Phytomedicine, Vol. 7(5), pp. 389–399

http://www.urbanfischer.de/journals/phytomed

Phytomedicine

Phyto-adaptogens protect against environmental stress-induced death of embryos from the freshwater snail *Lymnaea stagnalis*

E. K. Boon-Niermeijer[1], A. van den Berg[1], G. Wikman[2] and F. A. C. Wiegant[1]

[1] Department of Molecular Cell Biology, Utrecht University, Utrecht, The Netherlands
[2] Swedish Herbal Institute, Göteborg, Sweden

Summary

The main purpose of the studies presented in this paper is twofold: 1) to evaluate whether phyto-adaptogens (*Acanthopanax senticosus* and *Rhodiola rosea*) are able to exert a protective action against stress-induced death of embryos of the pond snail *Lymnaea stagnalis*; and 2) whether a possible protective action by phyto-adaptogens can be explained by the induction of heat shock proteins.
Enhancement in resistance by phyto-adaptogens was studied by applying plant extracts for a period of 20 hours to 3-day old larvae of the pond snail *Lymnaea stagnalis*. Subsequently they were exposed to a high and toxic dose of different environmental stressors. The following stress conditions were selected: a physical stress condition (heat shock: 43°C for 4 minutes), an oxidative stress condition (superoxide radicals induced by menadione (600μM for 2 hours)) and heavy metal-induced stress (copper (150μM for 1 hour) or cadmium (20μM during 1 hour)).
Both *Acanthopanax* and *Rhodiola* exert a strong protective action against a lethal heat shock. These adaptogens also significantly protect against the negative effect of superoxide radicals as induced by menadione. With respect to the protective action against exposure to heavy metals a small but significant protection was observed against intoxication with copper or cadmium by the phyto-adaptogens. In summary, there appears to be a difference in efficiency in enhancing resistance to the various stress conditions used (heat shock>menadione>copper>cadmium). Based on the results presented in this paper, we can conclude that phyto-adaptogens are able to enhance the resistance against the different stress conditions tested in developing individuals of *Lymnaea*. Although the degree to which resistance is enhanced appears to depend on the type of stressor applied, our results confirm the definition of phyto-adaptogens as being universal enhancers of non-specific resistance against different kinds of stress conditions.
With respect to the mechanism of enhanced resistance, the question was asked whether this protective action is caused by an induction of heat shock proteins (hsps), which are known to be involved in tolerance and adaptation. The phyto-adaptogens did not induce the synthesis of any of the hsps, nor did they modulate the normal heat shock induced synthesis of these stress proteins. We conclude that it is unlikely that hsps play a major role in obtaining an enhanced state of resistance provided by phyto-adaptogens.

Key words: Phyto-Adaptogens, stress induced *in vitro* death model, snail, *Lymnaea stagnalis*.

Introduction

An increase in non-specific resistance to various stress conditions including exposure to toxic conditions has been reported frequently when organisms were treated with phyto-adaptogens (Brekhman and Dardymov 1969, Brekhman 1980; Wagner et al 1994; Wagner 1995). Indeed a large number of experiments indicate that these adaptogens increase physical performance and adaptive ability when extracts from plants such as

0944-7113/00/07/05-389 $ 15.00/0

Acanthopanax senticosus and *Rhodiola rosea* were applied to a variety of model systems (Wagner et al 1994; Wagner 1995; Panossian et al 1999a for review).

In this paper we used the freshwater snail *Lymnaea stagnalis* as a model to extend our knowledge on the action of phyto-adaptogens. In the past decade, *Lymnaea* has increasingly been used as a model system to study the effect of different compounds in toxicity assays (Khangarot and Ray 1988; Szucs et al 1994; Gomot 1997, 1998).

Individual eggs from the freshwater snail *Lymnaea stagnalis* were incubated in water extracts of phyto-adaptogens and subsequently exposed to different concentrations of various environmental stressors (heat shock, menadione (an oxidative stressor) and heavy metals (copper and cadmium)). A number of stress conditions including heat shock have been used previously in larvae of *Lymnaea* to study the molecular basis of tolerance development (reviewed in Boon-Niermeijer 1991). An oxidative stress condition was selected since adaptogens were suggested to have anti-oxidant properties or to induce anti-oxidant defense. Menadione is frequently used in oxidative stress research as a compound that induces superoxide free radicals (Thor et al 1982; Chiou et al 1997). With respect to the heavy metals, copper is used as a molluscicid, whereas the effect of cadmium is frequently studied in toxicity assays on snails (Szucs et al 1994; Gomot 1998).

The modulatory effect of adaptogens was studied on stress-induced lethality. Survival or lethality is easily quantified as was shown previously in larvae of *Lymnaea* that were exposed to a lethal heat shock (Boon-Niermeijer and Van de Scheur 1984). Individuals that die, disintegrate completely within 24 hours after treatment.

Although various mechanisms have been postulated, it is essentially unknown how adaptogens exert their protective effect (Wagner 1995; Panossian et al 1999a). In the past decade, it became increasingly clear that the so-called heat shock or stress proteins (hsps) play a key role in protection and recovery at the cellular level (Welch 1992; Parsell and Lindquist 1994; Feige et al 1996). Their rapid induction under conditions of stress has been recognized as a vital cellular protective mechanism. Therefore, it has been suggested that adaptogens may influence the synthesis of heat shock proteins (Prodius et al 1996; Malyshev et al 1996; Afanasiev et al 1996), thus providing for an enhanced state of non-specific resistance.

The aim of our studies presented in this paper is two-fold

– to investigate whether exposure to phyto-adaptogens protects against a number of environmental stress conditions.
– to analyse whether heat shock proteins are involved in a protective action of these adaptogens.

Materials and Methods

Culture conditions

Adult specimens of the fresh water snail *Lymnaea stagnalis* (Gastropoda, Pulmonata, Basommatophora) were bred under standard laboratory conditions. The animals were housed in glass aquaria containing copper-free areated tap water at 20°C. They were kept under a 12h–12h light-dark cycle and fed a daily ration of lettuce. Egg laying was stimulated by transfer to clean water at 25°C. The eggs have been fertilized before laying and are deposited in egg masses, each containing 80 to 150 egg capsules. Each snail embryo is surrounded by an egg capsule. In Fig. 1 an impression is given of adult snails with an egg mass in between, as well as different stages of the embryos in their egg capsules (0, 3 and 7 days). The egg masses laid on the aquaria walls were collected 6 hours after stimulation and were kept in copper-free tap water at 24–25°C until the trochophore stage is reached after 3 days. At this stage, individual capsules were freed from the egg mass and transferred to plastic petri dishes (Greiner, Frickenhausen, Germany). All eggs from one egg mass were distributed among the different experimental groups in order to avoid effects due to slight differences in sensitivity between egg masses. For each experiment at least 3–5 egg masses were used in order to have triplicate samples, each sample consisting of at least 20 individual larvae. Each experimental group thus consisted of at least 60–100 individuals. The larvae remained within their capsules throughout the experiment.

Stress treatments

• *Heat shock:* Heat treatment was applied by transfer of the egg capsules from tap water of 25°C to glass tubes with tap water of 43°C. These were kept in a water bath heated with Thermomixes that provide an accuracy of 0.1°C. The temperature transition was abrupt, due to an excess of water used. Heat treatment was applied during 4 minutes which causes the death of 95 % of the population.

Groups of eggs were either exposed to phyto-adaptogens during 20 hours or they remained in tap water and were then subjected to heat. Immediately following heat treatment they were transferred again into plastic culture dishes with tap water of 25°C. Survival was evaluated the next day. As described previously, the lethal effect of heat shock on the trochophore stage is easy to determine (Boon-Niermeijer and Van de Scheur 1984; Boon-Niemeijer et al., 1988a). When the larvae

die they disintegrate completely within 24 hours after treatment. The survivors develop normally although their overall development shows some retardation (Boon-Niermeijer and Van de Scheur 1984). The effect of heat treatment was expressed as a percentage of survival on the day after the test treatment. Significance was tested by the Student's t-test.

• *Chemical stress conditions*. Cadmium chloride, copper sulphate and menadione were all obtained from Sigma, St.Louis, USA. Groups of 20 to 30 eggs at the 3-day-old larval stage were exposed for 1 hour to either 20 µM cadmium or 150 µM copper. Incubations were performed in plastic culture dishes containing a volume of 3 ml. Exposure to 600 µM menadione lasted for 2 hours. After completion of the stress period, the larvae were carefully rinsed and subsequently incubated in fresh tap water in order to evaluate survival during the subsequent period of four to six days. Survival was scored by daily inspection. The concentrations of adaptogens used did not affect survival. The effect of treatment is presented as a percentage of survival on the day that the death of 70–90 % of the population was observed. Significance was tested by the Student's t-test. The statistical procedures used were provided within the statistical package SPSS for Windows.

Protein synthesis and induction of heat shock proteins

The effect of adaptogens and of heat shock on the pattern of protein synthesis was analysed after one-dimensional SDS-PAGE. Protein synthesis in capsulated larvae was determined by incorporation of [^{35}S]-methionine and [^{35}S]-cysteine (SA of both amino acids: 1300 Ci/mmol, Amersham, Bristol, UK). 20 µCi of the radioactive tracers was used per ml. Embryos were incubated in radioactive medium either in the presence or absence of phyto-adaptogens during a period of 20 hours. After incubation, the larvae were decapsulated, carefully rinsed and solubilized in sample buffer (Laemmli 1970). Radioactivity was determined as hot trichloroacetic acid-precipitable fractions. Separation of proteins (samples adjusted to equal amounts of radioactivity) was performed by polyacrylamide gel electrophoresis (10 % gels) according to Laemmli (1970).

Adaptogens

The extracts from the adaptogens *Acanthopanax senticosus* and *Rhodiola rosea* were provided by the Swedish Herbal Institute (Göteborg, Sweden). Most experiments were performed with water extracts of *Acanthopanax* (EX20422), extr.spissum: containing

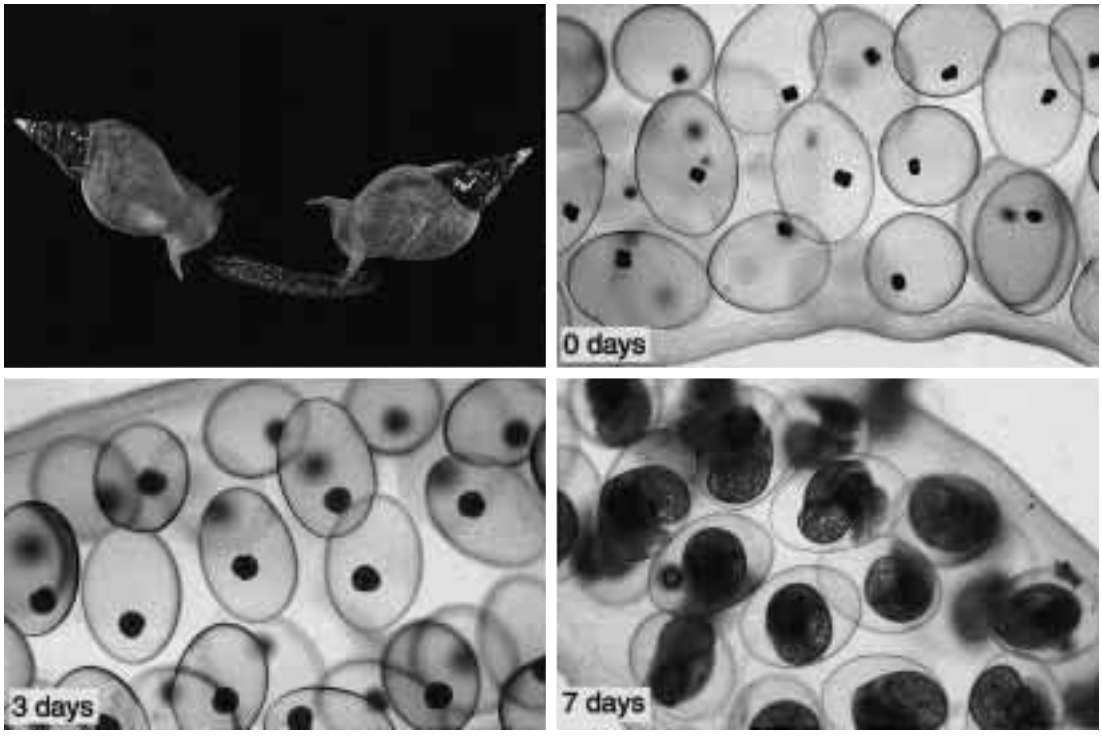

Fig. 1. Introduction to the model system. Adult specimen of the pond snail *Lymnaea stagnalis* just having produced an egg mass (A). Detail of the egg mass at day 0 showing individual encapsulated embryos (B). Individual embryos were freed of the egg mass and incubated in plastic petri dishes at day 3 (C). Morphology of the control embryos at day 7, just before hatching (D).

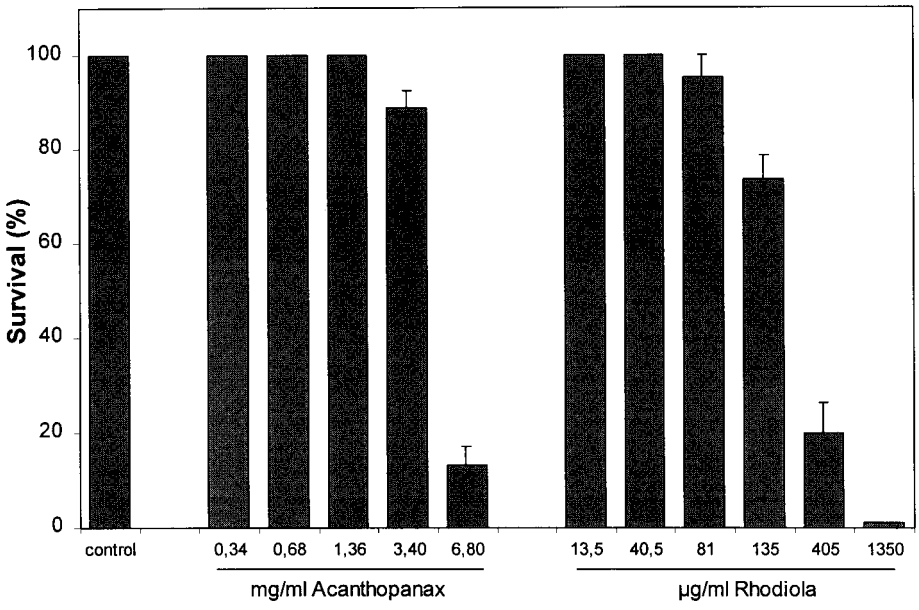

Fig. 2. Effect of increasing concentrations of phyto-adaptogens on survival of *Lymnaea* embryos. After a 24 hour exposure to the indicated concentrations of *Acanthopanax senticosus* or *Rhodiola rosea* survival was evaluated.

Eleutherosid-E (1 %) and Eleutherosid-B (0.34 %), and *Rhodiola,* SHR-5 (EX20404), extr.spissum: containing Rosavin (3.6 %); Salidrosid (1.6 %); p-tyrosol (< 0.1 %). Stock solutions of both extracts were diluted to the same ratio (1.9:1). Extracts of *Acanthopanax senticosus* were diluted in copper-free tap water to final concentrations ranging from 0.68 mg/ml to 68 µg/ml. The extract of *Rhodiola rosea* was used in a dilution ranging from 40.5 µg/ml to 4.05 µg/ml. In a number of experiments the effect was studied of ethanol extracts of the phyto-adaptogens.

Results

Effect of different concentrations of phyto-adaptogens on survival and development

In first instance the effect was determined of different concentrations of adaptogens on the survival of *Lymnaea* individuals. Larvae were exposed for 24 hours to increasing concentrations of *Acanthopanax senticosus* or *Rhodiola rosea*.

As is shown in Fig. 2, the highest concentrations tested are toxic. At a concentration of 1.35 mg/ml *Rhodiola* extract, all larvae are killed. At a concentration of 6.8 mg/ml *Acanthopanax* or 405 µg/ml *Rhodiola*, the death of about 80 % of the population is induced. Lower concentrations did not result in the death of individual larvae.

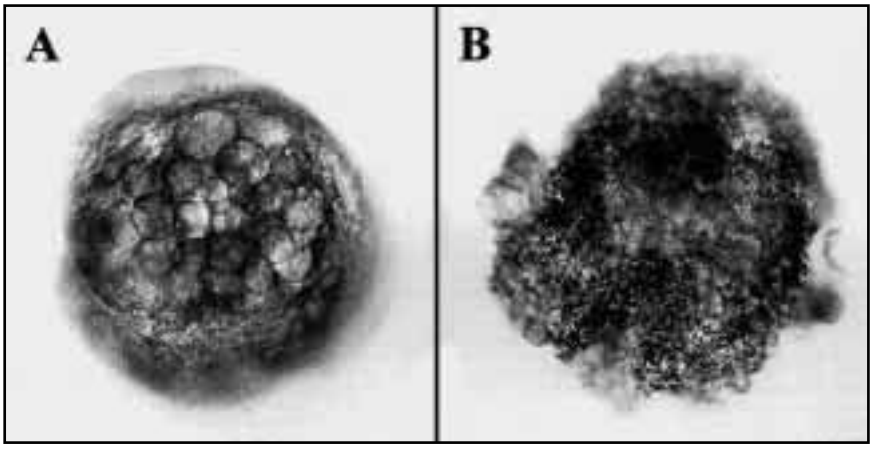

Fig. 3. Comparison of normal and heat shocked larvae of *Lymnaea stagnalis* at the 3-day old stage.
A) normal 3-day-old trochophore larva. B). Same larva shortly after heat shock, falling apart into loose, dying cells.

To study any possible detrimental effect of longer exposures, larvae were incubated up to 4 days in extracts from the phyto-adaptogens. The observations made during the experiments provided information on both the survival and development of the embryos up to the moment of hatching. A concentration of 1.36 mg/ml *Acanthopanax* or 81.2 µg/ml *Rhodiola* extract did not induce the death of any of the exposed larvae. However, at these concentrations development was somewhat retarded, leading to smaller individuals that hatched at a later time as compared to control conditions. It should be noted though that no deformations or abnormal individuals were observed. At a concentration of 0.68 mg/ml *Acanthopanax* and 40.5 µg/ml *Rhodiola* we did not observe any slowing down of growth and development. Embryos developed normally and hatched at the same time in comparison with non-exposed control embryos. For further experiments these concentrations were selected.

Effect of different stress conditions

In order to be able to study a possible protective effect of phyto-adaptogens, we first selected a number of stress conditions that were able to affect survival of *Lymnaea* embryos. As physical stress condition a short exposure to heat shock was chosen. Survival was quantified 24 hours later. Individuals that die, disintegrate completely within 24 hours after heat shock, as is shown in Fig. 3. As chemical stress conditions we selected menadione, a compound known to induce oxidative stress. In addition, two heavy metals were selected: cadmium (frequently used in toxicity studies in which snails are used) and copper (a frequently used molluscicid).

Like the effect of a heat shock, the effect of menadione could also be evaluated within 24 hours after treatment, since no more deaths occurred thereafter.

In contrast, the effect of a short exposure to heavy metals did not have an immediate effect. Survival was hardly affected within 24 hours after treatment, but decreased during the next couple of days. Therefore survival was monitored during a time period up to four days following exposure to these heavy metals. A possible protective action of phyto-adaptogens was evaluated at the moment that the control group reached a survival value of about 10–20 %.

For our further studies, stress conditions were selected that killed 80–90 % of the population at some moment during a subsequent period of 4 days. These conditions are: a heat shock at 43°C for 4 minutes, 600 µM menadione for 2 hours, a 1-hour exposure to 150µM copper sulphate or a 1-hour exposure to 20µM cadmium chloride.

Effect of phyto-adaptogens on the stressor-induced death

In order to determine whether phyto-adaptogens induce a non-specific resistance against stress-induced death, *Lymnaea* embryos were first incubated during a period of 20 hours with adaptogens, rinsed and subsequently exposed to one of the selected stress conditions mentioned earlier. After completion of the stress period, the embryos were rinsed again and transferred to fresh copper-free tap water in order to determine survival the next day (heat shock or menadione) or during a period of four days (copper and cadmium). At the moment that the control group reached a survival value of about 10–20 %, effects of pre-incubation with the phyto-adaptogens are evaluated.

- Protection against a lethal heat shock

In the next set of experiments we studied the effect of pre-incubation with phyto-adaptogens on the capacity of the 3-day-old larvae to withstand a lethal heat shock (4 minutes at 43°C). Without any pre-incubation, only 9 % of the control population survives this heat shock (Fig. 4).

As a reference, we studied the effect of a pre-exposure to a non-lethal hyperthermic temperature (38°C for 1 hour) known to induce a thermotolerant state to a subsequent lethal heat shock (Boon-Niermeijer et al 1986). The percentage of individuals that survive the lethal test treatment (43°C for 4 minutes) increased from 9 % to 84 % when the population of larvae were pre-exposed to the non-lethal hyperthermic temperature (38°C for 1 hour) 8 hours before the test heat shock was given (Fig. 4). This rise in survival percentage indicates the development of thermotolerance and is in agreement with previous data (Boon-Niermeijer et al. 1986).

Now, the question was asked whether the adaptogens (*Acanthopanax senticosus* or *Rhodiola rosea*) are also able to induce a state of thermotolerance. To this end, groups of larvae were incubated in the presence of different concentrations of these phyto-adaptogens during a period of 20 hours and subsequently exposed to the test heat shock. As is shown in Fig. 4, *Acanthopanax* or *Rhodiola* indeed provided a concentration dependent protection since the percentage of survival after heat rose from 9 % to about 90 % due to an incubation for a period of 20 hours in 0.68 mg/ml *Acanthopanax* or 40.5 µg/ml *Rhodiola*.

With respect to the duration of incubation with phyto-adaptogens we did not observe a significant difference in the degree of protection against a lethal heat shock between an incubation time of 8 hours or 20 hours (not shown).

In order to verify whether this protective effect depends on the type of extract (water or ethanol extract),

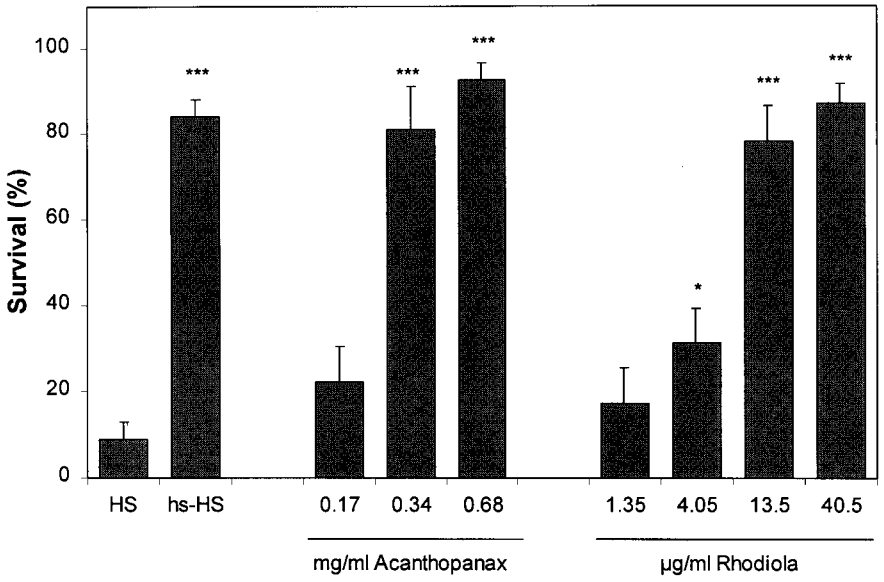

Fig. 4. Effect of pretreatment during 20 hours with different concentrations of an extract of *Acanthopanax senticosus* or *Rhodiola rosea* on survival percentages of individuals that were exposed to a lethal heat shock (43°C for 4 minutes). In addition, the effect is shown of a pre-treatment with a 1-hour hyperthermic exposure to 38°C, 8 hours before the lethal heat shock.

experiments were performed with an ethanol extract of *Acanthopanax*. Effects of a concentration range of 0.08 to 0.80 mg/ml *Acanthopanax* were tested. Since the stock solution of the extracts contain 40 % ethanol, a concentration range of the final ethanol concentration to which snail embryos were exposed was also tested (0.01–0.1 %). Although higher concentrations of ethanol (2 %) are known to induce thermotolerance (Boon-Niermeijer et al. 1988a), we did not observe any induction of tolerance by ethanol in the concentration range used in this study (not shown). Larvae were exposed to the indicated concentrations of *Acanthopanax* for 8 hours and subsequently exposed to a heat shock. As is shown in Fig. 5 and Table 1, a concentration-dependent increase in thermotolerance is also induced by ethanol extracts from *Acanthopanax*. A highly significant increase is observed in individuals that were exposed to 0.40 or 0.80 mg/ml *Acanthopanax* (survival percentage of 52.5 % and 88.7 % respectively) in comparison with non-pretreated individuals that showed a survival percentage of 4.5 %. Incubation in a concentration of 0.08 or 0.20 mg/ml *Acanthopanax* did not lead to a significant induction of thermotolerance.

An ethanol extract of *Rhodiola* also exerted a similar protective effect as a water extract of this phyto-adaptogen (not shown).

Since no significant difference in effectivity was observed between water extracts and ethanol extracts, we decided to use water extracts for our further experi-

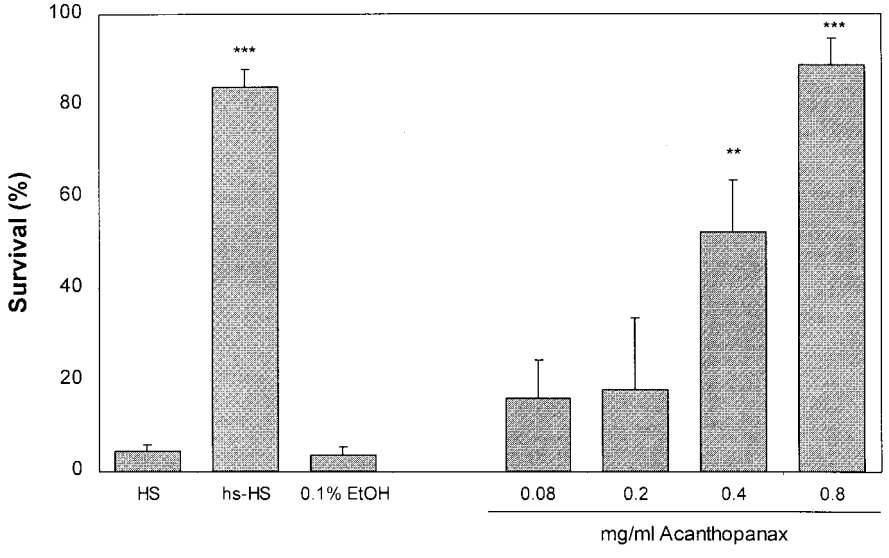

Fig. 5. Effect of pre-treatment during 8 hours with different concentrations of an ethanol extract of *Acanthopanax senticosus* on survival percentages of individuals that were exposed to a lethal heat shock (43°C during 4 minutes). In addition, the effect of an 8-hour incubation in the presence of 0.1 % ethanol before application of the heat shock is shown.

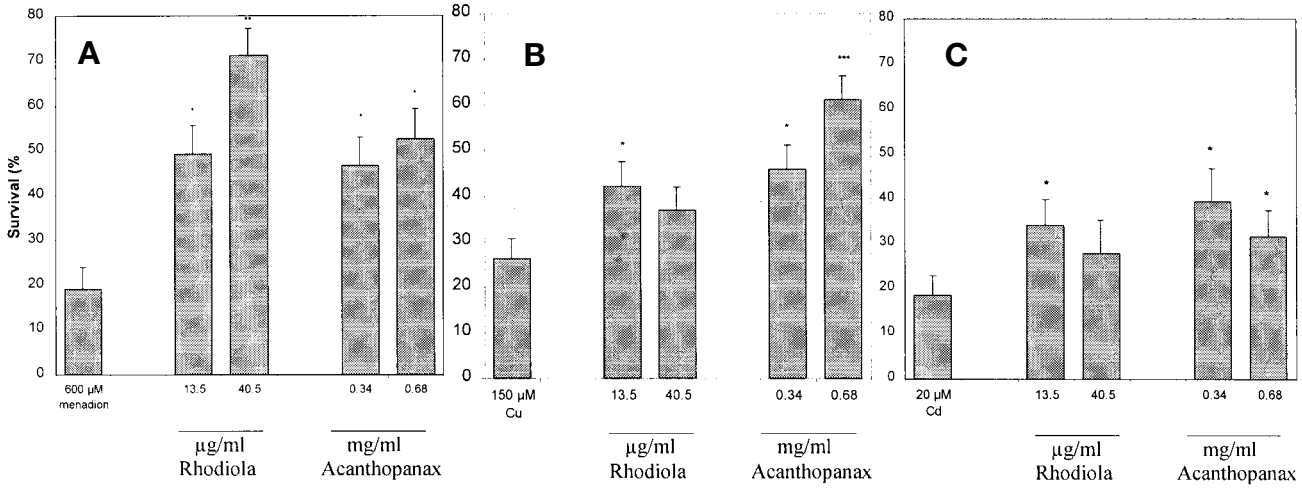

Fig. 6. Effects of preincubation for 20 hrs with *Rhodiola rosea* and *Acanthopanax senticosus* on survival after treatment with different stressors. Panel A represents survival after one day following incubation with 600µM Menadione for 2 hrs. Panel B shows survival two days after treatment with 150µM Copper for 1 hour. Panel C shows survival 5 days after exposure for 1 hour to 20µM Cadmium. Significance was tested by Students t-Test and is indicated by asterisks (* indicates $p < 0.05$; ** indicates $p < 0.01$ and *** indicates $p < 0.001$).

ments in order to exclude possible synergistic adverse effects between ethanol and the other stress conditions.

In conclusion, incubation of *Lymnaea* larvae in *Acanthopanax* or *Rhodiola* for a period of 8 or 20 hours protects them against a lethal heat shock. Nearly 90 % of the population is able to survive the lethal test treatment when pre-incubated with 0.68 mg/ml *Acanthopanax* or 40.5 µg/ml *Rhodiola*, whereas only about 5–10 % of the non-pretreated control population is able to survive this lethal heat shock.

For the next set of experiments two concentrations of each adaptogen were selected that were shown to be effective against heat shock. The question was asked whether these concentrations could also protect against other stress conditions such as menadione, copper or cadmium.

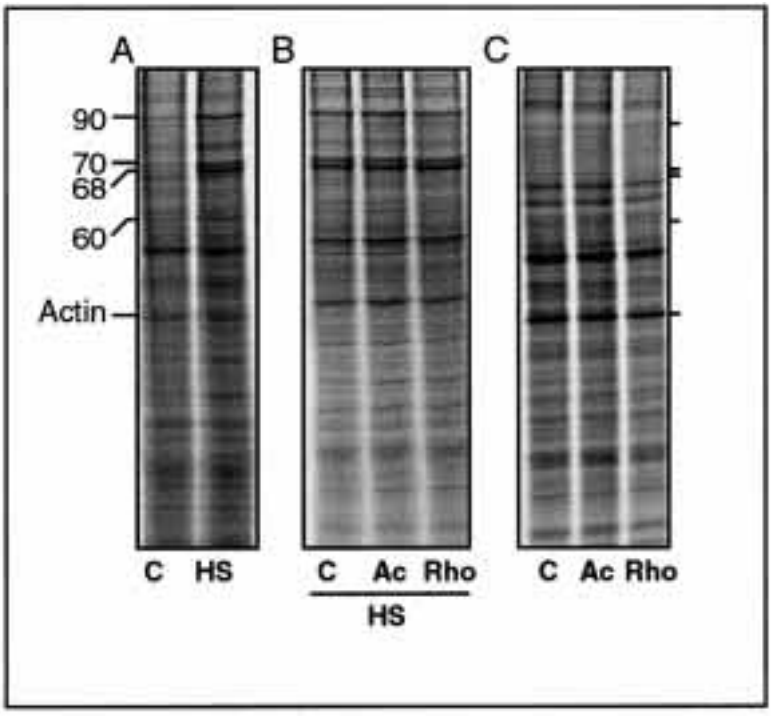

Fig. 7. Autoradiographs of gel electrophoretic pattern of synthesised proteins. Data of a number of experiments are shown.
A. the pattern of proteins that are synthesised during a period of 20 hours in control conditions (C) or following a heat shock challenge at 38°C during 1 hour (hs). Hsps are indicated with their molecular weight in kD.
B. The pattern of proteins synthesized following a heat shock. Groups of larvae were pre-exposed for 20 hours either in control conditions (C), in the presence of *Acanthopanax* (0.68 mg/ml)(Ac) or in the presence of *Rhodiola rosea* (40.5 µg/ml)(Rh). Following pre-exposure, groups of larvae were exposed to a heat treatment (38°C for 1 hour). During the subsequent 20 hours newly synthesized proteins were labelled using the radioactive aminoacids methionine and cysteine.
C. The pattern of proteins synthesized during a period of 20 hours in control conditions (C) or in the presence of *Acanthopanax* (0.68 mg/ml)(Ac) or *Rhodiola rosea* (40.5µg/ml) (Rh).

Table 1. Effect of pretreatments with hyperthermia, ethanol (0.1 %) or ethanol extracts of phyto-adaptogens on heat shock-induced death of 3-day old embryos of *Lymnaea stagnalis*.

	Survival (%)	SD	SEM	p-value
Control	4.5	4.7	1.4	
Hyperthermia(38°C)	84.0	12.0	4.5	.000
Ethanol (0.1 %)	3.6	5.6	1.7	.728
Acanth. 0.08 mg/ml	16.1	16.6	8.3	.253
Acanth. 0.20	18.0	31.3	15.7	.451
Acanth 0.40	52.5	27.2	11.1	.007
Acanth 0.80	88.7	16.1	5.7	.000
Rhodiola 4.05 µg/ml	30.9	4.7	2.7	.002

• Protection by phyto-adaptogens to menadione, copper or cadmium

Lymnaea larvae were incubated for 20 hours in the presence of phyto-adaptogens, rinsed and exposed to 600µM menadione for a period of 2 hours. After this oxidative stress challenge, larvae were rinsed again and transferred to clean tap water. Survival was evaluated 24 hours later. As is shown in Fig. 6A, both *Acanthopanax* (0.34 and 0.68 mg/ml) and *Rhodiola* (13.5 and 40.5 µg/ml) were able to protect significantly against menadione-induced death of the larvae (Table 2).

Fig. 6B shows that *Acanthopanax* as well as to *Rhodiola* have a protective effect against a subsequent toxic exposure for 1 hour to copper sulphate, as evaluated 2 days following intoxication. Although the degree of protection is lower in comparison with the effect of the adaptogens against heat shock or menadione-induced lethality, it is still significant (Table 2). Interestingly, no significant protective effect was observed in the presence of the highest concentration of Rhodiola.

As is shown in Fig. 6C, adaptogens hardly protect against a lethal exposure to cadmium chloride. Without pre-exposure, about 80 % of the embryos are killed as a result of cadmium intoxication. Pre-treatment with *Acanthopanax* or *Rhodiola* (13.5µg/ml) only slightly, but significantly, enhanced these survival values (Fig. 6C, Table 2).

Mechanism; induction of heat shock proteins

As a possible explanation of the protective effect of *Acanthopanax* and *Rhodiola*, we investigated whether extracts from these phyto-adaptogens are able to induce heat shock proteins. The effect was studied of a 20 hour exposure to *Acanthopanax* (0.68 mg/ml) or *Rhodiola* (40.5 µg/ml) since these concentrations were shown to be able to induce a significant protection against heat shock and menadione, without having any adverse effect on growth or development.

As references, we used the pattern of proteins synthesized during 20 hours under either control conditions or after 1 hour at 38°C. This heat treatment has previously been shown to induce a thermotolerant state as well as the expression of all the heat shock proteins (Boon-Niermeijer et al. 1986; 1988b). Fig. 7A shows that exposure to 38°C for 1 hour induces all heat shock proteins which confirms previous observations.

In order to study the effect of the phyto-adaptogens on gene expression, newly synthesized proteins were radioactively labelled during a period of 20 hours in the presence of *Acanthopanax* or *Rhodiola*. As can be observed in Fig. 7C, these adaptogens do not induce the synthesis of any of the heat shock proteins.

In order to determine whether an exposure for 20 hours to *Acanthopanax* or *Rhodiola* might modulate the synthesis of hsps induced by a mild heat shock, preincubated larvae were heat shocked and synthesis of hsps was monitored during the subsequent 20 hours. Exposure to the phyto-adaptogens did not influence the heat shock-induced pattern of synthesized proteins (Fig. 7B). It can be concluded that *Acanthopanax* and *Rhodiola* did not interfere with the synthesis of heat shock proteins.

Table 2. Protective effect of pretreatments with *Acanthopanax* or *Rhodiola* on chemical stress-induced death of *Lymnaea stagnalis*.

	Menadione Mean	p	Copper mean	p	Cadmium mean	p
Control	23.3 ± 10.7		25.3 ± 5.0		19.4 ± 6.3	
Acanth. 0.34 mg/ml	48.0 ± 6.7	.049	46.1 ± 2.8	.027	38.6 ± 11.2	.011
0.68 mg/ml	52.7 ± 11.8	.032	61.9 ± 4.0	.000	33.3 ± 6.4	.024
Rhodiola 13.5 µg/ml	51.7 ± 11.9	.046	41.3 ± 5.5	.045	35.6 ± 8.1	.036
40.5 µg/ml	72.0 ± 4.5	.001	36.9 ± 4.7	.132	29.5 ± 11.2	.282

The 'p-value' indicates significance of the protective action of the phyto-adaptogens against stress-induced death by the indicated conditions in the absence of adaptogens as evaluated with the Student's t-test

Discussion

The primary objective of this study was to examine the possible induction of a non-specific resistance against environmental stress conditions using extracts from phyto-adaptogens (*Acanthopanax senticosus* and *Rhodiola rosea*).

The protective effects of phyto-adaptogens were analyzed in 3-day old individuals of the freshwater snail *Lymnaea stagnalis* that were exposed to different environmental stress conditions. A pre-exposure for 20 hours to various concentrations of *Acanthopanax* or *Rhodiola* showed a dose-dependent protective action towards a subsequent lethal heat shock (43°C for 4 minutes). Whereas 9 % of a control population was able to survive this lethal heat shock, about 90 % of the larvae that were previously exposed to *Acanthopanax* (0.68 mg/ml) or *Rhodiola* (40.5µg/ml) were able to survive this lethal heat shock. Lower concentrations provided less protection. Concentrations of 68µg/ml or 4.05µg/ml of *Acanthopanax* and *Rhodiola* respectively did not protect at all. The observed protective effect appeared to be independent of the type of extract (being aqueous or ethanolic).

With respect to the possible underlying mechanism of this protective or preventive effect, we studied whether adaptogens were able to induce synthesis of hsps. Different authors have suggested that adaptogens might modulate induction of hsps and in this way stimulate the adaptive mechanism (Afanasiev et al. 1996; Prodius et al. 1996; Malyshev et al. 1996).

In the model system used in this study, no effect of *Acanthopanax* or *Rhodiola* could be observed on the induction of hsps. In addition, the adaptogens did not prevent the normal induction of hsps by a hyperthermic treatment.

The phyto-adaptogens did not induce the synthesis of any of the hsps, nor did they modulate the normal heat shock induced synthesis of these stress proteins in the described experimental conditions.

As an alternative mechanism that may explain the preventive potential of phyto-adaptogens, their anti-oxidative properties might be of relevance. In this paper we showed that *Acanthopanax* and *Rhodiola* are also effective against an exposure to menadione, an agent that induces superoxide free radicals. However, Wagner et al. (1994) suggested that this property is probably not the main action of adaptogens since many plants have anti-oxidative capacities without being adaptogenic.

The mechanism on which the beneficial protective effect is based remains essentially unknown. A number of mechanisms might be postulated:

– Gene products might be induced during the period of exposure to phyto-adaptogens that specifically enhance resistance against the denaturing and/or protein damaging conditions of heat shock and of oxidative free radicals. Although no indication was obtained to support this hypothesis, we could not exclude its possible relevance. In this paper we demonstrated that phyto-adaptogens did not induce hsps nor modulate heat shock induced hsp-synthesis.
– A mechanism that leads to a reduced sensitivity is related to the action of glycerol. Glycerol has been reported to enhance tolerance against heat shock in a non-specific way (Henle and Warters 1982). This enhancement of resistance has been postulated to occur by induction of structured water that is known to stabilize proteins and thus enhance tolerance (Lin et al. 1984; Piper et al. 1993). With respect to the action of phyto-adaptogens, it might be postulated that the glycosylated compounds in the extract enhance the degree of structured water thereby specifically protecting against heat denaturation, but not or hardly against heavy metal induced chemical stress.
– Recently it has been postulated that Panax ginseng might exert its beneficial effect by generation of nitric oxide (Gillis 1997). In this respect it is interesting that phyto-adaptogens were also reported to enhance the level of nitric oxide as determined in saliva of patients (Panossian et al. 1999b). In a recent overview evidence has been presented that low concentrations of nitric oxide are able to enhance the adaptive capacity of cells and organisms (Malyshev and Manukhina 1998). It remains to be determined whether a nitric oxide link for the phyto-adaptogens can also be established in different model systems.

In the definition of Brekhman (1980), adaptogens were described as having the following characteristics:

1. an adaptogen must show a non-specific activity; i.e. an increase in power of resistance against physical, chemical or biological noxious agents
2. an adaptogen must have a normalizing influence independent of the nature of the pathological state
3. an adaptogen must be innocuous and must not influence normal body functions more than required

In order to determine the non-specificity of the protective effect of phyto-adaptogens, we also studied the effect of *Acanthopanax* and *Rhodiola* on stress-induced death by the heavy metals copper and cadmium. These compounds did not exert an immediate effect. Only after a couple of days lethal effects became manifest. Apparently, irreparable damage was induced at the 3-day old stage that disturbed further normal development leading to the death of the embryo some days later. The adaptogens were able to prevent copper- and cadmium induced death to a small, but significant extent.

The question is why the adaptogens protect against heat in a highly efficient way, but to a lesser degree

against chemical stress. This might be due to the nature of the stress condition. Physical stress such as heat shock is only present during the stress period. After the stress period the embryos are immediately relieved of the higher temperature. Chemical compounds remain for a longer period in the cells of the embryo and it also takes time for the compound to diffuse out of the capsule fluid. In addition, it may be chelated or bound by proteins or other cellular compounds thereby leading to a longer presence of the compound in the period that the phyto-adaptogens are absent externally.

It can be concluded that larvae of the pond snail *Lymnaea* is an interesting model to evaluate the effect of phyto-adaptogens. In our opinion, this model might be interesting as an inexpensive, rapid and easy to handle bioassay to study the overall effectiveness of phyto-adaptogens in a protective and possibly also a curative context. Currently we are evaluating the protective action in conditions of chronic stress in order to determine whether phyto-adaptogens are able to postpone the exhaustion phase.

A drawback of the model is that it is less suitable to perform molecular analysis. Due to the fact that larvae are small and encapsulated it is quite complicated to obtain cellular material in sufficient quantities.

References

Afanasiev, S.A., Krylatov, A., Lasukova, T.V., Lishmanov, Yu.B. The role of inducible stress proteins in cardioprotective effects of Rhodiola rosea. *J. Biokhimiya (Moscow) 61:* 1779–1784, 1996

Boon-Niermeijer, E.K. Heat shock effects in snail development. *Results and problems in cell differentiation 17:* 7–28, 1991.

Boon-Niermeijer, E.K., Scheur, H. Thermosensitivity during embryonic development of Lymnaea stagnalis (Mollusca). *J. Therm. Biol. 9:* 265–269, 1984

Boon-Niermeijer, E.K., Tuijl, M.J.M., van de Scheur, M. Evidence for two states of thermotolerance. *Int. J. Hyperthermia 2:* 93–105, 1986.

Boon-Niermeijer, E.K., De Waal, A.M., Souren, J.E.M., Van Wijk, R. Heat-induced changes in thermosensitivity and gene expression during development. *Dev. Growth Differ. 30:* 705–715, 1988a.

Boon-Niermeijer EK, Souren JEM, De Waal AM, Van Wijk R. Thermotolerance induced by heat and ethanol. *Int. J. Hyperthermia 4:* 211–222, 1988b.

Brekhman, I.I. *Man and biologically active substance; the effect of drugs, diet and pollution on health*. Oxford: Pergamon Press, 1980:

Brekhman, I.I., Dardymov, I.V. New substances of plant origin which increase non-specific resistance. *Ann. Rev. Pharmacol. 9:* 419–430, 1969.

Chiou, T-J., Zhang, J., Ferrans, V.J., Tzeng, W-F. Cardiac and renal toxicity of menadione in rat. *Toxicology 124:* 193–202, 1997.

Feige, U., Morimoto, R.I., Yahara, I., Polla, B.S. (eds). *Stress-inducible cellular responses*. Basel, Birkhäuser Verlag, 1996.

Gillis, C.N. Panax ginseng pharmacology; a nitric oxide link? *Biochem. Pharmacol. 54:* 1–8, 1997.

Gomot, A. Dose-dependent effects of cadmium on the growth of snails in toxicity bioassays. *Arch. Environm. Contam. Toxicol. 33:* 209–216, 1997.

Gomot, A. Toxic effects of cadmium on reproduction, development, and hatching in the freshwater snail Lymnaea stagnalis for water quality monitoring. *Ecotoxicol. Environm. Safety 41:* 288–297, 1998.

Henle, K.J., Warters, R.L. Heat protection by glycerol in vitro. *Cancer Res. 42:* 2171–2176, 1982.

Khangarot, B.S., Ray, P.K. Sensitivity of freshwater pulmonate snails, *Lymnaea lutoela* L. to heavy metals. *Bull. Environm. Contam. Toxicol. 41:* 208–213, 1988.

Laemmli, U.K. Cleavage of the structural protein during the assembly of the head of the bacteriophage T4. *Nature 227:* 680–686, 1970.

Lin, P-S., Hefter, K., Ho, K-C. Modification of membrane function, protein synthesis, and heat killing effect in cultured chinese hamster cells by glycerol and D2O. *Cancer Res. 434:* 5776–5784, 1984.

Malyshev, I.Yu., Manukhina, E.B. Stress, adaptation and nitric oxide. *Biochemistry (Moscow) 63:* 992–1006, 1998.

Malyshev, I.Yu., Prodius, P.A., Arkhipenko, Yu.V., Wikman, G., Meerson, F.Z. A new approach to studying the mechanism of action and searching for criteria of adaptogen activity. *First Int Seminar on Adaptogens*, Göteborg, 1996.

Panossian, A., Wikman, G., Wagner, H. Plant adaptogens III; Earlier and more recent aspects and concepts on their mode of action. *Phytomedicine 6:* 287–300, 1999a.

Panossian, A., Oganessian, A.S., Ambartsumian, M., Gabrielian, E., Wagner, H., Wikman, G. Effects of heavy physical exercise and adaptogens on nitric oxide content in human saliva. *Phytomedicine 6:* 17–26, 1999b.

Parsell, D.A., Lindquist, S. Heat shock proteins and stress tolerance. In*: The biology of heat shock proteins and molecular chaperones*. Morimoto, R.I., Tissières, A., Georgopoulos, C., eds., New York, Cold Spring Harbor Laboratory Press0.457–494, 1994.

Piper, P.W. Molecular events associated with acquisition of heat tolerance by the yeast Saccharomyces cerevisiae. *FEMS Microbiol. Rev. 11:* 339–356, 1993.

Prodius, P.A., Manukhina, E.B., Bulanov, A.E., Wikman, G., Malyshev, I.Yu.. An adaptogen 'adapt' modulates stress-induced synthesis of hsp70 and enhances organismic resistance to heat shock. *J. Biokhimiya (Moscow)*. 1996.

Szucs, A., Salanski, J., Razsa, K. Effects of chronic exposure to cadmium- or lead-enriched environments on ionic currents of identified neurons in *Lymnaea stagnalis* L. *Cell. Mol. Neurobiol. 14:* 769–780, 1994.

Thor, H., Smith, M.T., Hartzell, P., Bellomo, G., Jewell, S.A., Orrenius, S. The metabolism of menadione (2-methyl-1,4-naphtoquinone) by isolated rat hepatocytes. *J. Biol. Chem. 257*: 12419–12425, 1982.

Wagner, H. Immunostimulants and adaptogens form plants.

Phytochemistry of medicinal plants. In: Arnason J, ed. New York, Plenum Press0.1–18, 1995.

Wagner, H., Nörr, H., Winterhoff, H. Plant adaptogens. *Phytomedicine 1:* 63–76, 1994.

Welch, W.J. Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and disease. *Physiol. Rev. 72:* 1063–1081, 1992.

Address:

F.A.C. Wiegant, Department of Molecular Cell Biology, Utrecht University, PO Box 80.056, 3508 TB Utrecht, The Netherlands
Phone: 0031-30-2533972/ 3184;
Fax: 0031-30-2513655;
E-mail: f.a.c.wiegant@bio.uu.nl