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Study of lipid constituents, glucosinolate –derived isothiocyanates and Antimicrobial activity of *Erucaria pinnata* growing in Libya

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Abstract:

Erucaria pinnata (family Brassicaceae) is growing in Sirt region, Libya. The plant was extracted with n-hexane in a soxhlet and fractionated into fatty alcohols, fatty acids and unsaponifiable matters. The GLC analysis of fatty acid methyl esters revealed the presence of six acids in which palmitic acid is the main one (65.10%). While, the GLC analysis of the unsaponifiable matters revealed the presence of a series of long chain hydrocarbons with n-C20 and n-C29 are the main ones (13.45% and 12.88%, respectively), in addition to three sterols. The total glucosinolates were extracted with aqueous methanol, purified and hydrolyzed with myrosinase enzyme. The obtained isothiocyanates after enzymatic hydrolysis were identified using GC/MS as 8-methylsulfinyl octylisothiocyanate, 4-methylsulfinyl-3-butenyl isothiocynate, 2-phenylethyl isothiocyanate and 3-methylthio-2-propenyl isothiocynate. The antimicrobial activity of the plant extracts in addition to total glucosinolates and isothiocyanates using disc diffusion method was studied against some Gram –ve, Gram +ve bacteria and fungi. The results proved that, the tested Gram –ve bacteria were resistance to all concentrations of all tested extracts with the exception of total isothiocyanates, glucosinolates and butanol extract respectively, and which exhibited different inhibition effects against all tested microorganisms.

Total isothiocyanates exhibited the highest inhibition activity against all tested microorganisms. However, ethyl acetate extract exhibited different inhibition effects against all tested Gram +ve bacteria only. While, the fatty alcohols and unsaponifiable matters showed no inhibition effects by any means against all tested microorganisms at all concentrations used. Above all, the results clearly showed that all inhibition effects exhibited were concentration dependant.

Key words: Crucifereae, Erucaria pinnata, fatty acids, unsapo -nifiable matters, glucosinolates, isothiocyanates, GC/MS and antimicrobial activity.

Introduction:

Brassicaceae (Crucifereae) family is one of largest families in plant kingdom, where it comprises about 300 genera and 1500 species. It includes vegetables, weeds, garden and wild flowers[1]. It is represented in Libya by 120 species, Erucaria pinnata is one of the most common species, which mainly grow in Sirt region and locally known as Saleekh[2]. Most of Brassicaceae plants have a characteristic flavor and aroma due to the presence of glucosinolates which is hydrolyzed by myrosinase enzyme to isothiocyanates; these compounds have many biological activities like antibacterial, antifungal, anticancer and exert potent effects on insects[3-6]. By reviewing the available literature, it was found that few studies about the chemical constituents and biological activities of this species. Hashem and Saleh [7] isolated many isothiocyanate after hydrolysis of E. microcarpa herb alcoholic extract with myrosinase which were identified as, isopropyl isothiocyanate, butyl isothiocyanate and benzyl isothiocyanate in addition to the non-volatile 8- methylsulphinyl octylisothiocyanate (hirsutin). The plant extracts contain high percentages of arachidonic and palmitic acids, nonadecane, cholesterol, stigmasterol and □-sitosterol. The volatile constituents of E. microcarpa showed higher activity against yeasts than Gram +ve, Gram -ve bacteria and fungi. The nonmethylated fatty acids of the plant showed higher activity against Gram +ve and Gram -ve bacteria than yeasts and molds. Hashem [8] isolated two steroids, namely, lanosterol and stigmasta-5, 22-dien-3-ol acetate; two coumarins, namely, corylidin and 2,3-dihydroxy-dihydrosubrosin; as well as four flavanoids, namely, quercetin-3-O-galactoside, lucenin-1, robinetin and isorhamnetin-3-O-galactoside were also isolated from the aerial

parts of Erucaria microcarpa Boiss., in addition to a chromone compound which identified as chromone 3-[2-(3,5-dimethomethoxy phenyl) ethenyl]-2-methyl. Testing this chromone for electron spin resonance, did not give any free radical alone, but with singlet oxygen (1O2), it gave one free radical, which rapidly combined with each other to give a stable compound. This chromone can thus be considered a singlet oxygen scavenger which changes (1O2) to ordinary oxygen.

This study aim to isolate, identify lipid constituents and isothiocyanates and also to evaluate the potency of different extracts of Erucaria pinnata as antimicrobial agents.

Materials and Methods:

Plant Material:

The plant was collected from Sirt region in February 2008 during the flowering stage. The plant was kindly identified by Prof. Mohamed El-Drawy, Faculty of Agriculture, Altahadi University. A voucher specimen has been deposited at the Herbarium of Faculty of science, Altahadi University, Sirt, Libya. The aerial parts of the plant (leaves, flowers and branches) were air dried and ground until they become as a fine powder.

Apparatus and Techniques:

1-Mass spectrophotometer GC/MS Jeol 500 Mass spectroscopy 70 ev.

2- Agilent Technologies 6890 N Network GC System

3-Gas liquid chromatography Hewlett Packard HP 6890 series.

4- Mass spectrophotometer GC-MS finnigan mat SSQ 7000, 70 ev.

Gas - liquid chromatographic analysis of unsaponifiable matters:

The following conditions were used for GLC analysis of the unsaponifiable matters Instrument: Agilent technologies 6890N (Network GC system) USA.

Oven: initial temp.: 80°C, initial time: 2min,Rate: 8°C/min., final temp.: 300°C, final time:25 min.

Injector temp. : 250°C (splitless), Detector temp. : 300 °C., Flow: 2ml/min

Column: Capillary column, HP-5.5% Phenyl Methyl Siloxane, Length = 30m, Diameter= 320µm, Film Thickness = 0.25µm, Carrier gas: N2: 30 ml/min.H2:30 ml/min. Air:300 ml/min

GLC analysis of fatty acid methyl esters was carried out according to the following conditions:-

Instrument: Agilent Technologies 6890 N Network GC System.

Oven: Initial temp.:70°C, Initial time: 2min, Rate: 10°C/min, Final Temp.:240°C, Final time, 25min.

Inlet temp.: 250 °C Splitless; Detector temp.: 300 °C (FID).

Flow rates: N2: 1 - 5 ml/min., Column: Capillary column HP-5.5% Phenyl Methyl Siloxane, Length = 30m, Diameter = 0.32µm, Film Thickness = 0.25µm

Carrier gas: N2: 30ml /min H2: 30ml /min Air: 300ml /min

GC/MS analysis of isothiocyanates:

Apparatus: GC-MS finnigan mat SSQ 7000. Column: DB5capillary column, I.D.0.25mm,

Ionization mode: Electronimpact ev 70,Temp.program: 50-250°C, 4°C/ min, Detector: MS

Sample volume: 2µl, Mass: 40-400, Work station: Digital DEC 3000.

Isolation of Lipids:

One kg of air dried powdered plant of Erucaria pinnata was extracted with n-hexane in a soxhlet for 24 hours until exhaustion. The hexane extract (dark greenish) was passed over fuller's earth to remove the colored pigments and then evaporated in vacuo at 40°C to give a yellowish residue (8.2g). The residue was dissolved in boiling acetone (100ml), cooled and the amorphous precipitate was separated out (acetone insoluble fraction= fatty alcohols fraction 2.1g). The acetone soluble fraction (5.1g) was saponified using N/2 alc. KOH, and the unsaponifiable matters were separated (3.2g) and analyzed by GLC. The liberated fatty acids mixture(1.35g) were

extracted and methylated (MeOH, HCl,5%) to afford methyl ester of fatty acids which were subjected to GLC analysis.

Extraction of the total Glucosinolates[4]:

About 950g of the defatted plant material were extracted with methanol (90%) for 48hours with continuous shaking from time to time (three times 2.5L each). The combined methanol extract was concentrated to 500ml, left for 24hrs at room temperature, the resulted sticky gummy precipitate was filtered, and the filterate was diluted with distilled water to 1 liter and then added onto an acidic alumina column (2.25kg). The column was first washed with deionized water (3.0L) and then the glucosinolates were eluted with potassium sulfate solution (2%) until the brown zone reached at the bottom of the column. The eluate was concentrated under reduced pressure till dryness, and the glucosinolates were extracted with hot methanol to remove the excess of potassium sulfate salt. The solution was evaporated till dryness and the total glucosinolates (2.3g) were subjected to further purification using cellulose column (300g) and elution was carried out using the upper layer of the solvent n-butanol: ethanol: water, (4:1:3). The eluant was evaporated and the residue was dissolved in distilled water, freeze dried to 0.75g in dry form.

Preparation of total isothiocyanates:

About 0.5g of total glucosinolates was subjected to enzymatic hydrolysis using myrosinase enzyme and little crystals of citric acid. The hydrolysate solution after saturation with NaCl was extracted with ether. The ether extract, after washing with water, dehydrated by passing over anhydrous sodium sulfate and evaporated in vaccuo leaving a small amount of semisolid yellow residue which was subjected to GC / MS analysis using the previous conditions.

Biological activity Study:

Preparation of the plant extracts for biological evaluation:

About 100g of the air dried powdered plant were first extracted (defatted) with n-hexane by maceration for 24 hours to afford hexane extract which tested as it is and fractionated to fatty alcohols, fatty acids and unsaponifiable matters. The defatted plant material was

then dried and after that extracted with methanol (70%) which tested as it is and partitioned to give chloroform, ethyl acetate, butanol and mother liquor (M.L.) extracts.

Three concentrations were prepared from each extract in addition to total glucosinolates (T.Gluc.) and total isothiocyanates (TITC) as a, b and c which were 50, 100 and 150 mg/ml respectively.

Antimicrobial activity:

The antimicrobial activity was determined using the sensitivity disk diffusion method of Kirby-Bauer and determination of inhibitory zone (I.Z.)[9].

Tested extracts: fatty alcohols, fatty acids, unsaponifiable matters, n-hexane, ethyl acetate, methanol, chloroform, butanol, mother liquor (M.L), total glucosinolates and total isothiocyanates and control (Standard Tetracycline and Miconazole).

Used microorganisms: listed in table.1 viz: Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Mycobacterium phlei, Enterobacter cloacae, Aspergillus niger and Candida albicans were obtained from the stock culture of Biotechnology Research Center, Tripoli, Libya. The microbiological media used were Mueller-Hinton agar for bacteria (Oxoid, UK), and Sabouraud dextrose agar (Oxoid, UK) for fungi.

Results and discussion:

The study of the lipid fraction of the aerial parts of the E. pinnata resulted in the identification of the unsaponifiable matters as well as fatty acid mixture. The GLC analysis of the unsaponifiable fraction table (1), revealed the presence of a mixture of normal hydrocarbons ranging from n-C15 to n-C29 in which n-C20 and n-C29 are the main ones (13.45% and 12.88%, respectively) with sterol fraction containing cholesterol (4.65%), stigmasterol (25.8%) and campasterol (14.84%). The GLC analysis of the fatty acid methyl esters, revealed the presence of six fatty acids in which palmitic acid is the main one (56.10%). The percentage of saturated fatty acids is (75.75%) while, the unsaturated acids constituted about (24.25%) in which the main unsaturated acid is oleic acid (17.51%). These findings are similar to

that reported by Hashem and Saleh [7] in 1999, who reported that, Erucaria microcarpa contain high percentages of arachidonic and palmitic acids, nonadecane (n-C19), cholesterol, stigmasterol and □-sitosterol but in this study of E. pinnata it was found that it does not contain □-sitosterol or arachidonic acid.

The obtained isothiocyantes after myrosinase hydrolysis of glucosinolates, were subjected to GC/MS. The results proved the presence of four isothiocyanates which were identified as:

Compound A: 8-methylsulfinyl octylisothiocyanate: the mass spectrum displayed a weak molecular ion peak M+ at m/z=233 (10%), in addition to very weak peak at m/z=234 (5%). The molecular formula adopted with this M+ was found to be C10H19NOS2. Another important ion peaks at m/z=216(M+- OH, 18%), m/z=170 (35%) due to the loss of mass 63 (CH3SO+) and the base peak at m/z=55 for C4H7+ ion. The cleavage which yielded the ion peak at m/z= 72 was noticed, these data were in accordance with that reported by Gayland and Melvin in 1980 [10] who investigated the GC/MS of isothioyantes derived from cruciferous glucosinolates.

Compound B: 4-methylsulfinyl-3-butenyl isothiocynate: this compound showed a molecular ion peak M+ at m/z= 175(24%) which correspond to the molecular formula C6H9NOS2. The fragmentation pattern displayed peaks at m/z=159(M+ - O+, 15%), 119(M+ - C4H8+, 25%) and 112(M+ - CH3SO+, 38%). The most pronounced peaks are at m/z=87(85%), and 72(100%) due to the fragments CH2CH2NCS++1 and CH2NCS+ respectively. This fragmentation pathway was found to be the same as that reported by Kjear et al [11], who studied the mass spectra of different isothiocyanates and found the same fragments.

Compound C: 2-phenylethylisothiocyanate: this compound is known as phenthyl isothiocyanate, the result of mass spectrum showed an intense molecular ion peak M+ at m/z= 163(65%) which was compatible with the molecular formula C9H9NS. The other important peaks are 105(M+ - NCS, 23%), 91(M+ - +CH2NCS, 100%), 77(M+ - +CH2CH2NCS, 18%) and 63(M+ - +CH2CH2CH2NCS, 12%).

Compound D: 3-methylthio-2-propenyl isothiocynate: the mass spectrum indicated that the molecular ion peak M+ at m/z= 145 (4%) which correspond to the molecular formula C5H7NS2 is very weak

but M+ +1 at m/z= 146 is more intense. A series of ion peaks is clearly seen at m/z=87(M+ - NCS, 25%), 73(M+ - +CH2NCS, 80%) and m/z= 60 due to [CH3SCH+] which is the base peak and equal to (M+ - +CHCH2NCS, 100%). These data were in agreement with that reported by VanEtten et al in 1976 [12], who investigated the hydrolysis products of glucosiolates which contain different isothiocyanates and nitriles among them.

The chromatographic investigation of different extracts by paper chromatography and thin layer chromatography revealed the presence of many classes of compounds such as glucosinolates, phenolics, saponnines and flavonoidal glycosides.

Biological evaluation:

Kirby-Bauer test results are interpreted using relates zone diameter to the degree of microbial resistance (Table. 3). The antimicrobial activity of different extracts with different concentrations (a=50mg/ml, b=100g/ml, c=150mg/ml) of Erucaria pinnata (Crucifereae) showed different effects against tested microorganisms (bacteria and fungi).

The results revealed that, the tested Gram –ve bacteria (E. coli and Ent. cloacae) were resistance to all concentrations of all tested extracts with the exception of total isothiocyanates, glucosinolates and butanol extract with inhibition zones of (I.Z. = 12.4, 10.3, & 8.4; and 9.9, 8.3, & 8.9mm, Conc.=150mg/ml), respectively. Additionally, total isothiocyanates exhibited the highest inhibition activity against all tested microorganisms (M. phlei, C. albicans, Staph. aureus, A. niger, E. coli, B. subtilis, and Ent. cloacae) with inhibition zones (I.Z. = 19.2, 17.3, 15.5, 12.9, 12.4, 11.34, and 9.9mm, Conc. = 150mg/ml), respectively. However, only total isothiocyanates, glucosinolates and butanol extract exhibited different inhibition effects against all tested organisms (E. coli, Ent. cloacae, Staph. aureus, B. subtilis, M. phlei, C. albicans, and A. niger) with inhibition zones (I.Z. = 12.4, 10.3, & 8.4; 9.9, 8.3, & 8.9; 15.5, 11.6, & 10.15; 11.34, 11.34, & 11.34; 19.2, 16.4, & 18.3; 17.3, 15.8, & 12.7; and 12.9, 10.1, & 11.57mm, Conc. = 150mg/ml), respectively. Also, ethyl acetate extract exhibited different inhibition effects against all tested Gram +ve bacteria only (M. phlei, B. subtilis and Staph. aureus) (I.Z. = 16.2, 11.2, and 10.95mm, Conc.=150mg/ml), respectively, these results are in accordance with

that reported by Pistelli et al. (2009)[13], who investigated the antimicrobial activity of flavonoids against different extracts of Inga fendleriana and proved that both the ethyl acetate and butanol extracts inhibited Gram +ve but not Gram -ve bacteria, and founded that both extracts contain flavonoidal glycosides. While, n-hexane, methanol and fatty acid extracts showed different inhibition effects only against two of the tested Gram +ve bacteria (M. phlei, and B. subtilis) (I.Z. = 15.2 & 11.46; 14.6 & 9.95; and 12.55 & 10.25mm, Conc.= 150mg/ml), respectively. However, the chloroform extract showed low inhibition effect against only one of the tested Gram +ve bacteria (B. subtilis) (I.Z.= 10.92mm, Conc.= 150mg/ml), respectively. Furthermore, the mother liquor (M. L) extract exhibited different inhibition effects against only one of the tested Gram +ve bacteria (M. phlei), and one of the tested fungi (C. albicans) (I.Z.= 14.0 & 10.9mm, Conc.= 150mg/ml), respectively. Meanwhile, the fatty alcohols and unsaponifiable matters extracts showed no inhibition effects by any means against all tested microorganisms at all concentrations used.

Above all, the results clearly showed that all inhibition effects exhibited were concentration dependant. These findings disagree with that reported by Mbosso et al (2010) [14] who proved that, the most inhibitory activity against Candida albicans was found with the mixture of linear aliphatic fatty alcohols (n-C24H50O to n-C30H62O), and also disagree with the results obtained by Al-Hela et al (2005) [15] who proved the antimicrobial activity of the unsaponifiable matters (which contain similar constituents to that of the tested one) isolated from Barbula convolute against some Gram +ve and Gram -ve bacteria, yeast and fungi. Also, Kitahra et al [16] proved the activity of some fatty acids and their amine derivatives against Methicillin-Resistant Staphylococcus aureus, and thus disagree with the findings of this study with regard to the none inhibition effects exhibited by fatty alcohols and unsaponifiable matters. However, this disagreement could be due to differences in chemical constituents present in each extract. Finally, the above results proved that all tested Gram –ve bacteria were resistance to all concentrations of all type of tested extracts with the exception of total isothiocyanates, glucosinolates and butanol extract which showed moderate to low inhibition effect against both tested organisms and only total isothiocyanates exhibited moderate inhibition effect against E. coli. Also, the tested fungi (C. albicans and A. niger) exhibited resistance against all concentrations of all type of extracts tested with the

exception of total isothiocyanates, glucosinolates, butanol and the mother liquor extracts (I.Z.= 17.3, 15.8, 12.7, & 10.9; and 12.9, 10.1, 11.57, & 0.0mm, Conc.= 150mg/ml), respectively, and from this it is clear that total isothiocyanates, and glucosinolates exhibited high inhibition effect against C. albicans, and moderate to low inhibition against A. niger, respectively, so, these results are in agreement with that found by Jahansson et al [17] who studied the growth inhibition of a spectrum of bacterial and fungal pathogens by sulforafane (an isothiocyanate product) found in broccoli and other cruciferous vegetables, and found that 23 out of 28 different microbial species were inhibited by sulforafane, and also found Candida albicans considered resistant at high concentration which is also in agreement with the results of this study. Finally, Nadarajah et al [18] who studied the use of mustard flour to inactivate Escherichia coli in ground beef under nitrogen flushed packaging, also reported that the activity was due to the presence of some isothiocyanates. In conclusion, the data obtained in this study were reported for the first time about this species and afford ground information for further investigation to isolate the most active pure compound.

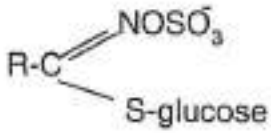

Fig.1: The general structure of glucosinolates

R-N=C=S

Fig.2: The general structure of isothiocyanates

Compound A: 8-methylsulfinyl octylisothiocyanate R= $CH_3SO(CH_2)_7CH_2$-

Compound B: 4-methylsulfinyl-3-butenyl isothiocynate = CH_3SOCH_2=CH- CH_2- CH_2-

Compound C: 2-phenylethylisothiocyanate = $CH_2(C_6H_5)$-CH_2-

Compound D: 3-methylthio-2-propenyl isothiocynate CH_3SOCH_2=CH- CH_2-

Table (1): GLC data of unsaponifiable matters of *E. Pinnata.*

Peak No.	R_t (min)	Relative (%)	Compounds
1	14.95	0.01	C_{15}
2	16.11	0.12	C_{16}
3	17.59	0.39	C_{17}
4	18.86	0.96	C_{18}
5	19.67	3.88	C_{19}
6	20.17	13.45	C_{20}
7	21.39	1.91	C_{21}
8	23.74	1.83	C_{22}
9	24.83	2.66	C_{23}
10	25.89	1.35	C_{24}
11	26.95	4.60	C_{25}
12	27.53	0.59	C_{26}
13	28.91	8.39	C_{27}
14	29.75	1.69	C_{28}
15	30.84	12.88	C_{29}
16	32.44	4.65	Cholesterol
17	34.19	14.84	Camposterol
18	35.43	25.80	Stigmasterol

R_t: Retention time (min.)

Table (2): GLC data of fatty acid methyl esters of *E. Pinnata*.

Peak No.	$R_{t\ (min)}$	Relative (%)	Compounds
1	18.11	10.18	Lauric ($C_{12:0}$)
2	20.24	9.47	Myristic ($C_{14:0}$)
3	22.31	56.10	Palmitic ($C_{16:0}$)
4	24.04	17.51	Oleic ($C_{18:1}$)
5	25.11	5.80	Linoleic ($C_{18:2}$)
6	25.95	0.95	Linolenic ($C_{18:3}$)

R_t: Retention time (min.).

Table (3): Antimicrobial activity of different extracts of *E. pinnata*

Extract	Conc.	Determination of inhibition zone (mm)						
		Gr. -ve bacteria		Gr. +ve bacteria			Fungi	
		E. coli	*Ent. cloaceae*	*Staph. aureus*	*B. subtilis*	*M. phlei*	*C. albicans*	*Asp. niger*
n-hexane	a	-	-	-	8.5±1.2	12.4±0.7	-	-
	b	-	-	-	9.8±0.9	14.1±1.0	-	-
	c	-	-	-	11.46±0.5	15.2±0.5	-	-
Fatty alcohol	a	-	-	-	-	-	-	-
	b	-	-	-	-	-	-	-
	c	-	-	-	-	-	-	-
Unsap. matters	a	-	-	-	-	-	-	-
	b	-	-	-	-	-	-	-
	c	-	-	-	-	-	-	-
Fatty acid	a	-	-	-	8.25±1.2	9.82±0.44	-	-
	b	-	-	-	9.7±0.40	11.0±0.0	-	-
	c	-	-	-	10.25±0.2	12.55±0.6	-	-

Methanol	a	-	-	-	7.89±1.0	12.0±1.2	-	-
	b	-	-	-	8.87±0.8	13.12±1.1	-	-
	c	-	-	-	9.95±0.6	14.6±0.7	-	-
Chloroform	a	-	-	-	8.45±1.2	-	-	-
	b	-	-	-	9.72±1.0	-	-	-
	c	-	-	-	10.92±0.2	-	-	-
Ethyl acetate	a	-	-	8.5±0.0	8.95±0.4	13.10±0.2	-	-
	b	-	-	9.65±0.22	10.0±1.0	14.9±0.4	-	-
	c	-	-	10.95±0.0	11.2±0.2	16.2±0.1	-	-
Butanol	a	-	-	8.5±1.1	9.32±0.2	15.7±1.0	9.8±0.3	9.4±0.2
	b	-	7.3±0.4	9.65±0.5	10.15±0.4	17.0±1.0	10.9±0.6	10.4±0.0
	c	8.4±0.8	8.9±1.0	10.15±0.4	11.34±0.2	18.3±1.1	12.7±0.2	11.57±0.2
M. L	a	-	-	-	-	11.0±1.20	8.5±1.2	-
	b	-	-	-	-	12.8±0.40	9.6±0.2	-
	c	-	-	-	-	14.0±0.15	10.9±0.2	-
T. gluc.	a	-	-	-	9.32±0.2	10.1±0.4	10.8±0.6	-
	b	9.1±0.2	-	8.4±0.8	10.15±0.4	13.6±1.3	13.6±0.9	-
	c	10.3±0.6	8.3±0.8	11.6±1.2	11.34±0.2	16.4±0.9	15.8±1.1	10.1±0.8
T ITC	a	-	-	9.4±0.8	9.32±0.2	16.5±1.4	11.6±0.4	-
	b	10.3±1.1	-	12.3±0.2	10.15±0.4	18.1±0.8	14.1±0.5	9.4±0.5
	c	12.4±0.5	9.9±1.0	15.5±0.9	11.34±0.2	19.2±1.3	17.3±0.8	12.9±1.5

a=50mg/ml, b=100mg/ml, c=150mg/ml, - = No Inhibition. T.gluc. =total glucosinolates and T.ITC =total isothiocyanates.

Standard Tetracycline: I.Z. = 19 mm, Standard Miconazole: I.Z. = 22 mm.

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