric juice and they will be stable for hours in the acidic environment. Among the phenolic compounds, the dialdehydic form of decarboxymethylligstroside aglycone showed the strongest bactericidal effect at a concentration as low as 1.3µg/ml (Romero et al., 2007).

Olive leaf extracts have shown a decreased risk of parasitic protozoan disease with an increasing consumption of olive products. Antiprotozoal activities of the competent oleuropein have been examined against *Echinococcus granulosus,* which causes Hydatid cysts.

Hydatidosis caused by *Echinococcus granulosus*is major zoonotic infection that is detrimental to both human and animal husbandries in many countries. Cystic *Echinococcus* affects mainly the intermediate host's viscera, including the liver, lungs, and less frequently, the spleen, kidneys, bone, brain and other organs. Currently the basic approaches for treatment of Hydatid disease are surgery and chemotherapy. However, operative leakage may lead to dissemination of viable protoscolices to adjacent tissue and thus, to intrapretoneal Hydatid disease.

The mortality rates of protoscolices of Hydatid cysts after exposure to different concentration of *O. europaea* extract 0.1% had strong scolicidal effect in 120 minutes, and 0.01% also revealed the same effects at the same time, but it decrease in 0.001 %(Zibaei et al., 2012).

Table 3. The protoscolicidal activity of *Olea europaea extract*

Concentration%	30min	60min	120min
	Rate of death (No. dead/No. tested)		
0.1	61.7±12.3 (386/627)	84.1 ±11.7 (523/622)	96.7±4.5 (593/613)
0.01	60.9±8.8 (374/614)	81.4±12.7 (512/628)	89.2±9.7 (533/619)
0.001	51.8±3.6 (336/612)	65.2±1.4 (405/621)	83.8±12.7 (533/634)

Results are expressed as mean ±SD.

3.2. Anti-inflammatory Activity

Inflammation occurs as a reaction to injurious stimuli such infection or in some cases auto-immunity. Vasoactive amine peptides and free radicals are some of the inflammation mediators. Inflammation is evidenced by increase temperature in site, redness, pain, and swelling. It is the body's attempt to eliminate exogenous, which infection and wounds would not be able to heal without. Macrophage, dendritic cells, histiocytes, kupffer cell and mastocytes initiate acute inflammation after undergoing activation and release of inflammation mediators. Vasodilation and its resulting increased blood flow cause the redness and increased heat. Increase permeability of the blood vessels results in an exudation of plasma protein and fluid into tissue which manifests itself as swelling. Some of the released mediators such as bradykinin increase the sensitivity to pain. The mediator molecules also alter the blood vessels for extravasation. The neutrophil migrate along a chemotactic gradient created by local cells to reach the site of injury and loss of function as the result of neurological reflex in response to pain. Eicosanoids are signaling molecules mainly involved in inflammation and as messenger for central nerves system, these molecules however are not performed in the tissue, they are generated from phospholipids. They are implicated in the control of many physiological processes and are among the most important mediator and modulators of inflammatory reaction.

The main source of Eicosanoids is arachidonic acid, a 20 carbon unsaturated fatty acid containing four double bonds. The main products of eicosanoids are prostaglandins, thromboxanes, and leukotrienes (Viljocn et al. 2012).

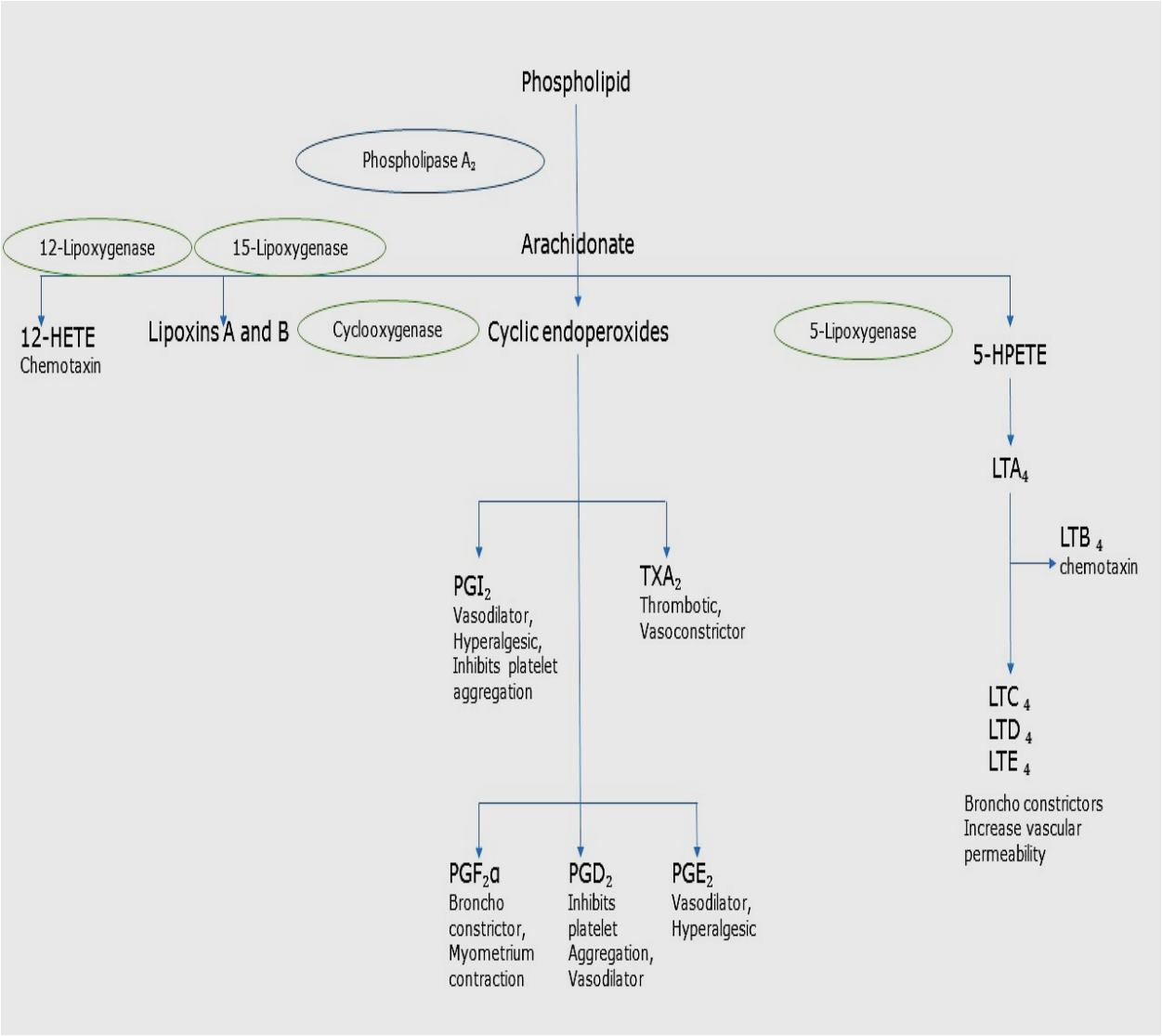


Fig. 7. Cyclooxygenase pathway (Viljoen at al., 2012)

Olive biphenols are now recognized as potential nutraceutical targets for the food and pharmaceutical industries. For example oleocanthal, the dialdehydic form of deacetoxyligstroside aglycone, is found in olive and does dependently inhibit COX 1 and COX2 as ibuprofen (Obied et al, 2007).

The anti-inflammatory effect of oleuropein; the major phenolic secoiridoids in *Olea europaea* was evaluated in an experimental model of chronic colitis in mice.

Ulcerative colitis and Crohn's disease are considered to be the principle inflammatory disorders included under the general category of inflammatory bowel disease (IBD). Although its exact etiology is known, genetic and environmental factors, an abnormal immune response and the contribution of intestinal micro flora seem to be essential for the development of IBD. Management of the disease involves pharmacological therapies (anti-inflammatory drugs, antibiotic and other biological agents) or even surgery in critical patients.

Oleuropein has been shown to possess anti-inflammatory properties. This compound was shown to lower the disease activity index (DAI) and to reduce pro-inflammation cytokine level, and also those of cyclooxygenase-2 inducible nitric oxide synthase (iNOS) and matrix metalloproteinase 9 (MMP9), in colon tissue, partly through inhibition of nuclear transcription factor Kappa B (NF-KB) activity.

Oleuropein reduce cell infiltration in chronic colitis. During mucosal inflammation a complex array of inflammation signaling involving prostaglandins and cytokine production impairs intestinal epithelial function and lead to the recruitment of inflammatory cells to the site of injury. Neutrophil, macrophage, and eosinophil infiltration and increase in myeloperoxidase (MPO), N-acetylglucosaminidase (NAG) and eosinophil peroxidase (EPO). The treatment with oleuropein reduce neutrophil, macrophage, and eosinophil accumulation in colon tissue, with reduction of 36%, 42%, and 46% respectively and by lowering the infiltration of cells, a significant lowering degree of mucosal injury and less edema. As well as oleuropein reduce interleukin (IL)-6 and IL-1β and decrease COX 2 and iNOS expression, which iNOS with oleuropein significantly down regulating the expression about 58% (Giner et al, 2013).

3.3. Antioxidant Activity

Oxidation processes are intrinsic in the energy management of all living organisms and are therefore kept under strict control by several cellular mechanisms. However, the excessive production of free radicals and the unbalanced mechanisms of antioxidant protection result in the onset of numerous diseases and accelerate ageing. Oxidation mediated by free radical reactions is also responsible for the rancidity of unpreserved food rich in unsaturated fatty acids (Sarikurkcu et al., 2009).

Among natural antioxidant, the olive tree has been widely accepted as one of the species with the highest antioxidant activity via its oil, fruits and leaves. It is well known that the activity of the olive tree byproducts, extracts in medicine and food industry are due to the presence of some important antioxidant phenolic compounds to prevent oxidative degradations (Jemai et al., 2009).

The spectrophotometric technique we used has measured the relative abilities of antioxidants to scavenge the 2,29-azinobis(3-ethylbenzothiazoline-6-sulphonate) radical cation $ABTS^{+}$ in comparison with the antioxidant activity of standard amounts of Trotox (6-hydroxy-2,5,7,8-tertramethylchroman-2-carboxylic acid). The radical cation $ABTS^{+}$, produced by the ferrylmyoglobin radical generated form metmyoglobin and in the presence of peroxidase (H_2O_2) is a blue/green chromogen with characteristic absorbance at 600nm. The high antioxidant activity of seed extracts is due to nuzhenide and related compounds (Silva et al., 2006).

2,2-diphenyl1-1picrylhydrazyl radical DPPH· assay test, which based on the theory that a hydrogen donor is an antioxidant, by DPPH· accept hydrogen from antioxidant. This assay applied on different parts of olive tree such as leaf and wood, and calculations are done on DPPH· scavenging percentage.

Table4. Olive leaf and wood extract scavenging activity

Part	Percentage of scavenging %
Wood ethyl acetate	**62.9**
Leaf ethanol	**60.7**
Wood ethanol	**52.4**

And by measuring the DPPH· scavenging on the compounds (Perez-Bonilla et al., 2006).

Table5. Olive phenolic compounds DPPH· scavenging

Compounds	Scavenging %
Hydroxytyrosol	**76.7**
Tyrosol	**3.7**
Oleuropein	**20.4**
Ligstroside	**7.4**
BHT	**13.6**
Rosmarinic acid	**58.3**

BHT and Rosmarinic acid as a control

Oleuropein, hydroxytyrosol and other compounds play important roles in diabetes disease as an antioxidant.

Diabetes mellitus (DM) is a chronic metabolic disease with the highest rate of prevalence and mortality worldwide. That is caused by an absolute or relative lack of insulin and/or reduce insulin activity. It is characterized by hyperglycemia and long term complications affecting the eyes, kidneys, nerves and blood vessels. It is the most common endocrine disorder.

Oxidation stress usually happens with DM patients. It has been suggested that oxidative stress may contribute to the pathogenesis of different diabetic complications. Furthermore, with diabetes, several features appear including an increase in lipid peroxidation, alteration of the glutathione redox state a decrease in

the content of individual natural antioxidants and finally reduction in the antioxidant enzyme activities.

The olive tree has long been recognized as having antioxidant molecules, such as oleuropein, hydroxytyrosol, oleuropein aglycone and tyrosol, these compounds have been proven to be potent scavenger of superoxide anion and hydroxyl radical.

The hepatic antioxidant enzyme activities, superoxide dismutase (SOD) and catalase (CAT) significantly decreased in diabetic rats compared with those fed a control diet. The decrease was significantly restored in the presence of oleuropein and hydroxytyrosol. Oleuropein makes hypoglycemic activity which is essentially due to its antioxidant potential. In fact, oleuropein as well as hydroxytyrosol have been shown to be scavengers of superoxide anions and inhibitors of respiratory burst of neutrophils and hypochlorous acid derived radicals. The eventual mechanism responsible of the hypoglycemic activity of oleuropein and hydroxytyrosol may result from potentiation of reduce glucose, is induced insulin release or increase peripheral uptake of glucose. These antioxidants could inactivate the circulating free radicals that produce NO before it reach pancreatic β-cells, where they induce their damage and/or death (Jemai et al., 2009).

This study is focused on the secoiridoid and phenolic compounds of *Olea europaea* which collected from Cyprus to see the extraction, isolation, biological and pharmacological activity of these compounds.

4. Materials and Methods

4.1. Instruments

Lyophilizator: Christ Alpha 1-4 LD plus

UV lamp: Camag

Vacuum: Rockk Vacuum

Electric Grinder: Retsch SK100

Plate Heater: Camag TLC plate heater II

Optical Rotation: SCHMIDT+ HAENSCH POLARTRONIC MHZ-8

NMR: ^{1}H- and ^{13}C-NMR (Brucker 500 and 125 MHz, respectively; CD_3OD)

4.2. Plant Materials

Olive leafs were obtained from the olive trees (*Olea europaea*) of Mesarya plain of North Cyprus in March 2014.

4.3. Chemical Solid Materials

1% vanillin\methanoland5% and Sulfuric acid H_2SO_4 (Merck) were used for Thin layer chromatography (TLC).

4.4. Solvents

Ethanol, t-butanol, distilled water, methanol, chloroform, dichloromethane (Merck, Fluka Analytical, Sigma- Aldrich).

4.5. Chromatographic Methods

4.5.1. Thin Layer Chromatography

Chloroform: methanol: water (61:32:7) was used a solvent system

Normal phase Silica gel (Kiesel gel $60F_{254}$ Aluminum 20x20cm, Merck 5554) was used as an absorbent.

1% vanillin/H_2SO_4 was used as an adsorbent.

4.5.2. Vacuum Liquid Chromatography

Water: Methanol 100:0 ⟶ 0:100 was used as a solvent system Lichroprep RP-18 (25-40µm), Merck was used as an adsorbent, column dimension 105x42mm.

4.5.3. Gel Chromatography

Sephadex (Lipophilic Sephadex Lit-20100 bed size 25-100µm, Sigma-Aldrich) was used as a gel. Methanol: water 1:1 was used as a solvent system.

4.6. Plant Extraction

200g of air dried and powdered leaves were extracted with 80% Ethanol 1000ml by shaking three days at room temperature and filtered. The filtrate was concentrated to 80ml in vacuum at 50°C and washed with chloroform 80mlx3 to remove lipophilic compounds

4.7. Fractionation and Isolation

4.7.1. Fractionation by Vacuum Liquid Chromatography

Concentrated 80 ml was applied to vacuum liquid chromatography (VLC) (LiChroprep RP-18, 25-40µm, and Column dimensions 105 x 42 mm) employing water: methanol mixture with increasing amount of methanol in water (0-100% methanol). For each 100 ml of eluent, the methanol ratio was increased by 5% of methanol. The fractions eluted with 45, 50 and 55% methanol were rich in oleuropein (958 mg, 793 mg and 302 mg respectively). Thin layer chromatography was used for control procedure of this study.

4.7.2. Final Purification of Oleuropein by Sephadex LH-20

400 mg of crude oleuropein was further subjected to Sephadex LH-20 column using a solvent system methanol: water 1:1 to yield pure oleuropein (OLE) 208mg.

4.8. Cell Culture Studies

Glioblastoma (U78MG), Human Cervical carcinoma (Hela), lungs carcinoma (A549), Mammary gland/breast cancer tissue (MDA-MB-241), Human prostate cancer (PC-3), Human embryonic kidney cells (HEK-239), Human breast adenocarcinoma (MCF-7), Caucasian colon adenocarcinoma (CaCo-2), Macrophage (RAW-264.7) and Leukemia cells (LH60) were provided from ATCC and they were stocked in liquid nitrogen in EGE University, Department of Bioengineering.

The cell lines were cultured in the RPMI 1640 (Lonza) medium supplemented with 10% heat inactivated fetal bovine serum (Gibco), 1% L-glutamine (Lonza) and 1% Gentamycin (Biochrome) in humidified atmosphere with 5% CO_2 at 37°C.

4.8.1. In-Vitro Cytotoxicity Assay

Cytotoxicity of compounds were determined with the general procedure used for the screening of cytotoxic agents based on metabolic cell viability using a modified MTT [3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H2tetrazolium bromide)] that effects the mitochondrial reductase activity of viable cells. The survival of viable cells after treatment of extracts in monolayer cultures cultivated for 24 hours in 96 well microplates with $1x10^5$ cells/ml. After that the cultured cells were treated with different dilution of compounds and incubate for 48 hours. The growth inhibition was compared with untreated controls and found the compound concentration inhibits growth by 50% (IC_{50}).

That assay based on cleavage of the yellow tetrazolium salt, MTT which forms water insoluble dark blue formazan crystals. This cleavage only take place in living cells by mitochondrial enzyme succinate dehydrogenase. The water insoluble dark blue formazan crystals are solubilized by using dimethyl sulfoxide (DMSO). The optical density of the dissolved material is measured at 570 nm (reference filter, 690nm) with UV visible spectrophotometer.

The plant-derived compound parthenolide was used as a positive cytotoxic control agent. Percentages of surviving cells in each culture were determined after treatment of oleuropein. The % viability was determined as formulated below:

$$\%\text{Viable cells} = \frac{[(\text{The absorbance of the treated cells}) - (\text{the absorbance of the blank})]}{[(\text{The absorbance of the control}) - (\text{the absorbance of the blank})]} \times 100$$

In this study 6.3 mg lyophilized oleuropein, 2.8 mg rosmarinic acid, 1.5 mg colragenic acid and 2.5 mg verbascoside dissolved in DMSO to prepare stock solution to make different concentrations (100 µg/ml, 50 µg/ml and 5 µg/ml).

Rosmarinic acid, colragenic acid and verbascoside are control compounds to compare their activities with oleuropein.

4.8.2. Morphological Studies

The morphological studies of the cells were done with inverted microscope (Olympus, Japan) comparing with the control group 48 h after treatment.

4.8.3. Data analysis

Values were presented as mean ±standard error of the mean (SEM). IC_{50}calculation and variance analysis (standard deviation calculation) were performed with Graph Pad Prism 5.

4.8.4. Induce Nitric Oxide Synthase (iNOS) Assay

Mouse macrophages (RAW 264.7) are cultivated in phenol red free, RPMI 1640 medium with fetal bovine serum. For the assay, cells are seeded in 96 well plate ($1x10^6$ cells/ml) and incubate for 24 hours. After inducing with lipopolysaccharide (LPS), test samples were added and cells are further incubated for 24 hours. The level of nitrite in the medium was measured by using Griess reagent. The absorbance was measured at 540 nm. Percent inhibition of nitrite production by sample was calculated in comparison to vehicle control and IC_{50} values were obtained from dose curves. (Erel et al, 2014).

In this study 6.3 mg lyophilized oleuropein, 2.8 mg rosmarinic acid, 1.5 mg colragenic acid and 2.5 mg verbascoside dissolved in DMSO to prepare stock solution to make different concentrations (100µg/ml, 50µg/ml and 5µg/ml).

Rosmarinic acid, colragenic acid and verbascoside are control compounds to compare their activities with oleuropein, in addition to parthenolide as positive control.

4.8.5. Antioxidant Assay

This method is based on a fluorimetric assay. The myelomonocytic cells (HL-60) $1x10^6$ cell/ml cultured of 3 hours in 96 well then treated with different concentration of compounds. Later, they are measured using 96 well fluorescense measurement system (ThermoScientific) (Excitation 485 nm and Emission 538 nm) after that incubate for 30 minutes.

Then cells were stimulated with PMA 100µg/well (Phorbol 12myristate 13 acetate) to form peroxyl radicals. After that incubate 30minutes to measure again with the same wave length.

Furthermore, cells were incubated for 15 min after the addition of 5 µg/ml 2, 7-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes). DCFH-DA is a non-fluorescent probe that diffuses into cells .Cytoplasmic esterases hydrolyze DCFH-DA to DCFH. Reactive oxygen species generated within HL-60 cells oxidize DCFH to the fluorescent dye2,7-dichlorofluorescein (DCF). The ability of the test materials to inhibit exogenous cytoplasmic ROS-catalyzed oxidation of DCFH in HL-60 cells was measured by PMA-treated control incubations with and without the test materials. And IC_{50} values were obtained from dose curves.

4.9. Antimicrobial Activity

4.9.1. Microorganisms

Gram positive, gram negative and yeast were used for antimicrobial activity study. The gram negative bacteria used were *E.coli ATCC 25922, E.coli 0157H7 and Salmonella typhimurium CCM 5445*. The gram positive bacteria used were *Staphylococcus aureus ATCC 25923, Staphylococcus epidermis ATCC 12228, Enterococcus faecium DMS 13590 and Enterococcus faecalis ATCC 29212 and Candida albicans ATCC10239* was used as yeast. The lyophilized bacteria and yeast were obtained from EGE University, Faculty of Science, Department of Basic and Industrial Microbiology.

4.9.2. Minimum inhibitory concentration (MIC) by micro-dilution susceptibility test

The microorganisms were growing in Mueller Hinton (MH) sterile Broth (Merck Darmstadt, Germany) for 5hours and adjusted to 0.5 McFarland turbidity standard (600 Absorbance), corresponding to $1.5x10^6$ CFU/ml. MIC serial dilution of lyophilized compounds after dilution with methanol. Starting from 250 µg/ml to 1.95 µg/ml (by dividing half) were prepared in 96 well microtiter trays, at final volume of 80 µl. then 20 µl of the adjusted bacterial inocula ($1.5x10^5$ CFU/ml) were added to each well and incubated at 37°C for 24 hours. Inhibition of microorganism's growth was determined by visual observation. The MIC was defined as the lowest concentration of these compounds to inhibit microbial growth. Ampicillin and Flucytocine were used as standard drugs for comparison. All assays were run using 3 replicates.

4.10. Antiprotozoal (*Echinococcus granulosus*) Activity

Hydatid cyst contained livers of sheep were collected from the governmental slaughterhouse and after local cleaning with povidone iodine, Hydatid fluid was aspirated from the cysts and collected in a sterile container under aseptic conditions. The cysts were opened and germinative membranes were extracted and put in a container to free additional protoscoleces and Hydatid sand. The protoscolex viability was confirmed under light microscopy by dye exclusion technique with 0.1% eosin.

Protoscoleces excluding the dye were considered as live and deeply colored ones were dead. Oleuropein solution was prepared in (0.1%, 1% and 2%) concentration. 2 ml from each solution was put in a test tube and a drop of viability confirmed protoscolex solution was added with a pipette. Then in 5, 10,15,20,25, and 30 minutes, specimens were taken from each solution and directly examined for protoscolex viability under light microscopy. Each experimental set is repeated for 0.1%, 1% and 2% concentration of oleuropein and protoscolex viability is evaluated.

5. Results And Discussion

5.1. Optical Rotation of OLE (Oleuropein)

Light can be viewed as normally oscillating throughout 360 degree at 90 degree to its direction of travel. The light source is usually sodium lamp with wave length 589nm, when the light cross the polarmeter curved, this curving depend on the substance, if the compound curved the light with clock way so our compound is Dexorotatory with positive signal and to the opposite direction it is Levorotatory and the signal is negative. 2.7mg of oleuropein give specific rotation about -133.33°. This result is closed to reference result which is -147° in the same solvent methanol (Leticia et al., 1980).

5.2. Structure Elucidation of OLE (Oleuropein) by NMR

All iridoids and secoiridoids exhibit complex ^{1}H-NMR spectra. The proton signals of oleuropein as a secoiridoid glycoside are discussed briefly below.

The ^{1}H-NMR spectrum of OLE (oleuropein) (**Spectrum 1**) can be evaluated in three molecular fragments, secoiridoid unit, sugar and tyrosol (3,4-dihydroxy-phenethyl alcohol) moieties. The signal observed at 1.20 ppm as a singlet belongs to tertiary-butanol which was used for lyophilization and was not taken into account in the structure elucidation. All proton signals arising from these three structural subunits were assigned by using 2D-NMR experiment, COSY (**Spectra 3A&B**). The ^{13}C-NMR spectrum of OLE exhibited 25 carbon resonances which were determined as twelve methyne (CH), four methylene (CH_2), two methyl groups (CH_3) and seven quaternary C atoms by DEPT-135 experiment, confirming the expected structure of oleuropein.

The signal observed at 4.79 ppm (d, J = 8.0 Hz) was consistent with β-configuration of the glucose unit. ^{1}H- and ^{13}C-NMR spectra and DEPT-135 spectrum (**Spectra 1** and **2A&B**, resp.) supported the presence of β-D-glucopyranose. The assignments of all proton and carbon resonances were aided by COSY and HSQC (**Spectra 4A&B**) experiments. Apart from the anomeric proton, clearly showed the all other protons of glucose unit in the same spin system (**Spectrum 3B; Table 1**). The corresponding

carbon signals of the β-D-glucopyranose established by HSQC experiment supported its terminal position. Because, no substituent chemical shifts were observed for glucose unit.

The proton signals arising from the 3,4-dihydroxy-phenethyl alcohol moiety were found to be as two spin systems. The former was consisted of three aromatic protons exhibiting an ABX system at δ 6.52 (d, 2.0 Hz; H-4'') and 6.66 (2H; dd 2.0, 8.5 Hz, H-7'' and d 8.5 Hz, H-8''). The latter was established as two methylene protons of the side chain of the dihydroxy-phenethyl alcohol at 2.75 ppm (2H, dd"t" 7.0 Hz, H_2-2'') and 4.10 and 4.18 ppm (each 1H, m, H_2-1''). The corresponding carbon resonances of the tyrosol unit were determined using HSQC (**Spectrum 4A**) and HMBC experiments (**Spectrum5**) (**Table 1**). The long range correlations from C-3'' (δ 130.76) to H-1'' (δ 4.10 and 4.18), H-2'' (δ 2.75) and H-4'' (δ 6.06) showed the connectivity of subunits, aromatic and aliphatic moieties of tyrosol.

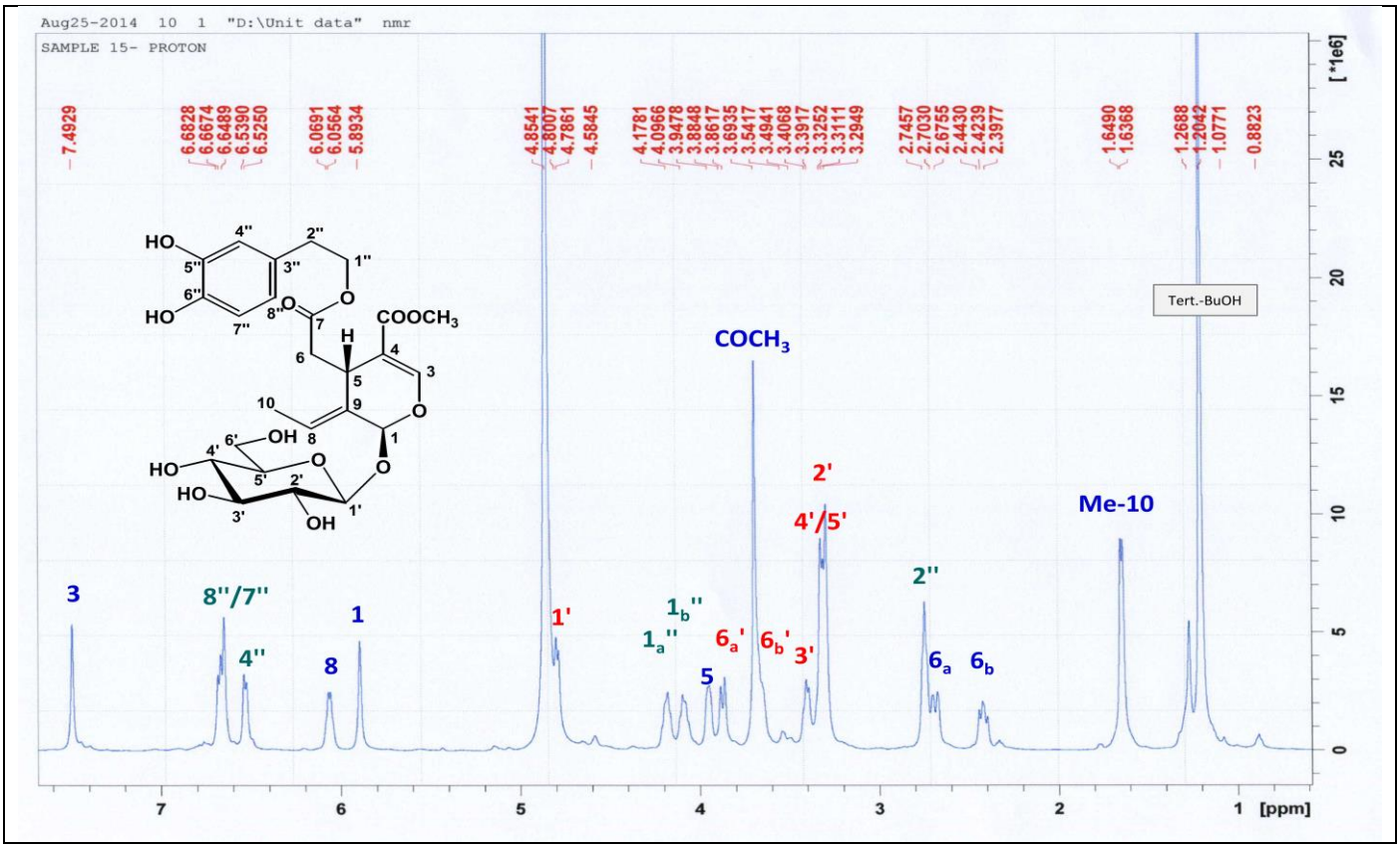
The remaining proton resonances arising from the secoiridoid aglycone were assigned to H-1 (δ 5.89 d, 1.5 Hz), H-3 (δ 7.49 s), H-5 (δ 3.95 m) of which the latter was found to be X part of an ABX system. COSY experiment showed the AB protons at 2.42 and 2.69 ppm (H_2-6). H-1 also showed a weak correlation to Me-10 (W-coupling).

The signals due to an olefinic proton at δ 6.06 dq and a methyl group δ 1.64 d were observed in the same spin system and assigned to H-8 and H_3-10, respectively. Additional three proton singlet signal at 3.69 ppm was attributed to the presence of carbomethoxyl.

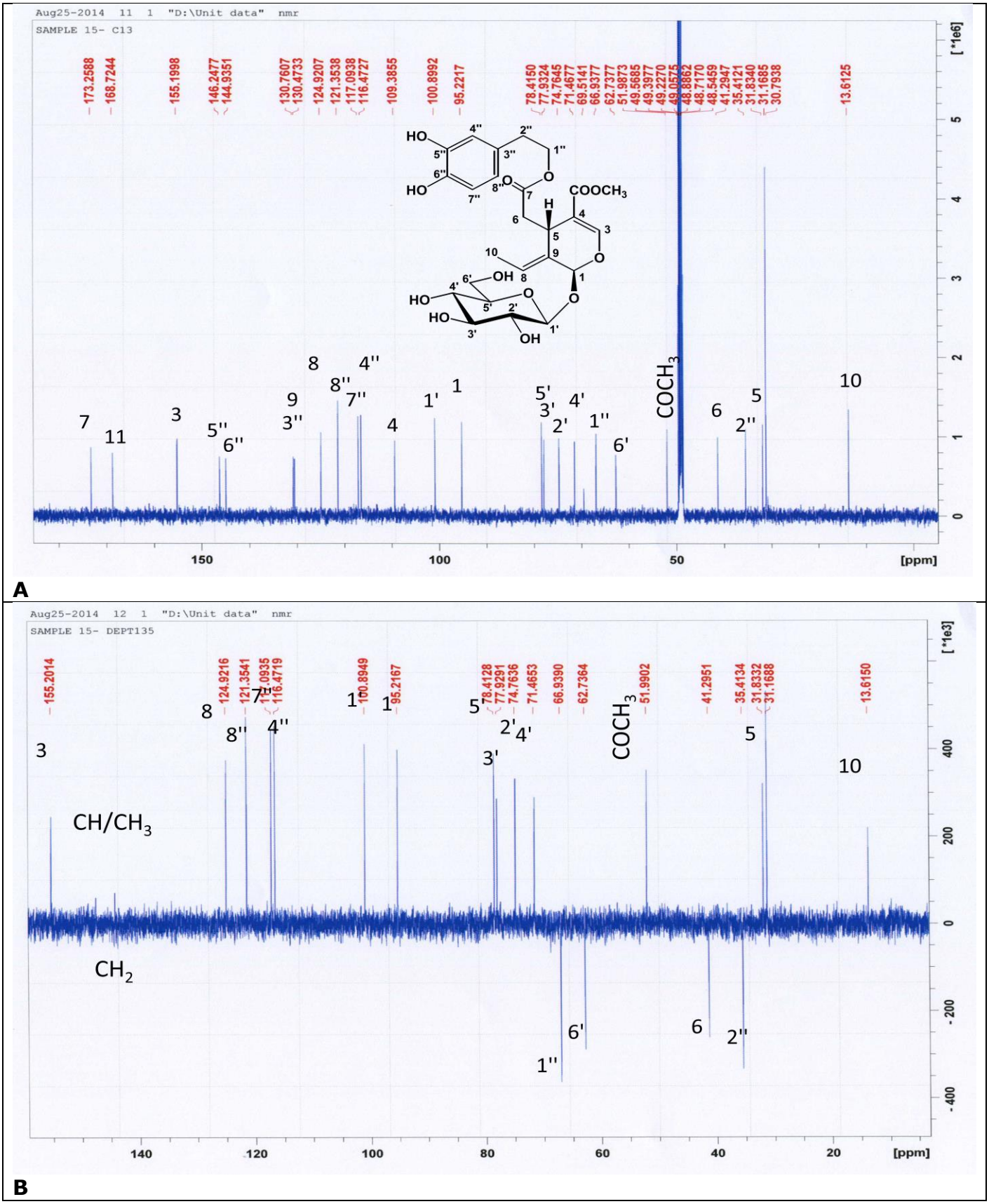
Finally, HMBC experiment (**Spectrum 5**) allowed to show intermolecular connectivities among the molecular fragments, secoiridoid unit, β-D-glucopyranose and tyrosol (3,4-dihydroxy-phenethyl alcohol) moieties. The expected heteronuclear ^{1}H, ^{13}C long-range couplings were observed from C-1 (δ 95.22) to H-1' of glucose, H-3 and H-8 indicating the site of glycosidation. The long-range correlations from C-7 (δ 173.26) to H_2-1'', H_2-6 and H-5 confirmed the ester linkage between the 3,4-dihydroxy-phenethyl alcohol and secoiridoid aglycone. The further significant long-range correlations from C-10 (δ 13.61) to H-8, from C-9 (δ 130.47) to H-5, H_2-6 and Me-10, from C-4 (δ 109.39) to H-3, H-5 and H_2-6 permitted the assignments of the remaining fragments of the secoiridoid aglycone.

Consequently, the NMR data of OLE was identical to those of published for oleuropein (Gariboldi, 1986) except the signals C-4 and C-3''. The chemical shift value of C-4

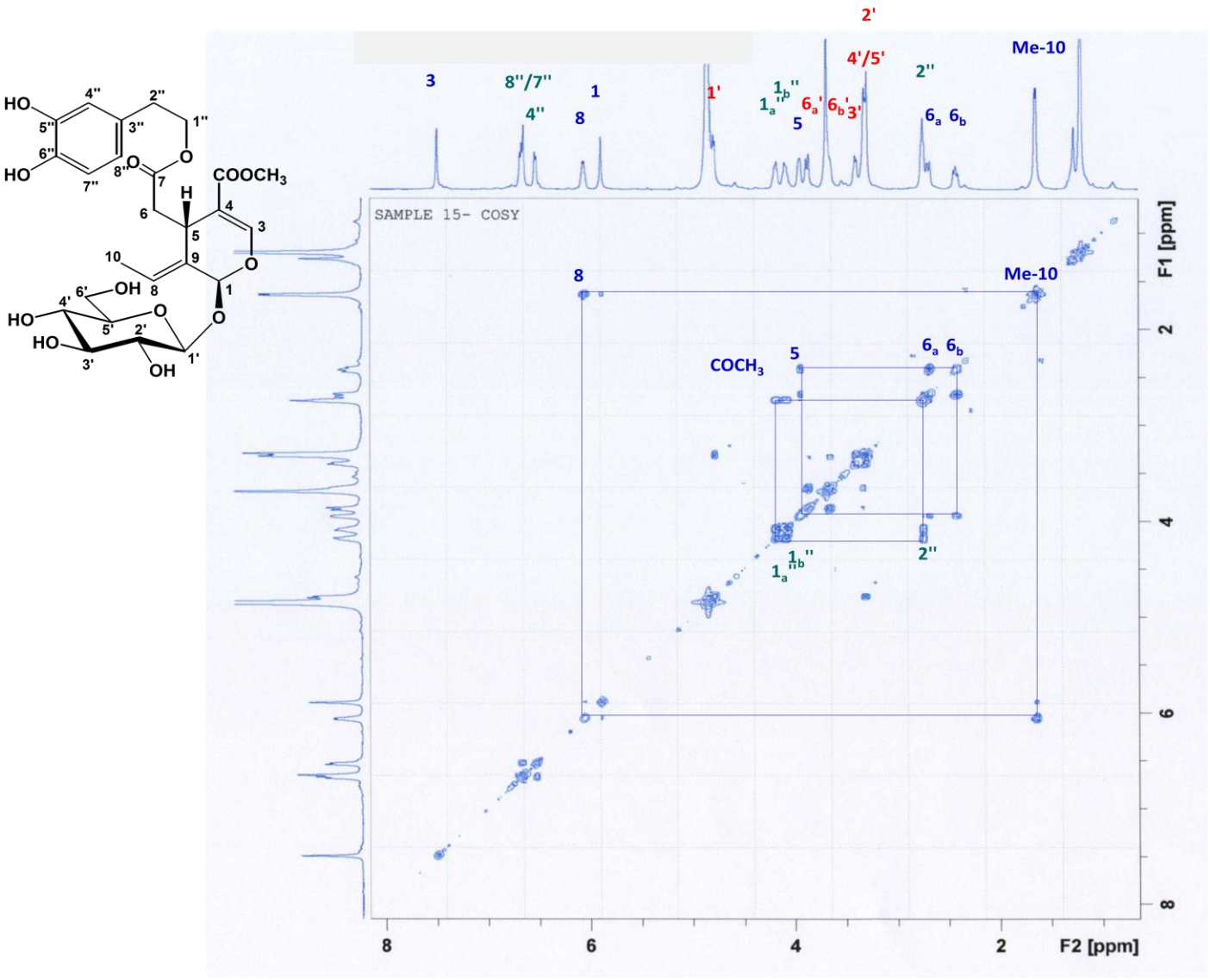
reported as 129.85 ppm by Gariboldi et al. (1986) has been corrected as 109.39 ppm in the present work. Similar corrections were also realized for C-3'' (*see* Table 1). The present NMR data were in good agreement with those of reported for oleuropein and similar glycosides from *Fraxinus angustifolia* (Çalış, 1993).



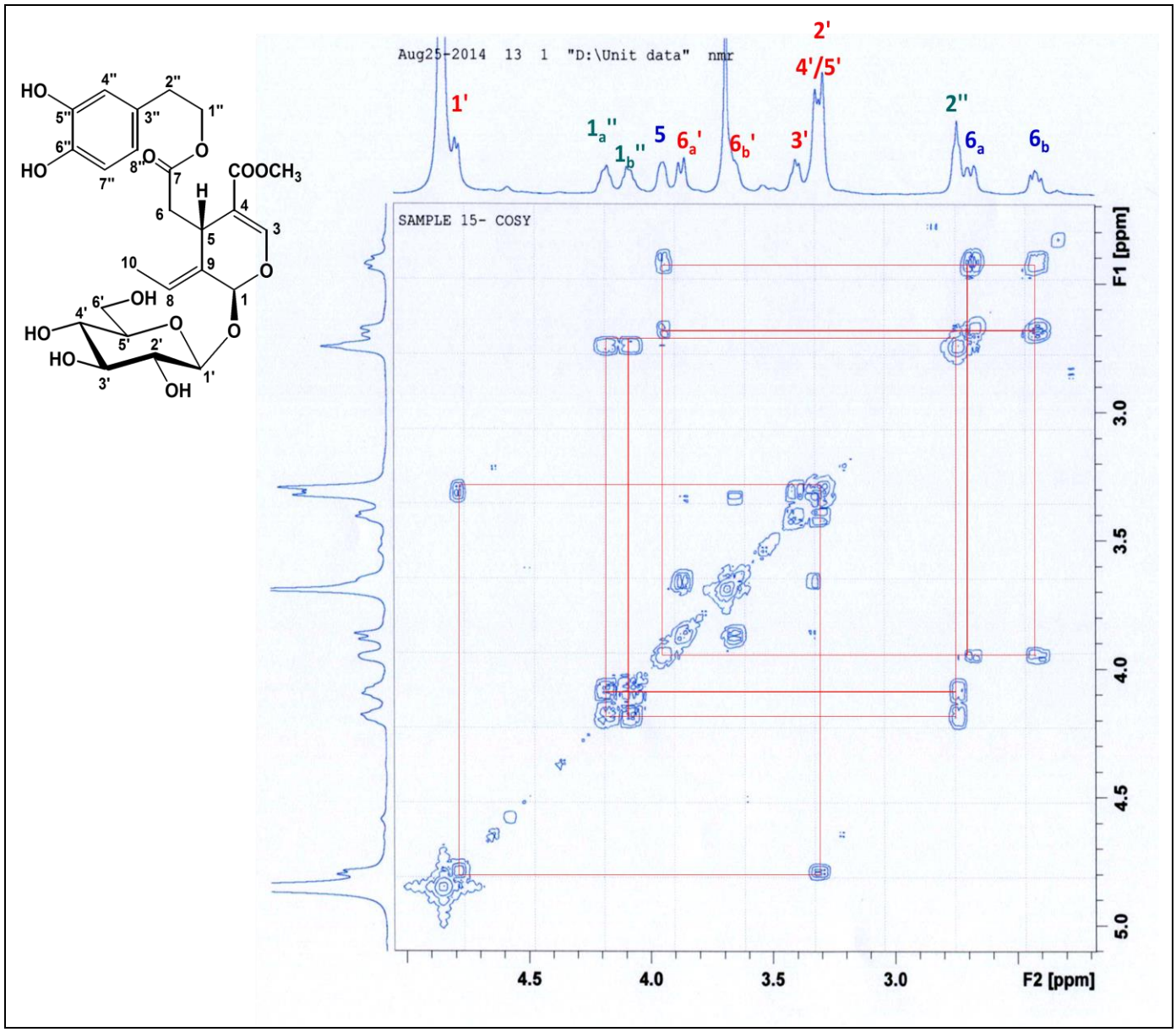
Spectrum 1. The ^{1}H-NMR Spectrum of Oleuropein (OL-1) (CD_3OD; 500 MHz)



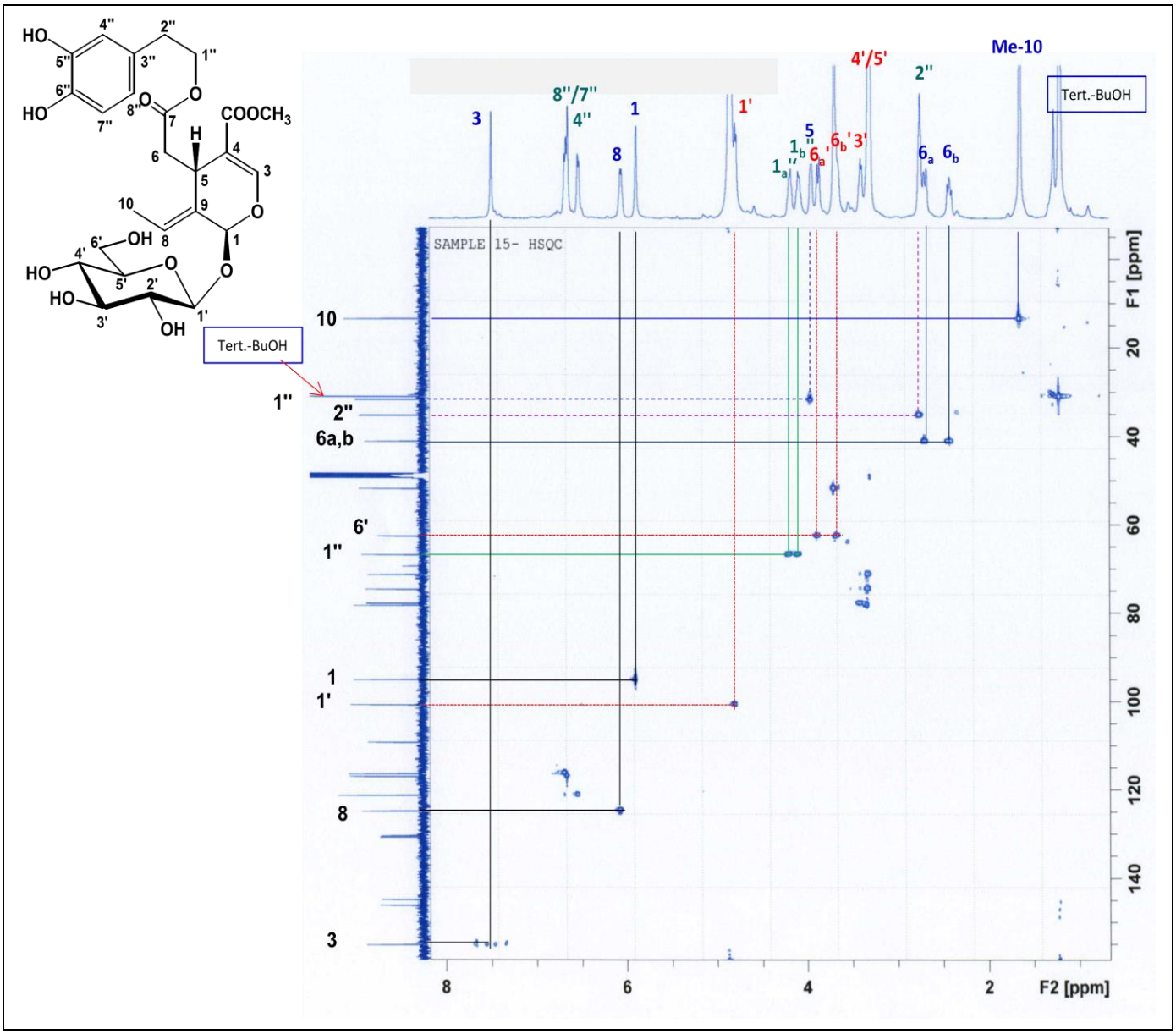
Spectrum 2A&B. The ^{13}C-NMR (A) and DEPT135 Spectra of Oleuropein (OL-1) (CD_3OD; 125 MHz)



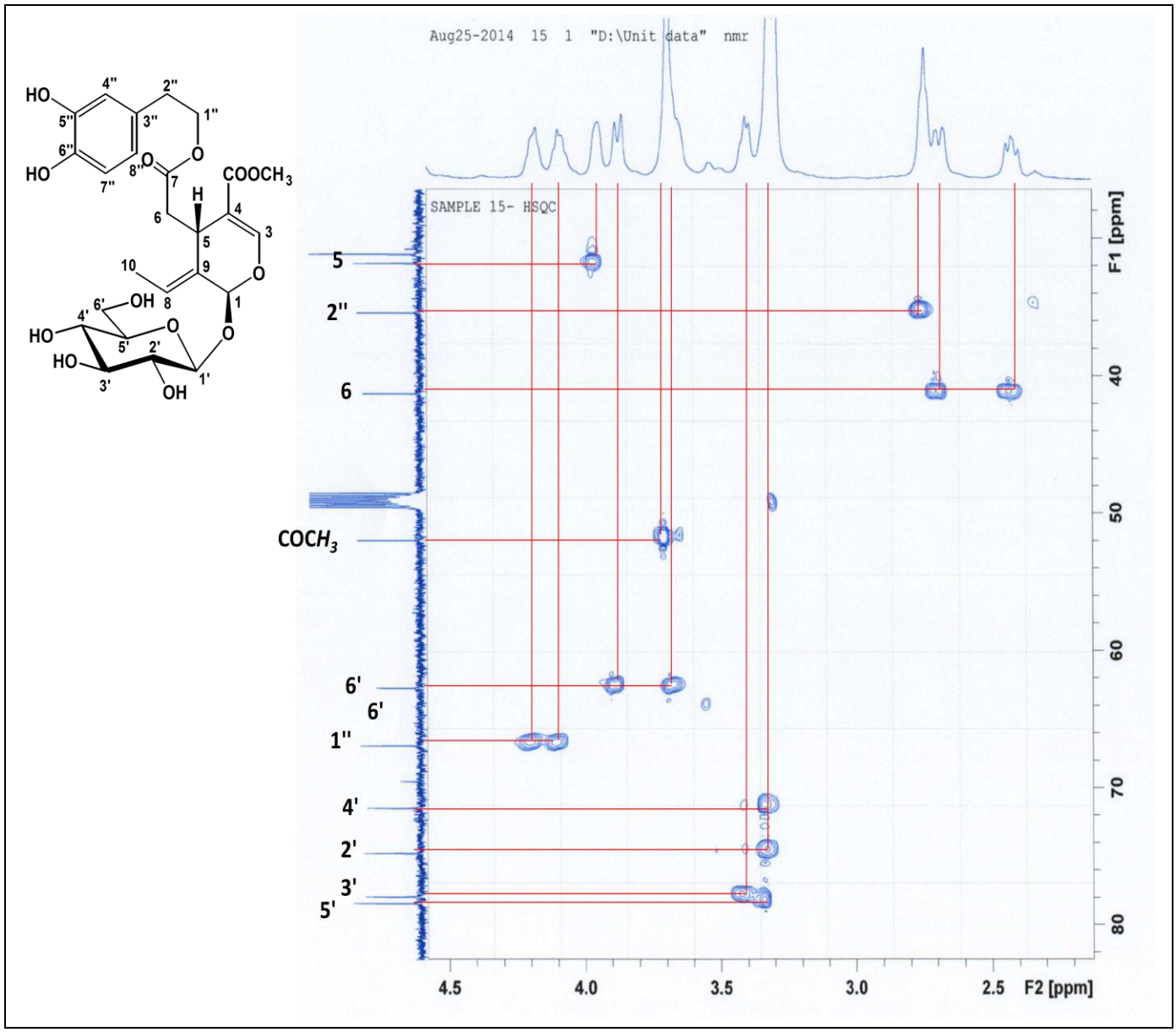
Spectrum 3A. The ^{1}H, ^{1}H-Homonuclear Correlated Spectrum (**COSY**) of Oleuropein (OL-1) (CD_3OD)



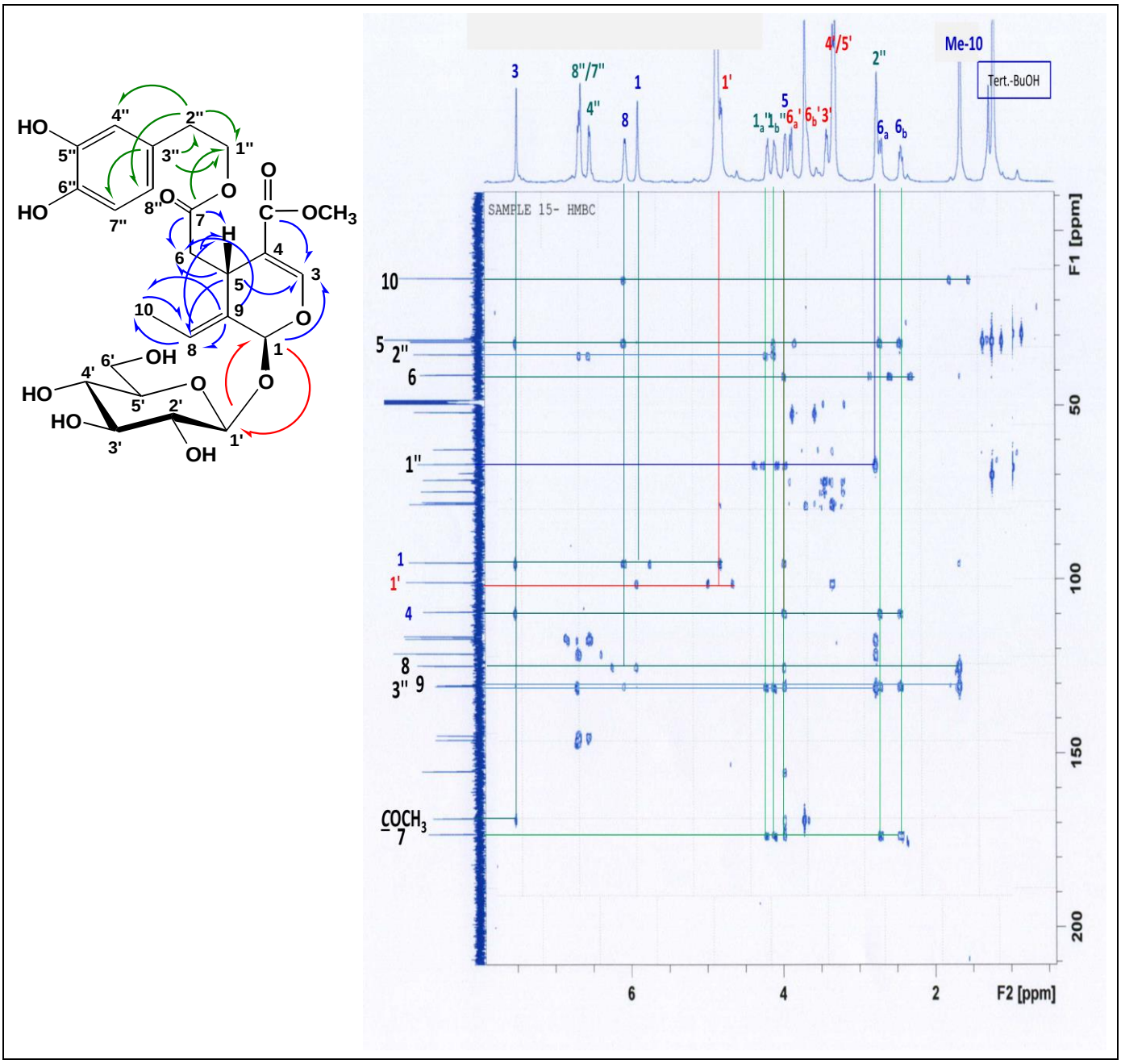
Spectrum 3B. The ^{1}H, ^{1}H-Homonuclear Correlated Spectrum (**COSY**) of Oleuropein (OL-1) (CD_3OD) (2.2 – 5.0 ppm)



Spectrum 4A. The ^{1}H, ^{13}C-Heteronuclear Single Quantum Coherence (**HSQC**) of Oleuropein (OL-1) (CD_3OD)



Spectrum 4A. The ^{1}H, ^{13}C-Heteronuclear Single Quantum Coherence (**HSQC**) of Oleuropein (OL-1) (CD_3OD)



Spectrum 5. The ^{1}H, ^{13}C-Heteronuclear Multiple-Bond Coherence Spectrum (**HMBC**) of Oleuropein (OL-1) (CD_3OD)

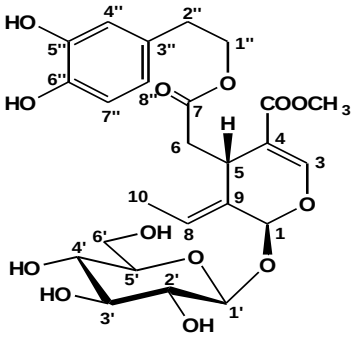


Table 6. Comparison of ^{1}H and ^{13}C-NMR data of oleuropein*isolated in the present study with those of reported (Gariboldi et al., 1986).

		OL-1*		Gariboldi et al., 1986	
		CD_3OD		DMSO-d_6	
C/H		δ_C ppm	δ_H ppm, *J* (Hz)	δ_C ppm	δ_H ppm, *J* (Hz)
1	CH	95.22	5.89 *d* (1.5)	93.96	5.80 *br s* (1.5 Hz)
3	CH	155.20	7.49 *s*	154.10	7.46 *s*
4	C	109.39	-	129.85	-
5	CH	31.83	3.95 *m*	30.88	3.86 *dd* (9.0, 3.0)
6	CH_2	41.29	2,69 *dd* (7.0), 2.42 *dd "t"* (7.0)	40.43	2.62 *d* (14.0) 2.40 *d* (14.0)
7	C	173.26	-	171.50	-
8	CH_2	124.92	6.06 *dq* (8.0, 1.5)	123.90	5.92 *dq* (8.0, 1.5)
9	C	130.47	-	129.49	-
10	CH_3	13.61	1.64 *d* (1.5)	13.55	1.64 *d* (1.56)
COCH_3	C	168.7	-	167.02	-
CO*CH_3*	CH_3	51.98	3.69 *s*	51.88	3.64 s
1'	CH	100.90	4.79 *d* (8.0)	99.79	4.66 d (8.0)
2'	CH	74.76	3.30†	73.87	
3'	CH	77.93	3.39 *dd "t"* (9.0)	77.08	
4'	CH	71.46	3.30†	70.61	
5'	CH	78.42	3.30†	77.69	
6'	CH_2	62.73	3.87 dd (12.0, 2.0), 3.68†	61.77	
1''	CH_2	66.94	4.10 m, 4.18 m	65.76	4.06 m
2''	CH_2	35.41	2.75 *dd "t"* (7.0)	34.37	2.63 *br t* (7.0)
3''	C	130.76	-	108.46	-
4''	CH	116.47	6.06 *d* (2.0)	116.25	6.60 *d* (2.0)
5''	C	146.24	-	145.53	-
6''	C	144.94	-	144.17	-
7''	CH	117.09	6.66	116.82	6.64 *dd* (8.5, 2.0)
8''	CH	121.35	6.66	120.97	6.64 *d* (8.5)

*CD_3OD; ^{1}H: 500 MHz, ^{13}C: 125 MHz. All assignments are based on 2D-NMR spectra (COSY, HSQC and HMBC)

5.3. In Vitro Cytotoxic Activity

Oleuropein, verbascoside, chlorogenic acid and reosmarinic acid did not exhibit any cytotoxicity on all the cells. However, they showed proliferation effect for most of the cells at all the concentrations (100 µg/ml, 50 µg/ml and 5 µg/ml) in contrast to parthenolide which is a sesquiterpene lactone with strong activity as cytotoxic and anti-inflammatory.

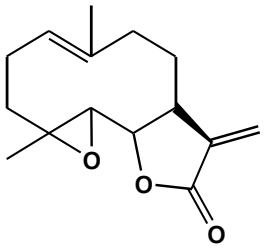


Fig.8. Parthenolide Structure

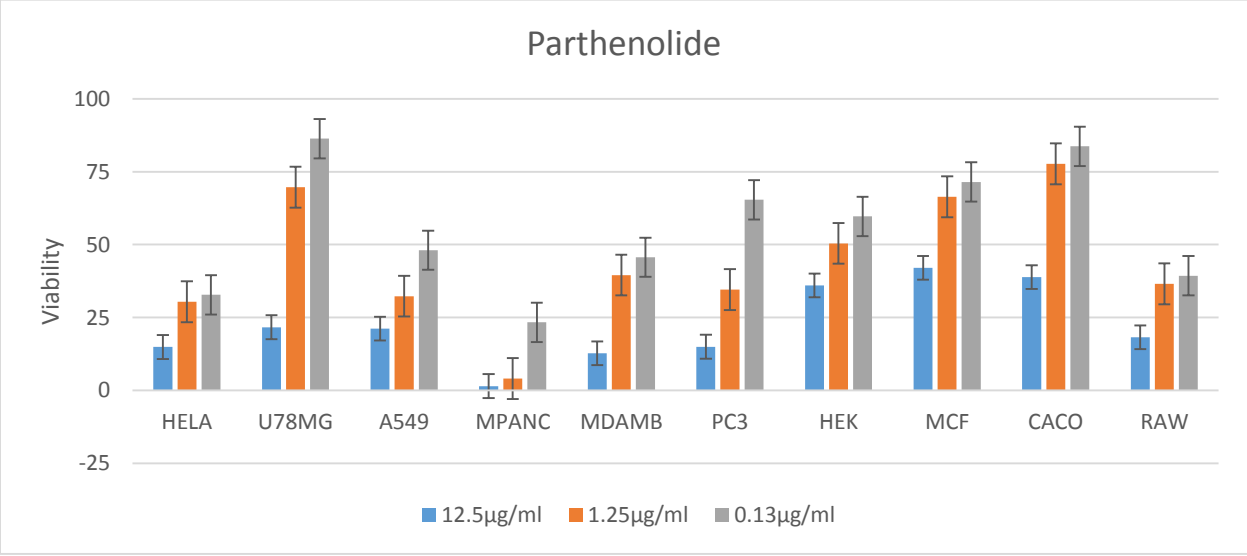


Fig.9. Effect of parthenolide on normal and various cancer cells

Oleuropein (OLE) showed inducing in cell growth (HELA, U78MG, A549, MPANC-69, MDA-MB-241, PC3, HEK-239, MCF-7, CaCo-2 and RAW-264.7) in all the concentrations especially the dose of 5µg/ml which exhibits this activity especially on prostate cancer cells, which have viability around 150%.

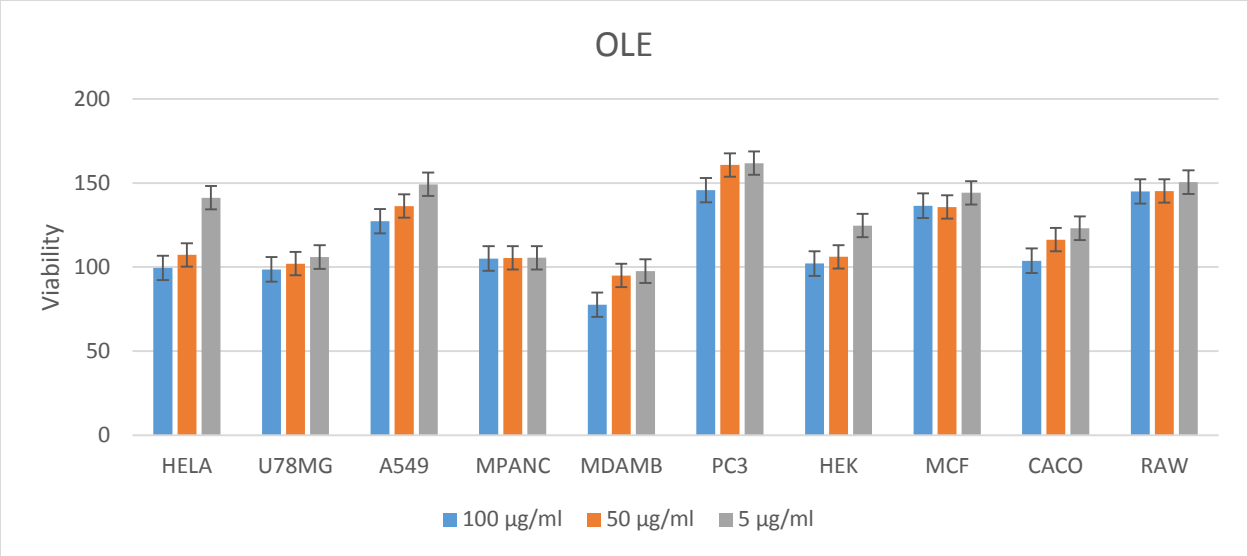


Fig.10. Effect of oleuropein on normal and various cancer cells

Chlorogenic acid (KLJA) showed cell proliferation with all the cells lines at all the concentrations particularly the dose of 5µg/ml as the lowest concentration and this activity effect mostly on (A549) and (PC-3) which the viability of these cells were 160% and 180% respectively.

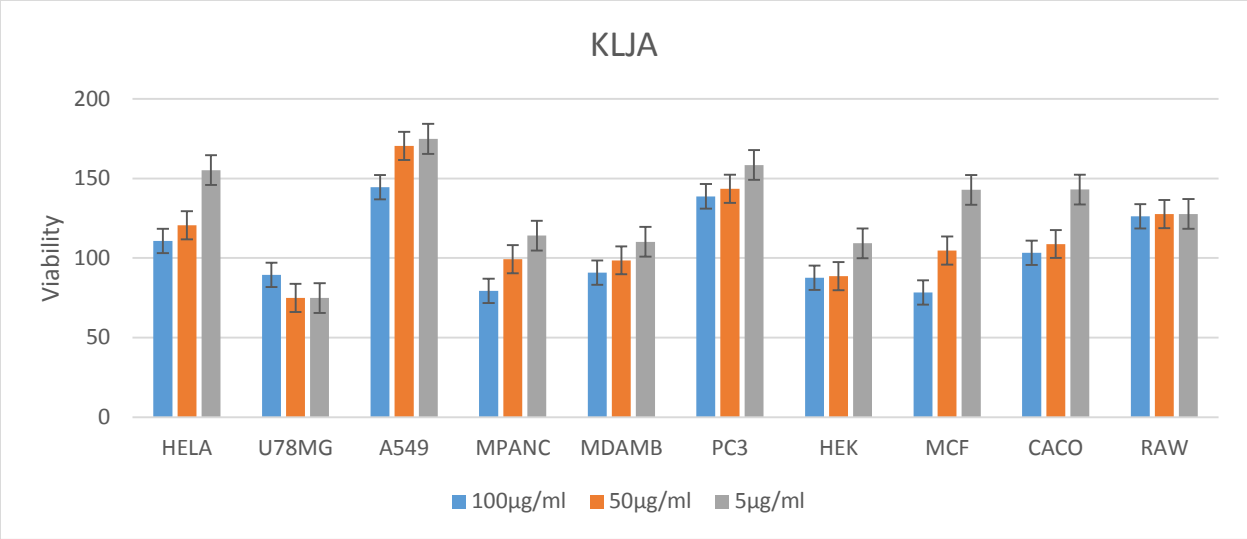


Fig.11. Effect of chlorogenic acid on normal and various cancer cells

Verbascoside (VER) showed the most cell growth inducing especially Hela, A549 and MCF-7 cells comparison with normal cell line HEK-239. In addition to U78MG which was the most sensitive cell with IC_{50} around 90%.

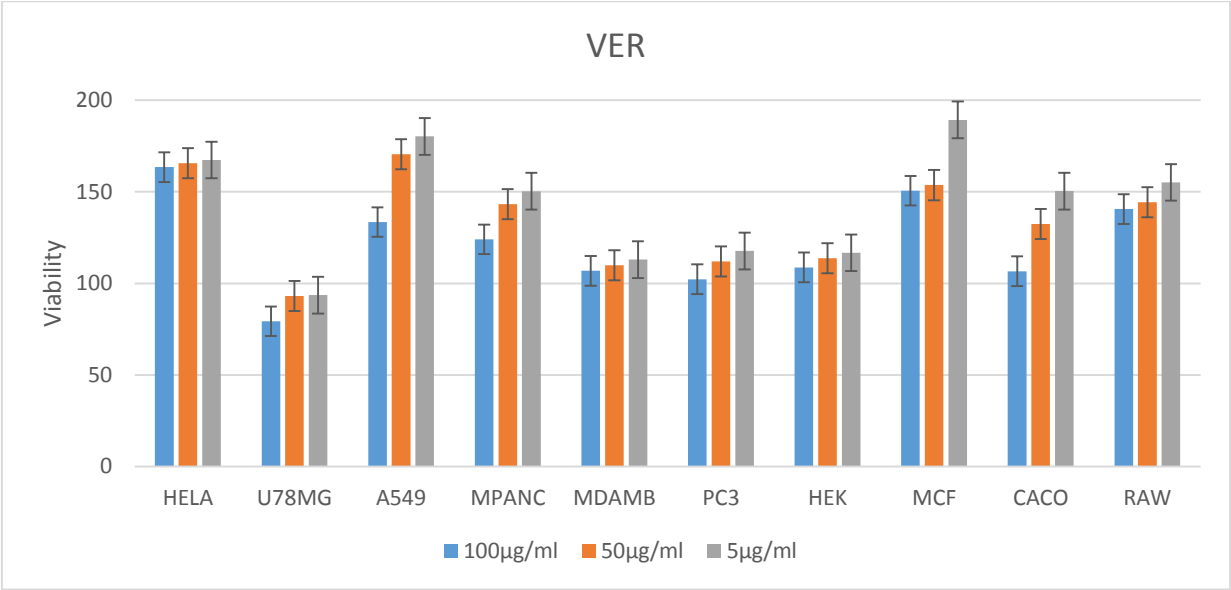


Fig.12. Effect of verbascoside on normal and various cancer cells

Rosmarinic acid (OSA) showed moderate proliferation activity comparison with previous compounds, which showed the most activity on A549, PC-3, and RAW-264.7

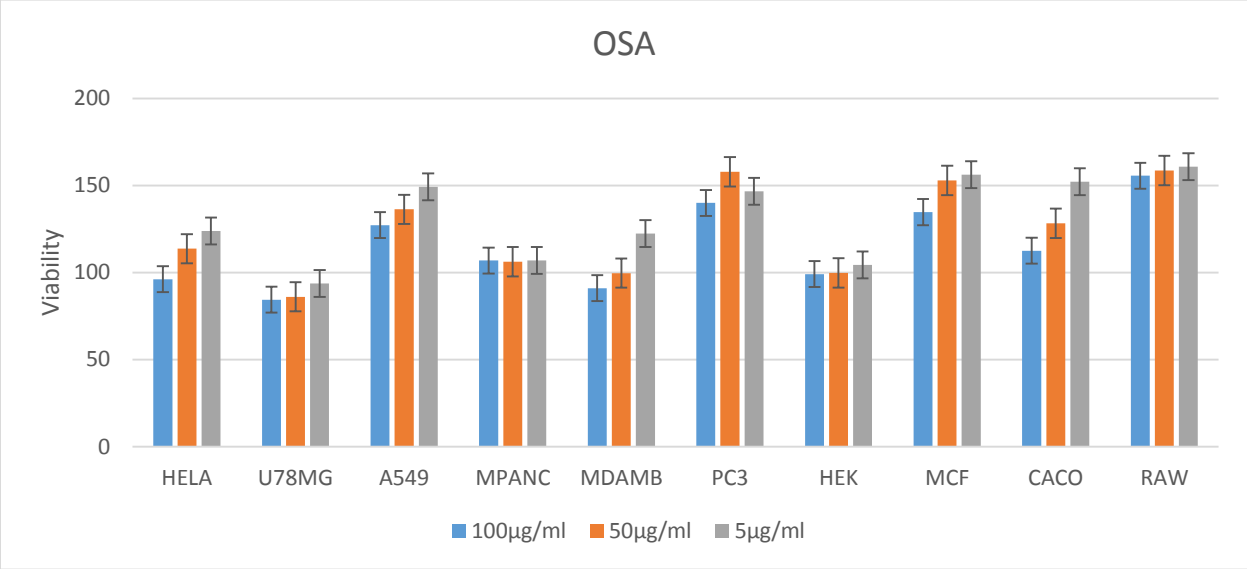


Fig.13. Effect of rosmarinic acid on normal and various cancer cells

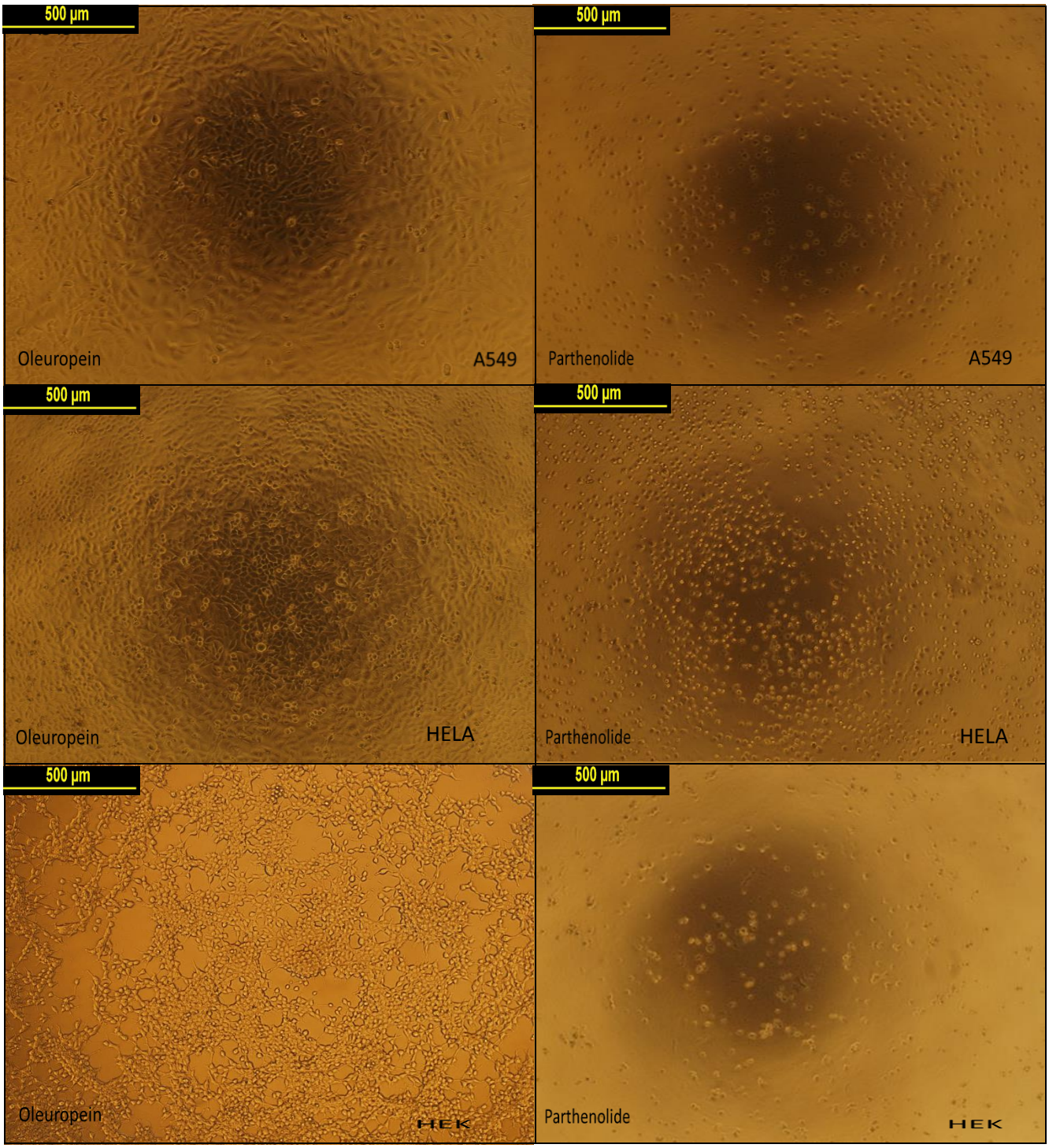


Fig. 14a. Effect of oleuropein with dose of 5µg/ml on different cancer cell lines and normal cell line observed by inverted microscope (cont.)

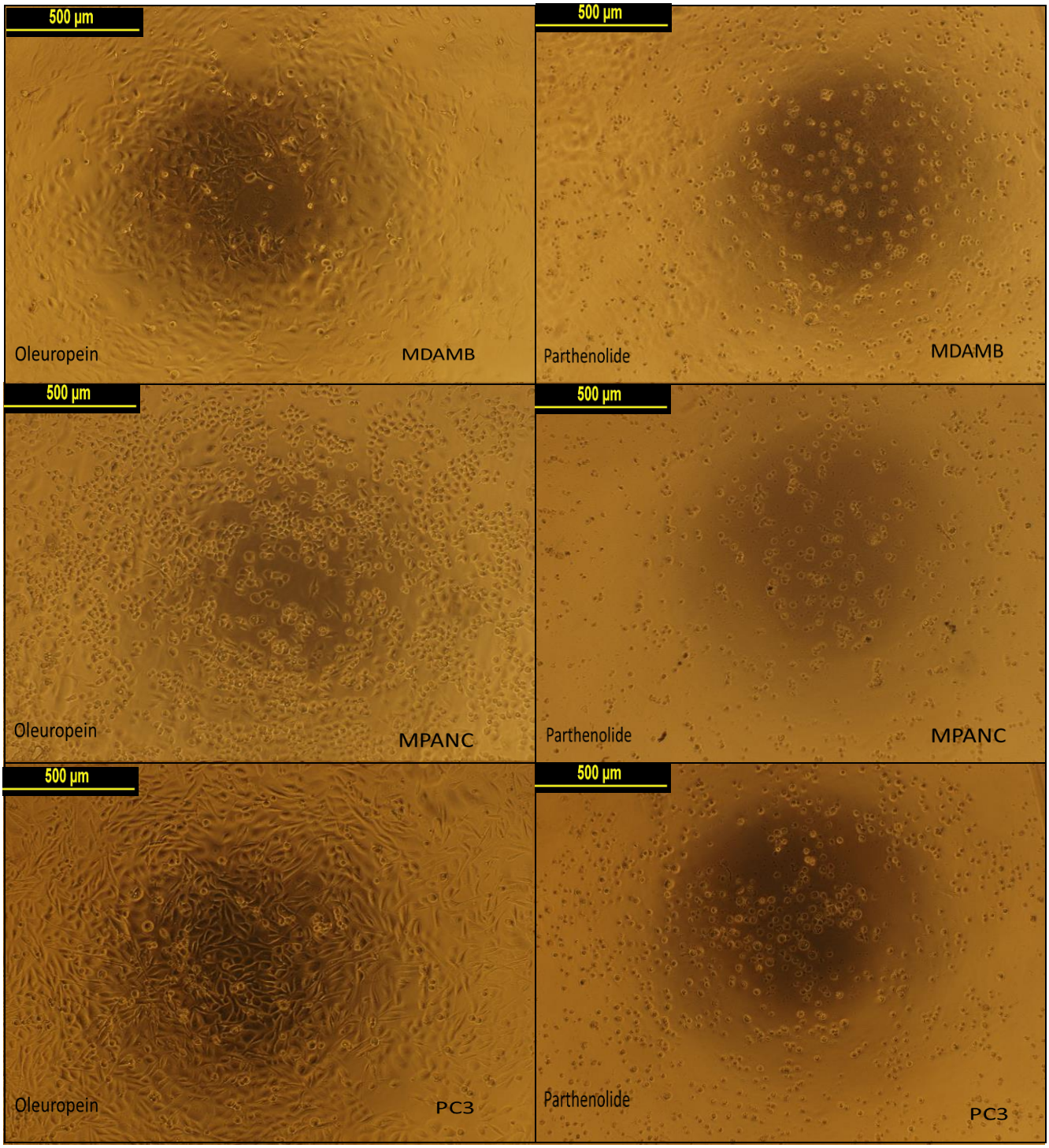


Fig. 14b. Effect of oleuropein with dose of 5µg/ml on different cancer cell lines and normal cell line observed by inverted microscope (cont.)

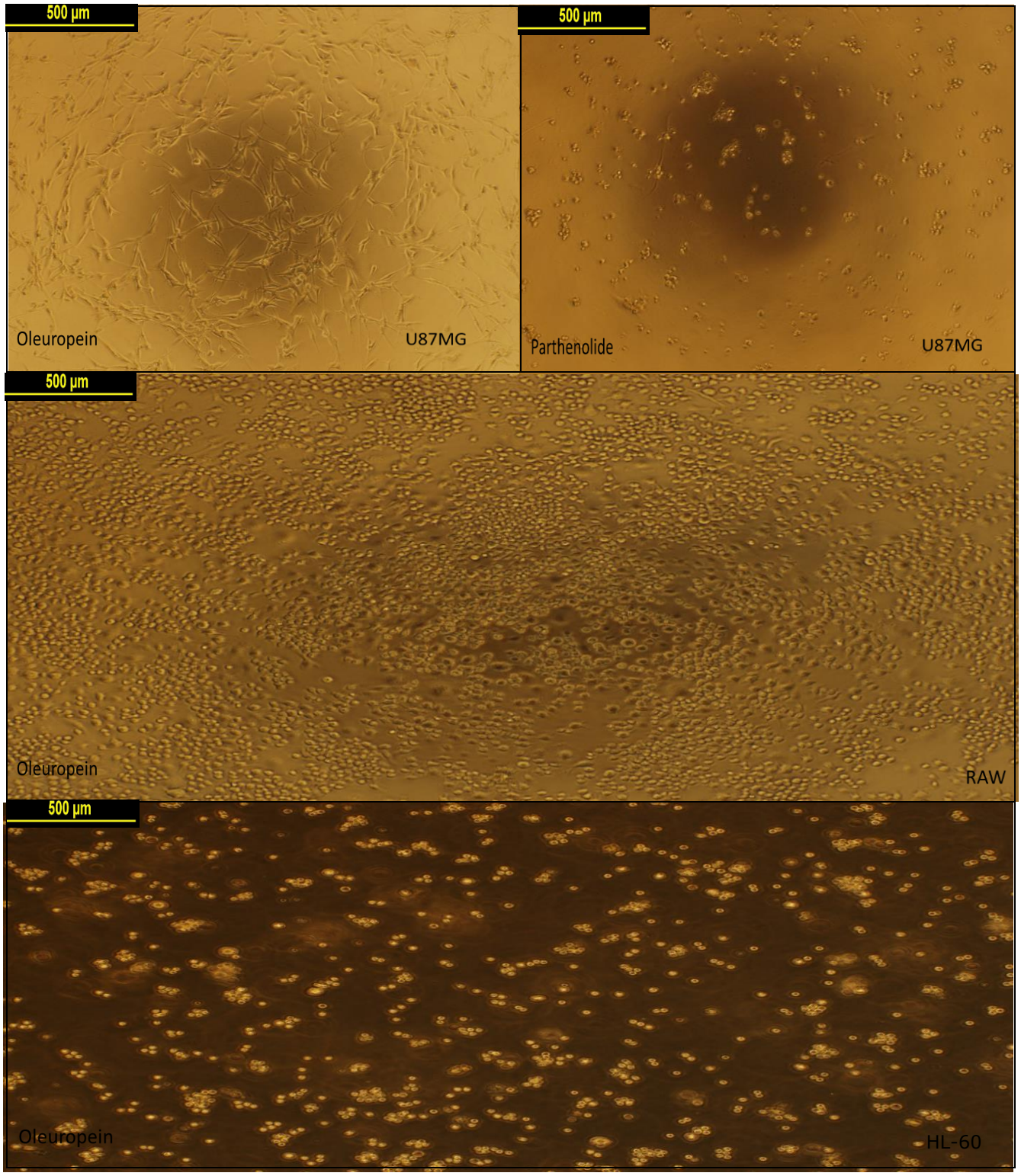


Fig. 14c. Effect of oleuropein with dose of 5µg/ml on different cancer cell lines and normal cell line observed by inverted microscope

5.4. Inhibition of iNOS And Antioxidant Activity

Induce of nitric oxide synthase which is indicator for inflammatory by producing nitric oxide as a result of macrophage excitation. Oleuropein, rosmarinic acid, chlorogenic acid and verbascoside showed significant iNOS inhibiting activity.

Oleuropein was the most active compound with IC_{50} of 1 µg/ml in comparison with parthenolide 0.38 µg/ml. Therefore, oleuropein can exhibit anti-inflammatory effect in low concentration, on the other hand rosmarinic acid, chlorogenic acid and verbascoside showed moderate activity as it is shown in Table 6.

Antioxidant activity of these compounds by prevention DCFH non-fluorescent compound to become DCF which was fluorescent. This transformation occurs due to reactive oxygen species (ROS).

Most of the compounds exhibit good antioxidant activity and this result correlate with growth inducing because these compounds protect the cells from free radical.

Oleuropein showed activity with IC_{50} 10.02µg/ml as it is shown in Table 6. And the others compounds showed good activity comparison with vitamin C which is considered as a control.

Table 7. Effect of OLE, VER, KLJA and OSA on iNOS and DCF production

Sample	iNOS inhibition µg/ml (IC_{50})	DCF production µg/ml (IC_{50})
OLE	1	10.02
OSA	2.5	6.35
KLJA	2	10.34
VER	9	6.187
Vit. C	-	0.6
Parthenolide	0.38	-

5.5. Antimicrobial Activity

Oleuropein, rosmarinic acid, chlorogenic acid and verbascoside have slight antimicrobial activity against gram positive and gram negative bacteria as well as against yeast. In table 7 shows the compounds activity comparison with ampicillin as antimicrobial and flucytosine as antifungal.

Table 8. The antimicrobial activity of OLE, VER, KLJA and OSA

	E. coli ATCC 25922 (G-) µg/ml	*E. coli 0157H7 (G-) µg/ml*	*S. aureus ATCC 25923 (G+) µg/ml*	*S. epidermidis ATCC 12228 (G+) µg/ml*	*E. faecium DSM 13590 (G+) µg/ml*	*E. faecalis ATCC 29212 (G+) µg/ml*	*Salmonella typhimurium CCM 5445 (G-) µg/ml*	*Candida albicans ATCC 10239 µg/ml*
OLE	62.5	62.5	62.5	62.5	31.2	62.5	62.5	31.2
VER	62.5	62.5	62.5	62.5	62.5	15.6	62.5	62.5
KLJA	125	125	62.5	62.5	62.5	62.5	62.5	31.2
OSA	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5
AMPICILLIN	1.9	3.9	3.9	1.9	3.9	7.8	3.8	-
FLUCYTOSINE								7.8

5.6. Antiprotozoal Activity

Oleuropein showed activity against *Echenococcus granulosus* which cause hydatid cysts. 0.1% oleuropein did not show any activity in 30 minutes, while 1% showed activity in 20minutes and the best activity with 2% which showed scolicidal activity in 5 minutes.

Table 9: Different oleuropein concentrations and their scolicidal activity

Oleuropein Concentration	Exposure Times in Minutes				
	5	10	15	20	30
0.1%	L	L	L	L	L
1%	L	L	L	D	D
2%	D	D	D	D	D

L: Solutions containing live protoscoleces, D: All protoscoleces are inactivated

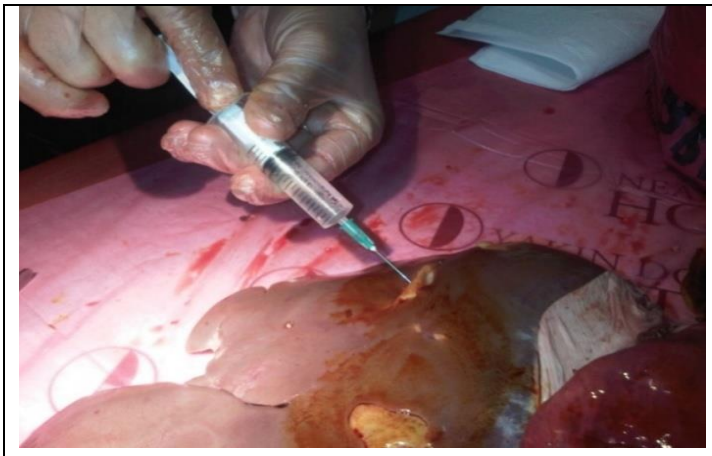
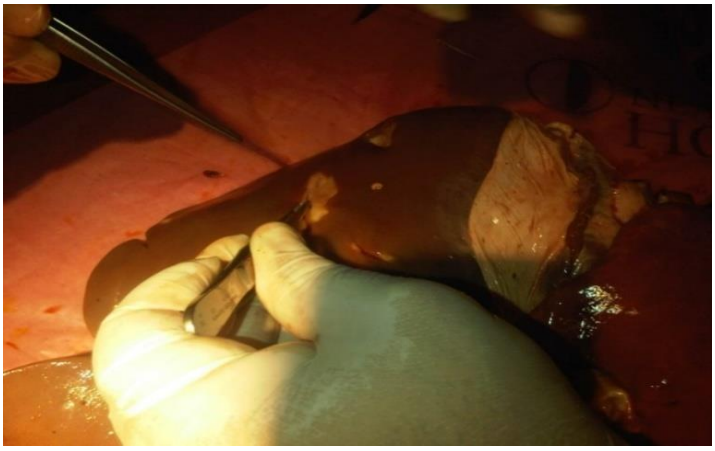
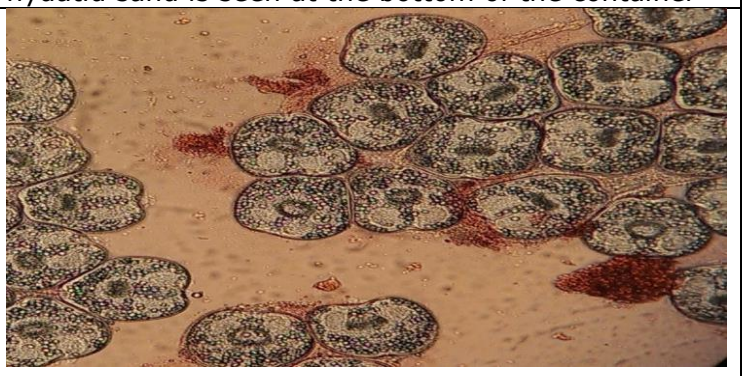
	
1.Cyst containing sheep liver are stained with povidone iodine and cysts are punctured and aspirated.	2.Cysts are opened and its contents are evacuated under aseptic conditions.
	
3.Hydatid fluid and cyst contents are filled in a sterile plastic container.	4.Protoscolex and daughter cysts are mixed and hydatid sand is seen at the bottom of the container
	
5.A drop of protoscolex solution is investigated for viability testing	6.Protoscoleces of *E. granulosus* stained with 0.1% eosin examined under light microscopy

Fig.15. Aspiration and viability testing of hydatid fluids containing metacestode form of *Echinococcus granulosus*, obtained from cyst containing sheep liver as an intermediate host.

Conclusion

Oleuropein is an olive secoiridoid, which was isolated from the ethanolic extract of the olive leaves. The extraction of *Olea europaea* were performed by soaking the leaves with 80% ethanol for three days in room temperature. After concentration, the extract was fractionated of by using reverse phase vacuum liquid chromatography (RP-VLC). Crude oleuropein was finally purified by Sephadex LH-20 gel chromatography. The purity of oleuropein and structure elucidation was established by 1D (^{1}H and ^{13}C NMR) and 2D-NMR (COSY, HSQC and HMBC) analysis and optical rotation. Oleuropein was subjected to a series of biological activity assays. It was found that it has shown the good activity against gram positive bacteria such as *S. aureus, S. epidermidis and Enterococcus faceium, Enterococcus faecalis*, gram negative such as *E.coli and Salmonella tyohimurium*, yeast such as *Candida albicans* and protozoa such as *Echenococcus granulosus.* In addition it showed significant anti-inflammatory activity through inhibition of iNOS production and antioxidant activity by preventing the formation of ROS which convert DCFH to DCF. Moreover, it has the ability to induce cell growth on Hela, A549, MCF-7, U78MG, MPANC-69, MDA-MB-241, HEK-239, CaCo-2, RAW-264.7 and PC-3.

In conclusion, these results open the possibility of considering oleuropein as a chemo preventing agent for bacteria, yeast, protozoa, inflammation, and oxidation as well as wound healing.

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