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Article

Antioxidant Activity and Isolation of β-Sitosterol from Ethyl Acetate Extract of Aerial Parts of *Consolida orientalis*

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Abstract: General phytochemical screening of the aerial parts of *Consolida orientalis* revealed the presence of steroids, terpenes, phenolic compounds, saponins, fatty acids, alkaloids. This study was conducted to investigate the bioactivities of extracts, isolation and identification the compounds from aerial parts of *C.orientalis.* The main goal of the present study is identifying and characterizing the antioxidant activity of the *Consolida orientalis* and biological isolation of active terpenoid. Aerial parts of the plant were dried at room temperature and reduced to small pieces, followed by using extraction with ethyl acetate percolation. Tree complementary analysis system was used, DPPH free radical scavenging test, total phenolic metabolites and FRAP. The total phenolic content was 38.83±2.09 mg gallic acid corresponding to g-1 extract with regarding to standard curve (y=0.0054x+0.0488, r2=0.995). IC50 value for DPPH radical – scavenging was 987.11±28.66 mgml-1. The extract was exhibited a medium reducing power compared with Vit C. The isolation and purification was afforded white crystalline powder which was subjected to physical, chemical and spectral identification by IR, ^{1}H- and ^{13}C- NMR and GC-MS. Isolated compound was identified as *β-sitosterol*. That is a terpenoid with melting point 133.4-134.5 ^{o} c and with molecular formula $C_{29}H_{50}O$.

Keywords: *Consolida orientalis*; ethyl acetate extract; antioxidant activity; *β-sitosterol*

INTRODUCTION

Thousand years ago, people had already known about the usage of natural source as medicinal agents, nowadays, with rapid development of science and technology, there are a lot of modern drugs with less side effects which were derived from natural source (1). Every year, abundant new compounds were isolated from traditional medicine or herbal (2). This also indicates that the isolated compounds from herbal plants play an very important role in pharmaceutical industry,

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however, a lot of herbal plants still have not been explored for their phytochemical constituents (3,4). Free radicals cause the oxidation of biomolecules which causes cell injury and death (5). Moreover, the oxidative stress caused by imbalance between the generation and the neutralization of free radicals by antioxidant mechanism is responsible for many human diseases, including aging, cancer and neurodegenerative disorders such as Alzheimer's disease, Parkinson's and Huntington's diseases(6).

Consolida orientalis Rech.f.(Ranunculaceae)is a native plant of southern and south-western Europe, central Asia and northern Africa. It acts mostly as a weed of winter crops, especially of winter wheat and less frequently in winter oil seed rape and winter barley. In locations where it is well naturalized and where the share of winter crops are higher the occurrence of *C. orientalis* is more homogenous. This weed species can easily gradate in crop stands when efficient control is absent.

Regarding the past studies, there are just a few reports on phytochemical analysis of the Iranian *Consolida orientalis*(7). The *C.orientalis* growing in the Turkey has been determined benzoxazolinone precursors from methanolic extract of Turkish *C.orientalis* (8). Norditerpenoid and diterpenoid alkaloids have been determined from ethanolic extract of Turkish *C.orientalis* (9,10). New norditerpene alkaloid (18-demethylpubescenine) has been determined from ethanolic extract of fresh whole plants of *Consolida orientalis* (11). Essential oils of foliar of *C.orientalis*,was collected from the Kojour, area of Iran, were studied using GC and GC-MS. The compounds Adipic acid, Phytol, Tericosan and Hexadecadine were the most abundant compounds (7). we report here the separation and identification of some terpenoid compounds from the aerial parts of *Consolida orientalis* which has not been previously reported. Thus, the main of present study is determining the antioxidant activity of ethyl acetate extraction and isolation terpenoid of *C. orientalis* aerial parts extract in order to understand the ability of this plant to be uses as a herbal medicine.

MATERIAL AND METHODS

Plant material

The aerial parts of *Consolida orientalis* were collected from the Kojour, Noshahr, in Northeastern of Iran, during in spring stage on April 2012. The Herbarium specimen was identified by Dr. Bahman eslami jadidi from the faculty of sciences, Azad University of Qaemshahr and deposited at the mentioned Herbarium. The foliar segment of plants were separated manually and were powdered after being air dried, and were stored for further analysis.

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The aerial parts of *C.orientalis* (600g) were dried at room temperature and were reduced to small pieces, then were followed by using extraction with ethyl acetate percolation. 12g (yield 5.3 % w/w) of dark green shoot extract which was stored at 4ºC.We have poured the remain of primary extract inside decanter and have done extraction of ethyl acetate extract with n-hexane (non-polar), ethyl acetate and distilled water (polar), respectively. So we have been able to separate the polar and non-polar materials of ethyl acetate extract.

Bioactivity Assays:Antioxidant activity

Analysis of total phenolics content (TPC)

Total phenolic contents were determined using the Folin-Ciocalteu method (Ragazzi and Veronese(12)). 0.4 ml of the extract was added to about 3.0 ml of phenol reagent of Folin-Ciocalteu (Merck-Schuchardt, Hohenbrun, Germany). The mixture was incubated at room temperature for 5 min and after addition of 3.0 ml of sodium carbonate, the solution incubated at room temperature for 90 min. Using spectophotometry ,the absorbance rate of the reaction was measured at 725nm. The total phenolics were expressed as mg Gallic acid antioxidant capacity.

Study of Ferric reducing antioxidant power (FRAP)

A modified method (13) was used to study FRAP rate of each solution. The FRAP reagent consisted of mixture of 0.1 M acetate Buffer (pH 3.6), 10 Mm TPTZ, and 20 Mm ferric chloride (10:1:1, v/v/v) was made. About 80µl of each extract was added to 3.6ml of reagent. Every 15 second reading were taken at 593nm (absobtion maximum) by eppendorf UV-Visible spectrophotometer (measuring was continued to 10min). calibration curves were drawn using FeSO4.

Scavenging activity against DPPH radical

1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for radical-scavenging assay of the extracts (14) .Different concentrations of extracts (240,480,720 and 1200 µg ml-1) at equal volumes were added to a 100Mm solution of DPPH . After 15min at room temperature, the absorbance was recorded at 517 nm. The experiments were repeated four times. Vitamin C and BHT were used as standard controls. IC50 values indicated the concentration of sample required to scavenge 50% of DPPH free radicals.

Extraction and Isolation Process

Silica gel column chromatography (CC mesh 60-120) with dichloromethane: petroleum ether (50:50, 70:30, 80:20, 90:10,100:0) was applied for the ethyl

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acetate extract (7g) giving eight fractions (E_1-E_8). The fractions E_6, E_7 and E_8 (700 mg) were identical and added to SPE column with dichloromethane to obtain 15fractions (A_1-A_{15}). The fractions A_{11}, A_{14} and A_{15} (240 mg) were identical and added to silica gel CC with dichloromethane: ethyl acetate (100:0, 95:5, 90:10, 85:15 and 80:20) to give 25 fractions (B_1-B_{25}). The fractions B_{10} and B_{11} (170 mg) were identical and added to SPE column with dichloromethane: petroleum ether (50:50, 80:20,90:10, 95:5, 100:0) and dichloromethane: ethyl acetate (95:5, 93:7, 90:10) to obtain 25fractions (C_1-C_{25}). The fractions C_{15}-C_{20} (36 mg) were identical and were controlled by TLC (Aluminum sheet whit silica gel 60 F_{254} and spraying Anisaldehyde-H_2SO_4 as reagent.

Instrumentation

The IR spectra measured on SHIMADZ and the ^{1}H-NMR and ^{13}C-NMR spectra were measured on a BRUCHER AVANCE 500DRX (500 MHz for ^{1}H and 125 MHz for ^{13}C) spectrometer with $CDCl_3$ as the solvent and chemical shifts are given in δ (ppm). The MS data was recorded on an Agilent Technology Detector (MS model). For TLC analysis silica gels Aluminum sheets (MERCK) were used and Anisaldehyde-H2SO4 sprays, followed by heating, were applied to detect spots on sheets.

Statistical Analysis

Experimental results are expressed as means ± SD. All measurements were replicated three times. The data were analyzed by an analysis of variance ($p < 0.05$) and the means separated by Duncan's multiple range tests. The EC50 values were calculated from linear regression analysis.

Results and Discussion

The total phenolic content was estimates as 38.83±2.09 mg Gallic acid equivalent g-1 extract referencing to standard curve ($y=0.0054x+0.0488$, $r^2=0.995$) .Figure 1 shows calibration curve for Gallic acid as standard.

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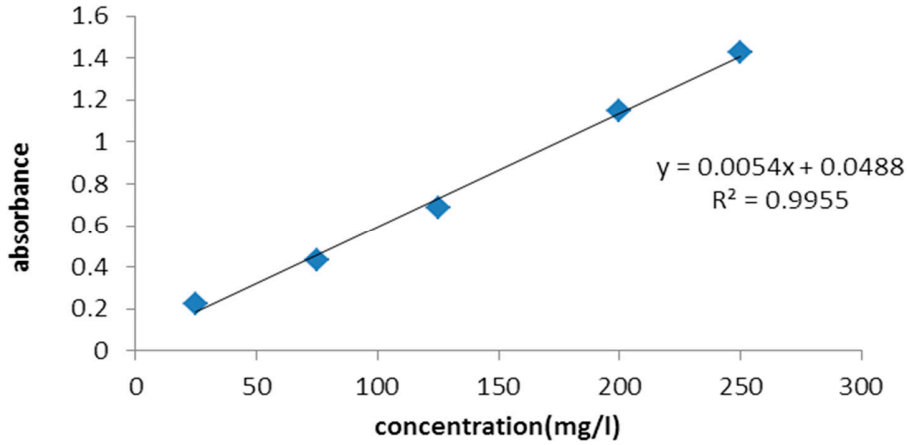

Fig. 1. Calibration curve for Gallic acid as standard.

In DPPH method, IC50 values of BHT, Vitamin C and extract were 15.28 ±0.37, 3.67±0.028 and 987.11±28.66 mgml^{-1}, respectively. Figure 2 shows percent of inhibition at concentration of extract. The extract showed a high reducing power at 1200 mg ml^{-1}.

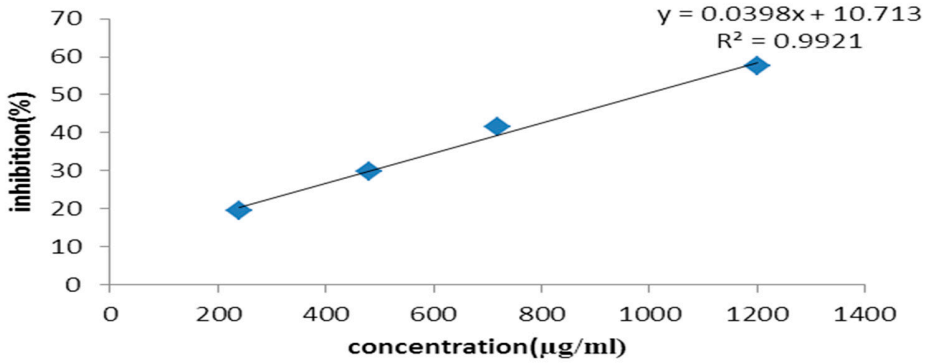

Fig .2. Percent of inhibition at concentration of *Consolida orientalis* extract.

Figure 3 shows curves for the reducing powers of Extract. Vitamin C and BHT as controls.

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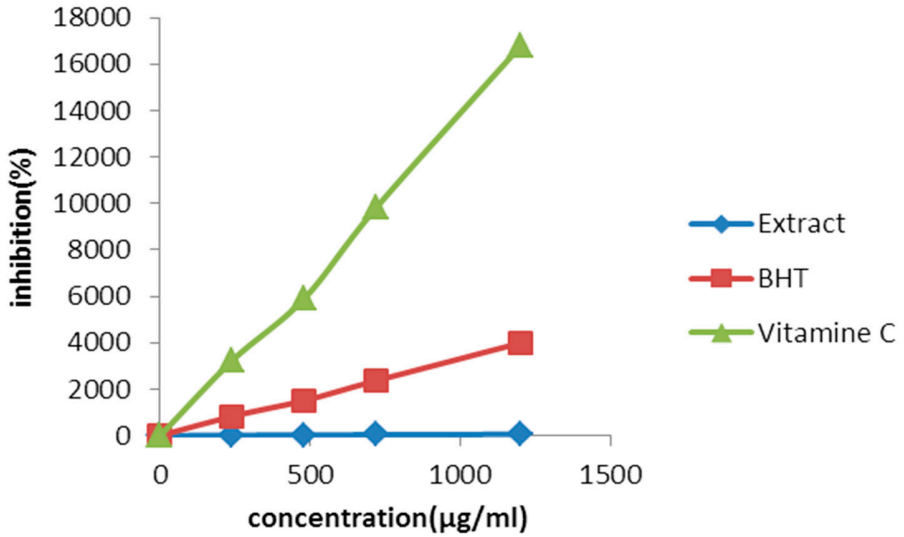

Fig.3.Reducing power of *Consolida orientalis* .Vitamin C and BHT was used as control.

In FRAP method percent of inhibition for ethyl acetate extract is 21.97, 21.36, 21.19 and 21.305. FeSO4 was used as standard. Figure 4 shows calibration curve for FeSO4 as standard.

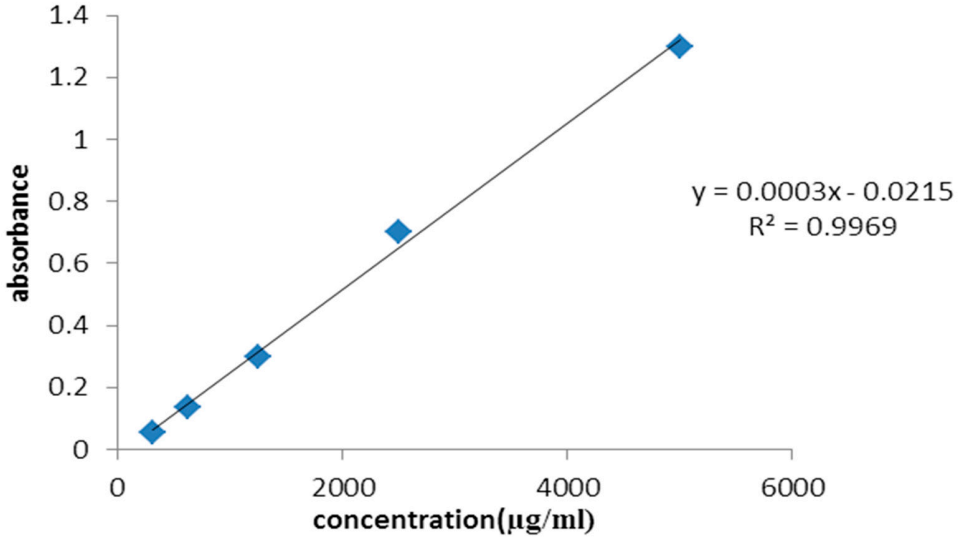

Fig. 4. Shows calibration curve for FeSO4 as standard.

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Table-1: shows inhibition, absorbance and IC_{50} for extract, vitamin C and BHT in DPPH and FRAP methods.

Method		Absorbance	Inhibition (%)	IC_{50}
DPPH	Extract		19.36-29.64-41.5-57.5	987.11
DPPH	VitaminC		26.95-36.89-54.6-83.92	3.67
DPPH	BHT		27.89-37.8-46.07-51.44-51.91	15.28
FRAP	Extract	0.374-0.363-0.36-0.362	21.97-21.36-21.19-21.305	
FRAP	VitaminC	0.374-0.363-0.36-0.362	2031.66-2008.33-1998.33-2015	

Table 1. Inhibition, absorbance and IC_{50} for extract vitamin C and BHT in DPPH and FRAP method.

Total phenol compound, as determined by the Folin Ciocalteau method, was reported as gallic acid equivalents and total flavonoid content was reported as the quercetin equivalent/g of extract powder by AlCl3 colorimetric method. This plant showed high total phenol and flavonoid contents. Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities (15). Studies have shown that increasing levels of flavonoids in the diet could decrease certain human diseases (16).

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples (17). DPPH is a stable nitrogen-centered free radical, the color of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron- donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers (18). It was found that the radical-scavenging activity of the extracts increased with increasing concentration. The high total phenol and flavonoid contents of this plant may lead to its good DPPH-scavenging activity.

According to Hodzic *et al.* (19), FRAP assay had been used to determine antioxidant activity as it is simple and quick. Besides that, the reaction is reproducible and linearly related to molar concentration of the antioxidants. However, some disadvantage was found in this method as FRAP assay does not react fast with some antioxidants such gluthathione (20). Schafer and Buettner (21)

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stated that FRAP assay still can be used for assessment of antioxidant activity in plants materials as humans only absorb limited amount of gluthathione. Higher FRAP values give higher antioxidant capacity because FRAP value is based on reducing ferric ion, where antioxidants are the reducing agent. Antioxidants are compounds capable of donating a single electron or hydrogen atom for reduction.

On subjection to IR spectroscopic analysis, the absorption band 3440.77 cm^{-1} can be observed, that is characteristic of O-H stretching. Absorption at 2869.88 cm^{-1} and 2960.88 cm^{-1} is due aliphatic C-H stretching. Other absorption frequencies include1465.80 cm^{-1} as resulted C=C stretching for cyclic $(CH_2)_n$.

^{1}H-NMR (fig.7): δ_H 0.69 (3H, s, H-18), 0.81 (3H br s, H-26), 0.83 (3H, br s, H-27), 0.84 (3H, s, H-24), 0.92 (3H, d, J=5.8Hz, H-21), 1.02 (3H, s, H-19), 3.53 (1H, m, H-3), 5.36 (1H, m, H-3). ^{13}C-NMR (fig.8 and 9): δ_C (from C-1 to C-27) 37.21, 31.6, 71.75, 42.24, 140.71, 121.67, 31.86, 31.86, 50.08, 36.11, 21.04, 39.73, 42.24, 56.72, 24.27, 28.21, 56.01, 11.82, 19.79, 33.9, 18.74, 33.9, 26.02, 45.78, 29.1, 19, 19.36.

The strong molecular ions were given at m/z 415 and the characteristic peaks were given at m/z 400 that corresponds to [m- CH_3]. One weak ion peak at 370m/z that corresponds to loss of HO^+ =CH-CH_3 and Other ion peak at m/z 382 that corresponds, to [m- (H_2O+CH_3)]. Other ion peaks at m/z 274 due to the formation of carbocation by β bond cleavage of side chain leading to the loss of $C_{10}H_{21}$. The characteristic peaks were given at m/z 330 that corresponds to [m- C_6H_{13}]. The molecular weight and fragmentation pattern indicate that the compound is β-*sitosterol* (fig.5). That is a terpenoid with melting point 133.4-134.5^o c and with molecular formula $C_{29}H_{50}O$ (P= .02). compering the MS and NMR data with the results of other researchers.

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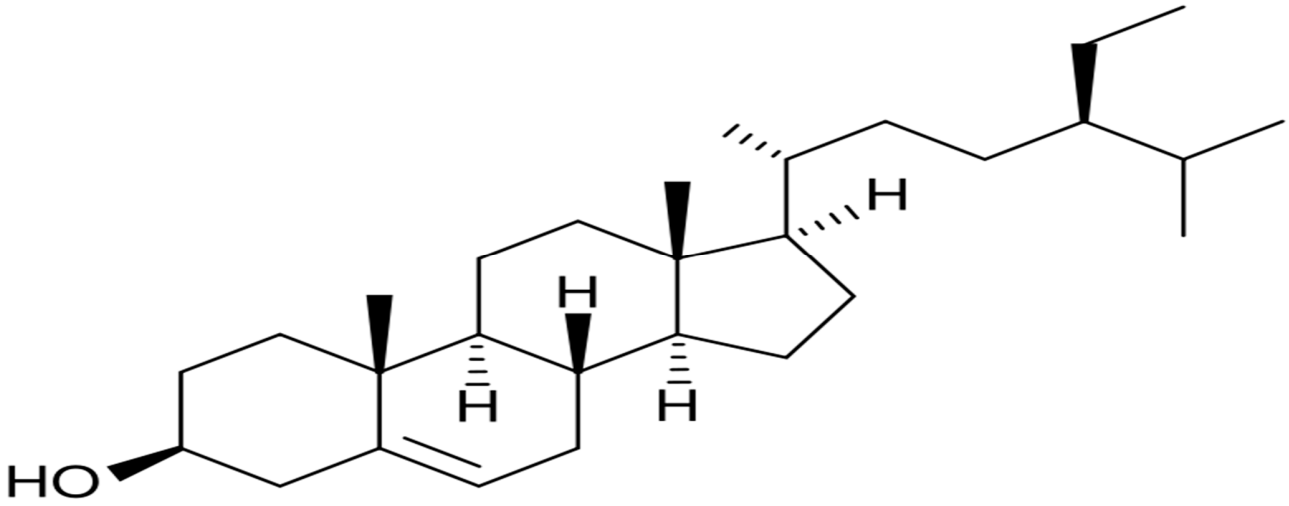

Fig. 5. The structure of isolated compound(βsitosterol (C29H50O; Mol.Wt: 414.71)) from *C.orientalis*

CONCLUSION

In general, pharmacological assessment of *C.orientalis* ethyl acetate extract indicates few interesting activities like Antioxidant activity of this plant. Since, ethyl acetate extract of *C.orientalis* showed Antioxidant activity, it can be assumed that there are various secondary metabolites in the solution and some of them may function synergistically. In fact, extract evaluations must be performed to understand the real mechanism of this effect.

From the above findings, β-sitosterol was isolated from ethyl acetate extract of the aerial parts of *C.orientalis* **(**It was carried out by means of various physical (solvent extraction, TLC, Column chromatography) and spectral techniques)that is a known phyto estrogen with many reported bio activities including anticancer and antibacterial and etc(22).

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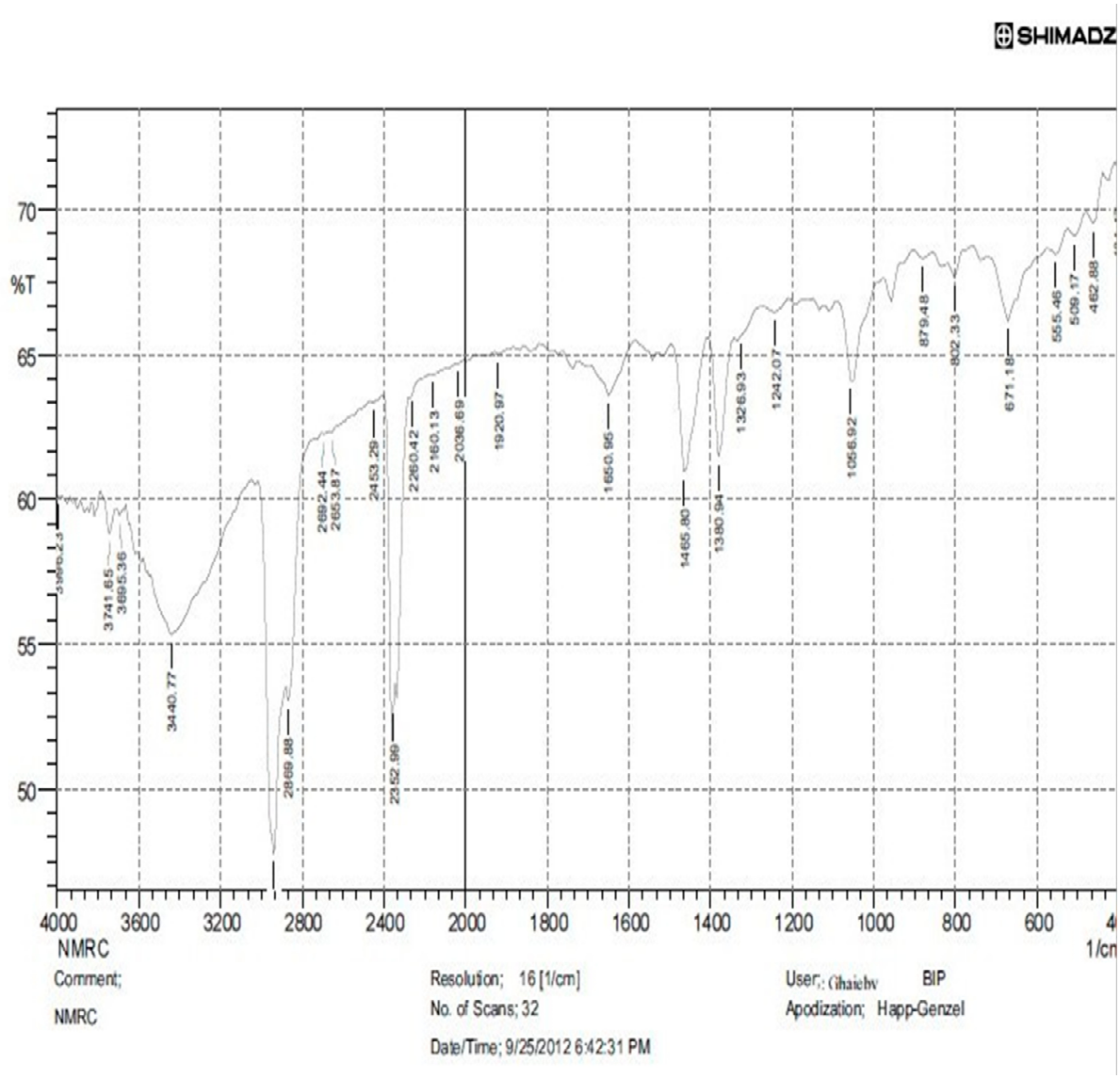

Fig. 6.IR Spectrum of Isolated compound from the ethyl acetate extract of *C.orientalis.*

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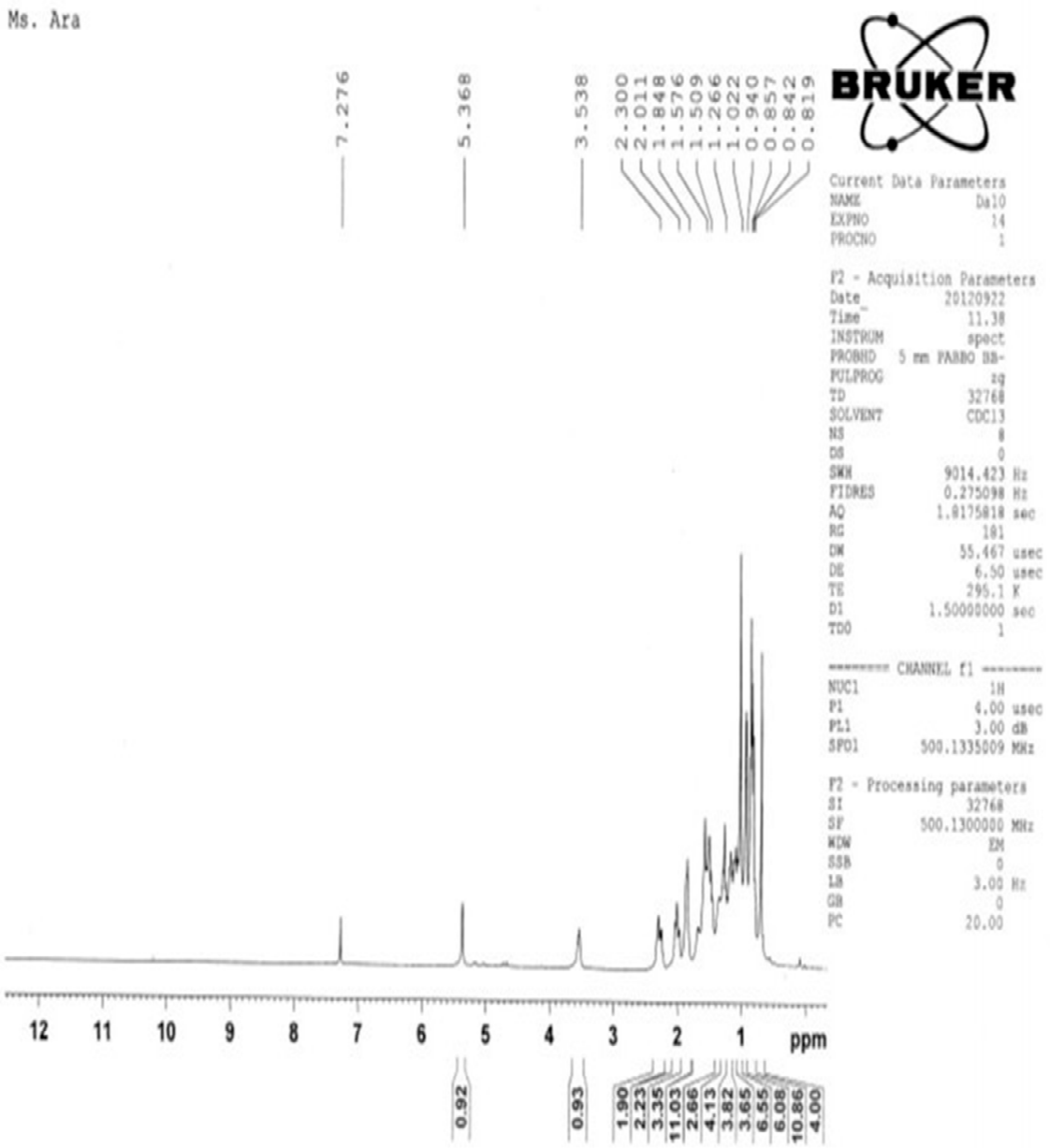

Fig. 7.^{1}H-NMR Spectrum of Isolated compound from the ethyl acetate extract of *C.orientalis.*

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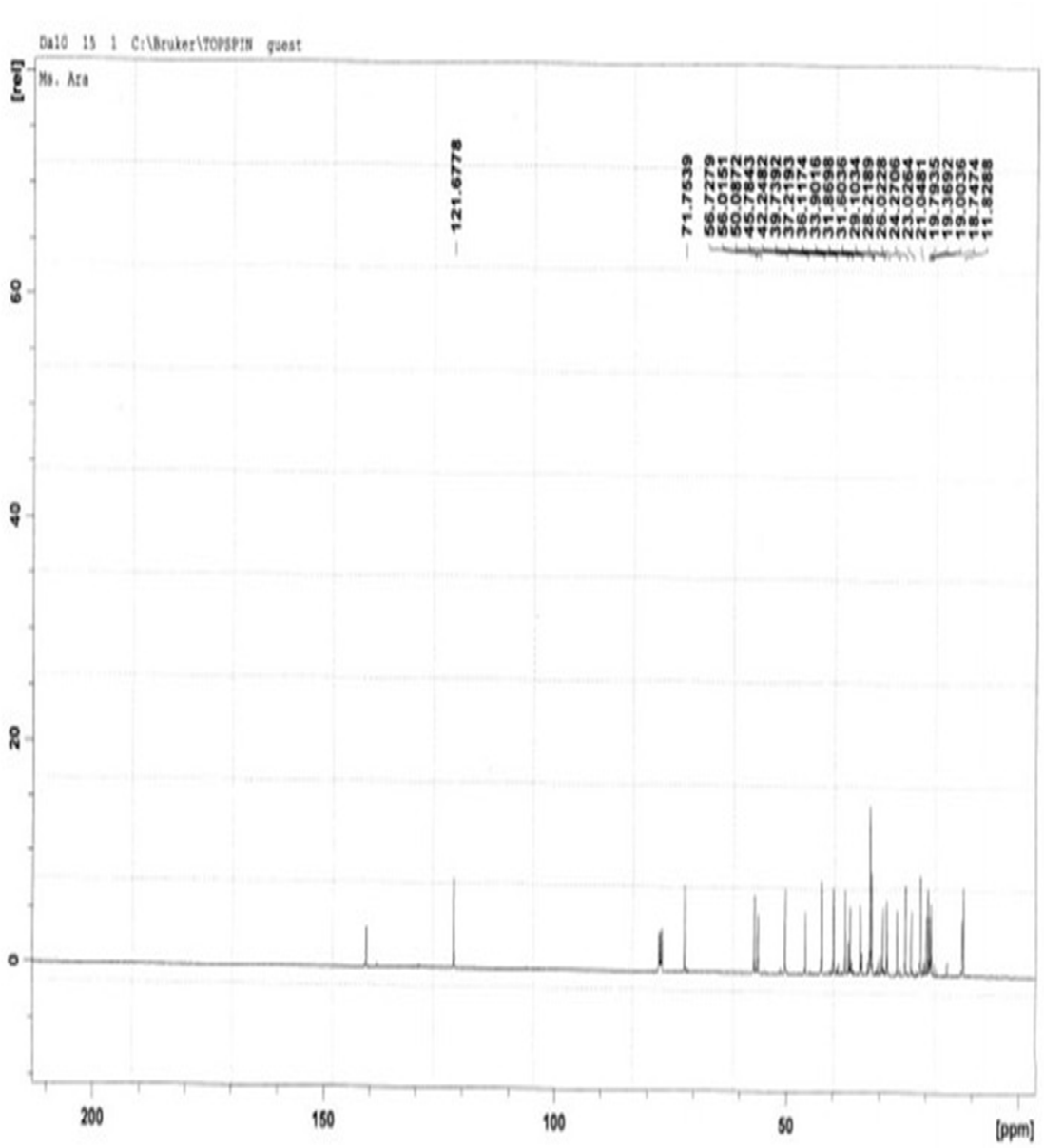

Fig.8.^{13}C-NMR Spectrum of Isolated compound from the ethyl acetate extract of *C.orientalis.*

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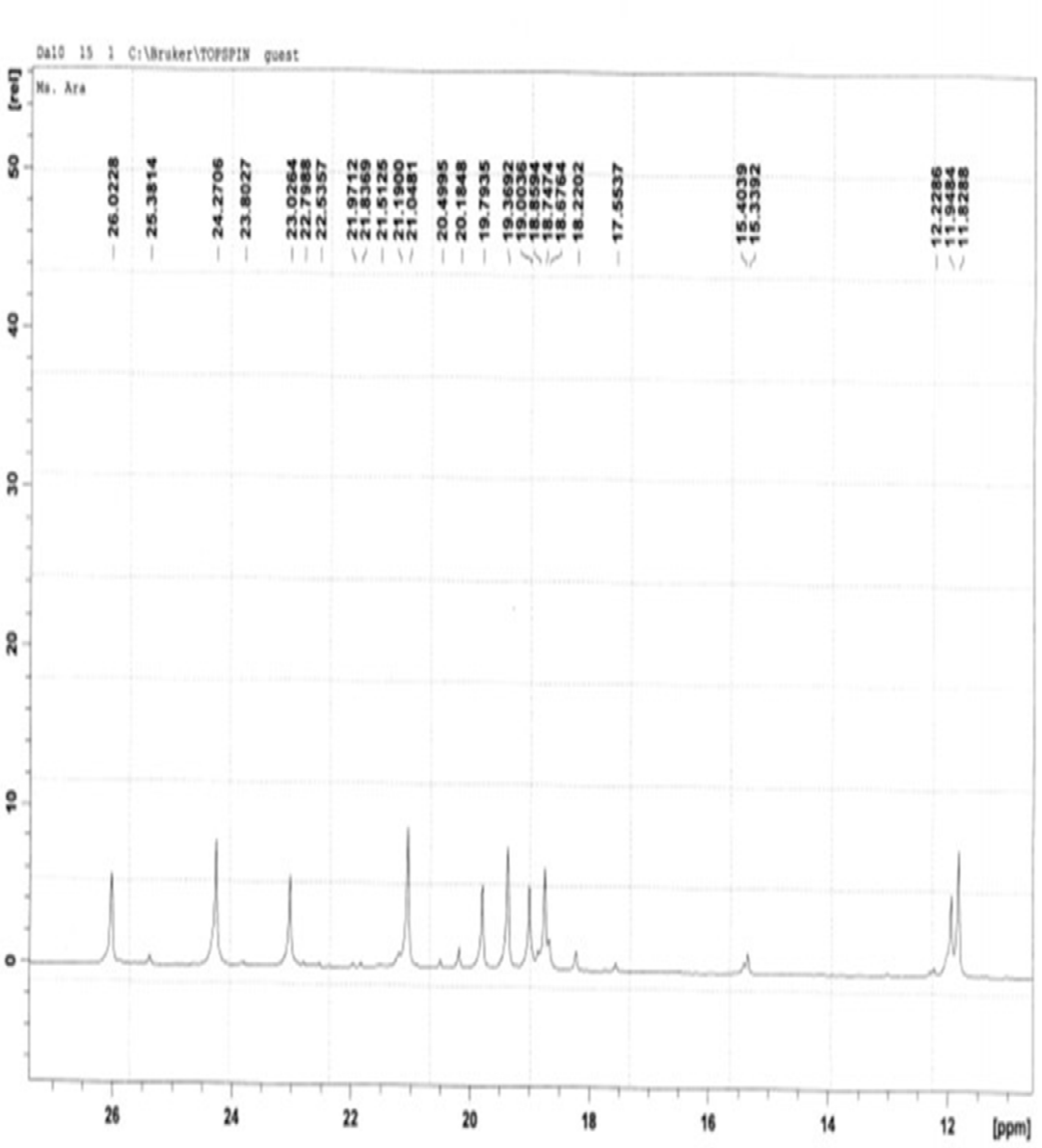

Fig. 9. Expanded ^{13}C-NMR Spectrum of Isolated compound from the ethyl acetate extract of *C.orientalis*.

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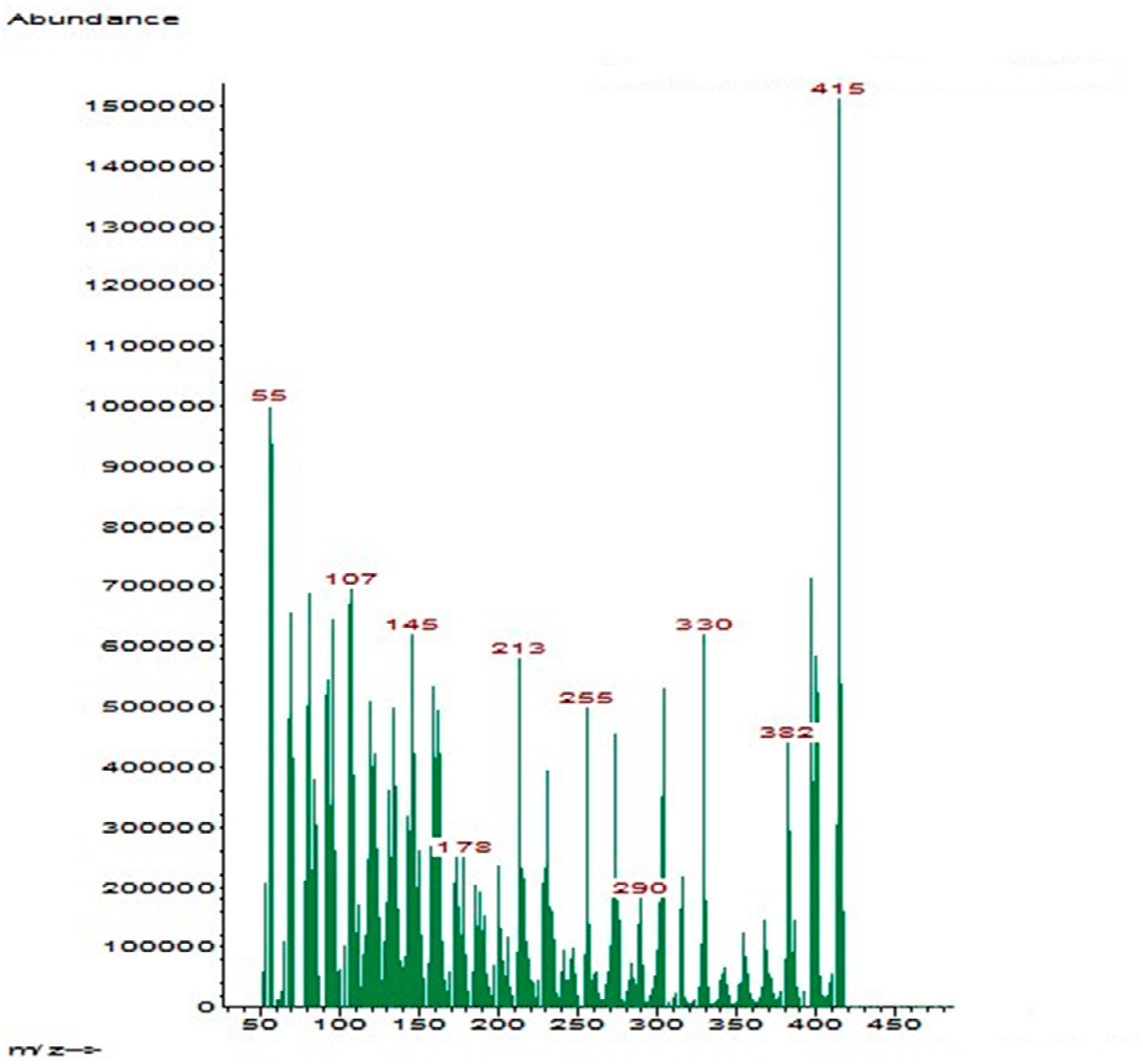

FiFig.10.GC-MS Spectrum of Isolated compound from the ethyl acetate extract of *C.orientalis*.