

PHYTOCHEMICAL CHARACTERIZATION AND PRELIMINARY FORMULATION OF  
*ACALYPHA INDICA* AS A NATURAL MOOD ENHANCER FOR CATS.Dasanayaka Arachchilage Arosh Lakshitha<sup>1\*</sup>, Jayantha Wijayabandara<sup>1</sup>, Walisinghe Pathirana<sup>2</sup><sup>1</sup>Department of Pharmacy and Pharmaceutical Sciences, Faculty of Allied Health Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka.<sup>2</sup>Department of Pharmacology and Pharmacy, Faculty of Medicine, University of Colombo, Kynsey Road, Colombo, Sri Lanka.

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22–29.**ABSTRACT**

Cats are well known to frolic around fresh roots of *Acalypha indica* plant. This study explores the use of root extract of the plant as a natural formulation for cat mood elevation. It aims to promote getting over states of depression, particularly cats living in isolation such as in high rise apartments together with the cat owner himself. Fresh *Acalypha indica* roots were extracted and their phytochemical profiling was conducted using standard chemical tests and UV spectroscopy. Cat-attractant compounds in the extract were identified via GC-MS analysis. A zebrafish embryo toxicity assay was performed to determine the LC<sub>50</sub>, based on which a spray formulation was developed. Short-term marker-based analysis of the highest concentration formulation was conducted using solid-phase microextraction (SPME). Physical evaluation of final formulations was also carried out. Phytochemical screening confirmed the presence of alkaloids, phenols, flavonoids, terpenoids, tannins, and steroids. GC-MS analysis revealed the presence of Iridomyrmecin and Dihydronepetalactone, which are the known cat-attractant compounds. The LC<sub>50</sub> of the extract was determined to be 10.70% (w/w). After one month, SPME analysis showed a reduction of Iridomyrmecin and Dihydronepetalactone by 50.95% and 58.84%, respectively. The identification of feline attractants in *Acalypha indica* root extract supports its potential use in veterinary formulations. Through cat-based animal studies and improved stabilization, the formulation may be successfully developed for commercial applications.

**KEYWORDS:** *Acalypha indica*, Iridomyrmecin, Dihydronepetalactone, mood elevator, cats, GC-MS.**INTRODUCTION**

*Acalypha indica* belonging to the family Euphorbiaceae, is an annual herb that grows up to a height of around 30cm – 50cm. In English, it is known by many names including Indian Copper Leaf, Indian *Acalypha*, Poison Bush, or Indian Nettle. In Sinhala it is known as *Kuppameniya*, and in Tamil, *Kuppa-mani* or *Kuppaman-cheti*. (Fig. 1) *Acalypha indica* has many therapeutic uses, including antibacterial activity,<sup>[1]</sup> anticancer activity,<sup>[2]</sup> lowering blood glucose levels, tissue restorative properties,<sup>[3]</sup> and stress-relieving.<sup>[4]</sup>

**Fig. 1: Young *Acalypha indica* plant with roots exposed.**

When felines approach plants such as catnip (*Nepeta cataria*) and silver vine (*Actinidia polygama*), they exhibit a typical behavioural response that is similarly seen towards *Acalypha indica*.

The compounds that act as cat attractants in *Nepeta cataria* were found to be volatile nepetalactone stereoisomers and non-volatile iridoid glucosides. Iridoids are widespread in flowering plants, including the mint family (Lamiaceae) to which *Nepeta* belongs.<sup>[5,6]</sup>

Domestic cats respond to all iridoids, including dihydroactinidiolide, but the response to actinidine is found to be rare.<sup>[7]</sup>

The Iridoid compounds that caused catnip response in *Nepeta cataria* are observed in *Acalypha indica* roots as well. This attracts domestic cats towards the fresh roots of *Acalypha indica*. The iridoid compounds that had the cat attractant property present in *Acalypha indica* were found to be Isodihydronepetalactone and Isoiridomyrmecin (Fig. 2, 3).<sup>[8]</sup> These herbal compounds not only attract domestic cats, but they also reduce their stress.<sup>[6]</sup>

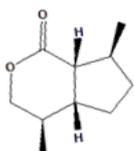


Fig. 2: Dihydronepetalactone

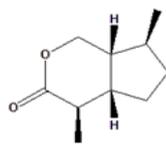


Fig. 3: Isoiridomyrmecin

A suitable formulation has to be an olfactory stimulating product. These cat-attracting compounds have proven to be of no use when ingested by the cat.<sup>[9]</sup> A prefabricated spray formulation would be more feasible and less complicated for cat owners to use.

## MATERIALS AND METHODS

### Testing materials

Chemicals and reagents: Methanol (100%), Sodium Hydroxide, Glacial Acetic Acid, Sodium Chloride,

Glycerine, Folin ciocalteu's reagent, Gallic Acid, Dragendorff's Reagent, Quercetin Dihydrate, Lead Acetate, Ferric Chloride, Liebermann Reagent, PEG.

Equipment used include: a rotary vacuum evaporator (BUCHI interface 1-100 (BUCHI Labortechnik AG, Flawil, Switzerland), GC-MS system (Agilent Technologies 7890A GC system, Autosampler 7693, with an Agilent 5975C inert XL EI/CI MSD with triple-axis detector (Agilent Technologies Inc., Santa Clara, CA, USA), Solid-Phase Microextraction (SPME) fibre (DVB/Carbon WR/PDSM (Supelco, Sigma-Aldrich, Bellefonte, PA, USA), and a UV spectrometer (Agilent Cary 60 UV-vis (Agilent Technologies Inc., Santa Clara, CA, USA).

### Preparation of extracts

Fully grown *Acalypha indica* plants were collected from the Colombo district, Kottawa area. Herbarium specimen was prepared and the specimen was authenticated by the Bandaranaike Memorial Ayurvedic Research Institute, Navinna, Sri Lanka.

Plants were collected around 30-40 minutes before the extraction. Plucking of plants was done carefully after wetting the soil to loosen roots to minimize damage. The roots and leaves were decontaminated and washed with distilled water and were patted with a towel to dry.

Crude plant material was ground separately in a blender. Cold extraction was carried out using plant material: methanol in a 1:5 ratio by weight for 48 hours. Filtrates were then stored in hermetically sealed glass containers at 2 °C.<sup>[10]</sup> A rotary vacuum evaporator was used at 150 mbar and 40°C water bath to obtain semi-solid extracts, and stored at 2 °C until used.

### Qualitative phytochemical investigation of *Acalypha indica*

A summary of all the reagents used and the tests followed is shown in table 1.

Table 1: Reagents and Chemicals Required for Phytochemical Screening.

Chemical constituent	Type of test	Reagent(s)
Alkaloid	Wagner's test	Wagner's reagent
Flavonoid	Alkaline reagent test	20% Sodium hydroxide. Dilute Hydrochloric acid
Phenolic compounds	Lead acetate test	10% Lead acetate
	Ferric chloride test	5% Ferric chloride
Terpenoids	Salkowski's test	Chloroform. Concentrated Sulfuric acid
Tannins	Braymer's test	Alcoholic Ferric chloride
Phlobotanins	Precipitate test with HCl	1% Hydrochloric acid
Steroid	Liebermann – Burchard test	Acetic anhydride. Concentrated Sulfuric acid

### Quantitative analysis of phenols

The standard curve was obtained using Gallic acid dilution series, 25 ppm, 30 ppm, 40 ppm, 50 ppm, 65 ppm, and 70 ppm. The reaction mixture was made with 0.5 ml of different dilutions mixed with 10% folin-ciocalteu (0.3 ml) and 10% sodium carbonate (0.3 ml). The reaction mixture was incubated at room temperature for 30 minutes before taking the absorbance at 765 nm with UV spectrometer.

Two sample stock solutions were made using 40mg of leaf and root extract and dissolved in 2 ml of methanol. The reaction mixture was made with 0.5 ml of sample stock solution mixed with 10% folin-ciocalteu (0.3 ml) and 10% sodium carbonate (0.3 ml). The reaction mixture was incubated at room temperature for 30 minutes before taking the absorbance at 765 nm.

All the test samples were triplicated to obtain average absorbance using UV spectrometer.

### Quantitative analysis of flavonoids

The standard curve was obtained using Quercetin, at 30 ppm, 40 ppm, 50 ppm, 60 ppm, 80 ppm, and 100 ppm. The reaction mixture was made with 0.5 ml of different dilutions of quercetin mixed with 5% NaNO<sub>3</sub> (0.3 ml), 10% AlCl<sub>3</sub> (0.3 ml), and 4% NaOH (2 ml). The reaction mixture was incubated at room temperature for 6 minutes before taking the absorbance at 510 nm using UV spectrometer.

Two samples of stock solution were made using 60 mg of leaf and root extract and dissolved in 2 ml of methanol. The reaction mixture was made with 0.5 ml of sample stock solution mixed with 5% NaNO<sub>3</sub> (0.3 ml), 10% AlCl<sub>3</sub> (0.3 ml), and 4% NaOH (2 ml). The reaction mixture was incubated at room temperature for 6 minutes before taking the absorbance at 510 nm. All the test samples were triplicated to obtain average absorbance using UV spectrometer.

### Identification of cat attractants using chromatography

From freshly prepared methanolic extracts as described above, an amount was dissolved in 10 ml of 100% methanol, to obtain 100 ppm of methanolic extract of both leaf and root for Gas Chromatography-Mass Spectrometry (GC-MS) analysis.<sup>[8,11]</sup>

GC system was used with BPX-5 column (non-polar) (5% phenyl polyphenylene-siloxane, 30 m × 0.25 mm × 0.25 μm film thickness). Helium was used as the carrier gas with a constant flow rate of 1.0 mL/min. A scan range of m/z 45 – 400 and a solvent delay of 5 min were used with split-less injections of 1.0 μL for 1.0 min. The ion source was set to 230 °C, and the transfer line temperature to 250 °C. The oven temperature program was 40 °C, held for 1 min, then ramped at 7 °C /min to 250 °C, and held for 10 min.

### Zebrafish embryo toxicity assay

Freshly spawned embryos were obtained from the Medical Research Institute, Danister De Silva Mawatha, Colombo, Sri Lanka.

In the assay, test solutions were in petri dishes. Five embryos were placed in each petri dish with test solutions after observing through the light microscope for selecting viable embryos.<sup>[12,13]</sup>

Test sample solution - A dried extract without methanol was obtained by further evaporating the extract under reduced pressure. Then it was used for the preparation of test solutions. Five test solutions were prepared by dissolving crude dried extract in distilled water such that 2.5%, 5%, 10%, 15 %, and 25% (w/w) test solutions were made. Petri dishes were used to hold the test solutions for the assay.

**Negative control** - Distilled water free of any chemicals was used as the negative control.

**Positive control** - Ethanol 50% was used, to achieve 100% motility.

**In the Assay layout - Embryos were distributed in 28 petri dishes as follows.**

- Four petri dishes with 5 embryos in each, a total of 20 embryos for each of the five test concentrations.
- Four petri dishes with 5 embryos in each, a total of 20 embryos for positive control.
- Four petri dishes with 5 embryos in each, a total of 20 embryos for negative control.

### Development of spray formulation

The LC<sub>50</sub> was obtained by carrying out zebrafish embryo toxicity assay. According to previous studies, it has been concluded that 1/3 of LC<sub>50</sub> provides a safe exposure level, as a margin of safety that ensures non-lethal effects.<sup>[14]</sup> Therefore, plant extract levels were used for the formulation to be lower than 1/3 of LC<sub>50</sub>.

The formulae of the three sprays are shown in table 2.

**Table 2: Suggested formulation of spray solution.**

Ingredients	% Weight		
	A	B	C
Root extract	3	2.5	2
PEG 4000	5	5	5
Ethanol	12	12	12
Glycerin	5	5	5
Distilled water	75	75.5	76

### Preparation of formulation

PEG is dissolved in ethanol and glycerin using a magnetic stirrer. The dry crude extract was gradually added to the above solution. The mixture is stirred by a magnetic stirrer for a further 5 minutes. Distilled water was slowly added to the required volume and stirred for

2 minutes. The prepared formulation is filled into spray containers.

#### Evaluation of spray formulation- Marker-based short-term Stability

To check the stability of the final spray formulations, the availability of the two cat-attracting compounds identified in the methanolic extract was used as the marker compounds.

SPME was done to identify the cat attractants in formulation A, just after a month of preparation. It was stored at room temperature ( $25 \pm 1$  °C), without direct sunlight.

SPME was performed with DVB/Carbon WR/PDSM fiber with a thickness of 80  $\mu$ m. Of the sample formulation, 10 ml was placed in the SPME vial. The head space of the vial was saturated overnight with 10 ml of the formulation and was kept on a hotplate at 40 °C for 15 minutes before exposing the fiber to the headspace of the vial. The Fiber was placed for 30 minutes. Afterwards, the fiber was withdrawn into the housing, the SPME device was removed from the sample vial and the fiber was desorbed into the GC injector.<sup>[15]</sup> GC-MS

analysis was carried out similarly to the method used for the analysis of methanolic extraction.

#### Physical parameters of the spray

**pH** - The pH of the spray solution was measured using a digital pH meter by assessing each formulation three times and calculating the mean pH.

**Evaporation Time**- Evaporation time refers to the duration of time required for the spray film to dry. This was measured by spraying the formulation onto a petri dish and recording the drying time.

#### Stickiness of the spray after evaporating the solvent -

The spray bottle was kept 5 cm away from the petri dish and sprayed. After solvent evaporation, to find out how sticky the residue is, it is pressed with a ball of cotton wool. The degree to which the film retains cotton fibres determines the stickiness rating. Stickiness is assessed as high if there is a dense coating of fibres stuck on the residue, medium if there is a thin layer of fibres, and low if there is little to no fibre adherence.<sup>[16]</sup>

## RESULTS

### Results of qualitative phytochemical screening

The results are shown in table 3.

**Table 3: Result of phytochemical screening of leaves and roots.**

Phytochemical	Tests	Methanolic root extract	Methanolic leaf extract
Alkaloids	Wagner's test	+	+
Flavonoids	Alkaline reagent test	+	+
Phenols	Lead acetate test	+	+
	Ferric chloride test	+	+
Terpenoids	Salkowski's test	+	+
Tannins	Braymer's test	+	+
Phlobotanins	Precipitate test with HCl	-	-
Steroid	Burchard test	+	+

+ Present, - Absent

#### Quantitative analysis of phenols and flavonoids using UV spectroscopic analysis

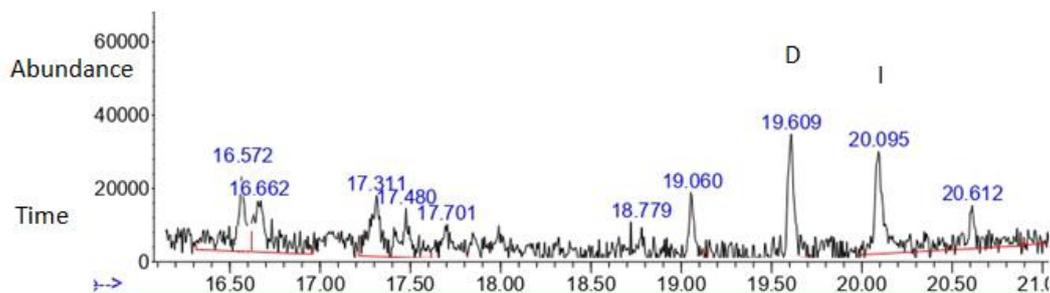
Gallic acid and quercetin standard curves were used for quantitative determinations. For the phenols, the interpolated concentration of the leaf extract was found to be 29.89 ppm. Therefore, Gallic acid equivalent per gram of leaf extract (GAE/g extract) is 1.499 mg and the interpolated concentration of the root extract sample was found to be 23.91 ppm. Therefore, Gallic acid equivalent per gram of root extract (GAE/g extract) is 1.195 mg.

For flavonoids, the interpolated concentration of the leaf extract sample was found to be 116.77 ppm. Therefore, quercetin equivalent per gram of leaf extract (QE/g extract) is 3.892 mg and the interpolated concentration of the root sample was found to be 65.55 ppm. Therefore, quercetin equivalent per gram of root extract (QE/g extract) is 2.185 mg.

#### Cat attractant identified using chromatography

In the GC-MS profile obtained for the leaf extract of *Acalypha indica*, cat attractants Iridomyrmecin or Dihydronepetalactone could not be identified.

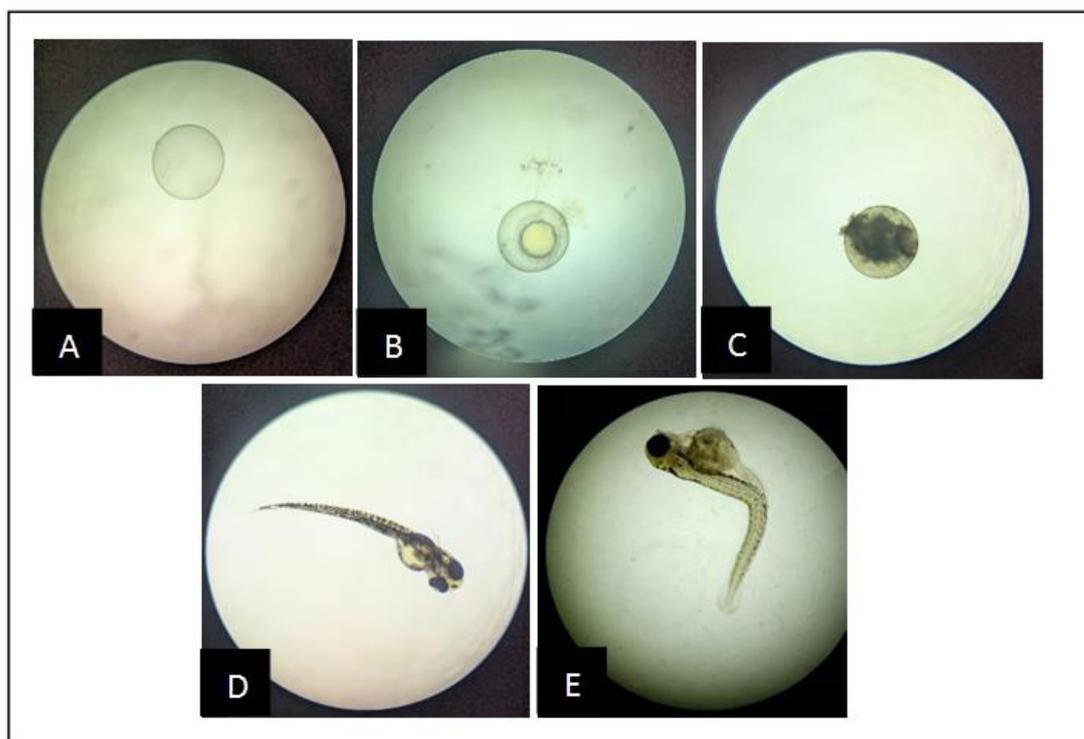
For the root extract, the first compound (Peak D) with a retention time of 19.609 minutes was in good agreement with the library spectrum of the NIST database, 2011 to be Dihydronepetalactone (NIST database, 2011) with a percentage total of 0.486%, as was the molecular formula of C<sub>10</sub>H<sub>16</sub>O<sub>2</sub>, as indicated by high-resolution mass spectrometry (HRMS) (Fig. 4). The second compound (Peak I) with a retention time of 20.095 minutes also had the formula C<sub>10</sub>H<sub>16</sub>O<sub>2</sub> and gave a good match to Iridomyrmecin (NIST database, 2011), identified with a percentage total of 0.577% (Fig. 4).



**Fig. 4: GC-MS profile of root extract of *Acalypha indica*. (D – Dihydronepetalactone, I – Iridomyrmecin)**  
**Results of the zebrafish embryo toxicity assay**

LC<sub>50</sub> was calculated to be 10.7098 (w/w) %. Abnormalities of zebrafish embryos until 96 hours - In addition to mortality rates, a variety of zebrafish

abnormalities were observed apart from the apical points in several concentrations during the observational period of 96 hours (Fig. 5).



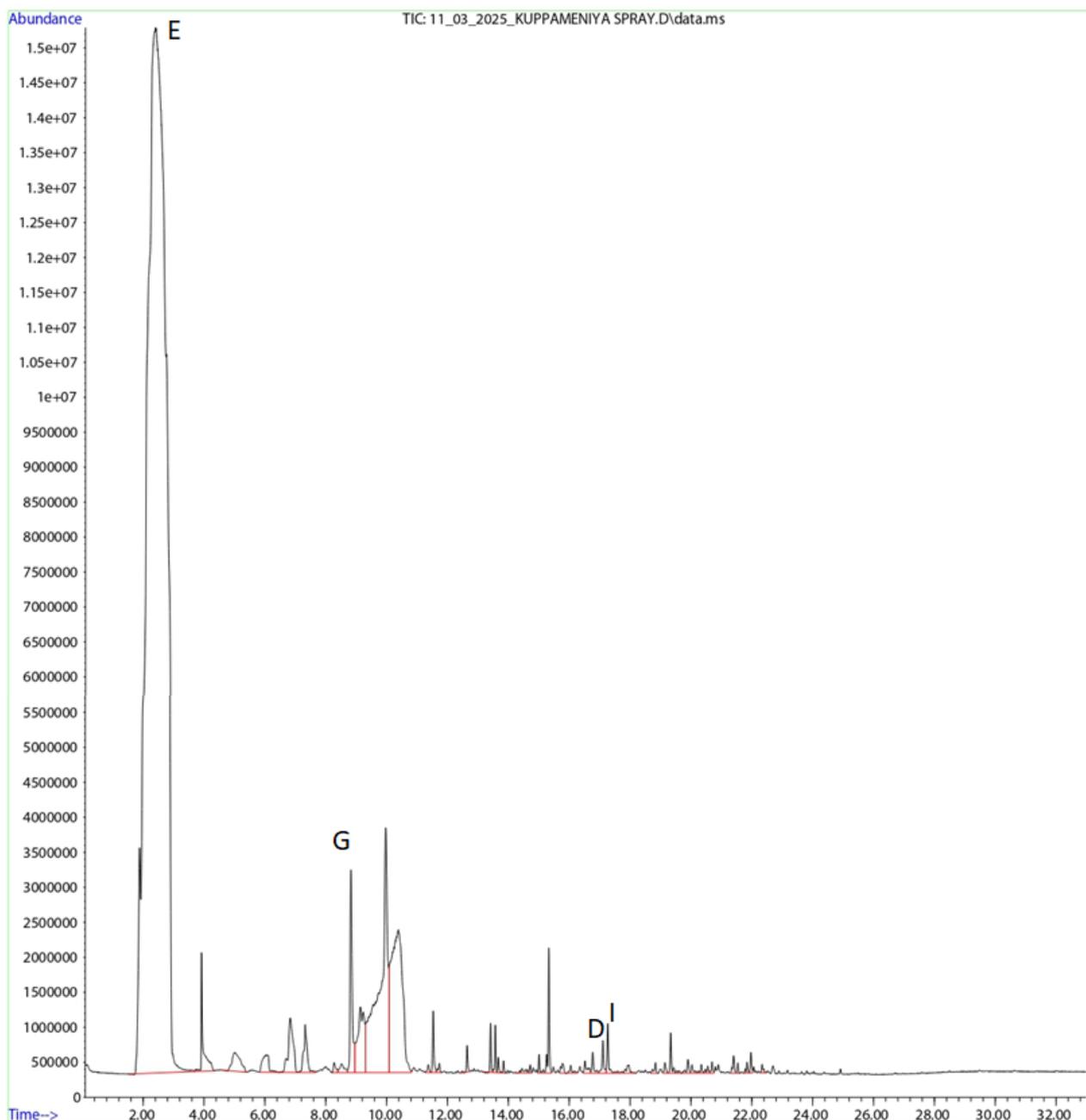
**Fig. 5: Abnormalities and apical points observed in zebrafish.**

*A – Lack of somite formation, B – No tail detachment, C – Coagulation, D – Bent tail, E – Bent tail with pericardial oedema.*

#### Marker-based short-term stability

In Fig. 6, obtained for formulation A, peak D with a retention time of 17.110 minutes was in good agreement with the library spectrum of the NIST database 2011, to be Dihydronepetalactone that had the formula C<sub>10</sub>H<sub>16</sub>O<sub>2</sub>, with a percentage total of 0.200% (w/v). The second compound (Peak I) with a retention time of 17.225 minutes also had the formula C<sub>10</sub>H<sub>16</sub>O<sub>2</sub> and

gave a good match to Iridomyrmecin (NIST database, 2011) with a percentage total of 0.283% (w/v).



**Fig. 6: GC-MS profile obtained by carrying out SPME for formulation A.**

*E – Ethanol, G - Glycerin, D – Dihydronepetalactone, I – Iridomyrmecin*

#### Physical parameters of the spray

The prepared spray solutions had good transparency and were yellowish-orange.

The following parameters in table 4 were used to evaluate the formulated sprays.

**Table 4: Physical evaluation of the spray formulations.**

Formulation	pH	Evaporation time	Stickiness of spray after evaporating the solvent
A	6.76	4 min 50 sec	No stickiness
B	6.46	5 min 04 sec	No stickiness
C	6.63	5 min 14 sec	No stickiness

*Extract strengths: A - Formulation with 3% (w/w), B - Formulation with 2.5 (w/w), C - Formulation with 2% (w/w)*

## DISCUSSION

Qualitative phytochemical analysis of both root and leaf extracts of *Acalypha indica* helped to identify the availability of commonly found phytochemicals. The standard tests carried out to identify alkaloids, phenols, flavonoids, terpenoids, tannins, phlobatanins, and steroids gave significant positive results to many screening tests.

Quantification of phenols and flavonoids was done for both the extracts of *Acalypha indica* root and leaves. Due to a lack of previous studies in this regard for *Acalypha indica*, standard methods available and followed in other studies were used.<sup>[17]</sup> Phenol gallic acid equivalent per gram of leaf extract (GAE/g extract) was 1.499 mg. In the root extract, it was 1.195 mg. Similarly, spectroscopic analysis for flavonoids, quercetin equivalent per gram of leaf extract (QE/g extract) was 3.892 mg and for root extract was 2.185 mg. Here, it was identified that flavonoids are available in higher concentrations in leaves than in roots.

In a previous study for the identification of cat attractants Iridomyrmecin and Dihydronepetalactone, solvents such as ethanol or dichloromethane were used for the extraction of *Acalypha indica* roots, leaving the extraction overnight. After filtering directly into a GC-MS vial, the sample was used for GC-MS analysis. That method was successful in identifying Iridomyrmecin and Dihydronepetalactone using the NIST database. 2011.<sup>[7,8]</sup>

In the current study, fresh roots and leaves were used as in the above study but methanol was used for extraction and was extracted for 48 hours. After evaporating methanol under reduced pressure, 100 ppm solutions of leaf and root extracts were made. Before filling the GC-MS vial, samples were treated with anhydrous Na<sub>2</sub>SO<sub>4</sub>, to remove any traces of water, since if water is present, it can damage the column. The samples ran through the column with similar parameters as used in the above study with slight modifications, with the ion source temperature at 250 °C and an initial wash of the column with methanol before injecting the sample.<sup>[7,8]</sup> With a retention time of 19.609 minutes and 20.095 minutes, it was possible to identify Iridomyrmecin and Dihydronepetalactone only in the root extract. This identification was also done using the NIST database.

The Zebrafish embryo toxicity assay was conducted under the OECD guidelines.<sup>[13]</sup> Standard curve was developed using 2.5% (w/w), 5% (w/w), 10% (w/w), 15% (w/w) and 25% (w/w) concentrations of the root extract dissolved in distilled water.

LC<sub>50</sub> with 50% mortality of zebrafish embryos was found to be 10.71% (w/w). Mortality percentage was determined by various apical points and abnormalities observed on zebrafish embryos.

Based on a review of 209 published papers, LC<sub>50</sub> (Lethal Concentration 50%) studies covering 96 different chemicals, using one-third (1/3) of the LC<sub>50</sub> value is a conservative estimate for non-lethal exposure levels. This means an exposure level that does not cause significant mortality in organisms upon inhalation.<sup>[18]</sup>

This means non-lethal and safe concentrations will be values less than 3.569% (w/w). Therefore, three test formulations were developed with 2% (w/w), 2.5% (w/w) and 3% (w/w) of *Acalypha indica* root extract.

In combination, ethanol 12% by weight and 5% glycerin by weight can be used as antimicrobial preservatives.<sup>[19]</sup> However, percentages can be varied upon further studies of the formulation. The Minimum Lethal Doses (MLD) of alcohols in cats showed that MLD for ethyl alcohol is 5.0 mL/kg.<sup>[20]</sup> Polyethylene glycol (PEG) is widely used as a fixative in sprays and air fresheners.<sup>[21]</sup> Another study confirms that cats can safely and palatably take PEG orally as a long-term laxative.<sup>[22]</sup> SPME Results showed there is Iridomyrmecin by 50.95% and Dihydronepetalactone by 58.84 % over a month after formulation. This may be because only basic excipients were used in the formulations. Several physical parameters were considered and tested for evaluating the final formulation with reference to previous studies.<sup>[16,23,24]</sup>

## CONCLUSION

Freshly obtained methanolic extracts of *Acalypha indica* leaf and root both contain alkaloids, phenols, flavonoids, terpenoids, tannins and steroids. Comparatively, the quantity of flavonoids and phenols in leaf extract is higher than in root extract according to the UV spectroscopic analysis data. The Iridomyrmecin and Dihydronepetalactone, which are cat attractants according to the literature, are available only in the root extract at 0.577% and 0.486% (w/w) respectively. Therefore, the root extract can be used for the formulation of a cat mood elevator.

LC<sub>50</sub> of root extract is 10.71% (w/w), determined by zebrafish embryo toxicity assay. Therefore, the final formulation was made with concentrations less than 1/3 of LC<sub>50</sub> which is considered the Minimum Lethal Dose (MLD).

According to marker-based short-term analyses, the reduction of Iridomyrmecin and Dihydronepetalactone is 50.95% and 58.84% respectively after a month of formulation. The stability can be radically improved with further modifications of the spray formulation for a marketable veterinary product.

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