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Quantitative and Qualitative Analysis by HPLC of Major *Peganum harmala* Alkaloids at Different Stages of Development

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Abstract
The analysis of major *Peganum harmala* alkaloids (harmaline, harmine, harmol and harmalol) has shown that the content of these secondary metabolites depends on the plant development stage. The qualitative and quantitative profile of major alkaloids change significantly between stems, leaves and roots. The roots and seeds are the richest and most diversified organs in these metabolites. The concentration of the major alkaloids in all organs increases during the first three weeks and stabilizes at a constant level during vegetative growth phase and then decline at the beginning of flowering. On the scale of the whole plant, major alkaloids concentration of *P. harmala* varies with the developmental stage of the plant between 21.16 and 26.96 mg/g and harmine remains the most abundant molecule. Optimum exploitation of these natural products from the *P. harmala* plant is possible if the harvest of plant material occurs during the vegetative growth phase and at the appropriate stage for the richest organ.
Keywords: *Peganum harmala*, Plant growth, Alkaloids, Harmine, Harmaline, Harmol, Harmalol

1. Introduction

Peganum harmala L. is an herbaceous xerophytic known for its wide geographical distribution and its toxicity against several species of animals and microorganisms (Bellakhdar, 1997; Idrissi Hassani, 2000; Mahmudian, 2002). Al-Shamma report (Al-Shamma *et al.*, 1981) that this plant has an ancient reputation as an antiseptic as well as being used for treatment of dermatological conditions; besides the fact that the fume of seeds was used as a strong disinfectant. As for the seeds, they have got a central nervous system stimulant properties, anti-inflammatory agents, anthelmintics, analgesics, narcotics, emetics, bronchodilatory and antispasmodic (reported by Al Yahya, 1986). Sijilmassi (1996) described their emmenagogue effect, anthelmintic, antispasmodic, antiparkinsonian, antibacterial, especially hallucinogens. Injections of seed extracts in humans cause a nervous breakdown (Duke, 1985). The extracts of different organs exhibit antimicrobial activities (Ross *et al.* 1980; Adday *et al.* 1989; Prashanth *et al.*, 1999), inhibition of reproduction (Shapira, 1989, Nath *et al.* 1993; Adday, 1994) and insecticidal activities (Idrissi Hassani *et al.*, 2002). It has been shown in vivo that the extract of *Peganum harmala* L. shows cytotoxicity on cancer cell lines (Lamchouri *et al.*, 1999a) and an antimitotic effect (Lamchouri *et al.*, 1999b). The cancer activity has been shown also in rats (Mharzi and Zaid, 1997) and mouse (Lamchouri *et al.*, 1999a). Recent studies showed that *P. harmala* seed-based-products have a potential antinematodes effect against *Meloidogyne javanica* and can be used as component of an integrated management system to control this root knot nematode (Mayad et al., 2013). In most cases, these effects are due to alkaloids type β-carbolines (Figure 1). Ross *et al.* (1980) attributed the antibacterial activity to harmaline and harmalol while Al-Shamma *et al.* (1981), concludes that harmine is the most active among the other β-carboline alkaloids on the tested microorganisms. Ayoub *et al.* (1994) link the proliferation inhibition of tumor cell lines to the presence of harmalol. Other effects are attributed to vasicine (Duke 1985, Gupta *et al.*, 1978), harmaline and harmine (Sepulveda and Robinson, 1974; Queshi *et al.* 1975; Sokolove and Roth, 1978). Bais and his collaborators reported that harmaline and harmine are endowed with antimicrobial activity vis-à-vis terrestrial pathogenic microorganisms and that his two molecules are formed and released from the plant *Oxalis tuberosa* in the rhizosphere to meet the microbial attack (Bais *et al.*, 2003). As the toxicity of *P. harmala* has often been linked to its alkaloids and their derivatives, the determination of changes in the content and the chemical nature of these "active products" in the plant are valuable data to improve the effectiveness of substances based on *Peganum harmala* L. when biological activity is desired. According to Wieser (1955), biological activity depends on the stage of plant growth. This result is due to the high concentration of toxic compounds at a given stage (Fujii, 2000; Oka, 2010). In this context, we sought to achieve qualitative and quantitative monitoring of major alkaloids along the growing cycle of the plant.

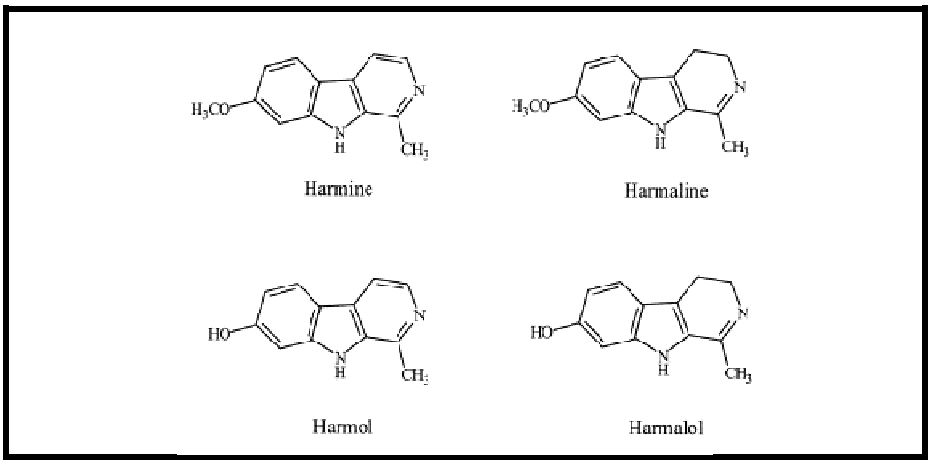

Figure 1: Major Alkaloids of *P. harmala*: harmaline, harmine, harmalol and harmol.

2. Materials and Methods

2.1 Preparation of plant material

Plants grown from seeds in black plastic bags of 2 liters of volume on a specific substrate (1 / 3 peat: 1 / 3 clay: 1 / 3 sand) according to Mayad et al., 2003 were harvested (at least 30 seedlings in homogeneous stage to minimize the effect of individual variation) at different stages of development (from 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 weeks post-germination until fruit stage) at the same hour of the day (4 p.m), dried for a week at 40 ° C, then the roots, stems and leaves have been sorted. The plant material is powdered and kept in the dark. The resulted powder is used to analyze the different desired compounds in plants.

2.2 Analysis of alkaloids majority by high performance liquid chromatography

Quantitative determination of major alkaloids was performed by high performance liquid chromatography, diode array at the national center of scientific and technical research (CNRST) in Rabat, according to the method of Sasse (1980). The mobile phase consisted of 0.5% formic acid, 17% water in methanol and buffered with triethylamine. The flow rate is 0.8ml/mn. The column support is silica bonded C18 ODS-type length 150mm, with a 4.6 mm in diameter and 3 microns as particle size. The standard curves were established for harmine, harmaline, the harmalol the harmol and harmane (SIGMA) in concentrations ranging from 1µg/ml and 3µg/ml.

2.3 Statistical Analysis

The results collected were presented as mean ± standard deviation after having been analyzed statistically by SPSS 11.5. To highlight the effect of developmental stage on the major alkaloids content in each organ, one way ANOVA and means comparison by Newman and Keuls test ($P \leq 5\%$) were performed using statistical software SPSS 14.0.

3. Results and discussion

Content of majors alkaloids, harmaline, harmine harmalol, harmol in *P. harmala* showed significant changes during its development cycle in the roots, stems and leaves (Figure 2, 3, 4). The root is the richest organ and most diversified molecules alkaloids while the stem is the organ that contains less.

3.1 Analysis of roots

In the root, the major alkaloids content varies between 2.96 and 26.44 µg/mg of dry weight. In general, these compounds appear very early and follow the same trend. Whatever the stage, harmine is the most abundant molecule while harmalol is the lowest abundant. For all the studied molecules, the concentration increases during the first weeks and stabilize along the growth cycle between 18.813 and 24.511 µg/mg of dry weight. For the most abundant molecules in the root, the amount varies between 2.225 and 17.570 µg/mg for harmine, and between 0.624 and 5.655 µg/mg for harmol. Towards the beginning of flower bud stage, the concentration of alkaloids decreases for a short period to regain its value at the last studied stage (capsule stage). The stage of maximum production in these majority alkaloids varies from one molecule to another, but in general, a high accumulation occurs during the vegetative growth phase of the plant between the fourteenth and eighteenth week post- germination for harmaline, harmol and harmalol (Figure 2).

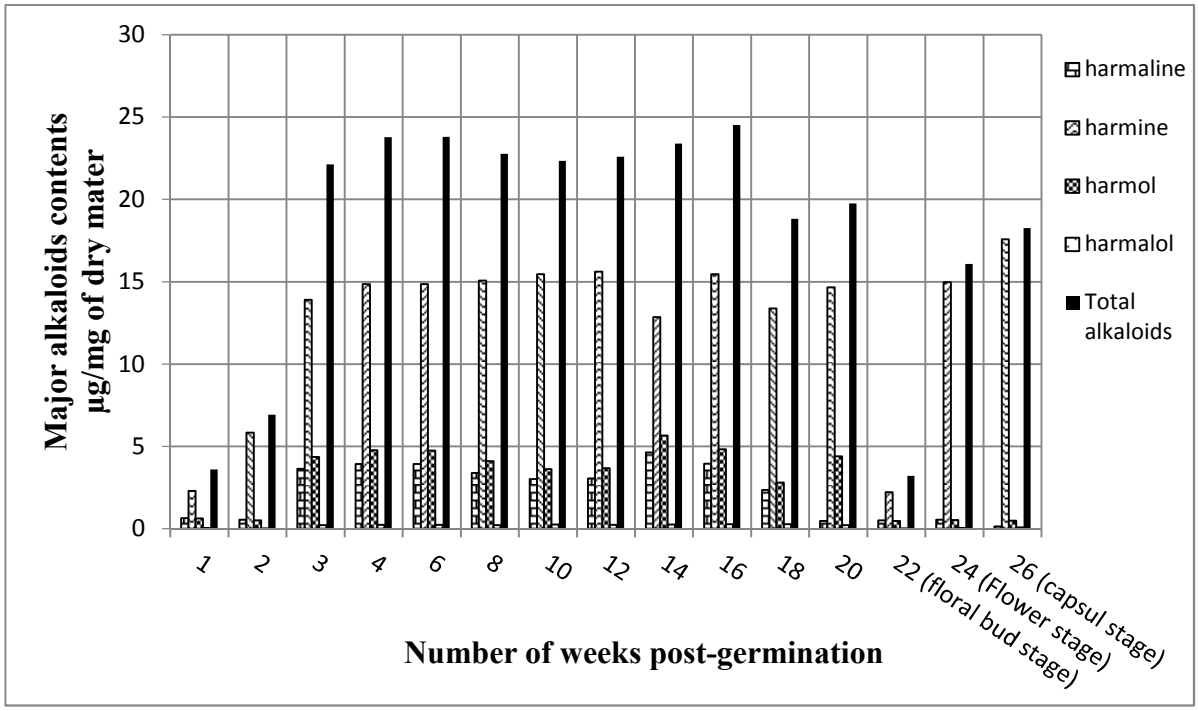

Figure 2: Majors alkaloids change in roots of *P. harmala* at different developmental stages.

3.2 Analysis of stems

In stems, major alkaloids are lower in quality and quantity compared to roots and their contents vary between 0.57 and 1.24 µg/mg. These compounds showed almost the same evolution. The harmalol seems to be absent and was detected only at some stages in small quantities (0.016 and 0.026 µg/mg). In the majority of stages, harmine and harmaline showed the highest levels and their changes during the vegetative growth phase are slightly similar. Their contents vary between 0.27 and 0.47 µg/mg for harmol; between 0.06 and 0.47µg/mg for harmaline and between 0.13 and 0.84 µg/mg for harmine. However, during the early phase of flower bud, there is a change in the opposite direction: the harmol and harmaline undergo a significant temporary decrease and then resumed its increases beyond the flowering stage while harmine decrease (Figure 3).

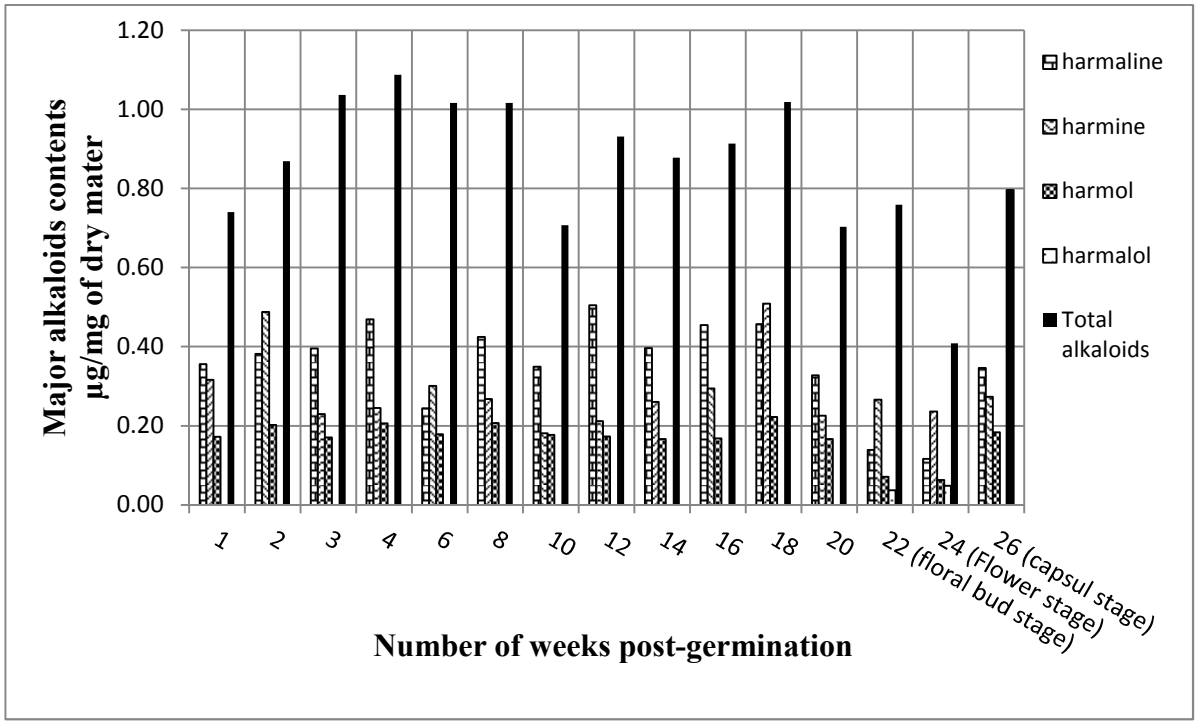

Figure 3: Majors alkaloids Change in stems of *P. harmala* at different developmental stages.

3.3 Analysis of leaves

The accumulation of the major alkaloids remains constant along the growth cycle in leaves of *P. harmala* from the cotyledon stage. The total concentration of these products in leaves depending on the stage is between 0.74 and 1.09 µg/mg for the vegetative growth phase. A remarkable drop was observed during the reproductive phase where the content of these metabolites reaches to 0.41 µg/mg. The harmalol is observed only toward the "bud" and "flower" stage at very low levels respectively at 0.04 and 0.05 µg/mg. The harmine, harmaline and harmol are generally accumulated in the same manner during the plant growth although the harmine and harmaline are slightly produced over the harmol in almost all stages (Figure 4).

3.4 Variation of major alkaloids in the whole plant

On the scale of the whole plant of *P. harmala*, the overall accumulated amount of major alkaloids studied increased during the first two weeks and remained stable along the growth cycle. This slight stabilization, which is between the third week and the previous one before the bud stage, occurs between 21.16 and 25.96 µg/mg. Towards the beginning of the floral development phase, a remarkable decrease occurs for the majority of studied molecules. The harmine, the most abundant in most periods of development accumulates between 2.75 and 18.20 µg/mg. The quantity of harmol and harmaline are relatively identical and take the same path across the cycle of development. The minimum and maximum content of harmaline are respectively of 0.78 and 5.42 µg/mg and harmalol are respectively of 0.25 and 0.27 µg/mg (Figure 5).

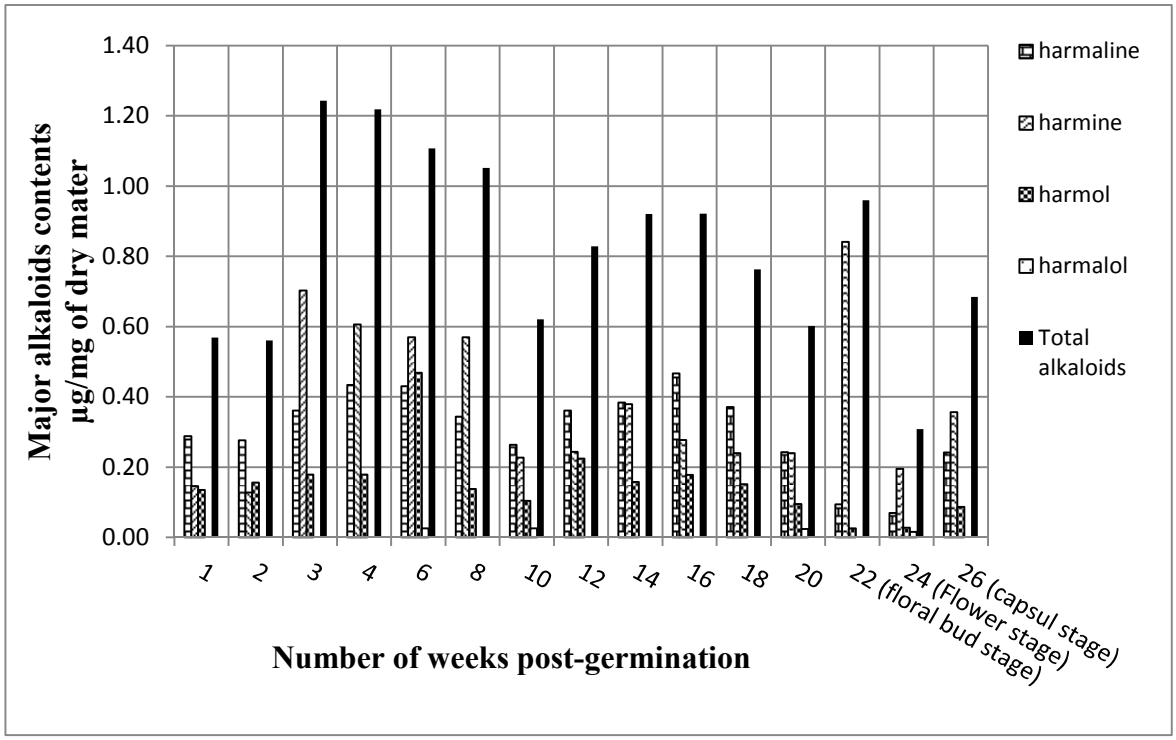

Figure 4: Majors alkaloids change in leaves of *P. harmala* at different developmental stages.

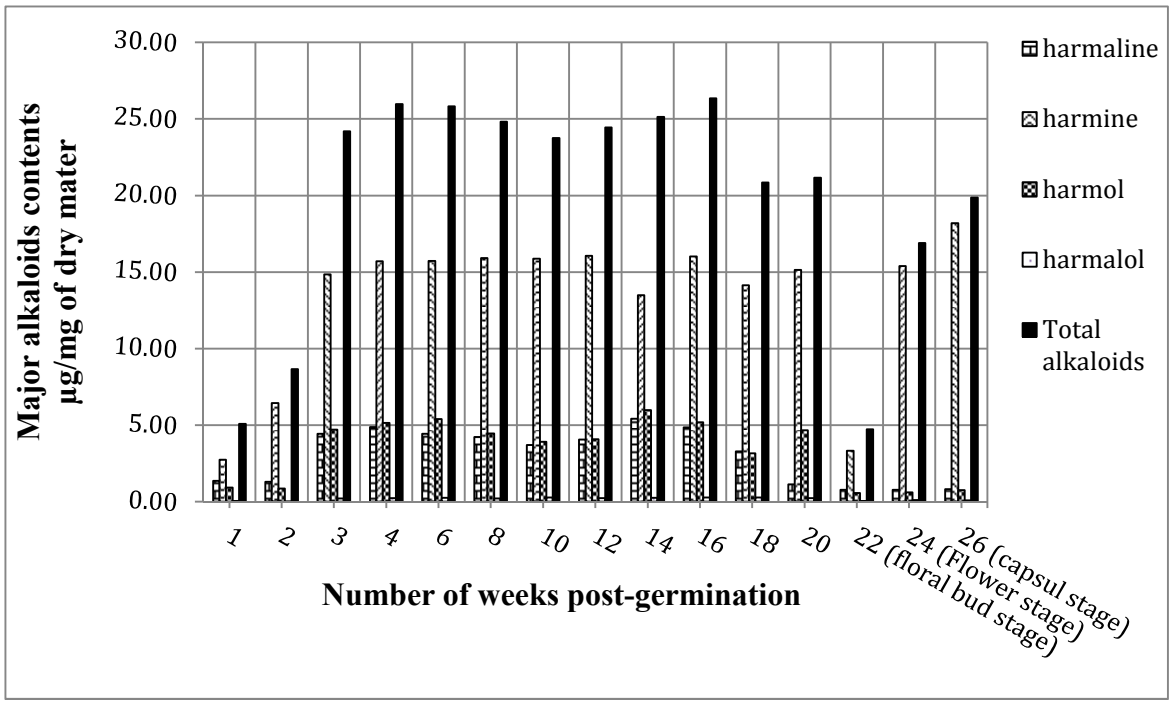

Figure 5: Majors alkaloids change in whole plants of *P. harmala* at different developmental stages.

5. Analysis of seeds in P. harmala

Among the four molecules of studied alkaloids, harmol was not detected in seeds. This could be due to the presence of this molecule in trace amounts below the existing limits of detection by the used method or it was reduced into harmalol. Anyway, the harmine is the most abundant (8.514 ± 0.521 mg/g dry weight) followed by harmalol (2.774 ± 0.164 mg/g). As for the concentration of harmaline, it is about 0.874 ± 0.016 mg/g. The total content of these major alkaloids in seeds is then approximately 1.22% (Table 1).

The alkaloids profile differs from one organ to another but generally remains constant in each organ in terms of quality along the different analysed stages. Quantitatively, the alkaloid content changes significantly with the stage of development. However, the root remains the richest organ in alkaloids qualitatively and quantitatively

surveyed, followed by leaves and then stems. The harmine and harmaline are the dominant compounds in the plant, they are present in all organs.

Table 1: Amount of major alkaloids harmaline, harmine harmol and harmalol in seeds of *P. harmala*

Alkaloids	Harmine	Harmaline	Harmol	Harmalol	Total of major alcaloïdes
Retention time (RT)	3.10	2.48	2.21	1.98	-
Seed content (mg/g)	8.514±0.521	0.874±0.016	-	2.774±0.164	12.162±0.637

While harmane is absent in all plants organs and prospected stages, it is in general, the high production of these major alkaloids by the whole plant occurs during the vegetative growth phase and varies between 20,85 and 25.96 µg/mg. The falling rate of alkaloids from the first week can be accounted for by the fact that molecules, brought by the seed, are metabolized and only from the second week does the renewal of the alkaloids stock occur when chlorophyll synthesis allows it. The last three stages that coincide with the ripening of fruit marke a rapid decline in alkaloid content in different organs. This decrease was due to a mobilization of alkaloids from root to fruit. Ben Salah *et al.* (1986) reported that alkaloid content rises sharply in summer, during the ripening phase of fruit at harvest of the seed and they concluded that the rate of alkaloids is much higher in the seed (3-4%) than in the root, stem (0.36%) and leaf (0.52%). Jado *et al.* (Jado *et al.*, 1979) reported that an ethanol extract of *Peganum harmala* L. leaves of Egyptian and Saudi lacks harmaline but contains harmalol with harmine. This is not the case in this study as our results are consistent with those obtained by Idrissi Hassani (Idrissi Hassani, 1998, 2000). Analysis of alkaloids from *P. harmala* in stems, dry leaves, green leaves, capsules, roots and seeds by HPLC have showed that the harmaline content in theses organs is respectively 0; 16; 18; 82; 85; 251µg/mg while the amount of this molecule was observed in the oil extracted from seeds of *P. harmala* varies between 2.314 µg/mg to 7.151 µg/mg of oil (Idrissi Hassani, 2000). However this controversy in the reported results by Jado *et al.* (1979) could be due to environmental or other factors related to the plant (varieties, cultivars ...). On the other hand, the absence of harmine in the leaves could be due to oxidation as the harmalol is only present in the root and seeds. The harmine and harmaline were reported to be as the most abundant molecules in the whole plant with a high concentration in roots and seeds. The same result was reported by Sasse *et al.* (Sasse *et al.*, 1980) who also states that the harmalol is less abundant. Sobhani and colleagues (Sobhani *et al.*, 2002) estimated that every hundred grams of seeds of *P. harmala* contain, respectively, 38.85 mg and 55.3 mg harmine and harmaline.

4. Conclusion

From these results, it appears that the *P. harmala* plants in the early stages of growth rapidly produce alkaloids in sufficient quantities and maintain a stable rate of their biosynthesis along the vegetative phase to ensure its protection. This chemical strategy could be the cause of repellency and anti-feedant vis-à-vis the herbivores. At the end of the development cycle, these molecules are stored in high concentrations in seeds and roots, since these are the components that provide the continuity of the plant during winter. In addition, while the roots contain harmaline and harmine in higher amount compared to their content found in stems and leaves reported in the literature, the seeds are organs that contain the high amount of harmaline and harmine at any stage of growth. Thus the seeds seem so, the best source for the extraction of indole alkaloids of *P. harmala*. Nevertheless, it is important to think in terms of productivity of dry biomass (kg/ha/year) for each organ to reach such a conclusion. A comparison of productivity for each member could help answer the question. Therefore, optimum exploitation of these molecules from the *P. harmala* plant could be possible if the collection of the plant material occurs during the vegetative growth phase and at the appropriate stage for the richest organs in alkaloids and having the higher biomass productivity.

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