Identification of the chemical identities in calli of different explants from*Munroniapinnata* (Wall.)Theob.

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Abstract- *Munroniapinnata*,also known asground neem/king bitter in English, is a valuable medicinal plant that is widely used in Ayurveda in Sri Lanka. It has shown number of medicinal properties like, antimicrobial, anti-inflammatory, anti-oxidant and anti-malarial. The objectives of this study were, to obtain calli from different explants of *M.pinnata* and to identify the chemical identities extracted from these calli. This study was conducted with an aim to confirm the availability of chemical classes produced by the natural plant in its callus stage and to see whether the same chemical classes were extracted from all three explants.
In vitro culture of *M.pinnata* was done using three different explants, leaf discs, petals and immature seeds. The calli obtained were extracted into methanol and water. These three samples were subjected to TLC to identify alkaloids and terpenoids, HPTLC, GC-MS and preliminary phytochemical tests, in order to identify glycosides, tannins, saponins, flavonoids and steroids. The chemical compounds extracted from the samples of leaf and petal calli and the mother plant were identified via the GC-MS study. A HPTLC fingerprint of the methanol extracts of all three samples and that of the mother plant was also obtained.Callus induction rate was highest in both leaf discs and petals. Highest yield of callus was from petals and lowest yield was from immature seeds. The data obtained from TLC depicted that all three calli samples gave two spots each, to show the presence of terpenoids. (Rf values: 0.18, 0.34 for petal and leaf calli samples and 0.20, 0.37 for seed calli sample). The results from the preliminary studies showed that, aqueous extracts of leaf, petal and seed calli samples were positive to flavonoids and aqueous extracts of leaf and seedcalli were positive to tannins. The methanol extracts of petal and seedcalli samples were positive to steroids.

Index Terms: *M.pinnata, in-vitro* culture, callus, HPTLC, GC-MS, TLC.

1 INTRODUCTION

Munronia pinnata is a native plant to Sri Lanka and also a very valuable medicinal plant. The whole plant has been widely used in Ayurveda medicine in treating ailments and diseases like, fever, dysentery, hemorrhoids, eczemas, asthma, cough, blood disorders and malaria [3]. Due to this high medicinal value, *M.pinnata* has a high demand in the market. It has been considered as one of the most expensive medicinal plants used in traditional medicine in Sri Lanka [5]. As a result this plant has been overexploited and it has been considered as a threatened species, according to the IUCN (International Union for Conservation of Nature) Red Data list.

M.pinnata is a small perennial shrub that belongs to the family Meliaceae.This plant's conventional mode of propagation by seeds cannot meet its increasing demand because of low germination rate and reduced viability.Best method to overcome the demand and overexploitation of this plant, is *in vitro* culture as it can propagate the plant in large scale. Objective of this study was to identify the chemical identities in thecalliobtained from different explants and to do a comparative study with the mother plant. As this plant is highly rich in medicinal properties, it has found to be enriched with a wide range of chemical compounds like glycosides, flavonoids, tannins, terpenoids, steroids and fatty acids [1].

In this study, calli from different explants from *M.pinnata*were obtained and the chemical compound families extracted at its callus stage were determined. Up to date, only few studies were done on the calli obtained from leaves of *M.pinnata*.In the present study, various chemical tests, TLC (Thin Layer Chromatography) andanalytical instruments likeGC-MS (Gas Chromatography-Mass Spectrometry) have been used to identify these chemical constituents. HPTLC (High Performance Thin Layer Chromatography) was done to obtain a chromatographic fingerprint of the calli samples and the mother plant.

2 METHODS AND MATERIALS

The mother stock of *M.pinnata* was maintained in a greenhouse and it was well watered and Captan® fungicide was sprayed at two weeks interval.

2.1 *In vitro* culture of *M.pinnata*.

Three month old, healthy and three leaflet type mother plants were selected and explants were collected from them. Leaf discs, petals of flower buds and seeds were used as explants. Leaf discs (4-5 mm in diameter) were obtained from second fully opened leaves [6]. Petals were also collected from flower buds and immature seeds were collected from young green pods after one week of maturity.

Surface sterilization of all types of explants were done as follows, initially they were dipped in dilute Dettol™ soap water for five minutes, and then they were washed under running tap water for one hour. Next, they were dipped in 10% Clorox for three minutes, washed twice in sterile distilled water, dipped in 70% ethanol for thirty seconds and finally washed twice with sterile distilled water.

All three types of explants were cultured separately on Murashige and Skoog (MS) [4] medium supplemented with 1.1 mg/L 2-4 – dichlorophenoxy acetic acid (2, 4-D) and 0.5 mg/L 6 – benzylaminopurine (BAP) [6].

The culture vessels were incubated in dark inside the culture room at 25±1 ^{0}C. Sub culturing was performed in every five weeks interval. Time taken for callus induction from each type of explant was recorded.

2.2 Preparation of the methanol and aqueous extracts.

Three month old calli from all three explants were collected separately and the fresh weights were measured. They were dried until a constant weight was achieved and their final dry weights were measured. A soxhlet extraction was done to obtain the methanol extracts of each dried and powdered calli samples. Some of the dried calli were used to prepare the aqueous extract. For that, each dried calli sample was extracted into water (10.0 mL) while being kept on the magnetic stirrer for 12 hours at 80 ^{0}C.

For the preparation of the methanol extract of the *M.pinnata* mother plant (preparation of the standard sample), leaves collected from the mother stock of *M. pinnata* were air dried and coarsely powdered (1.1 kg). The traditional decoction method of extraction practiced in Ayurveda was used, in the preparation of the standard sample. To the freeze dried samplea series of extractions were carried out in n-hexane, chloroform and methanol respectively. Finally the concentrated methanol extract was obtained using rotary evaporator at a temperature of 70^{0}C and was used as the standard sample in further chemical analyses.

Some of the freeze dried sample was kept aside to make the aqueous extract of the mother plant. A small amount of freeze dried sample was dissolved in 20.0 mL of water and was filtered. The filtrate was used as the positive control in preliminary chemical tests.

2.3 Identification of chemical identities in the calli samples using preliminary chemical tests

To the methanol and aqueous extracts of the three calli samples and the standard sample, following phytochemical tests were done [7], [9].

Glycosides - Liebermann test
Tannin - Braymer's test

Steroids - Salkowski test

Saponins - Foam test

Flavanoids - Lead acetate test.

Presence of respective phytochemicals were identified based on the colour changes or precipitation observed with all tested reagents.

2.4 Identification of phytochemicals present in the calli samples using Thin Layer Chromatography (TLC)

Previously obtained methanol extracts of the calli samples were subjected to a TLC. About 5.0 µL aliquot from each sample was spotted on a TLC plate, pre-coated with silica gel. It was developed using Ethyl acetate: methanol (4:1) solvent system. The resultant TLC was observed under UV (ultra violet) light (366 nm) and fluorescence light (254 nm). Then the chromatograms were sprayed with Anisaldehyde-sulphuric reagent and was heated until spots were appeared. Rf values of each spots were calculated. To determine the presence of alkaloids, another TLC was developed using the same solvent system and it was also sprayed with Dragendorff reagent.

2.5 Determination of the chromatographic fingerprint of the calli samples using High Performance Thin Layer Chromatography (HPTLC)

From each of the methanol extracts of calli samples and the methanol extract of the standard sample of *M. pinnata*, 5 µL aliquots were spotted on a TLC plate pre-coated with silica gel. It was developed in the solvent system of Ethyl acetate: methanol (4:1). Developed TLC plates were scanned using the Camag TLC scanner operated by winCATS Software. Its operating conditions were set as, scanning mode - re-emission (D2 and W lamp), measurement wavelength - from 200-800 nm and slit dimension at 6.00 × 0.30 mm.

2.6 Identification of phytochemicals present in calli samples using Gas Chromatography-Mass Spectrometry (GC-MS)

The methanol extracts of the two calli samples (obtained from leaf discs and flower buds) and the standard sample of *M. pinnnata* were subjected to a GC-MS analysis. The GC-MS analysis of the above mentioned samples were carried out with Agilent 7890A. The column used was HP-5 capillary column of 30m × 0.25mm and with a thickness of 0.25µm composed of 5% phenyl methyl siloxane. The Helium gas was used as the carrier gas at a flow rate of 14mL/min. The split ratio was 10:1. The sample size of 2 µL was injected from each sample. The inlet temperature was maintained at 300^0 C. The oven temperature was initially programmed at

70^{0}C for 40 min, then gradually it was increased at a rate of 10^{0}C/min up to 280^0 C for 20 min. The total run time for GC was 30 min. The MS transfer line was maintained at 230^0 C. The mass spectrum was taken at 70 eV. Mass scan parameters include the mass range (m/z) from 33-550.The compounds were identified in reference to the GC-MS library

3 RESULTS AND DISCUSSION

3.1 Development of callus cultures from *Munroniapinnata*

Callus induction was seen in all three explants that were inoculated on MS medium supplemented with 1.1 mg/L 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 0.5 mg/L 6-benzylaminopurine (BAP).

According to Table 1, it could be seen that the callus induction rate was highest in leaf discs and petals. It took four weeks for a fully grown callus to be produced from leaf discs and petals while seeds took five weeks to produce a full grown calli.

Table 1: Time period taken for callus induction from the three explants

Explant	1st week	2nd week	3rd week	4th week	5th week
Leaf discs	-	√	√ + w	+ + w	+ + lb
Petals from flower buds	-	√	√ + w	+ + w	+ + lb
Immature seeds	-	-	√ + w	+ w	+ + lb

- No callus √: Swelling +: callus initiation ++: callus w: white colorlb: light brown color

In previous studies, it has taken six days for callus initiation [6]. In the present study it took more time for callus initiation. Seeds were not used as explants in previous studies and from this study it was seen that immature seeds gave calli but its callus induction rate was lower than that of other two explants.

The mean fresh weights and dry weights of calli obtained from the three explants are shown in Table 2. There was a significant difference in callus production from different explants. Although leaf discs also produced significantly high amount of calli,petals from floral buds found to be the best explant to obtain calli. Immature seeds produced lowest amount of calli. Previous studies have stated that leaf discs are the most successful in producing highest amount of calli [6].

Table 2: Mean fresh weights and dry weights of calli from different explants after 12 weeks

Explant	Mean callus fresh wt (g)	Mean callus dry wt(g)
Leaf discs	1.47 ± 0.36	0.374 ± 0.10
Petals from Flower buds	1.62 ± 0.42	0.408 ± 0.11
Immature seeds	0.65 ± 0.10	0.148 ± 0.04
LSD %	0.01	0.02

3.2 Identification of phytochemicals present in the calli of *M. pinnata*

The results obtained in the phytochemical screening of aqueous and methanol extracts of the calli samples have been summarized in Table 3 and Table 4 respectively.

Flavanoids were found in all three samples but Tanins were found only in leaf calli and seed calli samples. In the study conducted by Dharmadasa *et al* [1] on phytochemical screening of roots, stem and leaves of *M.pinnata*, it was found out that glycosides, tanins, steroids, saponins and flavonoids were present.

Table 3: Phytochemicals extracted in aqueous extracts from the calli samples of *M.pinnata*.

Chemical compound	**Leaf calli sample**	**Petal calli sample**	**Seed calli sample**
Glycosides	Solution turned yellow	Solution turned yellow	Solution turned yellow
Tannins	**Brown precipitate**	No change	**Brown precipitate**
Steroids	No change	No change	No change
Saponins	No foam formation	No foam formation	No foam formation
Flavanoids	**Light brown precipitate**	**Light brown precipitate**	**Light brown precipitate**

Table 4: Results of the chemical tests conducted on the methanol extracts of the calli

Chemical compound	**Leaf calli sample**	**Petal calli sample**	**Seed calli sample**
Glycosides	Solution turned	Solution turned	Solution turned

	yellow	yellow	yellow
Tanins	No change	No change	No change
Steroids	No change	**Light brown ring**	**Light brown ring**
Saponins	No foam formation	No foam formation	No foam formation
Flavanoids	No change	No change	No change

Methanol extracts of petal calli and seed calli gave positive results for steroids while other compounds were absent in all three samples. There were no studies carried out on the phytochemical screening of methanol extracts of calli from*M.pinnata.*

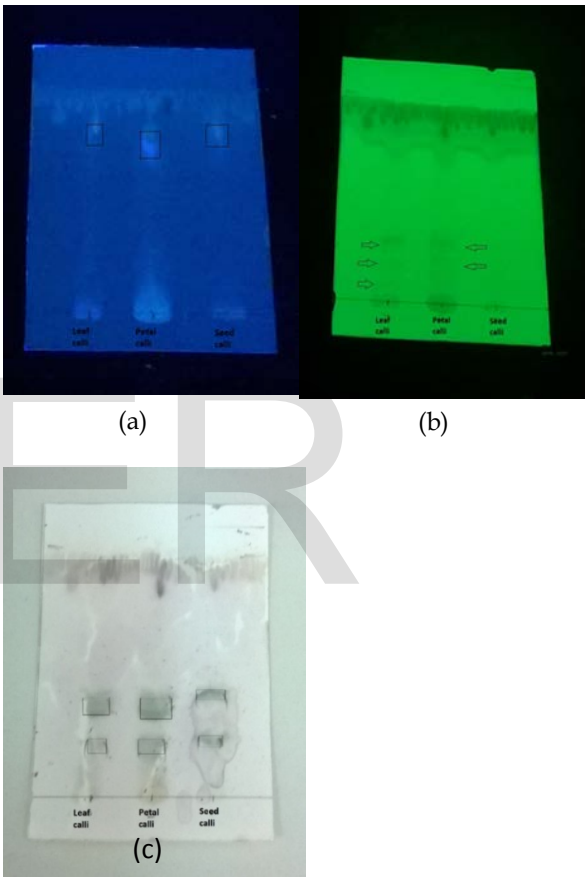
3.3 Identification of phytochemicals present in the calli samples using Thin Layer Chromatography (TLC)

The TLC plate was developed and it was observed under UV and Fluorescence light. (Figure 1). Under UV light (366 nm) there were three spots with Rf values, 0.815, 0.76 and 0.83 visualized in leaf, petal and seed calli samples respectively(Table 5). More of the spots were visualized under fluorescence (254 nm) light. Rf values of the three spots of calli sample from leaf discs were, 0.308, 0.154 and 0.077.There were two spots from the calli sample from petals that were with similar Rf values to the first two spots of calli sample from leaf discs, which were 0.308 and 0.154. (Table 5).The calli sample from immature seeds didn't show any spots under fluorescence. There were no studies done up to date to identify UV and fluorescence active compounds in the callus of *M.pinnata.* Therefore this study has shown that calli obtained from leaf discs and petals consist of UV active and fluorescence active compounds while calli obtained from immature seeds contain only UV active compounds.

The same TLC plate after been sprayed with Anisaldehyde-sulphuric reagent gave two dark blue spots each in all three samples. These spots confirmed the presence of terpenoids in all three calli samples (Fig. 1). Literature has shown that, presence of terpenoids, as appearance of dark blue /violet spots after spraying with Anisaldehyde-sulphuric is an indication for the presence of terpenoids[8]. When the other TLC plate was sprayed with Dragendorff reagent, it failed to give any spots. Thereby, it was concluded that alkaloids were not present in neither of the three calli samples.

Table 5: Calculated Rf values of the spots observed under UV (366 nm), fluorescence (254 nm) and after spraying with Anisaldehyde-sulphuric reagent for the methanol extracts of calli samples.

Sample	R_f value		
	UV	Fluorescence	Anisaldehyde Sulphuric reagent
Leaf calli	0.815	(1) 0.308 (2) 0.154 (3) 0.077	(1) 0.34 (2) 0.18
Petal calli	0.76	(1) 0.308 (2) 0.154	(1) 0.34 (2) 0.18
Seed calli	0.83	-	(1) 0.37 (2) 0.20



(a) (b)

(c)

Fig 1: TLC plate observed under (a) UV 366 nm (b) fluorescence 254 nm and (c) TLC plate after sprayed with Anisaldehyde –Sulphuric reagent (Ethyl acetate: methanol (4:1) was used as the solvent system).

3.4 Determination of chromatographic fingerprint of the calli samples using High Performance Thin Layer Chromatography (HPTLC)

A distinct chromatographic fingerprint of these calli samples were obtained using HPTLC. This provides a comparative study between mother plant and the three calli samples.Therefore this could be used in future studies related to *M.pinnata*, as HPTLC provides more accurate data than TLC. Up to date there has not been any HPTLC chromatographic fingerprint for neither the callus cultures

nor plant of *M.pinnata*. Fig 2 shows the HPTLC profile of the standard sample and the calli samples. Single peak was seen in all three calli samples while the same peak was also seen in the mother sample. There was also another distinct peak in the mother sample but that was absent in all three calli samples.

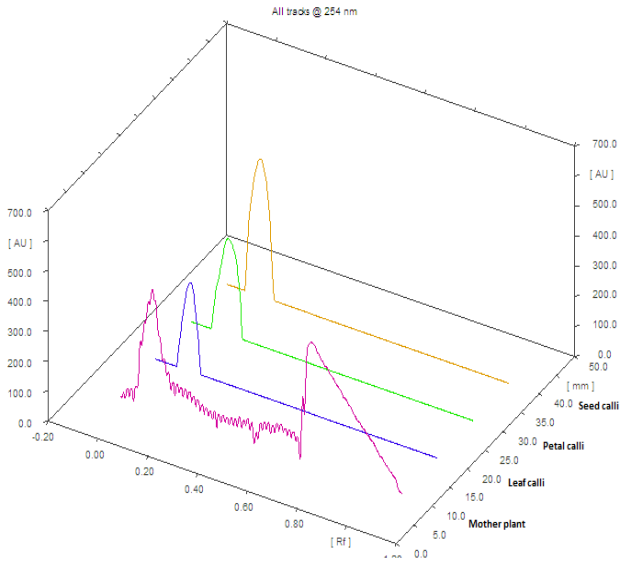


Fig 2: HPTLC profile for the extracts from leaves of mother plant and three calli samples in 3D view.

The compounds identified in this chromatogram were the ones active between the wavelength ranges from 200-800 nm. The Rf values and maximum absorption (in means of maximum height) for each peak has been given in Table 6. It could be concluded from the Rf values, that similar compounds are found in mother plant and seed calli sample due to similar Rf values and same could be seen between leaf calli and petal calli, as they also have similar Rf values.

Number of compounds detected in calli were lesser than in the mother plant, due to the fact that at this particular stage of calli other compounds might not have being produced yet or the quantity of calli used might not be enough so the concentration of those compounds might be below the detection level.

3.5 Identification of phytochemicals present in calli samples using Gas Chromatography-Mass Spectrometry (GC-MS)

Results of the chromatogram obtained from GC-MS analysis have been summarized in Table 7. The GC-MS analysis of two calli samples (leaf and petal) showed only one peak for the methanol extracts and the mother plant sample gave seven different peaks. Hydroxylamine, was the only compound found in the mother plant and as well as in the two calli samples. Other compounds identified in the mother plant sample were phytol, Decanoic acid-methyl ester, Heptadecanoic acid-16-methyl-methyl ester, 2-Heptadecanone, 2-Nonadecanone and cholesterol.Napagoda [5], identified 19 compounds from the GC-MS analysis of the n-hexane crude extract of *M.pinnata*. Phytol, dodecanoic acid, hexadecanoic acid were also included in the components found during that study. So far callus extracts of *M.pinnata* have not been subjected to any GC-MS analysis, therefore it is difficult to confirm the exact number of volatile components in calli extracts.

Table 6: Rf values and maximum absorption values for the methanol extracts of the calli samples and mother plant sample of *M. pinnata*.

Sample	**Peak number**	**Rf value**	**Maximum absorption/ AU**
Mother plant	3	0.08	390.9
Leaf calli	1	0.09	295.6
Petal calli	1	0.09	321.1
Seed calli	1	0.08	456.5

Table 7: Compounds identified in the methanol extracts of the mother plant, leaf calli sample and petal calli sample of *Munroniapinnata*, using the GC-MS analysis.

No.	Name of the compound	Mother plant	Leaf calli	Petal calli
1.	Hydroxylamine	√	√	√
2.	2-Heptadecanone	√	-	-
3.	Decanoicacid,methyl ester	√	-	-
4.	2-Nonadecanone	√	-	-

5.	Phytol	√	-	-
6.	Heptadecanoic acid,16-methyl-methyl ester	√	-	-
7.	Cholesterol	√	-	-

There are reasons for the identification of lesser number of compounds in the calli samples and mother plant. One reason is the quantity of calli obtained from each explant being low. Therefore enough concentration of compounds to be detected might not have been present in the samples. Depending on the polarity of the solvent used for the extraction, different compounds get extracted. This same issue with the solvent applies to the two calli samples. Sometimes the boiling point of the compounds also affect the results. GC-MS analysis identifies volatile compounds, and the mother plant was initially extracted into water and was boiled up to 100^0C. As a result, many volatile compounds might have been evaporated, therefore only the compounds with a high boiling points were identified.

CONCLUSION

Leaf discs, petals from flower buds and immature seeds were successful in producing callus. Callus induction rate was highest in both leaf discs and petals. Highest yield of callus was from petals, then from leaf discs and lowest yield was from immature seeds. The preliminary chemical tests gave positive results for flavonoids in the aqueous extracts of all three calli samples. Tanins were present only in calli produced from leaf discs and seeds. Steroids were present in the methanol extracts of the calli from petals and seeds. TLC conducted was able to identify terpenoids in all three calli samples. Two spots given by each sample showed the presence of two subclasses of terpenoids in calli. Negative results were given for the alkaloid test in all three calli samples. The chromatographic fingerprint of three calli samples and mother sample were obtained from HPTLC technique. This HPTLC profile could be used as a reference when it comes for standardization and authentication of any herbal drug produced from active constituents in *M.pinnata.* The results from the GC-MS analysis showed the presence of hydroxylamine in the calli obtained from leaf discs and petals. As this is a basic study on determining the chemical compounds in the calli of *M.pinnata*, there are improvements to be made. The main requirement is to have a large quantity of calli, and more advance techniques than preliminary chemical tests to obtain more accurate results. In order to go for a commercial level production of these secondary metabolites in calli, the drawbacks in this study needed to be addressed and the protocol for micro propagation could be further optimized. Considering all these improvements and drawbacks, it is possible to extract out the essential compounds at the callus stage of *M.pinnata*, without destroying the whole plant.

5 APPENDICES

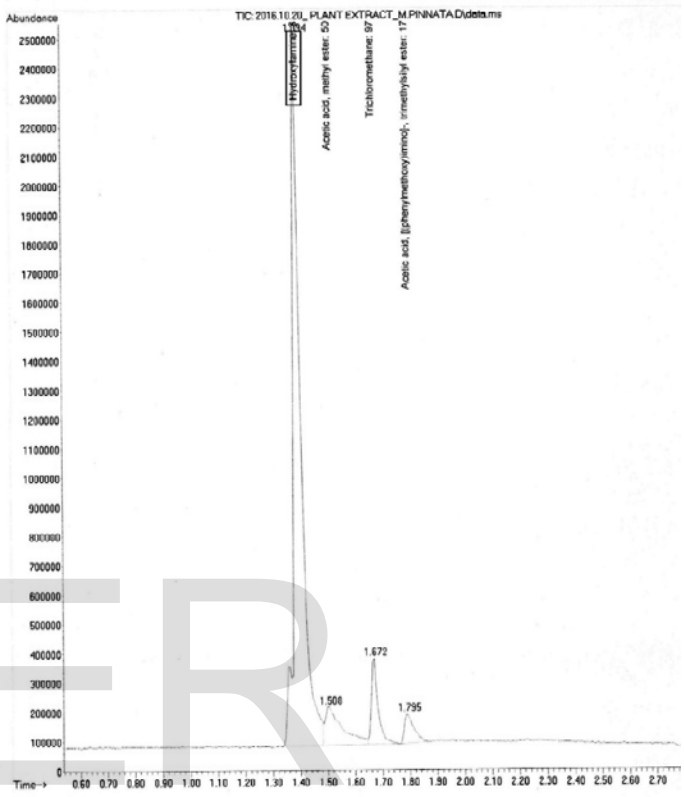


Fig 3: GC Spectrum of methanol extract of *M.pinnata*. The figure shows the peak for hydroxylamine.

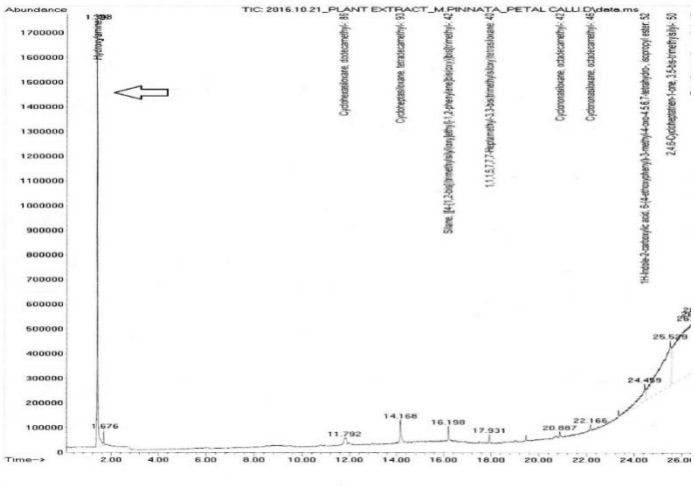


Fig 4: GC Spectrum of methanol extract of petal calli of *M.pinnata.* The single strong peak corresponds to the hydroxylamine compound. It is in high abundance in the calli sample. Arrow head shows the hydroxylamine peak.

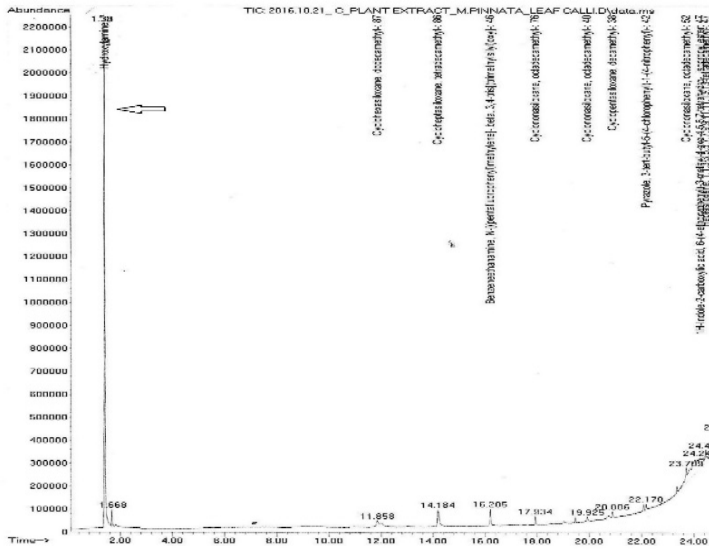


Fig 5: GC Spectrum of methanol extract of leaf calli of *M.pinnata*. The single peak gives the hydroxylamine compound that was identified in this calli sample. Arrow head shows the peak for hydroxylamine

6 ACKNOWLEDGMENTS

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