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HPLC ANALYSIS FOR METHANOLIC EXTRACT OF *JUSTICIA ADHATODA* UNDER ELEVATED CO_2

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ABSTRACT

Justicia Adhatoda is a well-known medicinal plant in Ayurvedic and Unani medicine that was included in the family of *Justicia*. It has gained a great interest from the pharmaceutical industry; the alkaloids vasicine from its sap has been shown to be an effective treatment for suppressant, cough and bronchodilator. HPLC analytical methods for the screening of the major in dole alkaloids of *J. Adhatoda* and their iridoid precursors have been developed. A five month study was carried out in *J. adhatoda* to see the changes in different concentrations of CO_2 with respect to vasicine. UV detector and L-6200 Intel pumps were used for the study. The separation was achieved on C 18 column. The data of first month and the fifth month were compared. When compared to other treatment, control was higher in first month (0.1572 mg ml^{-1}) and it decreased at the end of the fifth month (0.1157 mg ml^{-1}). The vasicine was found to be decreased in the other chambers with 600 ppm (0.1351 mg ml^{-1}), 600 ppm+RH (0.1248 mg ml^{-1}) and 900 ppm (0. mg ml^{-1}) in first month. Whereas at the end of fifth month, it was found that vasicine was present only in control chamber.

KEYWORDS: *Justicia Adhatoda*, HPLC, elevated CO_2, vasicine.

INTRODUCTION

Increased atmospheric CO_2 concentration associated with increasing temperatures is predicted to have profound impacts on terrestrial ecosystem (Ward and Strain, 1999).The important area of research which has been largely neglected is the effect of elevated levels of

atmospheric CO_2 towards growth of medicinal plants which leads to changes in physiology, productivity and their variation in the production of secondary metabolites both in terms of quality and quantity.

The use of traditional medicine and medicinal plants in most developing countries, as a normative basis for the maintenance of good health, has been widely observed (UNESCO, 1996). Furthermore, an increasing reliance on the use of medicinal plants in the industrialised societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants as well as from traditionally used rural herbal remedies (UNESCO, 1998). Moreover, in these societies, herbal remedies have become more popular in the treatment of minor ailments, and also on account of the increasing costs of personal health maintenance. Indeed, the market and public demand has been so great that there is a great risk that many medicinal plants today, face either extinction or loss of genetic diversity. Medicinal plants are of great importance to the health ofindividuals and communities. The medicinal value ofthese plants lies in some chemical substances thatproduce a definite physiological action on the humanbody. The most important of these bioactive constituentsof plants are alkaloids, tannins, flavonoids, and phenoliccompounds (Hill, 1952). Many of these indigenousmedicinal plants are used as spices and food plants.They are also sometimes added to foods meant forpregnant and nursing mothers for medicinal purposes (Okwu, 1999 and 2001).

Phytochemicals are naturally occurring,biologically active chemical compounds in plants. In plants, phytochemicals act as a natural defense system for host plants and provide colour, aroma and flavour. More than 4000 of these compounds have been discovered to date and it is expected that scientists will discover many more. Any one serving of vegetables could provide as many as 100 different phytochemicals. Phytochemicals are protective and disease-preventing particularly for some forms of cancer and heart diseases. The most important action of these chemicals with respect to human beings is somewhat similar in that they function as antioxidants that react with the free oxygen molecules or free radicals in our bodies. Freeradicals can damage the cells of our bodies and must be removed. Phytochemicals are found in all plant products. It is advised that we consume a wide variety of fruits and vegetables in order to gain maximum benefit from the nutrients and phytochemicals they contain.

There is increasing evidence that the current changes in climate across the temperate regions will continue into the future and affect temperature, precipitation, and atmospheric concentration of CO_2. The atmospheric CO_2 concentration has constantly increased since the pre-industrial era from about 280 ppm to the present concentration of nearly 400 ppm, mainly due to human activities, including combustion of fossil fuels, landuse, and land-cover change, and is expected to double by the year 2100 (Alley *et al.*, 2007; Houghton *et al.*, 2001). This change is believed to be largely irreversible for several centuries even after CO_2 emissions stop (Solomon *et al.*, 2009).

Climate change and crop production are intimately connected. Atmospheric CO_2 concentration has increased by more than 28% since the beginning of the industrial revolution mainly of increased utilization of fossil fuels and deforestation (IPCC, 2001). The CO_2 in the atmosphere is projected to double from the current 370 ppm by the end of the current century (IPCC, 2001). Elevated atmospheric CO_2 generally enhances leaf and canopy photosynthesis, especially in C3 crops, because the present CO_2 is not sufficient to saturate Rubisco and because high CO_2 inhibits the competing process of photorespiration (Lawlor and Mitchell, 2000). Numerous studies have demonstrated that crop growth and yield are favoured by elevated CO_2 (Kimball, 1983; Amthor, 2001; Kimball *et al.*, 2002).

One important subject that has been largely neglected is the effect of elevated levels of atmospheric CO_2 on the growth of medicinal plants and their production of secondary metabolites of therapeutic value. Although several studies have investigated the effects of atmospheric CO_2 enrichment on the production of various carbon-based compounds (Penuelas and Estiarte, 1998), antioxidants (Badiani *et al.*, 1993, 1996, 1997; Rao *et al.*, 1995; Schwanz *et al.*, 1996), and some have evaluated the effects of elevated CO_2 on plant vitamin and mineral contents (Madsen, 1975; Knecht and O'Leary, 1983; Tajiri, 1985; Penuelas *et al.*, 1997), few have considered the consequences of atmospheric CO_2 enrichment for specific plant compounds of direct medicinal value, it is very important to conduct such a study on the malabar nut *Justicia Adhatoda* leaves of which contain bronchodilator alkaloids, mainly vasicine.

Justicia Adhatoda (Acanthaceae) commonly known as vasaka or malabar nut. The genus which is synonym to *Justicia*, has about 420 species. Itis a small, evergreen, sub-herbaceous plant widely spread throughout the tropical region of south-east Asia and it is indigenous to India.It is one of the highly reputed plant species, utilized in indigenous systems of medicines

in India for over 2000 years. The plant is valued for containing bronchodilator alkaloids, mainly vasicine. All parts of the plant are used in herbal medicine and particularly the leaves are credited with insecticidal and parasiticidal properties. The root is useful in strangury, leucorrhoea, bronchitis, asthma, bilious vomiting, sore eyes, fever and gonorrhoea. It is a valuable antiseptic, antiperiodic and anthelmintic.

MATERIALS AND METHODS

The experiment has been conducted at the open top chambers (OTCs) located in the research nursery at Institute of Forest Genetics and Tree Breeding, Coimbatore, India (11°00'59.4" N, 76°57'02"E and elevation of 437 M above MSL). The seeds are sown in the mother bed and transferred in to poly bags and kept under the chambers with various concentrations and compared with the ambient and control. The plants were grown inside the OTC whose diameter is 3 m and the height is 10 m with transparent PVC sheets (0.125 mm thickness) and the for the treatment, the CO_2 levels of 600 ppm, 600+RH ppm and 900 ppm. Pure CO_2 gas was used for the enrichment. The plants were maintained in outside OTCs under ambient condition. To nullifying the chamber effect plants are kept under the chamber without CO_2 supply called as control. The CO_2 was provided throughout the day and night (24 h period). This experiment was studied for 6 months. A software facility called Supervisory Control and Data Acquisition (SCADA) was used to continuously control record and display the actual and desired CO_2 level in each OTC by feedback control loop passing through Programmable Logical Controllers (PLC) (Buvaneswaran *et al.*, 2010). The set that was maintained in the open served as the control under ambient conditions while the set maintained inside the chamber under ambient CO_2 conditions was used to eliminate the effects of the chamber on the response of the plants.

Chromatographic Conditions

Hitachi L-4000 high performance liquid chromatography (HPLC) instrument equipped with UV detector L 6200 Intel pump, Octadecylsilane C-18 5 V size,250 x 4.6 mm (Supelco) column was used for studying the estimation of alkaloid vasicine.Mobile phase: Buffer: Acetonitrile: Tetrahydrofuran (92:5:3) Wave length: 280 nm. Flow rate: 1 ml min^{-1}. Inject volume: 20 µL.

Standard preparation

10 mg of vasicine were accurately weighed and dissolved in 50 ml of methanol, sonicated for 5-10 minutes, cooled and the volume was made up to 100 ml using methanol.

Sample preparation

150 mg of sample (equivalent to 10 mg of vasicine) was weighed and dissolved in 50 ml of methanol, sonicated for 5- 10 minutes, cooled and the volume was made up to 100 ml using methanol.

Procedure

The instrument was set as per the chromatographic condition as prescribed above.10 µl of the prepared standard was injected and the relative standard deviations was recorded in the chromatogram. The procedure was repeated four times and the mean area and relative standard deviations were calculated. 20 µl of sample was injected and the relative standard deviations were recorded in the chromatogram. The percentage of vasicine content was calculated from the peak area.

25 seedlings in each chamber I (600 ppm), chamber II (Control), chamber V (900 ppm), chamber VI (600 ppm+ RH), and ambient condition were kept for the elevated CO_2 study for five months continuously. First month sample were collected and processed in all the treatment and processed for HPLC analysis, in the same way, fifth month sample was also collected and estimated for the alkaloid compound vasicine.

RESULTS AND DISCUSSION

The study was conducted to see the alkaloid vasicine in all the five treatments at the first month and the fifth month. The study at the first month revealed that the presence of alkaloid vasicine was recorded less in 900 ppm (0.1321 mg ml^{-1}) and the same was found more in control condition (0.1572 mg ml^{-1}).But the vasicine was found to be present in 600 ppm (0.1351 mg ml^{-1}) and 600 ppm + rh (0.1248 mg ml^{-1}).

At the end of 5^{th} month, the vasicine content was found only in control conditions (0.1157 mg ml^{-1}) but surprisingly the vasicine content was totally absent in the other treatments. In ambient condition, the vasicine was absent in both the first and the fifth months.

The present study showed that the alkaloid was higher in control at first month followed by 600 ppm (0.1351 mg ml^{-1}) and 900 ppm (0.1321 mg ml^{-1}). The least was observed in 600 ppm+rh (0.1248 mg ml^{-1}). This similar result was proposed by Mosaleeyanon *et al.,* (2005) that the total plant biomass was fully 30 times greater in the high-light, high-CO_2 controlled-

environment treatment, while under the same conditions the concentrations of hypericin and pseudohypericin were 30 and 41 times greater.

Schonhof *et al.*, (2007) reported that roughly 65% increase in atmospheric CO_2 concentration increased the fresh weight of the broccoli heads by approximately 7%, while it increased the total glucosinolate concentration of the broccoli inflorescences by 14%, due primarily to identical 37% increases in two methylsulfinylalkylglucosinolates: glucoiberin and glucoraphanin.

Nautiyal and Purohit (2000) noticed that in case of aconites found 8 to 11- fold higher production of tubers in plants grown inside poly house (4-5^0 C higher temperature than open) compared to open field grown plants. They also concluded that percentage of alkaloids were also higher in plants grown in polyhouse.

The similar results proposed by Ziska *et al.*, (2005) that it was grown well watered and fertilized tobacco and Jimson weed plants from seed in controlled-environment chambers maintained at atmospheric CO_2 concentrations of either 378 ppm (ambient) or 690 ppm (elevated) and mean air temperatures of either 22.1 or 27.1°C for 50 and 47 days after planting for tobacco and Jimson weed, respectively, while sampling the plants at weekly intervals beginning at 28 days after planting for tobacco and 16 days for jimson weed, in order to determine the effects of these treatments on three plant alkaloids possessing important pharmacological properties: nicotine, in the case of tobacco, and atropine and scopolamine, in the case of jimson weed. And in following these protocols, they found that at the time of final harvest the elevated CO_2 had increased the aboveground biomass production of tobacco by approximately 89% at 22.1°C and 53% at 27.1°C, and to have increased that of jimson weed by approximately 23% and 14% at the same respective temperatures. The extra CO_2 was also found to have reduced the concentration of nicotine in tobacco, increased the concentration of scopolamine in jimson weed, but to have had no significant effect on the concentration of atropine in jimson weed.

Ziska, *et al.*, (2005) reported that the productionand concentration of atropine and scopolamine (strong anticholinergic chemicals that block the transmission of nerve impulses)was stimulated with both recent and projected carbon dioxide increases.

Mooney *et al*., 1991 stated that when the plants are stressed, secondary metabolite production may increase because growth is often inhibited more than photosynthesis, and the carbon fixed not allocated to growth is instead allocated to secondary metabolites

Zobayed *et al.*, (2005) subjected 70-day-old *H. perforatum*plants, grown under controlled environment to differenttemperature treatments of 15, 20, 25, 30 and 35°C before harvesting for 15 days. They observed that high temperature 35°C treatment increased the hypericin, pseudohypericin and hypericin concentrations in the shoot tissues.The total hypericin yield per plant (hypericin +pseudohypericin) was the highest in plants grown under25°C, then followed treatment at 30°C temperature. Thebest treatment for hyperforin content per plant was at 30°C.

Sinden and Sanford (1981) reported that temperature and light, other factors can influenceglycoalkaloid content in potato plants. Stage of development is onesuch factor. The level of solamarine was higher in young foliage than in mature or senescent foliage.

Table-1: The amount of vasicine present in *Justicia Adhatoda* from elevated CO_2 levels

	First month					Fifth month				
Sample	**Injectionvolume**	**Retention time**	**Area %**	**Height %**	**content of vasicine (mg)**	**Injectionvolume**	**Retention time**	**Area %**	**Height %**	**content of vasicine (mg)**
Standard vasicine	20ul	2.321	100	100						
Ambient	20ul	-	-	-	-	20ul	-	-	-	-
600 ppm	20ul	2.293	13.24	13.51	0.1351	20ul				
control	20ul	2.321	19.80	15.72	0.1572	20ul	2.326	7.71	11.57	0.1157
900 ppm	20ul	2.304	10.58	13.21	0.1321	20ul	-	-	-	-
600ppm+RH	20ul	2.31	10.25	12.48	0.1248	20ul	-	-	-	-

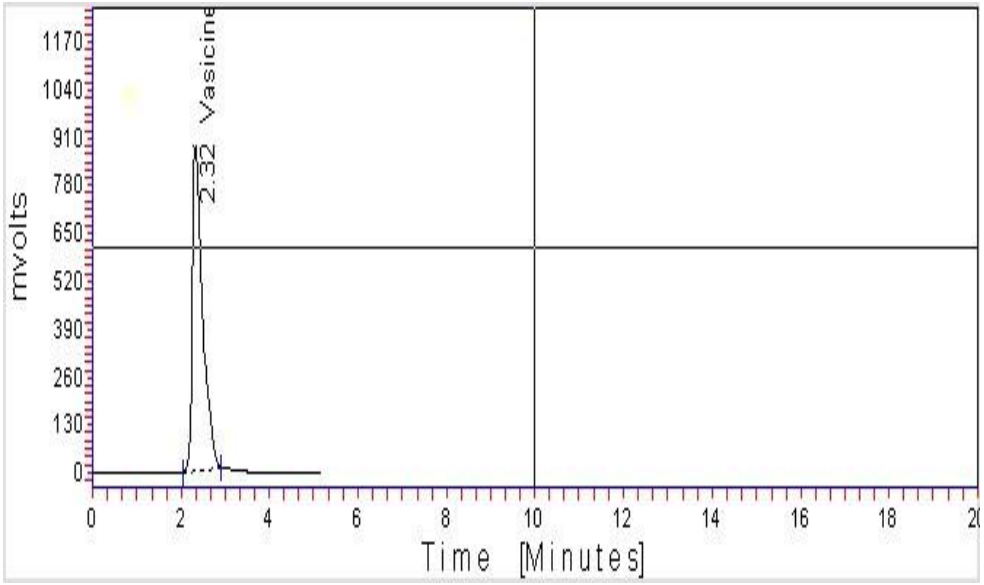

Fig. 1: HPLC Chromatogram of standard vasicine in methanol.

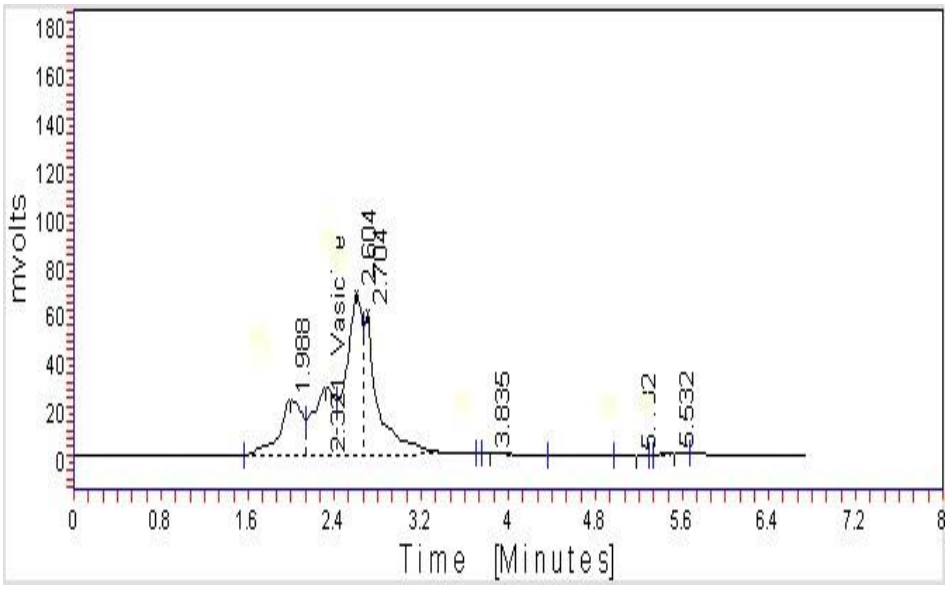

Fig. 2: HPLC Chromatogram of *Justicia Adhatoda* at first month on control of CO_2 concentration.

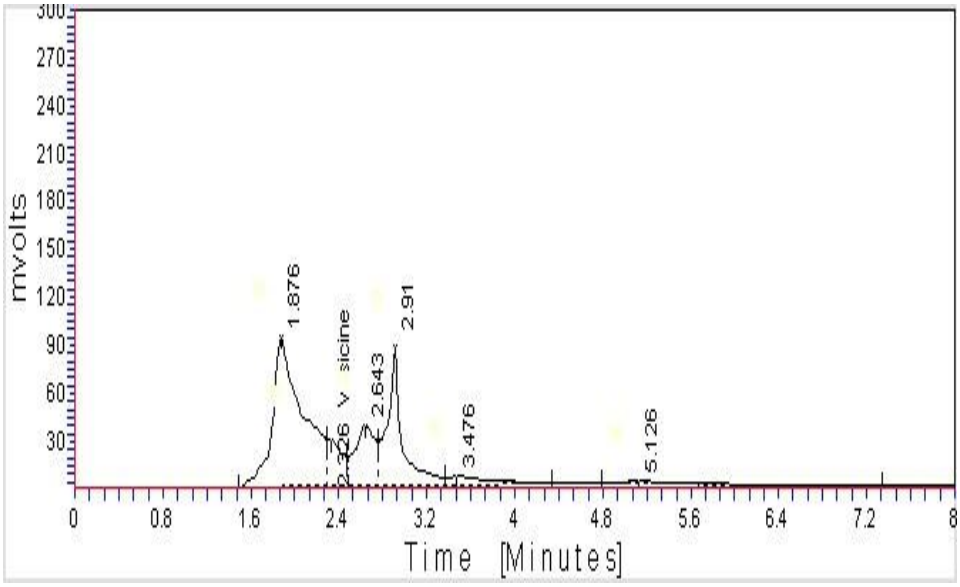

Fig. 3: HPLC Chromatogram of *Justicia Adhatoda* at fifth month on control of CO_2 concentration.

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