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Isolation, Identification, Thermal Analysis, DFT Calculations and Antioxidant Activity Studies of Lichen Metabolites Norstictic Acid and Evernic Acid

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Abstract

This manuscript deals with successful trials to isolate two lichen acids (Noristicic and Everinic acids) from natural sources. The isolated compounds were purified, crystallized from suitable solvent, and their melting points were measured. Their structures were identified by elemental analyses, spectroscopic tools (Electronic spectra, FT-IR), Mass and finally using thermal analyses (TG, DTG and DTA). Their thermal fragmentation pathways were compared with mass fragmentation. These fragmentation pathways were compared with the theoretical molecular orbital calculations (DFT- MOCs). The molecular structures of the title compounds in the ground state were optimized by a DFT method using B3LYP functional combined with 6-311G** (d) basis set. Calculations were carried out using GAUSSIAN 09 suite of programs. In addition, antioxidant activity of norstictic acid and everinic acid were determined. The results showed that both compounds exhibited antioxidant activity, but that the norstictic acid showed several times higher activity than evernic acid. This study refers to successful obtaining of these biologically active acids from natural sources and of wide biomedical applications.

Key words: Isolation, Norstictic acid, Evernic acid, Spectroscopic studies, Thermal Analyses, DFT-MOCs, Biological Activities.

1. Introduction:

Lichens are biosynthesize unique secondary phenolic compounds that possess different types of biological activities Ranković, 2015 [1]. They often contain compounds from the group of phenols, depsides, depsidones, dibenzofurans, xanthones and anthraquinones. Extract of some lichens and their secondary metabolites have many biological activities, primarily including antioxidant, antimicrobial, anti-inflammatory, anticancer, antigenotoxic, antipyretic and analgesic activities Ranković, 2015 [1].

Depsides and depsidones are common and very important secondary lichen metabolites Calcott et al. 2018 [2]. Many of these compounds are unique to lichens only and are not found in higher plants. Unlike many higher plants, chemistry of lichen has not been widely investigated. The reason why photochemistry and biological activity of lichens are not much examined by scientists is because they grow in inaccessible areas, are difficult to remove from the rootstock on which they grow, and often can't be found in large quantities. For this reason, lichen metabolites are of interest for testing. Evernic acid is depside which has been isolated from various lichens including lichen Evernia prunastri also known as oakmoss Kosanic et al. 2013 [3]. This lichen is commercially harvested are often used as perfume fixatives and form the base notes of many fragrances. Previous research has shown that evernic acid exhibits various biological activities including antimicrobial, antioxidant and antitumor activities Burlando et al. 2009 [4], Kosanic et al. 2013 [3], Emre et al. 2015 [5] and clastogenic effect on human lymphocytes Stojanović et al. 2014 [6]. Norstictic acid belongs to the depsidones and could be found in some lichens like Toninia candida where presents the major phenolic compound Ranković, 2015 [1]. Until now, different kind of biological activities of this compound are known such as antioxidant and

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antimicrobial activities and also anticancer activity Rankovic et al. 2012 [7].

Thermal analytical methods have thus become important tools for the development of modern medicines [8-12]. These are precise and accurate techniques with low sample requirements, and can provide detailed information about new chemical entities even at the very earliest stages of discovery and development of the new compositions and drugs [13-16]. Thermogravimetric TG/DTG analysis used to provide quantitative information on weight losses due to decomposition and /or evaporation of low molecular materials as a function of time and temperature. In conjunction with mass spectrometric analysis [17-19], the nature of the released volatilize may be deduced, thus greatly facilitating the interpretation of thermal degradation processes. On the other hand, computational quantum chemistry can provide additional information about the atoms and bonds, which can be used successfully in an interpretation of experimental results [20]. Application of computational quantum chemistry in addition to experimental results (MS and TA) gives valuable information about the atoms and bonds which helps in the description and prediction of primary fragmentation site of cleavage and subsequent one [21,22].

The aim of the present study was to identify the secondary metabolites of lichens Parmelia conspersa and Evernia prunastri from Serbia using HPLC-UV analysis, isolate and purity of their major metabolites and finally do their thermal analyses (TG, DTG and DTA). Their thermal fragmentation pathways were compared with mass fragmentation. These fragmentation pathways were compared with the theoretical molecular orbital calculations (DFT-MOCs).

Experimental

2.1. Material and compounds

2.1a. Lichen materials

Samples of the lichens were collected in various places during the spring 2018. Parmelia conspersa was collected from Mt. Golija and Evernia prunastri was collected from Mt. Suva planina in Serbia. The voucher specimens (Parmelia conspersa FSDB-221 and Evernia prunastri FSDB-222) were determined by prof. dr Perica Vasiljevic and the species are stored in the Mycological Herbarium of the Department of Biology, Faculty of Science, University of Nis.

2.1b. Preparation of the lichen extracts

The lichen materials were dried ten days at room temperature (26 °C), after which it was ground to a uniform powder. Then, 100 g dry powdered lichen material was soaked in 1000 mL of an appropriate solvent (acetone) at room temperature for three days with stirring. After that extracts were filtered through a Whatman no. 42 (125 mm) filter paper and concentrated in a rotary evaporator. In the same way, both extracts were prepared.

2.2. Procedures and Measurements

2.2.1. High performance liquid chromatography (HPLC) analysis

The extract samples were dissolved in 1 ml of methanol and analyzed on an Agilent 1200 System HPLC (Agilent Technologies) instrument with C18 column (C18; 25 cm × 4.6 mm, 10 m). UV spectrophotometric detector with methanol–water–phosphoric acid (80:20:0.9, v/v/v) solvent was used. The sample injection volume was 10 µl with a flow rate of 1.0 mL/min. Methanol was of HPLC grade and was purchased from Merck (Darmstadt, Germany). Phosphoric acid was analytical-grade reagent. Deionized water used throughout the experiments was generated by a Milli-Q academic water purification system (Milford, MA, USA). The standards used were obtained from the following sources: stictic acid (tR = 2.75 ± 0.10) from lichen Xanthoparmelia conspersa, norstictic acid (tR = 2.97 ± 0.10) was isolated from lichen Ramalina furinacea, evernic acid (tR = 3.12 ± 0.10) and usnic acid (tR = 8.22 ± 0.20) was isolated from lichen Evernia prunastri, atranorin (tR = 9.16 ± 0.10) and chloroatranorin (tR = 11.07 ± 0.20) from lichen Parmelia sulcata.

2.2.1 a. Isolation of norstictic acid from Parmelia conspersa

The dried acetone extract of the lichen Parmelia conspersa (600 mg) was fractioned on a silica gel column (0.149-0.074 mm; 100-200 mesh). The column was eluted with methanol-chloroform gradient solvent (10:1, 5:1 and 1:1) yielding twelve fractions. After TLC analysis and merging the fractions with the same components, norstictic acid (80 mg), was isolated, further purified by crystallization and used for structure identification and further studies. The structure of norstictic acid (1,3-Dihydro-1,4,10-trihydroxy-5,8-dimethyl-3,7-dioxo-7H-isobenzofuro(4,5-b)(1,4) benzodioxepin-11-carboxaldehyde) was confirmed by spectroscopic

data Huneck and Yoshimura, 1996 [23]. The purity of the isolated compound was determined by HPLC-DAD and amounted to 97.2% for norstictic acid.

2.2.1b. Isolation of evernic acid from Evernia prunastri

The dried acetone extract of the lichen P. perlata (500 mg) was dissolved in acetone and separated on a chromatographic column (silica gel, 0.149–0.074 mm; 100–200 mesh) with methanol-chloroform (10:1, 5:1 and 1:1 v/v), as solvent systems. Ten fractions were obtained. After TLC analysis, norstictic acid (95 mg), was isolated, further purified by crystallization and used for structure identification and further studies. The structure of norstictic acid (2-hydroxy-4-[(2-hydroxy-4-methoxy-6-methylbenzoyl)oxy]-6-methylbenzoic acid) was confirmed by spectroscopic data Huneck and Yoshimura, 1996 [23] and others [24-25]. The purity of evernic acid was determined by HPLC-DAD and amounted to 97.9%.

2.2.2. Analyses Measurements and Instruments:

The thermo spectronic Nicolet evolution300BB UV spectrophotometer was used for absorbance values measurements in the wavelength range 190 – 600 nm. Weights measurement was performed by using sensitive analytical balance of 0.0001g max 120 g d = 0.1mg, Shimadzu model. Stirring and heating were performed by using magnetic stirrer made in Europe, hot plate (VELP-made in UK). The pH values were measured using pH meter, ORION Triode model 420A with Ag/AgCl internal reference system of thermistor for ATC, model 915BN. The instrument is calibrated using standard buffer solution, 4.00, 7.00 and 10.00 before direct measurement. Micro-Accupipette, USA (100 – 1000) µL was used to measure the very small volumes, whereas glass pipettes (2, 5 mL) were used to measure the large volumes. Elemental micro-Analyses of the solid acids for C, H, N and Cl were performed at the Microanalytical Center, Cairo University, using CHNS-932 (LECO) Vario Elemental Analyzers. FT-IR spectra were recorded on a Perkin-Elmer FT-IR type 1650 spectrophotometer in wave number region 4000 - 400 cm-1. The spectra were recorded as KBr discs. The 1H-NMR and 13C-NMR spectra were recorded by 300 MHz Varian-Oxford Mercury. The used duterated solvent was dimethylsulphoxide (DMSO-d6) and the spectra extended from 0 to 15 ppm. The thermal analyses (TG, DTG and DTA) were carried out in dynamic nitrogen atmosphere (20 mL. min-1) with a heating rate of 10 °C min-1 using TG-60 H Shimadzu simultaneous DTA-TG instrument.

2.3.3. Theoretical calculations

The molecular structure of the title compounds in the ground state was optimized by a DFT method using B3LYP functional [26, 27] combined with 6-311G** (d) basis set. Calculations were carried out using GAUSSIAN 09 [27] suite of programs. The vibrational frequencies and the corresponding normal modes were evaluated at the optimized geometry [27, 28]. Vibrational modes were analyzed using GAUSSVIEW software [3]. Natural bond orbitals (NBO) analysis and frontier molecular orbitals were performed using NBO 3.1 program [29] at the same level of theory.

2.2.4. Biological Activity

2.2.4a. Determination of DPPH free radical scavenging activity

Determination of the ability to neutralize DPPH• (1,1-diphenyl-2-picrylhydrazyl) radical was analyzed using a spectrophotometric method Kumarasam 2007[30], by preparing a methanolic solution of DPPH radical concentrations of 40 µg/mL in a dark room. The sample solutions and DPPH˙ were then mixed with 3 mL of the DPPH radical solution and 2 mL of the sample solution and such a mixture was left for 30 minutes at room temperature in the dark, after which the absorbance was measured at 517 nm. Ascorbic acid and BHT were used as reference standards. The free radical neutralization capacity was calculated according to the following formula (1):

Inhibition capacity of the DPPH radical (%) = (Ac -As)/Ac × 100,…… (1)

Where the Ac is the absorbance of the control solution (negative control) and AS is the absorbance of the sample solution or standard.

The IC50 value (µg/mL), defined as the concentration of extract needed to reduce the DPPH concentration of the radical by 50%, and was obtained from the linear regression equation.

2.2.4b. Superoxide Anion Radical Scavenging Activity

The superoxide anion radical scavenging activity of samples was detected according to the method of Nishimiki et a l [24]. Briefly, 0.1 mL of test sample (1000, 500, 250, 125 and 62.5 µg/mL) was mixed

with 1 mL nitro blue Tetrazlouim (NBT) solution (156 µM in 0.1 M phosphate buffer, pH 7.4) and 1 mL nicotinamide adenine dinucleotide (NADH) solution (468 µM in 0.1 M phosphate buffer, pH 7.4). The reaction was started by adding 100 µL of phenazine methosulphate (PMS) solution (60 µM in 0.1 M phosphate buffer, pH 7.4). The mixture was incubated at room temperature for 5 min,and the absorbance was measured at 560 nm in spectrophotometer ("Jenway" UK) against blank samples. Decreased absorbance indicated increased superoxide anion radical scavenging activity. Ascorbic acid was used as positive control. The percentage inhibition of superoxide anion generation was calculated using the following formula (2):

Superoxide anion scavenging activity (%) = [(A0 − A1)/A0] × 100 …… (2)

Where A0 is the absorbance of the negative control and A1 is the absorbance of reaction mixture or standards.

The inhibition concentration at 50% inhibition (IC50) was the parameter used to compare the radical scavenging activity.

3. RESULTS AND DISCUSSION

3.1. HPLC isolation and Identification:

Lichens *Parmelia conspersa* and *Evernia prunastri* were used as a natural source of secondary metabolites used in this paper. Phytochemical analysis of the lichens was done by HPLC-UV chromatography. The HPLC chromatogram for standards and the acetone extracts of the tested lichens are represented in Figure 1-3. In the investigated extracts, depsidones, depsides and dibenzofuran were present as the most abundant substances classes. In addition to the major secondary metabolite norstictic acid (NOR, t_R = 2.97 ± 0.10), usnic acid (USN, t_R = 8.22 ± 0.20), stictic acid (STI, t_R = 2.75 ± 0.10) and atranorin (ATR, t_R = 9.16± 0.10) were also identified in acetone extract of *P. conspersa*. The acetone extract of *E. prunastri* showed that the most intense peak belongs to the evernic acid (t_R = 3.12 ± 0.10), in addition to the peaks of usnic acid (t_R = 8.22 ± 0.20), atranorin (ATR, t_R = 9.16 ± 0.10) and chloroatranorin (CHL, t_R = 11.07 ± 0.20). Identification of these compounds was achieved by comparison of their t_R values and

2.2.4c.Reduction capacity

Reducing capacity or reducing power was first described by Oyaizu. One milliliter of samples was mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide (1%). Then 2.5 mL of trichloroacetic acid was added to the mixture and spinning the mixture at 3000 rpm for 10 minutes. Take 2.5 mL of the upper layer (supernatant), add 2.5 mL of distilled water and 0.5 mL of iron three chlorides. The absorbance of the solution was measured at 700 nm on the spectrophotometer compared to the blank test. Ascorbic acid was used as a positive control. Increasing the absorption of the solution shows how much the reducing power is increased.

2.2.4d. Statistical analysis

All measurements are repeated three times, and the results are displayed as the mean ± standard error (mean ± SD). Statistical analyses were performed using Microsoft Excel and SPSS software (version 20) package. One way ANOVA was used to determine differences between mean measurement values, with a statistical significance of $p < 0.05$ UV spectra with the standard substances previously isolated from lichens (Table 1). Although norstictic acid (bryopogonic acid) and evernic acid have a completely different structure and belong to different classes of compounds, the two compounds have similar values of retention times. The UV absorbance spectral data (200–400 nm) also corresponded with those of standards and found in Yoshimura et al [31]. The UV spectra of depsidones have 3 absorption maxima and are dissimilar from those of depsides and monocyclic compounds. So, depsidone norstictic acid has absorbance maxima at 212, 239, 310 nm. On the other hand, evernic acid belonging to depside has a completly different spectrum appearance with absorbance maxima at 213, 270, 304 nm. Two major secondary metabolites of the lichens belonging to different classes of compounds (one depside and other depsidone) were isolated and used in further studies. Norstictic acid was identified for the first time in lichen *P. conspersa*.

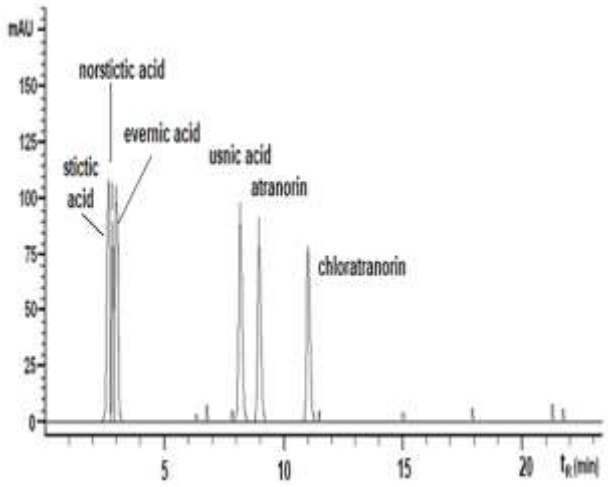


Fig. 1 HPLC chromatogram of mixed standards used for identification of the lichen compounds**. (**Stictic acid; Norstictic acid; Salazinic acid; Usnic acid; Atranorin; Chloroaranorin**)**

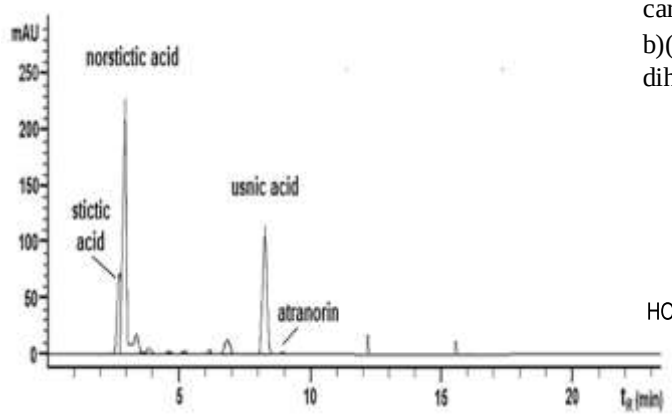


Fig. 2 HPLC chromatogram of the acetone extracts of *P. conspersa* at 254 nm.

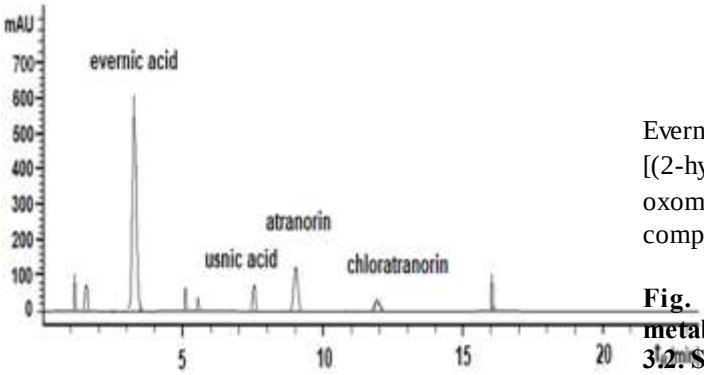
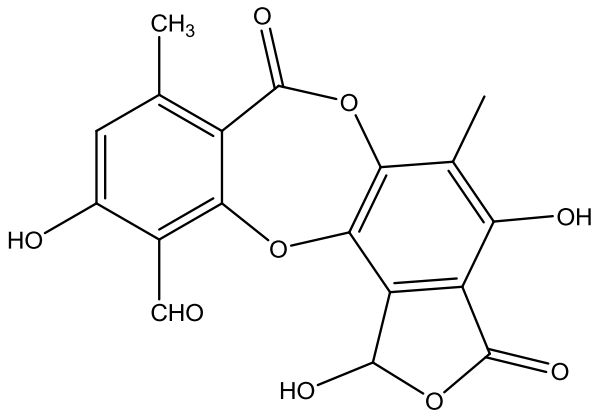
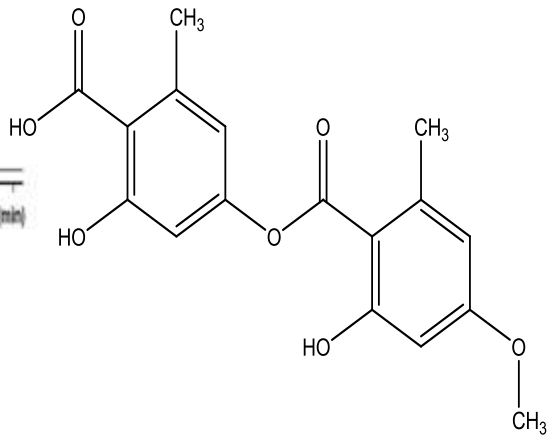


Fig. 3 HPLC chromatogram of the acetone extracts of *E. prunastri* at 254 nm.

The structures of the isolated lichen metabolites are shown in Figure 4.



Norstictic Acid one of its IUPAC name is 1,3-Dihydro-1,4,10-trihydroxy-5,8-dimethyl-3,7-dioxo-7H-isobenzofuro(4,5-b)(1,4)benzodioxepin-11-carboxaldehyde or 7H-Isobenzofuro(4,5-b)(1,4)benzodioxepin-11-carboxaldehyde, 1,3-dihydro-1,4,10-trihydroxy-5,8-dimethyl-3,7-dioxo-



Evernic Acid one of its IUPAC name is 2-hydroxy-4-[(2-hydroxy-4-methoxy-6-methylphenyl)-oxomethoxy]-6-methylbenzoic acid is a carbonyl compound.

Fig. 4. The structures of the isolated lichen metabolites

3.2. Scavenging activities and reduction power:

Table 1. Retention time of the isolated lichen substances, their absorbance maxima (nm), HPLC purity, DPPH radical scavenging and superoxide anion scavenging activities

Compound	Retencion time ($t_R \pm SD$)[a] (min)	Absorbance maxima (nm) UV spectrum	HPLC Purity (%)	DPPH radical scavenging IC_{50} (µg/mL)	Superoxide anion scavenging IC_{50} (µg/mL)
Norstictic acid	2.97 ± 0.10	212, 239, 310[m]	97.2	101.95 ± 1.02	132.89 ± 2.31
Evernic acid	3.12 ± 0.10	213, 270, 304	97.9	319.84 ± 4.07	561.05 ± 7.78
Ascorbic acid				6.05 ± 0.34	115.61 ± 1.09

[a] Values are the means of three determinations ± SD, m - minor absorbance maximum.

Table 2. Reducing power of norstictic acid and evernic acid

Lichen compound	Absorbance (700 nm) 1000 µg/mL	500 µg/mL	250 µg/mL	125 µg/mL	62.5 µg/mL
Norstictic acid*	0.8781±0.006	0.6639±0.003	0.0867±0.002	0.0419±0.002	0.0271±0.007
Evernic acid*	0.1121±0.004	0.0908±0.003	0.0480±0.006	0.0302±.0.005	0.0190±0.002
Ascorbic acid	2.114±0.031	1.653±0.020	0.0955±0.008	0.0479±0.005	0.0250±0.005

*Values are expressed as mean ± SD of triplicate measurements

After isolation and identification, the antioxidant activity of these compounds was determined. The values obtained for Noristicic acid and everinic acid was compared. The results of DPPH radical scavenging and superoxide anion scavenging activities (IC_{50}) for norstictic acid and evernic acid are shown in Table 1. The IC_{50} values for norstistic acid and evernic acid are = 101.95 ± 1.02 and 319.84 ± 4.07 µg/mL, respectively. As it can be seen, evernic acid has about three times large the value of IC_{50} then norstictic acid. On the other hand, the IC_{50} values of superoxide anion scavenging activity for norstictic acid and evernic acid are = 132.89 ± 2.31 and 561.05 ± 7.78 µg/mL, respectively. Evernic acid has about four times higher IC_{50} values than norstictic acid. The results of reduction power for norstictic acid and evernic acid are shown in Table 2. Higher absorbance indicates higher reducing power. Results showed that norstictic acid has significantly higher reducing power than the evernic acid. At lower concentrations (250 µg/mL, 125 µg/mL and 62.5 µg/mL), the reducing power values for norstictic acid were similar to those for the standard (ascorbic acid). On the basis of these results, it can be concluded that both tested compounds showed antioxidant activity, but that the norstictic acid showed several times better activity than evernic acid.

3.3. Microanalytical and spectral analysis:

The microanalyses of the isolated pure acids refer to the results of Evernic Acid of general formula $C_{17}H_{16}O_7$ of mol mass 332.2 g/L (m.p. =165-175^oC) (calculated % C= 61.39, H= 4.81 an O=33.70 and Found % = 61.29, H= 4.90, O= 33.61), Norstictic Acid of general formula $C_{18}H_{12}O_9$ of mol mass 372.0 g/L (m.p. = 275 ^oC) (Calculated % C= 58.018, H= 3.22, O=38.67, Found % C= 58.23, H=3.18, O=38.63).

3.3.2. Spectral measurements

3.3.2.1. Mass spectra of isolated acids:

The measured Negative LDI mass Spectra [32] of norstictic and evernic acids are given in Figs (5,6).

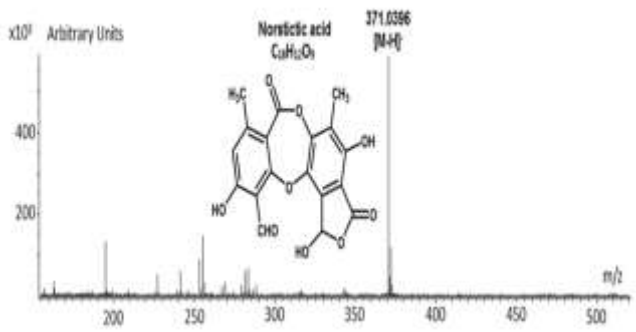


Fig (5): Negative LDI mass of Norstaictic Acid

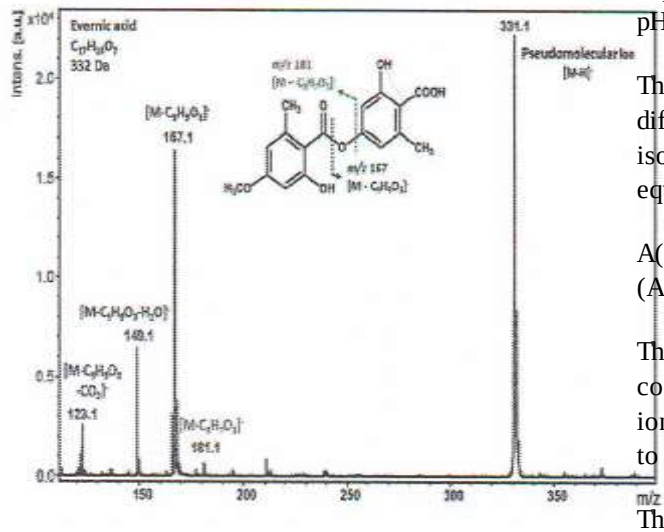


Fig (6): Negative LDI mass of Evernic Acid

These figures [32] refer to the obtained negative molecular ion $[M-H]^-$ of m/z = 371.0396 of the isolated Norstictic Acid; which confirm its elementally analized data of its general formuala $C_{18}H_{12}O_9$ and negative molecular ion [M-H]- of m/z = 331.1 of the isolated Evernic Acid; which confirm their elementally analyzed data of its general formula $C_{17}H_{16}O_7$ consequently the purity of the isolated acids from their natural source under the referred HPLC effective conditions.

3.3.2.3. UV spectra: Spectra of Norstictic Acid

The UV spectra of norstictic acid at different pH values (5.71-9.20) is depected in Fig (7).

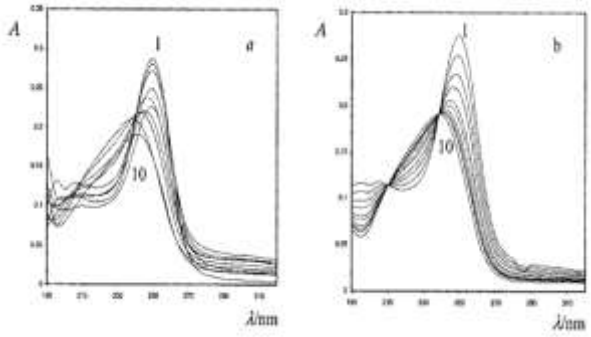


Fig.(7). UV of 2.1·10 –5 mol/dm 3 norstictic acid (a) experimental spectra and (b) reconstructed spectra, pH from 5.71 to 9.20.

This UV spectral measurement norstictic acid at different pH values in NaOH medium refers to two isosbestic points which may refer to the following equilibrium of the given acid:

$A(OH)_3 = A(OH)_2^- + H^+ = A(OH)^{2-} + 2H^+ = (AO)^{3-} + 3H^+$, …….. (3)

This equilibrium may refer to three dissociation constants of the, pK_1, pK_2 and pK_3 of the three ionized phenolic groups at different pH values (5.71 to 9.2).

The UV spectra of evernic acid at different pH values (5.83-9.48) in NaOH medium is given in Fig (8).

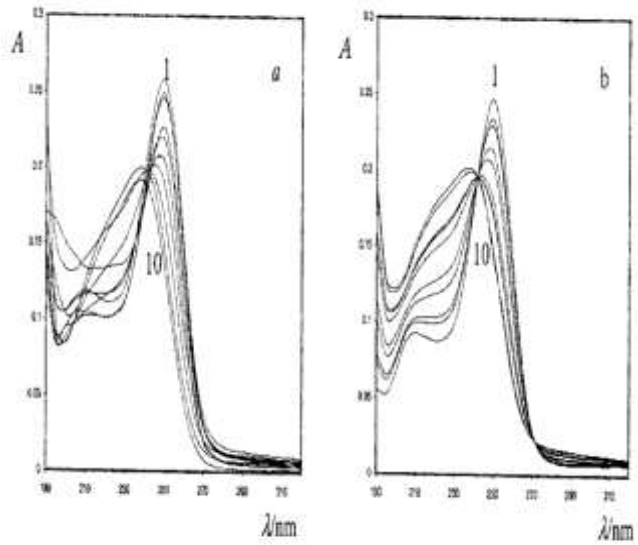


Fig.(8). UV spectra of 1.9·10 –5 mol/dm 3 evernic acid (a) experimental spectra and (b) reconstructed spectra, pH from 5.83 to 9.48.

This UV spectral measurement of evernic acid at different pH values in NaOH medium refers to two isosbestic points which may refer to the following equilibrium of the given acid:

$A(OH)_2COOH = [A(OH)_2COO]^- + H^+ = [A(OH)COO]^{2-} + 2H^+ = [(AO)COO]^{3-} + 3H^+$, ..(4)

This equilibrium may refer to three dissociation constants of the, pK_1, pK_2 and pK_3 of the carboxylic group and two ionized phenolic groups at different pH values (5.71 to 9.2).

3.3.2.3. IR spectra of Evernic Acid and Norstictic Acid:

The studied obtained FT-IR spectra of depside and depsidone isolated and analyzed compounds refer to 3425m 1754s, 3053 w 1724 sh, 2924w 1695 s, 1639 s, 1613 sh, 1563 m 1543m, 1527 m, 1481 sh, 1445 m, 1429m, 1379m, 1342 m, 1282 s, 1242m, l190m, 1156 s, 1136 sh, 1087m,1058m., a strong and sharp band occurs between 3425, and 3333 cm^{-1} possibly due to the presence of the OH group and the low frequency may be due to hydrogen bond formation with the, oxygen of diphenyl ether linkage. The carbonyl groups appear in region (1780-1600 $cm.^{-1}$). Carboxyl carbonyl, the chelated carboxyl group of the P-part shows a strong band near 1650-1630 cm^{-1} and this together with a number of weak absorption bands in the region 3000-2500 cm^{-1}. The chelated aldehyde carbonyl absorption appears near 1650-1640 cm^{-1}. The aromatic region appears at (1600-1500cm^{-1}). CH_3 region occurs near 1400 cm^{-1}. With all methoxy compounds the bands are present near 1460, 1340, 1250, 1180, 1125 and 1028 cm^{-1} due to the C-O-C group. The FT-IR of evernic acid is given in Fig (9).

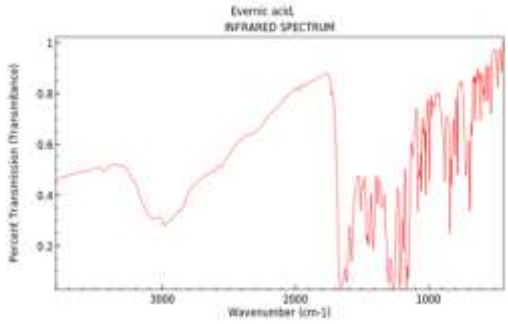


Fig (9): The FT-IR of evernic acid

3.3.2.4. ^{1}HNMR and ^{13}CNMR Spectra of Norstictic Acid and Evernic Acid:

The ^{1}H-NMR and ^{13}C-NMR spectra of norstictic acid obtained are given in Fig (10):

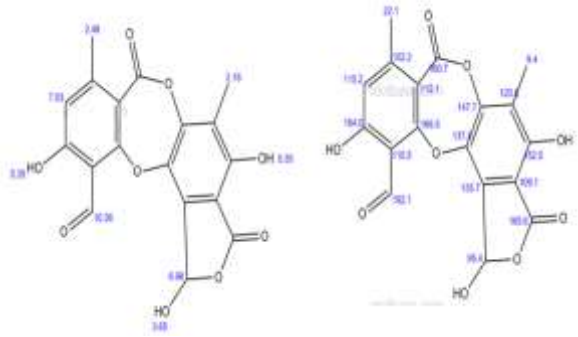


Fig (10): The ^{1}H-NMR and ^{13}C-NMR spectra of norstictic acid.

Evernic acid showed the presence of ^{l}H-N M R (200 M H z, CDC13): 2.27 (3 H , s, - M e) , 3.77 (3 H , s, - O M e) , 6 .2 -Ö .4 (3 H , m, 3 x arom . H) [33].

All of the above spectral data confirm to great extent the purity of the isolated acids from the studied natural source and also elucidate their structural formulae. Their structures can also confirmed by their thermal degradation behavior in comparison with mass fragmentation and also confirmation of these comparative studies by theoretical molecular orbital calculation.

3.4. Thermal Analyses measurements and calculations

To our knowledge, until now, there are very few scientific papers describing thermal analysis of lichen metabolites. Melo et al. 2008 [25] described the physicochemical characterization, thermal analysis and antinociceptive property of atranorin obtained from Cladina kalbii (DESABB.) Ahti. The TG/DTG curves of atranorin indicate that the thermal decomposition process of UA occurs in two stages in the following temperature ranges (mass loss): 193.4—280°C (% loss = 63.4%) and 280—900 °C (% loss = 36.5%). In the first stage partial thermal decomposition of atranorin occurs with elemental

carbon formation due to sample carbonization. Between 350 and 900 °C the elemental carbon is released slowly.

3.4.1. Thermal Analyses of Evernic Acid:

The thermal decomposition evernic acid is given by Figs (11-13).

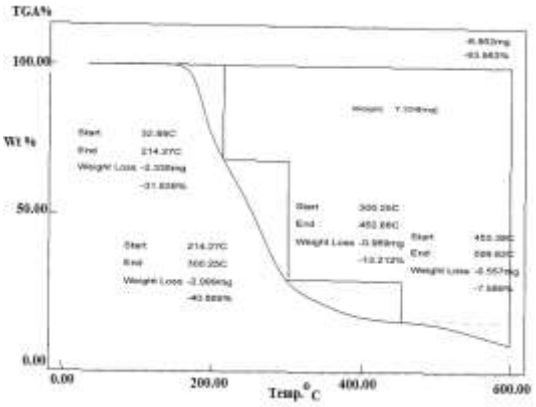


Fig (11): TG of evernic acid ND1

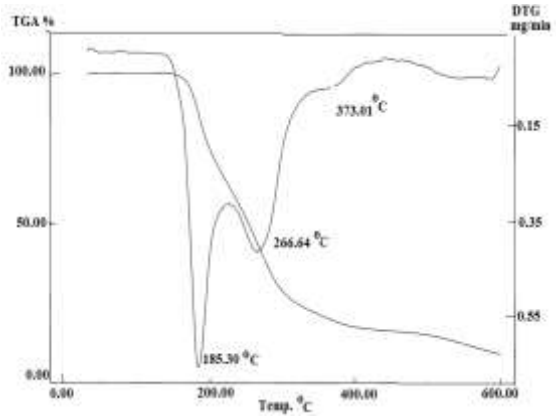


Fig (12): DTG of Evernic Acid ND1

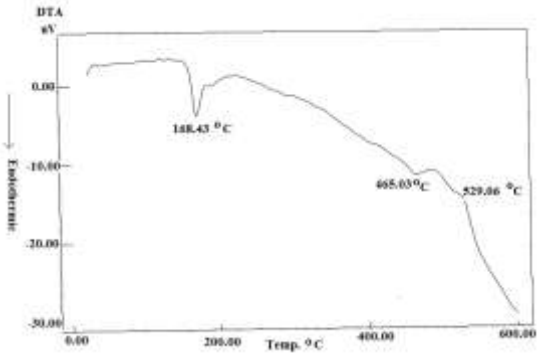
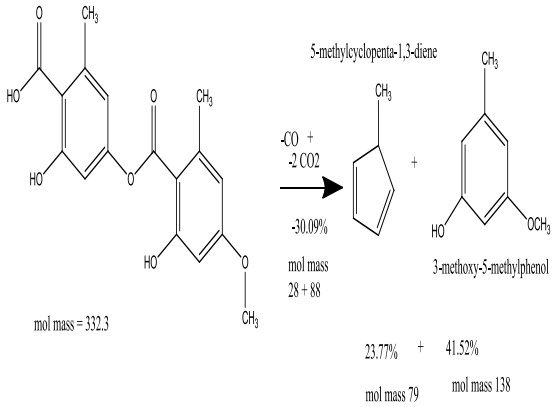


Fig (13): DTA of Evernic Acid ND1

The explanation of thermal degradation of Evernic acid coming from TGA and DTG (Figs 11-12), is given by Table (3) and thermal decomposition scheme (1).

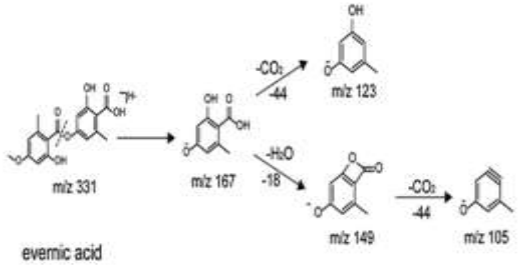


Scheme (1): The proposed thermal decomposition of evernic acid.

These results refer to the thermal degradation of this acid in two steps at temperature ranges of 23.55-214.27°C exactly at 185.30°C and at 214.30-300.25°C exactly at 373.61°C of mass losses 31.8 % (calcd = 30.9 %) and 40.88% (calcd = 41.52%) respectively. This degradation is given by the proposed Scheme 1. The DTA of evernic acid (Fig 13) refer to an endothermic peak at 168.43°C; which approximately equal to the average of m.p. of the isolated acid (165-175°C). The energy required for each decomposition step and corresponding thermodynamic parameters are calculated by Koat-Redfern equation [34] and given in Table (3). These data refer to endothermic nature of thermal decomposition of evernic acid. The proposed thermal decomposition scheme 1 is very similar to the previously published proposed mass scheme 2 [35].

Table (3): Thermodynamic parameters of thermal decomposition of (Evernic Acid)

Decomp. Temp. Range (^oC)	E* KJ $mole^{-1}$	A (S^{-1})	ΔS^* $J.K^{-1}.mole^{-1}$	ΔH^* KJ $mole^{-1}$	ΔG^* KJ $mole^{-1}$
306.04-487.7	35.5	1.288E+07	-112.40	31.7	83.2
487.27-573.3	112.02	1.311E+10	-56.16	107.53	137.83
573.4-872.82	2.25	5.337E+08	-84.28	-3.13	51.3



Scheme 2: Proposed mass fragmentation pathways of evernic acid (REF: 35).

3.4.2. Theoretical Calculations for Evernic Acid:

Table (4): Theoretical calculated bond order and bond length of selected bonds evernic acid

Quantum parameter	Neutral
Total energy (eV)	-4371.845
E_{HOMO} (eV)	-9.280
E_{LUMO} (eV)	-0.546
ΔE_{gap} (eV)	-8.734
Ionization energy (I)	9.280
Electron affinity (A)	0.546
Mulliken electronegativity (χ)	4.913
Hardness (η)	4.367
Heat of formation (kcal/mol)	-268.571

The MOCs for evernic acid molecule using the molecular formula and numbering system is given in Fig (14).

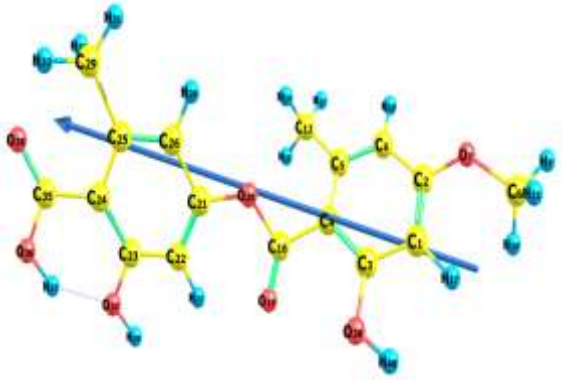


Fig (14): Optimized geometry, numbering system, and vector of dipole moment for the studied evernic acid using B3LYP/6-311G .**

The theoretically calculated selected bond length and bond order of the different bonds of the evernic acid are given in Table (4).

The bonds ruptured in evernic acid at thermal decomposition (scheme 1) are those bonds of long bond length and of less bond order values. These decomposed bonds lead to the formation of CO and CO_2 evolved gases; which confirm the thermal decomposition scheme (1). Both TA and mass spectral fragmentation schemes (1 and 2) are confirmed by calculated quantum parameters of evernic Acid in Table (5).

Table (5): Theoretical calculated quantum parameters of evernic acid

The theoretically calculated and obtained total energy (eV) = -4371.845 and heat of formation (kcal/mol) = -268.571of evernic acid refer to the high stability of evernic acid; which is correlated with its thermal stability concluded from TA study. In TA it required high ΔH^* = 107.53 KJ $mole^{-1}$ in step of CO and CO_2 gases formation as result of ruptured bonds. The high values of energy gap ΔEgap (eV) = -8.734, Ionization energy (I) = 9.280, Hardness (η)= 4.367 and energy difference between EHOMO (eV) = -9.280 and ELUMO (eV) = -0.546 energy levels refer to the high stability of evernic acid and its low polarizability (Fig 15).

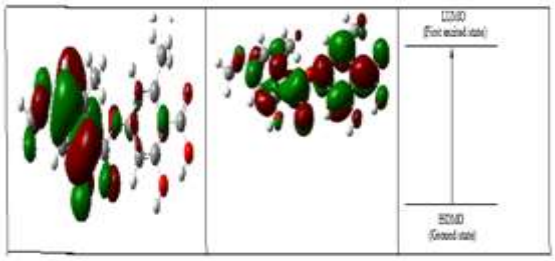


Fig (15): HOMO and LUMO charge density maps of the investigated evernic acid using B3LYP/6-311G**

This conclusion is confirmed by the low value of the calculated Softness (S) = 0.229 and high energy values required to its spectra appeared at UV range (Fig 8) absorption at λ_{max} of log ε 267 (4.29); 300 (4.06) respectively. These conclusions are also confirmed by theoretical calculated charges on atoms of evernic acid in both neutral and cationic forms (Table 6 and Fig16). The neutral form refer to the shape of acid molecule in thermal analyses (Scheme 1); while cationic form refer to acid in mass study (Scheme 2). The rupture of certain bonds such as C15-C19, C4-C5, C5-C6,…etc may be attributed to electrostatic repulsion and/or low electrostatic attraction between positively charged atoms and/ or negatively charged red labeled atoms; which leading to formation of evernic acid thermal and mass fragments and /or evolved CO and CO_2 gases.

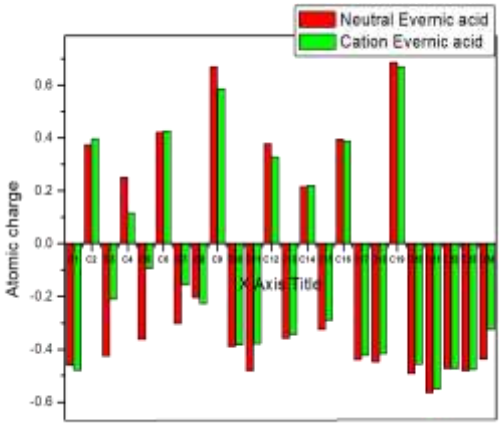


Fig (16): Graphical representation of theoretical calculated charges on atoms of everinic acid in both neutral and cationic forms.

3.4.3 Thermal Analyses of Norstictic acid:

The thermal analyses of norstictic acid are given in Figs (17-19):

Table (6): Theoretical calculated charges on selected atoms of evernic acid in both neutral and cationic forms

Atom	Charge	
	Neutral	Cation
O10	-0.390807	-0.383725
C15	-0.324554	-0.289207
C19	0.685845	0.669449
O20	-0.492366	-0.456194
O21	-0.565273	-0.550475

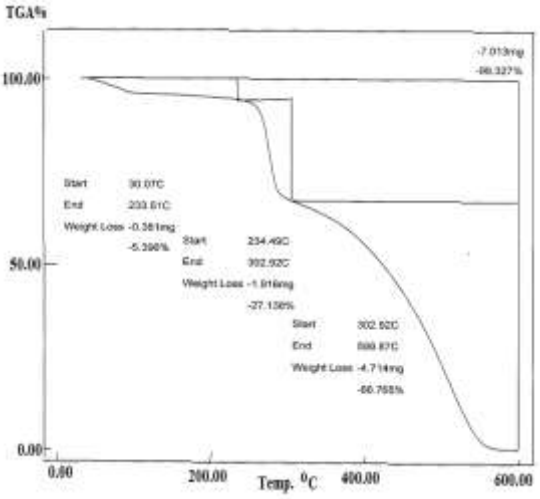


Fig 17: TG of norstictic Acid ND 2

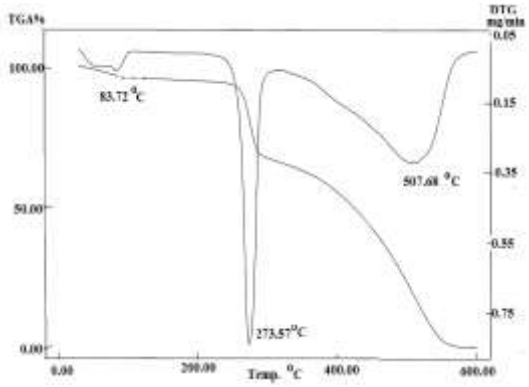


Fig 18: DTG of norstictic Acid ND 2

From the TGA and DTG curves of norstictic acid (Figs 17, 18); it is clear that the thermal decomposition of this acid occurs in three steps. The first one occurs at the temperature range 30.07 to 233.51°C and exactly at 83.72°C as indicated by DTG. It may be explained by the mass loss of 5.39%.

The second one occurs at 234.49- 302.92°C and exactly at 273.57°C. It refers to a mass loss of 27.1%. The third one occurs at 302.92 to 599.87°C and exactly at 507.68°C. It gives a mass loss of 4.714%. These mass losses are attributed to the loss of two CO and two CO_2 gaseous groups of total practical mass of 37.24% (Calcd% = 38.7), leaving remainder stable part of practical% = 62.76 % (Calcd% = 61.3) These mass losses are explained by the proposed scheme 3. The first step as endothermic peak appred at 49-64°C as given by DTA of acid (Fig 18). It followed by an endothermic sharp peak at 275.55°C; which may be attributed to the milting point of norstictic acd (m.p. = 275 °C). This sharp peak refers to the purity of the isolated acid. It is followed by an exothermic peak at 285.55°C and at 481.33°C; which may be explained by chemical rearrangement of acid skeleton after its final mass loss to give stable form.

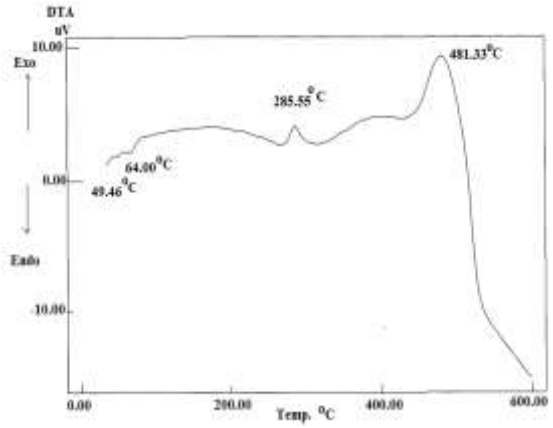
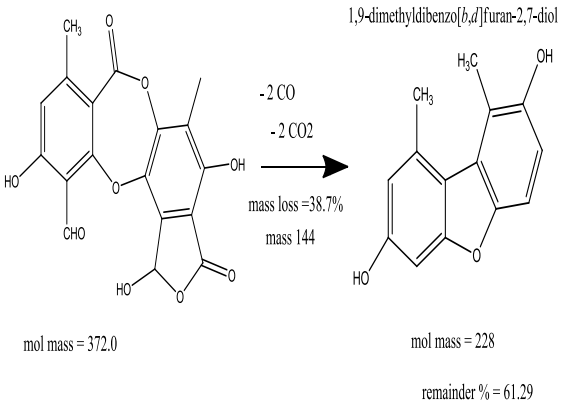


Fig 19: DTA of norstictic Acid ND 2

The calculated energy values and the thermodynamic parameters of thermal degradation of norstictic acid applying Koat and Redfern equation [34] are given in Table (7). These data refer to endothermic and exothermic mutual processes occur in thermal decomposition of norstictic acid to leave rearranged chemical stable compound.

The thermal decomposition of norstictic acid is explained by the following proposed scheme (3).



Scheme (3): The thermal decomposition of norstictic acid

3.5. Theoretical Calculation results:

According to Koopman's theorem [36], the ionization energy (I) and electron affinity (A) can be expressed through the energies of HOMO and LUMO as I = $-E_{HOMO}$ and A = $-E_{LUMO}$. Alternatively, the chemical hardness and softness of a molecule are good indicators for the chemical reactivity of a given molecule. From energy gap, one can find whether the molecule is hard or soft. The soft molecule is more polarizable than the hard one because it needs small energy to excitation. Softness (S) is a property of molecule that measures the extent of chemical reactivity. It is defined as the reciprocal of hardness (η) and η = (I - A)/2 [37]. As shown in Table 8, the dimeric PQC is softer than the monomeric one. In addition, the chemical potential (μ) and electronegativity (χ) of a molecule can be calculated

Table (7): Thermodynamic parameters of thermal decomposition of (Norstictic Acid)

Decomp. Temp. Range (°C)	E* KJ mole^{-1}	A (S^{-1})	ΔS^* J.K^{-1}.mole^{-1}	ΔH^* KJ mole^{-1}	ΔG^* KJ mole^{-1}
305.18-506.4	18.7	3.016E+07	-87.95	18.3	23.2
506.7-575.92	275.53	4.572E+25	241.32	270.98	139.08
575.92-872.87	78.65	4.636E+05	-144.47	72.16	184.9

as follows: $\mu = -(I + A)/2$, and $\chi = (I + A)/2$, i.e. $\mu = -\chi$. The electrophilicity index (ω), $x = \mu^2/2\eta$, [38] is a measure of energy lowering due to maximal electron flow between donor and acceptor.

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3.5.1. Norstictic acid:

The structure of Norstictic acid and numbering system used for theoretical molecular orbital calculation is given in Fig (19):

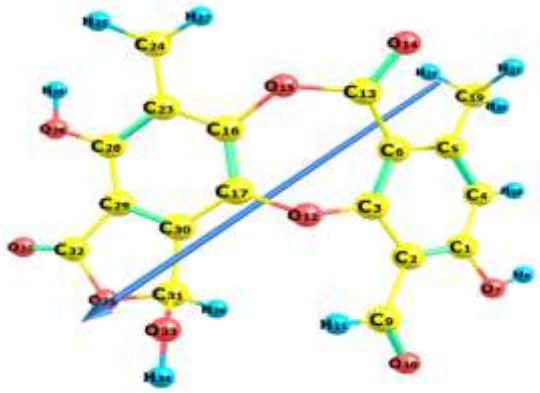

Fig (20): Optimized geometry, numbering system, and vector of dipole moment for the studied norstictic acids using B3LYP/6-311G**

The obtained results are in Tables (8-10):

Table 8: The calculated frontier orbital energies and related molecular properties of norstictic acid

Quantum parameter	Neutral
Total energy (a.u.)	-1370.165
E_{HOMO} (eV)	-6.848
E_{LUMO} (eV)	-2.363
ΔE_{gap} (eV)	-4.485
Ionization energy (I)	6.848
Electron affinity (A)	2.363
Mulliken electronegativity (χ)	4.606
Softness (S)	0.446
Hardness (η)	2.243
Electrophilicity index (ω)	
Total dipole moment (D)	2.140

The calculated total energy of norstictic acid molecule = -1370. 165 (a.u) is actually correlated with that calculated from practical thermal analyses (E* = 275.55 KJ mole^{-1} Table) and refer to its high stability and cosequenly its its difficult thermal decomopostion. Only terminal caboxylic groups are evolved as cabonageous gases (CO and CO_2) at high temperature range 302-599^{o}C. The value of Hardness (η) = 2.243 and low dipole moment D = 2.14, means that Norstictic acid is a hard compound and less chemically reactive and less polariazable compound as confirmed by its ionization at high pH values as given by UV spectral and chemical ioniyation under alkalinity (Fig 7 and eqilirium No 1). The HOMO and LOMO energy levels of the given acid are grahically represented in Fig (20).

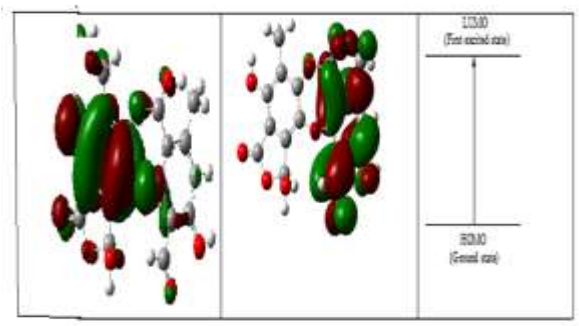

Fig (21): HOMO and LUMO charge density maps of the investigated Norstictic acid using B3LYP/6-311G**

The high energy gap value (ΔEgap (eV) 4.485) between EHOMO (eV) -6.848 and ELUMO (eV) -2.363 energy level of the given norstictic acid molecule molecule explain its stablity and why its electronic transions occure in short wavelenghtes in the UV range of λ_{max} and logε 239(4.33); 320(3.40) (Fig. 7). These conclusions are confirmed by the high vlues of Ionization energy (I) = 6.848 and Mulliken electronegativity (χ) = 4.606 and low values of Electron affinity (A) = 2.363 and Softness (S) = 0.446 of Norstictic acid molecule. The evolved CO and CO_2 gas molecules from Norstictic acid during thermal decompositin are due to long and weak red labled bonds rupture as results of Mulliken Charge(e) values that theretically calculated in Table (9) and graphically represented in Fig (21).

Table (9) : Selected bond lengths (Å), angles (°) and Mulliken charge (e) for Noristicic acid

Bond Length (Å)		Bond Angle (°)		Mulliken Charge(e)	
C3-C26	1.458	C4O7C8	115.516	C6	0.132916
C4-C5	1.397	O7C8C9	120.302	O7	-0.583855
C4-O7	1.383	C8C9O10	120.512	C8	0.229459
C5-C11	1.488	O7C8C12	121.799	O10	-0.512723
C6-C19	1.509	C8C12C13	119.261	C11	0.561394
O7-C8	1.388	C12C13C14	123.587	C12	-0.026087
C10-C11	1.386	C1C6C19	118.243	O17	-0.486733
C16-O17	1.463			C26	0.201867
C16-O25	1.384			O27	-0.470867
O17-C18	1.366				
C18-O24	1.216				
C26-O27	1.236				

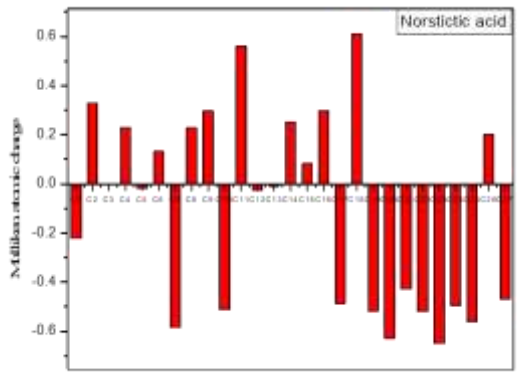

Fig (22): Mulliken Charge(e) on atoms of norstictic Acid.

4. Conclusions:

From the presented research it is concluded that, successful trials were made to isolate two lichen acids (norstictic and everinic acids) from natural sources. The isolation was successfully done by using effective HPLC. Their structures were proved via elemental, spectroscopic, and thermal analyses. In addition, antioxidant activity of norstictic acid and everinic acid were determined. The practical results obtained were successfully correlated with the theoretical calculations. The importance of this research stems from the importance of the isolated Lichen compounds from natural specimen and their possible applications in medical field.

Acknowledgments

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Authors declared that there is no conflict of interest

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الملخص باللغة العربية

دراسة فصل وتعريف و التحليل الحرارى و الحسابات النظرية و النشاط المضاد للأكسدة لبعض مشتقات الليشن (حمض نورستاكتيک و حمض ايفيرنيك)

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يتحدث هذا البحث عن محاولات ناجحة لعزل بعض مشتقات الليشن (حمض نورستاكتيك وحمض الأيفيرنيك) من مواد طبيعية كثل الكوزبرة بواسطة كروماتوجرافيا السوائل بالضغط والتعرف على نقاوتها وتركيبها بطرق القياس المختلفة كتحاليل العناصر المكونة لتلك الحماض والقياسات الطيفية والتحاليل الحرارية وحسابات الدوال الحرارية ودرجة ثباتها والحسابات النظرية واستخدامها كمواد مضادة للإكسدة. وتضمنت الحسابات النطرية أستخدام برنامج جاويسن ٩. وتم تجربتها بنجاح كمواد مضادة للأكسدة توطئة لأستخدامها في العديد من التطبيقات الطبية والصناعية.