Olive Leaf Extract Trigger Defense Physiological Markers in Datura metel against Tobacco Mosaic Virus

Article *in* Journal of Pure and Applied Microbiology · February 2014

CITATIONS
2

READS
568

4 authors, including:

Nagwa Mohamed Amin Aref
Ain Shams University
167 PUBLICATIONS **250** CITATIONS

SEE PROFILE

JOURNAL OF PURE AND APPLIED MICROBIOLOGY, FEBRUARY 2014. Vol. **8**(1), p. 825-842

Olive Leaf Extract Trigger Defense Physiological Markers in *Datura metel* against Tobacco Mosaic Virus

Asma A. Al-Huqail, Faheema Khan, Nora Al-Zahim and Nagwa Mohamed Aref*

Department of Botany and Microbiology, College of Science, King Saud University 11495 Riyadh, P.O. Box 22452, Saudi Arabia.

(Received: 09 November 2013; accepted: 16 December 2013)

Plants have been used as medicines since the time immemorial, among which *Olea europaea* products are widely available. Six essential oil extracts were tested for antiviral activities in *Datura metel*. The selected oils were Olive Leaf Extract (OLExt), Cinnamon, Clove, Black seed, Cedar, and Walnut oil. *Datura metel* was used as model host for TMV strain. The highest antiviral activity was observed in OLExt triggering physiological markers in *Datura metel* and showing reduced number and size of necrotic local lesion. Gas chromatography-mass spectroscopy (GC-MS) revealed the presence of three main compounds Iridoid glycosides, polysaccharides and phenolic acids in OLExt. *Datura* leaves were further analyzed for physiological markers. Among amino acids; alanine and serine found increased along with a significant rise in glutamine up to 7.16 mol/Kg DWT and methionine 0.128 Kg DWT in OLExt treated. There was an obvious increase in the lead, zinc, Chlorophyll A (from 1.75 to 1.93 mg/gm), total protein (from 2.8 to 3.12 mg/gm) and DNA (from 273.52 to 313.09 mg/gm). Fixing nucleus activity was assessed by comet assay in the value of tail moment unit from 18.870 in TMV to 12.314. These physiological markers were found to be parameters for virus host interaction in plant defense relay on Iridoids as an antiviral compound in OLExt.

Key words: Virus host interaction, Phyto active materials, Antiviral activity, TMV, Physiological Markers, Iridoids, Immunizing plant.

Many screening efforts have been made to find antiviral agents from natural sources. Plants have long been used as remedies, and many are now being collected and examined in an attempt to identify possible sources of antivirals (Abad *et al*., 2000). In the last decades and as an alternative to conventional chemical agents, a large number of phytochemicals have been recognized as a way to control infections caused by viruses (Kalvatchev *et al*., 1997); (Yamasaki *et al*., 1998) and (Abad *et al*., 1999a,1999b and 2000). Leaves from tree, *Olea europaea*, are rich in biophenols (BPs), such as oleuropein (Ole) verbascoside, ligstroside, tyrosol or hydroxytyrosol. These compounds have shown several biological activities such as antioxidant (Visioli *et al*., 1998.); (Benavente-Garcia *et al*., 2000); antithrombotic, and even skin photoprotective properties (Saija and Uccella 2000). Furthermore, some of these compounds have demonstrated antimicrobial activity by inhibiting the growth of a wide variety of bacteria, fungi and viruses (Renis, 1969) and (Hirschman, 1972). Ole has been claimed in a U.S. patent to have potent antiviral activities against herpes mononucleosis, hepatitis virus, rotavirus, bovine rhinovirus, canine parvovirus and feline leukemia virus (Fredrickson, 2000). Many studies have been conducted to prove

* To whom all correspondence should be addressed.
Phone: +966-1-4789585 Ext.1439
Fax: +966-1-4768171 & +966-1-4765598
E-mail: narif@ksu.edu.sa & nagwa_aref@hotmail.com

its potential as anti-oxidant, anti-viral, anti-microbial. Hydrolysis products obtained from oleuropein were: (i) l-3, 4-dihydroxyphenylethyl alcohol prepared by acid hydrolysis of oleuropein; (ii) elenolic acid obtained by methanolysis of oleuropein, isolation of the intermediate acetal, and subsequent acid hydrolysis; and (iii) oleuropein aglycone formed by the action of β-glucosidase on the parent glucoside (Walter *et al.,* 1973.). The mechanism of action of the antiviral activity is reported to include an ability to interfere with critical amino acid production essential for viruses, an ability to contain viral infection and/or spread by inactivating viruses or by preventing virus shedding, budding, or assembly at the cell membrane, ability to directly penetrate infected cells and stop viral replication. In the case of retroviruses, it is able to neutralize the production of reverse transcriptase and protease (Khan *et al.,* 2007). Bioactive compounds, such as flavonoids, phenolic compounds, tannins, or aromatic compounds, have been identified and their mechanisms of antiviral action during the pathogenesis for reducing membrane fluidity, inhibit fusion of the viral membrane; interfering with viral entry by reducing the receptor binding activity through virus adsorption, preventing both the release of virus from infected cells and the formation of viral aggregates after release from host cells; and inhibiting viral replication at the initial stage of viral infection (Rahman, 2012). Glycosides, polysaccharides membrane structure affect its mechanism of transporter; no sugar-binding site is observed in the absence of ligand, and deprotonating of the key residue Glu269 seems associated with ligand binding. Thus, substrate induces formation of the sugar-binding site, as well as the initial step in H+ transduction (Abramson *et al.,* 2003). Prevention of free radical formation by oleuropein occurs through its ability to chelate metal ions, such as Cu and Fe, which catalyze free radical generation reactions(Andrikopoulos *et al.,* 2002). The application of the pathogen-derived resistance PDR concept in plant revealed unknown sequence-specific RNA-degradation mechanism in plants which helped to design antiviral strategies to engineer viral resistant plants in the last 25 years. An attempt had been made to study antigenotoxic activity of the isolated Castasterone against H_2O_2 -induced DNA damage in human blood lymphocytes using Single cell gel electrophoresis assay (Comet Assay) (Sondhi *et al.,* 2010). The Comet assay, a relatively fast, simple, and sensitive technique for the analysis of DNA damage in all cell types, has been applied for the screening of chemicals, biomonitoring and intervention studies (Cemeli *et al.,* 2009). Applications of RNA silencing to protect crop plants against viral diseases of agronomic relevance called MicroRNAs in viral gene regulation (Simon'-Mateo *et al.,* 2011). Resistance to plant disease is often specific and metabolites and receptors contributing to this specificity may have specific structure. However; another structurally -unrelated compounds could induce systemic resistance (ISR) in unrelated plants to diverse pathogen. Both of them are associated with a rapid accumulation of the same structurally unrelated putative defense compound that have diverse functions(Ku, 2001) . The movement protein (MP) of *Tobacco mosaic virus* interacts with microtubules during infection. Although this interaction is correlated with the function of MP in the cell-to-cell transport of viral RNA, A direct role of microtubules in the movement process and the involvement of microtubules in viral RNA trafficking was proved (Seemanpillai *et al.,*2006.). In addition; olive leaf oil has antioxidant activity through the scavenging of superoxide (Ghisalberti, 1998). Over expressing defensins; small cysteine-rich peptides with a molecular mass of 5 kDa (45–54 amino acids) which belongs to a group of pathogenesis-related defense mechanism proteins; confer resistance to fungal pathogen in transgenic plants(Abdallah *et al*., 2010). The zinc finger C_{36}-X1-C_{38}-X7-C_{46}-X6-H_{53} of the unclearly localized C2 protein of *Tomato yellow leaf curl virus* China is involved in pathogenicity and suppression of posttranscriptional gene silencing (PTGS). Zinc finger is indispensable for the C2 protein to bind zinc and DNA. Mutation of cysteine residue C_{36}, C_{38}, or C_{46} reduced the zinc and DNA binding capacity of C2 protein. When expressed from potato virus X, all three mutants, C2-C_{36}R, C2-C_{38}N, and C2-C_{46}I, tagged with a green fluorescent protein (GFP) were still capable of transporting GFP into but aggregated abnormally in nuclei. It was establish that zinc- and DNA-binding activity correlates with C2-mediated pathogenesis and PTGS suppression (Van Wezel, *et al.,* 2003). Biological activities of naturally occurring iridoids

which resemble iridoid glycoside and acetal esters were reviewed that can best be considered as pro-drugs. The possibility that the activity of some iridoids is determined by their conversion to pyridine monoterpene alkaloids (PMTA) is also considered (Ghisalberti, 1998). Inducible defense-related proteins have been described in many plant species upon infection with oomycetes, fungi, bacteria, or viruses, or insect attack. Several types of proteins are common and have been classified into 17 families of pathogenesis-related proteins (PRs). Others have so far been found to occur more specifically in some plant species. Most PRs and related proteins are induced through the action of the signaling compounds salicylic acid, jasmonic acid, or ethylene, and possess antimicrobial activities in vitro through hydrolytic activities on cell walls, contact toxicity, and perhaps an involvement in defense signaling (Van Loon *et al.*, 2006).

Viruses on the other hand are intracellular obligatory parasitic pathogenic agents which infect other living organisms. Human diseases caused by viruses include chickenpox, herpes, influenza, rabies, smallpox and AIDS. Although these are the viruses commonly known, the first virus ever described and from which the term was eventually derived was tobacco mosaic virus (TMV) which was discovered by Martinus W. Beijerinck, a Dutch microbiologist, in 1898. (TMV) caused an important disease infecting family *Solanaceae*, one of the most economically important veg-etable crops worldwide. Tobacco mosaic virus is a stable virus causes very difficult disease to control because it spreads easily. The disease is mechanically transmitted, resulting in quick and effective infection. The first systemic symptom to occur in newly infected plants is vein clearing. Soon after vein clearing, mosaic symptoms occur in the newer leaves. The young leaves infected with TMV are often deformed and wrinkled. TMV infection may also cause the production of nonviable seeds. In this respect, the current study was designed to evaluate the effect and antiviral activity of commercially available olive leaf extract against the TMV as a model virus that affects many plants and easily induced local lesions on *Datura metel* leaves as plant of choice in this study (Ara *et al*., 2012).

MATERIALS AND METHODS

Virus Source and essential Oil Treatments

TMV strain was obtained according to (Ara *et al.,* 2012) and (Peacock *et al.*,1991). Six essential oil extracts were tested for antiviral activity on *Datura metel* plant leaves. The whole *D. metel* plant was used for the application and the leaves were divided into three partitions each containing triplicates. The virus extract was mechanically inoculated alone to the first partition as a positive control. Each essential oil extracts was sprayed only to the second partition as a control. For the other treatments essential oil extracts were sprayed first followed by virus infection, one volume of crude sap of infected source of virus: one volume of essential oil extracts and applied to the third partition of leaves. Pre, post and neutralized treatments were chosen for OLExt only for testing its potential antiviral activity.

Local Lesion Numerical Assay for Identifying OLExt and Antiviral Activity

The antiviral activities of the tested essential oil extracts against TMV were determined using the local lesions count method (Ara *et al.*, 2012). After spraying the bioactive compound in the OLExt to the infected *D. metel* leaves, the number of local lesions caused by the TMV was enumerated in order to deduce the effect of the potential bio compound extracted from OLExt chosen for this study.

Gas Chromatography-Mass Spectroscopy (GC-MS) Analysis of *Olea europaea* Whole leaves Liquid Extract.

GC-MS analysis was performed to identify the active antiviral compound from *Olea europaea* whole mature leaves liquid herbal extract oil obtained from Herb Farm Company (USA), certified organically grown without the use of chemical fertilizer or pesticides or herbicides. To assure optimal extraction of *oleuropein* and other bioactive component, the leaves is hand harvested only in the winter months, is carefully shade-dried, and then thoroughly extracted, never fumigated or irradiated dry herb/menstruum ratio: 1:5, contains certified organic grain alcohol (85-95%) & Olive leaf extractives). Identification of several compounds was done by injecting 1 µl of sample into a RT x -5 column (30 × 0.32 nm) of GCMS model (Perkin Elmer, Clarus 500, USA) and helium

(3 ml/min) was used as a carrier gas. The following temperature gradient program was used (75°C for 2 min followed by an increase from 75 to 175°C at a rate of 50°C per min and finally 7 min at 175°C) (Pandey *et al.,* 2010)The chemical components of the extracts were analyzed in the central laboratory of King Saud University, Riyadh, Saudi Arabia. Identification of the chemical constituents of extracts were performed using Perkin Elmer (Clarus 500, USA) gaschromatography coupled with (Clarus 500, USA) mass spectrometer (MS) (Table 1).

DNA Content

DNA was determined by the diphenylamine assay (Buckton *et al*., 1968) of extracts prepared by grinding leaves and homogenizer in 20 volumes (ml/g) of 2 M NaCl, 10 mM EDTA, 1 % SDS,pH 7.0.DNA isolation was performed using 300 mg of fresh leaves in liquid nitrogen. The DNA content was checked at 260 nm by using UV-Spectrophotometer (Shimadzu 1700). The A 260/ A280 nm values were determined. The ratio of DNA solution absorption (260/280) ranged from 1.7 to 1.9, as desirable quality of obtained DNA. The following formula: DNA (µg) = (A260) (dilution factor) 50 was used for measuring DNA concentration. Each absorption unit in 260 nm wavelength is equivalent to 50 µg/µl of double-strand DNA. (Deshmukh *et al*;2007) and (Doyle, 1990.)

Chlorophyll Content

0.5 cm^2 leaf discs in liquid N_2 were quickly grind and then extracted in 1 ml 80% acetone. Samples were repeated for each treatment at a time. Because the longer the sample is in acetone the more chlorophyll leaches out and this may bias readings. The reading at spectrophotometer at 645 nm for chlorophyll B and 663 nm for chlorophyll A was calculated. Chlorophyll concentration can be calculated using this formula: Chl. Conc in µg/ml = (OD645 × 20.2) + (OD663 × 8). This value is then multiplied by 2 to obtain chlorophyll $conc^n$ per cm^2 or by dividing the weight in mg to obtain total chlorophyll concentration per mg (Arnon, 1949).

Comet Assay

According to (Juclimiuk *et al.,* 2006) isolation of nuclei was performed. Individual leaves were treated with 200µl of cold 400 mMTris-HCl buffer, pH 7.5 (on ice). Gently sliced into a "fringe" to release nuclei into the buffer under yellow light. A mixture of 55 µl of nuclear suspension and 55µl of low melting point (LMP) agarose was used. Single Cell Gel Electrophoresis (SCGE) slides with plant cell nuclei were treated with the mutagen solutions for 2 h at 26°C, and then they were rinsed three times for 5 min in cold distilled water. The slides with plant cell nuclei were placed in a horizontal gel electrophoresis tank containing freshly prepared cold electrophoresis buffer (300 mMNaOH, 1 mM EDTA, pH>13) and incubated for 15 min. Electrophoresis was performed at 16 V, 300 mA for 30 min16 V, 300 mA at 4°C. The electrophoresis conditions used in the study were optimal to provide low level of DNA damage in control cells and linear concentration-response for the induction of comets after chemical mutagenic treatment in these species in earlier treatments. Then the gels were neutralized by 400mM Tris-HCl, pH 7.5 and stained with ethidium bromide (20 µg/ml). After staining, in each slide, 50 randomly chosen cells were analyzed under the fluorescence microscope with an excitation filter of 546 nm and a barrier filter of 590 nm using computerized image analysis system (Komet Version 3.1. Kinetic Imaging Liverpool UK). The tail DNA (TD, %) and tail moment (TM) were used as parameters of DNA damage.

Protein Quantitation

The method of (Lowry *et al.,* 1951) and (Skakir *et al.,* 1994) is based on both the Biuret reaction according to (Waterborg and Mathews, 1984) in which the peptide bonds of proteins react with copper under alkaline conditions to produce Cu+, which reacts with the Folin reagent, and the Folin–Ciocalteau reaction, The reactions result in a strong blue color, which depends partly on the tyrosine and tryptophan content. The method is sensitive down to about 0.01 mg of protein/mL, and is best used on solutions with Concentrations in the range 0.01–1.0 mg/mL of protein. Four materials were prepared; 1. Complex-forming reagent by mixing the following stock solutions in the proportion 100:1:1 (by volume), respectively: Solution A: 2% (w/v) Na_2CO_3 in distilled water. Solution B: 1% (w/v) $CuSO_4 \cdot 5H_2O$ in distilled water. Solution C: 2% (w/v) sodium potassium tartrate in distilled water; 2. 2 *N* NaOH; 3.Folin reagent (commercially available): Used at 1 *N* concentration and 4. Standard solutions: Use a stock solution of standard protein (e.g., bovine serum albumin

fraction V) containing 2 mg/mL protein in distilled water, stored frozen at –20°C. Standard solutions were prepared by diluting the stock solution with distilled water to have protein conc. (mg/mL). For each 0.1 mL of sample or standard, 0.1 mL of 2 *N* NaOH was added and hydrolyzed at 100ÚC for 10 min in a heating block or boiling water bath. 0.1 mL of Folin reagent, was added and mixed, and stand at room temperature for 30–60 min (do not exceed 60 min). The absorbance value was recorded at 750 nm if the protein concentration was below 500 mg/mL or at 550 nm if the protein concentration was between 100 and 2000 mg/ml A standard curve of absorbance as a function of initial protein concentration was plotted and used to determine the unknown protein concentrations .Whole cells or other complex samples may need pretreatment, as described for the Burton assay for DNA Waterborg and Matthews (1984).Following (Peterson, 1983) had described a precipitation step that allows the separation of the protein sample from interfering substances and also consequently concentrates the protein sample, allowing the determination of proteins in dilute solution. By using 0.1 mL of 0.15% deoxycholate to 1.0 mL of protein sample as well as adding 0.1 mL of 72% trichloro acetic acid (TCA) plus sodium dodecyl sulfate (SDS)that added to solubilize membranes or remove interfering substances (Markwell *et al*., 1981). We followed the modifications to this basic assay that have been reported for increasing the sensitivity of the reaction. The Folin reagent is added in two portions, vortex-mixing between each addition, for achieving 20% increase in sensitivity (Hess *et al.*, 1978). Also; the addition of dithiothreitol 3 min after the addition of the Folin reagent increases the sensitivity by 50% (Larson *et al.*, 1986) For complete protein recovery; adding phosphotungstic acid (PTA)was required as 0.2 mL of 30% (w/v) TCA and 6% (w/v) PTA to 1.0 mL of protein sample.

Amino Acids

Amino acids were analyzed by pre column derivatization with FMOC-Cl[4] followed by gradient elution reversed phase HPLC (2). The HPLC system consisted of a model 720 System Controller, a model 730 Data Module, a model 7 1OB WISP Sample Processor, one model M45 and one model 6000A pump, all from Waters Associates Inc., USA. The amino acid derivatives were detected with a fluorescence monitor (Shimadzu model RF-530) and by absorption of UV (Waters Variable Wavelength Detector 450. The column (Amino Tag Amino Acid Analysis Column, Varian Associates Inc., USA) was placed in a column oven (temperature 32°C).Leaf samples were cut using the micro cautery technique of Unwin, and immediately after excision the exuding sap was collected in a 0.5 µL microcap (Drummond Scientific Inc., USA).

Then, the samples were dissolved in 10 µL HCl/ethanol (1:1) and stored in a freezer prior to analysis. Leaf exudate samples were taken sequentially, within 2 min, from the same leaf as the corresponding. Phloem sap sample. The leaf was cut off less than cm from the excised stylet and the cut end was immersed immediately in 100 µL 5.0 mm EDTA solution (pH 7.0) in a small vial and then placed for 90 min in a humid chamber (approximately 95% RH, temperature 25°C). These samples were also stored in a freezer until analyzed.

All amino acids were obtained from Merck, BRD and from FLUKA AG, Switzerland. The water used was run through an Aqua-Cleer MD 1000 reverse osmosis system and a Culligan DB 50S cation and anion exchanger unit, both from Culligan TEKO AB, Sweden and then through a Spectrum System A water purifier (ELGA Ltd, England). Internal Standard Phosphoserine was used as an internal standard since it was well separated from the other amino acids. One phloem sap sample and one exudate sample were analyzed to confirm that no phosphoserine was present in the samples.

Sample Preparation.

The samples were prepared in a 300 µL polypropene plastic insert (Millipore JWSW OP 109) in a glass screw cap vial fitted with a PTFE-lined seal (Waters No. 73008). For phloem sap samples, 1, µL of the sample was added to 39 ul of 0.2 M $NaHCO_3$ buffer with 2.5, µM phosphoserine. A 40µL amount of 4 mM FMOC-Cl in acetone (dried with anhydrous $CaCl_2$) was added and the vial was shaken. After 10 min the solution was extracted with 120 µL pentane. Fifteen µL of the lower phase was injected within 30 min of sample preparation. Exudate samples were prepared in the same way, except that 20 µL of the sample was added to 20µL of the buffer solution, and 30, µL of the lower phase was injected subsequent to the extraction with pentane. Some exudate samples had to be diluted

up to 100 times before analysis.

Separation the elution solvents used were: (A) 0.02 M sodium citrate, 0.005 M tetra methyl ammonium chloride adjusted to pH 2.85 with concentrated H_3PO_4; (B) 80% of buffer A adjusted to pH 4.50 with concentrated H_3PO_4 and 20% methanol; and (C) acetonitrile. Solvents A and B were connected to pump 6000A via an electromagnetically controlled switch valve. The gradient used was (A: B: C, linear shifts between all points):0 min, 73:0:27; 11.5 min, 58:0:42; 13 min, 58:0:42; 13.1 min, 0:63:37 (electromagnetic valve switched); 18 min, 0:62:38; 25min, 0:30:70; 27 min, 0:25:75; 32 min, 0:25:75. Flow rate: 1.4 mL/min. Quantification: For quantification the standard curves were assumed to be linear (2). Amino acid derivatives were quantified by integration (Data Module 730) of the fluorescence chromatogram (excitation 264 nm, emission 340 nm), except for tryptophan, which was quantified based on its peak height in the UV chromatogram (264 nm). The combined asparagine/glutamine peak was quantified assuming identical response factors for the asparagine and glutamine derivatives. Tyrosine was quantified on the basis of the mono-FMOC derivative peak. Amino acid standards were prepared for phloem sap samples in 0.1 M HCl:EtOH 1: 1(0.10 mm of each amino acid), while those for the exudate samples were prepared in 5.0 mM EDTA adjusted to pH 7.0 with 2 M NaOH (2.5 µM of each amino acid) according to (Einarsson *et al*., 1983) and (Unwin, 1978).

SDS- PAGE

The soluble leaf proteins were extracted from 0.5 g leaf material in ice-cold 100 mMTris-HCl buffer (pH 8.0), containing 20 mM MgCl2, 10 mM $NaHCO_3$, 1 mM EDTA, 2 mM phenylmethane-sulfonyl fluoride, 12.5 % glycerol (v/v), 20 mM ß-mercapthoethanol and 2 % (w/v) Polyclar. After centrifugation (15 min, 15 000 *g*, 4 °C), the supernatant was boiled in sample buffer for SDS-PAGE. The proteins were separated by 12 % SDS-PAGE with a *Mini Protean II Dual Slab Cell* (Bio-Rad) according to (Laemmli, 1970) . Samples with protein quantity equivalent to the same MW were loaded for all variants. Gels were stained with Coomassie brilliant blue R-250 (Yeang, 1998).

Determination of Micro Elements

Plant samples were prepared by washing with de-ionized water and oven dried at 80°C for 2 days and then subjected to ground for powder formation. Two grams powder were digested for each plant sample and dissolved in 10 ml of nitric acid (HNO3) for 12 h and then heated until the reddish brown fumes disappeared. About 4 ml of perchloric acid was added to the above solution and heated for 5 min then 10 ml of aqua regia was added and heated to small volume and marked up to 250 ml by adding deionized water .Using Atomic absorption spectrophotometer (AAS) the .Major and trace elemental contents were determined using flame atomic absorption spectroscopy by Perkin Elmer A Analyst 700.(Abdul Ghani *et al.*, 2012)

RESULTS

Chemical Composition of Essential Oil from *Olea europaea* Whole leaves Liquid Extract

The HC-MS total ion chromatogram (TIC) of the essential oils from OLE analysis by HC-MS HPLC-purified OLE was further subjected to GC-MS analysis to enhance mass resolution in the second dimension and detect multiple chemical species with the same retention time shows the GC-MS results of oleuropein standard. As seen in Fig.1. Of oleuropein is as pure as the standard. A single major peak was observed at 13.55 min in both samples. MS shows a single mass peak at [M–H] _ µ539, corresponding to oleuropein in both samples. A minor peak was observed at twice this mass at [M–H] µ1079 representing the oleuropein dimer. The mass peak [M–H] µ652 in the standard represents the trifluoroacetic acid (TFA) adduct of oleuropein.The major identified components of the extracts are listed in Table 1 using GC-MS and demonstrated Iridoid glycosides, polysaccharides and phenolic acids. Three high concentration compounds as common individual factor in the three pikes representing (92.6%) was Cyclohexasioxane, Dodecamethyl $C_{12}H_{36}O_6Si_6$; (84.8%) was Cycloheptasiloxane, Tetradecamethyl $C_{14}H_{42}O_7Si_7$, and (80.9%) was Cyclooctasiloxane, Hexadecamethyl $C_{16}H_{48}O_8Si_8$, respectively. In addition to many derivatives of Caffeine (1, 3, 7-trimethylxanthine) structure; like 10 Decamethyle-5-(Trimethylsilxy) Hexasiloxane,9Nonamethyl-3-(Trimethylsiloxy)tetrasiloxane and 3-Ethoxy-6Hexamethyle-3Tris(Trimethylesiloxy) Tetrasiloxan (Table 1).

Table 1. The GC-MS analysis for antiviral activity having different compounds in the Olive Leaf Oil Extract (OLExt), with high REV (percentage of compound peak in the whole sample)

S. No	Compound name	MW	M Formula	REV
1	Cyclohexasioxane,Dodecamethyl	444	$C_{12}H_{36}O_6Si_6$	926
2	10 Decamethyle-5-(Trimethylsilxy)Hexasiloxane	490	$C_{13}H_{42}O_6Si_7$	768
3	1,2-Epoxy-Dihydroxycyclohexano[A]Pyrene	530	$C_{32}H_{42}O_3Si_2$	705
4	2H-1,4Benzodiazepin-2-one,7-Chloro-1,3-Dihydro-5-phenyle- 1-(trim ethyl)	342	$C_{18}H_{19}ON_2C_lSi$	693
5	Cycloheptasiloxane, Tetradecamethyl	518	$C_{14}H_{42}O_7Si_7$	848
6	Trisiloxane,6Hexamethly-3-(trimethylsilyl)Oxy	384	$C_{12}H_{36}O_4Si_5$	826
7	9Nonamethyl-3-(Trimethylsiloxy)tetrasiloxane	384	$C_{12}H_{36}O_4Si_5$	797
8	3-Isopropoxy-6-Hexamethyl-3,5-5-tris(Trimethylsiloxy) Tetrasil	57	$C_{18}H_{52}O_7Si_7$	795
9	Silane,[[4-[1,2-Bis[(Trimethylsilyl)oxy]Ethyl]-1,2-Pyenylene] Bis(oxy)]bis[trimethyl]	458	$C_{20}H_{42}O_4Si_4$	811
10	Cyclooctasiloxane,Hexadecamethyl	592	$C_{16}H_{48}O_8Si_8$	809
11	N-(trifluoroacetyl)-N,O.O,O-Tetrakis(Trimethylsilyl) Norepinephrine.	788	$C_{22}H_{42}O_4NF_3Si_4$	553
12	3-Ethoxy-6Hexamethyle-3Tris(Trimethylesiloxy) Tetrasiloxan	562	$C_{17}H_{50}O_7Si_7$	783
13	Propane, 2-Fluoro-2-Methyl.	76	C_4H_9F	604
14	Dimethyl amine, N-(Neopentyloxy).	131	$C_7H_{17}ON$	464
15	Hydroxyethyl Butyl Sulfide	134	$C_6H_{14}OS$	448
16	Beta-L-Arabinopyranoside, Methyl.	164	$C_6H_{12}O5$	403

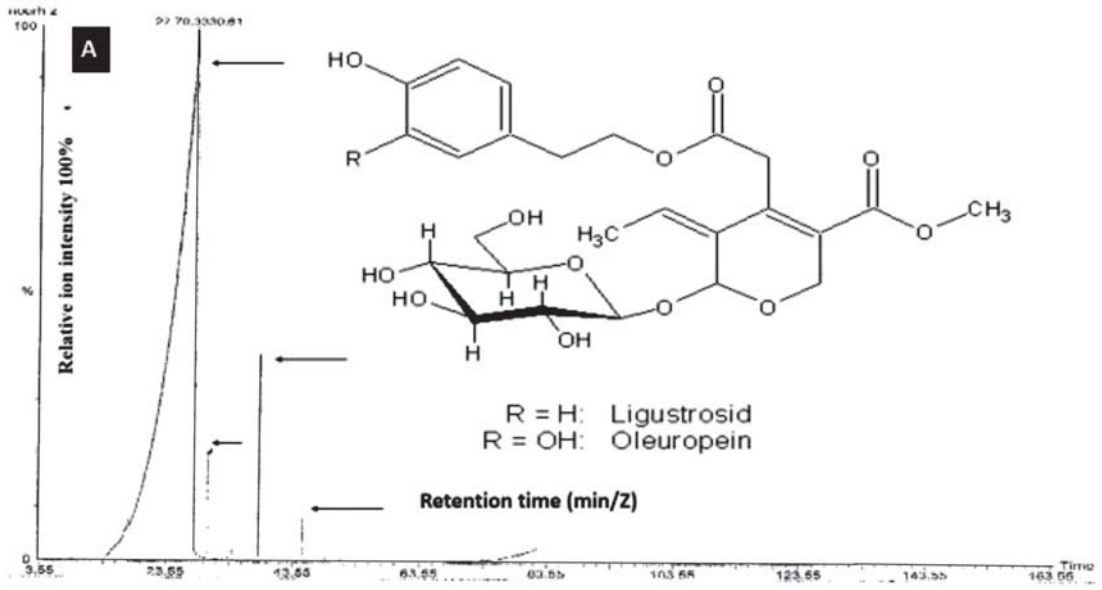

Fig. 1. A typical chromatogram**. A** of the constituents of essential Oil from the whole leaf of *Olea europeae*.GC-MS Chart with high REV % of the OLExt including bioactive chemical compounds analysis that resembles 4 peaks. The chemical structure of oleuropein, the major component of the olive extr. Indicated in the left. Highest first peak that have over 90% bioactive components illustrated and analyzed in chromatograms **A**

Antiviral Activity from Six Essential Oils and TMV Interaction on *Datura metel*

Symptomatology of TMV on *Datura metel* plants after application with six essential oils against the virus was observed (Fig. 2). OLExt had great antiviral effect; followed by; Cinnamon and Clove. While Black seed, Cedar, and Walnut had a very bad effects on the plants. Accordingly; OLExt was chosen to be analyzed and studied. Fig. 3.L & M. revealed the antiviral activity of OLExt on *Datura metel* in minimizing the size of necrotic local lesions NLL like pin point(Fig. 3.L) and then disappeared (Fig. 3.M) for the pretreated leaves which was so clear compared to the inoculated ones with the virus only after 6 days post inoculation (Fig. 3.M.) NLL on the inoculated leaves were clearly reduced with OLExt (Fig.2.C).compared to Cinnamon(Fig.2.E.) and Clove (Fig.2.G.) which showed moderate antiviral activities as a few more considered number of NLL.

DNA Content

It was obvious that antiviral components induced a highly significant increase of total DNA in all the treated plants (Fig.4.A); but with different levels. The most increased ones resembled in that treated with OLExt. It was 351.69 µg/gm in healthy plants and elevated from 273.52 µg/gm in the infected leaves with TMV up to 313.06 µg/gm in that treated with OLExt.

Chlorophyll Content

The total chlorophyll content in mg/gm of the chlorophyll a and b was dramatically dropped from 3.37 mg/gm in the healthy plants to 2.714 mg/gm in the infected with TMV. The best cured treatment from the infection appeared in that treated with OLExt (Fig. 4.C.) the best amount was noticed

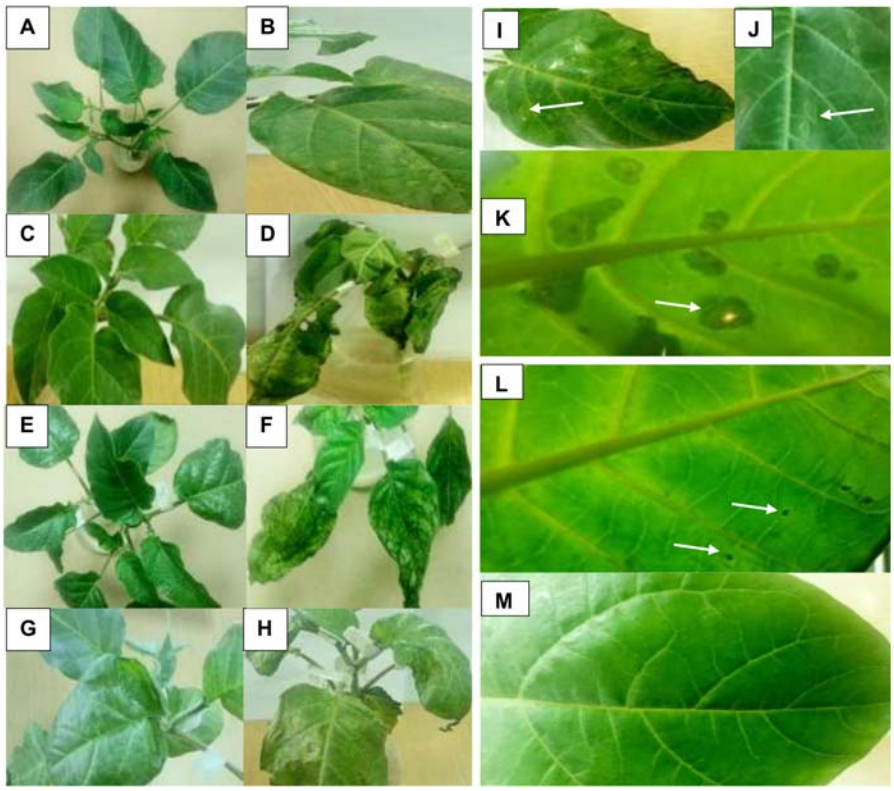

Fig. 2. Symptomatology of TMV on *Datura metel* plants after application with six essential oils against the virus as shown in C-H. Six were tested for antiviral activity. Olive Leaf Extract (OLExt) C. had great antiviral effects followed by; Cinnamon E; Clove G; Black seed H, Cedar F, and Walnut D. Healthy non-inoculated ones in A were compared to those inoculated with only virus in B. **Fig. 3.** Antiviral activity of Olive Leaf Oil Extract (OLExt) on *Datura metel* in L and M by minimizing the size of necrotic local lesions NLL (Arrows) for the pretreated leaves in both L &M which was so clear compared to the inoculated ones in I,J and K. NLL declared large size on the upper and lower blades (Arrows especially in K.

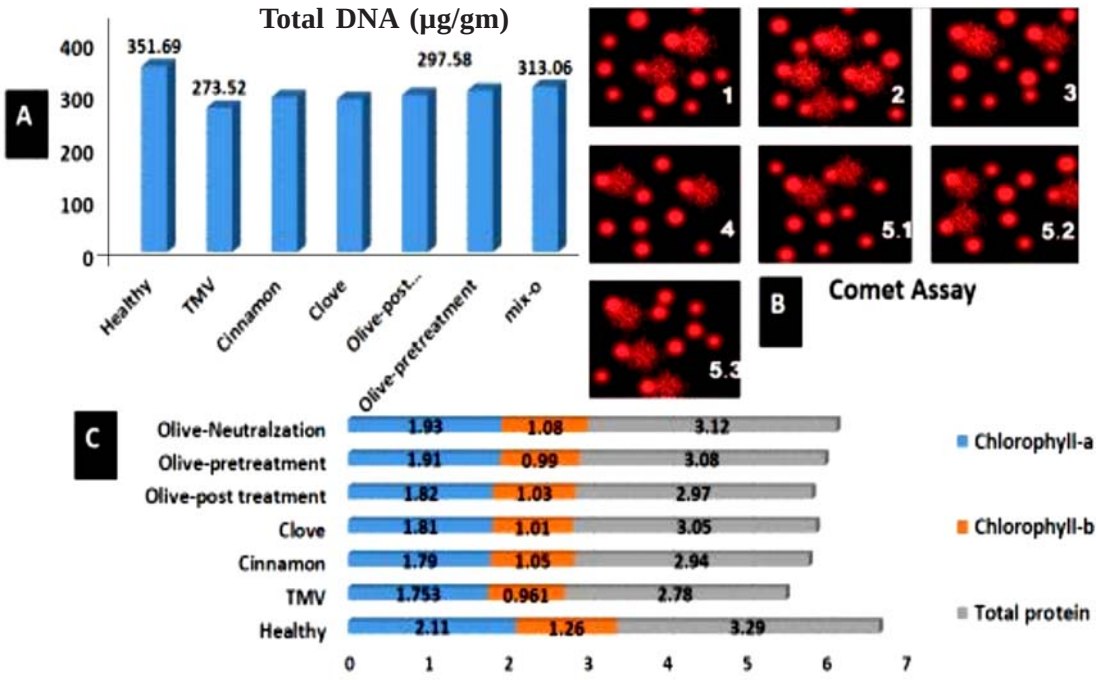

Fig. 4. Total DNA, A;numerical measurements by Comet Assay and Genotoxicity measuring the damages in the DNA using Tail Moment in Daturaplants for all treatments. B; and Total Protein Content and Chlorophyll; C as a physiological responses to the treatments with essential oils against Virus in chlorophylls and the Protein in Mg/gm.

in the treated ones was in olive –neutralization treatment which elevated the total chlorophyll content from 2.714 mg/gm up to 3.01 mg/gm.

Comet Assay or Single Cell Gel Electrophoresis (SCGE)

Comet Assay is a versatile and sensitive method for measuring single- and double-strand breaks in DNA as a routine test of the genomic DNA damage carried out with *Datuta metel* plant to evaluate the genotoxic effects of viral infection as well as genotoxic agents or compoundbased on the technique of micro electrophoresis. The general characteristics of our studied are summarized (Table 2 and Fig.4. B) The "tail moment"(TM), the product of the amount of DNA in the tail and the mean distance of migration in the tail, was the most informative feature of the comet image.The healthy value was 2.181 while the TMV infected cells had 18.870 i.e. ten times DNA damage**;** while after oil treatments the cells had repaired with

Table 2. The numerical measurements of the DNA damages by calculating the length of the tail and percentage of total DNA in the tail in 7 treatments , higher tail moments indicates grater DNA damages in Comet Assay

Treatments	1. Healthy	2. TMV	3. Cinnam-On	4. Clove	5.1 Olive Post-t.	5.2 Olive Pre-t	5.3 Olive Neutral
Tailed Cells%	5	22	19	15	17	14	12
Untailed Cells %	95	78	81	85	83	86*	88*
Tail Length µm	1.33	4.25	3.95	3.88	3.91	3.52*	3.43*
Tail Dna %	1.64	4.44	4.12	4.33	4.05	3.71*	3.59*
Tail Moment Unit	2.181	18.870	16.274	16.800	15.836	13.059*	12.314*

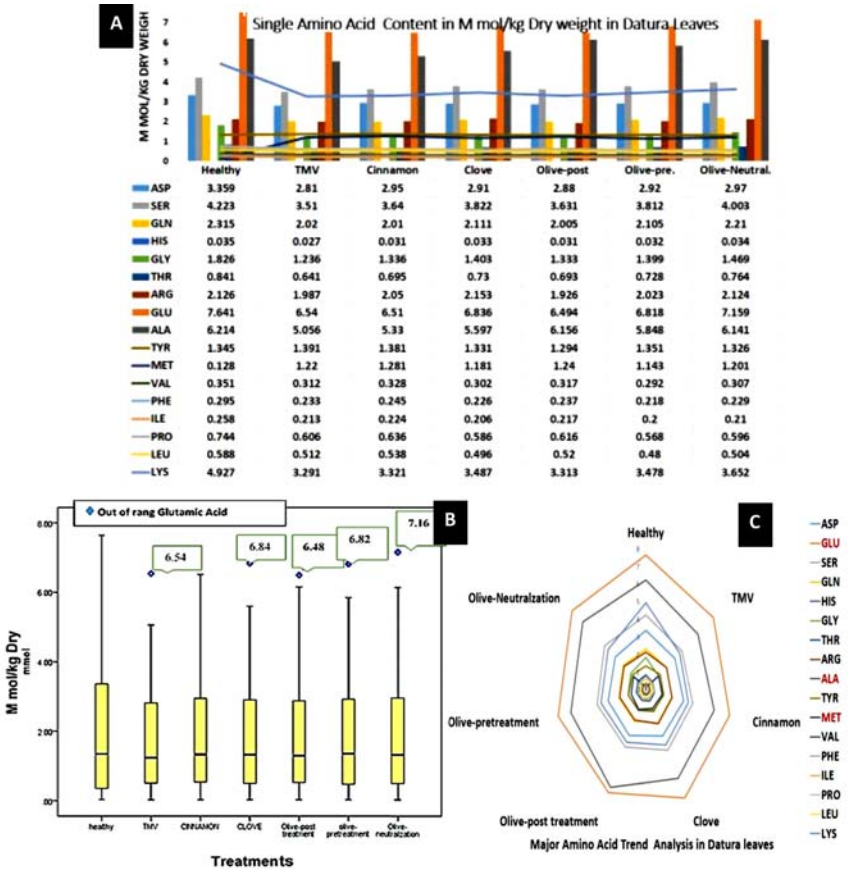

Fig. 5. Amino Acid Analysis from the Treated *Datura* Leaves with Essential Oils in A, B, and C.A.Displays each Single amino acid content in different treatments in Datura leaves B. shows the box plot of the median distribution values of the amino acid content M Mol/Kg dry W.in different treatments. Glutamic acid indicates high out range values. C. Demonstrates the trend behavior of the most changeable amino acids due to each treatment.

the most effective antiviral compound of OLExt/ TMV with 12.314/18.870 (TM), In addition, % tail DNA was12%/ 22% and tail lengthwas also 3.43 µm/4.25 µm. The percentage of the tailed in the infected was 22% and had great differences of 12% after the application with OLExt. (Fig 4. B.5.1) illustrated the tailed damaged cells in the treated plants by OLExt. Repairing effects elevated up to 83-86-88% in the DNA compared to infect with TMV with more damaged cells up to 22% (Fig. 4. B.2.)

Protein Quantitation and Amino Acids

The total protein content indicated high level amount in mg /gm with TMV infected leaves treated with all kind of studied essential oils (Fig. 4. C).The best results were with OLExt. That raised up to 3.12 mg /gm compared to 2.78 mg /gm in the infected leaves only with TMV; while total protein content in healthy plants showed 3.29 mg /gm.The median distribution values of the amino acid content measured with mmol/kg Dry was analyzed from the treated *Datura* Leaves with Essential Oils (Fig. 5). Fig.5.A shows the median values illustrated in the box plot diagram which had lower value of 110 mmol/kg Dry in TMV infected leaves compared to 139 mmol/kg Dry in healthy ones. In addition to the drop in the upper limit of the higher amino acid contents occurred in the box plot from 350 mmol / Kg dry W in healthy to 300mmol/kg Dry in the infected with TMV. While the maximum upper limits for the amino acids were repaired by increasing the number of amino acids that had high content values in the upper box plots for the treated plants with antiviral oil components. That was obvious in the higher limit of Glutamic acid which resembled out of range values of 7.16 mmol/kg Dry W,

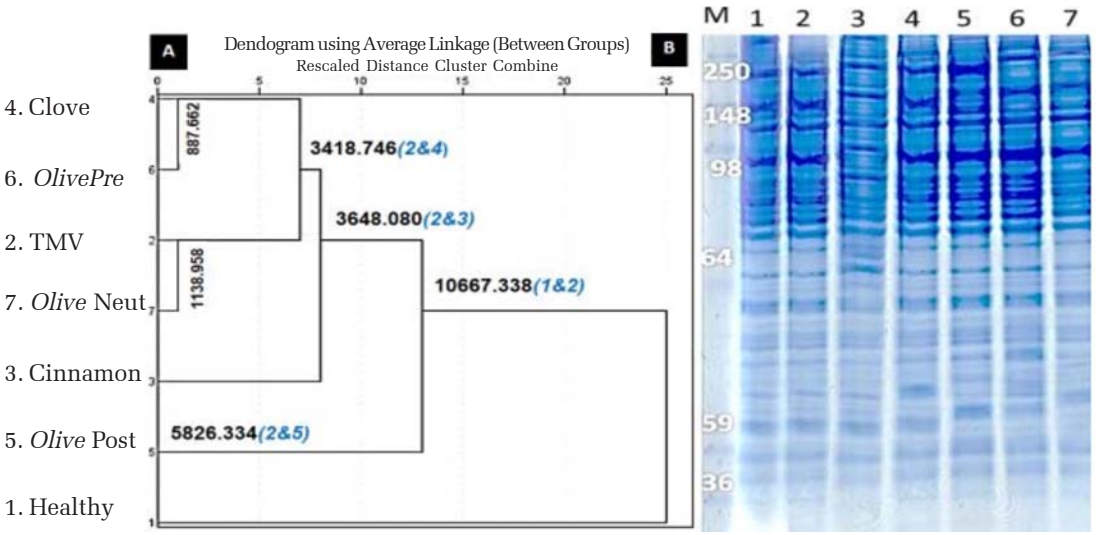

Fig. 6. SDS-PAGE Analysis A and B of the treated *Datura* leaves by different tested essential oils using dendrogram by average linkage, between groups, rescaled distance cluster combine

especially with OLExt treatment compared to 6.54 mmol /Kg dry in the infected ones as well as Alanine (Fig. 5.B&C). The only Methionine amino acid that had low value of 0.128 mmol/kg Dry in the healthy plants and was noticeably elevated in all treatments especially with OLExt. Up to 1.201 mmol/kg Dry. Asparagine, Lysine, Serine Arginine and Tyrosine indicated decrease values in the infected leaves with TMV similar to the other treatment compared to healthy plants. (Fig. 5.B&C).

SDS- PAGE

A comparative study was performed for having the results of protein contents from our studied treatments as well as separation of that protein to evaluate the effect of essential oil bio product on each protein profile compared to the control healthy and the infected ones with TMV inside each treatment according to the following

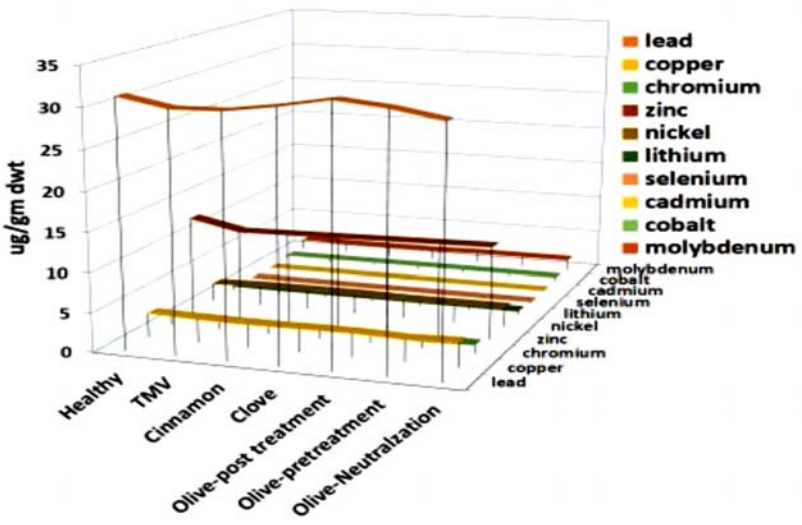

Fig. 7. The increased of some minerals due to the treatment with olive leaf extract

data: there were **26** separated resembled protein bands in the **7** studied treatments. They differed in their proportional protein percentage contents.22 protein bands occurred in the *Datura* healthy plants while 21bands in the TMV infected. At least 4 bands were extra in essential oil treatments (Fig. 6.A&B). In addition to different performance of the distributed bands with a new arrangement within high and medium M.W. with an increase in the percentage content. The new bands with 214.8KD and 211.8KD were noticed to be exist in the treated with OLExt. Which is missing in the infected withTMV. Small molecular weight bands were exhibited only in the treated with essential oils under 50 KD; all these separated small ones occurred between 43-47KD similar to healthy plants and were missing in the infected.

In Dendrogram (Fig. 6.A) distribution performance of protein separated data showed three main branches according to their similarities adjacent to the individual unique line of the healthy Datura leaves of group No.1. It was clear that healthy and viral TMV infected data had the most different separated protein bands with low similarity with coefficient was 10667.338. In OLExt; the closest treatment to healthy were No. 5 and 7 in addition to No 3 in the coefficient average link.in the percentage content.

Micro Elements

Data analysis of the indicated minerals (Fig. 7) and B revealed significant decrease in lead, copper and zinc by the following values of 30.08, 2.94and 11.28 ug/gm dwt. in the infected leaf content in ug/gm dwt.compared to 31.25, 3.16 and 12.58 ug/gm dwt. in the control healthy *Datura* respectively. After treatment by essential oils especially OLExt; these mineral showed increase in their contents which become 32.17, 3.11 and 11.885 ug/gm dwt of the treated leaves.

DISCUSSION

Natural products are a relevant source of antiviral drugs (Donia *et al*., 2003); (Abad *et al.*, 2000). The phenolic antioxidant content of olive oil has been under investigation for many years and recent studies have provided comprehensive identification and quantitation of these substances. (Owen *et al.*, 2003). But antioxidant property does not seem directly related to its antiviral effect (Ma *et al.*, 2001). The bitter principle of olives, oleuropein which intensely bitter glycoside give extra-virgin olive oil its bitter, pungent taste was isolated from green olive as well as the hydrolysis products. (Panizzi *et al*; 1960) showed that the oleuropein molecule contained glucose, β-3, 4-dihydroxyphenylethyl alcohol, and anacid.The compound, a bitter phenolic material, was considered to be an enzymatic degradation product of oleuropein (Fleming, 1969). It was proposed that oleuropein is hydrolyzed in vitro by β- glucosidase into glucose and a bitter tasting aglycone.

In our study we used commercial plant extract derived from olive tree leaf (*Olea europaea*) OLExt inhibited the in vivo infectivity of the TMV in *Datura metel* plants (Fig 3. M) Within 6-10 days. The used OLExt could effectively reduce the progression of TMV as well as therapeutic potential effect on the injured plant surface with NLLs (Fig. 3 L &M). It has many additives and hydrolyzed constituents as shown in Table 1. It was proved that the whole extract is a more convenient antiviral agent than pure Ole. Most of these derivatives have a similarity with the chemical structure of Caffeine (1, 3, 7-trimethylxanthine) which is a member of purine alkaloids and produced in over 80 plant species. It was proved that plants produced a low amount of caffeine (0.4–5 µg/g tissue) yet exhibited strong tolerance against herbivores and pathogens. Their self-defense system was autonomously activated without perceiving external stresses. This can be regarded as the priming of defense response, the feature resembles mammalian immunization or vaccination, and it was proposed that plants can also be immunized by expressing a mildly toxic 'antigenic' chemical such as caffeine *in planta*. The caffeine signal was predicted to be successively transduced through phosphodiesterase, cyclic AMP, calcium flux and salicylic acid (Hiroshi Sano, 2013). The higher antiviral activity of LExt rely on another possible additives or a synergistic antiviral effect among Ole and other minor components present in the OLExt. This probable additive or synergistic effect was in the LExt is under investigation (Micol *et al.*, 2005). Four antiviral mechanisms have been proposed by (Rahman, 2012) based on a series of *in vitro* and *in vivo* studies and effect on preventing both the release of virus from infected cells and the formation of viral aggregates after

release from host cells; and inhibiting viral replication at the initial stage of viral infection. Therefore; antiviral activity could differ than antibacterial and anti-fungal activities. Screening for various pharmaceuticals activities concerning plant virus infection; antiviral activities of bioactive material from *Streptomyces*'s included Cyclohexanol,4-ethyl(1methylethyl), (1.alpha, 3.alpha, 4. revealed strong potential activity against tobacco mosaic virus (TMV) in *Datura metel*. The isolates M3, M2, K1 and K2 showed very promising results in the form of the absence of all the local lesions (Ara *et al.*, 2012).

In our study on *Datura metel* infected with TMV the plants triggered with great physiological antiviral response after the treatment by OLExt. Concerning DNA content, genotoxic effects of viral infection,Chlorophyll Content, Protein Quantitation, Amino Acids,SDS- PAGE and Micro Elements.Considerable data and the best cured treatments from viral infection appeared (Fig. 4.A&C) of the infected leaves treated with OLExt. DNA contents elevated from 273.52 µg/gm in the TMV infected leaves up to 313.06 µg/gm in that treated ones. In addition the total chlorophyll content increased from 2.714 mg/gm up to 3.01 mg/gm.same trend raised also for total protein content up to 3.12 mg /gm compared to 2.78 mg /gm in the infected leaves. It was proved of the incidence of plant innate immunity relies on the recognition of pathogen effector molecules by nucleotide-binding-leucine-rich repeat (NB-LRR) immune receptor families. The N immune receptor, a member of TIR-NB-LRR family, indirectly recognizes the 50 kDa helicase (p50) domain of *Tobacco mosaic virus* (TMV) through its TIR domain. An N receptor-interacting protein, had been identified NRIP1, that directly interacts with both N's TIR domain and p50. NRIP1 is a functional rhodanese sulfurtransferase and is required for N to provide complete resistance to TMV. Interestingly, NRIP1 that normally localizes to the chloroplasts is recruited to the cytoplasm and nucleus by the p50 effector. As a consequence, NRIP1 interacts with N only in the presence of the p50 effector. The findings showed that a chloroplast protein is intimately involved in pathogen recognition. It was proposed that N's activation requires a precognition complex containing the p50 effector and NRIP1(Caplan *et al.*, 2008).

In respect to our results; it could be interpreted that application of OLExt.triger resistance protein and microbe-associated molecular pattern against TMVinfection in *Datura* leaves. It was proved that membrane-bound immune receptors, known to be pattern recognition receptors and exposed on the outside of the cell, recognize microbe-associated molecular patterns from pathogens. Intracellular immune receptors, also called plant disease resistance proteins, directly perceive pathogen-derived effectors or indirectly recognize the effector-mediated modification of host proteins inside the cells. The classes and functions of pattern recognition receptors were molecularly identified and summarized recent progresses in structural functions and molecular dynamics of the plant disease resistance proteins(Han and Jung, 2013).

It has been suggested by (Hirashima and Watanabe, 2003) that TMV viral proteins and two host proteins are involved in RNA virus replication. One protein (designated #3) belongs to a protein family of ATPase associated with various activities (AAA), while the second host protein (designated #13) is the 33K subunit of the oxygen-evolving complex of photosystem II. The two of them have affinity for the replicase proteins of TMV. The helicase domain region of the 126K protein not only plays a role in viral RNA replication, but is also involved in cell-to-cell transport of the virus in the infected plant (Hirashima and Watanabe, 2001)and (Seemanpillai *et al.*, 2006) both proteins affect the infection cycle of TMV and several other viruses. In this respect, the TMV replicase could be considered as a pathogenicity protein that inhibition of photosystemII enhances the levels of virus accumulation (Abbink *et al.*, 2002). According to the previous finding; it could be interpreted the loss of the total chlorophyll content with 2.714 mg/gm in the infected leaves compared to 3.37 mg/gm. In SDS-PAGE; the helicase domain region in our study could resemble the 122K in the SDS-PAGE infected treatment with 6.61% total protein which did not exist in the control as well as in the OLExt. Treatments (Fig. 6.C).

Comet assay was noticed to be useful and remarkable assay for evaluation of the effect of inducing damages on nucleus component which reflected highly percentage of tailed nucleus in Table 2. Infected, treated and normal cells showed

significant heterogeneity in damage. Similarly, DNA normal cells, and most of the cells had repaired the damage by 30 min following exposure to OLExt. throughout the cell cycle (Fig. 4.B.).The iridoids did not induce SOS repair of DNA, indicating a lack of genotoxic activity in *E. coli* PQ37. However, loganin, sweroside, and cornuside did reduce the amount of DNA damage caused by 4-nitroquinoline 1-oxide, suggesting potential antigenotoxic activity (Deng *et al.*,2013).

In our datarepairing effects elevated up to 83-86-88% in the DNA compared to the infected with TMV with more damaged cells up to 22% (Fig 4. B.2.). *Rubia cordifolia* leaves DMSO extracts leaves was very good antibacterial activity and minimum inhibitory concentrating of different virus. , has good minimum cytotoxic concentration activity and anti-oxidant(Prajapati and Parmar, 2011).Once a virus enters a cell, viral double-stranded RNA (dsRNA) is targeted by the RNA silencing machinery to initiate a cascade of regulatory events directed by viral small interfering RNAs (vsiRNAs). The contribution of vsiRNAs to antiviral defense and host genome modifications has profound implications for understanding of viral pathogenicity and host specificity in plants byrecent genetic and functional studies (Llave, 2010). An evidence proved by (Buchmann *et al.*, 2009) that viral chromatin methylation is an important host defense and allow us to propose that as a countermeasure, Gemini virus proteins reverse transcriptional gene silencingTGS by nonspecifically inhibiting cellular trans methylation reactions. They reported that viral proteins can inhibit TGS.

The mechanism of action of the antiviral activity of olive bio-phenols is suggested to include interference with viral amino acid production, prevention of virus shedding, inhibition of viral replication, neutralization of reverse transcriptase and protease in retroviruses, prevention of virus entry to cells, disruption of virus structure. Cell-to-cell transmission of HIV was inhibited in a dose-dependent manner with EC_{50}s of 0.2 µg/ml, and HIV replication was inhibited in an *in vitro* experiment(Lee-Huang *et al*; 2003). Ole blocks effectively HBsAg secretion in HepG2 2.2.15 cells in a dose-dependent manner (IC_{50} = 23.2 µg/ml). Ole (80 mg/kg, intraperitoneally, twice daily) also reduced viremia in DHBV-infected ducks (Guiqin Zhaoa *et al.*, 2009). Engineering herbicide resistance in plants by expression of a detoxifying enzyme. It consists of Phosphinothricin PPT, an analogue of L-glutamic acid, and two L-alanine residues. It is the only enzyme in plants that can detoxifyammonia released by nitrate reduction, amino acid degradation and cauliflower mosaic virus 35S promoter an activea phosphinothricin acetyltransferase PAT enzyme was produced in the plant cells (De Block *et al.*, 1987). Defense responses can be induced systemically in all parts of the plant by pathogens, although these N-terminal domains share no explicit sequence homology, both contain stretches rich in glutamic acid (ERC1, 10 Glu in 18 amino acids; EDS5, 9 Glu (Nawrath *et al.*, 2002)

In our study, some kind of amino acids and minerals interacts to induce significant protection markers due to OLExt application. Glutamic acid values with 7.16 mmol/kg Dry drastically increased especially with OLExt treatment compared to 6.54 mmol /Kg dry in the infected ones and also Alanine (Fig. 5.B&C). The only Methionine amino acid that had low value of 0.128 mmol/kg Dry in the healthy plants and was noticeably elevated in all treatments up to 1.201 mmol/kg Dry. Asparagine, Lysine, Serine Arginine and Tyrosine indicated decrease values in the infected leaves with TMV similar to the other treatment compared to healthy plants. (Fig. 5.B & C). In addition to minerals (Fig. 7) revealed significant decrease in lead, copper and zinc by the following values of 30.08, 2.94and 11.28 ug/gm dwt. in the infected leaf content in ug/gm dwt.and raised to 32.17, 3.11 and 11.885 ug/gm dwt in the treated leaves respectively. Oleuropein potently antioxidant activity and dose-dependently inhibits copper sulphate-induced oxidation of low-density lipoproteins (LDL)(Visioli F, 2002). oleuropein has both the ability to scavenge nitric oxide and to cause an increase in the inducible nitric oxide synthase (iNOS) expression in the cell. A scavenging effect of oleuropein was demonstrated with respect to hypochlorous acid (HOCl (De la Puerta R, 2001). Lead component called Acetohydroxyacid synthase (AHAS, EC: 2.2.1.6) is the key target enzyme of many kinds of herbicides, a-[N-nitro-N-(2, 4,6-triCl) phenyl] leucine ethyl ester urea (compound 3g), a novel N-nitrourea compound, was discovered a potential lead

compound for the AHAS target (Jingnan *et al*., 2012).

Compounds found in OLE have direct microbicidal activity against bacteria, mycobacteria and fungi (Garrido-Fernandez and Vaughn, 1978) and OLE also affects macrophage function and modulates the inflammatory response, two effects that may also contribute to activity against infectious agents. (Visioli *et al.*,1998) and; (De la Purerta *et al.*, 2000). OLE is known to contain a mixture of polyphenolic compounds, among them oleuropein and hydroxytyrosol, both of which are readily absorbed and bioavailable (De la Puerta *et al*; 2007) Iridoid glycosides have been associated with decreasedrisks of cancer, such as hepatocarcinoma (Rathee *et al.*,2013). Incubation of viral hemorrhagic septicemia virus VHSV with LExt or Ole before infection reduced the viral infectivity to 10 and 30%, respectively. Furthermore, LExt drastically decreased VHSV titers and viral protein accumulation (virucidal effect) in a dose dependent manner when added to cell monolayers 36 h post-infection. On the other hand, both the LExt and Ole were able to inhibit cell-to-cell membrane fusion induced by VHSV in uninfected cells, suggesting interactions with viral envelope. Therefore, we propose that *O. europaea* could be used as a potential source of promising natural antivirals (Micol *et al*., 2005).

In addition, Ole could be used to design other related antiviral agents. Our target of OLExt application on the plant leaves is for virus protection as well as inducing and trigger resistance against virus infection. The concepts and direction of induced systemic resistance in plants were classified the bio compounds into two categories; first: Putative defense compound/systems for disease resistance in plants; second: Agents reported to elicit systemic resistance (Ku, 2001).

Oleuropein is generally the most prominent phenolic compound in olive cultivars and can reach concentrations of 60–90 mg g”1of dry matter in the leaves(Le Tutour B, 1992). In the fruits, phenyl acids, flavonoids and secoiridoids have been reported, with phenolic compounds representing1–3% (w/v) of the olive(Brenes *et al.*,1993). In the leaves, oleuropein makes up 19% (w/w) and flavonoids make up 1.8% (w/w), of which 0.8% is luteolin 7-glucoside(Le Tutour B, 1992).The fruit of *Olea europaea* appears to accumulate only glucosylated derivatives of oleuropein. In contrast, dihydroxytyrosol and non-glucosylated secoiridoids derived from oleuropein have been found in the leaves (Amiot MJ, 1989)

Finally, there is an urgent need to fully appreciate the complexity of OLExt chemistry in olive products—especially the iridoids —where such complexity may impact on the interpretation of pharmacokinetic studies and could be applied as a green bio pesticide for immunizing the growing up plants against virus infection as well as other pathogens .

ACKNOWLEDGMENTS

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project no RGP- VPP-231.

REFERENCES

1. Abad MJ, Bermejo P, Gonzales E, Iglesias I, Irurzun A, Carrasco L. Antiviral activity of Bolivian plant extracts. *Gen. Pharmacol*. 1999a; **32**: 499-503.
2. Abad, MJ, Bermejo, P, Sanchez Palomino, S, Chiriboga, X., Carrasco, L. Antiviral activity of some South American medicinal plants. *Phytother. Res*.1999b; **13**: 142-146.
3. Abad, M. J., Guerra, J., A.Bermejo, P.Irurzun, A., Carrasco, L. Search for antiviral activity in higher plant extracts. [Research Support, Non-U.S. Gov't].*Phytother Res.* 2000; **14**(8): 604-607.
4. Abbink, T. E., Peart, J. R., Mos, T. N., Baulcombe, D. C., Bol, J. F., & Linthorst, H. J. Silencing of a gene encoding a protein component of the oxygen-evolving complex of photosystem II enhances virus replication in plants. *Virology.* 2002; **295**(2), 307-319.
5. Abdallah, N. A., Shah, D., Abbas, D., & Madkour, M. Stable integration and expression of a plant defensin in tomato confers resistance to fusarium wilt. *GM crops.* 2010; **1**(5): 344-350.
6. Abdul Ghani, Zulfaqar Ali, Muhammad Ishtiaq, and, M. M., & SairaParveen. Estimation of macro and micro nutrients in some important medicinal plants of Soon Valley, District Khushab. pp. *African Journal of Biotechnology*. 2012; **11**(78):14386-14391.

7. Abramson, J., Smirnova, I., Kasho, V., Verner, G., Iwata, S., & Kaback, H. R. The lactose permease of Escherichia coli: overall structure, the sugar-binding site and the alternating access model for transport. *FEBS letters*. 2003; **555**(1), 96-101.
8. Amiot MJ, F. A., Macheix JJ.. Phytochemistry. Accumulation of oleuropein derivatives during olive maturation. 1989; **28:** 67–70.
9. Andrikopoulos NK, K. A., Assimopoulou AN, Papageorgiou VP. Inhibitory activity of minor polyphenolic and nonpolyphenolic constituents of olive oil against in vitro low-density lipoprotein oxidation. *J Med Food.* 2002; **5:** 1–7.
10. Ara, I., Bukhari, N. A., Aref, N., Shinwari, M. M., & Bakir, M. Antiviral activities of streptomycetes against tobacco mosaic virus(TMV) in Datura plant: Evaluation of different organic compounds in their metabolites. *African Journal of Biotechnology.* 2012*;* **11**(8): 2130-2138.
11. Arnon, D. I.. Copper Enzymes in Isolated Chloroplasts. Polyphenoloxidase in Beta Vulgaris. *Plant Physiol.*1949; **24**(1): 1-15.
12. Benavente-Garc1a, O., Castillo, J., Lorente, J., Ortuño, A., & Del Rio, J. A. (2000). Antioxidant activity of phenolics extracted from Olea europaea L. leaves. *Food Chemistry*, **68**(4), 457-462.
13. Brenes M, G. P., Duran MC, Garrido A. Concentration of phenolic compounds change in storage brines of ripe olives. *J Food Sci.*1993; **58**: 347–350.
14. Buchmann, R. C., Asad, S., Wolf, J. N., Mohannath, G., & Bisaro, D. M. Geminivirus AL2 and L2 proteins suppress transcriptional gene silencing and cause genome-wide reductions in cytosine methylation. *J Virol.* 2009: **83**(10): 5005-5013.
15. Buckton, K. E., Langlands, A., Smith, P., & McLelland, J. Chromosome Aberrations Following Partial-and Whole-body X Irradiation in Man. Dose Response Relationships. 1968; Western General Hospital, Edinburgh.
16. Caplan, J. L., Mamillapalli, P., Burch-Smith, T. M., Czymmek, K., & Dinesh-Kumar, S. Chloroplastic protein NRIP1 mediates innate immune receptor recognition of a viral effector. *Cell.* 2008; **132**(3): 449-462.
17. Cemeli, E., Baumgartner, A., Anderson, D. Antioxidants and the Comet assay. *Mutation Res /Rev Mut Res.* 2009; **681**(1): 51-67.
18. De Block, M., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C., Gossele, V. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. *The EMBO journal.*1987; **6**(9): 2513.
19. De la Puerta, C., Carrascosa-Salmoral, M. P., García-Luna, P. P., Lardone, P. J., Herrera, J. L., Fernández-Montesinos, R.. Melatonin is a phytochemical in olive oil. *Food Chem.* 2007; **104**(2):609-612.
20. De la Puerta, E., Martinez-Dominguez, V., Ruiz-Gutierrez. Effect of minor components of virgin olive oil on topical anti-inflammatory assays. *Z. Naturforsch.* 2000; **55**: 814-819.
21. De la Puerta R, D. M., Ruiz-Guttierrez V, Flavill JA, Hoult JRS. Effects of olive oil phenolics on scavenging of reactive nitrogen species and upon nitrergic neurotransmission. *Life Sci.* 2001; **69**: 1213–1222.
22. Deng, S., West, B. J., & Jensen, C. J. UPLC-TOF-MS Characterization and Identification of Bioactive Iridoids in Cornus mas Fruit. *J Analyt Methods in Chem.* 2013; 2013.
23. Deshmukh, V. P., Thakare, P. V., Chaudhari, U. S., & Gawande, P. A. A simple method for isolation of genomic DNA from fresh and dry leaves of Terminalia arjuna (Roxb.) Wight and Arnot. *Elec J Biotechnol.* 2007; **10**(3): 468-472.
24. Donia, M., Hamann, M.T., Marine. Natural products and their potential applications as anti-infective agents. *Lancet Infect. Dis.* 2003; **3**: 338–348.
25. Einarsson, S., Josefsson, B., & Lagerkvist, S. Determination of amino acids with 9-fluorenylmethyl chloroformate and reversed-phase high-performance liquid chromatography. *J Chromatograp A.*1983; **282**:609-618.
26. Fleming, H. P., W. M. Walter, Jr., and J. L. Etchells. Isolation of a bacterial inhibitor from green olives. *Appl. Microbiol.*1969; 18: 856-860.
27. Fredrickson, W. R. Method and composition for antiviral therapy with olive leaves. *U.S. patent.* 2000; **6**: 117, 844.
28. Garrido-Fernandez, A., & Vaughn, R. H. Utilization of oleuropein by microorganisms associated with olive fermentations. *Can J Microbiol.*1978; **24**(6): 680-684.
29. Ghisalberti, E. L. Biological and pharmacological activity of naturally occurring iridoids and secoiridoids. *Phytomedicine.*1998;**5**(2): 147-163.
30. Guiqin Zhaoa, b., Zhifeng Yinb, Junxing Donga. antiviral efficacy against hepatitis B virus replication of oleuropein isolated from Jasminum officinale L. var. grandiflorum. *Journal of Ethnopharmacology.*2009; **125**(2):265–268.
31. Han, S.-W., & Jung, H. W. Molecular sensors for plant immunity; pattern recognition receptors and race-specific resistance proteins. *J Plant Biol.* 2013; **56**(6): 357-366.

32. Hess, H. H., Lees, M. B., & and Derr, J. E. A linear Lowry-Folin assay for both watersoluble and sodium dodecyl sulfate-solubilized proteins. *Analyt.Biochem.*1978; **85**: 295-300.
33. Hirashima, K., & Watanabe, Y. Tobamovirus replicase coding region is involved in cell-to-cell movement. *J Virology.* 2001; **75**(18): 8831-8836.
34. Hirashima, K., & Watanabe, Y. RNA helicase domain of tobamovirus replicase executes cell-to-cell movement possibly through collaboration with its nonconserved region. *J Virology*.2003; **77**(22):12357-12362.
35. Hiroshi Sano , Y.-S. K., Yong-Eui Choi. Chapter Ten – Like Cures Like: Caffeine Immunizes Plants Against Biotic Stresses. *Advances in Botanical Research.* 2013; **68**: 273–300.
36. Hirschman, S. Z. Inactivation of DNA polymerases of murine leukaemia viruses by calcium elenolate. *Nat New Biol.*1972; **238**(87): 277-279.
37. Jingnan Jin a, H. M., Xiufang Cao a, Shengzhen Xu a, Jianhong Li b, Qingye Zhang a,Changshui Chen a., The discovery of the novel lead compound of N-nitroureas target on acetohydroxyacid synthase. *Pesticide Biochemistry and Physiology.*2012; **104** :218–223.
38. Juchimiuk, J., Gnys, A., & Maluszynska, J. DNA damage induced by mutagens in plant and human cell nuclei in acellular comet assay. *Folia Histochemica et Cytobiologica.*2006; **44**(2): 127-126.
39. Kalvatchev, Z., Walder, R., & Garzaro, D. Anti-HIV activity of extracts from Calendula officinalis flowers. *Biomedicine & pharmacotherapy.* 1997; **51**(4): 176-180.
40. Khan, Y., Panchal, S., Vyas, N., Butani, A., & Kumar, V. Olea europaea: A phyto-pharmacological review. *Pharmacogn Rev.* 2007; **1**(1): 114-118.
41. Ku, J. Concepts and direction of induced systemic resistance in plants and its application. *European Journal of Plant Pathology.* 2001; **107**(1): 7-12.
42. L., D. J. Isolation of plant DNA from fresh tissue. *Focus.*1990; **12**: 13-15.
43. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.*1970; **227**(5259): 680-685.
44. Larson, E., Howlett, B., Jagendorf, A. Artificial reductant enhancement of the Lowry method for protein determination. [Research Support, U.S. Gov't, P.H.S.]. *Anal Biochem.*1986;**155**(2): 243-248.
45. Le Tutour B, G. D. Antioxidant activities of Olea europaea leaves and related phenolic compounds. *Phytochemistry.*1992; **31**: 1173–1178.
46. Lee-Huang S, Z. L., Chang YY, Huang PL. Anti-HIV activity of olive leaf extract (OLE) and modulation of host cell gene expression by HIV-1 infection and OLE treatment. *Biochem Biophys Res Commun.*2003; **307**:1029–1037.
47. Llave, C. Virus-derived small interfering RNAs at the core of plant–virus interactions. *Trends in plant science.* 2010; **15**(12):701-707.
48. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. Protein measurement with the Folin phenol reagent. *J Biol Chem.* 1951; **193**(1): 265-275.
49. Ma, S. C., He, Z.D., Deng, X.L., But, P.P., Ooi, V.E., Xu, H.X., Lee, S.H.,Lee, S.F. In vitro evaluation of secoiridoid glucosides from the fruits of Ligustrum lucidum as antiviral agents. *Chem. Pharm. Bull.(Tokyo).* 2001; **49**: 1471–1473.
50. Markwell, M. A., Haas, S. M., Tolbert, N. E., Bieber, L. L. Protein determination in membrane and lipoprotein samples: manual and automated procedures. *Methods Enzymol.* 1981; **72**: 296-303.
51. Micol, V., Caturla, N., Pérez-Fons, L., Más, V., Pérez, L., & Estepa, A. The olive leaf extract exhibits antiviral activity against viral haemorrhagic septicaemia rhabdovirus (VHSV). *Antiviral Research.* 2005; **66**(2): 129-136.
52. Nawrath, C., Heck, S., Parinthawong, N., Métraux, J.-P. EDS5, an essential component of salicylic acid–dependent signaling for disease resistance in Arabidopsis, is a member of the MATE transporter family. *The Plant Cell Online.*2002; **14**(1): 275-286.
53. Owen, R., Haubner, R., Mier, W., Giacosa, A., Hull, W., Spiegelhalder, B. Isolation, structure elucidation and antioxidant potential of the major phenolic and flavonoid compounds in brined olive drupes. *Food and Chemical Toxicology.* 2003; **41**(5): 703-717.
54. Pandey, A., Milind, M., & Naik, D. S. Organic metabolites produced by Vibrio parahaemolyticus strain An3 isolated from Goan mullet inhibit bacterial fish pathogens. *Goa, India. AJB.* 2010; **9**(42): 7134-7140.
55. Panizzi, L. M., J. M. Scarpati, and E. G. Oriente. Nota H. Gazz.l. Constituzione della oleuropeina, glucoside, glucoside amaro e ad azione ipotensiva dell 'olivo. *Chim. Ita.* 1960; **90**: 1449-1485.
56. Peacock, J. W., Walker, J. C., Dennis, E., & Howard, E. A. Plant anaerobic regulatory element: 1991;Google Patents.
57. Peterson, G. L. Determination of total protein. [Research Support, U.S. Gov't, P.H.S.].

*Methods Enzymol.*1983; **91**: 95-119.
58. Prajapati, S. N., & Parmar, K. A. Anti-viral and in-vitro free radical scavenging activity of leaves of rubia cordifolia. *International Journal of Phytomedicine.* 2011; **3**(1): 98-107.
59. Rahman, A.-u. *Studies in natural products chemistry* . 2012; 38:Access Online via Elsevier.
60. Rathee, D., Thanki, M., Bhuva, S., Anandjiwala, S., & Agrawal, R. Iridoid glycosides-Kutkin, Picroside I, and Kutkoside from Picrorrhiza kurroa Benth inhibits the invasion and migration of MCF-7 breast cancer cells through the down regulation of matrix metalloproteinases: 1st Cancer Update. *Arabian Journal of Chemistry.* 2013; **6**(1): 49-58.
61. Renis, H. E. In vitro antiviral activity of calcium elenolate. *Antimicrob Agents Chemother (Bethesda).* 1969; **9**:167-172.
62. Saija, A., & Uccella, N. Olive biophenols: functional effects on human wellbeing. *Trends in Food Science & Technology.*2000; **11**(9): 357-363.
63. Seemanpillai, M., Elamawi, R., Ritzenthaler, C., & Heinlein, M. Challenging the role of microtubules in Tobacco mosaic virus movement by drug treatments is disputable. *Journal of virology.*2006; **80**(13): 6712-6715.
64. Shakir, F. K., Audilet, D., Drake, A. J., and Shakir, K. M. A rapid protein determination by modification of the Lowry procedure. *Analyt.Biochem*, 1994; ***216***, 232-233.
65. Simón-Mateo, C. G., Juan Antonio. Antiviral strategies in plants based on RNA silencing. [Research Support, Non-U.S. Gov't Review]. *Biochimica et Biophysica Acta (BBA)-Gene Regul Mechns.*2011; **1809**(11): 722-731.
66. Sondhi, N., Bhardwaj, R., Kaur, S., Chandel, M., Kumar, N., & Singh, B. Inhibition of H_2O_2-induced DNA damage in single cell gel electrophoresis assay (comet assay) by castasterone isolated from leaves of *Centella asiatica*. *Health.*2010; **2**(6): 595-602.
67. Unwin, D., A versatile high frequency microcautery. *Physiol Entomol.*1978; **3**:71-73.
68. Van Loon, L., Rep, M., & Pieterse, C. Significance of inducible defense-related proteins in infected plants. *Annu. Rev. Phytopathol.*2006; **44**: 135-162.
69. van Wezel, R., Liu, H., Wu, Z., Stanley, J., & Hong, Y. Contribution of the zinc finger to zinc and DNA binding by a suppressor of posttranscriptional gene silencing. *J Virol.* 2003; **77**(1): 696-700.
70. Visioli, F., Bellomo, G., & Galli, C. Free radical-scavenging properties of olive oil polyphenols. [Research Support, Non-U.S. Gov't]. *Biochem Biophys Res Commun.*1998; **247**(1): 60-64.
71. Visioli F, G. C., Galli G, Caruso D. Biological activities and metabolic fate of olive oil phenols. *Eur J Lipid Sci Technol.* 2002; **104**: 677–684.
72. Walter, W., Fleming, H., & Etchells, J. Preparation of antimicrobial compounds by hydrolysis of oleuropein from green olives. *Appl Microbiol.* 1973; **26**(5): 773-776.
73. Waterborg, J. H. a., & Matthews, H. R. The Burton assay for DNA, in Methods in Molecular Biology. *Nucleic Acids (Walker, J. M., ed.), Humana Press,Totowa.*1984; 2: NJ pp. 1-3.
74. Yamasaki, K., Nakano, M., Kawahata, T., Mori, H., Otake, T., Ueba, N. Anti-HIV-1 activity of herbs in Labiatae. *Biol Pharm Bull.* 1998; **21**(8): 829-833.
75. Yeang, H. Y., Yusof, F., and Abdullah, L. Protein purification for the Lowry assay: acid precipitation of proteins in the presence of sodium dodecyl sulfate and other biological detergents. *Analyt.Biochem.* 1998; **265**: 381-384.