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Full Length Research Paper

High temperature stress decreases root iridoid glycosides biosynthesis of *Scrophularia ningpoensis* during florescence

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Radix Scrophulariaceae is an herbal medicine used as antipyretic and antibacterial. The objective of this study was to investigate the effect of high temperature stress on the secondary metabolites of *Scrophularia ningpoensis* Hemsl (*S. ningpoensis*). Potted *S. ningpoensis* plants of three ecotypes in florescence were subjected to high temperature stress for 15 days. Results showed that under high temperature stress, contents of iridoid glycosides and cinnamic acid in *S. ningpoensis* roots were significantly lower than the control in all ecotypes. The present study indicates that high temperature stress has an obvious inhibitory effect on the production of iridoid glycosides and cinnamic acid.

Key words: *Scrophularia ningpoensis*, secondary metabolites, cinnamic acid, iridoid glycosides.

INTRODUCTION

Radix Scrophulariae dried roots from some species of the *Scrophularia* genus, present curing capabilities such as trypanocidal, antipyretic, leishmanicidal, antiprotozoal, antimycobacterial, antimalarial and plasmodia FabI enzyme inhibiting properties (Nguyen et al., 2005; Tasdemir et al., 2005, 2008; Bas et al., 2007). Since ancient times, the dried roots of *Scrophularia ningpoensis* Hemsl (*S. ningpoensis*) have been used medicinally in East Asia (Wang et al., 2004; Xu et al., 2004), and it has been cultivated in many provinces of China for hundreds of years. Roots of the Scrophulariaceae family are characterized by containing many types of iridoid glycosides such as harpagoside, aucubin, catalpol and harpagide, which are defined as secondary metabolites (Bowers, 1991; Qian et al., 1992; Nass and Rimpler, 1996; Li et al., 1999).

Understanding the physiological mechanisms of iridoid glycosides accumulation in response to environmental stresses is critical for the scale-up production of iridoid glycosides. Temperature is one of the major environmental factors affecting plant growth and development. It probably alters the secondary metabolite production in plants. It is the hottest period in China from August to September, when the *S. ningpoensis* is just right in its florescence phase. Hence, *S. ningpoensis* plants during florescence are usually exposed to high temperature stress (higher than 35°C), especially in recent years due to the global climate change. However, there has been

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Table 1. The root contents of catalpol, harpagoside, aucubin, harpagide and cinnamic acid in *Scrophularia ningpoensis* Hemsl of three ecotypes after 15 days of high temperature stress during florescence.

Treatment	Catalpol (mg g^{-1} DW)	Harpagide (mg g^{-1} DW)	Aucubin (mg g^{-1} DW)	Cinnamic acid (mg g^{-1} DW)	Harpagoside (mg g^{-1} DW)
AG CK	1.31±0.01^{e}	9.38±0.05^{b}	12.54±0.14^{b}	0.76±0.01^{b}	4.50±0.06^{c}
AG HS	0.48±0.01^{f}	4.19±0.04^{d}	9.84±0.10^{c}	0.35±0.01^{d}	1.22±0.03^{e}
NC CK	2.02±0.04^{c}	4.60±0.03^{c}	7.25±0.07^{d}	1.21±0.02^{a}	4.43±0.05^{c}
NC HS	1.78±0.01^{d}	4.34±0.03^{cd}	5.79±0.06^{e}	1.15±0.03^{a}	2.70±0.02^{d}
DY CK	3.90±0.04^{a}	10.07±0.06^{a}	18.70±0.25^{a}	0.71±0.01^{b}	8.89±0.12^{a}
DY HS	2.41±0.01^{b}	9.77±0.05^{ab}	13.59±0.16^{b}	0.62±0.01^{c}	5.21±0.06^{b}

AG, NC, DY represented roots of *Scrophularia ningpoensis* Hemsl plantings from Anguo (Hebei province) ecotype, Nanchuan (Chongqing city) ecotype, and Dongyang (Zhejiang province) ecotype, respectively. CK and HS represented the *Scrophularia ningpoensis* Hemsl plants under temperature regimes of 28/23 and 45/38°C, respectively. Means ± standard deviation (SD) (n = 3) were shown. The different letters in the same column indicate significant differences among treatments.

there has been little research regarding the production of secondary metabolites in *S. ningpoensis* under high temperature stress. Therefore, the objective of this study was to investigate the effect of high temperature on root contents of secondary metabolites in *S. ningpoensis* during florescence.

MATERIALS AND METHODS

The plant materials of three ecotypes were the same as that in Wang et al. (2010). On 5th March 2008, well-preserved buds of *S. ningpoensis* from Anguo (AG) in Hebei Province, Nanchuan (NC) in Chongqing City, and Dongyang (DY) in Zhejiang Province were planted into pots filled with acid-washed sand in a greenhouse. Sixty milliliter 1/4 Hoagland nutrition was applied to each pot at three-day intervals. On 5th May, plants with uniform height and growth status were selected and transferred into plastic pots (30 cm diameter, 20 cm depth) filled with 2.1 kg vermiculite. Each pot was planted with 4 seedlings and filled with 0.9 L 1/4 strength Hoagland and Arnon solution. The pots were transported to a controlled growth chamber (light/dark regime of 13/11 h at 28/23°C, relative humidity of 50 to 55%, photosynthetic photon flux density of 150 to 200 µmol m^{-2} s^{-1}). Hoagland and Arnon solution (1/4 strength) was added at two-day intervals to provide the nutritional and water requirements for seedlings.

For each ecotype, 12 pots were chosen and divided into two equal groups randomly. Almost all young plants blossomed out on 3rd August. After one week, high temperature stress treatment was applied to the 120-day-old seedlings. For different temperature treatments, two groups of each ecotype were equally separated and transferred into two growth chambers. One chamber was set on a light:dark regime of 13:11 h and a coinciding thermoperiod of 45:38°C (high temperature stress), while the other was set on a light:dark regime of 13:11 h and coinciding thermoperiod of 28:23°C (control). After 15 days, the roots of all treatments were gathered, washed carefully and dried at 50°C for 96 h for determination of catalpol, harpagoside, aucubin, harpagide and cinnamic acid. These metabolite contents were determined according to the method of Wang et al. (2010) through high-performance liquid chromatography (HPLC). The results presented were the mean of three replicates. Means were compared by one way analysis of variance Duncan's multiple range test at the 5% level of significance.

RESULTS AND DISCUSSION

As stated in the introduction, *S. ningpoensis* accumulate lots of iridoid glycosides such as catalpol, aucubin, harpagide and harpagoside (Jessen et al., 1993; Li et al., 2000; Tasdemir et al., 2008; Jeong et al., 2008). A survey of the contents of the secondary metabolites in the dry roots of three *S. ningpoensis* ecotypes showed that the high temperature stress tended to decrease the secondary metabolite (that is, catalpol, harpagoside, aucubin, harpagide, and cinnamic acid) contents in dry roots of *S. ningpoensis* during florescence. The root contents of those five secondary metabolites in *S. ningpoensis* plants under control condition were higher than those under 45/38°C treatment in all ecotypes (Table 1). Compared with the controls, the high temperature stress decreased the contents of catalpol, harpagide, aucubin, cinnamic acid and harpagoside by 63.30 ± 2.35, 55.36 ± 1.89, 21.55 ± 0.69, 50.85 ± 3.04 and 72.89 ± 2.93%, respectively, in the roots of AG ecotype, by 11.86 ± 0.99, 5.74 ± 0.67, 20.13 ± 1.03, 4.65 ± 0.57 and 39.20 ± 1.37 %, respectively, in NC ecotype, and by 38.23 ± 1.99, 3.02 ± 0.16, 27.33 ± 0.91, 12.68 ± 2.04 and 41.34 ± 2.67%, respectively, in DY ecotype. The highest contents of catalpol, harpagide, aucubin and harpagoside were found in the control plants of DY ecotype, indicating the best quality of DY ecotype. In contrast with our results, Zobayed et al. (2005) reported that high temperature (35°C) treatment increased the hypericin, pseudohypericin and hyperforin concentrations in the shoot tissues of St. John' s wort. This difference might be explained by the fact that there are different secondary metabolite pathways in the two species tested. The highest cinnamic acid content wasobserved in the control plants of NC ecotype, suggesting that the NC ecotype is more suitable for the cinnamic acid production. Among the five secondary metabolites, the harpagoside contents showed the biggest reductions in all ecotypes, implying that the harpagoside metabolism is the most sensitive to high temperature stress.

The secondary metabolites of *S. ningpoensis* were sensitive to high temperature during florescence. The high

temperature has an inhibitory effect on accumulation of secondary metabolites in *S. ningpoensis* at florescence stage. Therefore, effective cooling measures should be taken to improve Radix Scrophulariaceae quality during florescence. Further research elucidating the iridoids glycoside biosynthesis pathway would provide a better understanding regarding the effect of temperature on iridoids glycoside production.

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Full Length Research Paper

Medicinal characteristics of *Smyrnium cordifolium* Boiss. plant extract in rats

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The most common sleep disorder is insomnia and about 35% of all people suffer from this disturbance. A trend in using herbal medications for this disorder is increasing globally. Current study aimed to investigate the hypnotic characteristics of *Smyrnium cordifolium* Boiss (SCB) plant extract. From an experimental study, 96 healthy male Wistar rats were equally divided into 12 groups. For hypnotic evaluations, a negative control with normal saline (10 mg/kg), a positive control with diazepam (1 mg/kg) and 3 treatment groups with different dosages of plant extract (100, 200 and 300 mg/kg) were selected and injected intraperitoneally. Sleep start time and sleep duration time were then measured in different groups. For relaxant effects of the plant, a negative control with normal saline (10 mg/kg), 3 positive controls with diazepam (0.125, 0.25 and 0.5 mg/kg) and 3 treatment groups of plant extract (200, 300 and 400 mg/kg) were allocated and injected intraperitoneally. Finally, the mean time of sleeping, sleep start time and holding bar (marker of muscle relation) were compared between treatment and control groups appropriately. SCB plant extract (300 mg/kg), compared to diazepam and normal saline (N/S) shortened the sleep start time significantly ($p < 0.001$). All 3 dosages of SCB plant extract (100, 200 and 300 mg/kg), compared to diazepam and normal saline, increased sleep duration significantly ($p < 0.001$) and as the SCB dosage was increased the sleep duration was increased too. The relaxant effects of the plant extract did not indicate a significant difference. SCB plant extract as a natural product showed hypnotic effects even better than diazepam and needs to be investigated in human by further studies.

Key words: *Smyrnium cordifolium* Boiss, hypnotic, insomnia, relaxant, Iran.

INTRODUCTION

One of the essential needs for human body is sleep phenomenon. Sleep is a dynamic time, necessary for mental and physical restoration during which some physiological processes such as recovering, remaking of

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(a) (b) (c)

Figure 1. Natural picture of *Smyrnium cordifolium* plant.

cells and structural growth occur.

A study reported that sleep deprivation caused some abnormal signs in rats, including a weak and slimsy appearance, weight loss, dermal lesions, increment of food and energy consumption, decrease in body temperature and sudden death (Zavesicka et al., 2008).

Sleep duration among 90% of adults is between 6 to 9 h and a sleep duration < 3.5 and > 9 h compared to those with 7 h increases the chance of death by 15% (Sadock et al., 2002). Insomnia is defined as the presence of long sleep latency, frequent nocturnal awakenings, or prolonged periods of wakefulness during the sleep period or even frequent transient arousals (Thomas, 2007). Drug based methods, for insomnia, are used when non medicinal methods are failed. Medications used for insomnia have a calmative effect on central nervous system (CNS). A good treatment for insomnia is characterized by a good start and continuity of sleep, high quality of sleep and the least side effects on CNS and also a lack of dependency during interruption (Sadock et al., 2002).

Benzodiazepines are the most common used drugs for insomnia. These kinds of medications have some disadvantages including withdrawal syndrome, drug accumulation, daily acedia, amnesia and relapse of insomnia (Pena and Krauss, 1999). Barbiturates are other types of medications used for insomnia with almost similar complications to benzodiazepines (Gillian and Byerley, 1990). Some kinds of plants have been traditionally used for insomnia and a trend in use of herbal products has been increased recently.

Smyrnium cordifolium Boiss is a spring plant which was traditionally used as diuretic and expellant for kidney stones (Ahvazi et al., 2012) and also an antibacterial effect of this plant has been reported (Ulubelen et al., 1985). This plant is grown in Central Asia, Iran, Turkey and West of Afghanistan and it is widely distributed in Iran, particularly in Ilam Province and is used as an herbal medication in human and as a feed by herbivorous animals (Mehrabi and Mehrabi, 2011). As consumption of this plant caused a moderate drowsiness and confusion among local consumers, the author decided to investigate the hypnotic and relaxant effects of *S. cordifolium* Boiss in rats in comparison with the current medications used for these purposes (Figure 1).

METHODOLOGY

The spring samples of *S. cordifolium* plant were collected from hillsides of Zagross mountains of Ilam province in west of Iran and then were confirmed by the Pharmacy Faculty of Mashhad and the Agricultural researches centre of Jahad Keshavarzi Directorate of Ilam Province. By an experimental study, 96 healthy male Wistar rats (weights 250 to 300 g), prepared from Pastor Institution in Iran and kept at the same conditions, dampness (38% ± 2), light (12 h darkness and 12 h lightness intermittently), temperature (22 ± 2°) and freely access to food and water, were divided into 12 equal groups among them, 5 groups were allocated for hypnotic evaluation and 7 groups for investigation of the relaxant effects of the plant. For hypnotic evaluations, a negative control with normal saline (10 mg/kg), a positive control with diazepam (1 mg/kg) and 3 treatment groups with different dosages of plant extract (100, 200 and 300 mg/kg) were selected and injected via insulin syringes intraperitoneally. Sleep start time and sleep duration time were then measured in different groups. Rats in the negative group were first injected by 10 mg/kg N/S intraperitoneally and 60 min later by another 10 mg/kg N/S and 90 min from the second injection a dosage of 30 mg/kg pentobarbital was applied intraperitoneally (Venkateswarlu and Rama, 2013).

For those in the positive control, 1 mg/kg diazepam was firstly injected intraperitoneally and 60 min later the second dosage of diazepam (1 mg/kg) was injected and 90 min from the second injection a dosage of 30 mg/kg pentobarbital was applied by the same method with negative control. Rats in treatment groups were firstly injected with the different dosages of plant extract (100, 200 and 300 mg/kg appropriately) and 60 min later the next dosages (similar to the previous dosages) of plant extracts were injected for the relevant groups. 30 mg/kg of pentobarbital was injected for rats in different groups, 90 min after the second injection (Table 1). After these steps, rats were put upwards on a cottony and warm (37°)

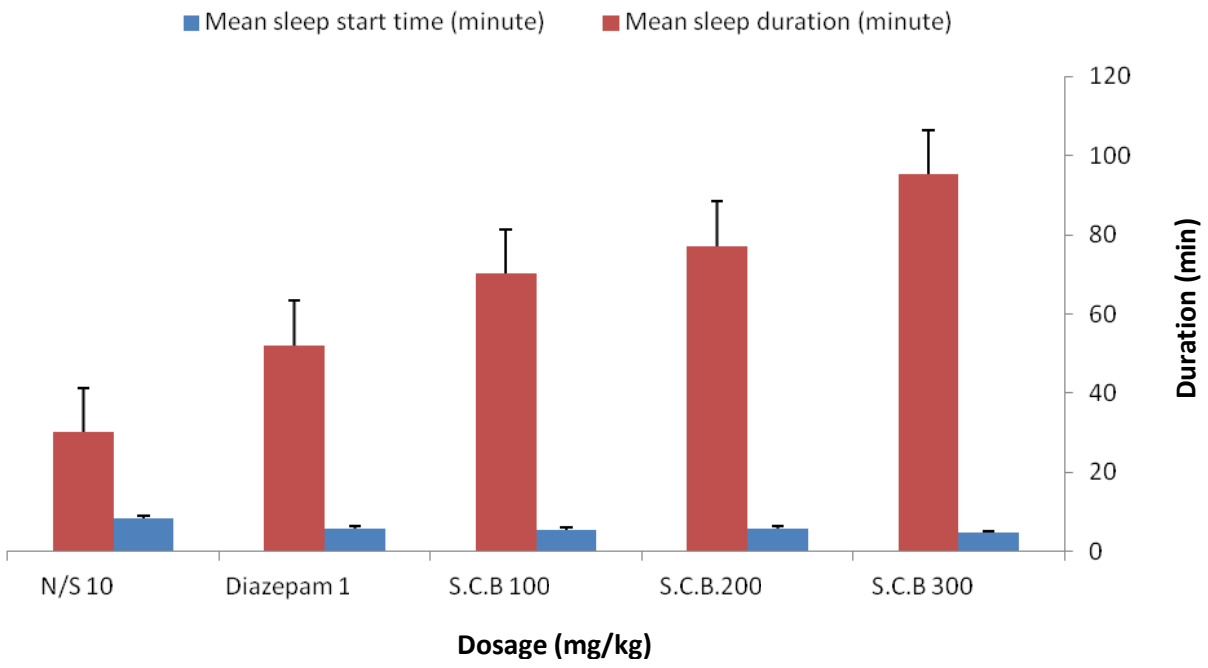

Figure 2. Comparison between mean sleep start time and sleep duration among rats due to different dosages of *S.C.B* plant extract and other tested variables. N/S: Normal saline, SCB: *Smyrnium* cordifolium Boiss. Bars: standard error.

surface. Time spent for righting (a duration in which rats could not amend their upwards positions) was considered as the sleep start time. Sleep termination time was considered if rats could amend their upwards positions, at least 3 times in a minute, remained on their feet and walked.

For relaxation test, a bar with 100 cm length and 50 mm diameter and a height of 30 cm from the surface was fixed in each cage and rats were hanged by two hands on the bar. If the animal could return to the normal position during 5 s, and hold is able to the bar with hands, it meant that the drug did not show any relaxant effect. Lacking the ability of holding the bar was considered as a relaxant effect of the drug. The mean duration of holding the fixed bar, as the marker of muscle relaxation, was measured among different rats groups. For relaxant effects of the plant, a negative control with normal saline (10 mg/kg), 3 positive controls with diazepam (0.125, 0.25 and 0.5 mg/kg) and 3 treatment groups of plant extract (200, 300 and 400 mg/kg) were allocated and were injected intraperitoneally. A score between 0 to 5 was considered as the marker of relaxation in rats and those that held the bar strongly and were not affected by medications, were given zero score and those that left the bar quickly were given a score of 5.

The score of zero meant that the drug did not show a relaxant effect. Monitoring for holding the bar was performed 30 and 60 min after injections and finally the mean time of sleeping, sleep start time and holding the bar were compared between treatment and control groups appropriately. Data was analysed using statistical package for social sciences (SPSS) version 16.0 and the mean and standard deviation of sleeping period, sleep start time and bar holding time among different rats groups were compared by paired T-test or analysis of variance (ANOVA) and Bonferroni test appropriately. A p-value less than 0.05 was considered as significant.

This research was conducted in accordance with the Principles of Laboratory Animal Care (NIH publication, revised in 1985) and was approved prospectively by Ethics Committee of Ilam University of Medical Sciences.

RESULTS

According to the results of this study, SCB plant extract (300 mg/kg), compared to diazepam (1 mg/kg) and N/S (10 mg/kg), shortened the sleep start time significantly ($p < 0.001$). The shortest time for sleep start was related to SCB at the dosage of 300 mg/kg. Using N/S (10 mg/kg) as negative control, the mean time for sleeping start was 8.25 min; however, using diazepam (1 mg/kg) as positive control, this time was lower (5.85 min). Using the dosages of 100, 200 and 300 mg/kg of SCB plant extract, in different groups of rats, the mean sleep start time was reduced to 5.55, 5.6 and 4.6 min, respectively. All 3 dosages of SCB plant extract (100, 200 and 300 mg/kg), compared to diazepam and normal saline, increased the mean sleep duration time significantly ($p < 0.001$) and as the SCB dosage was increased the sleep duration was increased too (Tables 1 and 2). Diazepam at the dosages of 0.25 and 0.5 mg/kg showed a significant relaxant effect; however, different dosages of plant extract did not indicate a significant relaxant effect in rats (Table 3 and Figure 2).

Table 1. Mean and 95% confidence intervals of sleep start time and sleep duration by rats in different groups.

Treatment	Dosage (mg/kg)	Mean (95%CI) of sleep start (min)	Mean (95%CI) of sleep duration (min)
Normal saline	10	8.25 (7.6-8.9)	30.02 (28.4-31.7)
Diazepam	1	5.85 (5.02-6.7)	52.1(50.8-53.4)
S.C.B extract	100	5.55 (4.9-6.2)	70.2 (69.6-70.7)
	200	5.62 (5.2-6.1)	77.2 (76.6-77.8)
	300	4.61 (4.2-5.03)	95.3 (94.8 -95.8)

Group N/S: Normal saline 10mg/kg (start) + N/S 10 mg/kg (60 min later) + pentobarbital 30 mg/kg (90 min after 2nd injection). Group Diazepam: Diazepam 1mg/kg (start) diazepam 1mg/kg (60 min later) + pentobarbital 30mg/kg (90 min after 2nd injection). Group Extract 100: Extract 100 mg/kg (start) + extract 100mg/kg (60 min later) + pentobarbital 30mg/kg (90 min after 2nd injection). Group Extract 200: Extract 200 mg/kg (start) + extract 200mg/kg (60 min later) + pentobarbital 30mg/kg (90 min after 2nd injection). Group Extract 300: Extract 300mg/kg (start) + extract 300 mg/kg (60 min later) + pentobarbital 30 mg/kg (90 min after 2nd injection).

Table 2. Comparison between hypnotic effects of different dosages of *S.C.B* plant extract and other tested variables.

Comparison variable		Mean (min)	Mean difference (min)	P-value	Significant
Sleep start time	N/S vs. diazepam	8.25 vs. 5.85	2.40	0.0001	Yes
	N/S vs. Extract 100	8.25 vs. 5.55	2.60	0.0001	Yes
	N/S vs. Extract 200	8.25 vs. 5.62	3.63	0.0001	Yes
	N/S vs. Extract 300	8.25 vs. 4.61	4.64	0.0001	Yes
	Diazepam vs. extract 100	5.85 vs. 5.55	0.30	1.0	No
	Diazepam vs. extract 200	5.85 vs. 5.62	0.23	1.0	No
	Diazepam vs. extract 300	5.85 vs. 4.61	1.24	0.02	Yes
	Extract 100 vs. extract 200	5.55 vs. 5.62	-0.07	1.0	No
	Extract 100 vs. extract 300	5.55 vs. 4.61	0.94	0.16	No
	Extract 200 vs. extract 300	5.62 vs. 4.61	1.01	0.09	No
Sleep duration	N/S vs. diazepam	30.02 vs. 52.1	-22.08	0.0001	Yes
	N/S vs. Extract 100	30.02 vs. 70.2	-40.18	0.0001	Yes
	N/S vs. Extract 200	30.02 vs. 77.2	-47.18	0.0001	Yes
	N/S vs. Extract 300	30.02 vs. 95.3	-65.28	0.0001	Yes
	Diazepam vs. extract 100	52.1 vs. 70.2	-18.1	0.0001	Yes
	Diazepam vs. extract 200	52.1 vs. 77.2	-25.1	0.0001	Yes
	Diazepam vs. extract 300	52.1 vs. 95.3	- 43.2	0.0001	Yes
	Extract 100 vs. extract 200	70.2 vs. 77.2	-7.0	0.0001	Yes
	Extract 100 vs. extract 300	70.2 vs. 95.3	-25.1	0.0001	Yes
	Extract 200 vs. /extract 300	77.2 vs. 95.3	-18.1	0.0001	Yes

Table 3. Comparison between relaxant effects of SCB plant with diazepam and normal saline.

Variable	Dosage mg/kg	Mean relaxant score	P value
Normal saline	10	0	-
Diazepam	0.125	0	-
	0.25	2.6	0.02
	0.5	5	0.0001
Extract	200	0.05	-
	300	0.1	-
	400	0.5	-

DISCUSSION

About one third of human life is spent by sleeping and any disturbance of this phenomenon would be strongly annoying and needs interventional issues by physicians or psychologists. The most common sleep disorder is insomnia, with a prevalence rate of 35% among general population (Mellinger et al., 1985). The most important drugs used for insomnia were benzodiazepines; however, some older medications such as barbiturates, glutethimide meprobamate, chloral hydrate and paraldehyde or new drugs such as zolpidem and zaleplon are also applied for this purpose (Sadock et al., 2002). Herbal medications have also been used for insomnia and because of their natural sources as well as physiological adaptation to human body, are interested globally (Uprety et al., 2012; Asadollahi et al., 2010). Row forms of *S. cordifolium* Boiss plant are consumed commonly in Ilam province as a spring plant and its application is usually accompanied with mild confusion and drowsiness.

The current study investigated the hypnotic and relaxant effects of this plant compared with routine drugs among rats. The current study showed that, using diazepam (1 mg/kg) as positive control reduced the sleep start time to 5.55 min; however, using plant extract at the dosages of 100, 200 and 300 mg/kg reduced this time to 5.85, 5.62 and 4.61 min, respectively, all statistically significant in comparison with normal saline ($p < 0.0001$). In comparison with diazepam, the only dosage of plant which showed a significant difference for sleep start time was 300 mg/kg ($p < 0.02$); however, all 3 dosages of plant extract increased the length of sleep significantly ($p < 0.0001$). Increasing the dosage of SCB plant extract caused a rise at the sleep duration but more than750 mg/kg was toxic and caused rats' mortality.

We could not find a similar study in the literature to compare the hypnotic or relaxant effects of SCB plant extract; therefore, the current study is the first investigation of the hypnotic or relaxant effect of this plant. One of the most common used herbal medications for insomnia was *Valerian officinalis* L. (Schulz et al., 1994). A study indicated that *V. officinalis* applied for 28 days had good and very good effects on insomnia compared to placebo (Kuhlmann et al., 1999). Another study reported a reduction of sleep start time by *V. officinalis* at the dosages of 450 and 900 mg/kg (Wheatley, 2001) which was in accordance to the effects of SCB plant. However, a systematic review of *Valerian* reported that evidences in support of the effectiveness of this plant are uncertain (Taibi et al., 2007). Some other herbal medications such as *Kavakava* (Cupp, 1999), *Rhodiola sachalinensis* (Muller and Ehrenstein, 1977), *Passiflora incarnate* (Speroni and Minghetti, 1988; Delaveau et al., 1989), *Lavandula angustinfolia* (Gyllenhaal et al., 2000), *Mimosa blossoms* (Muceniece et al., 2008), *Matricaria chamomile* (Perez-Ortega et al., 2008), *Tilia Americana var, Mexicana inflorecences* (Li et al., 2008), *Spikenarol* (Adeyemi et al., 2007), *Sanseviera liberica* Gerome (Herrera-Arellano et al., 2007), *Galphimia gluca* (Rakhshandah and Hosseini, 2006), *Rosa damascene* (Huang et al., 2008) and *Keampferia galangal* (Linfang et al., 2008) have been used and tested for insomnia by various studies in different societies including European, Asian and American countries.

The relaxant effects of SCB plant extract was compared to diazepam and there was not found a significant difference. Diazepam did not show any relaxant effect at the dosages of 0.125 mg/kg; however, at the dosages of 0.25 and 0.5 mg/kg showed mean relaxant effects of 2.6 and 5, respectively. A study performed in 2007 showed that a China plant (*Rhodiola sachalinensis*) caused loss of righting reflex in rats at the dosage of 150 mg/kg. The method used in their study was similar to ours but they reported a positive relaxant effect (Muller and Ehrenstein, 1977). The reason behind this difference may be associated with different cell receptors of extracts or their central or peripheral effectiveness.

Conclusion

SCB plant extract as a natural product showed hypnotic effects even better than diazepam and needs to be investigated in humans by further studies; however, the relaxant effects of this plant did not show a significant effect.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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ABBREVIATIONS

SCB, *Smyrnium cordifolium Boiss*; **N/S,** normal saline; **Ip,** intraperitoneally.

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Effect of methyl jasmonate on isoflavonoid accumulation and antioxidant enzymes in *Pueraria mirifica* cell suspension culture

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The effect of methyl jasmonate (MJ) on the isoflavonoid accumulation and activities of three antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) were studied in *Pueraria mirifica* cell suspension culture after MJ elicitation for up to 6 days. Treatment with MJ at a concentration of 1.0 µg/ml induced the highest isoflavonoid contents at the first day after elicitation, when compared to the control. This optimum concentration of MJ induced an oxidative stress in *P. mirifica* cells. The activity levels of superoxide dismutase and glutathione peroxidase were both increased, whilst that for catalase was decreased in the MJ-treated cells. Thus, *P. mirifica* cells have a defense mechanism against superoxide and hydrogen peroxide stress and MJ is one such potential stimulator of this defense mechanism.

Key words: *Pueraria mirifica*, isoflavonoid, methyl jasmonate, superoxide dismutase, catalase, glutathione peroxidase.

INTRODUCTION

The use of various herbs and medicinal plants in folklore medicine has a long history where they have been used since ancient times, especially in oriental countries (Sheng-Ji, 2001). However, the advent of antibiotics in the early 20th century led to a decline in their usage and a waned interest in providing scientific bases to their effects. The adverse effects of using antibiotics and other synthetic compounds on human health and on product quality and safety, plus increasing resistance to them, have regenerated interest in the fields of phytochemistry,

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phytopharmacology, phytomedicine and phytotherapy during the last decade (Makkar et al., 2009). Thus, more recently such folklore-based plants and their extracts are being evaluated for the chemical basis behind the treatment for adaptation to more conventional pure drug approaches or bioinformatic based modification, as well as for optimization of cultivar/cultivation conditions in agricultural rearing of the plants or biotechnological based mass production of the active component(s) (Chattopadhyay et al., 2004).

Plants produce numerous secondary metabolites that have historically been used as pharmaceuticals, fragrances, flavor compounds, dyes and agrochemicals (Sakuta and Komamine, 1988). An increasing number of these secondary plant metabolites are proving to be a major source of new drugs. Because their profiles vary among (even closely related) species, and also among cultivars and cultivation conditions, extracts from both known and newly discovered plants are subjected to screening for novel medical applications (Zhao et al., 2005). However, secondary metabolites are usually produced *in vivo* at only low concentrations, so large-scale production systems have been developed for use in plant cell culture.

Despite the enormous commercial efforts over several decades, only a few compounds have successfully been produced at a lower cost than by production by direct plant extraction or through chemical synthesis (Liu et al., 2002). Nevertheless, studies on secondary metabolite biosynthesis can increase the yield of active compound production in plants using biotechnology. Tissue culture techniques have been used to prepare primary cell cultures of plants in the form of either callus or cell suspension cultures, and from this, secondary metabolite production has been potentially developed via these cell culture techniques (Rao and Ravishankar, 2002).

P. mirifica, a Thai indigenous herb, with the local name of White Kwao Keur, has long been used among Thai women in a similar manner for modern hormone replacement therapy (Malaivijitnond, 2012). Products of White Kwao Keur are well known in international markets, such as China, Japan, Korea, Singapore, Malaysia, USA, Australia and Europe. Each year, the export value of "Kwao Keur" products from Thailand is approximately 1,500 million baht (~ 47.4 million US dollars). *P. mirifica* tuberous root contains a relatively rich diversity of isoflavonoids, including daidzein, daidzin, genestein, genistin and puerarin (Cherdshewasart et al., 2007). *P. mirifica* is classified as a medicinal plant but requires cultivation due to the limited sources of wild plants and habitat loss in their natural environment including soil erosion and deforestation (Sahavacharin, 1999). In addition, the amount of active components in the tuberous root has been reported to be dependent on various physical and chemical cultivation factors, such as the geographical location, climate and disease, as well as on the plant genetics (as in cultivar type) (Thanonkeo and Panichajakul, 2006). The variations in the level of active components, and their relative proportions, imposed by these factors have generated considerable interest in the use of the more controlled environment of plant tissue culture technologies for the production of isoflavonoids, so as to optimize and control these factors. These include the cultivation of specific organ cultures and suspension cultured cells selected for high production of secondary metabolites (Dicosmo and Misawa, 1995).

One way to increase the production level of secondary metabolites is by elicitation, which is the induction of secondary metabolite production by other molecules or treatments (the elicitors). Elicitors can be grouped into three categories. The first is the biotic elicitors, which are usually microbe-derived molecules that stimulate secondary metabolism. Biotic elicitors include polysaccharides, glycoproteins, low molecular weight organic compounds, and bacterial and fungal cell walls. Second are the abiotic elicitors, such as ultraviolet irradiation, salts of heavy metals and various chemicals. Thirdly are the endogenous elicitors, which are chemicals produced within the cell as secondary messengers, such as methyl jasmonate (MJ) (Fu et al., 1999). The optimal inclusion of elicitors in cell culture can lead to a pronounced increase in the activities of biosynthetic enzymes including differential effects on the enzymes involved in conjugate metabolism (Barz and Mackenbrock, 1994).

The effect of the elicitors depends on many factors, such as their concentration, the growth stage of the culture at the time of elicitation, period of contact and time course of elicitation. MJ has been found to enhance secondary metabolite production in plant cell cultures. This compound plays a key role both outside and inside plant cells. In exogenous application, MJ binds to the receptor on plant cell wall to stimulate biosynthesis of secondary metabolites, while intracellular MJ is one of the endogenous components in the jasmonic acid signaling pathway that is regarded as a transducer or mediator for elicitor signaling, leading to accumulation of plant secondary metabolites (Zhao et al., 2005).

The elicitation process also induced the reactive oxygen species (ROS) including superoxide (O_2^-) and hydrogen peroxide (H_2O_2). ROS may cause organelle, membrane and cell damage via oxidation of DNA, RNA, lipids and proteins. Plant cells have various antioxidant enzymes to restrict or reduce, and so act in defense of oxidative stress, such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and glutathione peroxidase (GPx, EC 1.11.1.12). SOD catalyzes the dismutation of O_2^- to H_2O_2 and O_2, whilst CAT and GPx breakdown H_2O_2 into water and O_2 (Ali et al., 2006). Understanding the control of these enzyme activities in plant cells, as well as their biological and biochemical activities could also help explain and overcome

the enzymatic and non-enzymatic activities during cell cultivation and elicitation. The information would be useful for optimizing the efficient large-scale production of isoflavonoids in *P. mirifica* cell culture. Accordingly, it would be interesting to know the effect of elicitation with MJ upon the isoflavonoid accumulation and antioxidant enzyme activity of *P. mirifica* cell cultures. In this study, we investigated the role of MJ on isoflavonoid production and antioxidant enzymes in *P. mirifica* cell suspension culture.

MATERIALS AND METHODS

Plant material, cell culture and treatment procedure

P. mirifica seeds were obtained from Assoc. Prof. Yuthana Smitasiri (Guest lecturer, Graduate School, Chiang Mai University). The specimens were then authenticated by Mrs. Jantrararuk Towaranonte, a lecturer of the School of Science, Mae Fah Luang University and kept as voucher specimens No. MFLU-307. The seeds were sterilized by treating first with 95% (v/v) ethyl alcohol for 30 s, followed by 15% (w/w) Clorox® for 15 min and then rinsed three times with sterile distilled water. Seeds were germinated on Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose. Germinated seedling roots were placed on MS medium supplemented with 3% (w/v) sucrose, 0.5 µg/ml naphthylacetic acid (NAA) and 0.5 µg/ml benzyl adenine (BA) for callus induction. Cultures were maintained at 25 ± 2°C with a photoperiod of 16 h daylight at a photosynthetic proton flux intensity of 90 µmol m^{-2} s^{-1}. Cell suspension cultures were initiated by inoculation with 10 mg/ml of 4 weeks-old friable calli into a 125 ml Erlenmeyer flask containing 25 ml of liquid MS medium with 3% (w/v) sucrose, 0.5 µg/ml NAA and 0.5 µg/ml BA. The cultures were placed on a rotary shaker (125 rpm) under the same conditions as for the callus culture. The MJ solution was filter-sterilized before use and was aseptically added to the culture medium at one of three final concentrations (0.1, 0.5 or 1.0 µg/ml, equivalent to 0.45, 2.23 or 4.46 µM, respectively) during the 12th day of the cultivation. The control treatment contained sterile distilled water in the same quantities as the added MJ. Cells were collected everyday for 6 days after the addition of MJ (elicitation).

Growth measurement, extraction and analysis of isoflavonoids

The growth of cells was measured in terms of their dry weight (DW). The DW was measured after drying the fresh cells in an oven at 60°C for 3 days or until a constant weight was attained. The dried samples (10 mg) were ground and extracted with methanol (10 ml) for 24 h in room temperature, filtered through 0.45 µm filter paper and the methanol solvent removed from the extract in a hot air oven (60°C). The solid extract residue was then dissolved in methanol to a final volume of 1 ml before analysis. The isoflavonoid content was quantified by high performance liquid chromatography (HPLC) as described by Cherdshewasart et al. (2007). The system was operated on HPLC model SpectraSystem (Thermo Fisher Scientific, USA) equipped with autosampler model AS3000, Photodiode array UV detector model UV6000LP and Chromquest Software. The C18 column (250 mm × 4.6 mm, Luna 5U, Phenomenex, USA) was used with rational gradient eluding condition (86:14 to 68:32) (v/v) ratio of 0.1% (v/v) acetic acid: acetonitrile at a flow rate of 1 ml/min. Isoflavonoids were detected at 254 nm. For quantitative analysis, the system was calibrated with five authentic isoflavonoids (daidzein, daidzin, genistein, genistin and puerarin), purchased from Sigma-Aldrich Co. Standard curves were fitted with linear regression.

Extraction and assay for antioxidant enzyme activities

Fresh *P. mirifica* cells were ground in a mortar and pestle under liquid nitrogen and then extracted at 0 to 4°C in extraction buffer (50 mM phosphate buffer pH 7.0) at 0.2 mg/ml. The homogenates were clarified by centrifugation (10,000 × g, 4°C, 20 min), the supernatant harvested and used for enzymatic activity assays and determination of the protein content according to Bradford (1976), using bovine serum albumin as a standard. SOD activity was evaluated using the ribloflavin/nitrotetrazolium blue chloride (NBT) assay according to Lai et al. (2008). The inhibition of NBT reduction was determined by measuring the absorbance at 560 nm. One unit (U) of SOD activity was defined as the amount of enzyme that provides a 50% inhibition of the riboflavin-mediated reduction of NBT. CAT activity was measured at 240 nm by the H_2O_2 decomposition assay according to the method of Aebi (1974) in 50 mM potassium phosphate buffer (pH 7.0) containing 20 mM H_2O_2 and 0.1 ml enzyme extract (at a total protein level of 0.5 mg/ml). GP_x activity was assayed by the oxidation of NADPH as described by Wendel (1980).

Statistical analysis

All parameters are shown as the mean ± standard error of the mean (SEM) of at least three independent experiments. The influence of various treatments on the plant cell biomass and isoflavonoid contents was processed statistically by analysis of variance (ANOVA) and the difference between means of the samples analyzed by Duncan's multiple range tests (DMRT) at a probability level of 0.05.

RESULTS AND DISCUSSION

The cell growth profile of *P. mirifica* cell suspension culture was established in preliminary trials, with the best cell growth being obtained in liquid MS medium supplemented with 0.5 µg/ml NAA and 0.5 µg/ml BA (data not shown). Under these conditions the biomass reached a maximum level at the 15th day (14.14 ± 0.17 g/L DW) of cell culture and decreased slightly thereafter, although the isoflavonoid production peaked after 9 days of culture (5.5 mg/g DW) before dropping at 12 days and then increasing from 18 days to a maximum level (6.0 mg/g DW) at 21 days of cell culture (Figure 1).

MJ treatment at 0.5 or 1.0 µg/ml increased the isoflavonoid accumulation with a greater response to the higher MJ concentration, in comparison with the control group. The maximum was 4 days after 0.5 µg/ml of MJ elicitation (5.2 mg/g DW and 1.24 times the control group), and it was only 1 day after 1.0 ug/ml of MJ elicittation (6.27 mg/g DW and 1.45 times of the control group) (Figure 2A). In contrast, at a concentration of 0.1 µg/ml MJ had no stimulatory effect upon isoflavonoid levels but

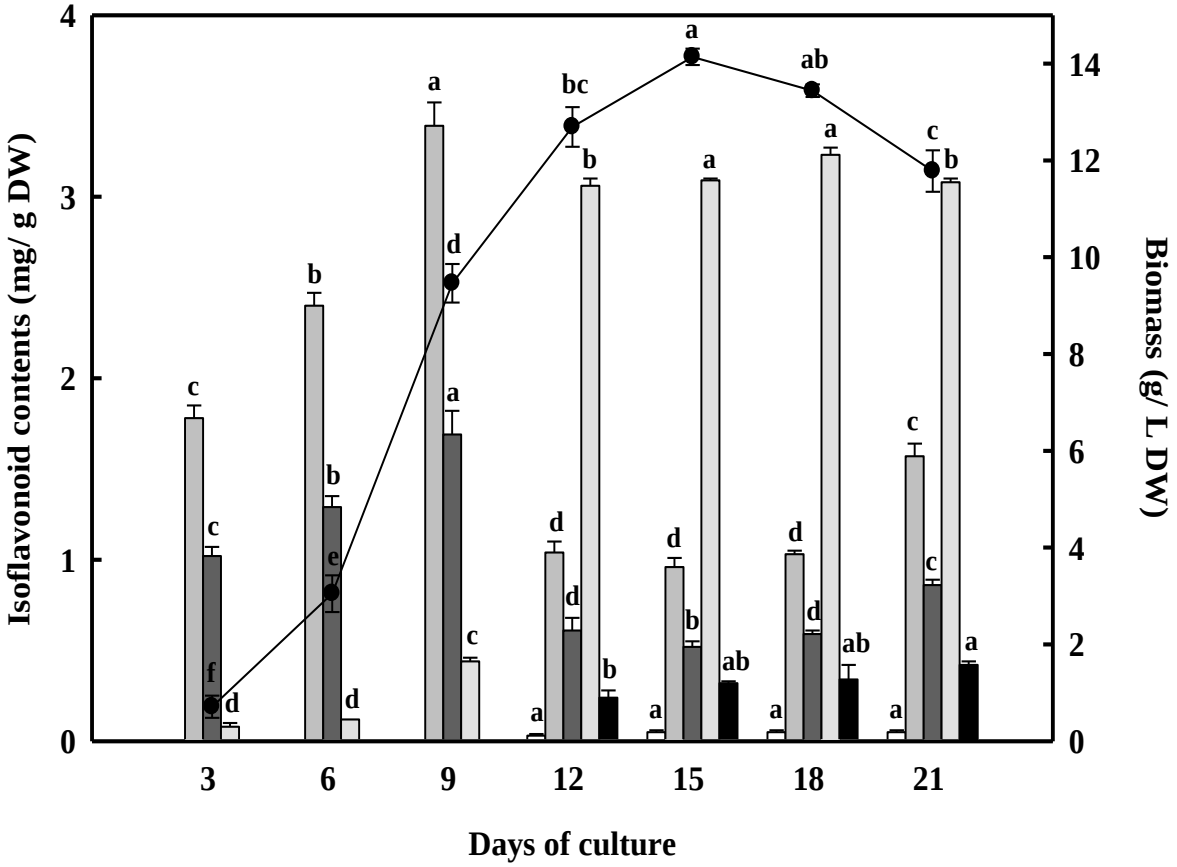

Figure 1. Cell growth (●) and isoflavonoid production: puerarin (white), daidzin (gray), genistin (dark gray), daidzein (light gray), and genistein (black) of *P. mirifica* cell suspension culture. Results are means ± SEM. Different letters in different bar differ significantly among samples (DMRT test, $P \leq 0.05$).

rather decreased the level at some time periods (especially at 6-day after MJ treatment). Knowledge of such periods of elicitation are useful for large scale production, such as in fed-batch or continuous bioreactors (Thanh et al., 2005; Ali et al., 2007; Prakash and Srivastava, 2008). This supports that MJ acts as a signaling molecule to induce the biosynthesis of isoflavonoid in *P. mirifica* cells, in agreement with the report of Korsangruang et al. (2010). However, the observed response on the total isoflavonoid levels is neither simply time nor dose-dependent. MJ treatment at all three tested doses did not affect the cell biomass of the *P. mirifica* cell culture (data not shown).

Bhagwath and Hjortso (2000) demonstrated that the production of thiarubrine A, a potential pharmaceutical, in the hairy root culture of *Ambrosia artemisiifolia* was enhanced eight-fold by elicitation with 50 µg/ml vanadyl sulphate, reaching 569 µg/g of biomass after 72 h. However, a higher maximum yield (647 µg/g of biomass) was achieved when the cultures were exposed to 5 mM autoclaved cell wall filtrates of *Protimyces gravidus*, a pathogenic fungus, for 48 h. The treatment of plant cells with biotic and abiotic factors is potentially a simple and effective means for stimulating secondary metabolite production in plant cell cultures. A variety of chemical and biochemical elicitors have been widely used to enhance isoflavonoid production in leguminous plant cultures. Kneer et al. (1999) reported that in the root cultures of *Lupinus leteus*, the genistin level was increased to 44.7 µg/g (8.94 times of the control group) when it was stimulated by 100 µM MJ. In addition, the production of genistin in the hairy root cell culture of *Psoralea* sp. were greatly enhanced with the addition of 30 µg/ml chitosan at the end of the exponential phase (Bourgaud et al., 1999).

MJ and its related derivatives have been implicated as signal transduction molecules with a multifaceted effect on plant growth, development and stress responses (Starwick, 1999). Moreover, the intracellular signal cascade that begins with the interaction of an elicitor molecule with the plant cell surface results, ultimately, in

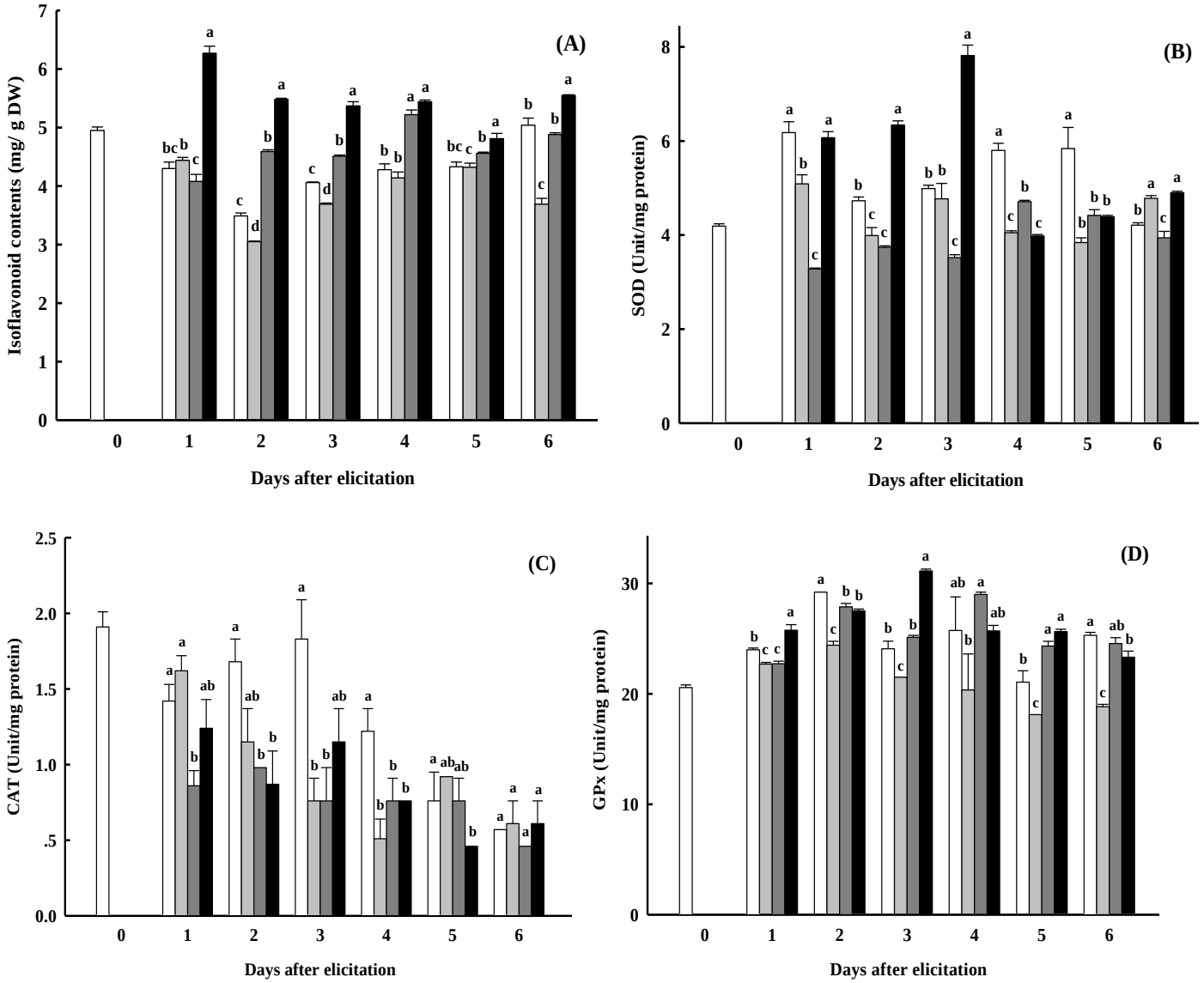

Figure 2. Effects of methyl jasmonate (MJ) addition at different concentrations on the (A) isoflavonoid production, and the enzymic activity levels of (B) superoxide dismutase (SOD), (C) catalase (CAT) and (D) glutathione peroxidase (GP_x); control (white), 0.1 µg/ml (gray), 0.5 µg/ml (dark gray), and 1 µg/ml (dark) in *P. mirifica* cell suspension culture.
Results are shown as the mean ± SEM. Different letters in different bar differ significantly among treatments (DMRT test, $P \leq 0.05$).

the accumulation of secondary metabolites (Gundlach et al., 1992). The role of MJ as an endogenous elicitor for stimulating secondary metabolite production in cell suspensions, and hairy root cultures of various secondary plants has been reported, such as for the lignans pinoresinol, metarisinol (Schmitt and Petersen, 2002), saponin (Lu et al., 2001) and ginsenoside (Yu et al., 2000), and alkaloids (Zabetakis et al., 1999). In addition, Furmanowa and Syklowaka-Baranek (2000) reported that paclitaxel was accumulated at high levels in hairy root cultures *of Taxus x media* var. *hicksii* after being elicited by 100 µM (22.43 µg/ml) MJ. Similarly, the production of terpenoid indole alkaloids in the hairy root cultures of *Catharanthus roseus* were increased by the same concentration of MJ (El-Sayed and Verpoorte, 2002).

The effect of MJ on the SOD, CAT and GP_x activities in the *P. mirifica* cell suspension culture are shown in Figure 2B to D. At a concentration of 1.0 µg/ml, MJ significantly

increased the SOD activity after two and three days exposure, reaching a maximum at the third day after elicitation (~ 7.8 U/mg protein), whereas in contrast, at a concentration of 0.1 or 0.5 µg/ml, MJ decreased the SOD activity compared to the control at all time points (Figure 2B). All MJ concentrations significantly decreased the CAT activity in the initial 1 to 4 days (or 1 to 5 days for 1.0 µg/ml) after elicitation (Figure 2C). The activity of GPx was significantly decreased by 0.1 and 0.5 µg/ml MJ, but was increased erratically over time after elicitation by 1.0 µg/ml MJ with a maximum level of GP_x activity on the third day after elicitation (Figure 2D).

Elicitation, especially at the highest dose of 1.0 µg/ml MJ causes oxidative stress via the production and accumulation of ROS. In this study, we focused on the enzyme activities of SOD, CAT and GPx since they are involved in the detoxification of ROS. MJ treatment at 1.0 µg/ml induced SOD and GPx activities by up to 56 and 30%, respectively, compared to the control treatment at the third day after elicitation. Whether the induction of SOD and GPx activity was correlated to an increase in the H_2O_2 and/or O_2^- levels, and so for the protection of the cell system (Jabs et al., 1997), is not investigated in this study. In contrast, the CAT activity was suppressed in MJ treatment while the GPx level was increased, which could suggest that the CAT functions in H_2O_2 removal were compensated by the GPx enzyme. A reduction in the level of CAT activity was also observed in *Panax ginseng* (Ali et al., 2006) and *C. roseus* (Zhao et al., 2001), with the activities of these enzymes reverting back to normal levels 6 days after elicitation.

Conclusion

This paper has described, for the first time, a procedure for initiation and establishment of cell suspension of *P. mirifica* which was able to accumulate a relatively high level of isoflavonoids and antioxidant enzyme activities. The treatment *P. mirifica* cell suspension culture have the highest isoflavonoid content and antioxidative enzyme levels on 1 and 3 days of culture with 1.0 µg/ml MJ, respectively. The results demonstrated the importance of the MJ (elicitor) concentration and time after addition in the growth medium. However, it seems that the increase of the isoflavonoid and antioxidative levels was not much in this study, the multiple elicitor additions over time or the addition of the multiple elicitors need to be tested. In addition, the cost of MJ compared to the value of the increased isoflavonoids obtained remains to be equated. Increasing the production level of isoflavonoid contents may be achieved by means of cultivation of *P. mirifica* in a large scale bioreactor, but this requires evaluation using the cell suspension cultures from our callus collection for confirmation and optimization of the production production of isoflavonoids under bioreactor conditions.

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ABBREVIATIONS

BA, Benzyl adenine; **CAT,** catalase; **GPx,** glutathione peroxidase; **MJ,** methyl jasmonate; **MS,** Murashige and Skoog's medium; **NAA,** naphthylacetic acid; **NADPH,** nicotinamide adenine dinucleotide phosphate; **NBT,** nitrotetrazolium blue chloride; **ROS,** reactive oxygen species; **SEM,** standard error of the mean; **SOD,** superoxide dismutase.

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The influence of extraction solvents on the anticancer activities of Palestinian medicinal plants

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Palestine has a rich and prestigious heritage of herbal medicines. To investigate the impact of variable extraction techniques on the cytotoxic effects of medicinal plant extracts, 5 well-known medicinal plants from Palestine were extracted with 90% ethanol, 80% methanol, acetone, coconut water, apple vinegar, grape vinegar or 5% acetic acid. The resulting 35 extracts were screened for cytotoxic activities against three different cancer cell lines (B16F10, MCF-7 and HeLa) using a standard resazurin-based cytotoxicity assay and Nile Blue A as the positive control. Highly variable toxicities and tissue sensitivity were observed, depending upon the solvent used for extraction. The acetone extract of *Salvia officinalis* L. exhibited the most potent cytotoxicity (IC_{50} = 14 to 36 µg/ml), but very little sensitivity between the three cell lines. More moderate cytotoxicity with improved tissue sensitivity was observed with coconut water extract of *S. officinalis* L. (IC_{50} = 114 µg/ml) and methanol extract of *Teucrium polium* L. (IC_{50} = 104 µg/ml). In this study, acetone consistently gave lower extraction yields but higher cytotoxicity, whereas other solvent systems gave much higher extraction yields with lower cytotoxicity. These results demonstrate how the cytotoxicity of plant extracts can be inversely proportional to the yield, and that solvent selection plays an important role in both factors.

Kew words: Plant extract, natural products, anticancer drug, cytotoxicity.

INTRODUCTION

Cancer is one of the most devastating diseases in both developing and developed countries. Due to a global increase in life expectancies, the incidents of cancer and related mortality rates are dramatically increasing. Treatment options are typically expensive and unavailable in developing countries. New and widely available drugs are therefore needed to provide treatment options. Natural products have provided some of the

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Table 1. List of screened plants, collected part and their uses in Palestinian traditional medicine

Scientific name	Common name	Family	Collected part	Preparation	Traditional Uses	Voucher specimen
Ficus carica L	Fig	Moraceae	Fruit and leaf sap	Direct use	Anti viral (warts treatment)	03/04/2012
Olea europaea L	Olive	Oleaceae	Leaves	Decoction of leaves	Reduces hypertension	03/04/2012
Salvia officinalis L	Sage	Lamiaceae	Leaves and stems	Decoction of leaves and stems	Antispasmodic, antibacterial	04/04/2012
Teucrium polium L	Felty germander	Lamiaceae	Leaves and stems	Decoction of leaves and stems	Antispasmodic	04/04/2012
Vitis vinifera L	Grape	Vitaceae	Liquid sap of the stem	Direct use	Skin problems, hair loss	06/04/2012

most important cancer chemotherapeutics, largely because they provide structurally complicated molecules that are difficult to access in significant quantities by total synthesis (Mukherjee et al., 2001; Raymond, 2004; Efferth, 2009, 2010; Filip et al., 2011; Siu 2011). The extraction of drug candidates from natural product sources requires a proper selection of plant, extraction method and screening method for discovering bioactive molecules.

Palestine has a rich and prestigious heritage of herbal medicines. More than 700 species of medicinal plants are known to exist, and approximately 63 of these are actively used for the preparation of traditional medicines (Ali-Shtayeh et al., 1998; Sawalha et al., 2008; Ali-Shtayeh and Jamous, 2012). The majority of these plants have already been subjected to chemical analyses. Gas chromatography mass spectrometry (GC MS) spectroscopy, high performance liquid chromatography (HPLC) and other methods have revealed that terpenoids and phenolic compounds are the two main families of secondary metabolites present (Hassan et al., 1979; Aron and Kennedy, 2007; Waterman and Lockwood, 2007; El Hadri et al., 2010; Conforti et al., 2012).

Although many efforts have been focused on deciphering the chemical composition and biological effects of these plants, a systematic study of the effects of variable solvents for extract preparation has not been reported. In this study, variable solvents were used to prepare extracts from 5 Palestinian plants (*Olea europaea, Vitis vinifera, Ficus carica, Salvia officinalis and Teucrium polium*) and screened for cytotoxic activities. These particular plants have been used in traditional medicine for the treatment of various diseases such as inflammation (Surh et al., 2001; Kaileh et al., 2007), hypertension (Suleiman et al., 1988), and diabetes (Table 1) (Baluchnejadmojarad et al., 2005; Orhan et al., 2006; Eidi et al., 2009). Palestinians have used *T. polium* for abdominal pain, *S. officinalis* for relief menstrual pain, *V. vinifera* for weight loss, *F. carica* for ulcer treatment and *O. europaea* for destroying urinary and gall stones. Most of the medicinal plants in Palestine are sold in herbal shops, where most patients seeking herbal therapy are elderly (age of > 55 years) who usually suffer from multiple diseases and cannot afford to buy expensive medications.

One of the key steps in natural product processing is the selection of extraction solvent (Taamalli et al., 2012). The most commonly used solvents are water, methanol, ethanol and acetone. Those solvents are used in neat form or as mixtures. In this study, we used apple vinegar, grape vinegar and coconut water as widely-available and inexpensive replacements for pure organic solvents. The non-flammable and non-volatile nature of these solvents also makes their handling safe and environmental friendly for scale-up of production in developing countries (Diaz-Reinoso et al., 2006; Fontana et al., 2009; Yapo, 2009; Min et al., 2011).

MATERIALS AND METHODS

Plant

The leaves of *S. officinalis*, *O. europaea*, *F. carica*, *V. vinifera* and *T. polium* were collected from the Hebron area of Palestine (Coordinates: 31° 32′ 00″N 35° 05′ 42″E) on April, 2012. Plant characterization was conducted by Dr. Rami Arafeh and voucher specimens were deposited in the Biotechnology Research Center at the Palestine Polytechnic University (Table 1). The fresh leaves were separated and cleaned from dust by tissue paper and placed in the shade inside a well-ventilated room until a

constant weight was obtained. Dried leaves were grounded to a fine powder and the powder was stored at 4°C.

Solvents and chemicals

All solvents were of American Chemical Society (ACS) grade and purchased from Merck. Vinegars were purchased from a local grocery store in Hebron city. Coconut water was collected from coconut fruit and stored at 4°C. Nile Blue A was purchased from Fluka.

Preparation of crude extracts

Extracts were prepared by adding the specified solvent (30 ml) to 1 g of dry powdered material in a corning centrifuge tube (50 ml). The mixture was shaken for 24 h at room temperature (23°C), centrifuged, and the supernatant was filtered through cotton. The filtrate was dried under reduced pressure, and stock solutions of 50 mg/ml in dimethyl sulphoxide (DMSO) were prepared at room temperature and stored at -20°C. Extracts prepared with natural solvents (apple vinegar, grapes vinegar, coconut water) were likewise dried and the extraction yields were calculated by subtracting the dry weight of the natural solvent residue from total weight of natural product extract.

Cell lines

Murine metastatic B16F10 melanoma, breast cancer MCF-7, and cervical cancer HeLa cell lines were obtained from American Type Culture Collection, USA (ATCC), cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen. Carlsbad, CA, USA) supplemented with 10% heat-inactivation fetal calf serum (FCS), 2 mM L-Glutamine, 100 U/ml of penicillin (Sigma), and 100 µg/ml of streptomycin (Sigma) and incubated in 5% CO_2 at 37°C.

Cytotoxicity assays

"Alamar Blue" resazurin reduction assays were conducted as described (O'Brien et al., 2000). Cell suspended in 100 µl of DMEM were seeded in 96-well plates at a density of 5×10^3 cells per well and incubated for 24 h. All extracts were serially diluted into supplemented media using a separate 96-well plate, applied to the cells, and incubated for 48 h. Following the incubation, 100 µl of fresh media, (containing 10% (v/v) of a 860 µM solution of resazurin in PBS) was added to the cells, and incubated for 2 to 4 h. The fluorescence intensity of the dye was then quantified by a SpectraMax M5 plate reader using excitation at 560 nm. IC_{50} values were calculated from the fluorescence intensity values, by using an exponential decay curve fit. DMSO was used as a negative control, whereas Nile Blue A (Lin et al., 1991) was used as a positive control.

Statistical analysis

IC_{50} values are defined as the concentration of the extract where there is a 50% loss of total metabolic activity as compared to untreated controls and are reported as mean ± standard deviation (SD). IC_{50} values with 95% confidence limits were calculated using GraphPad Prism 3.3 software (GraphPad Software, Inc., San Diego, CA). *p* Values less than 0.05 were considered to be significant. All experiments have been conducted in duplicate.

RESULTS

Extract yields

Five Palestinian plants were extracted with seven different solvents to yield 35 extracts in total (Table 2). The isolated yields of the extracts were corrected for non-volatile residues present in the natural solvents. The maximum extraction yields ranging between 63 to 91% were consistently obtained when coconut water was used, suggesting the presence of a "green" surfactant effect. Methanol and ethanol extracts gave yields in the range of 12 to 34%, while the acetic acid solution and vinegars gave highly variable yields ranging between 9 to 41%. Acetone extractions consistently gave lowest percentage yields ranging between 4 to 13%, suggesting greater extraction selectivity.

IC_{50} values in cell cultures

The plant extracts were screened for their cytotoxic activities in three different cancer cell lines using the "Alamar Blue" resazurin reduction assay (O'Brien et al., 2000). This assay reports the combined effects of proliferation and metabolism on total cellular respiration. In general, the least toxic extracts were prepared using the aqueous solvents: 5% acetic acid, natural vinegars and coconut water, while the most toxic extracts were prepared using alcohol or acetone. Little or no cytotoxic effects were exhibited by *F. carica* or *T. polium* extracts, irrespective of the type of solvent used for extraction. In contrast, extracts of *S. officinalis* prepared using organic solvents exhibited exceptionally potent activities with IC_{50} values ranging between 14 to 64 µg/ml in all three cell lines tested (Table 2). In contrast, acetone and ethanol extracts of *O. europaea* exhibited good selectivity between the cell cultures, with IC_{50} values ranging between 43 to 63 µg/ml for MCF-7 cells, and 170 to 510 µg/ml for B16F10 and HeLa cells.

Acetone extracts of all five plants generally exhibited the highest cytotoxicity as compared to the other extraction solvents used (Table 2, Figure 1). Since acetone extracts of *S. officinalis* exhibited the most potent cytotoxic activities, we characterized the time dependency of its cytotoxicity in MCF-7 cell cultures. As shown in Figure 2, the rapid action of metabolism inhibition indicates that the extract exhibits a cytotoxic,

Table 2. Extraction yields and IC_{50} values for 35 different extracts

Extract #	Plant	Solvent	Extraction Yield (%)	MCF-7 IC_{50} µg/ml	B16F10 IC_{50} µg/ml	HeLa IC_{50} µg/ml
1		90% ethanol	34	63±18*	321	490
2		80% methanol	32	400	190±18*	440
3		acetone	10	43±13*	170±28*	510
4	*Olea europaea*	5% acetic acid	26	430	>1000	>1000
5		apple vinegar	41	>1000	>1000	>1000
6		grape vinegar	28	530	>1000	>1000
7		coconut water	91	860	>1000	>1000
8		90% ethanol	12	440	880	>1000
9		80% methanol	26	186±4*	>1000	>1000
10		acetone	4.0	400	720	690
11	*Ficus carica*	5% acetic acid	36	>1000	>1000	>1000
12		apple vinegar	35	>1000	>1000	>1000
13		grape vinegar	21	>1000	>1000	>1000
14		coconut water	63	>1000	>1000	>1000
15		90% ethanol	25	870	686	610
16		80% methanol	21	400	908	620
17		acetone	5.6	62±9*	137±3*	336
18	*Vitis vinifera*	5% acetic acid	24	950	>1000	>1000
19		apple vinegar	25	>1000	993	>1000
20		grape vinegar	11	>1000	>1000	>1000
21		coconut water	65	>1000	>1000	>1000
22		90% ethanol	19	27±11	35±9*	53±8*
23		80% methanol	24	34±7*	51±2*	64±5*
24		acetone	13	16±3*	14±2*	36±4*
25	*Salvia officinalis*	5% acetic acid	24	540	>1000	820
26		apple vinegar	17	400	436	>1000
27		grape vinegar	11	390	542	>1000
28		coconut water	86	114±4*	>1000	845
29		90% ethanol	17	184±37*	803	420
30		80% methanol	20	104±32*	426	460
31		acetone	7.4	140±56*	129±16*	173±3*
32	*Teucrium polium*	5% acetic acid	23	360	>1000	>1000
33		apple vinegar	25	400	>1000	>1000
34		grape vinegar	9	360	>1000	>1000
35		coconut water	53	650	>1000	>1000
36	Nile Blue A	Positive control		3±1*	3±1*	0.8±0.2*

Cell viability was determined using a resazurin reduction assay. Results are expressed as mean ± S.D (N= 2). *denotes statistically significance of $p < 0.05$.

rather than cytostatic activity.

MCF-7 cells exhibited the highest sensitivity to the plant extracts, with most lower IC_{50} values than the other cell lines evaluated (Daoudi et al., 2013). As compared to

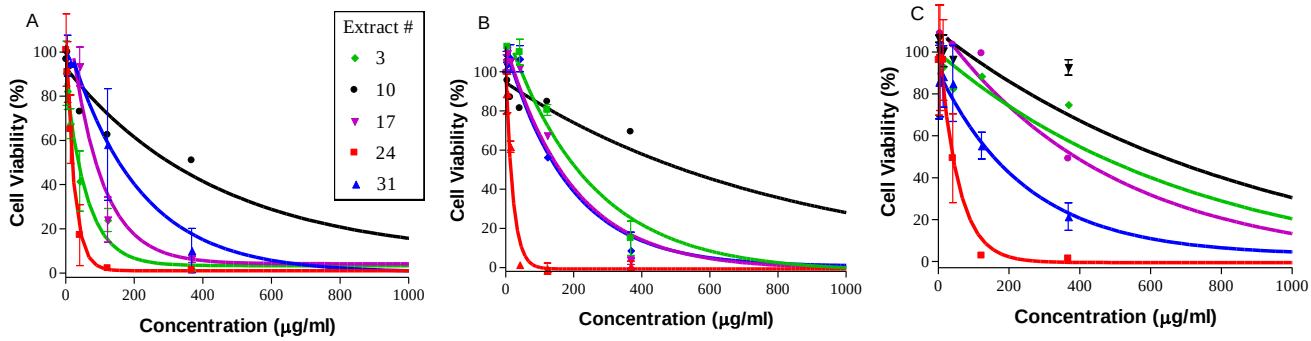

Figure 1. Cell viability according to total metabolic activities of MCF-7 (A), B16F10 (B), or HeLa (C) cells after a 48 hour incubation with extracts prepared from acetone. 3: *O. europaea*, 10: *F. carica*, 17: *V. vinifera*, 24: *S. officinalis*, 31: *T. polium*. Cell viability was determined using resazurin reduction assay. Results are expressed as mean ± S.D (N= 2).

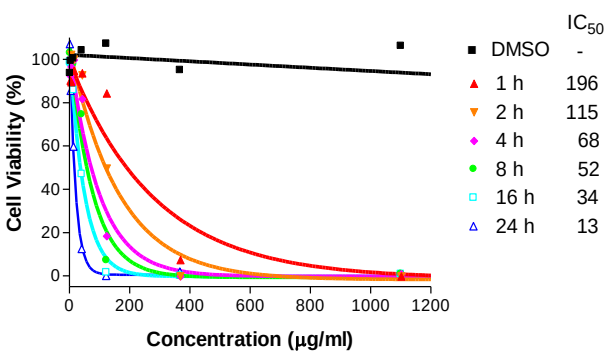

Figure 2. Time-dependent viability of MCF-7 cells incubated with variable concentrations of the acetone extract from *S. officinalis* (#24).

HeLa and B16F10 cells, five to 10-fold lower IC_{50} values were observed in MCF-7 cells for diverse extracts including No.: 1, 3, 9, 17, 28, 29, and 30 (Table 2). In contrast, HeLa cells generally exhibited the lowest sensitivity to the plant extracts. The acetone extract of *S. officinalis* exhibited the most potent activities in HeLa cells with an IC_{50} of 36 µg/ml followed by ethanol and methanol extracts with an IC_{50} value of 53 and 64 µg/ml, respectively. Moderate activities were observed from the acetone extract of *T. polium* with an IC_{50} of 173 µg/ml, whereas the acetone extract of *V. vinifera* gave only weak activity with an IC_{50} of 336 µg/ml. The 14 extracts of *O. europaea* and *F. carica* were inactive against HeLa cells.

Since acetone extracts of all five plants exhibited the highest cytotoxicity and *S. officinalis* exhibited the most potent cytotoxic activities, we further evaluated the effect of different acetone extracts of the five plants and the effect of different solvents of *S. officinalis* to MCF-7 cells at fixed extract concentration (40 µg/ml). The MCF-7 sensitivity to different plants were in the following order: *S. officinalis* > *O. europaea* > *V. vinifera* > *F. carica* > *T. polium* and the MCF-7 sensitivity to *S. officinalis* extracts were in the following order: acetone > 90% ethanol > 80% methanol > coconut water > 5% aceticacid, apple vinegar, grape vinegar (Figure 3).

Conflict of Interests

The author(s) have not declared any conflict of interests.

DISCUSSION

Most of the currently used anticancer drugs are highly toxic, expensive, and resistance mechanisms pose a significant problem (Lippert et al., 2008; Petrelli and Giordano, 2008; Hait and Hambley, 2009). There is a continuing need to identify new drug candidates that are more effective, widely available and less toxic. Plants extracts are an important source of potentially useful compounds for the development of new anticancer drugs. Here we investigated solvent extraction effects of five Palestinian medicinal plants for cytotoxic activities in three cancer-derived cell lines. Among the 35 extracts tested, a few exhibited potent activities with IC_{50} values of ≤ 100 µg/ml (Table 2).

The acetone, ethanol and methanol extracts from *S. officinalis* exhibited highest cytotoxicity against all cell lines tested, with acetone extract being the most cytotoxic (Figure 3). *S. officinalis* is not currently used for anticancer treatments in traditional Palestinian medicine, but the cytotoxicity of *S. officinalis* has been previously

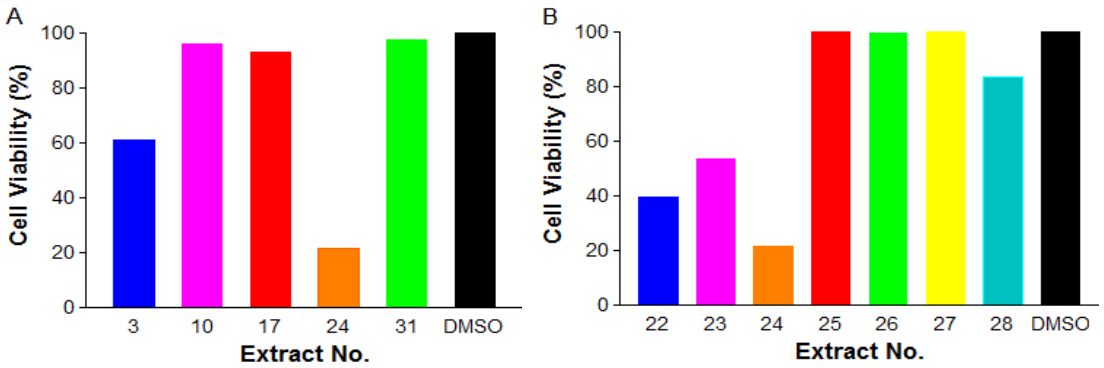

Figure 3. Cell viability of MCF-7 at fixed extract concentration (40 µg/ml). (A) extracts prepared from acetone. 3: *O. europaea*, 10: *F. carica*, 17: *V. vinifera*, 24: *S. officinalis*, 31: *T. polium*. (B) *S. officinalis* extracts prepared from different solvents. 22: 90% ethanol, 23: 80% methanol, 24: acetone, 25: 5% acetic acid, 26: apple vinegar, 27: grape vinegar 28: coconut water. Cell viability was determined using resazurin reduction assay. Results are expressed as mean ± S.D (N= 4).

reported (Xavier et al., 2009; El Hadri et al., 2010). An essential oil prepared by sub-fractionation of *S. officinalis* by hydrodistillation has previously been tested against cell lines of murine macrophage, colon cancer, and breast cancer cell lines (El Hadri et al., 2010). The reported IC_{50} values against murine macrophage, colon cancer and MCF-7 cell lines were reported to be 41.9, 77.3, 213.1 µg/ml, respectively.

Our studies demonstrated extracts of *S. officinalis* exhibit potent cytotoxicities that are dose, time, and solvent dependent. The exceptional cytotoxicities of acetone extracts of *S. officinalis* is reproducible even when the extract solution was kept for one week at room temperature. Other extracts like *O. europaea*, in contrast, exhibited diminished activities if the extract was kept at room temperature for few days. To maintain the cytotoxic activities of *O. europaea* extracts, stock solutions must be freshly prepared and stored at -20°C. Oxidation of phenolic compounds from *O. europaea* might be responsible for this loss in activity (Alu'datt et al., 2011; Kontogianni and Gerothanassis, 2012). The stability and reproducibility of *S. officinalis* extracts suggest the involvement of compounds that are resistant to oxidation. The chemical composition of *S. officinalis* has previously been evaluated, sesquiterpenes α-humulene and trans-caryophyllene were found to be major components (Loizzo et al., 2007; El Hadri et al., 2010). The cytotoxic activity of α-humulene against MCF-7 is reported to be 81 µg/ml, whereas trans-caryophyllene was reported to be less cytotoxic (IC_{50} > 100 µg/ml). This activity is not correlated with the exceptionally high activity of acetone extract reported here; where the combined effects of various compounds with different cellular targets is likely responsible for the high activity.

Natural apple and grapes vinegars and coconut water are natural solvents which could be used for green technologies to replace organic solvents (Chemat et al., 2012). Although high extraction yields were obtained from natural solvents, almost no cytotoxic activities were observed for the extract with unusual exception of coconut water. Coconut water extracts of *S. officinalis* exhibited high activities against MCF-7 cells with an average IC_{50} of 114 µg/ml and good selectivity as compared to B16F10 and HeLa cells (Figure 4). More study is needed therefore to evaluate the *S. officinalis*-coconut water mixture as a potential chemopreventive agent against breast cancer.

As compared to the vinegars and coconut water, acetone consistently gave lower extraction yields but higher cytotoxicity. These results demonstrate that high extraction yield is not a key factor for achieving high cellular activity. While *in vitro* cytotoxicity can be an initial indicator of *in vivo* antitumor activities, a wide range of phytocompounds are capable of exhibiting nonspecific cytotoxicity. According to American National Cancer Institute (NCI) (Suffness and Pezzuto, 1990) guidelines, an IC_{50} < 30 µg/ml is considered to be a promising cytotoxicity, therefore plant extracts with significant cytotoxic activity such as extract No. 22, 23, and 24 should be further assessed using animal models.

Conclusion

The results of the present study demonstrated that a number of Palestinian medicinal plants have promising anticancer activities in cell cultures. Depending on the extraction solvent used, these plants exhibited moderate

to highly potent cytotoxic activities. The cytotoxicity of acetone extract of *S. officinalis* L. was highly reproducible, as the potency remained unchanged even when the extract was left in the presence of oxygen for one week at room temperature. Interestingly, coconut water was found to offer a potential alternative to classical organic solvents; it gave consistently highest extraction yields, and in the case of *S. officinalis* L., highly toxic extracts towards MCF-7 cells derived from human breast cancer. To our knowledge coconut water has never been utilized for the purpose of natural product extraction. Taken together, these results demonstrate how the cytotoxicities of plant extracts depend on the solvent used, and that traditional Palestinian medicinal plants can serve as a source for the discovery of new anticancer agents.

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Full Length Research Paper

Byrsonima intermedia preparations inhibits trypsin and chymotrypsin activities from *Aedes aegypti* larval gut

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This work describes the larvicidal activity of the ethanolic crude extract (EEB) as well as the hexanic (FH), chloroform (FCL), ethyl acetate (FAE) and remaining (FR) fractions obtained from *Byrsonima intermedia* leaves and bark against *Aedes aegypti.* Hexanic and remaining fractions from the leaves demonstrated larvicide activity against *A. aegypti* larvae on the 3rd stage, with LC_{50} values of 250 and 125 µg/ml^{-1}, respectively, with less mortality as compared to the other fractions. Hexanic and remaining fraction from the leaves demonstrated larvicide acitivity against *A. aegypti* larvae on the 3rd stage, with LC_{50} values of 250 and 125 µg/ml^{-1}, respectively, with less mortality as compared to the other fractions. Trypsin activities from gut of larvae treated with the extract or the fractions were significant ($p < 0.05$), and the major effects occurred with hexanic fraction (0.06 µmol min^{-1}) and remaining fraction (0.09 µmol min^{-1}) in the 1000 µg/ml^{-1} concentration, respectively. The same fractions inhibited chymotrypsin activity in 0.06 µmol/ml^{-1} (hexanic fraction) and 0.13 µmol/ml^{-1} (remaining fraction) at 1000 µg/ml^{-1}. The inhibition was dose-dependent. The results show the inhibitory effects of the hexanic and remaining fractions from the leaves of *B. intermedia* on trypsin and chymotrypsin activity, which may be responsible for the deleterious effects on larvae mortality. The crude ethanolic extract and fractions of the bark does not inhibit the synthesis of trypsin compared to control. The results show the inhibitory effects of the hexanic and remaining fraction from the leaves of *B. intermedia* on trypsin and chymotrypsin activity, which may be responsible for the deleterious effects on larvae mortality.

Key words: Digestive system, dengue fever, larvicidal activity, natural products.

INTRODUCTION

Classical and hemorrhagic dengue epidemics have reappeared in the past 25 years in tropical regions which present a warm and humid weather and propitious socio-environmental conditions for the proliferation of the mosquito vector *Aedes aegypti* (Silva et al., 2008). *A. aegypti* control has been performed using synthetic chemicals with larvicidal effect (Luna et al., 2004; Lima et al., 2006). However, its frequent use is costly and can lead to environmental contamination, public health damage and the arising of resistant insects (Oga, 2003; Braga and Valle, 2007). In this sense, the use of natural bioactive products with insecticide potential emerges as

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aviable alternative. *Malpighiaceae* is one of the major families of angiosperms, comprising about 66 genus and 1,200 species, spread in tropical and subtropical regions (Anderson, 1990). In Brazil, 55 genus occured, with emphasis on *Byrsonima.*

Although not very studied in scientific works, *Byrsonima intermedia* is known as murici anão-do-campo and murici-anão. It occurs with predominance in the *Cerrado* region of Mato Grosso do Sul, Brazil and it shows adstringent activity against diarrheas and dysenteries when used as an infusion from the bark stark (Rodrigues and Carvalho, 2001). In the literature, anti-inflammatory and anti-nociceptive activities are reported for the bark extract (Orlandi et al., 2011), and the leaves show gastroprotective and antidiarrheal activities (Santos et al., 2012). The floral oil of *B. intermedia* is rich in free fatty acids and the main constituent is byrsonic acid (Reis et al., 2007).

From the leaves of *B. intermedia*, it was possible to isolate some chemical substances, such as (+)-catechin, (-)-epicatechin, quercetin-3-O-beta-d-galactopyranoside, metyl gallate, gallic acid, quercetin-3-O-α-L-arabinopyranoside, amentoflavone, quercetin, quercetin-3-O-(2''-O-galoil)-beta- galactopyranoside and quercetin-3-o-(2''-o-galoil)-α-arabinopyranoside (Sannomiya et al., 2007). It has been reported that plant extracts can be toxic to *A. aegypti* larvae by killing them and inhibiting enzymes from larval gut such as trypsin and chymotrypsin (Venancio et al., 2009; Melo-Santos et al., 2010). The *Moringa oleifera* flower extract was considered a larvicidal agent against *A. aegypti* of 4th ínstar, mainly on the last developmental stage, and inhibited proteases from gut (Pontual et al., 2012). Enzymatic inhibitors, when added to the diet of insects, interfere with their digestive process by decreasing the assimilation of nutrients, leading to delayed development and mortality (Napoleão et al., 2012).

Current strategies based on the elimination of breeding sites and applications of chemical insecticides for larval and adult mosquito control have resulted in development of resistance without eliminating the constant risk of dengue epidemics (Lima et al., 2011). Thus new approaches are urgently needed. Interest on possible use of environment friendly natural products such as oils of plants or plant parts increased for vector control. Plant derived products have received increased attention from scientists and more than 2000 plant species are already known to have insecticide properties (Pankaj and Anita, 2010; Kamaraj et al., 2011). Considering the advancement of researches on the botanical larvicies and their potential for larvae control, the objective of this work was to evaluate, under laboratory conditions, the bioactivity of crude ethanolic extract (CEE), hexane (FH), chloroform (FCL), ethyl acetate (FAE) and remaining fractions (FR) from the leaves and barks of *Byrsonima intermedia*, against *A. aegypti*, through assays of mortality, trypsin and chymotrypsin activities.

MATERIALS AND METHODS

Vegetal material

B. intermedia was collected in the *Cerradinho* of the Universidade Federal do Mato Grosso do Sul, from July to August, 2011, and a species sample was identified by comparison with material deposited in the Campo Grande, Mato Grosso do Sul herbarium, under number 21176.

Crude extract and fractions obtainment

The collected material was dried at 25°C, milled and submitted to exhaustive extraction with ethanol in a Sohxlet apparatus, consisting of a vertical glass cylindrical extraction tube that has both a siphon tube and a vapor tube, that is fitted at its upper end to a reflux condenser and at its lower end to a flask so that the solvent may be distilled from the flask into the condenser whence it flows back into the cylindrical tube and siphons into the flask to be distilled again. After extraction, the ethanolic crude extract was dried and submitted to fractioning with solvents of different polarity degrees, obtaining the hexanic (FH), chloroform (FCL), ethyl acetate (FAE) and remaining (FR) fractions.

Aedes aegypti mortality

The larvicide activity under *A. aegypti* was assessed following the World Health Organization (1981) methods, with some modifications. Eggs from *A. aegypti*, Rockefeller strain, were provided by the Fundação Oswaldo Cruz – Rio de Janeiro. For egg eclosion, they were put in a plastic tray and 500 ml of chlorine-lacking water was added, and then taken to a BOD incubator in 27 ± 2°C temperature and 80 ± 5% relative humidity. Regarding the assays, we chose to evaluate the extract activity using 3rd stage larvae, as they are considered more resistant (Silva and Silva, 1999). Larvae feeding was prepared with Fish's diet (Aldon Basic™, MEP 200 Complex) from the eclosion period to the 3rd larvae stage, and solutions of the extract and its fractions in the concentration of 1000 µg ml^{-1} were prepared by solubilizing the samples with 0.5% dimetilsulphoxide (DMSO) and diluted in water in the concentrations of 1000, 500, 250 and 125 µg ml^{-1} for the assays. The stearic acid activity was evaluated as well. The same method was used to prepare a 50% concentration.

Samples containing 15 larvae at the 3rd stage were put in plastic cups and the volume was completed to 5 ml. The assays were performed in triplicates, totaling 45 larvae. A 0.5% DMSO aqueous solution was used in triplicate, as a negative control. Temephos™ was used as a positive control, technical grade of 90%, lot #005/2011, manufactured by Fersol Mairinque Laboratories, São Paulo. The calibration was made following the protocol recommended by the World Health Organization described in World Health Organization (2009), Lima et al. (2003) and Braga et al. (2004) using 0.060 mg ml^{-1} as a diagnostic concentration (DC), which is twice the lethal concentration that causes 99% mortality in a susceptible strain, as defined by World Health Organization (2009). The larvicide activity was evaluated after 24 h, by counting the number of dead larvae in each sample. Moribund larvae, unable to reach the water surface when touched, were considered dead (World Health Organization, 2009).

Larvae homogenate preparation

A. aegypti larvae homogenates, submitted to the extract and fractions during 6 h were prepared according to Macedo et al. (1993), with modifications. Live larvae were homogenized by hand

Table 1. Effect of different concentrations of the crude ethanolic extract (CEE) and hexanic (HF) chloroform, ethyl acetate (EAF) and remaining (RF) fractions of leaves and barks of the *B. intermedia* in the mortality and LD_{50} against *A. aegypti* larvae.

Parameter	Control*	125 µg ml^{-1}*	250 µg ml^{-1}*	500 µg ml^{-1}*	1000 µg ml^{-1}*	DL_{50}
HF	0.0±0.0^a	5.7±0.8^b	7.0±1.2^b	9.7±0.7^b	11.9±1.5^b	<250
CLF	0.0±0.0^a	3.0±1.2^b	1.3±0.7^a	1.7±0.9^b	7.7±1.6^b	<500
EAF	0.0±0.0^a	1.0±1.3^a	1.3±0.9^a	1.3±0.5^a	6.0±1.1^b	<500
RF	0.0±0.0^a	7.0±1.8^b	9.0±1.3^b	10.7±0.9^b	11.7±0.7^b	<125
CEE	0.0±0.0^a	2.3±1.1^b	4.0±1.6^b	5.0±1.4^b	8.3±0.3^b	>500
Temephos: 15.0±1.3						≤60
Barks						
CEE	0.0±0.0^a	1.7±0.5^a	2.3±1.1^b	4.0±1.5^b	7.7±0.8^b	>500
HF	0.0±0.0^a	0.0±0.0^a	0.0±0.0^a	0.7±1.7^a	0.7±1.2^a	>1000
CLF	0.0±0.0^a	1.0±0.5^a	1.7±1.1^a	1.7±1.3^a	1.7±1.6^a	>1000
EAF	0.0±0.0^a	2.7±1.4^b	4.0±0.6^b	5.0±1.1^b	5.0±1.3^b	>500
RF	0.0±0.0^a	0.7±1.2^a	0.7±1.5^a	1.7±1.4^a	1.7±1.8^a	>1000
Temephos: 15.0±1.3^b						≤60

*Means followed by the same letter from the control have no significant difference among them, according to Dunnet test ($p < 0.05$).

in a porcelain mortar, with 1.0 ml of 0.05 M Tris/HCl Buffer (pH 8.0) and centrifuged at 17,000 rpm during 20 min at 4°C. The supernatants were collected and an additional 1 ml of Tris/HCl Buffer was added.

Determination of protein content

The protein content in the homogenate preparations from larvae was determined according to Lowry et al. (1951), using serum bovine albumin (25 to 500 µg/ml) as standard.

Protease activity assay

The total trypsin activity was determined using the N-benzoyl-D, L-arginine-ρ-nitroanilide (BAPNA) reagent as substrate. About 500 µl of the larvae homogenate (0.8 µg of protein) were added to BAPNA and left to rest for 20 min at 37°C; after this, the reaction was stopped by adding 30% acetic acid (v/v). The trypsin activity was read in a microplate reader at 410 nm (ε, 10.0 mM^{-1} cm^{-1}) in accordance with Silva et al. (2009). The chymotrypsin activity was determined using N-succinyl-Ala-Ala-Pro-Phe-p Nitranilide (SAAP) as substrate. About 50 µl (0.08 µg of protein) of the homogenates were incubated in 450 µl of Tris-HCl Buffer 0.1 M (pH 8.0) for 10 min. Afterwards, 1.0 ml of SAAP 2 Mm dissolved in pure DMSO was added. The reaction mixture was incubated at 37°C for 20 min and then the reaction was ended by the addition of 30% acetic acid (v/v). The absorbance was read at 405 nm (ε, 8.8 mM^{-1} cm^{-1}) (Silva et al., 2009).

Statistical analysis

Values of lethal concentration (LD_{50}) in µg/ml were determined using the Probit analysis method (Finney, 1974). For each evaluated sample, triplicates were used and data was submitted to analysis of variance, and when a difference was detected, the averages were compared by the Dunnet test, with 5% of probability.

RESULTS

The mortality of *Aedes aegypti* larvae varied according to extract and concentration tested. The hexanic (LD_{50} < 250 µg ml^{-1}) and remaining fractions (LD_{50} < 125 µg ml^{-1}) from the leaves demonstrated more activity, showing a mortality of 11.9 ± 1.5 and 11.7 ± 0.7 individuals (mean ± SD), respectively for the 1000 µg ml^{-1} concentration. Chloroform and remaining fractions showed lower activity with a LD_{50} < 500, with mortality of 7.7 ± 1.6 (mean ± SD) and 6.0 ± 1.1 (mean ± SD), respectively. Larvae treated with fractions from the barks showed less mortality, with a LD_{50} > 1000 µg ml^{-1}. The crude ethanolic extract demonstrated larvicide activity with a LD_{50} > 500 (Table 1). No concentration had an effect comparable to that of the diagnostic concentration (DC) of 0.060 mg mL^{-1} of the pesticide Temephos. The inhibition in trypsin activity was also observed and a dose-dependent relationship was verified, with larger inhibitory effects for the 1000 µg ml^{-1} concentration. The hexanic and remaining fractions from the leaves inhibited the trypsin synthesis in 0.06 and 0.09 µmol min^{-1}, respectively, demonstrating more activity of the fractions when compared to crude ethanolic extract (0.13 µmol min^{-1}), chloroform (0.20 µmol min^{-1}) and ethyl acetate fractions (0.21 µmol min^{-1}). Only the ethanolic crude extract (EEB) from the barks caused an accentuated inhibition in trypsin synthesis (0.21 µmol min^{-1}). The fractions did not lead to alterations in trypsin synthesis (Figure 1).

The EEB and all the fractions from the leaves inhibited the chymotrypsin synthesis. A higher inhibitory effect was verified for the hexanic (0.06 µmol min^{-1}) and remaining fractions (0.13 µmol min^{-1}) in the 1000 µg ml^{-1} concentration

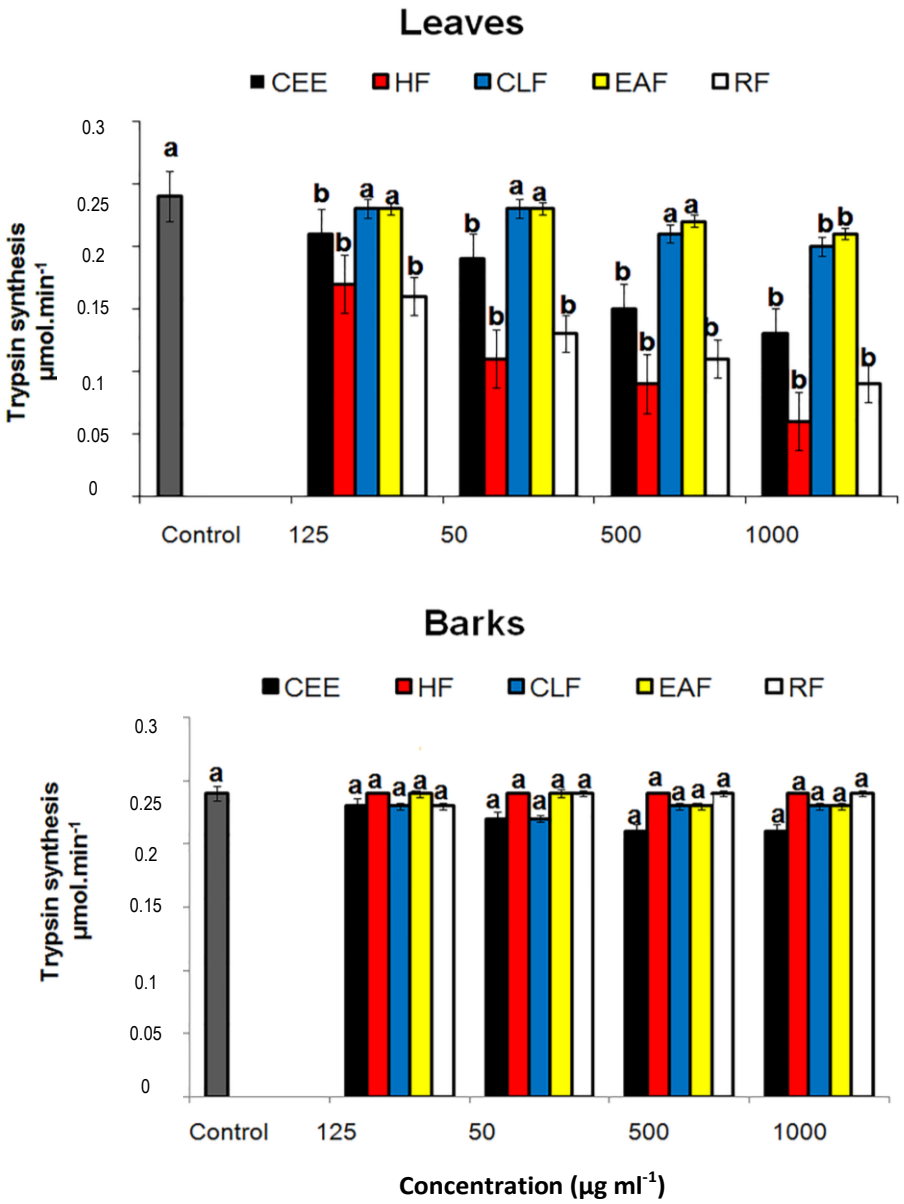

Figure 1. Effects of different concentrations of the crude ethanolic extract (CEE) and hexanic (HF) chloroform, ethyl acetate (EAF) and remaining (RF) fractions of leaves and barks of the *B. intermedia* on trypsin synthesis in *A. aegypti* larvae. *Means followed by the same letter from the control have no significant difference among them, according to Dunnet test ($p < 0.05$).

The crude ethanolic extract, chlorophorm and ethyl acetate fractions affected the chymotrypsin activity as well, albeit in a less proportion. The crude ethanolic extract and fractions of the barks did not alter the activity of chymotrypsin when compared to control (Figure 2).

DISCUSSION

The results of the larvicidal assay demonstrated that the crude ethanolic extract and fractions of *B. intermedia* was effective against the last A. *aegypti* larval stage. Secondary metabolites have been reported as active principles from insecticidal plant extracts.

Compounds such as quercetin may be involved in larvicidal activity since they are able to kill mosquito larvae (Rahuman et al., 2008; Ochieng et al., 2010). Quercetin can bind to trypsin S1 region through hydrogen bonds and electrostatic interactions and promote enzyme inhibition (Maliar et al., 2004). Secondary metabolites have been reported as active components of vegetal

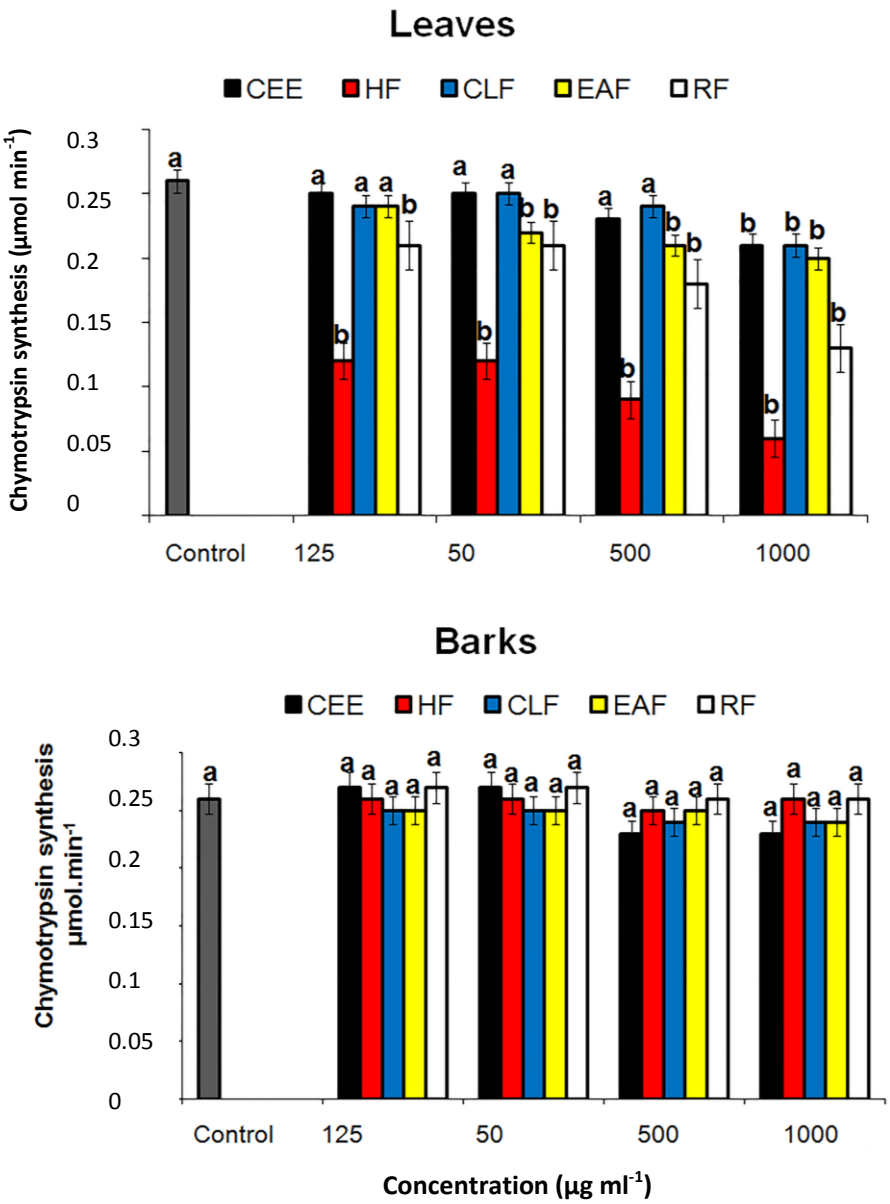

Figure 2. Effects of different concentrations of the crude ethanolic extract (CEE) and hexanic (HF) chloroform (CLF), ethyl acetate (EAF) and remaining (RF) fractions of leaves and barks of the *B. intermedia* on chymotrypsin synthesis in *A. aegypti* larvae. *Means followed by the same letter from the control have no significant difference among them, according to Dunnet test ($p < 0.05$).

extracts which exhibit insecticide activity. Detected substances such as quercetin may be involved in the larvicide activity because they are able to kill the mosquito larvae in all its development stages (Rahuman et al., 2008; Nikkon et al., 2010; Ochieng et al., 2010).

Toxic substances interact specifically with apical membrane receptors of the median gut, causing serious damage to the epithelium that culminate with larvae death (Charles, 1981; Gill et al., 1992). The hexanic and remaining fractions from the leaves were more active when compared to the other fractions. These results demonstrate that it is possible that one of the action mechanisms occurs by ingestion of the extract and fractions which are solubilized in the intestinal lumen and cleaved by trypsin and chymotrypsin, then becoming toxic.

Trypsin and chymotrypsin may play various important roles in food digestion, immune defense and zymogen activation in insects (Ge et al., 2012). Despite several studies concerning adult *A. aegypti* digestive biochemistry and molecular biology, very few studies have been performed to elucidate the digestion in *A.*

aegypti larvae. Trypsin-like and chymotrypsin-like activities are known in *A. aegypti* larvae (Mesquita-Rodrigues et al., 2011). Enzymatic inhibitors, when added to the diet of insects, interfere with their digestive process by decreasing the assimilation of nutrients, leading to delayed development and mortality (Napoleão et al., 2012).

Zhang et al. (2010) reported what enzymes involved in the food digestion; it has to be secreted into the interspaces between the epithelium and peritrophic membrane or the lumen of the gut, where it digests the ingested food proteins, and the results showed that digestive enzymes was present not only in the epithelium of the anterior, middle and posterior midgut, but also in the lumen food residues of the anterior, middle and posterior midgut, as well as the feces of the larvae, suggesting that the protein was secreted into the lumen of the gut.

Current strategies based on the elimination of breeding sites and applications of chemical insecticides for larval and adult mosquito control have resulted in development of resistance without eliminating the constant risk of dengue epidemics (Lima et al., 2011). Thus new approaches are urgently needed. Interest on possible use of environment friendly natural products such as oils of plants or plant parts increased for vector control. Plant derived products have received increased attention from scientists and more than 2000 plant species are already known to have insecticide properties (Pankaj and Anita, 2010; Kamaraj et al., 2011). The protease inhibitors show insecticide activity because of the damage in processes related to digestion and absorption of nutrients (Carlini and Grossi-De-Sá, 2002). The effects of trypsin inhibitors in insect larvae include reduction of body weight, decrease of survival rate, as well as delay or blockade of larvae development (Macedo et al., 2002, 2003; Bhattacharyya et al., 2007; Ramos et al., 2009).

It is plausible to infer that hexanic and remaining fractions obtained from the leaves of *B. intermedia* cause the same effects on larvae of A*. aegypti*, and these effects are associated with inhibition of gut enzymes. Thus, the use of hexanic and remaining fractions for the control of insects is an alternative way to minimize the harmful effects of pesticides used to control mosquitoes. Further, samples of locals where there has already been intensive use of pesticides by the control programs demonstrate that the larvae and mosquitoes became resistant by mechanisms such as increase in the synthesis of acetylcholinesterase (Pinheiro and Tadei, 2002). Extracts of plants could be used in stagnant water bodies which are known to be the breeding grounds for mosquitoes. However, further studies on the active principals involved and their mode of action and field trials are usually needed to recommend any of these plant materials as an anti-larvicidal product used to combat and protect people from mosquitoes in a control program. Plant could be an alternative source for mosquito larvicides because they constitute a potential source of bioactive chemicals and generally free from harmful effects. Use of these botanical derivatives in mosquito control instead of synthetic insecticides could reduce the cost and environmental pollution.

Taking this into consideration, the hexanic and remaining fractions of leaves represent an alternative to the larvae control, as they acted as inhibitors of digestive enzymes such as trypsin and chymotrypsin, demonstrating its potential as physiologic pesticide. The isolation of substances present in hexanic and remaining fractions, and new research on the isolated activity of these substances could improve our understanding on the popular indication of this plant as pesticide by the Cerrado population, and evolve to the developing of formulations that could be used for the control of larvae and mosquitoes.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Conclusion

The hexanic and remaining fraction of *B. intermedia* leaves can be considered as a larvicidal agent against *A. aegypti* on the last developmental 3rd stage, and this activity is related to trypsin and chymotrypsin inhibition. New insecticides of herbal origin discovered through ethnopharmacological studies have shown interesting results. Our laboratory has initiated and developed original investigations, and we have evaluated the larvicidal effect from natural extracts of plants. Purification of the bioactive component(s) from *B. intermedia* leaves is underway, and further investigations may improve our understanding of possible developmental changes from hexanic and remaining fraction of this plant used in folk medicine.

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