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Extraction, phytochemical screening, chemical quantification and identification of bioactive compounds from Lebanese *Urtica dioica*

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ABSTRACT

Plants are widely used by people in traditional and modern medicine all over the world. In fact, all of their remedies can be used safely and without the side effects of drugs. Phytochemical analysis of medicinal plants has revealed that numerous bioactive compounds in plants traditionally used for medicinal purposes have many therapeutically properties. Hence in the present study, a general chemical identification of a Lebanese medicinal plant *Urtica dioica* was carried out. The results obtained validate the traditional uses of nettle, and showed that this plant possesses an important pharmaceutical value and leads to the isolation and characterization of three compounds from hexane extract. Structures of these compounds were elucidated by spectral methods [FTIR, GC-MS, ^{1}H NMR] after column chromatography on silica gel.

Keywords: *Urtica dioica*, phytochemical screening, bioactive compounds, FTIR, GC-MS, ^{1}H NMR.

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INTRODUCTION

The use of plants as a source of medicine has been inherited from the onset of human civilization and is an important component of the healthcare system [1]. Herbal medicine is the major stay of about 75-80 % of the world population, mainly in the developing countries, for primary health care due to a better cultural acceptability, better compatibility with human body and few side effects [2]. The medicinal value of these plants lies in the presence of some chemical substances known as secondary metabolites that have a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds [3]. There is therefore the need to look inwards to search for herbal medicinal plants in order to validate the ethno-medicinal use and consequently the isolation and characterization of bioactive compounds which will be added to the potential list of drugs.

Urtica dioica (stinging nettle) are members of the Urticaceae family native to Eurasia [4].This plant is highly important because of its accelerated maturity level, and easily cultivated process [5].

Urtica dioica has been used since ancient times and is believed to be a galactogogue [6] with diverse functions including, stimulation of the digestive system [7], usage in the treatment of diabetes [8], being a diuretic agent in addition to having significant antimicrobial and antioxidant activities [9].

Recently, ultrasonic assisted extraction (UAE) technique has attracted an increased attention for being a novel technique for extracting plant tissue with high yield in a small interval of time in addition to be carried out at low temperatures which prevent thermal damage [10].

The main purpose of the present study was to quantify, purify and identify the chemical components present in the Lebanese *Urtica dioica* extracts through phytochemical screening of 5 different extracts and by using different chromatographic and spectrophotometric methods : gas chromatography-mass spectrometry (GC-MS), infrared spectroscopy (IR) and nuclear magnetic resonance spectroscopy (NMR).

MATERIALS AND METHOD

Plant collection and extraction

Fresh leaves and stems of *Urtica dioica* were gathered from the south region of Lebanon. 1 g of grinded leaves and stems was added to 100 ml of solvent in a flask, and the mixture was dipped in an ultrasonic bath for one hour at a temperature just below the boiling point of the solvent used. Five solvents were adopted: hexane, dichloromethane, acetone, ethanol and water. The extract was filtered through the Buchner funnel and then the filtrate was stored in sterile bottles at 5°C for further use.

Phytochemical screening

Preliminary qualitative phytochemical screening was carried out according to the method used by Muanda et al. [11].

Chemical quantifications of secondary metabolites

Saponin determination [12]

1 g of powdered plant has been added to 100 mL ethanol (20 %) and kept in a flask on stirrer for half hour and then heated for 4 h at 45 °C with mixing. The mixture was then filtered using a filter paper whatman N 1 and the residue was again extracted with another 100 mL ethanol (25 %). The combined extracts were concentrated by using rotary evaporator at 40 °C to get 40 mL approximately. The concentrate was then transferred into separator funnel and extracted twice with 20 mL diethyl ether. The ether layer was discarded while the aqueous layer was kept and then re-extracted with 30 mL n-butanol. The n-butanol extract was washed twice with 10 mL aqueous sodium chloride (5 %). The remaining solution was evaporated. After that, the samples were dried in the oven at 40 °C to a constant weight. The saponin content was calculated using the following formula:

$$\% \text{ Saponin} = [\text{final weight of sample} / \text{initial weight of extract}] \times 100$$

Total alkaloids [12]

100 mL of 10 % acetic acid in ethanol were added to 1 g of dry powdered plant and then the extracts were covered and allowed to stand for 4 h. After that, the extracts have been filtered and concentrated on a water bath to 25 mL of its original volume. The droplets of concentrated ammonium hydroxide were added to the extract until the precipitation and the whole solution was allowed to settle. Then, the precipitates were washed with dilute ammonium hydroxide and then filtered using filter paper whatman N 1. The residue was dried in the oven at 40 °C and weighed. The alkaloid content was determined using the following formula:

% Alkaloid = [final weight of the sample / initial weight of the extract] × 100

Determination of total lipids [12]

10 g of powders from leaves and stems were added in Soxhlet apparatus with 200 mL of petroleum ether (40-60 °C) and extracted during 8 hours. After that, the solvent was filtered using Büchner funnel under reduced pressure, and then an evaporation using a rotary evaporator at 40 °C has been done. Finally, the weight of lipids was calculated.

Estimation of the proportion of ash

Leaves and stems have been tested to estimate the proportion of ash using the standard methods of AOAC 923.03 (1980) [13]. 1 g of powders from nettle has been tested to estimate the proportion of ash. 1 g was put in and burnt in a furnace burning (muffle furnace) at 550 °C for 5 h until obtaining an ovary gray color of the powders. The residues were then weighted and the percentage of ash was determined according to the essential dry weight of plant powder.

Determination of protein content [14]

Protein content was determined according to Bradford's method using bovine serum albumin as standard.

Purification and identification

Bioassay guided isolation of active compounds

The hexane extract of *U. dioica* was fractionated using silica gel 60 (0.063 to 0.200 mm) column chromatography (CC). The column was eluted with a solvent gradient of hexane-ethyl acetate (EtOAc) in 10:0 and 0:10 ratios to give 11 fractions as follows:

10% EtOAc – hexane (3 fractions)

20% EtOAc – hexane (2 fractions)

30% EtOAc – hexane (2 fractions)

50% EtOAc – hexane (3 fractions)

100% EtOAc (1 fraction)

11 fractions were collected, analyzed by a thin layer chromatography (TLC) (analytical plate) on silica gel 60 PF254 (Merck) and pooled together due to similarity in TLC profile to give overall 3 fractions: FI, FII and FIII. Each fraction was then purified by a preparative plate of thin layer chromatography (TLC): FI gave 6 sub-fractions, FII gave 4 and FIII gave 3. All sub-fractions of both hexane and dichloromethane extracts were analyzed by GC-MS, NMR and IR.

GC-MS analysis

GC agilent of the hexane extract of *U. dioica* was done with Supelco Analytical, fused silica capillary column SP 2380 with 60m*0.25mm*0.25 um film thickness. Helium gas (99.999%)

was used as a carrier gas at a constant flow rate of 1 ml/min, and an injection volume of 2 µl was employed (a split ratio of 10:1). The injector temperature was maintained at 250 °C, the ion-source temperature was 180 °C, the oven temperature was programmed from 50°C (isothermal for 2 min), with an increase of 10 °C/min to 60°C, then 20°C/min to 80°C, with a 3 min isothermal at 80 °C, then 10°C/min to 90°C, with a 2 min isothermal at 90 °C, then 30°C/min to 120°C, with a 3 min isothermal, then 30°C/min to 150°C, with a 5 min isothermal, then 100°C/min to 250°C, with a 10 min isothermal at 250 °C, then 50°C/min to 300°C, ending with a 5 min isothermal. The GC was coupled with MS, an electron ionization system was operated in fast atomic bombardment (FAB) mode with ionization energy of 70 eV, the temperature of transfer line was 280°C; a scan interval of 0.5 s for all fragments mass. The solvent delay was 0 to 2 min, and the total GC/MS running time was 70 min.

Identification of phytocomponents

Interpretation on mass-spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62.000 patterns. The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library. The name, molecular weight, and structure of the components of the test materials were ascertained.

Infrared spectroscopy analysis (IR)

FTIR (Fourier transform Infra-red) (Model - Varian 3600; Range: 400-4000 cm^{-1}) was obtained for different sub fractions. Sample (1-2 mg) was crushed with KBr (3-4 mg) and pellet was formed with the help of mechanical pressure formed and was observed at the different coming wavelengths in FTIR instrument.

Nuclear Magnetic Resonance Spectroscopy analysis (^{1}H NMR)

Nuclear Magnetic Resonance (Avance-300 Megahertz Bruker) was obtained for each sub fraction. Sample was dissolved in respective deuteriated solvents ($CDCl_3$) and about 600 µl were poured in NMR tube and observed on the applied magnetic field.

RESULTS AND DISCUSSION

Phytochemical screening

The present study conducted on *U. dioica* showed the presence of active medicinal components. Bioactive compounds of *U. dioica* were qualitatively evaluated in the whole plant. The results are reported in Table 1. Phytochemical screening of alkaloids, phenols, glycosides, terpenoids, flavonoids, saponins and resins shows some differences in the components of the five extracts.

All extracts tested positive for phenolic compounds known to have antioxidant, anticarcinogenic, anti-inflammatory, antiapoptosis, antiaging, antiatherosclerosis, and cardiovascular protection effects [15,16]. Terpenoids, known to have significant pharmacological activities such as anti-viral, anti-bacterial, anti-malarial, anti-inflammatory, inhibition of cholesterol synthesis and anti-cancer activities [17], were found in hexane, dichloromethane and aqueous extracts; Flavonoids, a group of polyphenolic compounds having antioxidant and anti-inflammatory effects [18], were present in acetone and ethanol extracts. Alkaloids, known to exhibit analgesic and antibacterial activities [16], were found in ethanol extracts only. Glycosides, known to lower blood pressure [19], were found in hexane and aqueous extracts of *U. dioica*. Saponins, with various pharmacological effects such as anticholesterolemic, anti-inflammatory, anti-parasitic and anti-viral [20], were found in aqueous extracts only.

Table 1 Phytochemial screening of different extracts from *Urtica dioica*

Bioactive compound	Hexane	Dichloro-methane	Acetone	Ethanol	Aqueous
Phenols	+	+	+	+	+
Terpenoids	+	-	+	-	+
Alkaloids	-	-	-	+	-
Flavonoids	-	-	+	+	-
Glycosides	-	-	-	+	-
Saponins	-	-	-	-	+

(+)=presence of bioactive compound; (-)=absence of bioactive compound

Total content

Quantitative estimation of the percentage of the crude chemical components in this medicinal plant is summarized in Table 2.

Table 2 Percentage of active contents in *Urtica dioica*

Total saponin	8.14 ± 0.012
Total alkaloid	1.4 ± 0.084
Total ash	22 ± 0.006
Total lipid	2.51 ± 0.012
Total protein	6.76 ± 0.01

Lebanese *U. dioica* contained a high percentage of alkaloids (1.04 %) and saponins (8.14 %) in comparison to the results provided by Khan et al. [21] where the percentage of saponins was 0.49 %. On the other hand, the alkaloid was not detected. These results correspond to those provided by the preliminary phytochemical tests presented above. This plant shows a significant amount of minerals manifested by the high percentage of ash detected in the Lebanese nettle or other types of nettles. In addition, our plant contains high protein content relative to that exhibited by

Rutto et al. [22] (4.1 %). Several reasons may be involved in these changes such as physical and chemical properties of soil, environmental conditions and genetic factors [23].

Identification of Compound 1 (diisooctyl phthalate)

Compound 1 was obtained from the first sub-fraction from FI. The IR absorptions displayed a carbonyl group (1656 cm^{-1}) and 2 absorption bands frequencies for -C-C- bonds of an aromatic ring (1414 and 1451 cm^{-1} respectively) (Figure-1). IR results and examination of ^{1}H-NMR data suggested that compound 1 was a phthalate. Thus, its ^{1}H NMR spectrum revealed characteristic resonances of a symmetric disubstituted aromatic compound: two multiplets were observed at ǧ 7,50 ppm (m, H-2/H-2') and ǧ 7,65 ppm (m, H-1/H'-1) (Figure-2 and Figure-4). The identification of high peak area obtained by GC analysis at retention time RT 45.82 min (Figure-3) showed the presence of diisooctyl phthalate in the MS spectrum. The molecular formula, $C_{24}H_{38}O_4$ was obtained on the basis of the [MH^+] ion at *m/z* 390. Fragments observed at *m/z* 167 and *m/z* 104 (Figure-4) correspond to the loss of parts 1 and 2 of the molecule. This compound is characterized by an antimicrobial, anti-inflammatory and anti-fouling activity [24].

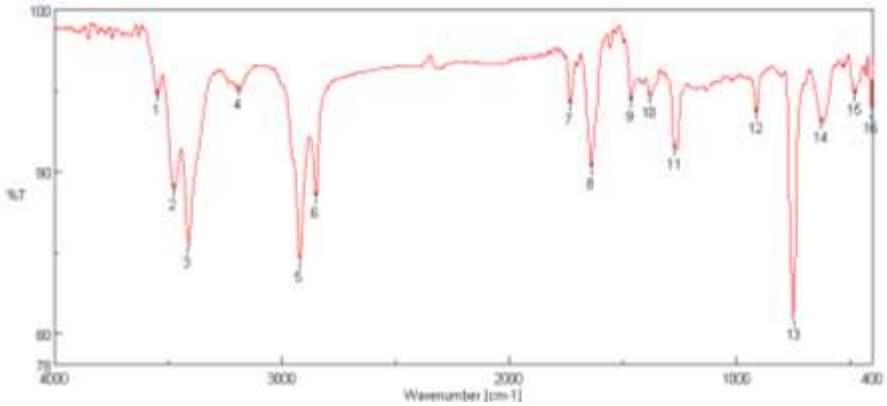

Figure 1 IR spectrum of compound 1

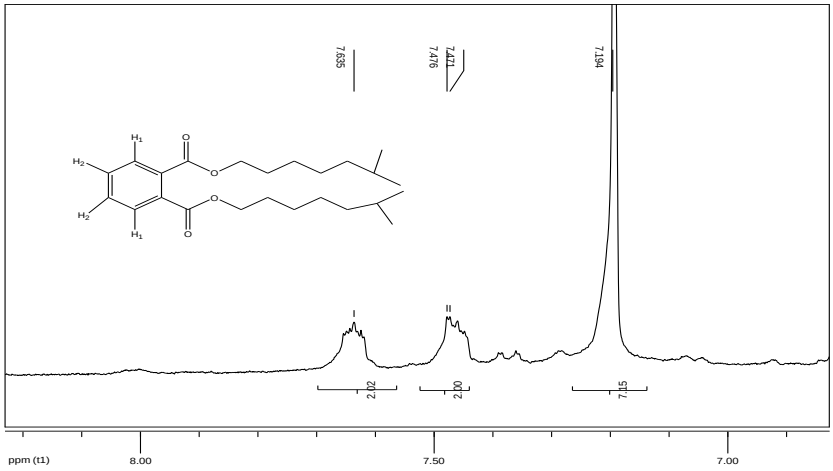

Figure 2 ^{1}H NMR spectrum in $CDCl_3$ of compound 1 (aromatic region)

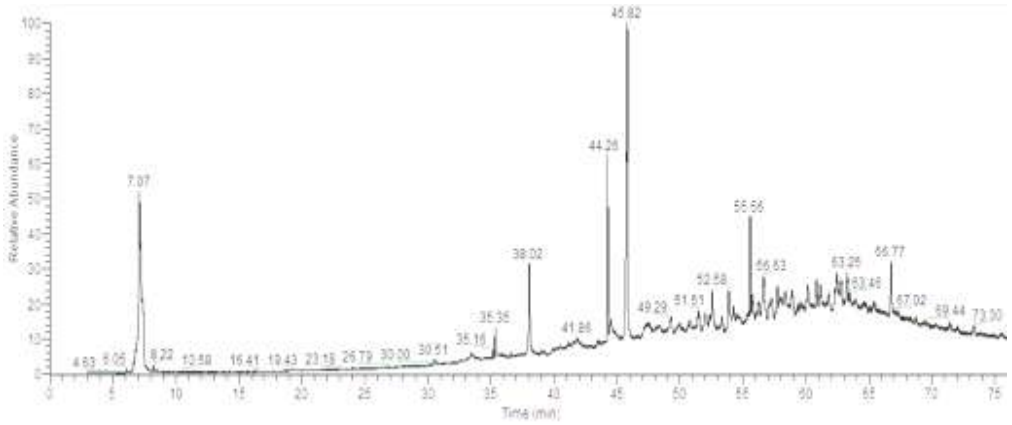

Figure 3 Chromatogram of the first sub-fraction obtained from FI

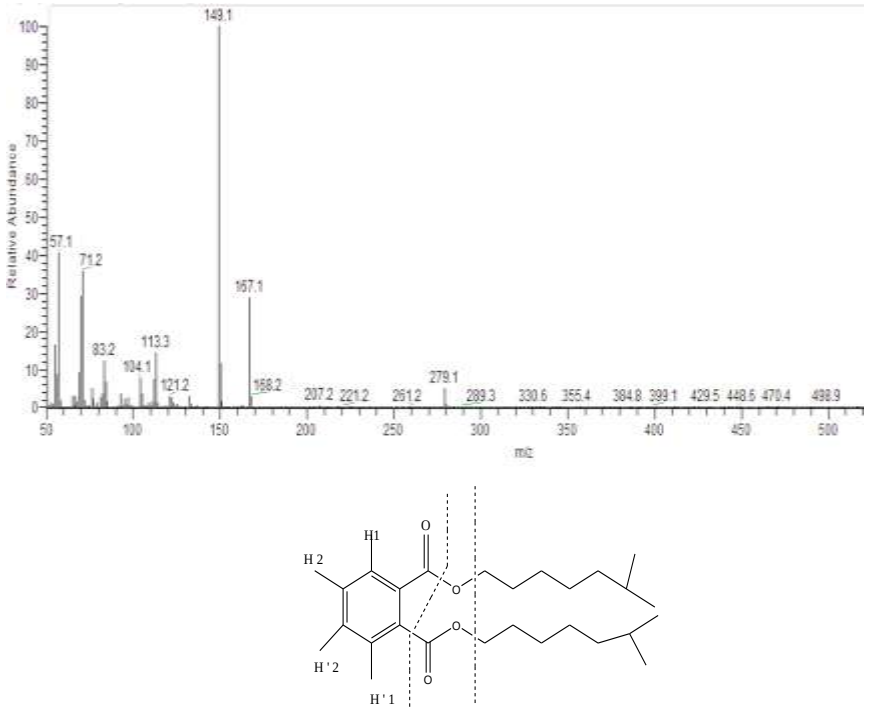

Figure 4 MS Spectrum of compound 1

Identification of compound 2 (2,2'-methylene bis (6-tert-butyl-p-cresol))

This compound was obtained from the second sub-fraction of FI. The structure of this compound after assignments of the ^{1}H-NMR signals and investigation of MS and IR spectrum was established as 2,2'-methylene bis(6-tert-butyl-p-cresol) (compound 2). The molecular formula, $C_{23}H_{32}O_2$, was obtained from the [MH^+] ion at *m/z* 340 (RT: 52.6 min) (Figure-7). The ion obtained at *m/z* 284 [M-56] refer to the loss of part 1 and the fragment observed at *m/z* 177 is attributed to the loss of part 2 [M-(56+107)] (Figure-8). The IR spectrum showed a hydroxyl group of a phenol at 3513 cm^{-1}, -C-C- bond of aromatic ring at 1631 and 1382 cm^{-1} respectively and -C-H- bond of aromatic ring at 747 cm^{-1} (Figure-5). In the ^{1}H- NMR spectrum, two doublets observed at 6.9 ppm (d, 2H, 2H, H_1) and 7,76 ppm (d, 2H, H_2) correspond to 4 symmetric

protons of two tetra substituted aromatic cycle, at 3.89 ppm (s, 2H, H_3), at 2.1 ppm (s, 6H, H_4) and the two *tert*-butyl integrating for 18 H at 1.25 ppm (H_5) (Figure-6). In the literature, this compound presents an important antioxidant activity [25].

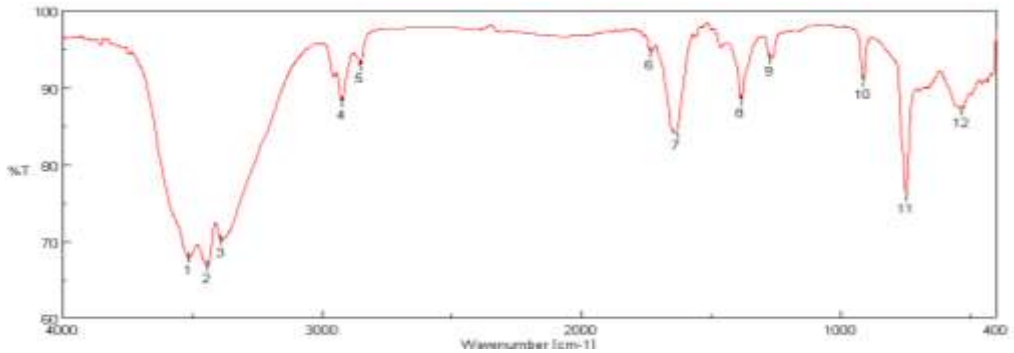

Figure 5 IR spectrum of compound 2

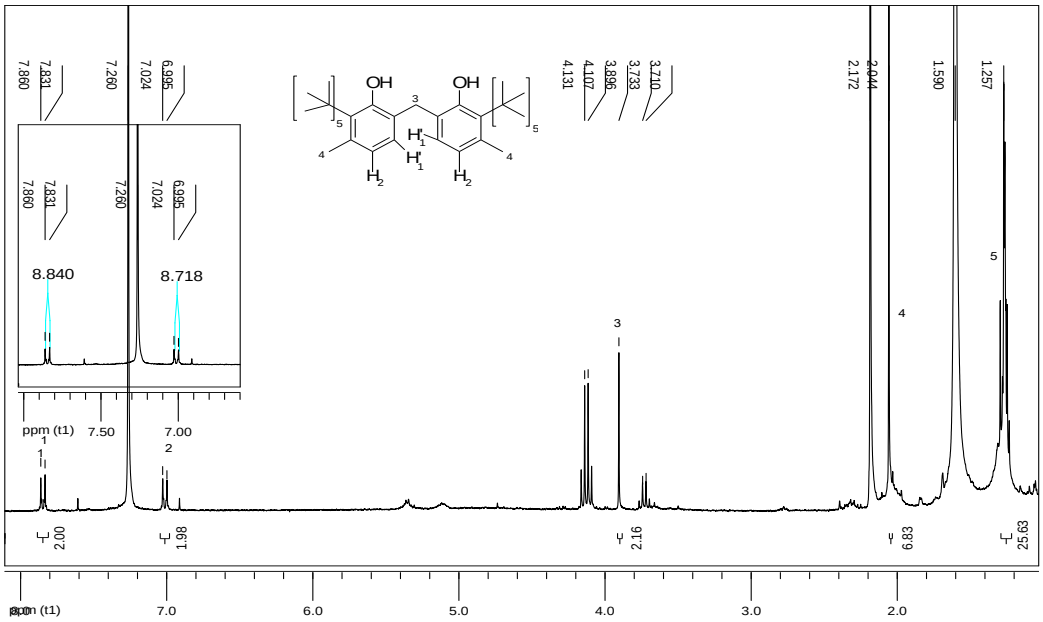

Figure 6 ^{1}H NMR spectrum in $CDCl_3$ of compound 2

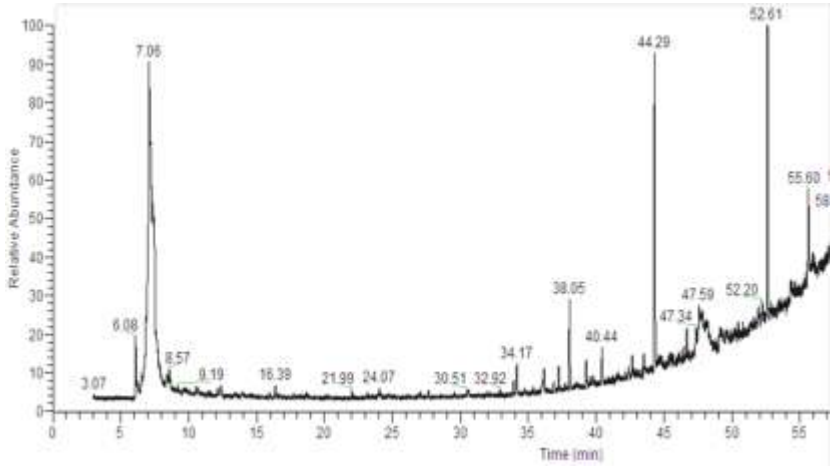

Figure 7 Chromatogram of the second sub-fraction from FI

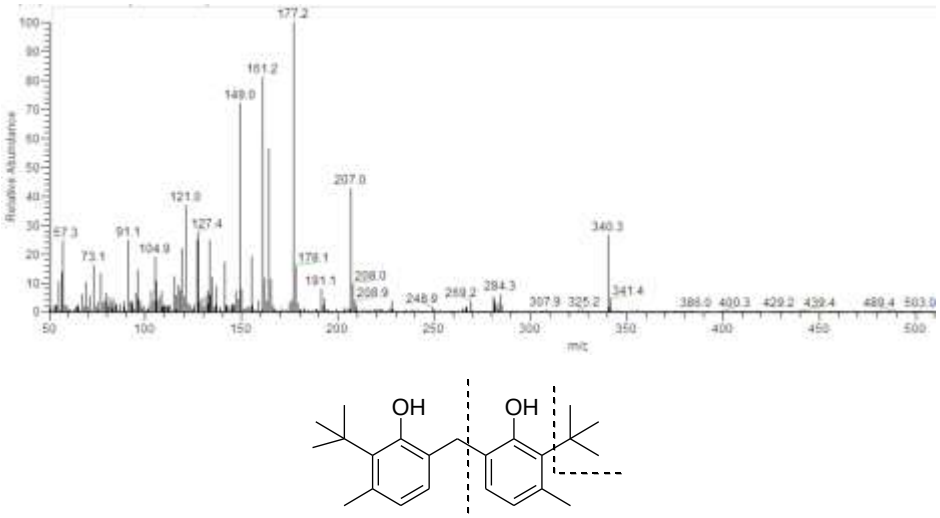

Figure 8 MS Spectrum of compound 1

Identification of compound 3 (octadecanoic acid, methly ester)

The structure elucidation of compound **3** is as follows**:** compound **3** (octadecanoic acid, methyl ester) showed IR bands for carbonyl groups at 1222 and 1091 cm^{-1} (bands 13 and 14 respectively), and -C-H- band of an alkene at 2965, 2924 and 2853 cm^{-1} (bands 4, 5 and 6) (Figure-9). The analysis of peak obtained in GC at 44.27 min (Figure-11) by MS gives these results: the ion peak was observed at *m/z* 298 corresponding to formula: $C_{19}H_{38}O_2$; the fragment observed at *m/z* 87 is attributed to the loss of $C_{13}H_{23}O_2$ chain of the molecule; the base peak is observed at *m/z* 74 and is attributed to the loss of the fraction $C_{14}H_{24}O_2$ (Figures-12 and 13). The investigation of ^1H spectrum data suggested that compound **3** also is a methylated fatty acid. Its ^1H -NMR spectrum showed the presence of 3 multiplets in the aliphatic region at 0.78 ppm (m, 3H, CH_3), at 1.3-1.9 ppm (m, 30 H, CH_2), and at 3.68 ppm (CH_3OCOCH_2-ester, 2H), The presence of a singlet at 3.8 ppm (s, CH_3OCO-, 3H) is attributed to the methyl ester (Figure-10). This compound shows antibacterial, anti-inflammatory, anti-fungal, sedative and analgesic properties [26].

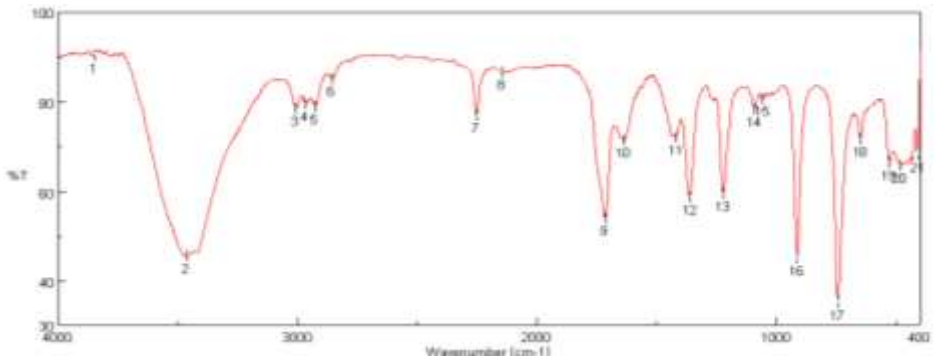

Figure 9 IR spectrum of compound 3

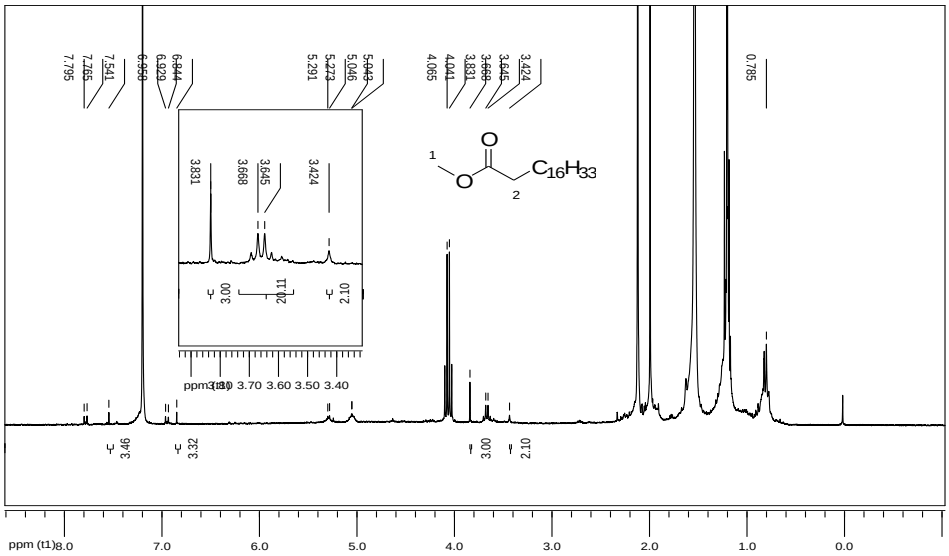

Figure 1 ^{1}H NMR spectrum in $CDCl_3$ of compound 3

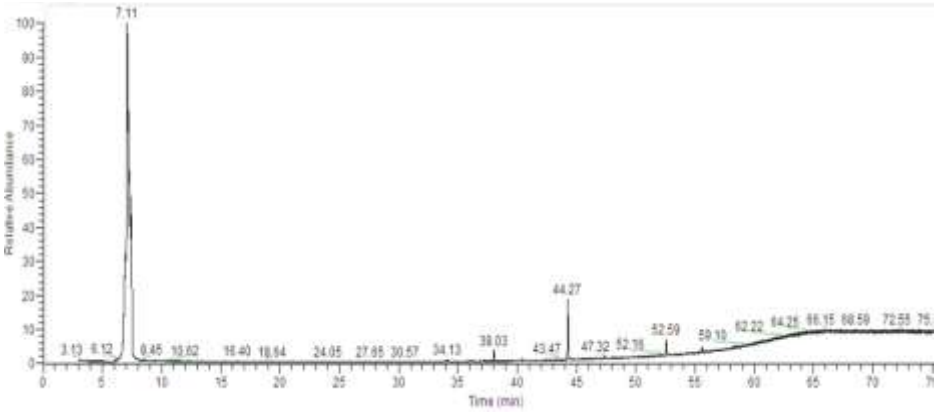

Figure 11 Chromatogram of the third sub-fraction from FI

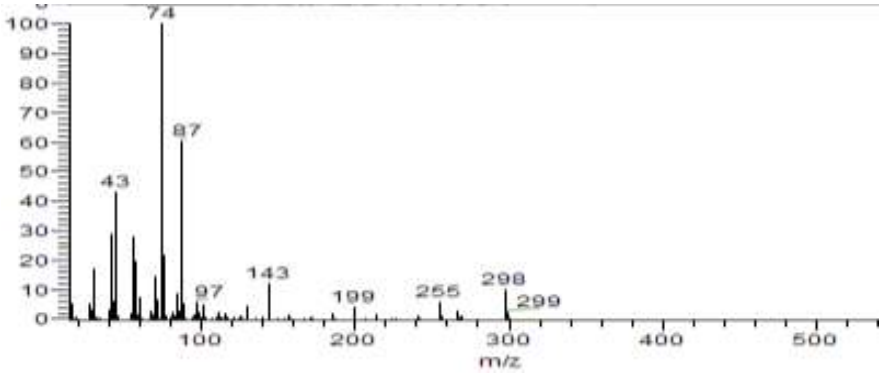

Figure 12 MS spectrum of compound 3

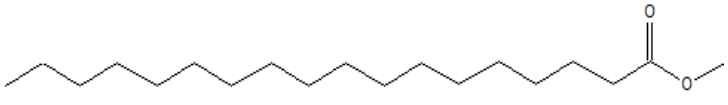

Figure 13 Chemical structure of compound 3

CONCLUSION

Phytochemical screening of *Urtica dioica* showed that there are several bioactive compounds present in the five plant extracts which could be used for therapeutic purposes. Three molecules were isolated and identified from hexane extract of *Urtica dioica*: diisooctyl phthalate, 2,2'-methylene bis(6-*tert*-butyl-p-cresol) and octadecanoic acid methyl ester; they showed an antioxidant, anti-inflammatory, anti-bacterial, analgesic and sedative activities. These results confirm the traditional uses of *Urtica dioica* in medicine and prove that this plant can be used as a source of multi resistant drugs in the future.

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