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Anxiolytic properties of botanical extracts in the chick social separation-stress procedure

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Abstract Rationale: The recent growth in sales of natural products labeled as dietary supplements in the United States has renewed scientific interest in the study of the therapeutic effects of multi-component botanical products. **Objectives:** This study sought to determine whether botanical extracts derived from the Rutaceae family, *Acori graminei*, the Magnoliaceae family, *Alchemilla vulgaris* and *Primula veris*, which had previously been identified in bioassays as having potential anxiolytic activity, were active in the chick social separation-stress procedure. **Methods:** Eight-day-old chicks received IP injections of test articles 30 min before being tested in the presence of two social companions or in isolation for a 3-min observation period. Dependent measures were: a) latency to adopt a ventral recumbent posture to index sedation, b) number of vocalizations to index separation-distress and c) a composite pain score (comprised of footlift frequency and footlift duration in response to 50 µl of 0.10% formalin injected into the plantar surface of the foot) to index stress-induced analgesia. **Results:** Proprietary extracts NPS00033 from the Rutaceae plant family and NPS00039 (Relora™) from the Magnoliaceae plant family screened positive in this chick model without causing sedation. **Conclusions:** These results suggest that botanical extracts NPS00039 and NPS00033 may be useful in modulating anxiety states.

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Introduction

The recent growth in sales of natural products labeled as dietary supplements in the United States has renewed scientific interest in the study of the therapeutic effects of multi-component botanical products. Unlike single entity pharmaceutical products, botanicals contain a large number of diverse chemical constituents that often act synergistically to exert a desired biological effect (Tyler 1994). The type of extraction process utilized and manner in which the formulation is standardized have dramatic effects on the pharmacological activity of the final product (Murray 1992). The development of new botanical products requires a multidisciplinary effort consisting of expertise in ethnobotany, natural product chemistry, analytical chemistry, pharmacology and natural product extraction.

Once promising botanicals have been identified, there is the need to screen these various extract fractions in valid animal models to determine efficacy and to estimate therapeutic doses for future human clinical trials. The purpose of this study was to determine whether various botanical extracts derived from the Rutaceae family, *Acori graminei*, the Magnoliaceae family, *Alchemilla vulgaris* and *Primula veris*, which had previously been identified in bioassays as having potential anxiolytic activity, were active in the chick social separation-stress procedure (Sufka and Weed 1994; Watson and Sufka 1996; Watson et al. 1999).

Materials and methods

Subjects

Cockerels (*Gallus gallus*; W36 strain, Cal-Maine Foods Inc., Mendenhall, Miss., USA) were obtained 1-day post-hatch and

Note: NPS00039 is the Magnolia extract in Relora and NPS00033 is the Phellodendron extract in Relora.

group housed in stainless steel cages at 12 chicks per cage. Food (Purina Start and Grow) and water were available ad libitum. Room temperature was maintained at 29±1°C, and overhead illumination was maintained on a 12:12-h light dark cycle. Chicks were handled briefly each day during daily maintenance prior to testing in order to reduce experimenter-related stress (Sufka and Hughes 1991). The Institutional Animal Care and Use Committee approved all research protocols, and studies were conducted under the ethical guidelines specified by the Animal Welfare Act and National Institutes of Health.

Apparatus

Three Plexiglas viewing chambers (25×25×22 cm) situated in separate sound attenuating boxes were used for data collection (see Sufka and Weed 1994 for complete details). Distress vocalizations were monitored via microphones located above the observation chambers, which were connected to sound activated relays that triggered counters.

Test materials

The following samples were developed by Next Pharmaceuticals to screen for anxiolytic activity: NPS00032, a hydroalcoholic extract from the Rutaceae family; NPS00033, an aqueous extract from the Rutaceae family; NPS00034, a hydroalcoholic extract of *Acori graminei*; NPS00035, an aqueous extract of *Acori graminei*; NPS00038, an aqueous extract from the Magnoliaceae family; NPS00039, a hydroalcoholic extract from the Magnoliaceae family; NPS00056, a hydroalcoholic extract of *Ladies Mantle*; and NPS00058, an alcoholic extract of *Primula veris*.

The vehicle for all preparations was 40% propylene glycol, 10% ethanol, and 50% water. Single-dose screenings to identify active botanical extracts were conducted in two test sessions (four extracts per session). All samples were tested at concentrations of 25.0 mg/ml per kg with the exception of samples NPS00033 and NPS00056, which were tested at concentrations of 28.0 mg/ml per kg. These initial screenings were followed by dose-response studies on the active compounds NPS00039 and NPS00033. Doses for NPS00039 were 12.5, 25.0, and 50.0 mg/2 ml per kg and for NPS00033 were 14.0, 28.0, and 56.0 mg/2 ml per kg.

Procedure

All tests were conducted at 8 days post-hatch. Drug injections or vehicle were administered IP 30 min before testing. The stress ma-

nipulation involved placing a chick into the observation chamber in isolation or in the presence of two conspecifics (social) for a 3-min test session. To index stress-induced analgesia, a 50 µl injection of 0.10% formalin was administered into the plantar region of the chick's foot immediately before placement in the chamber. Trained observers recorded footlift frequency and footlift duration in response to formalin and, to index sedation, ventral recumbent latency that resembles a sleep-like posture. A composite pain score (CPS) was derived from the following formula: $CPS = 4(z\text{-score footlift}) + [z\text{-score (duration/total number of lifts)}]$ (Roach et al. 1998).

The design for each screening session formed a single factorial design (vehicle plus four test articles in isolated test condition) with a single hanging control group (vehicle in social test condition). Sample sizes were $n=18$ per group. The experimental design for both dose-response studies formed a 2×4 factorial which combined two levels of the stress manipulation (social versus isolated) with four levels of dose (vehicle and three doses). Sample sizes in these studies were $n=15$ per group.

Data were screened for homogeneity of variance and analyzed using one- and two-way analysis of variance (ANOVA), Fisher's LSD and *t*-tests (Kirk 1982).

Results

Initial screening sessions

The results from the two initial screening sessions are summarized in Table 1. None of the eight test compounds affected ventral recumbent latency; nearly all chicks remained active through the entire observation period. These data indicate that samples NPS00058, NPS00038, NPS00034, NPS00032, NPS00056, NPS00039, NPS00035, and NPS00033 do not possess sedative properties at the doses tested.

In the first screening session, a one-way ANOVA on the vocalization measure revealed a significant main effect [$F(5,96)=5.14$, $P<0.001$]. Vehicle-isolated chicks exhibited significantly more distress vocalizations than vehicle-social chicks, illustrating the social separation-stress effect [$t(33)=4.70$, $P<0.001$]. At the doses tested, NPS00058, NPS00038 and NPS00034 tended to elevate

Table 1 Effects of botanical extracts on measures of ventral recumbency latency, distress vocalizations, and composite pain scores

	Dependent measures		
	Ventral recumbency latency	Distress vocalizations	Composite pain score
Session 1			
Social-Vehicle	172.28 (3.62)	10.78 (5.53)	2.41 (1.28)
Isolated-Vehicle	179.47 (0.53)	74.53 (12.69)*	0.66 (0.90)
NPS00058	173.75 (4.07)	104.06 (24.41)	-0.48 (0.76)
NPS00038	180.00 (0.00)	109.89 (17.33)	-1.14 (0.61)
NPS00034	176.41 (3.59)	113.59 (15.29)	-1.19 (0.74)
NPS00032	179.94 (0.06)	80.00 (24.01)	-0.82 (0.81)
Session 2			
Social-Vehicle	167.69 (6.10)	6.88 (2.12)	4.09 (1.43)
Isolated-Vehicle	174.07 (5.26)	125.60 (20.65)*	-0.37 (0.84)*
NPS00056	174.56 (4.99)	98.56 (17.98)	-0.77 (0.60)
NPS00039	171.61 (4.93)	62.00 (16.17)**	-1.36 (0.70)
NPS00035	170.38 (5.24)	104.75 (17.92)	-0.78 (0.65)
NPS00033	179.86 (0.14)	60.79 (17.31)**	-0.60 (0.68)

Values represent group means (SE in parentheses). *Denotes significant isolation stress effect. **Denotes significant attenuation of stress effect. $P<0.05$

and NPS00032 did not alter distress vocalizations. These data indicate that all four test articles in this screening session do not possess anxiolytic effects on this measure. A similar and significant isolation stress effect on vocalizations in vehicle-treated chicks was observed in the second screening session (see Table 1, bottom) [$t(29)=5.91$, $P<0.001$]. Fisher's LSD revealed that NPS00039 and NPS00033 produced a significant attenuation of distress vocalizations to approximately 50% of control ($P<0.01$). NPS00056 and NPS00035 did not affect this stress response. These data suggest that NPS00039 and NPS00033 may possess anxiolytic effects on this measure.

In addition to vocalizations, nociceptive measures were collected to index stress-induced analgesia. In the first screening session, vehicle-isolated chicks tended to have lower composite pain scores than vehicle-social chicks, a pattern consistent with stress-induced analgesia. However, large variability prevented detection of any statistically significant difference between these groups. All four of the test articles in this study tended to lower the nociceptive responses. These data indicate that NPS00058, NPS00038, NPS00034, and NPS00032 do not possess anxiolytic effects on this measure. In the second screening session, vehicle-isolated chicks had a significantly lower composite pain score than vehicle-social chicks, illustrating the social separation-stress effect [$t(29)=2.64$, $P<0.05$]. None of the four test articles significantly affected this measure. These data indicate that, at the doses tested, NPS00056, NPS00039, NPS00035, and NPS00033 do not possess anxiolytic effects on this stress measure.

Taken together, the results from these screening sessions were not conclusive. While NPS00058, NPS00038, NPS00034, NPS00032, NPS00056 and NPS00035 did not alter stress responses in the model, NPS00039 and NPS00033 attenuated distress vocalizations without affecting composite pain scores. These initial screens with limited dose evaluations prompted two follow-up dose-response studies to further investigate putative anxiolytic effects of NPS00039 and NPS00033 in the chick social-separation-stress procedure.

NPS00039 dose-response

NPS00039 did not affect ventral recumbent latency at any of the three doses tested in either isolated or social groups (see Table 2). These observations are consistent with our initial screenings and indicate that NPS00039 does not possess sedative properties within this dose range.

The results of NPS00039 on distress vocalizations are summarized in Fig. 1A. In vehicle control groups, isolated chicks (closed bar) vocalized significantly more than social chicks, illustrating the social separation-stress effect [$t(26)=5.56$, $P<0.05$]. In the isolated groups, NPS00039 produced a dose-dependent reduction in vocalizations, with the intermediate dose producing the largest effect. A two-way ANOVA revealed a significant main effects for dose [$F(3,49)=4.65$, $P<0.01$]. Fisher's LSD revealed that the 12.5 and 25 mg/kg doses produced a significant attenuation in distress vocalizations ($P<0.01$). NPS00039 did not alter vocalizations in the social groups; this is not unexpected, due to the floor effect of this measure in social-tested chicks under lower stress levels. This pattern of distress vocalizations is consistent with the notion that NPS00039 possesses anxiolytic effects on this stress measure. It is difficult to derive an accurate ED_{50} on this measure because of the limited number of doses tested in this trial. However, the pattern of response suggests an ED_{50} of approximately 10.0 mg/2 ml per kg.

The results of NPS00039 on composite pain scores are summarized in Fig. 1B. Vehicle-isolated chicks (closed bar) had significantly lower composite pain scores than vehicle-social chicks [$t(24)=-3.16$, $P<0.01$]. In both isolated and social groups, NPS00039 did not affect composite pain scores. The inability of NPS00039 to attenuate stress-induced analgesia is not an unexpected finding as we have previously observed this measure to be less sensitive than the vocalization measure to anxiolytic effects of BZ agonist manipulations (Watson et al. 1999). It is also possible that NPS00039 possesses both analgesic and anxiolytic properties. This profile of effects would show competing responses on the nociceptive measure: anxiolytic effects increase and analgesic effects decrease nociceptive responses.

Table 2 Ventral recumbency latency measures for samples NPS00039 and NPS00033

Sample	Dose							
	Vehicle		Low		Medium		High	
	Social	Isolated	Social	Isolated	Social	Isolated	Social	Isolated
NPS00039	167.07 (6.93)	174.80 (5.32)	151.21 (10.00)	166.62 (5.23)	177.36 (2.00)	171.53 (4.87)	168.71 (5.50)	180.00 (0.00)
NPS00033	172.67 (5.11)	174.67 (5.33)	170.25 (5.28)	176.53 (3.47)	166.20 (6.65)	169.73 (7.07)	169.87 (4.84)	173.31 (5.13)

Values represent group means (SE in parentheses)

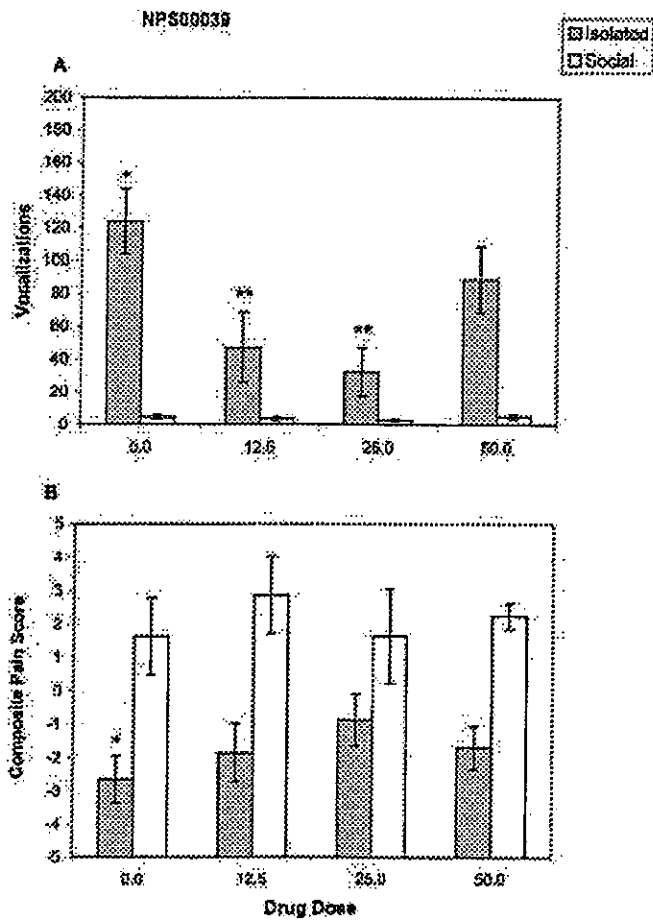


Fig. 1 Distress vocalizations (A) and composite pain scores (B) as a function of NPS00039 dose (mg/kg). *Indicates significant stress effect. **Indicates significant attenuation of the stress response. All $P < 0.05$

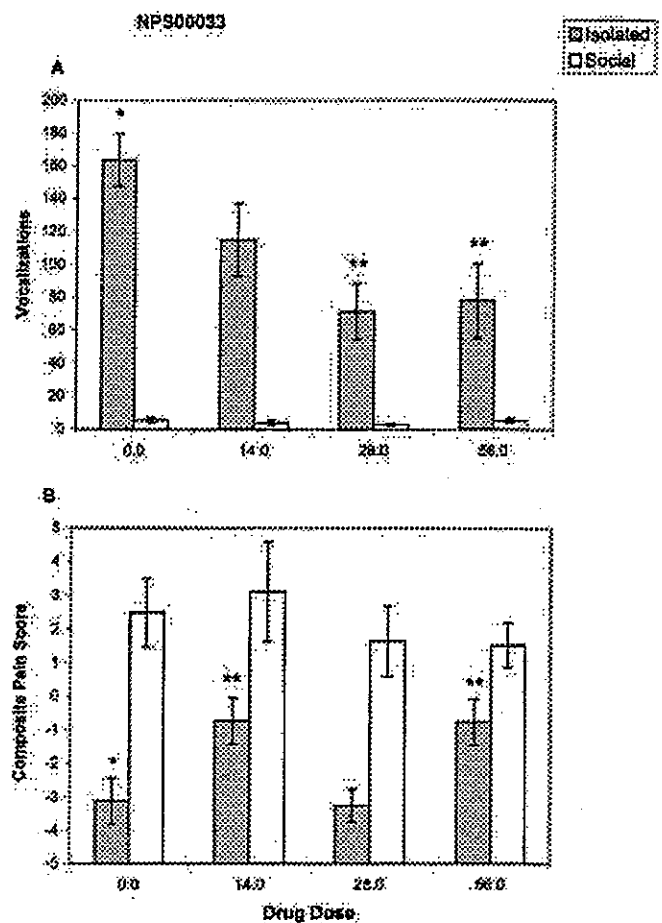


Fig. 2 Distress vocalizations (A) and composite pain scores (B) as a function of NPS00033 dose (mg/kg). *Indicates significant stress effect. **Indicates significant attenuation of the stress response. All $P < 0.05$

NPS00033 dose-response

NPS00033 did not affect the ventral recumbent latencies at any of the three doses tested in either the isolated or social chicks (see Table 2). These observations are consistent with findings from the initial screenings and indicate that NPS00033 does not possess sedative properties within this dose range.

The effects of NPS00033 on distress vocalizations are summarized in Fig. 2A. Vehicle-isolated chicks (closed bar) vocalized significantly more than vehicle-social chicks, illustrating the social separation-stress effect [$t(28)=9.86$, $P < 0.01$]. In the isolated groups, NPS00033 produced a significant dose-dependent reduction in vocalizations. A two-way ANOVA revealed a significant main effect for dose [$F(3,54)=5.45$, $P < 0.01$]. Fisher's LSD revealed that the 28.0 and 56.0 mg/kg doses significantly attenuated this separation-stress effect ($P < 0.01$). NPS00033 did not alter vocalizations in the social groups. These data are consistent with the notion that NPS00033 possesses anxiolytic

effects on this stress measure. Again, it is difficult to derive an accurate ED_{50} on this measure because of the limited number of doses tested in this trial. However, the pattern of response suggests an ED_{50} of approximately 12.0 mg/2 ml/kg.

The effects of NPS00033 on composite pain scores are summarized in Fig. 2B. Vehicle-isolated chicks (closed bar) had significantly lower composite pain scores than vehicle-social chicks, illustrating the social separation-stress effect [$t(26)=-4.58$, $P < 0.01$]. A two-way ANOVA revealed a significant effect for dose [$F(3,54)=4.35$, $P < 0.01$]. In the isolated groups, NPS00033 produced a significant attenuation of stress-induced at the 14.0 and 56.0 mg/kg doses ($P < 0.05$). NPS00033 did not affect the nociceptive responses in social chicks. These data demonstrate that NPS00033 possesses anxiolytic effects on this stress measure. Taken collectively, these results demonstrate that NPS00033 proved to test positive on both measures in the chick social separation-stress paradigm for anxiolytic effects.

Discussion

The purpose of the present research was to determine whether various botanical extracts derived from natural products possess anxiolytic properties. Extracts were screened using the chick social separation-stress procedure. This model utilizes vocalizations and nociceptive responses to index social separation-stress (Sufka and Weed 1994) and is sensitive to the anxiolytic properties of various benzodiazepine agonists (Watson and Sufka 1996; Watson et al. 1999). In addition, this model is cost effective in that animals are relatively inexpensive to purchase and maintain and that they require small quantities of drugs in the screening process. Moreover, the chick social separation-stress procedure measures species-typical responses rather than time- and labor-intensive conditioned responses. Thus, this chick procedure makes an ideal screening model in that it possesses not only construct and predictive validity but also high utility (Willner 1991).

The results from the initial screenings demonstrate the powerful effects of social separation-stress on chick behavior. Isolated chicks exhibited an increase in vocalizations and a decrease in nociceptive responses, the latter of which is indicative of stress-induced analgesia. Initial screenings indicated that two of the eight botanical extracts, NPS00039 and NPS00033, reduced isolation-induced distress vocalizations (see Table 1). These findings prompted a set of dose response screening sessions in order to examine more fully the putative anxiolytic effects of these two botanical extracts in the model.

The results from the dose-response screening trials for NPS00039 and NPS00033 demonstrate, as before, the powerful effects of social separation-stress on chick behavior. In both screening trials, isolated chicks exhibited an increase in distress vocalizations and a decrease in composite pain scores. NPS00039 and NPS00033 effectively attenuated separation-induced distress vocalizations (see Fig. 1A and Fig. 2A) without affecting ventral recumbent latencies (see Table 2). These data indicate that, at the doses tested, botanical extracts NPS00039 and NPS00033 possess anxiolytic but not sedative properties in the chick social separation-stress procedure.

Whereas both NPS00033 and NPS00039 attenuated distress vocalizations, only NPS00033 attenuated stress-induced analgesia (see Fig. 1B and Fig. 2B). This pattern of effects is consistent with that produced by various benzodiazepine anxiolytics in the model (Watson and Sufka 1996; Watson et al. 1999). First, separation-induced vocalizations seem much more sensitive to anxiolytic manipulations than nociceptive measures used to index stress-induced analgesia (Watson et al. 1999). Second, compounds that additionally possess analgesic properties can affect this nociceptive measure by masking a drug's anxiolytic effects. These factors may account for the absence of an effect of NPS00039 and the variable effects of NPS00033 in this paradigm.

Previous research in this chick model has determined ED₅₀ values for the benzodiazepine agonists chlordiazepoxide, lorazepam, and alprazolam in attenuating separation-induced DVoc. These values are 3.75 mg/kg for chlordiazepoxide (Watson 1996), 0.34 mg/kg for lorazepam, and 0.19 mg/kg for alprazolam (Watson et al. 1999). These ED₅₀ values are well within their respective daily recommended dose ranges for humans (Baldessarini 1996). Calculation of accurate ED₅₀ values in the present study is difficult due to the limited number of doses of NPS00039 and NPS00033 screened. However, the pattern of responses on the vocalization measure suggests approximate ED₅₀s of 10.0 mg/kg and 12.0 mg/kg for NPS00039 and NPS00033, respectively. Whether these novel botanical extracts have significant benzodiazepine agonist activity is currently being investigated.

The results of this study demonstrate that this chick model can be used to screen multi-component botanical extracts for anxiolytic activity. In addition, the model was able to differentiate test articles that were prepared from the same plant biomass but using different extraction processes. For example, NPS00033 was found to have anxiolytic activity and NPS00032 did not; these test articles were both derived from the Rutaceae plant family using different extraction techniques. A similar result was observed in test articles derived from the Magnoliaceae plant family: NPS00039 (*Retora*TM) was active and NPS00038 was not. Surprisingly, NPS00038, which did not have anxiolytic activity, seemed to enhance the stress response in this chick screening model.

In conclusion, the results of the chick model demonstrated that NPS00033 and NPS00039 have anxiolytic activity without sedation. These pharmacological characteristics are highly desirable and may play a role in modulating anxiety states.

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