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Assessment of the Contribution of Foliar Trichomes towards Allelopathy

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Abstract: Plant trichomes vary in their structure and cellular composition. Glandular trichomes contain a bulk of specific (secondary) metabolites of diverse nature. Trichomes are connected with various adaptive processes, which include protection against herbivores and pathogens as well. Our study investigates the allelopathic contribution of structures present on the leaf surface of *Nicotiana plumbaginifolia* Viv. against seedling growth of *Cicer arietinum* L. The infusion obtained after dipping *Nicotiana* leaves in Dichloromethane (DCM) for 10 seconds (s) was the most phytotoxic among all the infusions. The observed inhibition in *Cicer* growth was not only dependent on type of infusion but also the concentration. Scanning electron microscopy of the leaf samples showed the modifications in trichomes under the influence of the different concentration of DCM. Glandular trichomes were most dehydrated at 10 seconds in DCM, suggesting their role for the observed allelopathy. Such study on the biochemistry of trichomes and their phytotoxicity may develop highly valuable objects for plant metabolic engineering.

Keywords: Allelopathy; phytotoxicity; secondary metabolites; medicinal plants

1 Introduction

The secondary metabolites of plants are employed in several areas including pharmaceuticals, nutraceuticals, flavouring industry, perfumery, pest control and non-food or fiber sectors [1]. The production and storage of these compounds are mostly restricted to the plant surfaces [2]. However, they may also concentrate to take the shape of specialized cells namely glandular trichomes to deliver their maximum effect and improve their interactions with the external world. These cells protect plants from auto toxicity and possess several functions such as secretion. For secretion, they may take up different forms such as trichomes [3], stinging hairs, glandular hairs, epidermis [4] or glands on the outer surfaces of many plants. Secondary metabolites (allelochemicals) may either store in glandular trichomes reservoir or volatilize from leaf surfaces [5].

Secondary metabolites inhibit the seed germination of other plants and alleviate the competition between plants possessing metabolites in trichomes [6]. The allelochemicals stored in the trichomes of *Parthenium*

hysterophorus L. inhibited the early growth of *Triticum aestivum* L. seedlings [7]. Growth reductiion observed in seedlings of *Lactuca sativa* cultivar Nigra, *Hordeum vulgare* L., tomato *Lycopersicon esculentum* Mill. and *Triticum aestivum* L. on exposure to leaf extract of *Helianthus annuus* L. were analyzed for its source and the responsible allelochemicals were found to be sesquiterpene lactone present in the later [8].

Solanaceae trichomes are likewise known to synthesize various terpenes [9]. In tobacco, cembratrieneols and cembratrienediols (diterpenes), which have the reputation of providing defense, are secreted in the trichomes [10]. Diterpene synthase primarily synthesizes cembratrieneols from the precursor geranylgeranyl diphosphate (GGPP) which is later oxidized by another enzyme, cytochrome P450 into cembratrienediols [10]. The genes synthesizing these two proteins show their expression in the trichomes [11] or explicitly at its tip [12]. Probably, the whole terpene synthesis is dynamic. Likewise, linalool, a monoterpene is produced in the trichomes of *Lycopersicum* species by exposing the plant to methyl jasmonate [13,14].

Some recent studies have reported the allelopathic potential of *Nicotiana plumbaginifolia* Viv. [2,15,16] and chemical profile of its foliar part [16]. These allelopathic studies [2,15–17] found the beneficial or neutral effect at low doses and toxic effects at higher doses, such a condition is called hormesis. However, the role of trichomes towards the observed effect is still not clear. This investigation was therefore aimed at elucidating the potential of glandular structures of *N. plumbaginifolia* towards allelopathy, thereby contributing to available information on the (a) foliar structures of *Nicotiana*; to this end, imaging techniques such as scanning electron microscopy (SEM) was used and (b) influence of phenolics present on the *N. plumbaginifolia* leaf on the growth of *Cicer arietinum* L. seedlings.

2 Materials and Methods

2.1 Leaf-Dipping Experiments

The study for evaluating the impact of the allelochemicals absolutely found on the *N. plumbaginifolia* leaf on growth behaviour of *C. arietinum* was performed by means of different concentrations of an organic solvent, dichloromethane (DCM). In total, 05 plants were used. 04 plants were used in bioassays 1-4 separately and 04 mature leaves from each plant weighing around 04 grams in total were used, and 5th plant was used as control to study trichomes only. The leaves were washed 2-3 times with distilled water (DW) before exposing them to bioassays and/or fixing them for Scanning Electron Microscopy. The leaf samples were exposed to different concentrations of DCM for varying time durations as follows:

2.1.1 Bioassay 1: Leaf Dipping for Five Seconds

Four freshly collected leaves of *N. plumbaginifolia* at the flowering stage (weighing 4 g) were rinsed with DCM to extract the material and trichomes from the leaf surface [18,19]. The leaves were successively immersed in 100 ml DCM for 5 seconds to obtain a 100% concentrated solution. A portion of this solution (100%) was diluted to 25%, 50% and 75% concentration solutions. Pure DCM was used to serve as the control treatment. The whole assessment was carried in Petri dishes (15 cm diameter), covered with a thin layer of absorbent cotton at the bottom which was moistened with 5 ml of the corresponding test solution [20]. The DCM was let to evaporate in a laminar; the Petri dishes were thereafter provided with 10 ml of DW and 10 seeds of *C. arietinum* were placed in them. The seeds of *C. arietinum* were obtained from Indian Agricultural Research Institute; New Delhi, India Subsequent to fixing the Petri dishes with parafilm, the whole bioassay was setup in the growth incubator with temperature ± 26°C for eight days. The root/shoot length of *Cicer* saplings was resolved after eight days using a metric scale. Five replicates were maintained for each treatment solution.

2.1.2 Bioassay 2: Leaf Dipping for Ten Seconds

Four freshly collected *N. plumbaginifolia* leaves (total weight 4 g) were plungied in 100 ml DCM for 10 seconds to formulate a 4 g/100 ml concentrated solution (100%). The other concentrations (25%, 50% and

75%) were prepared through its dilution using DW. Pure DCM was used as control. The study was performed in glass Petri dishes (same dimensions as used in bioassay 1) lined with absorbent cotton (similar to bioassay 1) and moistened with 5 ml of the corresponding treatment solution [20]. After letting the DCM to evaporate, the Petri dishes were watered with 10 ml of DW and 10 seeds of *C. arietinum* were placed in each Petri plate. The whole arrangement was put in the growth incubator at ± 26°C for eight days. The root/shoot length of saplings was measured after eight days as discussed in bioassay 1.

2.1.3 Bioassay 3: Ten Second Dipping in DCM Followed by 24 Hours in DW

Four fresh leaves of *N. plumbaginifolia* (4 g) collected at the mature stage were dipped in DCM for 10 seconds and successive sopping in 100 ml DW for 24 hours. The *Nicotiana* leaf samples were thereafter taken for SEM analysis and the two solutions were mixed together in 1:1 ratio. The mixture was applied to Petri dishes as in the above bioassays.

2.1.4 Bioassay 4: Leaf Soaking in DW for 24 Hours

This bioassay is the standard/control. Four intact *N. plumbaginifolia* leaves (4 g) were soaked in 100 ml DW (100% concentration), agitated, covered and kept in a dark place for the duration of 24 hours. Thereafter the other solutions (25%, 50% and 75%) were prepared by dilution of the 100% concentration extract. 5 ml of respective concentration and ten *Cicer* seeds were added onto the Absorbent cotton. Five replicates were prepared for each concentration. The whole setup was maintained in a growth chamber and seedling measurements were done as discussed earlier.

2.2 Scanning Electron Microscopy Assessment

The different magnifications of the dorsal and ventral surfaces of *N. plumbaginifolia leaf* were seen by utilizing JEOL (JSM-6510LV) SEM at 10 keV acceleration voltage. *N. plumbaginifolia* was collected at its reproductive stage from the university outskirts of Aligarh Muslim University, Aligarh (India). The plant was identified by a specialist (plant taxonomist). The approach adopted by Mushtaq et al. was adopted [15]. The leaves were rinsed with DW 2-3 times and then fixed with 0.05 M sodium cacodylate (pH 7.5). The leaf samples were dehydrated with an ethanol series (10, 30, 50, 70, and 80%) 3 times at 5 minutes/washing. This was trailed by critical point drying with liquid carbon dioxide in Hitachi HCP-2 Critical Point Dryer. The dehydrated samples were then mounted on aluminium specimen stubs with adhesive discs coated with double-sided carbon and sputter-coated with gold-palladium (Eiko IB-3 ion coater). The JEOL (JSM-6510LV) SEM was driven at 10 keV and Microsoft Image software for Windows was employed to capture the distinguishing inspected structures.

SEM was accomplished in concurrence with every bioassay. Accordingly, SEM analysis was accomplished on leaf samples of *Nicotiana* that were:

(a) Dipped in DCM for

1. 05 seconds
2. 10 seconds
3. 10 seconds followed by soaking in DW for 24 hours

(b) Dipped in DW for 24 hours and

(c) Fresh leaves to serve as the control.

The selected leaf samples were roughly cut into 3 × 5 mm from the center of the laminas. The exposed leaf surfaces of the selected sections were gold coated with SEM Autoclaving unit E5200. Colloidal carbon (used for conductive purposes and as glue) was mounted on the leaf boundaries over gold coating. The photographs were taken through a JEOL (JSM-6510LV) SEM.

2.3 Statistical Analysis

The whole study was arranged in a Completely Randomized Block Design. The seedling length was measured with respect to control and analyzed by Duncan Multiple Range Test (DMRT) at, $P < 0.05$ [21].

3 Results

On both the dorsal and ventral leaf surfaces [22], long-glandular trichomes and stomata were observed (Fig. 1).

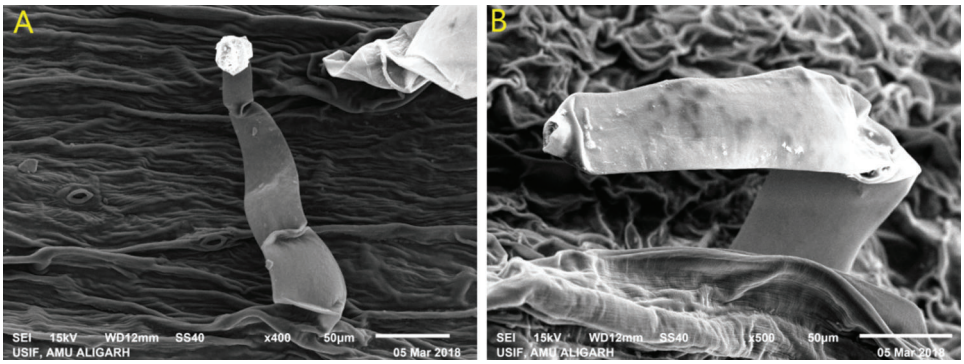

Figure 1: Ultramorphology of control trichomes (A) Well developed trichome and their relative origin to stomata on the leaf surface (B) Enlarged view of trichome

3.1 Bioassay 1

Introduction of the leaf to DCM for 5 seconds in order to indicate contrasts in morphology and ultrastructure of trichome, leaf epidermis and cuticle on the adaxial and abaxial side is shown by SEM in Fig. 2.

The trichomes on the upper surface appear to be more flattened with no alteration in the epidermal structure. The trichomes on the lower surface appear to be unaltered as well. However, the epidermal cells of lower surface experience distortion bringing about a rusty appearance. However, this bioassay did not demonstrate hormesis. The radicle length showed more affectability than plumule length (Fig. 3).

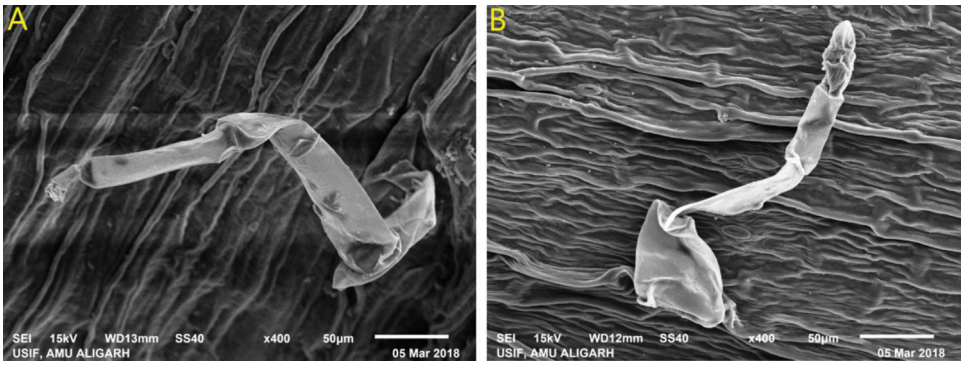

Figure 2: Ultramorphology of trichomes in Bioassay 1 (A) Upper, (B) lower surfaces of *N. plumbaginifolia* leaf

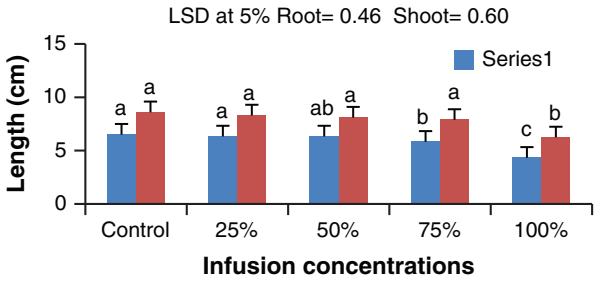

Figure 3: Root length and shoot length of *Cicer* seedlings exposed to different DCM solutions of Bioassay 1. Series 1: blue represents root; Series 2: red represents shoot. Means in each series followed by the same letters are statistically insignificant applying DMRT and bars over columns represent standard deviation

3.2 Bioassay 2

Albeit no significant inclination as far as seedling development restraint or hormesis is concerned (Fig. 4).

However, SEM presents (Fig. 5) that the majority of the trichomes on lower leaf surfaces on exposure to DCM for ten seconds get flattened with just a couple in the bloated condition. The flabby trichomes appear as a wispy structure. The state of the stomata additionally mutilated on abaxial surfaces with no articulated

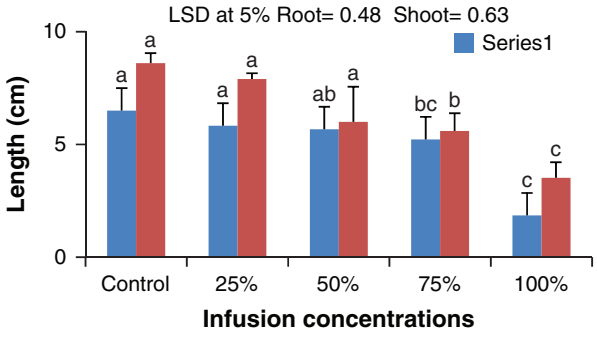

Figure 4: Root length and shoot length of *Cicer* seedlings exposed to different DCM solutions of Bioassay 2. Series 1: blue represents root; Series 2: red represents shoot. Means in each series followed by the same letters are statistically insignificant applying DMRT and bars over columns represent standard deviation

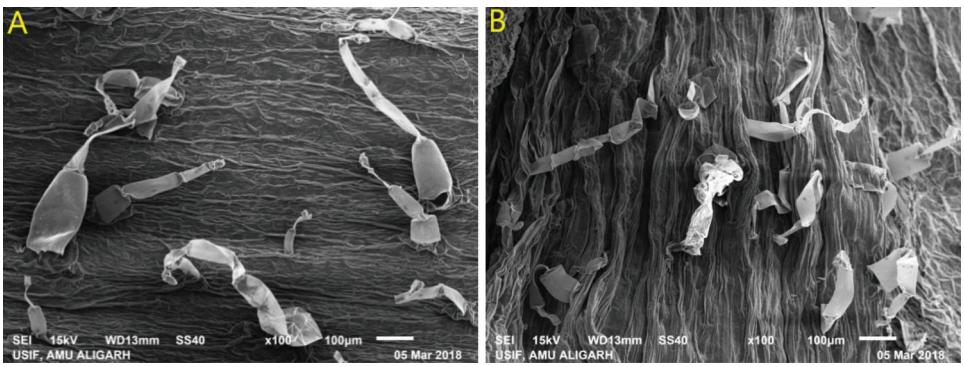

Figure 5: Ultramorphology of trichomes in Bioassay 2 (A) Upper, (B) lower surfaces of *N. plumbaginifolia* leaf

alteration on the leaf surface. On the adaxial side, the trichomes appear to be flabby as well. Nevertheless, no major modification was identified on the leaf surface and in stomata.

3.3 Bioassay 3

Fig. 6 shows that *C. arietinum* shoots demonstrated a characteristic hormesis reaction to the extract. The roots though demonstrated a linear reduction in growth along the concentration of the extract.

The impact of plunging the *N. plumbaginifolia* leaf in DCM for ten seconds and afterward drenching it for 24 hours in DW is shown in Fig. 7. The trichomes appear to be drooping discharging the substance alongside the modification in the epidermis and stomata shape. The epidermal cells contracted. Other than that, the trichomes look ruptured (Fig. 7A). All trichomes appear shrunk alongside the closure of stomata.

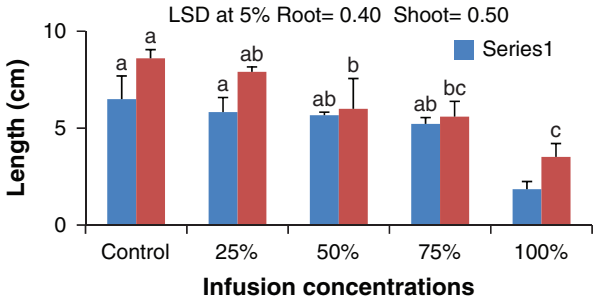

Figure 6: Root length and shoot length of Cicer seedlings exposed to different DCM solutions of Bioassay 3. Series 1: blue represents root; Series 2: red represents shoot. Means in each series followed by the same letters are statistically insignificant applying DMRT and bars over columns represent standard deviation

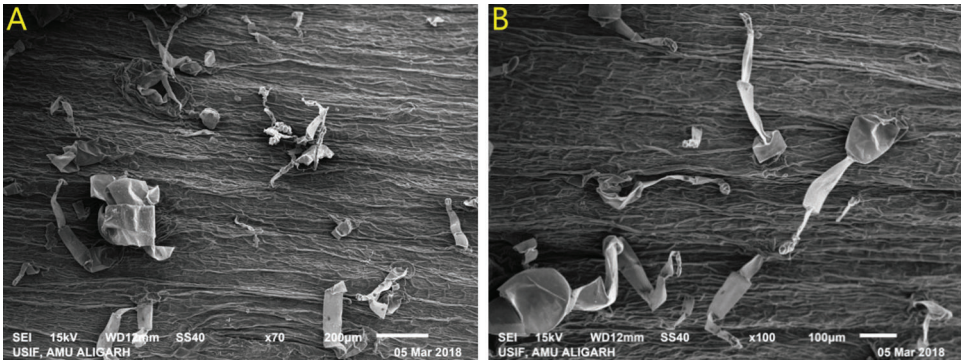

Figure 7: Ultramorphology of trichomes in Bioassay 3 (A) Upper, (B) lower surfaces of *N. plumbaginifolia* leaf

3.4 Bioassay 4

In this bioassay (Fig. 8), an incitement was observed in seedling growth at lower concentrations in contrast with the inhibition (hormesis) at higher concentrations. The impact on radicle length was more prominent than on plumule length, accordingly proposing that roots were more subtle to *N. plumbaginifolia* phytochemicals.

Fig. 9 shows more noteworthy variations in the morphology of trichome by dousing the Nicotiana leaf in DW. The trichome appears to be completely flattened on the epidermis.

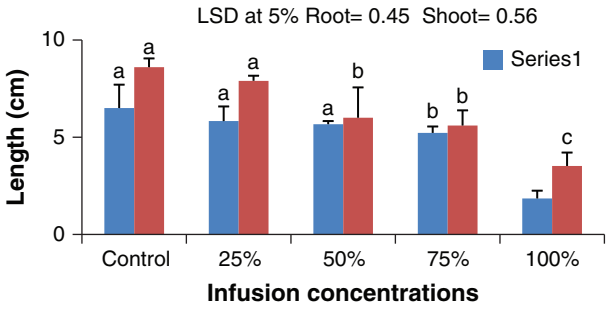

Figure 8: Root length and shoot length of *Cicer* seedlings exposed to different infusion concentrations of Bioassay 4. Series 1: blue represents root; Series 2: red represents shoot. Means in each series followed by the same letters are statistically insignificant applying DMRT and bars over columns represent standard deviation

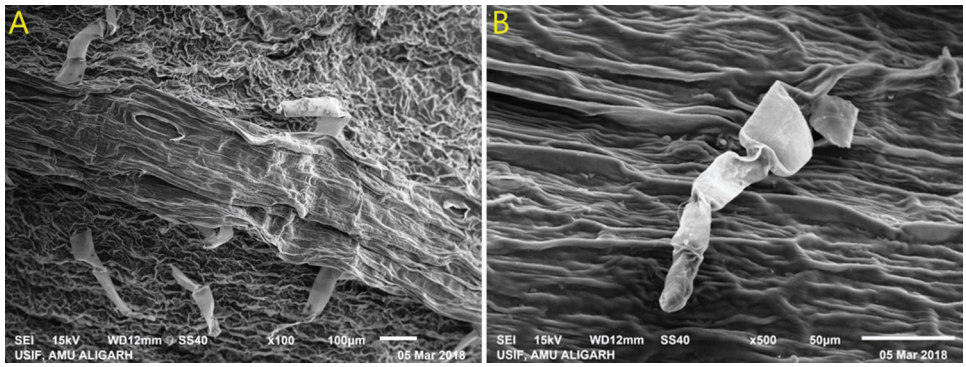

Figure 9: Ultramorphology of trichomes in Bioassay 4 (A) Upper, (B) lower surfaces of *N. plumbaginifolia* leaf

Fig. 1 demonstrates the SEM photographs of leaf slices at control. The trichomes appear complete with positively no harm showing up on the leaf surface, despite the detachment of an upper portion of trichome from the structure may contribute the phytochemicals to seedling development limitation at the site of rupture. There was not any modification of the leaf surface upon dipping of leaf in DW for 24 hours.

4 Discussion

When it was resolved that leaves held the most grounded allelopathic nature than other plant parts in past investigations, the *Nicotiana* leaf surface was analyzed through Scanning Electron Microscopy (SEM). Long glandular trichomes were seen in *N. plumbaginifolia* [23]. Such trichomes are recognized as 2-celled structure with uniseriate stalk and unicellular head [23]. SEM was executed to analyze if allelopathic chemicals arise and/or are deposited on the leaf superficially. Lovett [24] announced that some secondary metabolites discharged from trichomes are involved in allelopathy; the stimulatory or inhibitory impacts of the chemical compounds produced by one plant may apply to another [25]. For instance, the exudates collected from leaves of *Abutilon theophrasti* Medic. suppressed significantly the growth and development of *Lepidium sativum* L. [26]. The leaves of *Compuloclinium macrocephalum* (Less) D.C. have also been reported to possess glandular trichomes that release water-soluble allelochemicals which reduce the growth of *Lactuca sativa* [27]. Optimistic outcomes of our study demonstrated that trichomes are conceivable springs of phenolic chemicals on both dorsal and ventral surfaces of *N. plumbaginifolia* mature leaves, which is in line with the previous investigations [18,28,29].

Glandular trichomes discharge allelochemicals that display the phytotoxic intrusion. For instance, allelochemical 1,8-cineole associated with plant versus plant allelopathy [29] is present in the trichomes of *Artemisia annua* [30]. Similarly, other secondary metabolites, for example, terpenes confined to the glandular trichomes of *Salvia* species [26,31] suppress their target species [32]. These compounds may stretch out to their rivals through volatilization from ground litter. In like manner, artemisinin present in the glandular trichomes of *Artemisia annua* [33] has been accounted for to be phytotoxic [34,35] against other plants in the field, nonetheless even the dried leaves when mixed in the soil inhibit the growth pattern of plants growing in proximity. In addition, trichomes and pollen hold many secondary metabolites which they may release at different developmental stages (seedling to reproductive), under the discourse of various environmental factors, in many possible combinations of methods by different groups of plants [36]. The flavonoid allelochemicals showed a rich density in young leaves of *Paulownia tomentosa* (Thunb.) Steud as compared to mature leaves [37]. The chemicals in trichomes display phytotoxic obstruction on growth and development is shown by Vrchotova et al. [38] and Won et al. [39] which is in line with our study overall.

The effects of bioassay 1 did not demonstrate an explicit development propensity, i.e., hormesis or development hindrance instigated by phytochemicals discharged from trichomes, which stand in accordance with the study of Belz et al. [29,40]. However, Kraus [41] doesn't support our findings, where a similar bioassay demonstrated a sound biological action upon plunging of *Parthenium hysterophorus* leaves in the organic solvent, DCM.

In our case, as seen in the leaf sections of *N. plumbaginifolia* in Figs. 2A and 2B, DCM seems to have little impact on the leaf morphology, which maybe clarifies why no phenolic chemicals were evidently discharged into the DCM following five seconds.

Duke et al. [32] demonstrated that plunging *Artemisia annua* leaves in an organic solvent for a couple of seconds exiled the substance from the peltate glands but didn't cause any major disturbance except for crumbling the cuticle of these glands. More or less analogous results were seen by Reinhardt et al. [25] in the capitate-sessile glands of *P. hysterophorus* when leaves were dipped in DCM. Kraus [40] indicated immense biological action towards test species in bioassays utilizing these aqueous infusions. In our case, *N. plumbaginifolia* on the contrary as seen in Figs. 9A and 9B demonstrate no explicit development propensities, particularly hormesis, a critical pointer of the allelopathic capability [40]. In this way, it is expected that phenolic chemicals maybe present on the leaf surfaces of *N. plumbaginifolia* or may be insoluble in DCM.

In our case, one of the trichomes appears to have been ruptured (Fig. 1.4) which may be the site of discharge. Nevertheless, more such studies are expected to affirm the ultrastructure and capacity of trichomes. The impact where the solvent rupture the cuticle of trichomes was found in studies by Duke et al. [32] and Reinhardt [25] on *A. annua* and *P. hysterophorus* respectively.

Since trichomes jut from the epidermal cells and can frequently be smoothly isolated and collected, the mRNAs, proteins and other phytochemicals which they possess are remarkably open to investigation. A feature like this marks trichomes exceptional to trace pathways accountable for the production of the particular metabolites produced by such structures and in some cases somewhere else in the plant.

5 Conclusion

In this manner, in view of these observations, we can conclude that structures present on the leaves of the plant may contain the allelochemicals. The allelochemicals can suppress the growth of plants and are utilized by the donor plant to guarantee its fruitful intrusion. The allelochemicals giving the allelopathic character to *N. plumbaginifolia* are presumably water-soluble (polar compounds). Allelopathic effects may occur after the plant dies and releases its contents in the soil.

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Conflicts of Interest: The authors declare that there is no conflict of interest among them.

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