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New Compounds in Extract of *Eryngium Creticum Lam* and Preliminary Biological Activity Evaluation

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ABSTRACT

The identification of 6, not previously recorded, compounds, in an extract of *Eryngium creticum lam*, native in Jordan, have been observed using LC-MS/MS. The compounds are isobutyl 3-(diheptylcarbamoyl) benzoate, 3-Nitrophthalic anhydride, Metamitron, 1, 3-Diacetylindole, The baine and Clemizole. The extract was evaluated for cytotoxicity against human colorectal cancer cells HT29 and SW480.

Keywords: *Eryngium creticum lam*, LC-MS/MS, cytotoxicity, colorectal cancer cells.

1. INTRODUCTION

Eryngium is the largest and most taxonomically complex genus of the family Apiaceae (Calvino, Martinez, Downie, 2008). *Eryginium creticum Lam* is a perennial or biennial, glaucous, globous herb with a height of up to 20-50 cm and is widely distributed in Jordan (Dammous, et al., 2014). There is a current scientific interest in the plant because of its traditional and current use as a diuretic, a laxative and as a remedy for snake and scorpion bites in the rural areas (Alkofahi, et al., 1997) as well having hypoglycemic effects (Jaghabir, 1991, Kasabri et al., 2011) and antibacterial activity (Dirani et al., 2015).

Presently, there is an increasing interest, both in the industry and in the scientific research in medicinal herbs because many have strong biological properties. These properties are due to the presence of many substance types, including vitamins, flavonoids, terpenoids, carotenoids, phytoestrogens, and minerals. Over years, numerous plants have been studied chemically from the viewpoints of biosynthesis of active constituents (Cutler, 1999). The success of natural products in drug discovery is well documented (Lahlou, 2013; Koehn et al., 2005). Of the 877 small-molecule "New Chemical Entities" (NCEs) introduced between1981-2002, 49% were natural products, semi-synthetic natural product analogues or synthetic compounds based on natural-product pharmacophores (Newman et al., 2003) and also for cancer drugs from the 1940's to 2010 (Newman et al., 2012). Since 2002 the World Health Organization (WHO) has taken an interest in traditional medicine (WHO, 2002) and recently published its strategy on the subject for 2014-2023 (WHO, 2014). Arab countries, particularly Jordan, are distinguished by a great wealth of plant species with medicinal properties. Thus, it is very necessary to conduct scientific studies on these indigenous plants, especially those currently used locally as medicines.

Cancer is one of the current and on-going major world health concerns. In this study, we have investigated a plant extract in an effort to discover new and effective cancer drugs. The total extract was subjected to an initial biological activity screening by cytotoxicity assay and cell-based proteasome inhibition assay. In the future the extract will be fractionated and re-examined, until the active pure compounds are isolated.

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Previous work has shown the presence of a wide range of compounds in the genus *Eryngium* (Al-Khalil, 1994; Çelik, 2011; Wang, 2012; Mohammd hosseini, 2013). The present study, using LC-MS/MS, has identified 6 compounds not previously listed as being present in this plant's extracts.

Experimental

Plant collection and preparation of powders

Fresh plants were gathered in north Jordan at an altitude of 350 m, between September and November in 2013. The plants were cleaned physically, washed with water, and dried at room temperature in well ventilated air, after which the roots were transformed to powder by grinding. The powders were stored in sealed clean glass containers, away from light, refrigerated until use.

Soxhlet extraction

The sample of (1.5 kg) of dried roots was removed from the refrigerator and kept at room temperature (≈28°C) for 30 minutes. 3 x 50 g samples were placed in filter thimbles (22 x 90 mm, Advantec) and covered with cotton wool to retain all the material within the thimbles during the extractions. The dried root material was extracted, firstly by n-hexane, then exhaustively by methanol for about 9 hours following Alali et al.'s (Alali, 2013)] modification of Shafiee's method (Shafiee, 1979) . The combined methanol extracts were filtered and concentrated by rotary evaporation at 45°C, yielding 47 g of concentrate from the 150 g of root extracted. The extract concentrate was stored below 5 °C until analyzed by LC-MS/MS.

Liquid chromatography-MS/MS

The extracted crude with solvent was analyzed using an Agilent 1200 Liquid chromatography system equipped with an API 3200 triple quadrapole mass spectrometer detector with an electrospray ion source (A B Sciex, USA). The chromatographic column was a Unisol C18 column (46 x 150 mm) with 5 µm film thickness (Bonna-Agela Technologies, USA). Injection volumes were 10-50 µl, the mobile phase flow rate was 500 µl/min. The water and methanol eluent was programmed as shown in Table 1.

Table 1. Solvent Programme

Time (min)	Water (%)	Methanol (%)
0.00	90.0	10.0
5.00	90.0	10.0
40.00	10.0	90.0
55.00	90.0	10.0
60.00	90.0	10.0

Cell Viability Assays

The acute cytotoxic effect of the extract on human cancer cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assays as described previously (Alali, 2013; Wu, 2005). Human colorectal cancer cell lines SW480 and HT29 were generously provided by Dr. Rick F. Thorne (University of Newcastle, Australia) and were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS) (Bio Whittaker, Verviers, Belgium). Briefly, cells were seeded at 5000/well onto flat-bottomed 96-well culture plates and allowed to grow for 24 hours before the desired treatment. Cells were then labeled with MTT from the Vybrant MTT Cell Proliferation Assay Kit (Molecular Probes, Eugene, UR) according to the manufacturer's instructions and resulting formazan was solubilized with dimethyl sulphoxide (DMSO). Absorbances were read with a microplate reader at 540nm.

Results and Discussions

Effect of moisture content on the extraction process

Air dried ground samples, water content 7.8%w/w, with particle size of less than 150µm (roots) used rather than the undried materials. Moreover, wet samples can cause operational problems with analytical instruments such as LC-MS/MS. High moisture contents also resulted in lower extractions. The most favourable extraction time was found to be 9 hours (31.3% yield), after 5 hours the yield was much lower (17.3 %).

Qualitative Analysis

The identification of the compounds was based on the mass spectra of compounds in the peaks in the chromatogram of the extract when compared with those in NIST Standard Reference Data (National Institute of Standards and Technology). The total ion current chromatograms, 0-60 min. are shown below, (+)-ESI TIC (Figure 1) and (-)-ESI TIC (Figure 2).

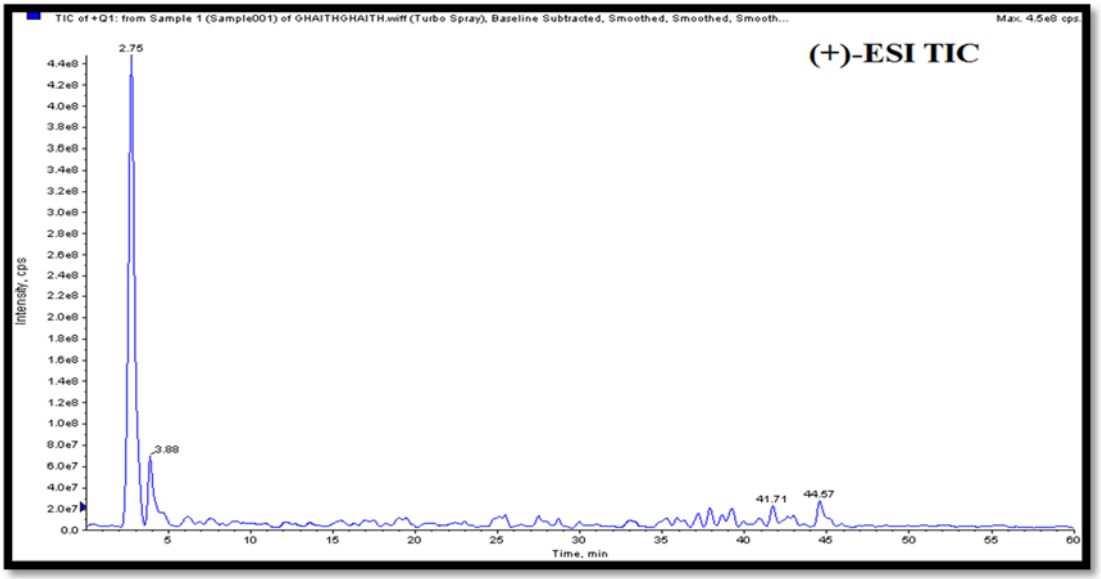

Figure 1: (+)-ESI TIC of MeOH extract of *Eryngium creticum Lam*

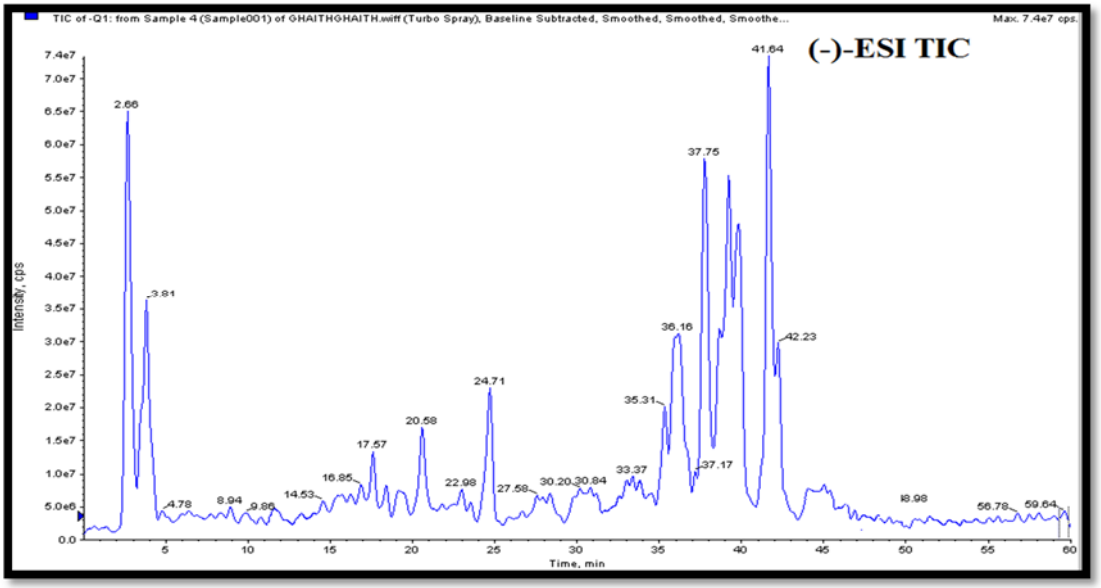

Figure 2: (-)-ESI TIC of MeOH extract of *Eryngium creticum Lam*

As can be seen the (-) –ESI TIC was more informative than the (+)-ESI TIC mode chromatogram.

The six compounds, not previously recorded as being present, in Eryngium creticum Lam are Isobutyl 3-

(diheptylcarbamoyl) benzoate, 4-Nitrophthalic anhydride, Metamitron, 1,3-Diacetylindol, Thebaine (paramorphine) and Clemizole, as characterized below.

3.2.1 Isobutyl 3-(diheptylcarbamoyl) benzoate

The (-)-ESI TIC of crude extract has a peak at 17.564 min. The MS data (Figure 3) confirms the presence of isobutyl 3-(diheptylcarbamoyl) benzoate. The molecular ion peak corresponded to a molecular formula of $C_{26}H_{43}O_3$ (m/z 417.0 $[M-H]^-$ calc. 417.63); the peak at m/z 373.0, was attributed to loss of CO_2; the peak at m/z 360.1, attributed to loss C_4H_9, the peak at m/z 317.9, attributed to loss of C_7H_{15}, and one at m/z 176.5, which was attributed to loss of $C_{15}H_{30}NO$. The fragmentation pattern is depicted in Figure 4. The compound has thus been identified as isobutyl 3-(diheptylcarbamoyl) benzoate, the first report of this compound in *Eryngium creticum Lam.*

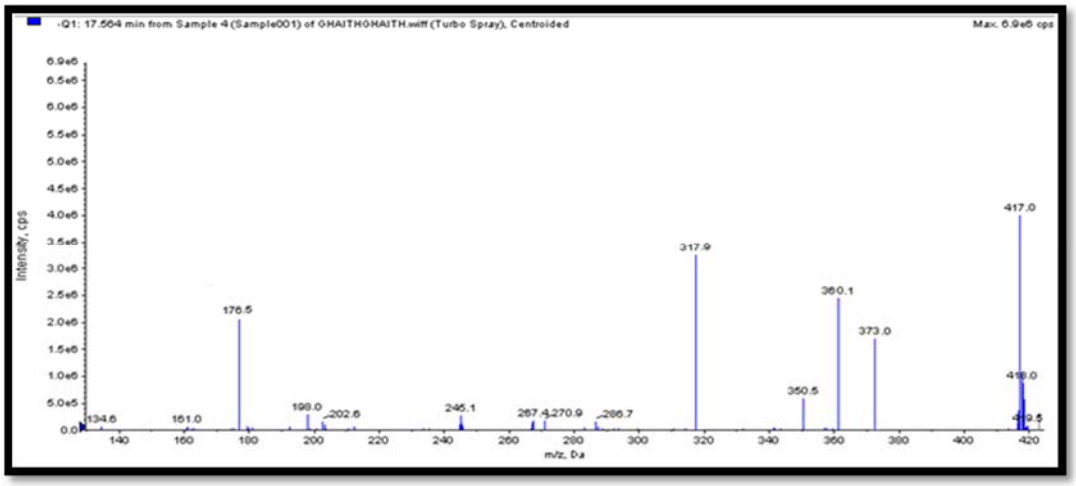

Figure 3: The mass spectrum of the molecular ion from the peak at 17.564 min

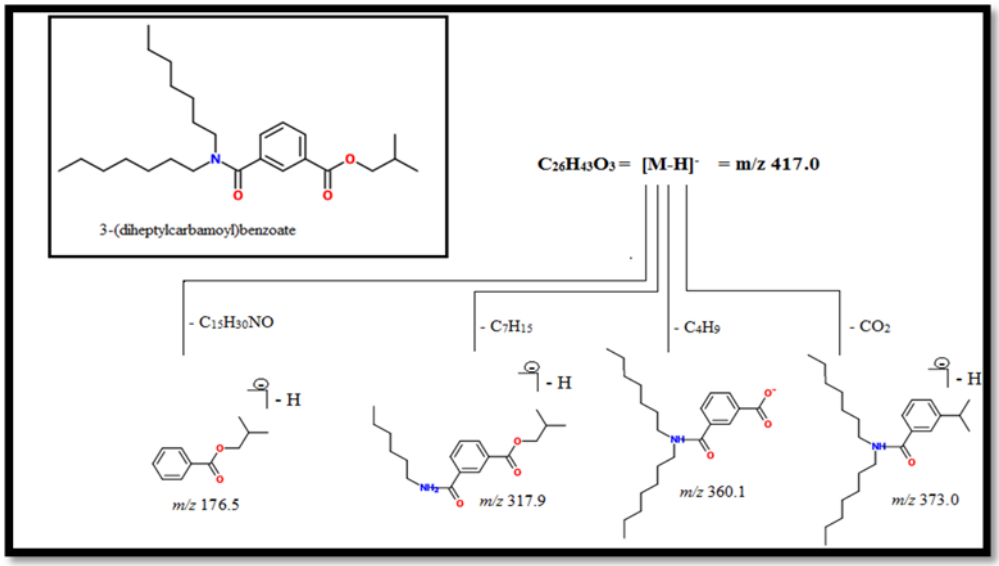

Figure 4: MS Fragmentation pathway of isobutyl 3-(diheptylcarbamoyl) benzoate

3.2.2 3-Nitrophthalic anhydride

The (-)-ESI TIC chromatogram of the extract revealed a peak at 20.590 min. The mass spectrum identified the compound as 3-Nitrophthalic anhydride. The molecular ion peak corresponded to a molecular formula of $C_8H_3NO_5$ (m/z 192.2 $[M-H]^-$). The mass spectrum (Figure 5) showed a peak at m/z 148.3, attributed to loss of CO_2; a peak at m/z 164.3, attributed to loss of CO; a peak at m/z 146.0, was attributed to loss of NO_2; a peak at m/z 140.2, was attributed to loss of NO_2 followed by loss of C_4H_4; a peak at m/z 153.2, attributed to loss of C_3H_3; a peak at m/z 120.6, attributed to loss of CO followed by CO_2. The fragmentation pattern is depicted in Figure 6. The compound has thus been identified to be 3-Nitrophthalic anhydride, the first report of this compound in *Eryngium creticum Lam.*

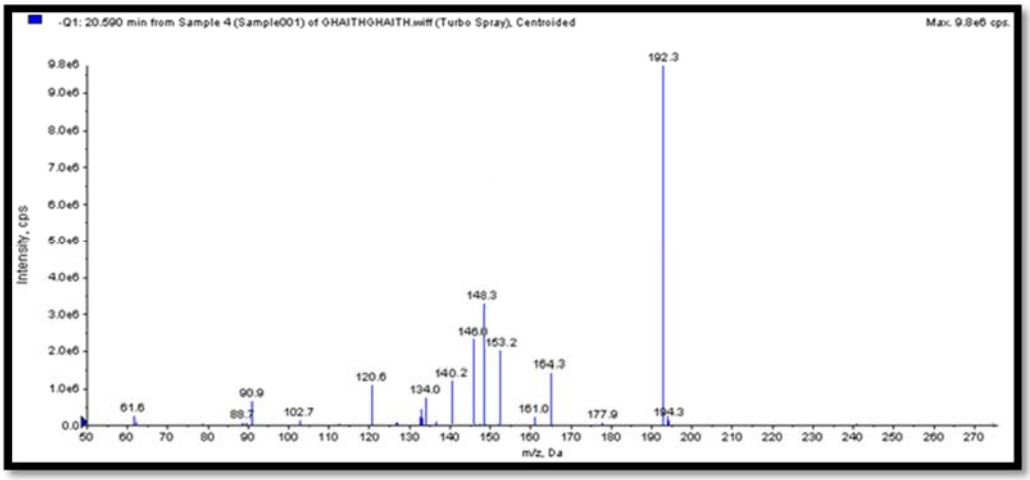

Figure 5: The mass spectrum of the molecular ion from the peak at 20.590 min

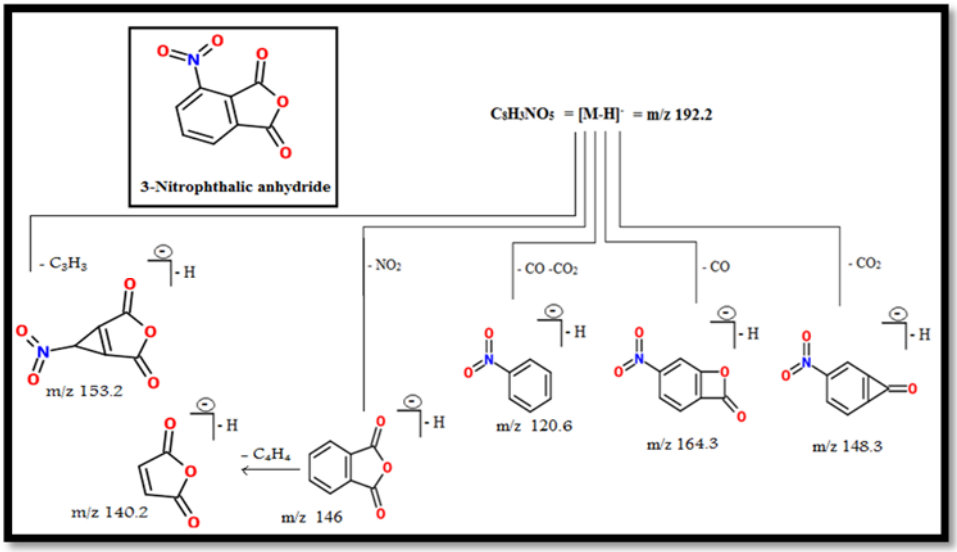

Figure 6: MS Fragmentation pathway of 3-Nitrophthalic anhydride

3.2.3 Metamitron

The (+)-ESI TIC chromatogram of the extract revealed the presence of a compound at 2.542 min. The molecular ion peak corresponded to $C_{10}H_{10}N_4O$ (m/z 203.5 $[M+H]^+$ calc. 202.2126). The mass spectrum (Figure 7) showed a peak at m/z 78.3, was attributed to loss of $C_4H_5N_4O$; a peak at m/z 104.2 was attributed to loss of $C_3H_5N_3O$; a peak at m/z 132.5 was attributed to loss $C_2H_5N_3$; a peak at m/z 175.3 was attributed to loss CO $[M+H-CO]^+$ or N_2. The fragmentation pattern is shown in figure 8. The compound has thus been identified as Metamitron, the first report of this compound in *Eryngium creticum Lam.*

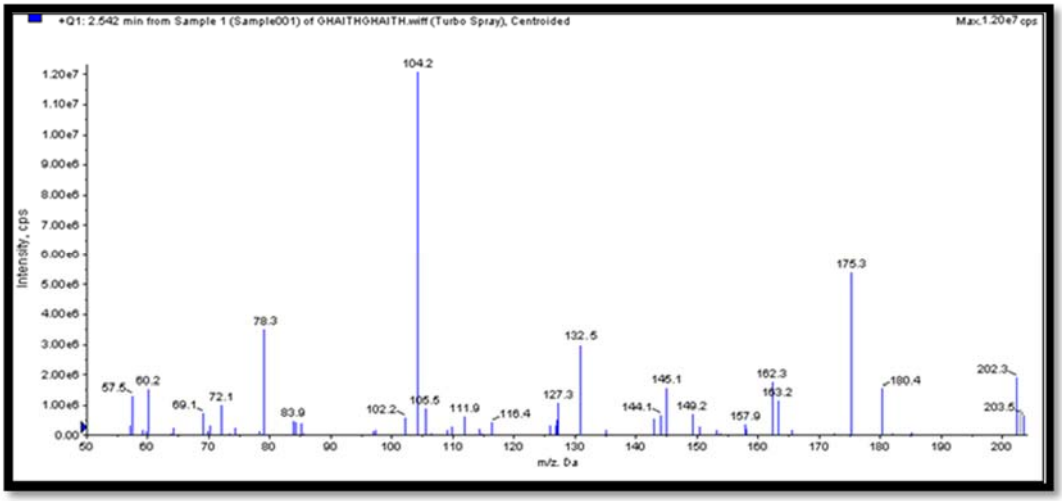

Figure 7: The mass spectrum of the molecular ion from the peak at 2.542 min

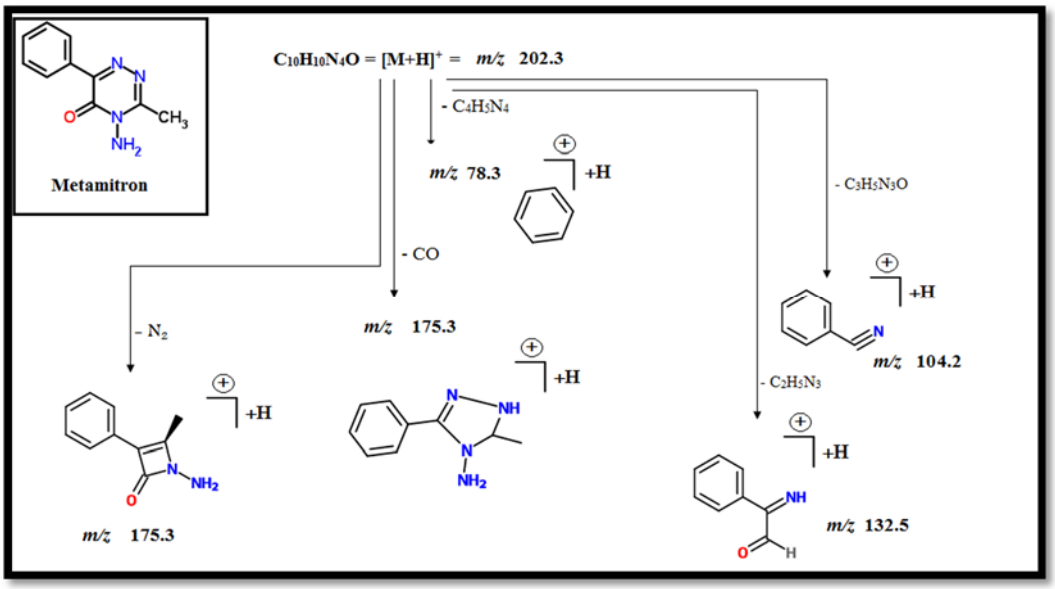

Figure 8: MS Fragmentation pathway of Metamitron

3.2.4 1,3-Diacetylindole

The (+)-ESI TIC chromatogram of the extract revealed the presence of a compound eluting at 3.884 min. The molecular ion corresponded to $C_{12}H_{12}NO_2$ (m/z 202.2

$[M+H]^+$), The spectrum (Figure 9) showed a peak at m/z 145.3, attributed to loss of C_2H_3NO; a peak at m/z 158.8 to loss of C_2H_3O; a peak at m/z 70.3 attributed to of C_9H_8O and peak at m/z 126.3 to loss C_6H_4. The fragmentation pattern of is depicted in Figure 10. This compound in this peak was identified as1, 3-Diacetylindole

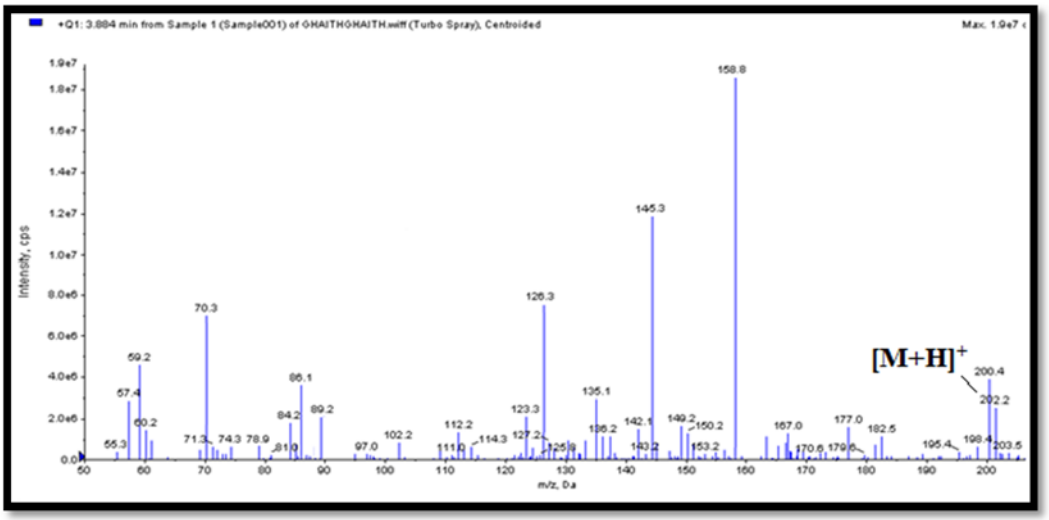

Figure 9: The mass spectrum of the molecular ion from the peak at 3.884 min

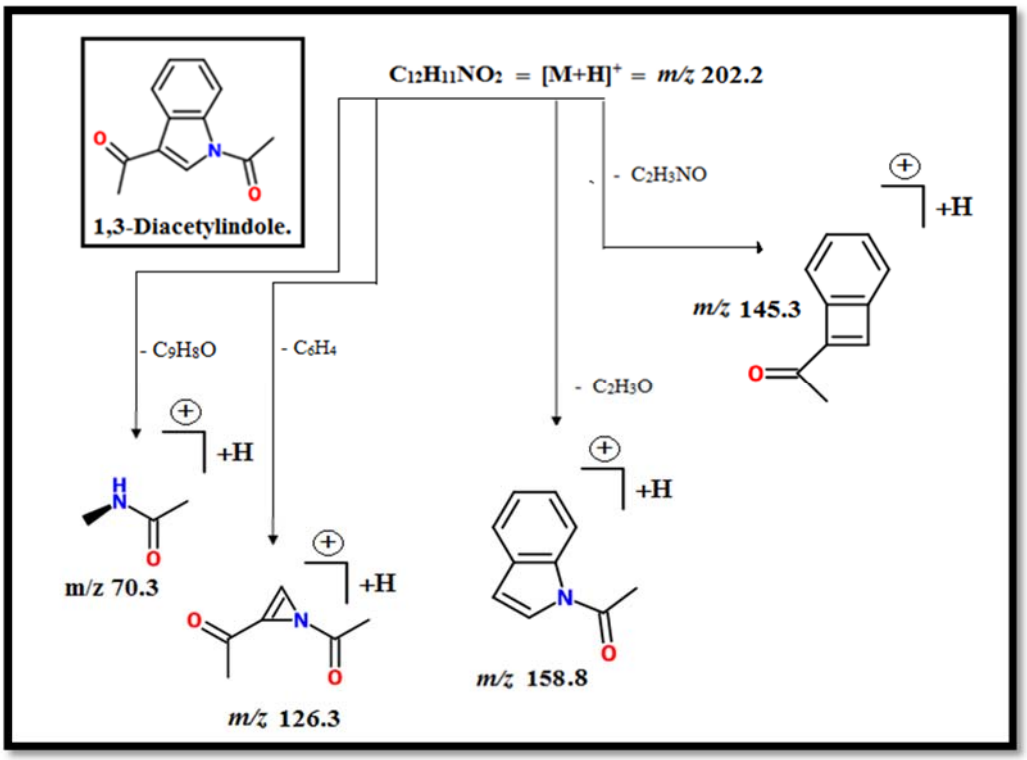

Figure 10: MS Fragmentation pathway of 1,3-Diacetylindole

3.2.5 Thebaine (paramorphine)

The (-)-ESI TIC chromatogram of the extract revealed the presence of a compound at 39.829 min.. The mas spectrum had a molecular ion for $C_{19}H_{21}NO_3$ (m/z 310.3 $[M-H]^-$ calculated for 311.37), with a peak at m/z 295.4 which was attributed to loss CH_3; a peak at m/z 279.3, which was attributed to loss CH_3O; a peak at m/z 69.1, which was attributed to loss $C_{15}H_{16}NO_2$. The fragmentation pattern is depicted in Figure 12 .This compound has been identified as thebaine, the first report of this compound in *Eryngium creticum Lam.*

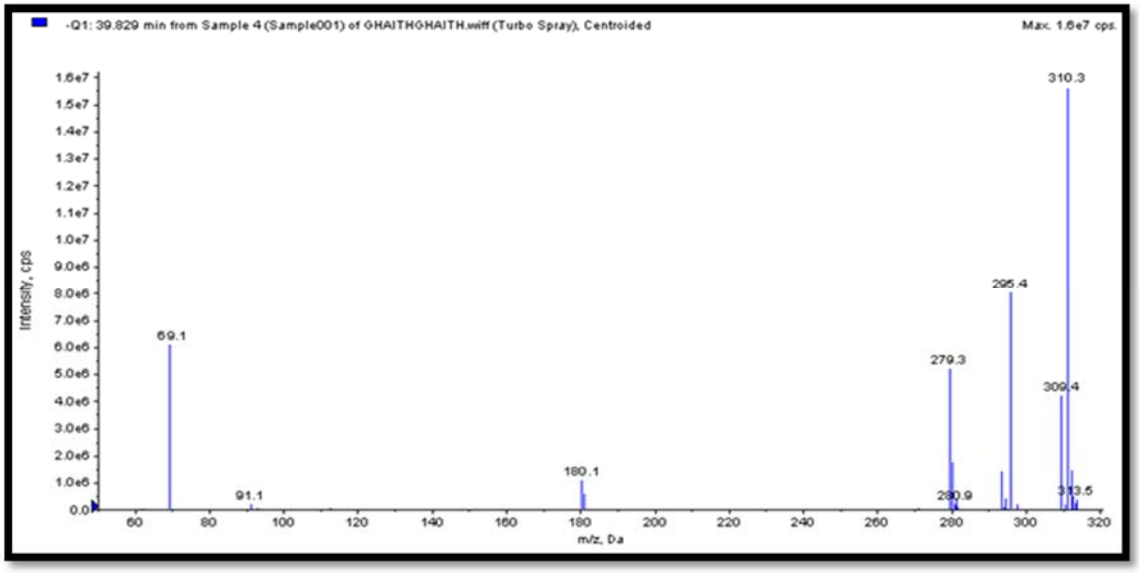

Figure 11: The mass spectrum of the molecular ion from the peak at 39.829 min

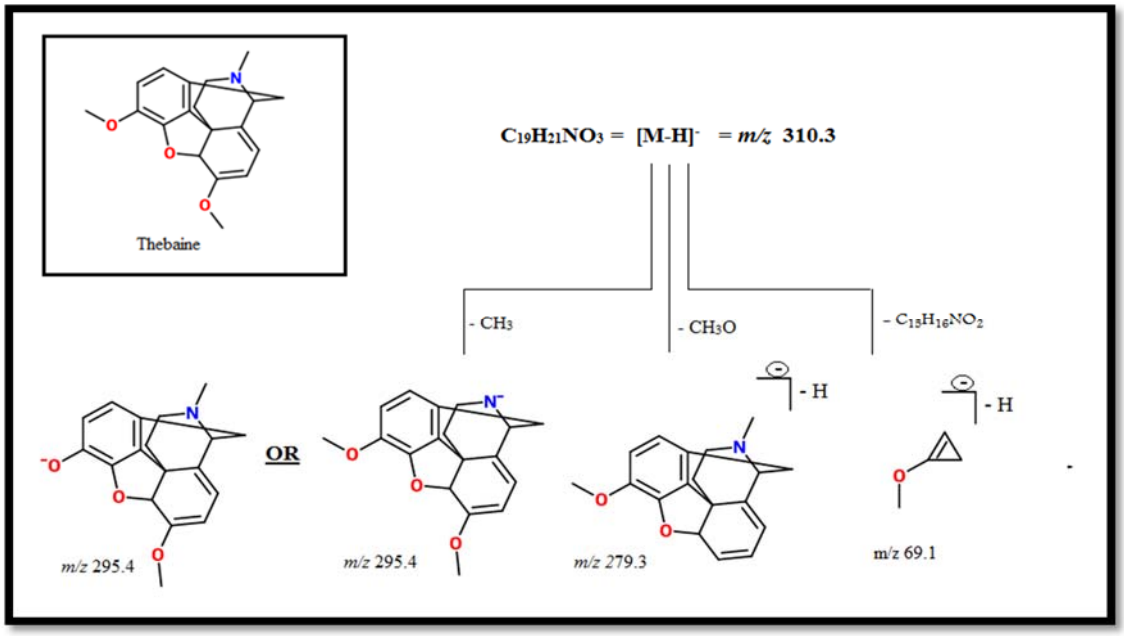

Figure 12: MS Fragmentation pathway of Thebaine

3.2.6 Clemizole

The (-)-ESI TIC chromatogram of crude extract revealed the presence of a compound eluting at 41.524 min for. The mass spectrum (Figure 13) had molecular ion peak of $C_{19}H_{20}N_3Cl$ (m/z 324.3 $[M-H]^-$ calculated for 325.67), a peak at m/z 254.1 which was attributed to loss C_4H_8N; a peak at m/z 248.2, which was attributed to loss C_6H_4; a peak at m/z 199.1, attributed to loss C_7H_6Cl;. a peak at m/z 70.3, attributed to loss $C_{15}H_{12}N_2Cl$; a peak at m/z 70.9, attributed to Cl_2. The fragmentation pattern of Clemizole is depicted in Figure 14. This compound was identified to be Clemizole, the first report of this compound in *Eryngium creticum Lam.*

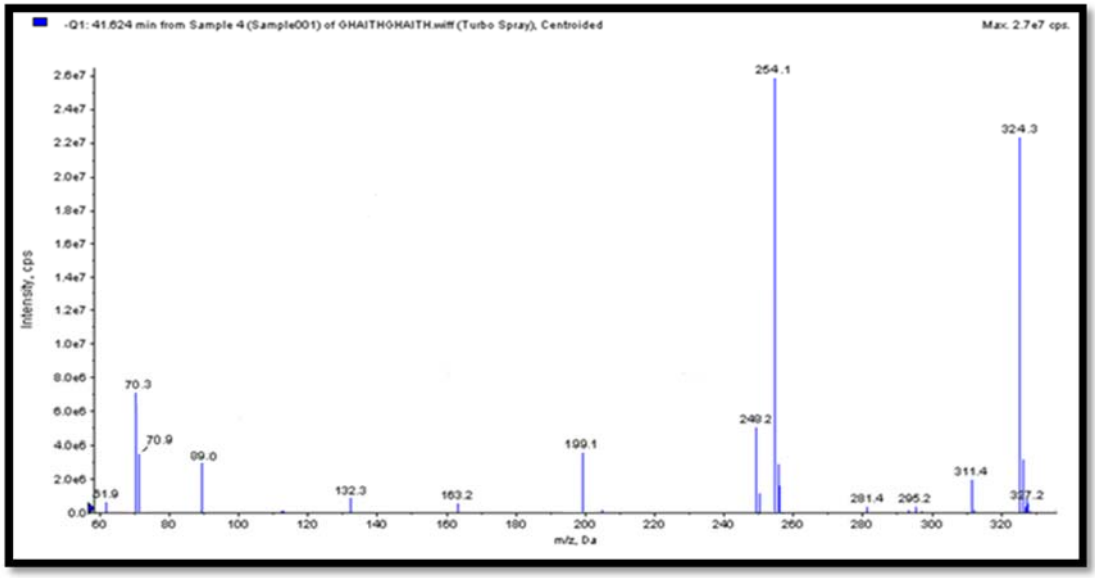

Figure 13: The mass spectrum of the molecular ion from the peak at 41.524 min

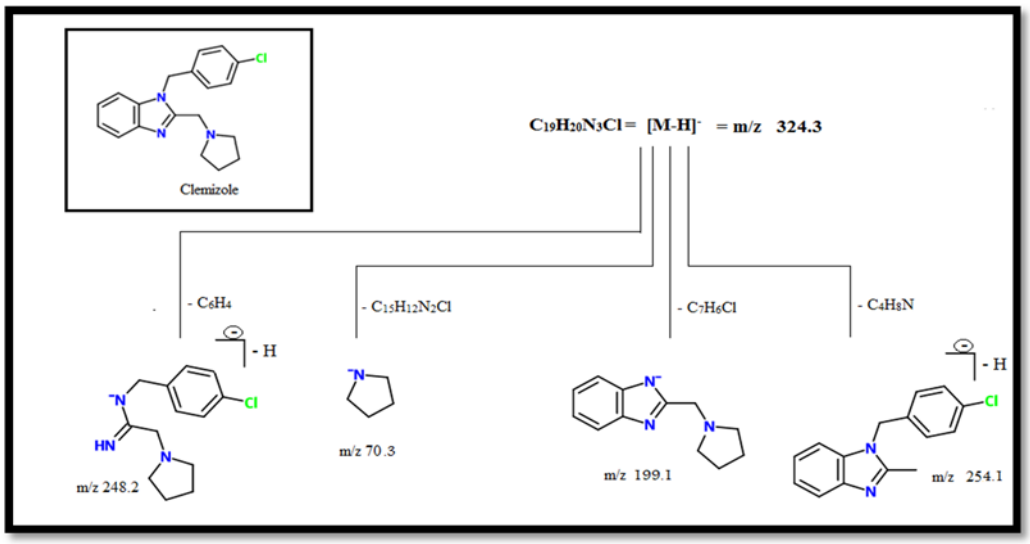

Figure 14: MS Fragmentation pathway of Clemizole

3.3 Biological Activity of Extract against Cancer cell lines

The antitumor potential of the extract was examined by cell viability analysis on colorectal cancer cell lines SW480 and HT29. Cells were incubated with a wide range of the compounds concentrations (0-200 µL/mL) for 72 hours and then cell growth was evaluated using the MTT assay. The results (Figure 15) revealed that the extract exhibited a dose-dependent toxicity against cancer cells. SW480 cells were more sensitive to the extract than were HT29 cells.

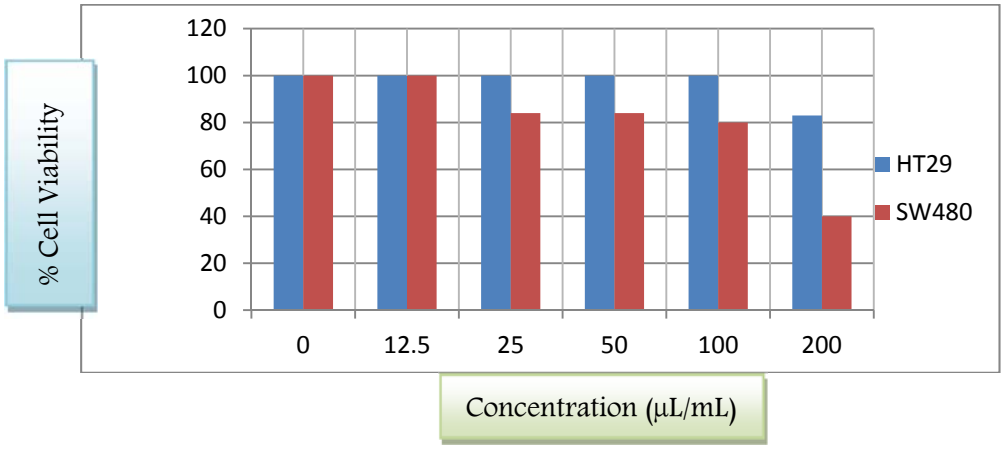

Figure 15: Cell Viability Assays

CONCLUSIONS

Conditions have been established for the efficient extraction of biologically active compounds from *Eryngium creticum Lam.*

The study has identified the presence of six compounds not previously recorded in an extract of *Eryngium creticum Lam*, namely, isobutyl 3-(diheptylcarbamoyl)benzoate, 3-Nitrophthalic anhydride, Metamitron, 1,3-Diacetylindole, Thebaine and Clemizole.

The total extract exhibited a dose-dependent toxicity against cancer cells. SW480 cells were more sensitive to the extract than the HT29 cells. Work to isolate significant amounts the individual biologically active compounds continues.

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المركبات الجديدة في نبات قرصعنة (*Eryngium Creticum Lam*) وتقييم الأنشطة البيولوجية

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ملخص

تم عزل وتحديد النشاط البيولوجي ل 6 مركبات جديدة من نبات قرصعنة creticum hg`d الذي ينمو بصورة عشوائية في الأردن باستخدام LC-MS/MS مع القطبية الإيجابية والسلبية E. creticum لام، المركبات المعزولة هي بيوتيل 3 (diheptylcarbamoyl)، –بنزوات، أنهيدريد 3Nitrophthalic-، Metamitron، 1، 3-Diacetylindole، الثيبايين وكليميزول. وتم تقييم سمية هذه المكبات على الخلايا السرطانية في المختبر. كان المستخلص المثيلي فعال ضد خلايا سرطان القولون الإنسان (HT29) و(SW480).

الكلمات الدالة: نبات قرصعنة (*Eryngium Creticum Lam*)، الأنشطة البيولوجية، الخلايا السرطانية.

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