Studies on the chemical constituents of fresh leaf of *Eruca sativa* extract and its biological activity as anticancer agent *in vitro*.

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Phytochemical investigations of the aqueous extract of *Eruca sativa* fresh leaves, afforded the presence of nine natural flavonoid compounds which were isolated and identified as kaempferol 3-O-(2''-O-malonyl-β-D-glucopyranoside)-4'-O-β-D-glucopyranoside (1), kaempferol 3,4'-O-diglucopyranoside (2), rhamnocitrin 3-O-(2''-O-methylmalonyl-β-D-glucopyranoside)-4'-O-β-D-glucopyranoside (3), 3-O-glucopyranoside (4), 4'-O-glucopyranoside (5), rhamnocitrin 3-O-glucopyranoside (6), 4'-O-glucopyranoside (7), kaempferol (8) and rhamnocitrin (9). Compounds (1) and (3) appear to be novel. Elucidation of the chemical structures of all the isolated compounds was determined by different spectroscopic methods in addition to the chemical and physical methods of analysis. *In vitro* antitumour study of *E. sativa* 70% ethanolic extract (*ES-EE*) as well as Compounds (1) and (3) proved their cytotoxic activity in 4 different human tumor cell lines: HepG₂ (liver carcinoma), MCF₇ (breast carcinoma), HCT₁₁₆ (colon carcinoma), and Hep₂ (larynx carcinoma). On the basis of these results, the *ES-EE* as well as Compounds (1) and (3) seem to have potential as a novel cancer preventive agent.

Key words: *Eruca sativa* (Mill.), cruciferae, fresh leaves, novel malonylated kaempferol glycosides, antitumour activity

INTRODUCTION

The American Institute of Cancer Research (AICR) together with the World Cancer Research Fund (WCRF) underlined that cancer is 30 to 40% preventable over time by appropriate food and nutrition (AICR, 2007). A significant part of the research on plant foods and cancer prevention suggests the potentially beneficial effect of a diet rich in cruciferous vegetables. (Ambrosone and Tang, 2009; Traka et al., 2008; Higdon et al., 2007; Tang et al., 2008; Thomson et al., 2007). These beneficial effects are attributed to a range of phytochemicals including flavonoids and glucosinolates, both of which are found in high levels in Brassicaceous crops (Jin et al., 2009). Among cruciferous vegetables, rocket salads, which are widely used in the Mediterranean diet and well-studied as source of healthy phytochemicals (Bogani and Visioli, 2007; Schaffer et al., 2005). The name “rocket” is commonly used to indicate different species belonging to the large family of Brassicaceae (also called Cruciferae) that are mainly represented by *Eruca sativa* Mill. Rocket species are well-known in traditional medicine for their therapeutic properties as an astringent, diuretic, digestive, emollient, tonic, depurative, laxative, rubefacient and stimulant (Uphof, 1968; Yaniv et al., 1998; Perry and Metzger, 1978). Its leaves are used tenderly when they are not matured in salads and sometimes cooked as a potherb. Ancient Egyptians and Romans both have considered the leaves in salads to be an aphrodisiac. It is also antiscorbutic, stimulant and rubefacient (Boulos, 1983). In terms of antioxidant compounds, rocket salad species are a good source of vitamins, like vitamin C, carotenoids, and polyphenols, which play a very important role among natural antioxidants (Martinez-Sanchez et al., 2008).

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Most attention has been focused on its seeds, in which thiocyanates, isothiocyanates and their precursors, the glucosinolates, have been extensively studied (Hamence and Taylor, 1978; Adhikari et al., 1989; Mahran et al., 1992), as well as in intact leaf and root powders where their antioxidant activity showed moderate results (Kin and Ishii, 2006). El-Missiry and his colleague suggested that E. sativa seeds exert a beneficial antidiabetic effect in cases of chemically induced diabetes mellitus in rats by reducing oxidative stress (El-Missiry and El Gindy, 2000). Recently, Sarwar et al. (2007) suggested that the ethanolic extract of E. sativa seeds possessed a potent antioxidant activity and exert a protective effect on mercuric chloride induced renal toxicity. In both mentioned studies, the health-promoting activities of rockets plants have been partially related to their strong antioxidant properties (Barillari et al., 2005; Heimler et al., 2007; Kim et al., 2004). Alqasoumi et al. (2009) concluded that, rocket (E. sativa) extract possessed anti-secretory, cytoprotective and anti-ulcer activities against experimentally-induced gastric lesions through prostaglandin-mediated activity and antioxidant properties. Results of Yehuda et al. (2009) imply that MTBI (4-methylthiobutylisothiocyanate), the major isothiocyanate in E. sativa seeds, may represent a new family of natural compounds possessing significant skin inflammation-preventive activities. In a study carried out by Lamy and Mersch-Sundermann (2009) there is evidence of the strong antigenotoxic effect of E. sativa in benzo[a]pyrene exposed human hepatoma (HepG2) cells. Recently, chemoprotective properties of rocket leaves on human colon cancer cells have been also investigated (Jin et al., 2009).

Until the last year in the previous decade, surprisingly, scarce information was found about the chemical constituents of E. sativa leaves. Three novel quercetin triglucoside, in addition to their acylated sinapoyl derivatives, have been isolated and identified from its leaves by Weckerle et al. (2001). It was found that the percentage of the aglycone kaempferol and its glycosides in the edible parts of E. sativa was more than that of the structure elucidation of two new malonylated glucopyranoside, 4'-O-glucopyranoside, rhamnocitrin 3-O-glucopyranoside, rhamnocitrin 3-O-glucopyranoside, kaempferol and rhamnocitrin. Structures of the isolated pure compounds were elucidated by chromatography, chemical degradation, UV and NMR spectroscopy. In addition, the examination of the medicinal value of its E. sativa 70% ethanolic extract (ES-EE) as well as compounds (1) and (3) as anticancer agent against 4 different human tumor cell lines, HepG2 (liver carcinoma), MCF7 (breast carcinoma), HCT116 (colon carcinoma) and Hep2 (larynx carcinoma) in a dose-dependent manner was studied in vitro.

**MATERIALS AND METHODS**

**Experimental**

1H (300 MHz) and 13C (75 MHz)-NMR: Jeol spectrometer in DMSO-d6 and reported at δ-value relative to TMS as internal standard. UV: Shimadzu spectrophotometer model UV-240. CC: Polyamide 6S and Sephadex LH-20. PC: was carried out on Whatman No.1 and 3MM using solvent systems 1- BAW (n-BuOH: HOAc: H2O, 6: 1: 2); 2- 2% AcOH (AcOH: H2O, 2:98); 3- 15%. AcOH (AcOH: H2O, 15:85).

**Plant material**

Fresh leaves of E. sativa (Mill.) (1 kg); origin Egypt, cultivar not specified; obtained by a local wholesaler.

**Extraction and isolation**

The fresh leaves of E. sativa were extracted with 70 % ethyl alcohol; the concentrated extract was applied onto a polyamide 6S column. Elution started with water, followed by increasing concentration of ethanol gradually, where six fractions (F1-6) were obtained. The two new compounds:- kaempferol 3-O-(2'-O-malonyl-β-D-glucopyranoside)-4'-O-β-D-glucopyranoside (11 mg) and rhamnocitrin 3-O-(2'-O-methylmalonyl-β-D-glucopyranoside)-4'-O-D-glucopyranoside (14 mg) were eluted from F2 and F3, respectively, while kaempferol 3,4'-di-O-glucopyranoside (17 mg) from F2, its 3-O-glucopyranoside (21 mg) and 4',0'-glucopyranoside (19 mg) from F3, rhamnocitrin 3-O-glucopyranoside (13 mg) and its 4'-O-glucopyranoside (9 mg) from F5 and the two aglycones; kaempferol (27 mg) and rhamnocitrin (23 mg); were isolated from the last fraction. All the isolated known compounds were purified using Sephadex LH-20 column and their chemical structures were identified through copaper chromatography, hydrolytic procedures, UV and NMR spectroscopy.

**Potential cytotoxicity measurements by SRB assay**

Four tumor cell lines - HepG2 (liver carcinoma), MCF7 (breast carcinoma), HCT116 (colon carcinoma), and Hep2 (larynx carcinoma) were obtained from the National Cancer Institute, Cairo, Egypt, by serial sub-culturing. Potential cytotoxicity of the fresh leaves of ES-EE as well as Compounds (1) and (3) were tested using (SRB) assay of cytotoxic activity according to the method of Skehan et al. (1990).
Principle

The sensitivity of the human tumor cell lines to thymoquinone was determined by the SRB assay. SRB is a bright pink aminoxanthrene dye with two sulphonic groups. It is a protein stain that binds to the amino groups of intracellular proteins under mildly acidic conditions to provide a sensitive index of cellular protein content.

Procedure

Cells were used when 90% confluence was reached in T25 flasks. Adherent cell lines were harvested with 0.025% trypsin. Viability was determined trypan blue exclusion using the inverted microscope (Olympus 1×70, Tokyo, Japan). Cells were seeded in 96-well microtiter plates at a concentration of 5×10^3-10^5 cell/well in a fresh medium and left to attach to the plates for 24 h. After 24 h, cells were incubated with the appropriate concentration ranges of drugs, medium and left to attach to the plates for 24 h. Following 24, 48 and 72 h treatment, the cells were treated with vehicle alone. For each drug concentration, 4 wells were incubated with the appropriate concentration ranges of drugs, completed to total of 200 µl volume/well using fresh medium and incubation was continued for 24, 48 and 72 h. Control cells were treated with vehicle alone. For each drug concentration, 4 wells were used. Following 24, 48 and 72 h treatment, the cells were fixed with 10 µl cold 50% trichloroacetic acid for 1 h at 4°C. Wells were washed 5 times with distilled water and stained for 30 min at room temperature with 50 µl 0.4% SRB dissolved in 1% acetic acid. The wells were then washed 4 times with 1% acetic acid. The plates were air-dried and the dye was solubilized with 100 µl/well of 0.1 N tris base (pH 10.5) for 5 min on a shaker (Orbital shaker OS 20, Boeco, Germany) at 1600 rpm. The optical density (OD) of each well was measured spectrophotometrically at 564 nm with an ELIZA microplate reader (Meter tech. E 960, U.S.A.). The mean background absorbances was automatically subtracted and mean values of each drug concentration was calculated.

Calculation

The percentage of cell survival was calculated as follows:

\[
\text{Survival fraction} = \frac{\text{O.D. (treated cells)}}{\text{O.D. (control cells)}}
\]

The IC_{50} values (the concentrations of thymoquinone required to produce 50% inhibition of cell growth. The experiment was repeated 3 times for each cell line after growth.

Spectral data of the two new isolated natural compounds

**Kaempferol** 3-O-(2''-O-malonyl-β-D-glucopyranoside)-4''-O-β-D-glucopyranoside (1)

R_f -values x (100): 24 (1), 50 (2), 32 (3).

UV/Vis λ_{max} (MeOH) nm: MeOH: 265, 282*, 341; +NaOMe: 275, 372; +NaOAc: 270, 310*, 355; + NaOAc/HBO_2: 265, 350; +AlCl_3: 275, 300*, 339, 405; + AlCl_3/HCl: 275, 298*, 335, 404. * = shoulder.

^1H NMR (300 MHz, DMSO-d_6): Aglycone moiety: δ(ppm) 8.15 (d, J = 9 Hz, H-2’ and H-6’); 7.10 (d, J = 9 Hz, H-3’ and H-5’); 6.20 (d, J = 2 Hz, H-6); 6.45 (d, J = 2 Hz, H-8).

Sugar moiety: δ (ppm) 5.49 (d, J = 7.5 Hz, H-1'' of 3-O-glucoside); 5.05 (d, J = 7.5 Hz, H-1''' of 4''-O-glucoside); 3.1 (s, CH_2 of malonic); 3.10-3.90 (m, rest of sugar protons).

^13C NMR (75 MHz DMSO-d_6): Aglycone moiety: δ(ppm) 156.55 (C-2); 133.76 (C-3); 177.63 (C-4); 161.31 (C-5); 98.89 (C-6); 164.40 (C-7); 93.88 (C-8); 155.70 (C-9); 104.19 (C-10); 123.77 (C-11); 130.72 (C-12); 115.88 (C-13); 158.30 (C-14); 130.72 (C-15). 3-O-β-D-glucoside: 100.85 (C-1''); 76.56 (C-2'''); 76.40 (C-3''); 69.93 (C-4'''); 77.69 (C-5'''); 60.91 (C-6'''); 4''-O-β-D-glucoside: 99.97 (C-1'''); 73.27 (C-2'''); 76.57 (C-3'''); 69.62 (C-4'''); 77.13 (C-5'''); 60.66 (C-6''). Malonyl moiety: δ (ppm) 166.0 (CO); 167.5 (CO); 41.5 (CH), 29.50 (CH).

RESULTS AND DISCUSSION

The 70% aqueous ethanolic extract of the *E. sativa* (ES-E) fresh leaves, after successive chromatographic separations using Polyamide 6S column and preparative paper chromatography, afforded six fractions where nine flavonoid compounds were isolated including the two new ones.

Compound (1) was obtained as light-yellow amorphous powder. It appeared as a dark spot under UV light and it did not change on exposure to ammonia vapours, it possesses UV absorption bands at λ_{max} at 265 and 341 nm in MeOH for Bands II and I, respectively. The addition of different diagnostic reagents proved that Compound (1) is substituted in 3 and 4' positions (Markham, 1982). Kaempferol and glucose were detected by Co-PC after complete acid hydrolysis (2N HCl for 45 min). Furthermore, controlled acid hydrolysis after 3 min liberated kaempferol 4''-O-glucoside which was identified by Co-PC and UV spectral data, indicating that Compound (1) may be the 3,4'-di-O-glucoside of kaempferol. Therefore, enzymatic hydrolysis using β-glucosidase was engaged which yielded the sugar glucose and another compound occupied in the 3-position and not identified as kaempferol 3-O-glucoside which on alkaline hydrolysis...
yielded kaempferol 3-O-glucoside plus malonic acid (CO-PC) indicating that it was attaching to the 3-O-glucose moiety, that is, this compound may be kaempferol 3-O-malonyl glucoside-4'-O-glucoside. To be ensured of this proposed structure, $^1$H and $^{13}$C NMR spectrum were studied. The first one showed signals of kaempferol skeleton with its H-3',5' signal appeared as doublet at $\delta$ 7.1 ppm ($J = 9$ Hz), indicating substitution of 4'-position and the appearance of two anomeric sugar proton signals at $\delta$ 5.49 and 5.05 ppm, both with $J = 7.5$ Hz, that is, two β-glucose moiety (Harborne, 1993); one at 3-position and the other at the 4'-position, in addition to a singlet signal appeared at $\delta$ 3.1 ppm of CH$_2$ of malonic acid (Kazuma et al., 2003). To know the link of this acid with the 3-O-glucose moiety, $^{13}$C NMR spectroscopy was engaged, where the two characteristic carbonyl carbon signals of the malonyl group were observed at $\delta$ 166.0 (RCOOR') and 167.5 ppm (RCOOH) while its methylene carbon observed at $\delta$ 41.5 ppm (Kazuma et al., 2003, Agrawal, 1989). All the other signals were assigned for the aglycone kaempferol substituted at 3 and 4'-O-positions with the two glucose moieties, where their signals were shifted upfield at $\delta$ 133.76 and 158.30 ppm, respectively, while the signals assigned for C-2,4 and 5 were shifted downfield (156.55, 177.66 and 161.31 ppm, respectively) with respect to that of the aglycone kaempferol. Also the C-2'' signal of that of 3-O-glucose was shifted downfield and appeared at $\delta$ 76.50 ppm (unsubstituted one at $\delta$ 74.40 ppm), as well as the upfield shift of 1'' and 3'' from 103.3 and 78.00 to 100.8 and 76.40 ppm, respectively, indicated its substitution with the malonic acid moiety. So, the above data, ensured that the chemical structure of Compound (1) is kaempferol 3-O-{2''-O-malonyl-β-D-glucopyranoside}-4'-O-β-D-glucopyranoside which is a new natural compound isolated for the first time in nature (Figure 1).

The difference between Compounds (1) and (3) [rhamnocitrin 3-O-{2''-O-methylmalonyl-β-D-glucopyranoside}-4'-O-β-D-glucopyranoside] is the presence of a methoxy group at Position 7 of kaempferol and methylmalonyl one at the 2''-position of glucose moiety instead of malonic, where, from the UV spectral data, no shift was produced in Band II upon the addition of NaOAc to the MeOH curve which proved its substitution at Position 7. By complete acid hydrolysis, rhamnocitrin was obtained (CO-Pc and UV spectral data) which ensured the substitution of Position 7 with the methoxy group.

On the other hand, the $^1$H NMR spectrum showed the presence of a singlet signal at $\delta$ 3.85 ppm of the methoxy group at Position 7 and that of the CH of methylmalonyl moiety was present in the region of the sugar moiety which its methyl group appearing as a doublet one at $\delta$ 1.22 ppm.

Its $^{13}$C NMR data proved the identity of its structure with the proposed structure, where the presence of these groups was assigned from the appearance of their signals at $\delta$ 56.16 ppm of a methoxy group at the 7-position of kaempferol, $\delta$ 168 and 170 ppm of the two carbonyl carbon (COOH) of methylmalonyl moiety, its CH and its methyl group signals at $\delta$ 50.0 and 29.50 ppm (Kazuma et al., 2003; Agrawal, 1989), respectively, where the downfield shift of the C-7 signal from $\delta$ 163.90 to 165.39 ppm and the upfield shift of the C-6 and 8 from $\delta$ 99.20 and 94.50 to 97.47 and 92.38 ppm, respectively; ensured its substitution with a methoxy group and the
Table 1. IC$_{50}$ (µg/ml) of 70% aqueous ethanolic extract of *E. sativa* as well as Compounds (1) an (3) on 4 different human cell lines, HepG$_2$, MCF$_7$, HCT$_{116}$ and Hep$_2$.

<table>
<thead>
<tr>
<th>IC$_{50}$ (µg/ml)</th>
<th>HepG$_2$</th>
<th>MCF$_7$</th>
<th>HCT-116</th>
<th>Hep$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES-EE</td>
<td>10.0</td>
<td>21.7</td>
<td>10.3</td>
<td>15.5</td>
</tr>
<tr>
<td>CPD 1</td>
<td>7.6</td>
<td>25</td>
<td>8.9</td>
<td>*</td>
</tr>
<tr>
<td>CPD 2</td>
<td>9.8</td>
<td>26.6</td>
<td>*</td>
<td>34.9</td>
</tr>
</tbody>
</table>

Figure 2. Cytotoxic activity of the 70% aqueous ethanolic extract of *E. sativa*, rhombus$
\text{(1)}$, Compound (1 , square$
\text{(3)}$) and Compound 3 (△) against HepG$_2$ human cell lines.

C-2'' of the β-D-glucose present in the 3-position moiety with the methylmalonic moiety (Figure 1).

Evaluation of cytotoxic activity on human tumor cell lines

The main objective of cancer therapy is to achieve maximum therapeutic destroy of tumor cells using the minimal concentration of the drug. This can be achieved, in principle, via selective antitumor preparations. While 100% selectivity may be impractical, achievement of reasonably high selectivity seems to be a feasible aim (Badawi et al., 2007).

The results of the cytotoxic activity on human tumor cell lines was determined according to the dose values of drug exposure required to reduce survival in the cell lines to 50% (IC$_{50}$). Cytotoxic activities were examined on 4 different human tumor cell lines: HepG$_2$, MCF$_7$, HCT$_{116}$ and Hep$_2$. Plots of surviving fraction vs concentration in micrograms of ES-EE, Compounds (1) and (3) are shown in Figures 2 to 5 while the results of their IC$_{50}$ were recorded in (Table 1).

Results in Table 1 showed that the 3 examined samples has strong cytotoxic effect against HepG$_2$ (Figure 2) and MCF$_7$ (Figure 3). The most effective sample at the lowest dose, in case of HepG$_2$, was Compound (1), since its IC$_{50}$ was 7.6 µg/ml, and, in case of MCF$_7$, it was the ES-EE. These results are in accordance with Tao et al. (2008) and Li et al. (2010), who found similar inhibitory activity of kaempferol towards HepG$_2$. Similar results were found by Alqasoumi (2010) who observed that ethanolic extract of *E. sativa* L. has hepatoprotective and antioxidant effect on hepatic injury through its potent antioxidant activity in rats. Our results are in accordance with that of Lamy et al. (2008) which assessed the chemopreventive potency underlying action mechanisms of extracts of *E. sativa* in HepG$_2$ cells. They found also that *E. sativa* extract reduced the benzo(a)pyrene-induced genotoxicity in a U-shaped manner. Results are also going with the findings of Cho et al. (2009) and Zhang et al. (2008), who found that kaempferol inhibits the proliferation of malignant human cancer HCT$_{116}$ cell line. Their results indicate that kaempferol, might be a
good strategy for chemopreventive or chemotherapeutic application. Figure 4 showed that both the ES-EE and Compound (1) has cytotoxic effect against colon cancer cell line (HCT116), while Compound (3) has no effect. Figure 5 showed that both the ES-EE and Compound (3) has cytotoxic effect against Hep2 cell line, regarding that the IC50 of the later was relatively high (34.7 µg/ml). These results illustrated that the ES-EE is more effective
against all of the examined cell lines and then each compound separately. This may lead to an assumption that their synergistic interaction is responsible for the strong cytotoxicity of the plant leaf extract.

Results also illustrated that Compound (1) is more effective than Compound (3) especially in HepG2 and HCT116. Inhibition potency of the flavonoids was attributed to their basic chemical structure, the flavan nucleus, depending on the number of OH groups and their side chains on the phenyl rings (Katalinić et al., 2009).

Conclusion

_**E. sativa**_ has powerful active components that might be effective in increasing human health and preventing cancer.

REFERENCES


