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Preliminary Phytochemical and Pharmacological Studies on Plantlets of *Alternanthera brasiliana* Cultured Under Different Spectral Quality of Lights

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SUMMARY. Methanolic extracts from *Alternanthera brasiliana* plantlets cultured under different spectral quality of lights were analyzed by two chemical nociception models in mice. The results showed a highest biomass yield in plantlets developed under blue and white light treatments. The methanolic extracts (blue and white lights) at 10 mg/kg body weight caused inhibition of 51 and 62.5% against writhing test and 22 and 45.5% against capsaicin test, respectively. Phytochemical analysis indicated the absence of alkaloids or phenolic compounds, but suggested the presence of steroids or terpenes.

RESUMEN. "Estudio Fitoquímico y Farmacológico Preliminar de Plántulas de *Alternanthera brasiliana* Cultivadas bajo Diferentes Cualidades Espectrales de Luz". Extractos metanólicos de plántulas de *Alternanthera brasiliana,* cultivadas bajo diferentes cualidades espectrales de luz, fueron analizadas en dos modelos químicos de nocicepción en ratones. Los resultados mostraron mayor biomasa en las plántulas cultivadas con los tratamientos de luces azul y blanca. Extractos metanólicos (luces azules y blancas) administrados en concentraciones de 10 mg/kg del peso corporal, causaron inhibición de 51 y 62,5% para el test de contracciones y de 22 y 45,5% para el test de capsaicina, respectivamente. El análisis fitoquímico indicó la ausencia de alcaloides o compuestos fenólicos, pero sugiere la presencia de esteroides o terpenos.

INTRODUCTION

Alternanthera brasiliana is a Brazilian plant occurring in several regions, being known as "penicilina" or "terramicina", and widely used by rural communities as a medicinal agent to cure different diseases, such as inflammations and dolorous or infectious processes [1]. Some experimental investigations have confirmed that this plant exhibits important biological activities, including antiviral and anti-inflammatory properties [2-4]. Previous studies conducted in our laboratories have demonstrated that the hydroalcoholic extract from the whole plant exerts promising analgesic effects in mice [5]. Other study revealed that this plant is ineffective as antiinfective against pathogenic bacteria [6]. We have also shown that two extracts from callus cultures of this plant also produce analgesic compounds, specially steroids or terpenes [7].

The present study extend our previous investigations on *A. brasiliana* focusing the influence of different kind of lights to produce compounds with possible analgesic action. Thus, we have analyzed the phytochemical profile of the methanolic extracts as well as the analgesic activity in two chemical models of pain in mice, writhing and capsaicin tests.

MATERIALS AND METHODS

Plant material and tissue culture

Seeds of *A. brasiliana* obtained from greenhouse-grown plants were collected in March 1999, sterilized [7] and sown into sterile MS [8] medium supplemented with 30g.L^{-1} sucrose and vitamins [7]. After 50 days the nodal explants of the plantlets were subcultured in MS medium without growth regulators. Finally, nodal explants were cultured in MS medium and grown

KEY WORDS: *Alternathera brasiliana* plantlets, Analgesic Activity, Phytochemical Analysis.
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under different light conditions by using various commercially available lamps or in dark. To avoid interference's in light treatments in the same room, each environment was divided by black plastic foils. For all treatments a 16h-photoperiod was used and the cultures were maintained at 25 ± 1 °C.

The following lamp types were used: Sylvania blue, white (daylight), red and green, type F20-T12-Color (1.6W.m^{-2}). Fresh and dry weighs of plantlets were used to analyzed photomorphogenesis. The aerial segments of the plantlets after 60 days of culture were immediately weighed after harvest from the culture flasks to obtain the fresh weight. In order to evaluate the dry weight, the plant material was dehydrated in a oven (Fanen) at 40 °C for 72 h, until sample weights did not change.

Phytochemical analysis

In vitro plants cultured under different spectral quality of lights were air-dried, cut in small pieces and separately macerated with methanol at room temperature for five days. The extracts were lyophilized after solvent removal under reduced pressure. The extracts yields were: 13.6% (red light), 27.7% (green light), 25.1% (blue light), 33.7% (white light) and 37.8% (without light). Each extract was dissolved in methanol to obtain the desired concentration (100 mg/ml). An aliquot of these extracts was then used for phytochemical evaluation (2 µl each spot). The chromatographic profile of all extracts was examined by thin layer chromatography (TLC) using Merck silica pre-coated aluminum plates, 200 µm in thickness, with several solvent systems. Spots were visualized by general and specific reagents, according to previously described methodologies [9,10]. Sitosterol used as standard was previously isolated from this same species [7].

The specific spray reagents employed included anisaldehyde-sulfuric acid (terpenes and steroids), iron (III) chloride (phenolic compounds) and Dragendorff (alkaloids), while the general reagents were ultra-violet irradiation and sulfuric acid-methanol [9,10].

Animals

Swiss mice of both sexes (25-35 g) were housed in automatically controlled temperature conditions (23 ± 2 °C, 12 h light-dark cycles). The animals were given access to water and food freely, and remained in the UNIVALI Bioterium until some hours before the experiments.

Pharmacological analysis

Abdominal constriction response caused by intraperitoneal injection of diluted acetic acid (writhing test)

The abdominal constriction induced by intraperitoneal injection of acetic acid (0.6%), was carried out according to the procedures described previously [5,11] with minor modifications. Animals were pretreated with methanolic extracts (10 mg/kg) from *A. brasiliana* intraperitoneally 30 min before the acetic acid injection. Control animals received a similar volume of 0.9% NaCl (10 ml/kg, i.p.). After the challenge, each mouse was placed in a separate glass funnel and the number of contractions of the abdominal muscles together with stretching, was cumulatively counted over a period of 20 min. Antinociceptive activity was expressed as the reduction of the number of abdominal contractions between control animals and mice pretreated with methanolic extracts.

Capsaicin-induced pain

The procedure followed was similar to that described previously [12]. The animals were placed individually in transparent glass cylinders. Following the adaptation period, 20 µl of capsaicin (1.6 µg/paw) were injected under the skin of the right hindpaw plantar surface using a microsyringe. The animals were observed individually for 5 min following capsaicin injection. The amount of time spent licking the injected paw was timed with a chronometer being considered as indicative of nociception. Animals were treated with the methanolic extracts (10 mg/kg, i.p.) or saline solution (10 ml/kg, i.p.) 1 h before administration of capsaicin. Control animals received a similar volume of 0.9% NaCl (10 ml/kg, i.p.).

Statistical analysis

The results are presented as mean ± s.e.m. and statistical significance between groups was analyzed by means of the *t* test or analysis of variance followed by Dunnett's multiple comparison test, when appropriate. *p* values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Growth, tissue differentiation and development of plant body determine the location and accumulation of secondary metabolites in the intact plant, in plant cell and tissue cultures [13]. When isolated from their host plants and puri-

fied, some secondary products provide very high value compounds useful as flavor and fragrances, and pharmaceuticals. The culture of plant cells on a large scale has long been regarded as a potential source of secondary metabolites, especially for those products with inaccessibility problems of the plant source [14], low concentration in plants and expensive chemical synthesis [15]. Some metabolic pathways accompany morphogenic responses in plants treated with light. The degradation of lipid reserves and enhancement in the rate of cell expansion in sunflower cotyledons is a well-documented example [16]. Alkaloid biosynthesis in *Catharanthus roseus* are also affected by light [17]. Cultivation of Digitalis lanata under continuous dark reduces leaf size of the shoot-cultures and their metabolic status changes in terms of cardenolide production [18]. Our objectives were to evaluate an improved and cost effective protocol, without the use of phytohormones, manipulating only light conditions, for *in vitro* mass propagation of *A. brasiliana*, with an optimization of their pharmacological effects by comparison with previous works [5,7].

The results indicated that highest biomass accumulation, after 60 days, was observed in plantlets developed under blue and white light treatments. On the other hand, dark, green and red treatments reduced the accumulation of dry and fresh weight on the same extent (Fig. 1 A and B). The Tukey Honest Significant Difference Test (HSD) for comparison of the different treatments indicated no significant statistical difference between white and blue light treatments or between green, dark and red treatments (α = 0.05). These results are in agreement with other studies on *Amaranthus paniculatus* callus culture, a species of the same family (Amaranthaceae), showing that blue light promoted callus proliferation [19].

In order to detect the main components present in the extracts, they were analyzed by thin layer chromatography (TLC) using several solvents systems and different specific spray reagents [9,10]. Similarly to those of callus and crude hydroalcoholic extract from whole plant previously described [5,7], all the methanolic extracts showed the absence of alkaloids and phenolic compounds (tannins, flavonoids, etc.). On the other hand, the methanolic extracts presented strong positive reaction with anisaldehyde-sulfuric acid and Lieberman-Burchard spray reagents, indicating the presence of steroids or terpenes. The chromatographic profiles for these

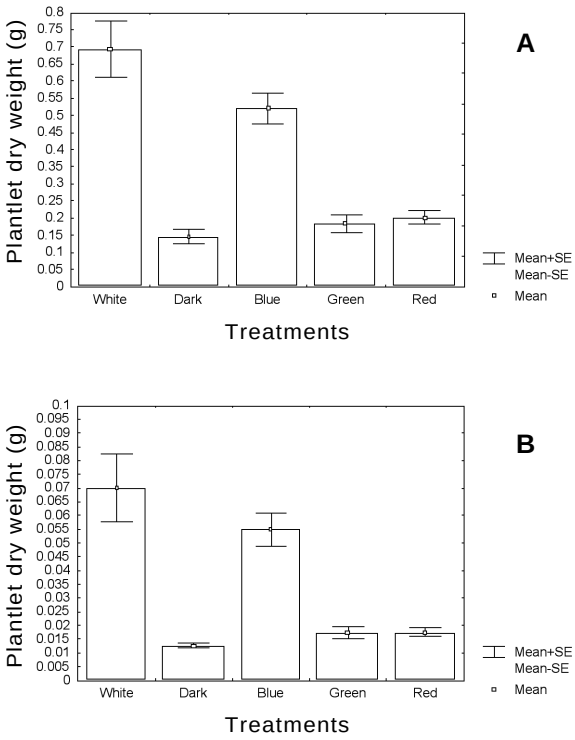

Figure 1. Fresh (**A**) and dry (**B**) plantlet weight accumulation after 60 days of "*in vitro*" culture under different light treatments. Results are for 10 replicates, repeated three times with mean + S.E. = standard error. Values of white and blue; dark, green and red treatments are significantly different between the two groups and similar into each one of the groups ($p < 0.05$) with Tukey test. ANOVA was performed on the results of each experiment, and after confirmation of the significance of the F-value, the data means were compared using the Tukey test.

compounds were different to those of callus and whole plant previously verified [7], showing different steroids or terpenes structures involved. For example, the well documented phytosterol, β-sitosterol, which was the main component of those extracts and partially responsible for the analgesic activity of several plants [20], was not found in this present study, suggesting that other components could be acting as analgesic in the extracts. Such compounds could eventually present structures related to brassinosteroids, since the steroids are their biosynthetic precursors [21,22], but more detailed phytochemical studies are required.

Two chemical models of pain were utilized because their wide used in evaluating the antinociceptive effects of synthetic and natural compounds [23]. Table I shows the analgesic activity of the extracts growing under different spectral light conditions. For comparison pur-

Treatment	Writhing Test Inhibition (%)	Capsaicin Test Inhibition (%)
Ext. MeOH (green light)	50.8 ± 0.6	38.0 ± 4.5
Ext. MeOH (red light)	85.0 ± 1.8	37.9 ± 3.7
Ext. MeOH (blue light)	51.0 ± 1.4	22.2 ± 6.4
Ext. MeOH (white light)	62.5 ± 2.6	45.5 ± 3.2
Ext. MeOH (without light)	26.0 ± 2.8	Inactive
HE [a]	68.8 ± 4.8	NT
Callus A [b]	66.6 ± 4.4	NT
Callus B [b]	50.4 ± 2.6	NT
Aspirin	38.0 ± 2.0	NT
Diclophenac	NT	43.0 ± 2.0

Table 1. Analgesic effect of methanolic extracts obtained from *A. brasiliana* growing under different lights and some reference drugs on acetic acid-induced abdominal constrictions and capsaicin test in mice (10 mg/kg, i.p.). Each group represents the mean ± s.e.m. of six experiments. Solvent used to dissolve the extracts did not caused any inhibition. [a] Data for comparison purposes from Souza *et al.*, [5] and [b] from Macedo *et al.* [7].

poses other extracts previously studied [5,7] or reference drugs were also included. When analyzed against writhing test at 10 mg/kg, given intraperitoneally, all the extracts (except that without light) significantly inhibited the abdominal constrictions, being more active than Aspirin, a well-known non-steroidal anti-inflammatory and analgesic drug, analyzed by the same experimental procedure. The most active extract was that one growing under red light, which caused 85.0% of inhibition, whereas the hydroalcoholic extract from the whole native plant and callus A or callus B caused 66.8, 66.6 and 50.4% of inhibition respectively. The analgesic effect of these extracts against the writhing test does not enable its mechanism of action to be elucidated, so further pharmacological studies are necessary.

When evaluated on capsaicin induced-pain at 10 mg/kg, the extracts growing under blue light and without light were practically inactive whereas those growing under red, green and white lights caused inhibitions at 37.9, 38.0 and 45.5% respectively. These results evidenced that some compounds could be acting on neurogenic pain. However, the mechanism by which the methanolic extracts of *A. brasiliana*, growing under different lights, produce analgesia in the models of pain studied remains unclear and requires further investigations.

CONCLUSIONS

Our results suggest that the production of plants of *A. brasiliana* by using different kind of lights, especially red light, may be an important strategy to obtain compounds with analgesic potential whose structures and mechanism of action should be studied more in detail.

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