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PHYTOALEXINS INDUCED IN *CICER ARIETINUM* CHARACTERIZED BY LC-MS TECHNIQUE

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SUMMARY

The elicitor activity of high molecular weight polysaccharides (HMWP) of *Hypnea musciformis* (red algae) of Karachi coast was determined and established in chickpea tissues. A simple extraction procedure and HPLC method was developed to analyze the major and minor components of induced phytoalexins. LC–Electrospray-mass spectrometry, characterized some of the individual components and as a result, four compounds (isoflavonoids) and their glycoconjugates likewise formononetin, maackiain, naringin and naringin melonate were identified by their mass spectra.

***Key words*: Elicitor, *Hypnea musciformis*, isoflavonoids, phytoalexin**

INTRODUCTION

As a result of infection or stress, plants exhibit some natural resistance responses, amongst which induced browning and phytoalexin production have especially gained attention (Bailey and Mansfield 1982, Nicholson and Wood 2001). Phytoalexins are low molecular weight, biologically active compounds produced by plants as defensive reaction to various exogenous stimuli, particularly fungal invasion, their role in disease resistance has been deduced from their antifungal activity (Arnold and Merlin 1990, Svabova and Lebeda 2005). It is reported that hypersensitive responses produced in plants after microbial attack may be triggered by elicitors, the compounds isolated from cell wall, culture filtrate and cytoplasm of parasitic and non parasitic plant pathogens. Elicitors are diverse in nature and are usually polysaccharides, proteins and fatty acids (Rao *et al.* 1996). Some of the reported phytoalexins in leguminosae species are alkaloids, coumarins and especially isoflavonoid derivatives (Grayer and Kokubun 2001). The biosynthesis of isoflavonoid phytoalexins has been intensively studied and their metabolic regulation is known to be different according to the inductor agent, plant cell type and type of elicitors (Smith and Banks 1986). Recently a relationship between phytoalexin accumulation and defense against pathogenic microorganisms in pea (*Pisum sativum*), alfalfa (*Medicago sativa*), barrel medic *(Medicago truncatula*) and chickpea (*Cicer arietinum*) has been reported in literature (Liu *et al.* 2006).

Chickpea (*Cicer arietinum* L.) is an important crop plant and have large economic and nutritional importance due to its high content of protein. *Ascochyta rabiei* and *Fusarium oxysporum* are the fungi commonly invade chickpea and produce Ascochyta blight and fusarium wilt diseases in the crop (Jimenz *et al.* 2004). Phenolics like isoflavones (formononetin and biochanin-A), isoflavanones (homoferreirin and cicerin) and the pterocarpans (medicarpin and maackiain) are the representative phytoalexins of chickpea (Barz and Mackenbrock 1994). Experiments have shown the genetic degradation of chickpea phytoalexins medicarpin and maackiain by the

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fungal pathogen *Nectria haematococca* and converted them into less toxic compounds (Enkerli *et al.* 1998). Chemicals like IAA, cycloheximide and cyocel (abiotic elicitors) showed very strong protective effect (45 to 57 %) against Fusarium wilt (Chowdhury 2000). Similarly phenolics like gallic acid and ferulic acid prevented fungal infection caused by *Sclerotium rolfsii* in seedlings of chickpea (Sarma and Singh 2003).

It is documented that in most cases elicitor activity is associated with polysaccharides fraction of various elicitor preparations when tested in chickpea and other plant tissues (Melotto and Lebavitch 1994, Daniel *et al.* 1990). In the present study, seaweed (*Hypnea musciformis*) polysaccharides were evaluated as an elicitor or inducer of plant defense responses in terms of induced browning and phytoalexin production. A simple HPLC separation method was developed to analyze the major and minor components of mixture of induced secondary metabolites and the attempts were made to identify the individual components by LC –MS technique.

MATERIALS AND METHODS

Plant collection, extraction and dry weight of high molecular weight crude elicitor polysaccharides (HMWCEP) from hot water extract of *Hypnea musciformis*, red algae of Karachi coast was described earlier. These polysaccharides were chemically analyzed for total sugar, protein and sulphate content (Fatima and Seema 1999). Elicitor activity experiments of chickpea were conducted in PCSIR Labs. Complex Karachi, Pakistan. HPLC and LC-MS analysis of induced phytoalexins were carried out in Royal Holloway College, University of London.

Elicitor activity: A general method of elicitor application was employed in all experiments, as previously described by Whitehead *et al.* (1982). Chickpea (*Cicer arietinum* L.) desi channa was purchased from local market, germinated in a tray on filter paper placed on a moist cotton bed and kept at 25 ^{0}C in the dark. The excised cotyledons (2-3 days) were surface sterilized by immersion in 1% sodium hypochlorite solution for 2 minutes and then washed extensively with distilled water, finally rinsed with sterile water. Elicitor preparation of 100 µg glucose eq/ml were used for elicitor activity. Treated and control samples were prepared by application of 20 µl of elicitor preparation and sterile water (control) on the cut surface of cotyledons placed on a moistened filter paper in a petri dish (10- 15 cotyledons) and incubated at 25 ^{0} C in the dark for a specified time period of 24 hours.

Ethanol extraction for induced secondary metabolites (ISM): After specified period of incubation, treated and control cotyledons were dipped into 20 ml distilled ethanol (95 %) and left over night for complete extraction. Illumination was avoided as much as possible. The extracts were filtered through Whatman filter paper No.1 and concentrated on rotary evaporator below 40^{o}C. Concentrated solutions were transferred to sample bottle and the remaining solvent was removed by N_2. The residues were stored at 20 ^{o}C in the dark.

HPLC separation and identification of phytoalexins by LC-Electrospray–MS technique: HPLC separation was accomplished on high chrom 2.1 x 300 mm reverse phase C_{18} column using branded instrument of waters with a gradient system and variable UV detector. A guard column of pellicular C_{18} hydrocarbon chemically bonded to glass beads was placed before the analytical column, flow rate was 1 ml/min. Initially a solvent system consist of 80 : 20 Water : Acetonitrile (both phases contain 1 % acetic acid) was run for 5 minutes, then a gradient of 40 : 60 reached into 15 minutes and further goes to 100 % acetonitrile in 5 minutes, stays there for another 5 minutes and returned to initial solvent system, the whole chromatogram was monitored at 254 nm. All data were recorded on a finnigan LCQ operated in the negative ion full scan MS mode. The electrospray ionization (ESI) probe was installed with sheath and auxilliary gases were run at 80 and 20 units respectively. The reproducibility of elution time of the separated components was found 2-3%, the integrated area of the peak was assumed to be proportional to the amount of ISM present.

Sample preparation: Dry alcoholic extracts of elicited and control tissues were dissolved into 2 ml of 40: 60 water and acetonitrile (both phases contain 1 % acetic acid). 100 µl of each solution was further diluted with 1 ml of above solvent, centrifuged and filtered through 0.2

ìm filters. A clear solution of 25 µl was applied to the column.

RESULTS AND DISCUSSION

Main purpose of this study is to evaluate seaweed polysaccharides as an elicitor of disease resistance responses in plants. A common indication of pathological metabolism is some discoloration/browning of the affected tissues. Isoflavones and related pterocarpans constitute a group of secondary metabolites primarily in leguminous plants and are known for their role as phytoalexins (Barz and Welle 1992). Results of the present study showed that sliced cotyledons of chickpea treated with HMWP (High Molecular Weight Polysaccharides) of hot aqueous extract of *H.musciformis* (red algae) responded differentially and produced a positive and definite elicitor activity in terms of induced browning and phytoalexin production (Fig. 1). After recording browning, alcoholic extracts of treated and control tissues of chickpea were prepared for the estimation of ISM polyphenols (isoflavonoids). An improved HPLC method in the present case provided separation and quantification of induced secondary polyphenols (isoflavonoids including glycones and aglycones). Samples treated with 100 µg elicitor concentration and 24 hour incubation time were the best conditions to induce metabolites at maximum level in the samples. Previous studies for time course and dose dependent elicitor activity revealed that elicitor from fungus *A.rabiei* induced large amount of pterocarpon phytopalexins medicarpin and macckiain in cell suspension cultures of chickpea within 8 hours in resistant varieties whereas in susceptible cultivar only small amount of phytoalexins were accumulated (Kessman *et al.* 1988).

Due to low amount of induced metabolites and non-availability of standard material, mass spectrometry appears to be the ideal technique for the identification of these secondary metabolites (Kite *et al.* 2006). The alcoholic extracts resolved by HPLC, monitored by UV at 254 nm and total ion current chromatograms in negative mode are displayed in Fig. 2. Not necessarily all the peaks appeared in total ion current chromatogram were characterized in this study. Some peaks are prominent at RT 1.31, 1.44, 16.67 and 19.99 in total ion current chromatogram which are also found in UV analog of the same as shown in Fig.2. The peaks were interpreted by the molecular ion mass [M – 1] in negative ion mode, as compared with the relative molecular mass [M] (Table 1). The molecular masses [M] of the identified compounds were calculated on the basis of the data generated by LC-MS analysis and the reported structure of the compound.

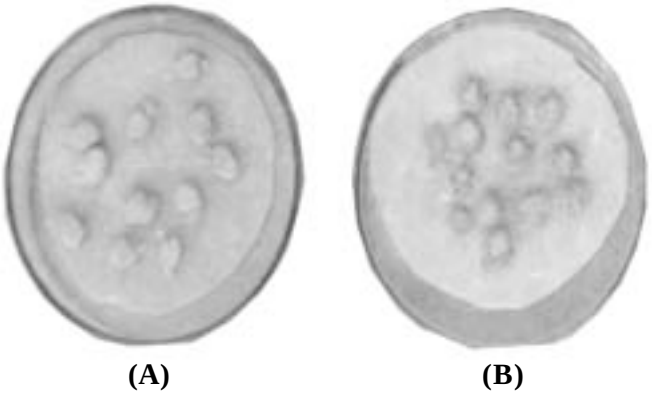


Fig. 1. (A) Control samples B) Browning induced in the etiolated cotyledons of chickpea treated with HMW polysaccharides of *Hypnea musciformis* (red algae) of Karachi coast at a concentration 100 µg glu eq/ml

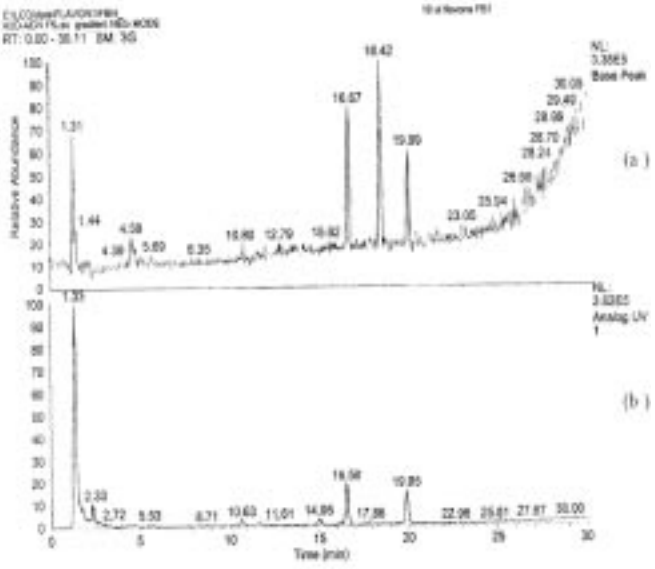
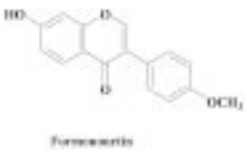
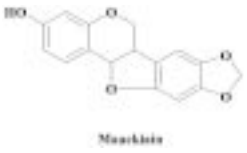


Fig. 2. Total ion current chromatogram in negetive mode (a), and LC-UV chromatogram (b), of induced secondary metabolites of chickpea in response to biotic elicitor of *H. musciformis* (red algae)

Table 1. Characterization of phytoalexins by LC-MS induced in *Cicer arietinum* (chickpea) by treatment of biotic elicitor of *Hypnea musciformis* (red algae)

Retention time (min)	[M - Na]$^-$	Molecular formula/ mass	Name / structure
1.31	683.4	$C_{30}H_{34}O_{18[683]}$	
1.44	579.7	$C_{27}H_{32}O_{14[580]}$	
	[M - 1]$^-$		
16.67	267.6	$C_{16}H_{12}O_{4[268]}$	
19.99	283.6	$C_{16}H_{12}O_{5[284]}$	

The metabolic grid of an enzyme sequence showing the conjugation reaction and the metabolic turnover of isoflavonoids in *Cicer arietinum* (chickpea) was established earlier (Hinderer *et al.* 1986) and explained the conjugation reaction of isoflavanone such as formononetin-7-O glucoside and formononetin-7-O-glucoside-6-O-melonate. Formononetin is referred as an intermediate component for the biosynthesis of pterocarpan phytoalexin maackiain and medicarpin in this system. Barz *et al.* (1988) reported formononetin with one sugar component. The present studies showed an early glycoconjugation reaction soon as the naringenin is formed in the primary biosynthetic pathway (Barz *et al.* 1988). LC-MS data showed that isoflavanone naringenin conjugation reaction provided two compounds correspond to peaks at RT 1.31 and 1.44, eluted in aqueous phase of the chromatogram (Fig. 3 and 4).

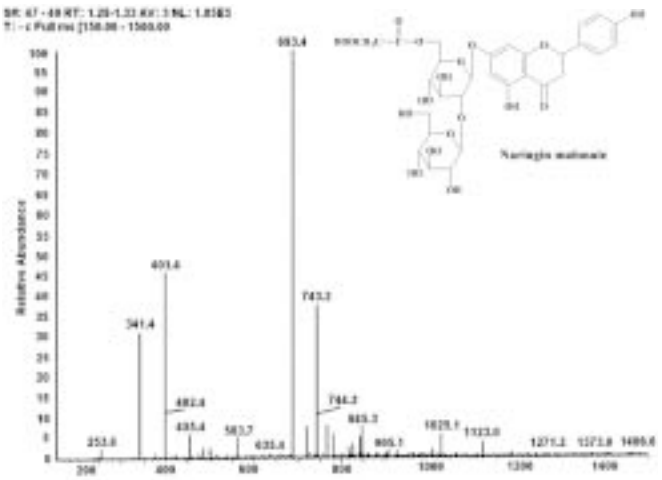


Fig. 3. Mass of negetive ion of naringin malonate, an induced phytoalexin labeled peak at RT. 1.31

These compounds were characterized as naringin malonate (naringin-7-O-diglucoside-6-O-melonate) of molecular mass [683] and naringin (naringin- 7- O-diglycoside) of molecular mass [580]. Mass calculation indicated the presence of two sugar moieties, glucose and rhamnose in these two compounds which were detected and confirmed by paper chromatography. These compounds were also characterized by Zhang and Brodbelt (2004) and first time identified during this interaction.

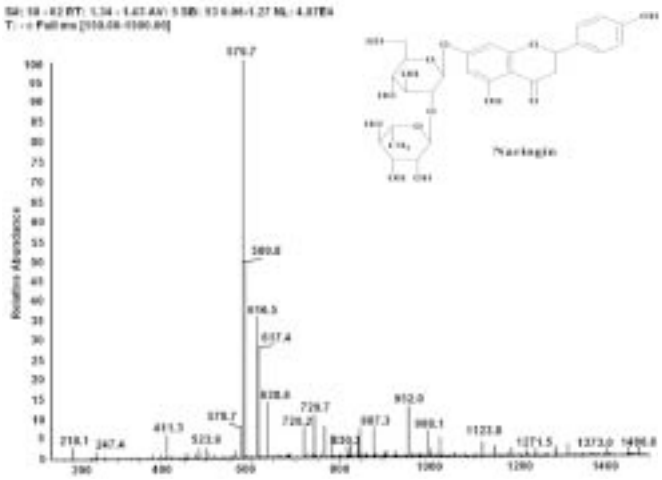


Fig. 4. Mass of negetive ion of naringin, an induced phytoalexin labeled peak at RT. 1.44

The compounds refers the peaks at RT 16.67 and 19.99, eluted in organic phase of the chromatogram were identified as formononetin with a molecular mass of [268] and the second component was maackiain of molecular mass [284] and considered as chickpea phytoalexins (Fig. 5 and 6). These results support the investigation of Sumner *et al.* 1988 that described the optimization parameters in the identification of flavonoid glucosides in alfalfa and chickpea extracts using High Performance Liquid Chromatography/ Continuous – Flow Liquid Secondary Ion Mass Spectrometric (HPLC/CF-LSIMS) technique.

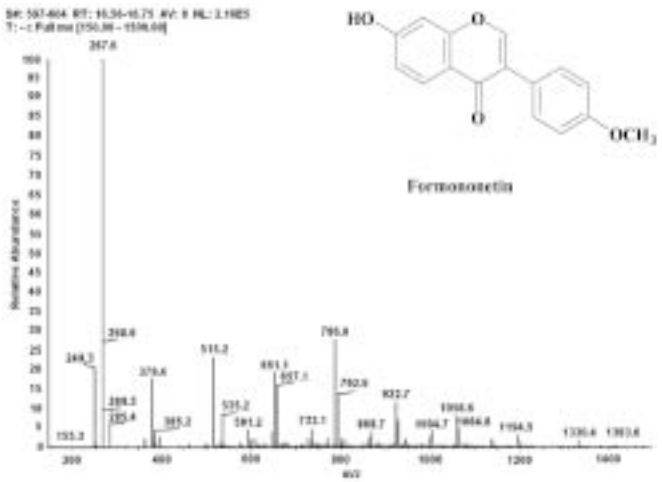


Fig. 5. Mass of negetive ion of formononetin, an induced phytoalexin labeled peak at RT. 16.67

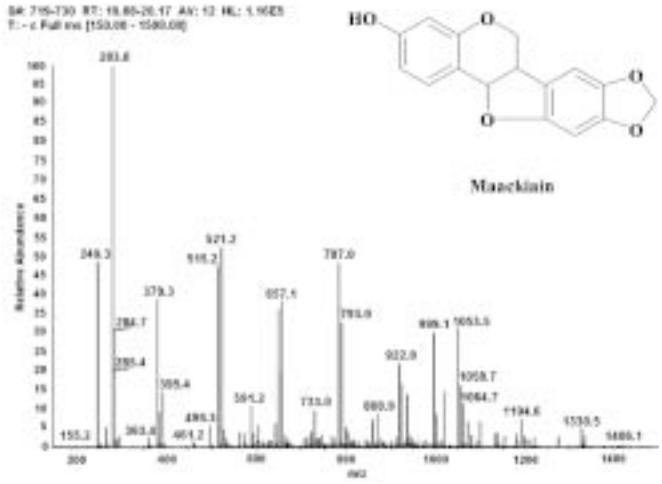


Fig. 6. Mass of negetive ion of maackiain, an induced phytoalexin labeled peak at RT. 19.99

The developed HPLC method produced a good separation and quantification of induced polyphenolic compounds, isoflavonoids and isoflavonoid glycoconjugates characterized as naringin, naringin melonate, formononetin and maackiain by using technique of LC-MS on ESI mode. Methods and techniques involved have an advantage of reduced time of analysis which make these techniques one of the promising tools available for looking at initial changes in a particular plant pathogen or plant elicitor interactions and also facilitates the routine processing of large number of elicited samples for quantitation and identification. High level induction of metabolites in chickpea tissues suggests that the seaweed polysaccharides especially from the red algal plant, *Hypnea musciformis* are active elicitor of plant defense responses and can be used as the potent plant protectant.

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