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Chemical Composition and Cytotoxic Activity of *Eruca sativa* L. Seeds Cultivated in Egypt

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Eruca sativa L. (family: Brassicaceae) is native to southern Europe and central Asia where it has been cultivated since classical times. It is sometimes referred to as a rocket, true rocket, rocket salad, arugula, roquette or white pepper. It is used for flavouring salads. Investigation of the 70 % alcoholic extract of *E. sativa* L. seeds led to the isolation of three flavonoids, quercetin (**I**), rhamnetin (**II**) and kaempferol-3-O-galactoside (**III**), in addition to 2-glucosinolates, 4-methylthio butyl glucosinolate (glucoerucin) (**IV**) and 3-methylsulfinylpropyl glucosinolate (glucoiberin) (**V**). The compounds were identified by the use of (TLC, PC, MS and GC/MS). The petroleum ether extract of *E. sativa* seeds was investigated. The fatty acid fraction was found to contain 9 fatty acids in which erucic acid represented the main component (44 %) of the total fatty acid mixture. The unsaturated fatty acids represent 90 % of the total mixture. The unsaponifiable matter constituted of mixture of *n*-alkanes from C_{16}-C_{26}, β-sitosterol, cholesterol, stigmasterol, campsterol. β-sitosterol are the major constituents, representing 28 % of the total mixture. Cytotoxic activity of total alcoholic extract of the defatted seeds and the isolated compounds were performed against several types of tumor cell lines using the SRB assay. The total alcoholic extract and the aglucones of the isolated compounds glucoerucin and glucoiberin exhibited significant cytotoxic activity for HCT116 (colon carcinoma cell line) (IC_{50} = 0.74, 2.42 and 0.94 μg/mL), respectively, while the IC_{50} was > 10 μg/mL for both Hela (Cervix carcinoma cell line), HEPG2 (liver carcinoma cell line), MCF7 (breast carcinoma cell line) and U251 (brain carcinoma cell line).

Key Words: *Eruca sativa*, Brassicaceae, Flavonoids, Quercetin, Rhamnetin, Kaempferol-3-O-galactoside, Glucosinolates, Cytotoxic activity, Glucoerucin, Glucoiberin.

INTRODUCTION

Eruca sativa (syn. *E. vesicaria* subsp. *sativa* (Miller) Thell, *Brassica eruca* L.), also known as rocket or arugula, is an edible plant. It is a species of *Eruca* native to the Mediterranean region, from Morocco and Portugal east to Jordan and Turkey[1]. It has been grown in the Mediterranean area since Roman times and is considered an aphrodisiac.

It is rich in vitamin C and potassium. It is frequently cultivated, although domestication cannot be considered complete. *Eruca sativa* seeds possess antioxidant activity and exert a protective effect on mercuric chloride induced renal toxicity[2]. Studies on taramira seed (*Eruca sativa* Lam.) proteins were carried out[3]. Sulforaphane, erucin and iberin from *Eruca sativa* Lam. Up-regulate thioredoxin reductase 1 expression in human MCF-7 cells[4]. Some constituents of brassicaceae have already been shown to induce apoptosis or block proliferation in cancer cells *in vitro* Huang *et al.*[5] showed that phenylethyl isothiocyanates (ITC) induces p. 53 transactivation in mouse epidermal cells in a dose- and time-dependent fashion, while Gamet-Payrastre *et al.*[6] demonstrated that sulforaphane induces cell-cycle arrest and apoptosis in HT29 human colon cancer cells. Naturally occurring glucosinolates in plant extracts of rocket salad (*Eruca sativa* L.) were identified by liquid chromatography coupled with negative ion electrospray ionization and quadrupole ion-trap mass spectrometry[7]. The present work illustrates the isolation and identification of the main constituents of *Eruca sativa* L. seeds grown in Egypt. Also cytotoxic investigation for the total alcoholic extract and the hydrolyzates of the isolated compounds on various tumor cells was carried out.

EXPERIMENTAL

The seeds of *E. sativa* were purchased from local market, germinated and the germinated plant was kindly identified by Prof. Dr. Salwa El-Kawashty, Department of Taxonomy, National Research Centre, Cairo, Egypt. A voucher specimen was kept in the herbarium of the National Research Centre No. 2-6-2005, *Eruca sativa* (Brassicaceae).

Shimadzu UV-PC 2401 spectrophotometer, mass spectrophotometer: GC-MS Jeol 500 mass spectroscopy 70 eV, agilent GC-system, 6890 N. Myrosinase enzyme E.C.3.2.1.147 and boron triflouride were purchased from sigma chemicals Co. St, Louis, MO, USA.

Neu's spray reagent (1 % diphenyl-boric acid ethanolamine complex) or 5 % $AlCl_3$ in MeOH were used to visualize flavonoids[8]. Modified silver nitrate reagent[9]. Solution (A): 0.75 g silver nitrate is dissolved in 10 mL H_2O and diluted up to 400 mL with acetone. Solution (B): 5 % of sodium hydroxide in methanol. The dried chromatograms were dipped quickly in solution (A), dried and sprayed on both sides with solution (B) and left for 5 min. The chromatograms are then washed with nitric acid solution (1 %) followed by washing with distilled water. The glucosinolates appeared as brownish spots on a yellowish back ground.

Conditions for GLC of lipid fraction

For the unsaponifiable matter: Column: HP-1 (methyl siloxane) 30 m (0.53 mm × 2.65 mm) Temp. program: Initial temp. 60 ºC, Initial time 2 min, program rate 10 °C/min, final temp. 280 °C, final time 0.5 h, injection temp: 260 °C, detector (FID), T = 300 °C, flow rate of carrier gas N_2: 30 mL/min, H_2: 35 mL/min, air: 300 mL/min.

For fatty acid methyl esters: Fatty acid methyl esters were analyzed on HP-6890 GC. Column: HP-5 (phenyl methyl siloxane) 30 m (0.32 mm × 0.25 mm). Temp. program: Initial temp. 70 °C, Initial time 2 min, program rate 8 °C/min, final temp. 270 °C, final time 27 min, injection temp: 270 °C, detector (FID), T = 300 °C, flow rate of carrier gas N_2: 30 mL/min, H_2: 35 mL/min, air: 300 mL/min.

Conditions for GC/MS analysis of isothiocyanates: Apparatus: GC/MS finningan SSQ7000, work station: digital DEC 3000, column: DB5 cappillary column, (0.25 mm i.d.). Ionization mode: EI at 70 eV. Temperature program: 50-250, 4 °C/min. Detector: MS. Sample volume: 2 µL, mass range 40-400.

Solvent systems: *n*-Butanol:acetic acid:water (4:1:5, v/v/v, upper layer (solvent a), 4:1:2 v/v/v (solvent b), *n*-butanol:ethanol:water (4:1:4) (solvent c). EtOAc: formic acid:acetic acid:water, 30:1.5:1.5:8 v/v) (solvent d), 15 % acetic acid (solvent e).

Isolation of lipids: About 1.5 kg of air dried of *E. sativa* powdered aerial parts of plant material *E. sativa* was extracted in a Soxhlet apparatus with petroleum ether (40-60 ºC). The extracts was filtered through fuller's earth and the filtrate was evaporated under vacuum at 40 ºC to give an oil (300 g). About (8.5 g) of the oil saponified (0.5 N methanolic KOH) and the unsaponifiable matter (3.9 g) was separated by extraction with ether. The liberated fatty acids mixture, after acidification of the saponifiable matter was extracted with peroxide-free ether, and then methylated (methanol, 12 % BF_3). Aliquots of the isolated unsaponifiable fraction and methyl esters of fatty acids were subjected to GLC analysis.

Isolation of flavonoids: The defatted powdered plant material (600 g) was extracted with methanol (70 %). The combined alcoholic extract was evaporated *in vacuo*, dissolved in hot distilled water and left overnight in a refrigerator then filtered. The aqueous filtrate was extracted with successive portions of chloroform followed by ethyl acetate. Each of combined solvent extracts was separately evaporated *in vacuo*. The ethyl acetate fraction (2.8 g) was subjected to column chromatographic fractionation using silica gel (100 g, silica gel, 230-270 mesh, Merck) packed in a glass column (80 cm × 2.5 cm i.d.) eluted with $CHC1_3$ then a gradient of $CHC1_3$/MeOH with an increasing amount of MeOH. The chromatic fractionation was monitored by (TLC GF_{254}, silica gel). The fraction eluted with $CHC1_3$/MeOH (80:20 v/v) was found by TLC (silica gel GF_{254} eluted by solvent **d**) to contain two main flavonoidal compounds. It was further purified by preparative paper chromatography using Whatman 3 mm irrigated with (solvent **a**) to afford compounds **I** and **II** . The fraction eluted with $CHC1_3$:MeOH (70:30) was found by (TLC, using solvent system **d**) to contain one main component with three minor ones. It was further purified using repeated column chromatography on silica gel followed by Sephadex LH-20 column eluted with methanol (90 %) to yield compound **III**.

Isolation of glucosinolates: The defatted powdered plant material (600 g) of *Eruca sativa* was extracted by boiling 70 % methanol. The methanolic extract was evaporated *in vacuo* at 40 °C till dryness. The residue (58 g) was dissolved in hot distilled water (700 mL), left overnight in a refrigerator for 24 h and filtered. The

filtrate was allowed to pass slowly through a column of acidic aluminium oxide aniontropic, (1 Kg, 70 cm × 5.4 cm), sigma chemical Co., activity grade-I type (WA-I acidic). The column washed with deionized water (3L), till the eluate became colourless, then the glucosinolates were eluted with potassium sulphate solution (2 %, 1.5 L). The eluate was evaporated *in vacuo* at 45 °C till dryness. The residue was extracted with hot methanol (3 mL × 300 mL), filtered though centered glass funnel (G3) and evaporated *in vacuo* at 45 °C till dryness to give a brown residue (1.76 g). The residue was subjected for further purification by passing through cellulose column (chromatographic grade, Merck, 64271 Darmstadt, Germany). Eluted by (solvent **c**) the solvent free residue (purified total glucosinolates), was extracted with distilled water, freeze dried and subjected to PPC, using solvent **a**, 3 runs as a developing solvent applying the descending technique. Two main compounds (RB 0.34 and 0.21) were isolated. They are subjected for further purification by passing through sephadex LH_{20} column eluted by 90 % methanol.

Screening for antitumor activity

Preparation of the samples: 50 g of seeds of *E. sativa* was extracted with petroleum ether in soxhlet apparatus, then the dried marc was percolated with 70 % ethanol till exhaustion. The total ethanolic extract was subjected to evaporation of the solvent *in vacuo* at 40 ºC, the remainder aqueous extract was lyophilized. 1 mg of this lyophilized powder was dissolved in 0.1 mL of DMSO and the volume completed to 1 mL with distilled water and sent to the National Cancer Institute.

The plant extract were screened *in vitro* using a single tumor (Ehrlich ascites carcinoma cells). The tumor cells were maintained in the laboratory by weekly intrapritonial transplantation in female albino mice from the animal house of National Cancer Institute. A set of sterile test tubes was used for each test solution, where 2.5×10^6 tumor cells per mL were suspended in phosphate buffer 0.1 mL of different dilutions of each test solution was added separately to the suspension, kept at 37 ºC for 2 h. Trypan blue dye exclusion test[10] was then carried out to calculate the percentage of non-viable cells, using a dose of 100, 50 and 25 µg/mL of each extract. Concentrations causing less than 30 % non-viable cells in the suspension were considered inactive, while those producing more than 70 % non-viable cells were considered active.

Assay for cytotoxic activity: Potential cytotoxic activity of the total ethanolic extract was performed in the National Cancer Institute reported method[11]. Cells were plated in (104 cells/well) for 24 h before treatment with the isolated compound to allow attachment of cell to the plate. Different concentrations of the compound under test (0, 1, 2.5 and 10 µg/mL) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated for 48 h at 37 ºC in atmosphere of 5 % CO_2, after 48 h cells were fixed, washed and stained with sulforhodamine B stain. Excess stain was washed with acetic acid and attached stain was recovered with *tris* EDTA buffer. Colour intensity was measured in an ELISA reader. The relation between surviving fraction and the drug concentration

is plotted (Fig. 1) to get the survival curve of each tumor cell line for the specified compound[11]. The effective dose required to inhibit cell growth by 50 % (IC_{50} μg/mL) was determined[11]. The total alcoholic extract and the hydrolyzates of the two glucosinolates were tested for the following human tumor cell lines at concentrations between 1.00-10.00 μg/mL using the SRB assay. (i) U251 (Brain tumor cell line). (ii) HEPG2 (Hepatocellular carcinoma cell line). (iii) Hela cell (cervix tumor cell line). (iv) HCT116 (Colon carcinoma cell line). (v) MCF7 (Breast carcinoma cell line).

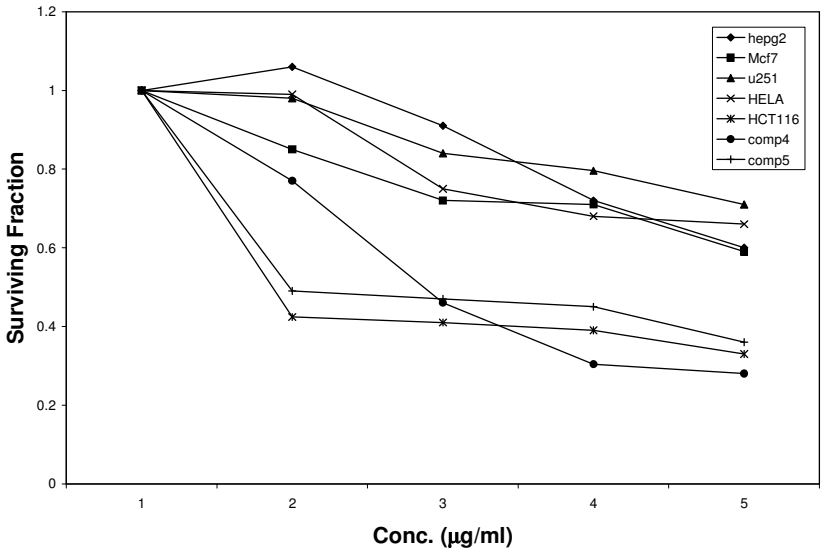


Fig. 1. Cytotoxic activities of total alcoholic extract, glucoerucin and glucoiberin isolated from *Eruca sativa* L. seeds

RESULTS AND DISCUSSION

GLC analysis of the unsaponifiable fraction proved to be a mixture of hydrocarbons and sterols. Identification of the compounds was carried out by comparison of their retention time with the available reference compounds. The identified compounds listed in Table-1 illustrated that β-sitosterol represents the main steroidal component (28.79 %).

GLC analysis of the fatty acid methyl esters resulted in the identification of 9 fatty acids Table-2 in which $C_{22(1)}$ erucic acid is the major component (44.86). Moreover, it was shown that the unsaturated fatty acids represent the major constituents of the total mixture (90.01 %) and total saturated fatty acids (9.09 %). The presence of high content of erucic acid makes the oil unsuitable for edible or medicinal use but high content of unsaturated fatty acids makes it reliable for some industries. It is mainly used in industries as a lubricant, for soap making, as an illuminating agent and in massaging[12].

TABLE-1
GLC ANALYSIS OF UNSAPONIFIABLE MATTER OF *Eruca sativa* SEEDS

Peak no.	RRT	Relative (%)	Constituents
1	0.31	3.52	*n*-Hexadecane C_{16}
2	0.38	9.37	Non identified
3	0.41	2.84	*n*-Heptadecane C_{17}
4	0.56	2.40	*n*-Octadecane C_{18}
5	0.57	6.37	*n*-Nonadecane C_{19}
6	0.59	2.21	*n*-Eicosane C_{20}
7	0.65	2.21	*n*-Heneicosane C_{21}
8	0.81	5.25	*n*-Tricosane C_{23}
9	0.86	2.19	*n*-Tetracosane C_{24}
10	0.92	8.99	*n*-Hexacosane C_{26}
11	0.95	6.05	Cholesterol
12	1.00	28.79	β-sitosterol
13	1.05	7.65	Stigmasterol
14	1.08	8.46	Campsterol
15	1.12	3.70	Non identified

RRT = Relative to retention time of β-sitosterol (36.51 min).

TABLE-2
GLC ANALYSIS OF FATTY ACIDS METHYL ESTERS OF *Eruca sativa* SEEDS

Peak no.	RRT	Relative (%)	Constituents
1	0.91	4.100	$C_{15(0)}$ Pentadecanoic acid
2	1.00	4.080	$C_{16(0)}$ Palmitic acid
3	1.09	9.900	$C_{18(1)}$ Oleic acid
4	1.10	12.30	$C_{18(2)}$ Linoliec acid
5	1.13	10.56	$C_{18(3)}$ Linolenic acid
6	1.22	0.980	Non identified
7	1.37	12.38	$C_{20(1)}$ Cosaenoic acid
8	1.40	44.10	$C_{22(1)}$ Erucic acid
9	1.66	1.600	$C_{24(1)}$ Nervonic acid

RRT = Relative to retention time of palmitic acid $C_{16(0)}$ (20.71 min).

Flavonoidal constituents

Compound I: Quercetin: The chromatographic behaviour in different solvents of this compound indicates that it is an aglycon in nature. It appears as a purple spot changed to yellow spot when exposed to ammonia vapour. The UV absorption spectra in the methanol displayed peak-I at λ_{max} (MeOH) at 368 nm which proved the flavanol nature of the compound in addition to bathochromic shift (53 nm) in peak-I with fast decomposition to 323 nm confirm the presence of the OH groups at C_3, $C_{3'}$, $C_{4'}$. The other shift reagents $AlCl_3$ and NaOAc and H_3BO_3 confirm the presence of free OH group at C_5 and C_7. The negative FABMS displayed molecular ion peak at m/z = 301 which corresponds to the molecular formula $C_{15}H_{10}O_7$. All these data were coincided with that reported for quercetin[8,13].

Compound II: Rhamnetin: The compound appeared as yellow dull spot in UV (366 nm) the colour intensified on exposure to NH_3 with R_f (0.69). TLC silica gel (solvent d) UV spectrum Table-3 showed λ_{max} (MeOH): 368, for band I, 269 (sh), 256 nm for band II, which justify the presence of a flavanol nucleus of 3', 4'-dihydroxy system which is further confirmed by bathochromic shift of (19 nm) with sodium acetate/boric acid diagnostic reagent.

TABLE-3
UV SPECTRA OF ISOLATED FLAVONOIDS

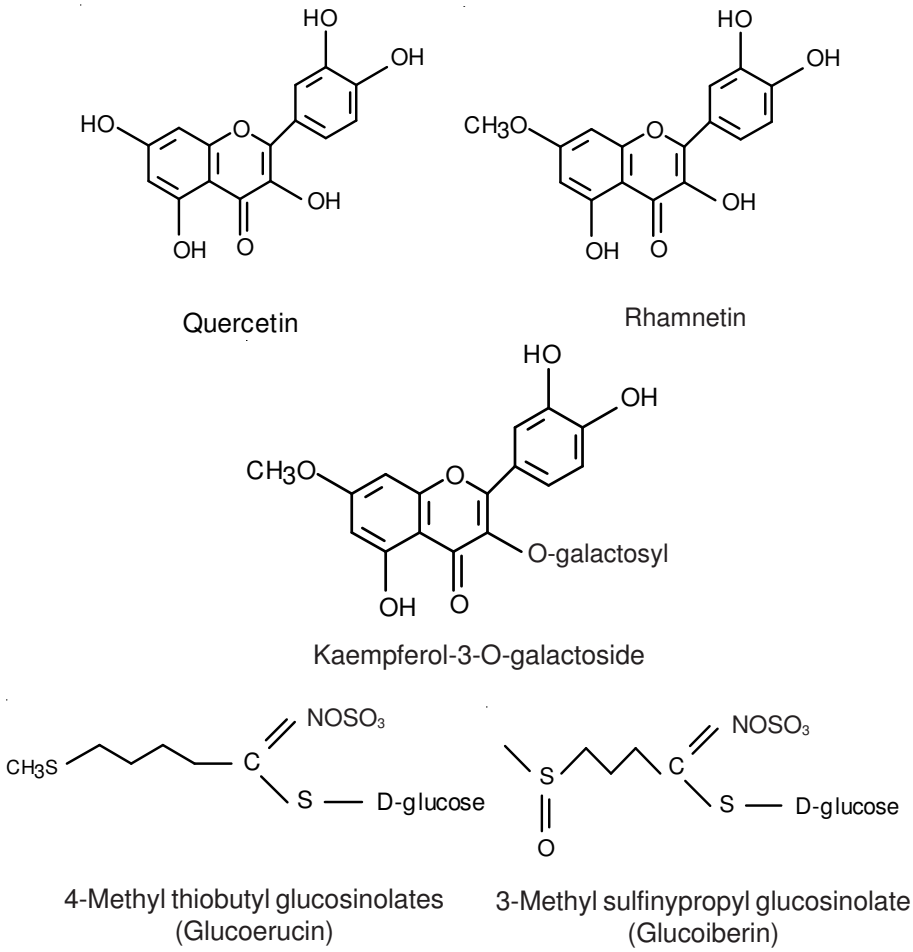
λ_{max} Comp.	MeOH	NaOMe	$AlCl_3$	$AlCl_3$/HCl	NaOAc	NaOAc/H_3BO_3
Comp. I	256, 267, 300 (sh), 368	277, 323 (sh), (Dec.)	271, 300 (sh), 350, 430	266, 360, 400	271, 320, 387	260, 370
Comp. II	256, 269, 292 (sh), 368	224, 286, 331, 428 (Dec.)	272, 300, 330 (sh) 449	268, 298 (sh), 363 (sh), 414	255, 292 (sh), 387, 418 (sh), (Dec.)	259, 387
Comp. III	266, 312 (sh), 351	270, 300 (sh), 351 (sh) 394	276, 301, 349, 401	279, 297 (sh) 346, 396	277, 312 (sh), 354, 403 (sh)	267, 316 (sh), 352

Compound I = Quercetin, Compound II = Rhamnetin,
Compound III = Kaempferol-3-O-galactoside.

It was also confirmed by the hypsochromic shift in band I of 35 nm of $AlCl_3$/HCl relative to $AlCl_3$ spectrum. There is no bathochromic shift of band II in the spectrum of sodium acetate shift reagent which indicates the absence of free 7-OH group. FAB/MS in a negative mode showed a molecular ion peak at m/z 315 and fragmentation pattern was found to be identical to that of rhamnetin. The previous data were found to be in accordance with that found in the literature for rhamnetin[13,14].

Compound III: Kaempferol-3-O-galactoside: The compound showed R_f values 0.38 and 0.39 [PC 3MM, (solvent a) and (solvent e)], respectively which suggest a monoglycosidic nature. From UV spectrum, Table-3 the compound showed two absorption maxima in methanol at 351 nm for band I and 266 nm for band II, suggesting a 3-substituted flavonol structure. The absence of *ortho*-dihydroxyl groups in ring B was confirmed by addition of $AlCl_3$ and $AlCl_3$/HCl mixture, there is no hypsochromic shift on addition of the acid[8]. The occurrence of 11 nm bathochromic shift of band II in the spectrum of sodium acetate indicates the presence of free 7- hydroxyl group. FAB/MS-ve mode showed a molecular ion peak at m/z 447 (M-1), at m/z 285, M-hexose. Acid hydrolysis revealed galactose in the aqueous layer, while the aglycone was detected in organic layer as kaempferol having the same UV spectra and R_f value of authentic sample. So the isolated compound was identified as a kaempferol 3-O-galactoside[8,13].

Compound IV: 4-Methyl thiobutyl glucosinolates (glucoerucin): The UV absorption spectrum of compound **IV** (R_B^* {*relative to glucotropaeolin} 0.34, in solvent c) in methanol showed λ_{max} at 225 nm and a hypsochromic shift to 220 nm



Structures of isolated compounds

by addition of NaOH, which indicates the glucosinolate nature of the compound[15]. The FAB/MS in a negative mode showed a molecular ion peak at m/z 420 (M^+-1) corresponding to the m.f. $C_{12}H_{22}O_9NS_3$. Enzymatic hydrolysis of the compound by myrosinaze enzyme produced an aglucone which subjected to GC/MS analysis according to the conditions mentioned before. The mass spectrum of the aglucone of compound **IV** showed a molecular ion peak at m/z 160 which corresponds to the m.f. ($C_5H_9NS_2$), 4-methylthiobutylisothiocyanate and base peak at m/z = 61 which corresponds to the loss of terminal methylthio group ($CH_3S^+=CH_2$) with fragment ion peaks at 146 (M^+-CH_3), 103 (M^+-NCS) and 75 (M^+-CH_3NCS). From this fragmentation pattern the aglucone was identified as 4-methylthiobutyl isothiocyanate (CH_3-S-CH_2-$CH_2CH_2CH_2NCS$). One mg of this aglycone was tested for the cytotoxic activity on HCT116 cell line (colon carcinoma cell line). The glucose was only the sugar detected in the enzymatic hydrolyzate by paper chromatography. The presence

of the SO_4^{2-} ions was also confirmed by addition of few drops of $BaCl_2$ solution to few mL of aqueous layer of the hydrolyzate and the formed precipitate of barium sulphate was noticed. From all the previous data the compound **IV** was identified as 4-(methylthio)butyl glucosinolate[16,17].

Compound V: 3-Methyl sulfinylpropylglucosinolate (glucoiberin): The UV absorption spectrum of compound **V** (R_B 0.21, in solvent c) in methanol showed λ_{max} at 223 nm and a hypsochromic shift to 219 nm by addition of NaOH, which indicates the glucosinolate nature of the compound[15]. FAB/MS in a negative mode showed a molecular ion peak at m/z 422 [M^+-1] which constituted to m.f. $C_{11}H_{20}NO_{10}S_3$ also base peak at m/z = 64 which corresponds to loss of terminal methyl sulfinyl group (M^+-CH_3S^+OH). The aglycone after enzymatic hydrolysis was subjected to GC/MS analysis using the same conditions. The mass spectrum of the aglucone showed a molecular ion peak at m/z = 162 which corresponds to m.f. $C_4H_7ONS_2$. Another important fragments at m/z = 161 (M^+-1), m/z = 146 (M^+-CH_4), m/z = 118 (M^+-CS) and at m/z = 104 (M^+-CNS). The glucose was only the sugar detected in the hydrolyzate. The presence of the SO_4^{2-} ions was also confirmed by addition of few drops of $BaCl_2$ solution to few mL of aqueous layer and the formed precipitate of barium sulphate was noticed. From all the previous data the compound **IV** was identified as 3-methylsulfinylpropyl glucosinolate[18].

The total alcoholic extract of the defatted powdered seeds of *Eruca sativa* was screened for antitumor activity, it showed 100, 95 and 90 % non viable count for the concentrations of 100, 50 and 25 µg/mL, respectively. So cytotoxic activity was performed against several types of tumor cell lines using the SRB assay. The total alcoholic extract, the aglucones of glucoerucin and glucoiberin exhibited significant cytotoxic activity for HCT116 (colon carcinoma cell line) (IC_{50} = 0.74, 2.42, 0.94 µg/mL), respectively, while the IC_{50} was > 10 µg/mL for both Hela (cervix carcinoma cell line), HEPG2 (liver carcinoma cell line), MCF7 (breast carcinoma cell line) and U251 (brain carcinoma cell line). This activity is due to the glucosinolate content. The isolated compounds showed relatively high IC_{50} than the total extract this may be due to sinergestic activity between total components. This result is potentiated by Wang *et al*.[4] they reported that sulforaphane, erucin and iberin from *Eruca sativa* Lam. Up-regulate thioredoxin reductase 1 expression in human MCF-7 cells. Gamet *et al*.[6] also found that sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells.

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