THE EVALUATION OF ANTI-OXIDANT, ANTI-BACTERIAL AND WOUND HEALING ACTIVITY OF METHANOLIC EXTRACT OF *EICHHORNIA CRASSIPES* IN MICE FIBROBLAST CELL LINE

A PROJECT REPORT

Submitted by

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In partial fulfilment for the award of the degree

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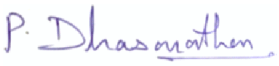
BONAFIDE CERTIFICATE

Certified that this project report **"THE EVALUATION OF ANTI-OXIDANT, ANTI-BACTERIAL AND WOUND HEALING ACTIVITY OF METHANOLIC EXTRACT OF *EICHHORNIA CRASSIPES* IN MICE FIBROBLAST CELL LINE"** is the bonafide work of **RAGHUNATH. M (Reg. No. 111416214027), PADMANABHAN. P. S (Reg. No. 111416214020)** who carried out the project work under my supervision.



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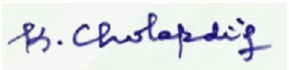
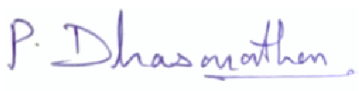


INTERNAL EXAMINER



EXTERNAL EXAMINER

DECLARATION

We hereby declare that the project report entitled **"THE EVALUATION OF ANTI-OXIDANT, ANTI-BACTERIAL AND WOUND HEALING ACTIVITY OF METHANOLIC EXTRACT OF *EICHHORNIA CRASSIPES* IN MICE FIBROBLAST CELL LINE (3T3)"** Submitted to the Department of Biotechnology, Prathyusha Engineering College, affiliated to the Anna University, Chennai, in partial fulfilment of the award of the degree Bachelor of Technology in Biotechnology is the record of the original work carried by us under the guidance of **Dr. A. J. A. RANJIT SINGH,** professor, Department of Biotechnology, Prathyusha Engineering College, during the period of December 2019 to March 2020. We further declare the results of the work have not been previously submitted for the award of any degree or diploma.

Place: Thiruvallur

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We solemnly express our earnest and humble thanks to our guide **Dr. A. J. A RANJIT SINGH,** Assistant professor, Department of Biotechnology, Prathyusha Engineering College and project coordinator, **Dr. Cholapandian,** Assistant professor, Department of Biotechnology, Prathyusha Engineering College for their guidance and valuable suggestions throughout our project work.

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ABSTRACT

Wound is the discontinutity or break in the surface of skin due to cuts, burns or due to pathological infections. Wound healing process can be effectively improved by shortening the healing time period and by avoiding pathological infection. In our study, Water hyacinth was found to serve as potential wound healers, by minimizing the healing time and inhibiting microbial infections, herein we aimed to assess the anti-oxidant and anti-bacterial effects of *Eichhornia crassipes* (Water hyacinth) and thereby evaluated the wound healing property of Water hyacinth in mice fibroblast cell lines by scratch assays. Air dried, powered Water hyacinth was used for extraction process with methanol. The methanolic extract of the Water hyacinth found to have anti-bacterial and anti-oxidant property, and wound scratch assay showed rapid migration of mice fibroblast cells from both the ends having 80.77% of wound healed within 76 hours. The results conclude by saying that, Water hyacinth plays a significant role in healing the wound. Thereby, effective way of minimizing the plant population in water bodies and making them used clinically.

Keywords: *Eichhornia crassipes*, anti-bacterial, anti-oxidant, wound scratch assay, wound healing.

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LIST OF ABBREVIATIONS

MEX	- Methanolic extract
DPPH	- 2, 2-diphenyl-1-picryl-hydrazyl-hydrate
MSP	- Mass spectrum
GC-MS	- Gas chromatography–mass spectrometry
FAO	- Flavin-containing anime oxidases
MIC	- Minimal inhibitory concentration
DMSO	- Dimethyl sulfoxide
MTT	- 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
CuAO	- Copper anime oxidases
H2O2	- Hydrogen peroxide
CFU	- Colony-forming unit
sp.	-Species
E. coli	- Escherichia coli
E. cloacae	- Enterobacter cloacae
P. aeruginosa	- Pseudomonas aeruginosa
E.crassipes	- Eichhornia crassipes
NaH2PO4	- Monosodium phosphate
Na2HPO4	- Disodium phosphate
NaCl	- Sodium chloride
ZOI	-Zone of ionhibition

AIM OF THE PROJECT

To evaluate the anti-oxidant and anti-bacterial property of E. crassipes (water hyacinth), and investigated the wound healing activity of Water hyacinth in vitro in Fibroblast cell lines using in-vitro wound scratch assay.

OBJECTIVES OF THE PROJECT

i. To extract the phytochemicals of water Hyacinth (*E. crassipes*) using methanol as solvent by cold percolation method.

ii. To evaluate the anti-bacterial activity of MEX of *E. crassipes.*

iii. To evaluate the anti-oxidant property of *E. crassipes*.

iv. To ensure the biocompatibility of MEX of *E. crassipes* in Fibroblast cell line (3T3).

v. To evaluating the wound healing rate using wound scratch assay.

vi. To screen and isolate the bioactive compunds and determine the composition of each components present in the MEX using GC-MS analysis method.

CHAPTER 1

INTRODUCTION

1.1 WOUND:

The incidence of accidents has steeply risen in this modern age, which is responsible for the different types of wounds. A wound is the anatomic discontinuity or cellular disruption which may occur due to accidents, cuts, burns or pathological infection. (Prafulla et.al., 2012). If wounds were not healed, it may activate many inflammatory mediators and may cause severe pain, which will brings about physical and mental illness to patients (Badri et.al., 2011). Unhealed wounds may develop in chronic wounds, which also lead to organ damage. Healing of wounds (i.e. process of repair) partly dependes on the depth of wounds, as well as the overall health and nutritional state of the individual.

1.2 TYPES OF WOUNDS

Based on their aetiology, location, type of injury or presenting symptoms, wound depth and tissue loss or clinical appearance, a wound can be classified as open and closed wound on underlying cause of wound creation; acute and chronic wounds on the physiology of wound healing (Prafulla et.al., 2012).

1.2.1 Open Wounds

Here, the blood escapes the body and there will be clear visible bleeding. Open wounds are further classified as: Incised wound, Laceration or tear wound, Abrasions or superficial wounds, Puncture wounds, Penetration wounds and gunshot wounds (Strodtbeck et al 2001).

1.2.2 Closed wounds

In closed wounds blood escapes the circulatory system, but remains in the body. It includes Contusion or bruises, heamatomas or blood tumor, Crush injury etc..

1.2.3 Acute wounds

Acute wound is a tissue injury that normally precedes through an orderly and timely reparative process that results in sustained restoration of anatomic and functional integrity. Acute wounds are usually caused by cuts or surgical incisions and complete the wound healing process within the expected time frame (Kumar et al 2007).

1.2.4 Chronic wounds

Chronic wounds are wounds that have failed to progress through the normal stages of healing and therefore enter a state of pathologic inflammation chronic wounds either require a prolonged time to heal or recur frequently (Kumar et al 2007; (Robert et al 1998)

1.3 WOUND HEALING

Wound healing is a biologically sequential event that takes place at the wound site in the name of four steps: haemostasis phase, inflammatory phase, proliferative phase and maturation phase.

1.4 PHASES OF WOUND HEALING

- **Haemostasis phase** is the first and fore most phases, take place within seconds when wound created. The blood vessel gets constricted and platelets stick on to the walls of the vessels, thus forming coagulation.

- In **Inflammatory phase**, where the transudate (made of water, salt and protein) leaks from the blood vessel wall which causes swelling. Swelling, pain, heat and redness takes place during inflammatory phase.
- **Proliferative phase**, here the wounds get rebuild with the new tissue and extra cellular matrix. And the wounds are kept moist and hydration takes place, so which epithelialization happens fast.
- **Maturation phase,** the last phase in wound healing where the type III to type II collagen remodalization takes place. The cross-linking of collagen reduces scar thickness and makes wounded skin area stronger. Consequently, the proper wound healing and scar formation get delayed if inflammatory response elongated or exacerbated (Van-Linh Nguyen et. al., 2017)

1.5 WATER HYACINTH [*E. crassipes*]

Figure 1.1 *E. crassipes* (Water Hyacinth)

1.5.1 THE SYSTEMIC POSITION:

Kingdom : Plantae

Family: Pontederiaceae

Order: Commelinales

Genus: Eichhornia

Species: crassipes

Common name: Water hyacinth

1.5.2 GEOGRAPHICAL DATA

E. crassipes, commonly known as water hyacinth, is native to the Amazon Basin in tropical South America, its entry into Africa, Asia, India, Australia, Central America North America (California and southern states) and New Zealand was largely facilitated by human activities. Its one of the world's most noxious aquatic weed. Lack of natural enemies together with nutrient enriched water bodies; continues to spread aggressively throughout temperate, tropical and sub-tropical climates (Dagno, Lahlali, Friel, Bajji, & Jijakli, 2007).

1.5.3 HABITUAL CONDITIONS

After first introduced into Bengal around 1896 as an ornamental plant, it has spread throughout India and occupies over 200,000 ha of water surface. It now occurs in all fresh water ponds, tanks, lakes, reservoirs, streams, rivers and irrigation channels. *E. crassipes* stabilizes pH levels and temperature in lagoons thereby preventing stratification and increasing mixing within the water column. It can tolerate pH values from 4 to 10. Optimal water temperature for growth is 28-30^{o}C while optimal air temperature is 21-30^{o}C. The plant mass doubles in two weeks if water temperature lie between 27^{o}C and 33^{o}C. Temperatures above 33^{o}C inhibit further growth (Center, Hill, Cordo, & Julien, 2002). Water hyacinth tolerates drought well because it can survive in moist sediments up to

several months (Center, Hill, Cordo, & Julien, 2002).Salinity is the main obstacle for growth of water hyacinth in coastal areas (Evans, 1963; De Groote, Ajuonua, Attignona, Djessoub, & Neuenschwandera, 2003).

1.5.4 CHARACTERISTICS

Water hyacinth has the following systematic, morphological, developmental, biological, and ecological characteristics (Gopal, 1987):

• A perennial plant whose average size is 40 cm but can reach up to 1 m high;

• Have a high rate of vegetative growth and multiplication;

• Produce seeds that remain viable for very long periods;

• Have a fairly wide ecological amplitude;

• Stems and leaves contain air-filled tissue which gives the plant its considerable buoyancy;

• Double its population in 15-18 days by asexual vegetative reproduction;

• Each mother plant produces 4 daughter plants which are capable of reproduction after 2 weeks;

• Growth is highly enhanced in nutrient-rich, eutrophic water bodies with high nitrate and phosphate content;

• Seeds can remain viable for up to 15 years;

• Have no known natural enemies for the seeds;

• It grows in mats up to 2 m thick; and

• When mature, it consists of long, pendant roots, rhizomes, stolons, leaves, inflorescences and fruit clusters.

1.5.5 EFFECT ON ENVIRONMENT

The weed has inflicted enormous negative effects not only on the environment but also on the health status and well-being of many people who seek livelihood from the infested waters and the economy in general (Dagno, Lahlali, Friel, Bajji, & Jijakli, 2007). Photosynthesis is limited beneath water hyacinth mats, and the plant itself does not release oxygen into the water as do phytoplankton and submerged vegetation, resulting in decreased dissolved oxygen concentration (Dagno, Lahlali, Diourte, & Haissam, 2012).Worryingly, climate change may allow the spread of water hyacinth to higher latitudes (Rahel & Olden, 2008; Patel, 2012).

1.5.6 EFFECT ON HUMANS

E. crassipes cause major public health problems such as malaria, schistosomiasis and lymphatic filariasis by presence Some species of mosquito. These plants also create prime habitat for mosquitos, classic vectors of disease, and a species of snail known to host a parasitic flatworm which causes schistosomiasis. It interferes with the production of hydro-electricity, blocks water flow in irrigation projects (40 to 95% reduction), prevents the free movement of navigation vessels, interferes with fishing and fish culture and facilitates. The weed is responsible for great water loss (1.26 to 9.84%) due to evapo-transpiration from the luxuriant foliage of water hyacinth (Sushilkumar 2011). So far, not even a single successful mycoherbicide has been employed against any aquatic weed in India in spite of many reports of fungal pathogen infesting many aquatic weeds severly (Aneja et al. 1993, Kauraw and Bhan 1994, Ray et al. 2008b). Ray et al. (2008c) studied the combined impact of various pathogens for integrated management of E. Crassipes (Mart.) Solms. Therefore, eradication of the weed is highly advocated all over the world

CHAPTER 2

REVIEW OF LITERATURE

Rupesh Thakur *et al.,* **(2000)** Study described about the creation of wounds on animal by using appropriate surgical equipment and handling of animals before the testing of animals. The surface of the animal which was to be handled must be shaved and then it was to be anasthesized by using suitable chemicals by using chloroform/ether and and wound was to be created with the help of surgical puncher and proper testing must be carried out.

Alessandra Cona *et al.,***(2008)** had performed the wound healing activity in plants by identifying the reaction of chemical such as CuAO and FAO and H_2O_2 producing enzymes which are primarily responsible for the oxidative de-amination of polyamines. These apoplastic anime oxidases are found to have a key role in plants as they tend to behave as a H_2O_2 delivering systems in the cell wall during cell growth and cell differentiation in the context of host-pathogen interactions. The H_2O_2 also plays a key role as a signalling molecule in defence mechanism and also acts as a co-substrate for peroxidase driven reaction during the process of cell-wall maturation. Experimental evidences also denote that PAO performs wound healing activity in *Zea mays.* It was also found that the same apoplastic PAO was found to have similar effect in *Nicotiana tabacum.* These wound healing activity was found to be decisive in some of the plants.

Chi-Bao Bui *et al.,***(2017)** have predicted the wound healing and anti-inflammatory activity by usimg calophyllolide-a major constituent from *Callophyllum inophyllum* which is reported to have anti-inflammatory, anti coagulant, anti-bacterial and even anti-cancer activities. In this process the isolated callophyllolide was made to test on HaCaT and RAW264.7 cell viability and was tested by MTT assay. It is found that the calophyllolide was found to have no effect on cell viability and also reduced the fibrosis formation

and effectively resulted in would closure in animal models without any weight loss. The calophyllolide was also checked whether it has the property of accelerating the process of wound healing through anti-inflammatory activity mechanism. From those findings it is shown that the calophyllolide have a effect of wound healing in experimental models.

Kofi Annan *et al.,***(2008)** performed the anti-oxidant and anti-bacterial activity of *Ficus asperifolia* and *Gossypium arboreum* by preparing their extracts. The extracts were prepared by collecting 30g of the sample and was introduced onto an cellulose thimble and was extracted for 48 hours. The anitoxidant and anti-bacterial assays were also performed. Microorganism innocula was prepared from the 24hr Mueller-Hinton broth and the suspensions were adjusted to 10^5 CFU. The Minimal Inhibitory Concentration(MIC) was predicted based on an micro-well diffusion method. Anti-oxidant assay was predicted on fibrobast cell lines in which the cells were treated with different concentration of extracts overnight and then they werr exposed to 10^{-4}M hydrogen peroxide and further incubated for 3 hours at 37^{o}C. Catalase was used as a positive control throughout the process. The cell lines were then stained with neutral red and was observed for any damage in the cell lines.

Lata Nuka et al., **(2010)** had used the plant methanolic extract of *E. crasspies* to predict the wound healing activity of water hyacinth. In this process the wound healing properties were tested on rats by preparing a gel matrix (10% and 15% of extract with the gel matrix ointment) and the wound healing activity was compared with respect to regular ointment. The gel matrix suspended with the plant extract had shown greater activity when compared to the standard (regular ointment) and with increase in concentration increases its effect considerably higher than before.

Venapani Dubey *et al.,* **(2010)** have identified the phytochemical properties and therapeutic activities of *E. crassipes.* The collected samples were powdered and soaked in distilled water for 12 hours. The extracts were filtered using whatman filter paper and used for tests. They have tested the plant samples tannins, pholobatannin, saponin, steroid, terpenoid, alkaloid,flavanoid, phenol,quinone, anthraquinone and cardiac glycosides. All the tests that have been performed showed positive results predicting the presence of such phytochemicals in *E. crassipes*.

Renu solanki *et al.,* **(2011)** had explained about the scaling up of plant extracts if they were to be developed into an novel product. One of the methods includes the development of gel matrix in which the plant extract was entrapped. The gel matrix was made up of 15% w/w polyethylene glycol-150,then 37.4-45% w/w ethoxydiglycol, 1-6% w/w polysorbate surfactant and a biocompartible liquid.

Ranjit singh *et al.,* **(2012)** explained about the preparation of methanoloic extract of *Desmodium gyrans* in which the shade dried leaves leaves were extracted with the help of soxhlet apparatus and then the extracts were evaporated in rotary vacumn evaporator and from the extracted samples, the antimicrobial and phytochemical screenings were performed. The wound healing studies were performed on *Orchtolacus sp* in which a wound was created and 0.5g of neomycin was added. In one rabbit, 1g of *Desmodium gyrans* was used to treat as it was applied over the region where the wound was created and it was observed for 7 days. The extract showed excellent activity in clearing the wound.

Abhay K. Pandey *et al.,* **(2014)** performed the invitro anti-oxidant, anti-bacterial and cytotoxic activity and invivo effect of leaf extracts of *E. crassipes* and *Syngonium podophyllum.* The phenolic contents of the prepared extracts were determined by dissolving the extracts with DMSO solution and the absorbance

was measured at 650nm against blank using spectrophotometer and the antimicrobial activity was performed against *Bacillus cerus, Streptococcus mutans, Proteus vulgaris* and *Salmonella typhi.* The plates were then saturated using DMSO and was incubated at 37ºC for 24 hours. Ampiciilin was used as positive control and the growth were recorded. The plant extract showed excellent inhibitory activity similar/more than the control that was used.

Lalitha et al., **(2014)** experimented on predicting the antimicrobial activity of E. crassipes. The ethyl acetate extract of E. crassipes was prepared and the extract was initially subjected to sonication, dipping and homogenization for 1hour and the anti-bacterial studies was initially performed on Corneybacterium strain. The antimicrobial studies were done by using disk diffusion method in which a sterile swab was introduced onto an previously developed inoculum and they were left to dry at room temperature with their lid closed. The plate was tehn divided into two quadrants in which the extracted samples were added on one column and ciprofloxacin was added to other column. The plates were then placed on refrigerator at 4ºC for 1hour and then incubated at 37ºC for 24 hours. The ZOI was then measured. No bacterial growth was observed on the extract coated sample thereby showing its antimicrobial ability.

F.Khosravitabar *et al.,***(2017)** have performed the extraction of *Achillea eriophora* by the performing the process of maceration in which the grinded dried leaves of plants were extracted with methanol (1:20 W/V) and the extract was then filtered using normal filter paper and then it was made to evaporate under vacuum to become a powdered extract.

Tyagi Tulika *et al.*, **(2017)** The studies that they performed on the plant *E. crassipes* have found that the plant had various phytochemical properties such as phenoloic compounds, alkaloids sterols and glycosides respectively. Also by performing paper disk diffusion assay, they have also found that the plant

species tend to have high antimicrobial activity and it is also found that the MEX of *E. crassipes* tend to show better wound healing activity in which the extracts were developed into an ointment (10% and 15% w/w of leaf extract in the ointment) and was investigated in animal model (rat) and it tend to show good wound healing activity.

Samuel Mesfin** et al.,(2018)** worked on various phtochemical tests for checking the phytochemical properties of the *E. crassipes.* The tests include wagner's test, Alkali reagent test, Ferric chloride test, Foam test, Chloride test, Tannins test to predict the phytochemical activity of the *E. crassipes.* The results were found to be positive on all the tests showing such phyochemicals were present within the plant system with various therapeutic properties.

***Agung Krismariono** et al.,* **(2019)** performed the anti-bacterial activity of *E. crassipes* by preparing the ethanoloic extract of the leaves by the process of maceration and the anti-bacterial activity is identified. Inhibition activity test was performed in which serial dilution of plant extracts were performed on various concentrations ranging from 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56% and 0.78%. In each test tube 1ml of *Aggregatibacter actinomycetemcomitans* was added and was incubated at 37ºC for 24 hours. It was then streaked with MHA agar and then incubated for further 24 hours. In the concentrations of 100%, 50%, 25%, 12.5% and 6.25%, there was no or less growth of bacteria in that concentration thereby showing excellent anti-bacterial activity.

***Abira Khan** et al.,* **(2018)** performed antimicrobial efficiency and phytochemical analysis of three aquatic plants namely *E. crassipes, Pistia stratiotes* and *Spirodela polyrrhiza.* The extract of *E. crassipes* was prepared from isolating the dried leaves of the sample and and was prepared in 95% ethanol and 95% ethyl acetate(20g of each in 200mL solvent) and filtered using

whatman filter paper and the extracts were finally concentrated using rotary evaporator and then store at -10^{o}C. The antimicrobial assay was performed against *Staphylococcus aureus*, *Salmonella typhi* and *Lactobacillus spp* and compared their efficiency against standard antibiotics and Well diffusion test was done to confirm the results. The inhibitory effects of ethanol and ethyl acetate extracts was calculated by measuring the activity index.

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1SAMPLE COLLECTION:

E. crassipes an aquatic weed was collected during the month of December, 2020 at madhuravoyal , chennai. The leaves of *E. crassipes* were cut removed from the stem . They were washed, shade dried and grinded to powered using mortar and pestle.

Figure 3.1 Leaf- Cut Removed

Figure 3.2 Leaf- Shade Dried and Powdered

3.2 PREPARATION OF EXTRACTS:

The phytochemicals were extracted using Cold Percolation technique **(Kalirajan et al., 2012)**. Powered test sample of 30g were taken in an conical flask and absolute methanol (gifted from Cavins care pvt. Ltd) was added and kept in a orbital shaker at 37 ° for 3 days. The extract was filtered using whatman filter paper and stored for later use.

Figure 3.3 Filtered Extract

Figure 3.4 Stored Extract

3.3 PHYTOCHEMICAL TEST ASSAY:

The phytochemical test were conducted with reference to the work by (Sahira Banu et al., 2015)

3.3.1 ALKANOIDS (Wagner's test):

1ml of the test extract and 2ml of Wagner's reagent (1.27g of iodine +2g of potassium iodide) was added in a test tube. Appearence of reddish brown colour indicates the presence of alkaloids.

3.3.2 CARBOHYDRATE (Fehling's test):

1ml of extract was boiled with fehlings solution A & B. The formation of red precipitate indicates the presence of carbohydrate.

3.3.3 GLYCOSIDES (Born trager's test):

2ml of test extract was added to 3ml of chloroform and once the chloroform layer separated, 10% ammonium solution was added. The appearence of pink colour indicates the pink colour.

3.3.4 SAPONINS (foam test):

1 ml of test extract was added to 2 ml of didtilled water. Formation of foam indicates the presence of foam.

3.3.5 PROTEINS (Biuret test):

To the 2 ml of test extract few ml of 2% copper sulfate solution was added, followed by addition of 1 ml ethanol along with potassium hydroxide pellets. Appearance of pink colour on ethanolic layer indicates the presence of proteins.

3.3.6 AMINO ACIDS: (Ninhydrin test):

2ml of test extrct was added to 2ml of ninhydrins (10 mg ninhydrin in 200 ml acetone). Appearance of purple colour indicates the presence of amino acids.

3.3.7 PHENOLIC COMPOUND (Ferric chloride test):

2 ml of distilled water and few drops of 5% ferric chloride solution was added to 1 ml of extract. Appearance green colour indictes the presence of phenolic compunds.

3.3.8 STEROIDS:

2 ml of chloroform was added to 1 ml of test extract, followed by addition of few drops of acetic acid and concentrated sulfuric acid. Appearance of blue green colour indicates the presence of steroids.

3.3.9 FLAVANOIDS:

1 ml of test extract was added to 2 ml of ammonia. Appearance of yellow colour indicates the presence of flavonoids.

3.4 ANTI-BACTERIAL ASSAY:

The anti-bacterial activity of the sample was tested using agar well diffusion assay against the Gram-positive bacteria, such as *Enterococcus sp.* and Gram-negative bacteria, such as Pseudomonas *aeruginosa, Enterobacter cloacae , Escherichia coli*. The pathogenic strains were obtained from wound and pus of patients, billroth hospital, Chennai. The freshly cultured pathogens were swabbed on nutrient agar plates and five equidistant wells were made using a sterilized cork borer. The wells were loaded with different concentrations (25, 50, 75, 100 µL) of sample. Gentamycin was also added in separate well as positive control. The plates were incubated at 37°C for 24 hrs and the zone of inhibition (ZOI; mm) appearing around the wells was recorded (Mosachristas et al., 2018).

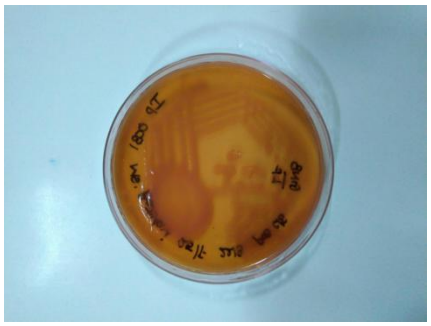


Figure 3.5 *E. cloacae*

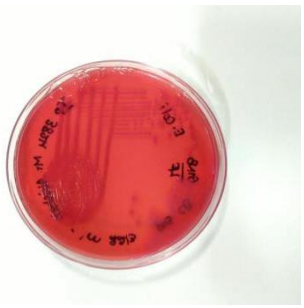


Figure 3.6 *E. coli*

Figure 3.7 *Enterococcus sp.*

Figure 3.8 *P. aeruginosa*

3.5 ANTI-OXIDANT ASSAY:

3.5.1 DPPH radical scavenging assay

In order to evaluate the anti-oxidant potential through free radical scavenging by the test samples, the change in optical density of DPPH radicals is monitored. According to Manzocco et al. (1998), the various concentrations of sample extract were mixed with 2 mL of DPPH solution (4 mM) and left in dark at room temperature. After 30 min, the absorbance is measured at 517 nm. The percentage of the DPPH radical scavenging is calculated using the equation as given below:

% inhibition of DPPH radical = Abs control – Abs sample x 100 / Abs control.

3.6 ANTI-INFLAMMATORY ASSAY:

3.6.1 Protein denaturation inhibition assay

Protein denaturation assay was done as decribed by (Jayasuriya et al., 2017) Egg albumin was the protein sample used here. In 1000 µL of methanolic extract, 200 µL of egg albumin and 1400 µL of phosphate buffered saline were added and incubated for 37 ° C for 15 min. Instead of methanolic extract, distilled water was used as negative control and ibuprofen (Brufen, Abbott India Ltd.) was used as positive control. The final mixture was heated at 70° C for 5 min in a preheated water bath. After cooling, the absorbances were measured at 660 nm.

$$\% \text{ Inhibition of denaturation} = (1\text{-}A1/A2) \text{ X } 100$$

Where A1 = absorbance of sample (test sample or positive control) and A2 = absorbance of negative control.

3.6.2 Heat induced hemolysis assay

i. Preparation of Erythrocyte Suspension: Erythrocyte suspension was prepared according to the method described in (Shin de et al., 1999), with some modifications. Whole human blood was collected from a healthy human subject. The blood was centrifuged at 3000 rpm for 5 min in heparinized centrifuge tubes, and washed three times with equal volume of normal saline (0.9% NaCl). After the centrifugation, the blood volume was

measured and reconstituted as a 10% (v/v) suspension with isotonic buffer solution (10 mM sodium phosphate buffer pH 7.4). Composition of the buffer solution (g/L) used was NaH2PO4 (0.2), Na2HPO4 (1.15), and NaCl (9.0).

ii. This test was carried out as described by (Gunathilake et al., 2018). Briefly, 0.05 mL of blood cell suspension and 0.05 mL of methanolic extracts of leaves were mixed with 2.95 mL phosphate buffer (pH 7.4). The mixture was incubated at 54 ◦C for 20 min in a shaking water bath. After the incubation, the mixture was centrifuged (2500 rpm for 3 min), and the absorbance of the supernatant was measured at 540 nm using a UV/VIS spectrometer (Optima, SP-3000, Tokyo, Japan). Phosphate buffer solution was used as a control for the experiment. The level of haemolysis was calculated using the following equation based on the (Okoli et al., 2008):

$$\% \text{ inhibition of hemolysis} = 100 \times (1 - A2/A1),$$

where A1 = absorption of the control, and A2 = absorption of test sample mixture.

3.7 GAS CHROMATOGRAPHY-MASS SPECTROMETRY:

The crude extract of E. crassipes was sent to Sophisticated Instrumentation facility, School of advanced sciences, Chemistry division, VIT university, Vellore for GC-MS analysis. During GC-MS analysis, the test extract was injected into a GC which volatiles the sample, then seperates the various components based on its size and polarity. The separated components then go into mass selective detector and the produced spectrum was correlated with standard reference libraries for the identification of the components (Jennifer Mathias, 2014).

3.7.1 GC-MS INFORMATION:

Make : Perkin Elmer
GC model : clarus 680
Mass Spectrometer : clarus 600 (EI)
Software : TurboMass ver 5.4.2
Library year : NIST-2008

3.7.2 INSTRUMENT ACQUISITION PARAMETERS:
Oven: Initial temp 60°C for 2 min, ramp 10°C/min to 300°C, hold 6 min,
Total Run Time: 32.00 mint
InjAauto=260°C, Volume=1 μL, Split=10:1,
Flow Rate: 1 mL/mint
Carrier Gas=He,
Column=Elite-5MS (30.0m, 0.25mmID, 250μm df)

3.7.3 MASS CONDITION (EI):
Solvent Delay=2.00 min,
Transfer Temp=230°C,
Source Temp=230°C,
Scan: 50 to 600Da.

3.7.4 GC-MS analysis:
The Clarus 680 GC was used in the analysis employed a fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250μm df) and the components were separated using Helium as carrier gas at a constant flow of 1 ml/min. The injector temperature was set at 260°C during the chromatographic run. The 1μL of MEX of *E. crassipes* was injected into the instrument. The oven temperature was initially held at 60°C for 2 min, followed by an increase in temperature at the rate of 10°C min−1 till it attained 300°C, where it was held for 6 min. The mass detector conditions were set with transfer line temperature at 230°C, ion source temperature at 230 °C; and ionization mode electron impact at 70 eV, along with a scan time of 0.2 sec and scan interval of 0.1 sec. The fragments from 40 to 600 Da were analysed. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2008) library.

3.8 WOUND HEALING ASSAY:

3T3 Swiss albino mice embryo cells were seeded in 6-well plates (8×10^5 cells/well) and grown until reached a confluence of 90%, in the optimum culture conditions. In the middle of cell monolayer, a scratch was made by a P10 pipette tip, to mimic a wound, and cell debris were removed by washing with fresh medium. The wound was exposed for 50 µg/mL of MEX of *E. crassipes* for 24-72 hours at 37°C in a humidified atmosphere of 5% CO_2. The cell grown in extract-free medium were used as control. Scratch wound closure was analyzed under the inverted microscope (Magnus INVI, Noida) equipped with a digital CCD camera, by acquiring digital images at different time 0^{th} (T0), 2^{nd} (T1), 3^{rd} (T2) and 4^{th} (T3) days (static imaging). In the static imaging modality, the closure of the scratch was quantified, as recently described (Felice *et al*., 2015), by measuring the difference between the wound width at T0 and T1/T2/T3, using the ImageJ processing software [http://rsbweb.nih.gov/ij/].

CHAPTER 4

RESULTS AND DISCUSSION

4.1 PHYTOCHEMICAL TESTS:

The phytochemical test were conducted, according to Sahira Banu et al (2015). The observations were recorded and tabulated as below. Similar test results were found in the phytochemical test conducted by Tyagi Tulika et al**.** (2015)**,** where they additionally got positive observations for Alkaloids and Terpeniods test with MEX of *E. crassipes.* Also the presence of Phenolic and Flavonoid compounds may support the fact that the plant extract may be used of its medicinal value.

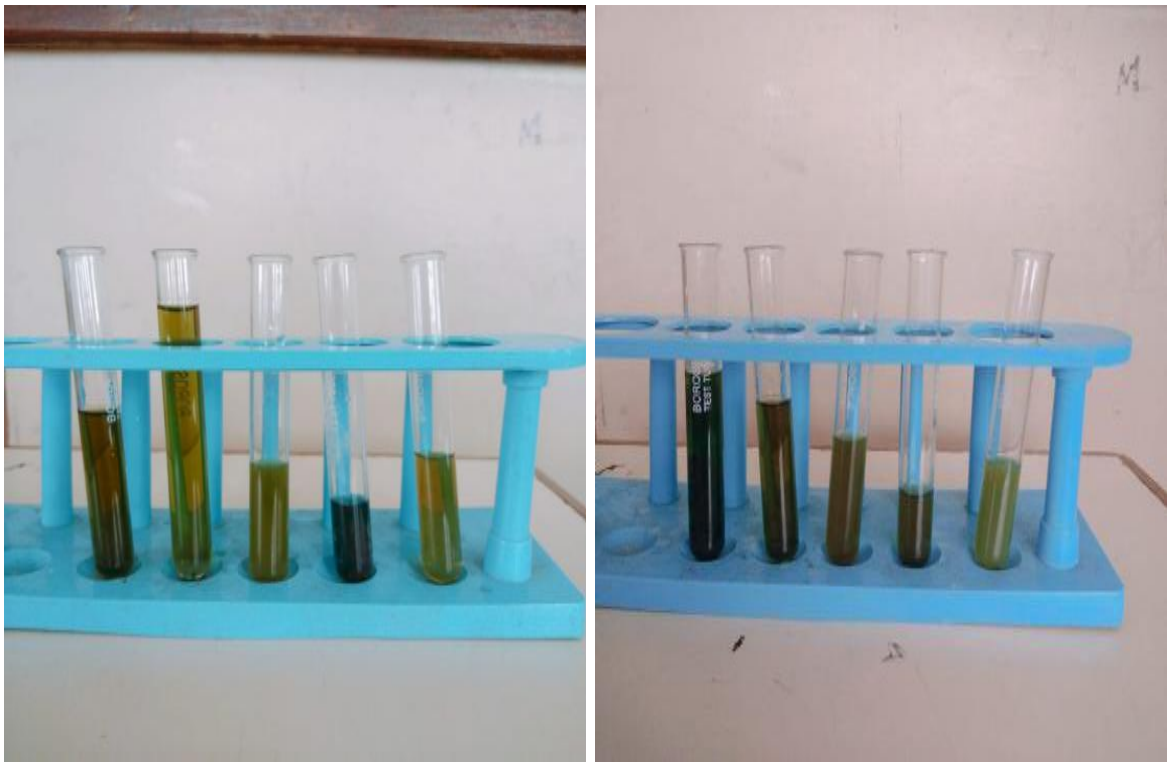


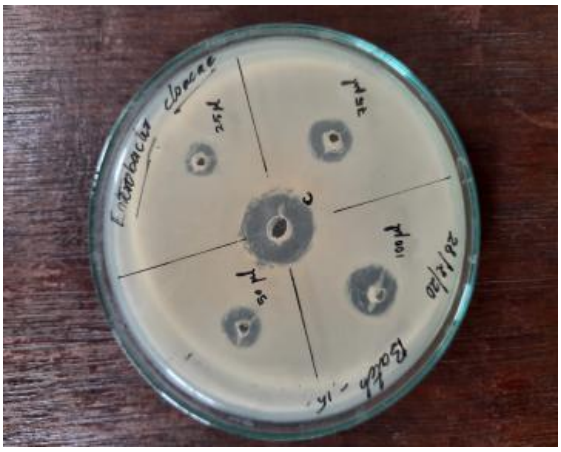
Figure 4.1 Phytochemical Test

Table 4.1 Phytochemical estimation of methanolic extract of *E. crassipes*.

S. No	Phytochemical	E. crassipes
1	Alkanoids	-
2	Carbohydrate	+
3	Gylcosides	-
4	Saponins	+
5	Proteins	-
6	Amino acids	-
7	Phenolic compounds	+
8	Steroids	+
9	Flavonids	+
10	Terpenoids	-

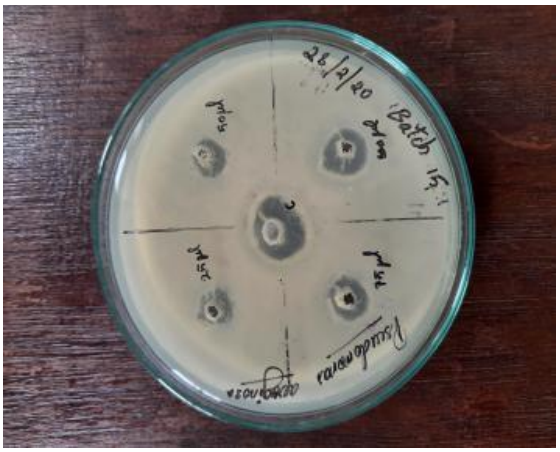
4.2 ANTI-BACTERIAL ASSAY:

The bacterial plates after 24 hours from plating and adding the extract through well diffusion, were observed to measure the ZOI created by the extract of *E. crassipes* against the bacterial strains. The extract shows more inhibition to *P. aeruginosa* and *Enterococcus sp* than to *E. cloacae* and *E. coli.* The zones of inhibition were measured in mm and were tabulated as in table 4.2.



(a) *E. cloacae*

(b) *Enterococcus sp*



(c) *P. aeruginosa*

(d) *E. coli*

Figure 4.2 ZOI of methanolic extract of *E. crassipes* against (a) *E.cloacae*, (b) *Enterococcus sp*., (c) *P.aeruginosa* and (d) *E.coli*.

TABLE 4.2 Representing the Zone of inhibition of various bacterial strains treated against MEX of *E. crassipes.*

Organisms	ZOI (mm in diameter)				
	25 µl	50 µl	75 µl	100 µl	Positive Control
P. aeruginosa	06	10	13	15	22
E. coli	-	-	03	04	20
E. cloacae	04	06	07	09	20
Enterococcus sp.	04	09	10	14	16

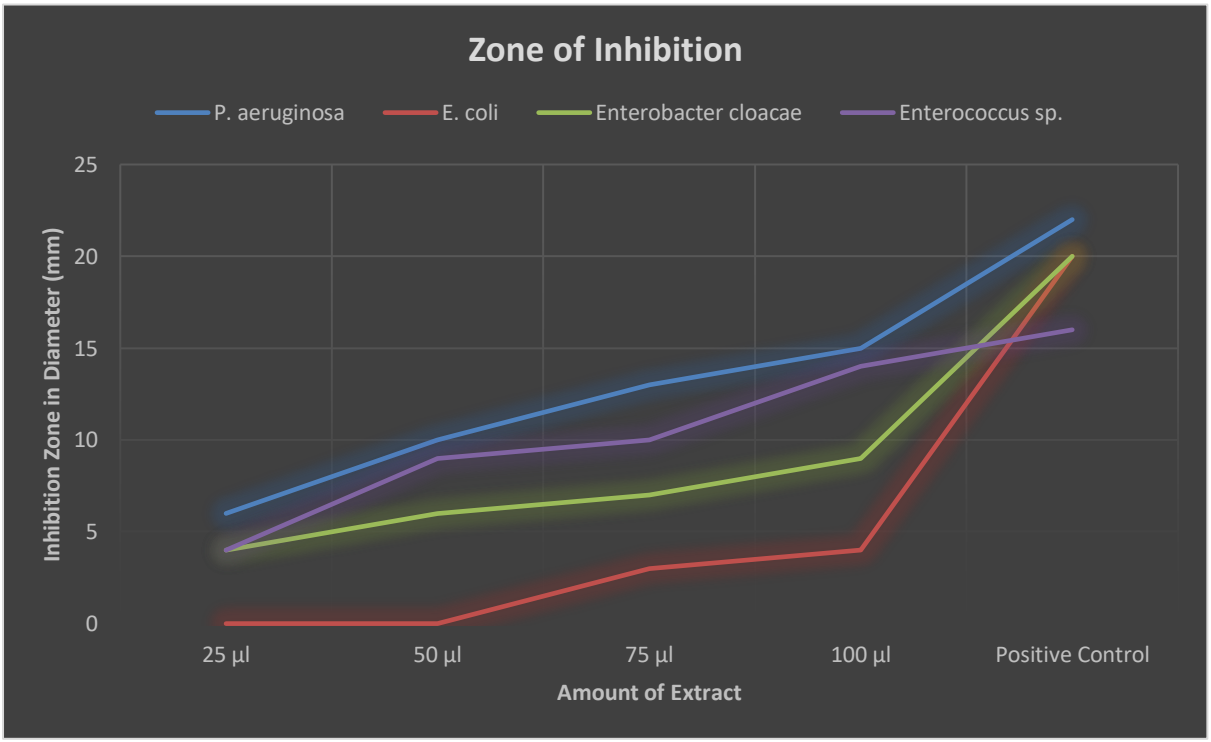


Figure 4.3 ZOI of various bacterial strains treated against MEX of *E. crassipes*

4.3 ANTI-OXIDANT ASSAY:

E.crassipes have well known anti-oxidant property that ensure its medical importance for using it for skin diseases and wound healing. It was found that Gallic acid (phenolic) and Rutin (flavonoids) were rich in *E. crassipes,* (**Ganesh et al., 2018)** which is responsible for their anti-oxidant and wound healing properties. Also found that, Hydroponical exposure of *E.crassipes* to various concentration of Ag, Cd, Cr, Cu, Hg, Ni, Pb And Zn for 21 days may positively induce their anti-oxidant property. From the test, it was observed that 20, 40, 60, 80 and 100 (µg/mL) could have inhibition to oxidation about 9.80, 19.65, 41.65, 64.48 and 81.04 (%) respectively.

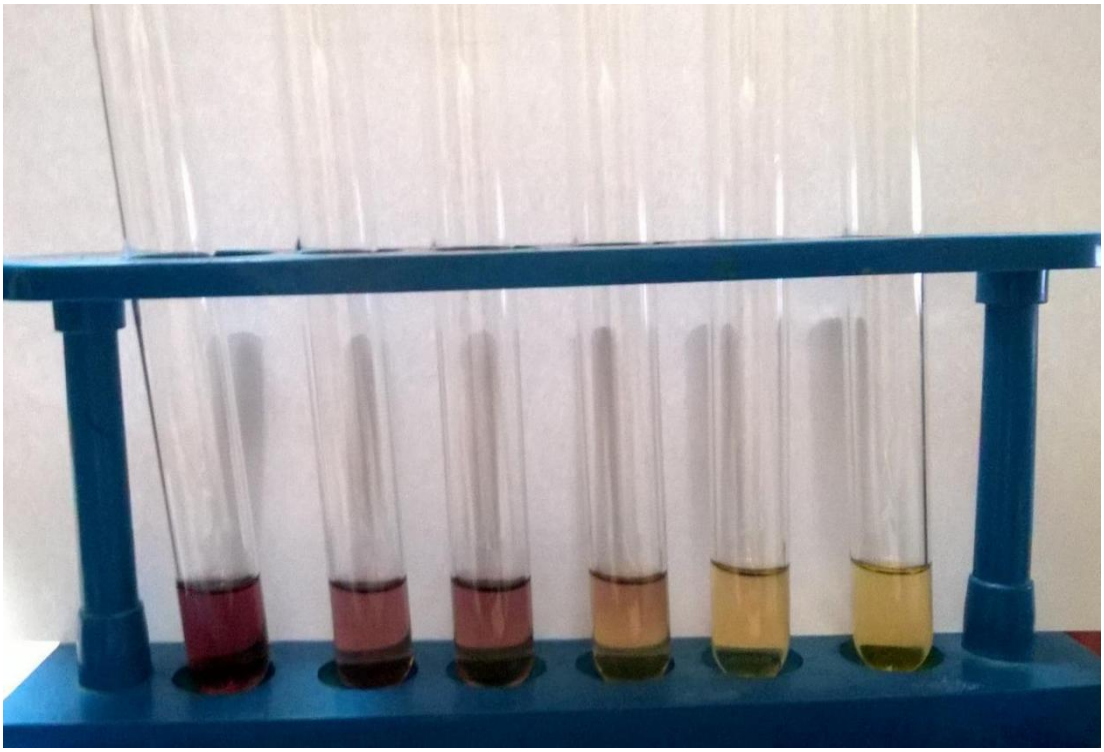


Figure 4.4 Anti-Oxidant Assay - DPPH Method

Table 4.3 Anti-oxidant activity of *E. crassipes*

Concentration (µg/mL)	**I**	**II**	**Average**	**Inhibition (%)**
Control	0.937	0.941	0.939	0
20	0.845	0.849	0.847	9.80
40	0.757	0.752	0.7545	19.65
60	0.555	0.55	0.5525	41.16
80	0.331	0.336	0.3335	64.48
100	0.177	0.179	0.178	81.04

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4.4 ANTI-INFLAMMATORY ASSAY:

The anti-inflammatory test was done in reference to *Jayasuriya et al.* (2017). Two different tests were conducted for determining the anti-inflammatory property of MEX of *E. crassipes*. In Protein denaturation inhibition assay, the % inhibition of denaturation was found to be 24% and in Heat induced haemolysis method, the % inhibition of haemolysis was calculated as 15.5%.

4.4.1 PROTEIN DENATURATION INHIBITION ASSAY:

% Inhibition of denaturation = (1-A1/A2) X 100

Where A1 = absorbance of sample (test sample or positive control) and A2 = absorbance of negative control.

we found that,

% Inhibition of denaturation = (1 - 1.69 / 2.00) * 100

= 24%

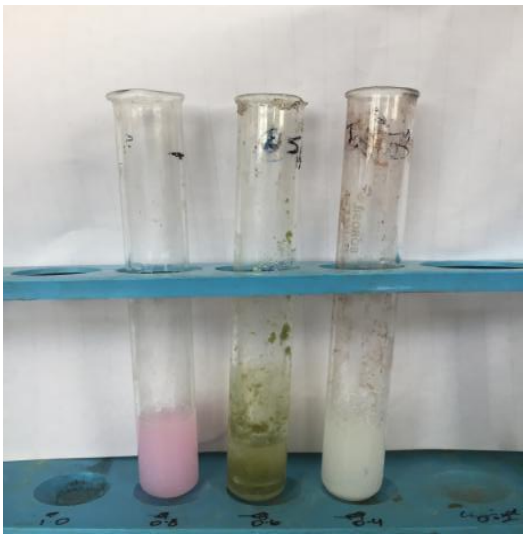


Figure 4.5 Protein denaturation

4.4.2 HEAT INDUCED HEMOLYSIS:

$$\% \text{ inhibition of hemolysis} = 100 \times (1 - A2/A1),$$

where A1 = absorption of the control, and A2 = absorption of test sample mixture.We found that,

$$\% \text{ inhibition of hemolysis} = (1 - 1.52 / 2.00)$$

$$= 15.5\%$$

Figure 4.6 Heat induced hemolysis

4.5 GAS CHROMATOGRAPHY-MASS SPECTOMETRY :

The resulting spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2008) library. These results were shown as follows

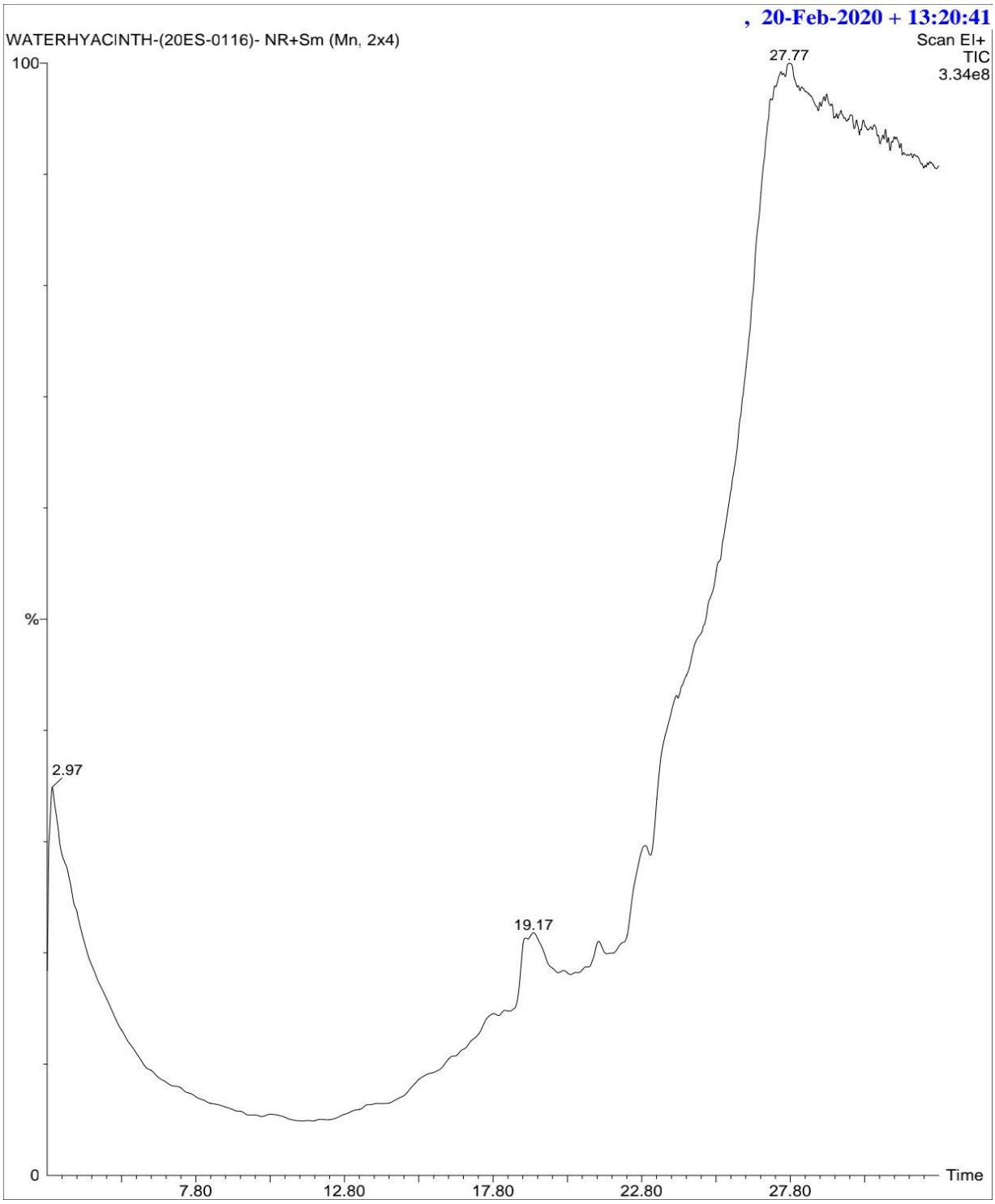
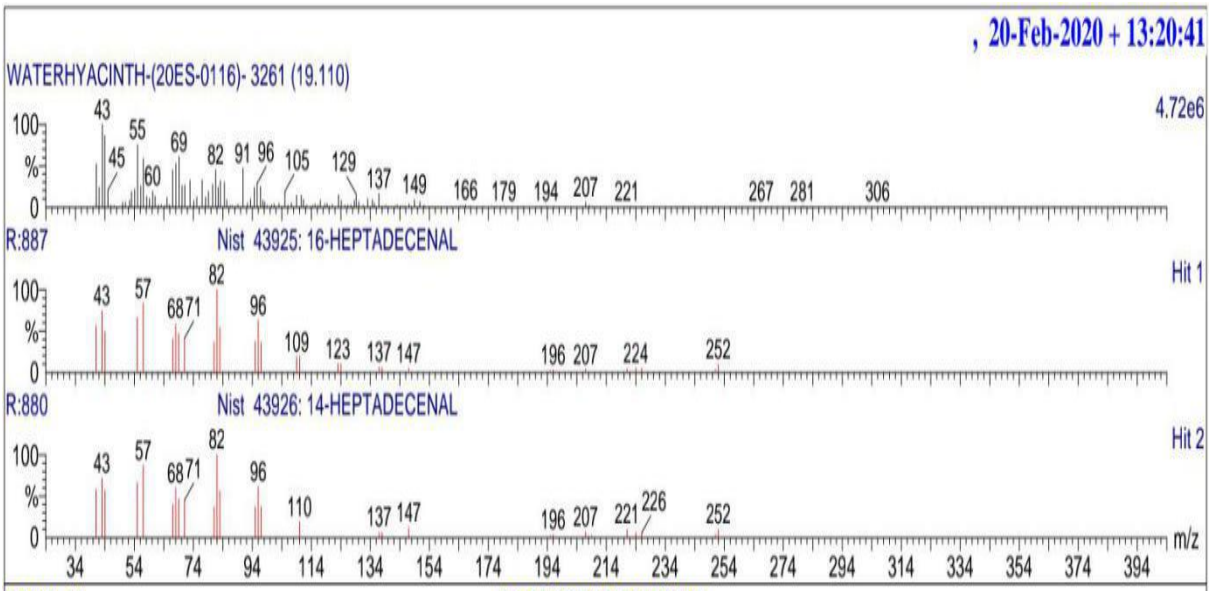
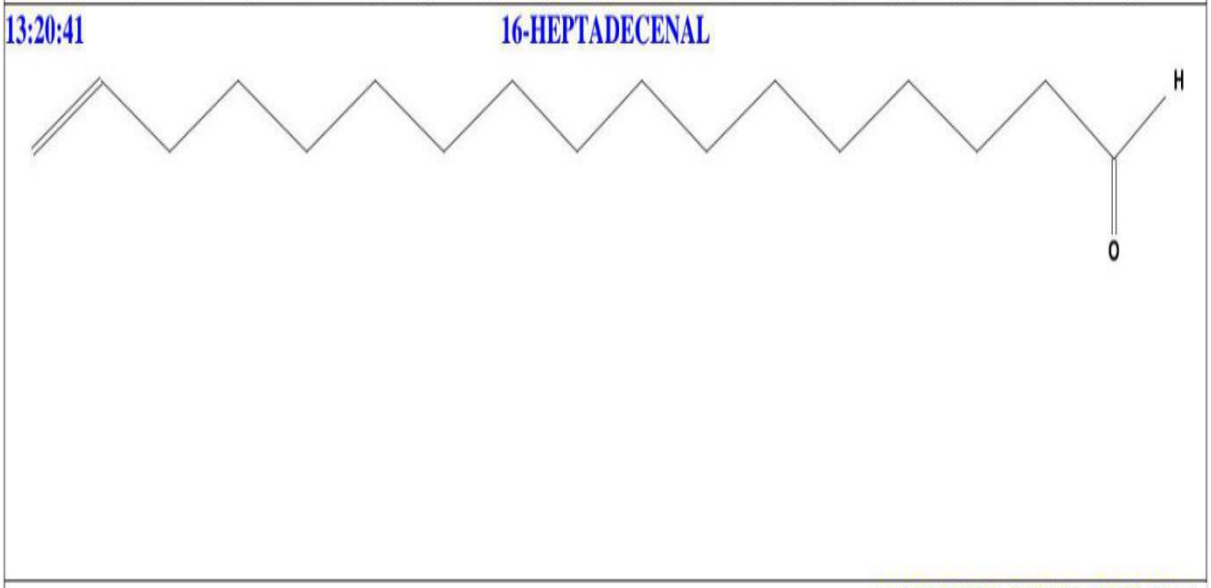


Figure 4.7 GC - *E. crassipes* -MEX

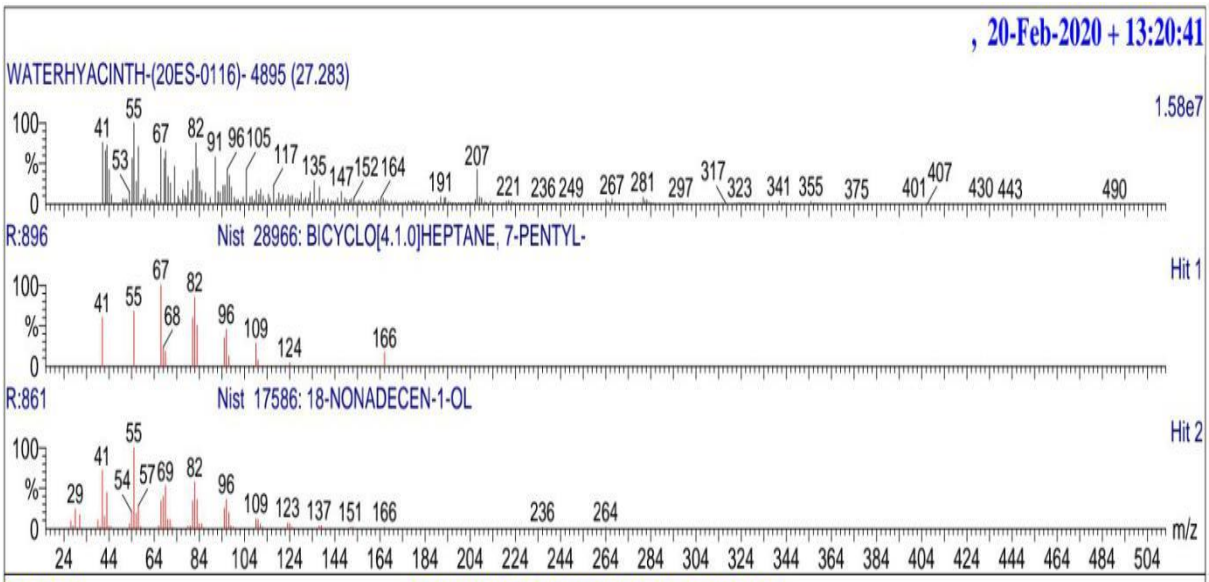


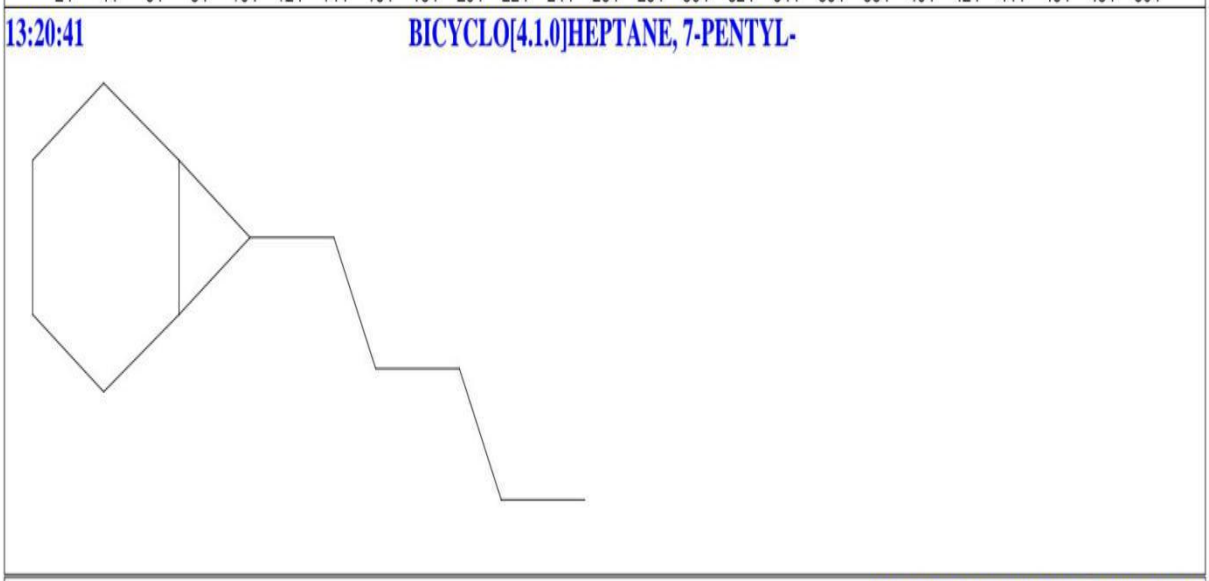


WATERHYACINTH-(20ES-0116)-

Hit	REV	for	Compound Name	M.W.	Formula	CAS
1	887	614	16-HEPTADECENAL	252	C17H32O	900144-57-9
2	880	587	14-HEPTADECENAL	252	C17H32O	900144-58-0
3	879	618	CIS-9,10-EPOXYOCTADECAN-1-OL	284	C18H36O2	13980-12-6
4	868	640	Z-3-OCTADECEN-1-OL ACETATE	310	C20H38O2	900131-08-1
5	865	617	18-NONADECEN-1-OL	282	C19H38O	900142-89-2
6	865	631	11-TRIDECEN-1-OL	198	C13H26O	900130-96-8
7	865	683	OCTADECANAL	268	C18H36O	638-66-4
8	860	613	OXIRANE, HEXADECYL-	268	C18H36O	7390-81-0
9	860	634	1,1'-BICYCLOPENTYL, 2-HEXADECYL-	362	C26H50	55334-11-7
10	859	612	OXIRANE, HEPTADECYL-	282	C19H38O	67860-04-2
11	855	649	1-OCTADECYNE	250	C18H34	629-89-0
12	854	662	DODECANAL	184	C12H24O	112-54-9
13	854	674	HEXADECANAL	240	C16H32O	629-80-1
14	853	608	OXIRANE, TETRADECYL-	240	C16H32O	7320-37-8
15	852	574	CYCLOPENTANE, HENEICOSYL-	364	C26H52	6703-82-8
16	852	672	TETRADECANAL	212	C14H28O	124-25-4
17	851	672	TETRADECANAL	212	C14H28O	124-25-4

Figure 4.8 Mass spectrum (RT-19.110) of *E. crassipes* - MEX

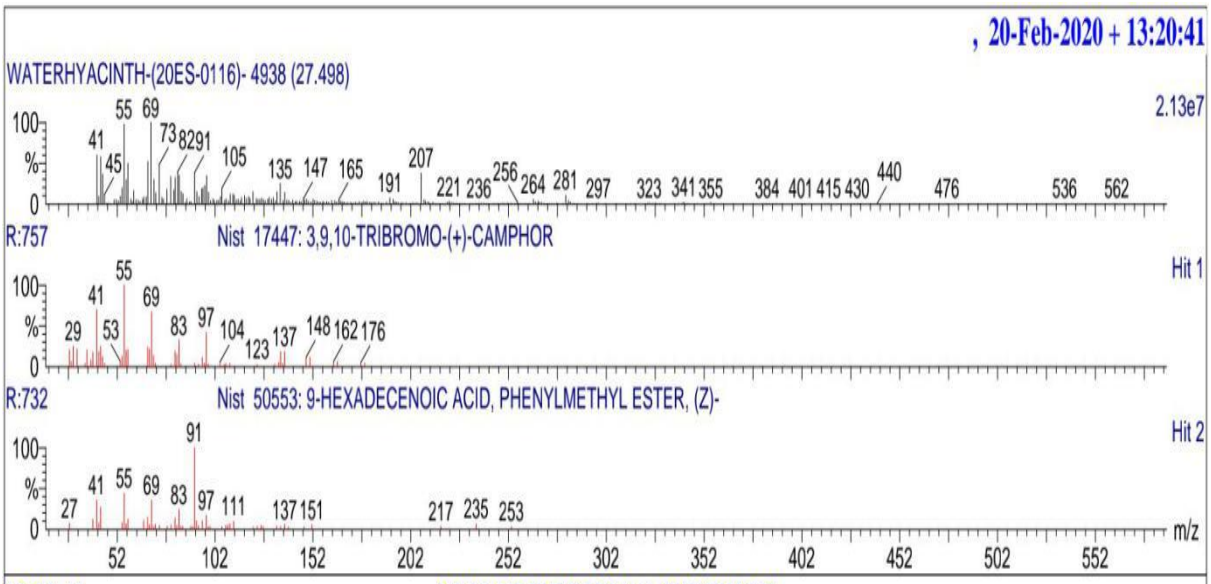




WATERHYACINTH-(20ES-0116)-

Hit	REV	for	Compound Name	M.W.	Formula	CAS
1	896	402	BICYCLO[4.1.0]HEPTANE, 7-PENTYL-	166	C12H22	41977-45-1
2	861	632	18-NONADECEN-1-OL	282	C19H38O	900142-89-2
3	853	613	3-DODECEN-1-OL	184	C12H24O	900164-12-4
4	851	642	11-TRIDECEN-1-OL	198	C13H26O	900130-96-8
5	850	625	1,19-EICOSADIENE	278	C20H38	14811-95-1
6	850	654	OXIRANE, TETRADECYL-	240	C16H32O	7320-37-8
7	850	666	E-2-TETRADECEN-1-OL	212	C14H28O	900130-83-7
8	850	625	1,12-DODECANEDIOL	202	C12H26O2	5675-51-4
9	850	659	13-OXABICYCLO[10.1.0]TRIDECANE	182	C12H22O	286-99-7
10	848	652	Z-10-PENTADECEN-1-OL	226	C15H30O	900245-48-5
11	848	648	OCTADECANAL	268	C18H36O	638-66-4
12	847	639	1-OCTADECYNE	250	C18H34	629-89-0
13	846	647	TETRADECANAL	212	C14H28O	124-25-4
14	845	662	PENTADECANAL-	226	C15H30O	2765-11-9
15	845	662	1,15-PENTADECANEDIOL	244	C15H32O2	14722-40-8
16	844	620	1,19-EICOSADIENE	278	C20H38	14811-95-1
17	843	632	E-3-PENTADECEN-2-OL	226	C15H30O	900130-83-8

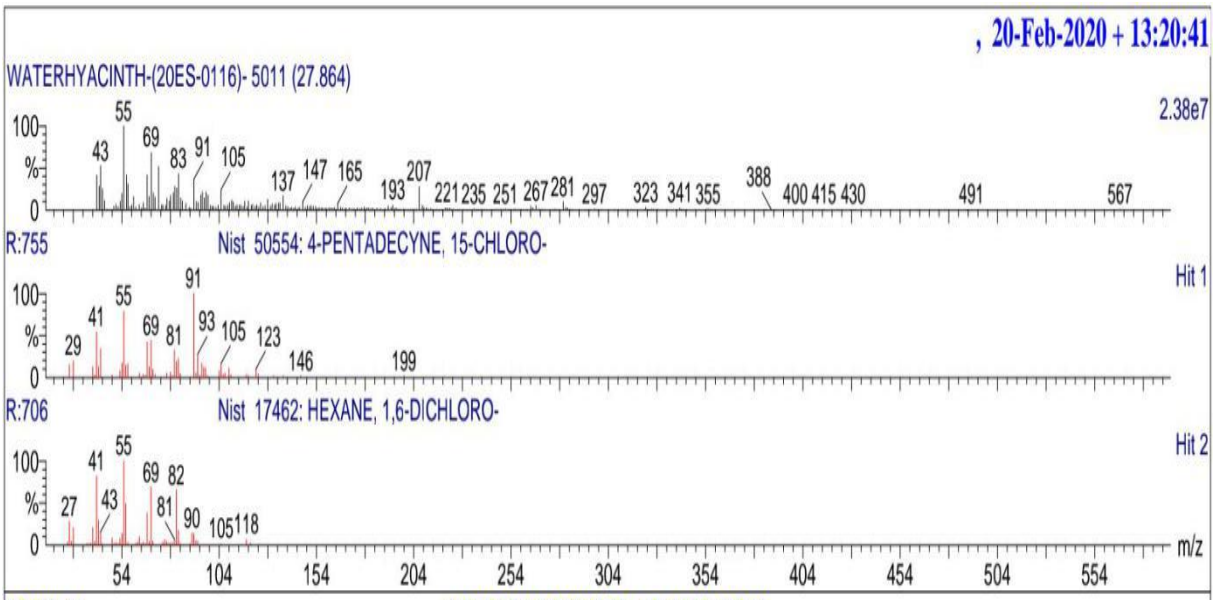
Figure 4.9 Mass spectrum (RT-27.283) of *E. crassipes* - MEX

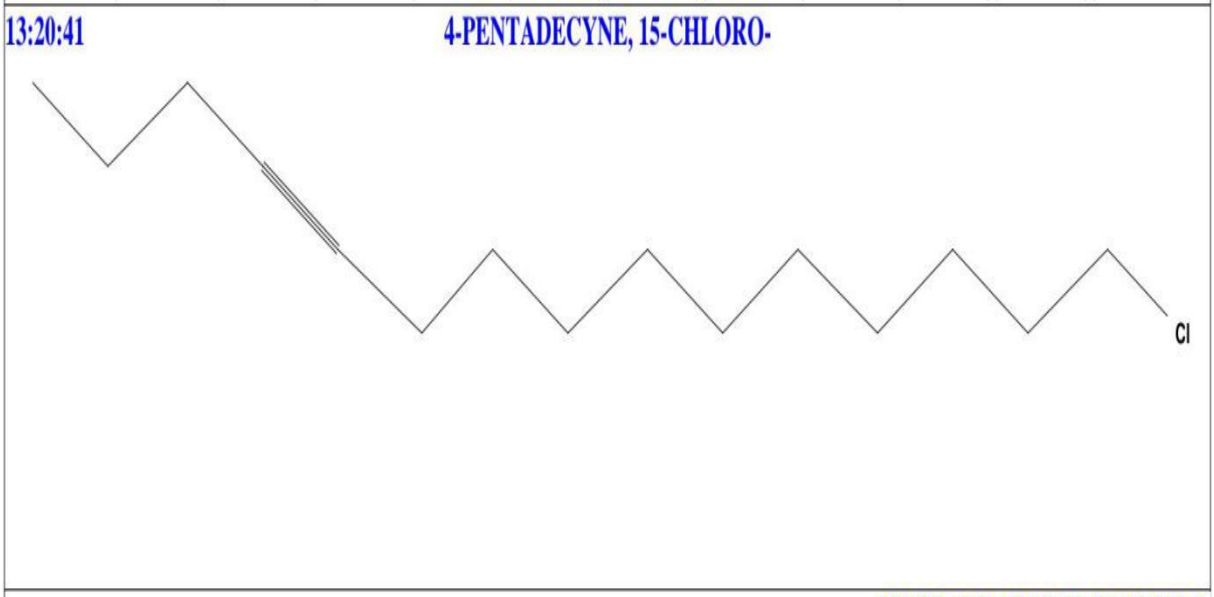


WATERHYACINTH-(20ES-0116)-

Hit	REV	for	Compound Name	M.W.	Formula	CAS
1	757	557	3,9,10-TRIBROMO-(+)-CAMPHOR	386	C10H13OBr3	85706-52-1
2	732	520	9-HEXADECENOIC ACID, PHENYLMETHYL ESTER, (Z)-	344	C23H36O2	77509-01-4
3	727	534	CYCLOPENTANE, 1,1'-[3-(2-CYCLOPENTYLETHYL)-1,5-PENTANEDIYL]BIS-	304	C22H40	55255-85-1
4	720	529	CYCLOHEXANE, 1,1'-(2-ETHYL-1,3-PROPANEDIYL)BIS-	236	C17H32	54833-34-0
5	717	527	CYCLOHEXANE, 1,1'-(2-METHYL-1,3-PROPANEDIYL)BIS-	222	C16H30	2883-08-1
6	716	511	2-BROMOPROPIONIC ACID, 1-(CYCLOPENTYL)ETHYL ESTER	248	C10H17O2Br	900293-39-1
7	711	542	1-NAPHTHALENOL, DECAHYDRO-4A-METHYL-	168	C11H20O	54972-52-0
8	706	467	DECANE, 1,10-DIBROMO-	298	C10H20Br2	4101-68-2
9	705	496	CYCLOHEXANE, 1,1'-(2-PROPYL-1,3-PROPANEDIYL)BIS-	250	C18H34	55030-21-2
10	689	475	1-(10,10-DIMETHYL-3,3-DIOXO-3-THIA-4-AZATRICYCLO[5.2.1.0(1,5)]DEC-4-YL)-3-ME	311	C16H25O3NS	900210-42-6
11	679	349	CARBONIC ACID, DITHIO-, S-METHYL O-(2-METHYLCYCLOHEXYL) ESTER, TRANS-	204	C9H16OS2	15288-13-8
12	673	481	1,3-DIOXOLANE, 4-PENTYL-5-PROPYL-2,2-BIS(TRIFLUOROMETHYL)-, CIS-	322	C13H20O2F6	38274-68-9
13	666	421	CHOLEST-3-ENO[3,4-D]PYRIMIDINE, 2'-CHLORO-	456	C29H45N2Cl	900210-43-1
14	661	440	1,3-DIOXOLANE, 4-PENTYL-5-PROPYL-2,2-BIS(TRIFLUOROMETHYL)-, TRANS-	322	C13H20O2F6	38274-69-0
15	646	363	I-PROPYL 5,9,19-OCTACOSATRIENOATE	460	C31H56O2	900336-60-3
16	644	407	ARACHIDONIC AMIDE, N-[5-HYDROXY-N-PENTYL]-	389	C25H43O2N	158840-60-9
17	637	484	1,2-DIHYDRO-8-HYDROXYLINALOOL	172	C10H20O2	900131-83-5

Figure 4.10 Mass spectrum (RT-27.498) of *E. crassipes* - MEX

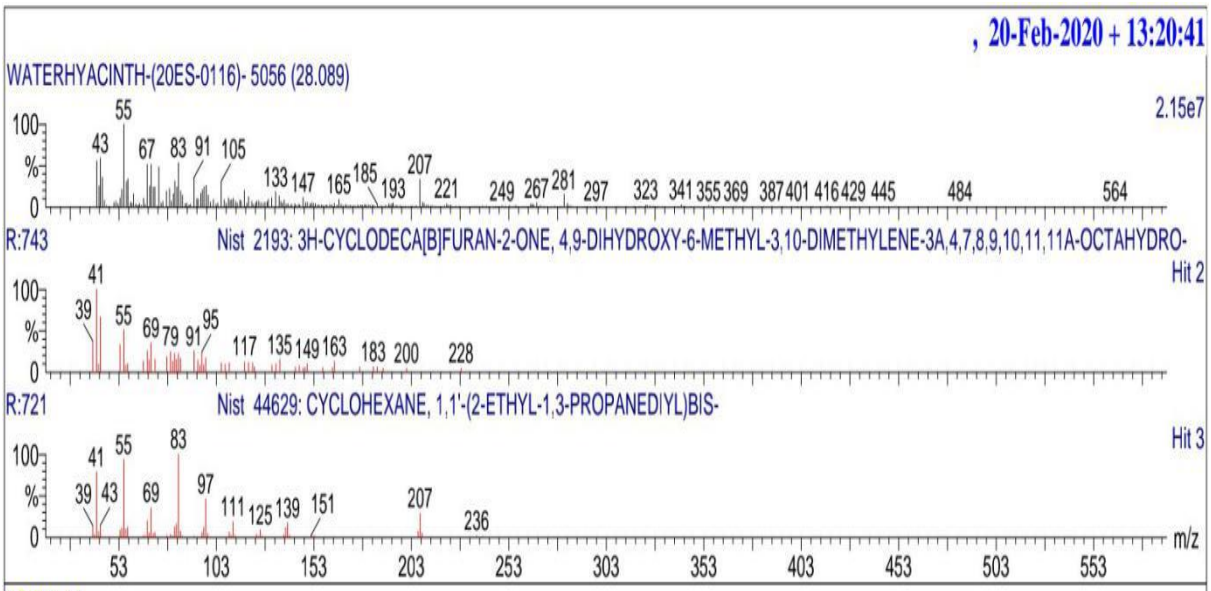




WATERHYACINTH-(20ES-0116)-

Hit	REV	for	Compound Name	M.W.	Formula	CAS
1	755	553	4-PENTADECYNE, 15-CHLORO-	242	C15H27Cl	56554-70-2
2	706	466	HEXANE, 1,6-DICHLORO-	154	C6H12Cl2	2163-00-0
3	705	495	1,9-DICHLORONONANE	196	C9H18Cl2	821-99-8
4	703	507	CYCLOHEXANE, 1,1'-(2-ETHYL-1,3-PROPANEDIYL)BIS-	236	C17H32	54833-34-0
5	700	505	CYCLOHEXANE, 1,1'-(2-METHYL-1,3-PROPANEDIYL)BIS-	222	C16H30	2883-08-1
6	699	359	1,4-DIBROMO-2-CYCLOHEXYLBUTANE	296	C10H18Br2	71052-99-8
7	690	465	TRANS-TRAUMATIC ACID	228	C12H20O4	6402-36-4
8	688	489	1,3-DIOXOLANE, 4-PENTYL-5-PROPYL-2,2-BIS(TRIFLUOROMETHYL)-, CIS-	322	C13H20O2F6	38274-68-9
9	687	387	STIGMASTERYL TOSYLATE	566	C36H54O3S	53139-42-7
10	687	496	CYCLOPENTANE, 1,1'-[3-(2-CYCLOPENTYLETHYL)-1,5-PENTANEDIYL]BIS-	304	C22H40	55255-85-1
11	683	437	9-HEXADECENOIC ACID, PHENYLMETHYL ESTER, (Z)-	344	C23H36O2	77509-01-4
12	681	368	2-N-BUTYLTHIOLANE, S,S-DIOXIDE	176	C8H16O2S	71053-03-7
13	677	476	CYCLOHEXANE, 1,1'-(2-PROPYL-1,3-PROPANEDIYL)BIS-	250	C18H34	55030-21-2
14	674	387	CHOLESTA-6,22,24-TRIENE, 4,4-DIMETHYL-	394	C29H46	900128-66-9
15	672	488	8-CHLORO-1-OCTANOL	164	C8H17OCl	23144-52-7
16	671	452	1,3-DIOXOLANE, 4-PENTYL-5-PROPYL-2,2-BIS(TRIFLUOROMETHYL)-, TRANS-	322	C13H20O2F6	38274-69-0
17	665	438	6-OCTEN-1-YN-3-OL, 3,7-DIMETHYL-	152	C10H16O	29171-20-8

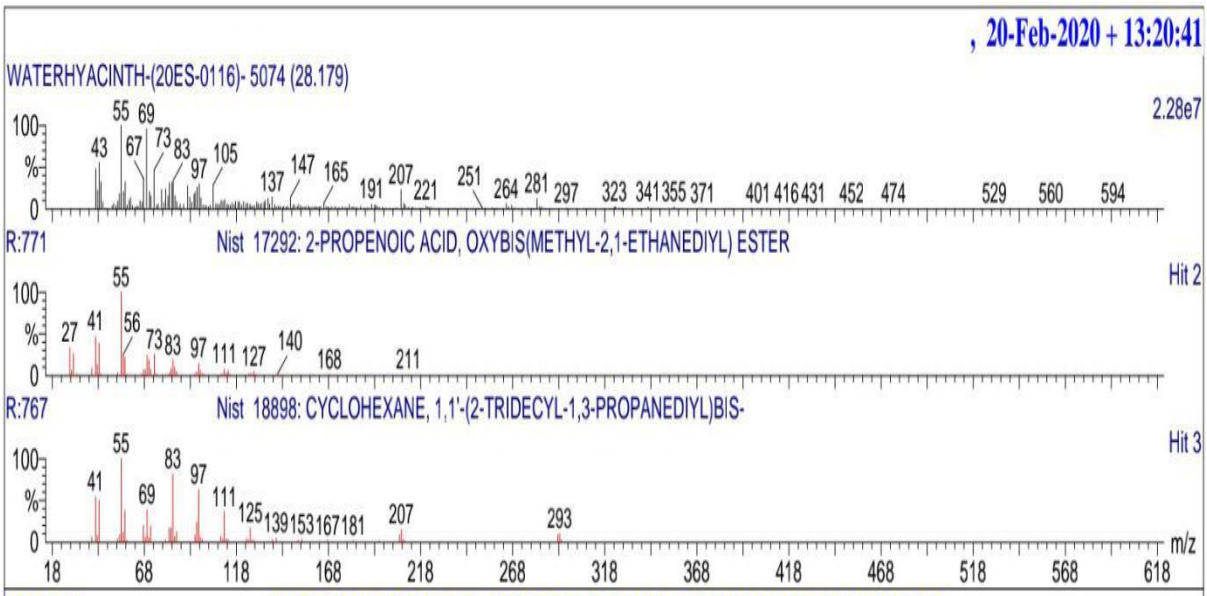
Figure 4.11 Mass spectrum (RT-27.864) of *E. crassipes* - MEX

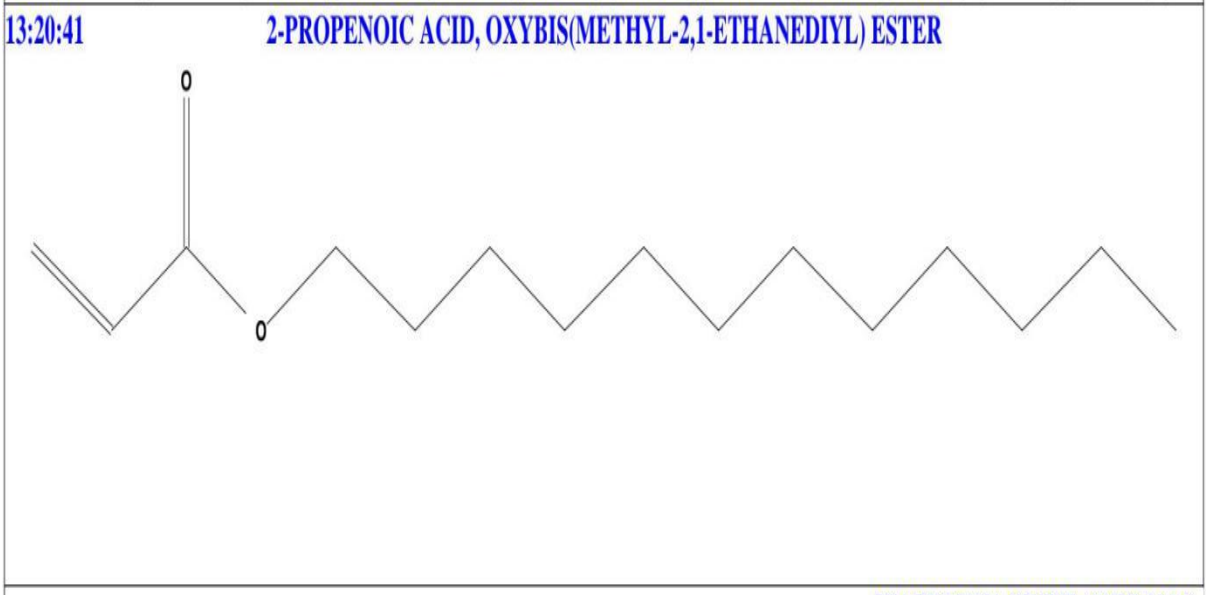


WATERHYACINTH-(20ES-0116)-

Hit	REV	for	Compound Name	M.W.	Formula	CAS
1	750	519	4-PENTADECYNE, 15-CHLORO-	242	C15H27Cl	56554-70-2
2	743	515	3H-CYCLODECA[B]FURAN-2-ONE, 4,9-DIHYDROXY-6-METHYL-3,10-DIMETHYLENE-	264	C15H20O4	900310-90-7
3	721	490	CYCLOHEXANE, 1,1'-(2-ETHYL-1,3-PROPANEDIYL)BIS-	236	C17H32	54833-34-0
4	718	488	CYCLOHEXANE, 1,1'-(2-METHYL-1,3-PROPANEDIYL)BIS-	222	C16H30	2883-08-1
5	715	470	TRANS-TRAUMATIC ACID	228	C12H20O4	6402-36-4
6	715	518	1,3-DIOXOLANE, 4-PENTYL-5-PROPYL-2,2-BIS(TRIFLUOROMETHYL)-, CIS-	322	C13H20O2F6	38274-68-9
7	701	476	CYCLOHEXANE, 1,1'-(2-PROPYL-1,3-PROPANEDIYL)BIS-	250	C18H34	55030-21-2
8	700	476	1,3-DIOXOLANE, 4-PENTYL-5-PROPYL-2,2-BIS(TRIFLUOROMETHYL)-, TRANS-	322	C13H20O2F6	38274-69-0
9	694	413	CHOLESTA-6,22,24-TRIENE, 4,4-DIMETHYL-	394	C29H46	900128-66-9
10	691	479	1,9-DICHLORONONANE	196	C9H18Cl2	821-99-8
11	691	445	9-HEXADECENOIC ACID, PHENYLMETHYL ESTER, (Z)-	344	C23H36O2	77509-01-4
12	690	392	STIGMASTERYL TOSYLATE	566	C36H54O3S	53139-42-7
13	686	368	1,4-DIBROMO-2-CYCLOHEXYLBUTANE	296	C10H18Br2	71052-99-8
14	683	450	1-NAPHTHALENEPROPANOL, .ALPHA.-ETHYLDECAHYDRO-5-(HYDROXYMETHYL)-.	308	C20H36O2	72401-52-6
15	683	448	CYCLOPENTANE, 1,1'-[3-(2-CYCLOPENTYLETHYL)-1,5-PENTANEDIYL]BIS-	304	C22H40	55255-85-1
16	672	458	3-HEXEN-1-OL, 2,5-DIMETHYL-, ACETATE, (Z)-	170	C10H18O2	900132-12-5
17	670	469	8-CHLORO-1-OCTANOL	164	C8H17OCl	23144-52-7

Figure 4.12 Mass spectrum (RT-28.089) of *E. crassipes* - MEX

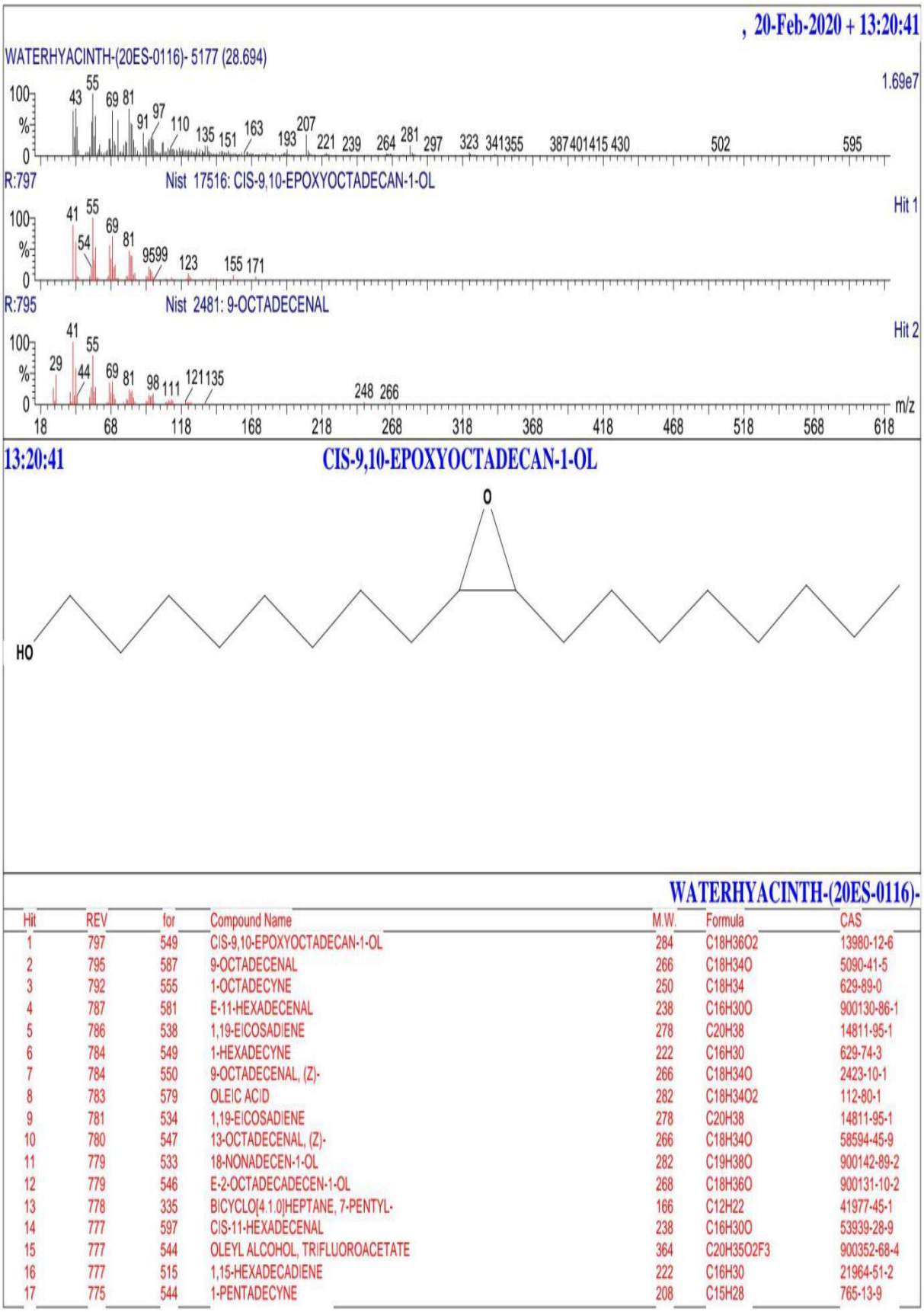




WATERHYACINTH-(20ES-0116)-

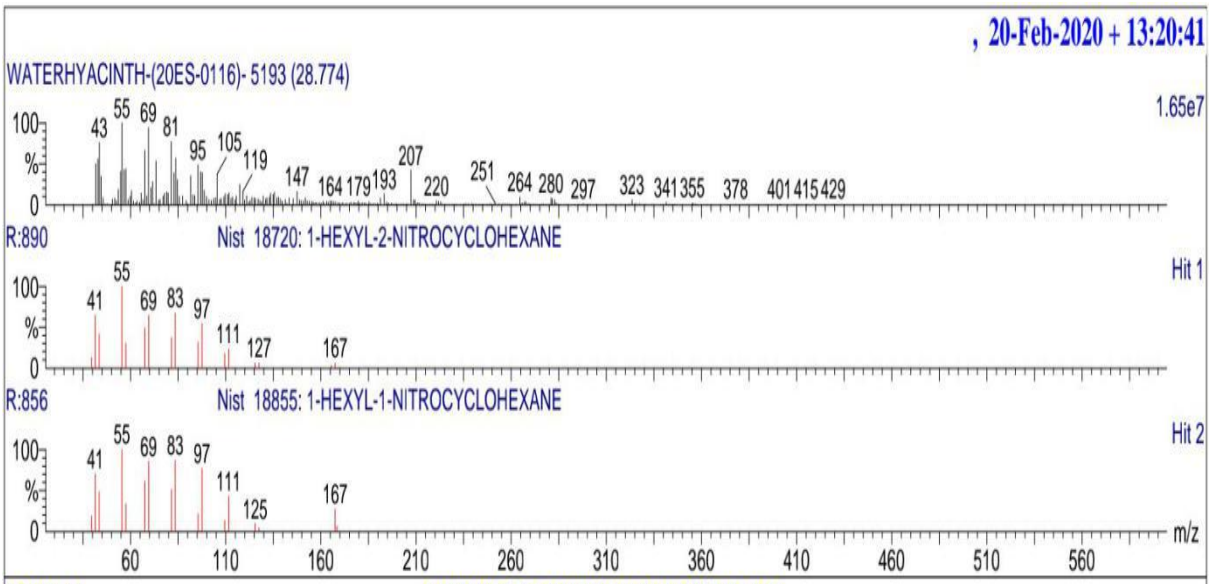
Hit	REV	for	Compound Name	M.W.	Formula	CAS
1	788	575	OLEIC ACID	282	C18H34O2	112-80-1
2	771	539	2-PROPENOIC ACID, OXYBIS(METHYL-2,1-ETHANEDIYL) ESTER	240	C15H28O2	57472-68-1
3	767	542	CYCLOHEXANE, 1,1'-(2-TRIDECYL-1,3-PROPANEDIYL)BIS-	390	C28H54	55255-74-8
4	765	554	14-PENTADECENOIC ACID	240	C15H28O2	17351-34-7
5	757	513	DODECYL ACRYLATE	240	C15H28O2	2156-97-0
6	746	502	4-TRIDECANOL	200	C13H28O	26215-92-9
7	740	543	4-TETRADECANOL	214	C14H30O	1653-33-4
8	740	541	CYCLOPENTANE, 1,1'-[3-(2-CYCLOPENTYLETHYL)-1,5-PENTANEDIYL]BIS-	304	C22H40	55255-85-1
9	737	551	HEXADECANE, 1,16-DICHLORO-	294	C16H32Cl2	7735-39-9
10	736	499	2-PROPENOIC ACID, TRIDECYL ESTER	254	C16H30O2	3076-04-8
11	734	536	CYCLOHEXANE, 1,1'-(2-ETHYL-1,3-PROPANEDIYL)BIS-	236	C17H32	54833-34-0
12	732	535	CYCLOHEXANE, 1,1'-(2-METHYL-1,3-PROPANEDIYL)BIS-	222	C16H30	2883-08-1
13	728	506	1,3-DIOXOLANE, 4-ETHYL-5-OCTYL-2,2-BIS(TRIFLUOROMETHYL)-, TRANS-	350	C15H24O2F6	38274-73-6
14	724	514	TRANS-2-HEXADECENOIC ACID	254	C16H30O2	929-79-3
15	721	501	1,3-DIOXOLANE, 4-ETHYL-5-OCTYL-2,2-BIS(TRIFLUOROMETHYL)-, CIS-	350	C15H24O2F6	38274-72-5
16	719	508	CYCLOHEXANE, 1,1'-(2-PROPYL-1,3-PROPANEDIYL)BIS-	250	C18H34	55030-21-2
17	717	520	UNDECYLENIC ACID	184	C11H20O2	112-38-9

Figure 4.13 Mass spectrum (RT-28.179) of *E. crassipes* - MEX

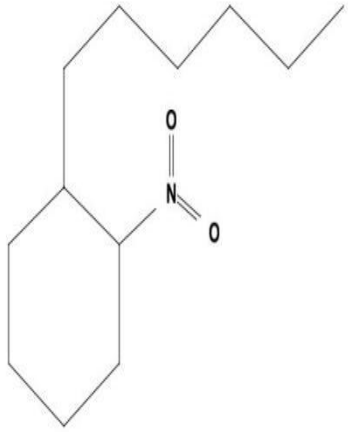


Hit	REV	for	Compound Name	M.W.	Formula	CAS
1	797	549	CIS-9,10-EPOXYOCTADECAN-1-OL	284	C18H36O2	13980-12-6
2	795	587	9-OCTADECENAL	266	C18H34O	5090-41-5
3	792	555	1-OCTADECYNE	250	C18H34	629-89-0
4	787	581	E-11-HEXADECENAL	238	C16H30O	900130-86-1
5	786	538	1,19-EICOSADIENE	278	C20H38	14811-95-1
6	784	549	1-HEXADECYNE	222	C16H30	629-74-3
7	784	550	9-OCTADECENAL, (Z)-	266	C18H34O	2423-10-1
8	783	579	OLEIC ACID	282	C18H34O2	112-80-1
9	781	534	1,19-EICOSADIENE	278	C20H38	14811-95-1
10	780	547	13-OCTADECENAL, (Z)-	266	C18H34O	58594-45-9
11	779	533	18-NONADECEN-1-OL	282	C19H38O	900142-89-2
12	779	546	E-2-OCTADECADECEN-1-OL	268	C18H36O	900131-10-2
13	778	335	BICYCLO[4.1.0]HEPTANE, 7-PENTYL-	166	C12H22	41977-45-1
14	777	597	CIS-11-HEXADECENAL	238	C16H30O	53939-28-9
15	777	544	OLEYL ALCOHOL, TRIFLUOROACETATE	364	C20H35O2F3	900352-68-4
16	777	515	1,15-HEXADECADIENE	222	C16H30	21964-51-2
17	775	544	1-PENTADECYNE	208	C15H28	765-13-9

Figure 4.14 Mass spectrum (RT-28.694) of *E. crassipes* - MEX



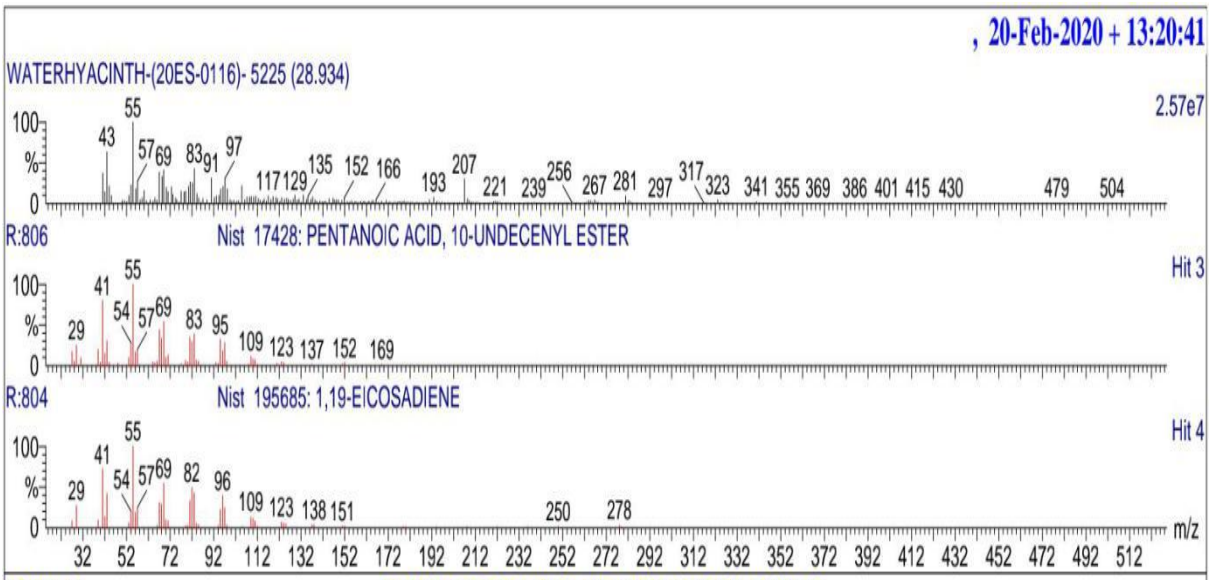
13:20:41 **1-HEXYL-2-NITROCYCLOHEXANE**

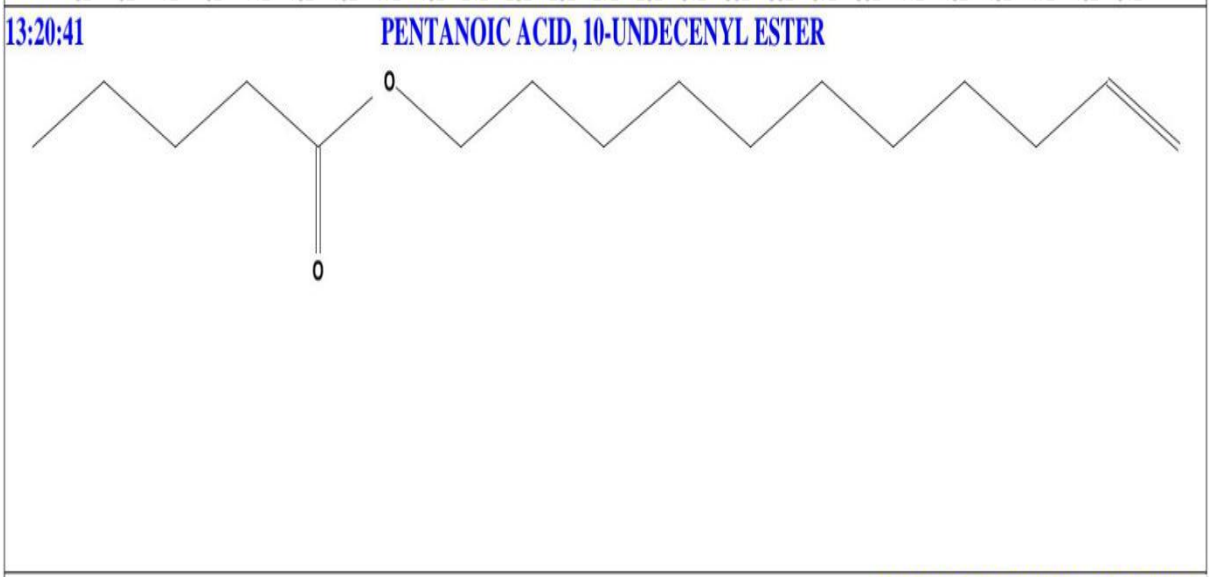


WATERHYACINTH-(20ES-0116)-

Hit	REV	for	Compound Name	M.W.	Formula	CAS
1	890	466	1-HEXYL-2-NITROCYCLOHEXANE	213	C12H23O2N	118252-04-3
2	856	449	1-HEXYL-1-NITROCYCLOHEXANE	213	C12H23O2N	118252-09-8
3	830	507	CYCLOHEXANE, 1-(1,5-DIMETHYLHEXYL)-4-(4-METHYLPENTYL)-	280	C20H40	56009-20-2
4	810	611	1-OCTADECYNE	250	C18H34	629-89-0
5	804	606	1,19-EICOSADIENE	278	C20H38	14811-95-1
6	804	596	9-HEXADECENOIC ACID, 9-HEXADECENYL ESTER, (Z,Z)-	476	C32H60O2	22393-97-1
7	798	602	1-HEXADECYNE	222	C16H30	629-74-3
8	798	567	CIS-9,10-EPOXYOCTADECAN-1-OL	284	C18H36O2	13980-12-6
9	798	602	PENTANOIC ACID, 10-UNDECENYL ESTER	254	C16H30O2	900159-93-4
10	795	605	18-NONADECEN-1-OL	282	C19H38O	900142-89-2
11	791	380	BICYCLO[4.1.0]HEPTANE, 7-PENTYL-	166	C12H22	41977-45-1
12	790	596	1,15-PENTADECANEDIOL	244	C15H32O2	14722-40-8
13	790	596	E-2-OCTADECADECEN-1-OL	268	C18H36O	900131-10-2
14	789	595	1-PENTADECYNE	208	C15H28	765-13-9
15	789	595	1,19-EICOSADIENE	278	C20H38	14811-95-1
16	787	627	1,14-TETRADECANEDIOL	230	C14H30O2	19812-64-7
17	783	591	CYCLODODECANEMETHANOL	198	C13H26O	1892-12-2

Figure 4.15 Mass spectrum (RT-28.774) of *E. crassipes* - MEX

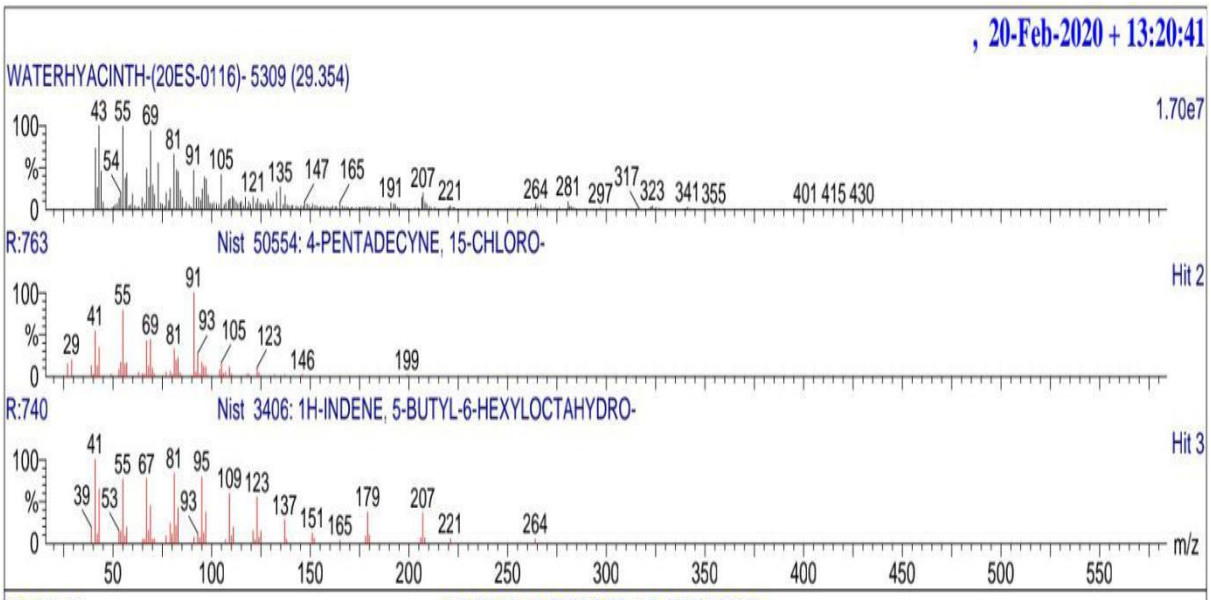


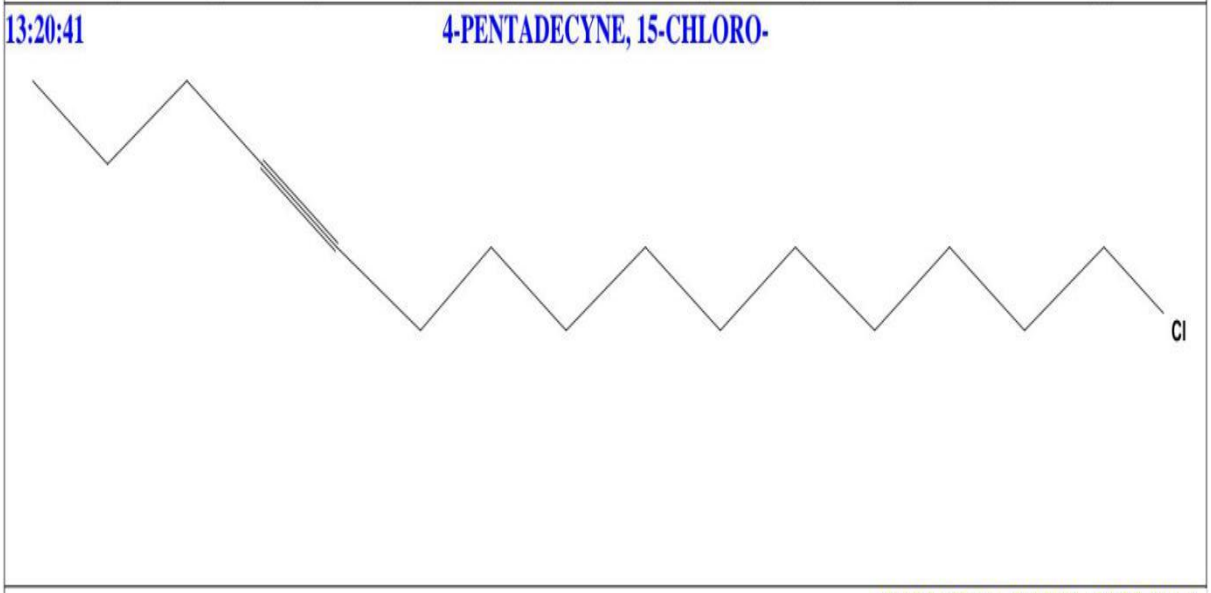


WATERHYACINTH-(20ES-0116)-

Hit	REV	for	Compound Name	M.W.	Formula	CAS
1	860	448	1-HEXYL-2-NITROCYCLOHEXANE	213	C12H23O2N	118252-04-3
2	811	422	1-HEXYL-1-NITROCYCLOHEXANE	213	C12H23O2N	118252-09-8
3	806	605	PENTANOIC ACID, 10-UNDECENYL ESTER	254	C16H30O2	900159-93-4
4	804	596	1,19-EICOSADIENE	278	C20H38	14811-95-1
5	800	570	1,1'-BICYCLOPENTYL, 2-HEXADECYL-	362	C26H50	55334-11-7
6	796	590	1,19-EICOSADIENE	278	C20H38	14811-95-1
7	794	478	CYCLOHEXANE, 1-(1,5-DIMETHYLHEXYL)-4-(4-METHYLPENTYL)-	280	C20H40	56009-20-2
8	792	627	1-CYCLOHEXYLNONENE	208	C15H28	114614-84-5
9	787	598	9-OCTADECENAL, (Z)-	266	C18H34O	2423-10-1
10	782	593	OLEYL ALCOHOL	268	C18H36O	143-28-2
11	780	541	9-HEXADECENOIC ACID, 9-HEXADECENYL ESTER, (Z,Z)-	476	C32H60O2	22393-97-1
12	780	570	(S)(+)-Z-13-METHYL-11-PENTADECEN-1-OL ACETATE	282	C18H34O2	900130-84-8
13	779	592	2-METHYL-Z,Z-3,13-OCTADECADIENOL	280	C19H36O	900130-90-5
14	778	557	(Z)-14-TRICOSENYL FORMATE	366	C24H46O2	77899-10-6
15	776	572	EICOSEN-1-OL, CIS-9-	296	C20H40O	112248-30-3
16	775	589	CIS-9-HEXADECENAL	238	C16H30O	56219-04-6
17	775	555	1,16-HEXADECANEDIOL	258	C16H34O2	7735-42-4

Figure 4.16 Mass spectrum (RT-28.934) of *E. crassipes* - MEX





WATERHYACINTH-(20ES-0116)-

Hit	REV	for	Compound Name	M.W.	Formula	CAS
1	800	612	OLEIC ACID	282	C18H34O2	112-80-1
2	763	586	4-PENTADECYNE, 15-CHLORO-	242	C15H27Cl	56554-70-2
3	740	519	1H-INDENE, 5-BUTYL-6-HEXYLOCTAHYDRO-	264	C19H36	55044-36-5
4	739	450	2-METHYL-6-METHYLENE-OCTA-1,7-DIEN-3-OL	152	C10H16O	22459-10-5
5	729	577	HEXADECANE, 1,16-DICHLORO-	294	C16H32Cl2	7735-39-9
6	721	503	9-BROMONONALDEHYDE	220	C9H17OBr	124388-97-2
7	719	439	PHOSPHINE, BENZYLCHLOROMENTHYL-	296	C17H26ClP	900157-40-7
8	718	521	9-HEXADECENOIC ACID, PHENYLMETHYL ESTER, (Z)-	344	C23H36O2	77509-01-4
9	718	411	1-NAPHTHALENEPROPANOL, .ALPHA.-ETHENYLDECAHYDRO-5-(HYDROXYMETHY	308	C20H36O2	900143-81-8
10	716	445	4,22-STIGMASTADIENE-3-ONE	410	C29H46O	20817-72-5
11	714	507	1-NAPHTHALENEPROPANOL, .ALPHA.-ETHYLDECAHYDRO-5-(HYDROXYMETHYL)-.	308	C20H36O2	72401-52-6
12	714	455	CHOLESTA-8,24-DIEN-3-OL, 4-METHYL-, (3.BETA.,4.ALPHA.)-	398	C28H46O	7199-92-0
13	712	422	26-HYDROXYCHOLESTEROL	402	C27H46O2	13095-61-9
14	709	441	STIGMASTEROL	412	C29H48O	83-48-7
15	708	549	9-OCTADECENOIC ACID (Z)-, PHENYLMETHYL ESTER	372	C25H40O2	55130-16-0
16	702	495	.BETA. CAROTENE	536	C40H56	7235-40-7
17	702	492	3H-CYCLODECA[B]FURAN-2-ONE, 4,9-DIHYDROXY-6-METHYL-3,10-DIMETHYLENE-	264	C15H20O4	900310-90-7

Figure 4.17 Mass spectrum (RT-29.354) of *E. crassipes* - MEX

Table 4.4 Proposed Retension Time, Compound, Molecular weight and molecular formula of MEX of *E. crassipes*

S.No.	RT	Compound	Mw	Formula
1.	**19.110**	**16-HEPTADECENAL**	**252**	**$C_{17}H_{32}O$**
2.	**27.283**	**BICYCLO[4.1.0]HEPTANE, 7-PENTYL**	**166**	**$C_{12}H_{22}$**
3.	**27.498**	**3,9,10-TRIBROMO-(+)-CAMPHOR**	**386**	**$C_{10}H_{13}OBr_3$**
4.	**27.864**	**4-PENTADECENLY, 15-CHLORO**	**242**	**$C_{15}H_{27}Cl$**
5.	**28.089**	**3H-CYCLODECA[B] FURAN-2-ONE, 4,9-DIHYDROXY-6-METHYL-3,10-DIMETHYLENE**	**264**	**$C_{15}H_{20}O_4$**
6.	**28.179**	**2-PROPENOIC ACID, OXYBIS (METHY-2-1-ETHANEDIYL) ESTER**	**240**	**$C_{15}H_{28}O_2$**
7.	**28.694**	**CIS-9,10-DIMETHYLENE**	**284**	**$C_{18}H_{36}O_2$**
8.	**28.774**	**1-HEXYL-2-NITROCYCLOHEXANE**	**213**	**$C_{12}H_{23}O_2N$**
9.	**28.934**	**PENTANOIC ACID, 10-UNDECENYL ESTER**	**254**	**$C_{16}H_{30}O_2$**
10.	**29.354**	**4-PENTADECENLY, 15-CHLORO**	**242**	**$C_{15}H_{27}Cl$**

4.6 WOUND HEALING ASSAY:

From the obsevation of scratch wound assay, it is noted that the application of MEX of *E. crassipes* enhances the rate of wound healing. The assay showed that with the application of extract the cells take only 76 hours to get healed by 80.77% proving that the MEX of *E. crassipes* have wound healing property. The images of the were given below:

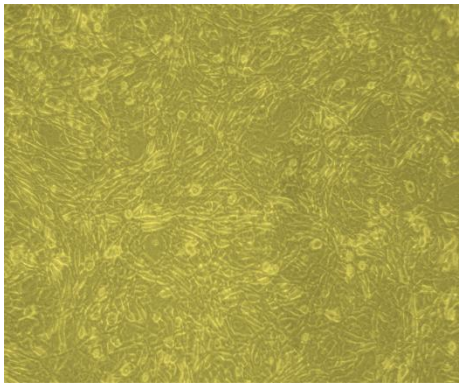


Figure 4.18 control

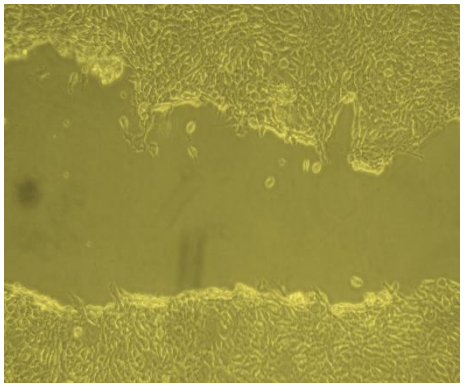


Figure 4.19 wounded cells

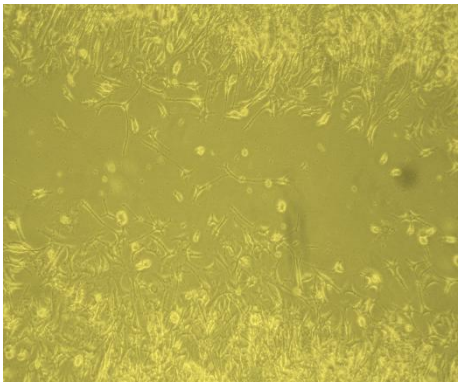


Figure 4.20 wound healing at 24 hr (07.69%)

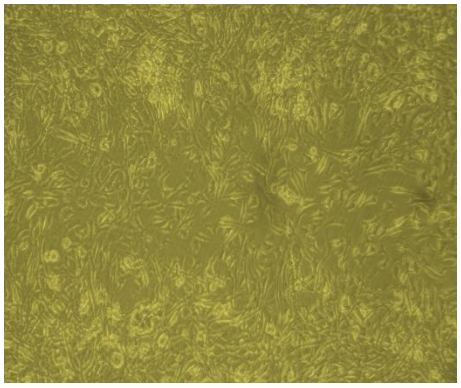


Figure 4.21 Wound healing at 48 hr (42.31%)

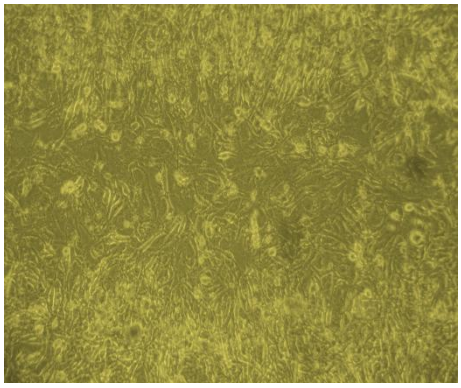


Figure 4.22 Wound healing at76 hr (80.77%)

Table 4.5 Rate of Wound Healing with Extract Treatment

S.No.	**Hours**	**Percentage wound healing**
1.	0	0 %
2.	24	07.69 %
3.	48	42.31 %
4.	76	80.77 %

CHAPTER 5

SUMMARY & CONCLUSION

This project has its soul concern to focus on the properties that adds up value to *E. crassipes* for being medically used to treat wounds as well as to minimise the environmental pollution by using the abundant resouce of water hyacinth plant found in ponds and lakes. As the primary step, the leaves of water hyancith were collected . These leaves were shade-dried for 3 days and ground to powder using mortar and pestle. The 30g of powder was used to extract the phytochemicals present in the water hyacinth leaves where methanol is used as solvent. Then the MEX was stored in Conical flask with the mouth wrapped with aluminium foil. Meanwhile to prove the anti-bacterial property of *E.crassipes* the bacterial strains of *P. aeruginosa, E. cloacae, E. coli, Enterococcus sp.* were collected from the patients wound sample at billroth hospital, amanjikarai. The bacterial strains were plated and the MEX were introduced into the plates through well diffusion method. After 24 hours the plates were observed to measure the ZOI of MEX against the bacterial strains. The ZOI measured were 6mm ,10mm, 13mm and 15mm for *P. aeruginosa,* 0mm ,0mm, 3mm and 4mm for *E.coli,* 4mm ,6mm, 7mm and 9mm for *E. cloacae* and 4mm ,9mm, 10mm and 14mm for *Enterococcus sp.* Having positive result for anti-bacterial assay then the next criteria to test for the Anti-oxidant and antiinflammatory property of *E. crassipes.* The anti-oxidant activity of *E.crassipes* was determined using DPPH assay, where it was observed that 20, 40, 60, 80 and 100 (µg/mL) could have inhibition to oxidation about 9.80, 19.65, 41.65, 64.48 and 81.04 (%) respectively. Thus it had a positive result for anti-oxidant test. Then, inorder to conduct the anti-inflammatory assay, two methods were considered namely Protein denaturation inhibition assay and Heat induced haemolysis method. Egg albumin was used as protein sample for protein denaturation inhibition assay and the methanolic extract was found to

have 24% of denaturation inhibition. For the second method (i.e) Heat induced hemolysis method, an erythrocyte suspension was prepared to induce hemolysis through heat. In this assay the MEX was found to possess 15.5% inhibition for hemolysis. So these assay ensured that the MEX of *E. Crassipes* have anti-oxidant and anti-inflammatory property. While the studies have been going on, we would also like to have a GC-MS anaylsis to find out what really were those componnents present in the MEX of *E. crassipes.* So, the powdered sample was sent to to Sophisticated Instrumentation facility, School of advanced sciences, Chemistry division, VIT university, Vellore for GC-MS analysis. As a final step to prove and to evaluate the wound healing ability of the extract, The Wound Scratch assay was conducted using 3T3 mice cells. The wound was created in the culture using P10 pipette tip and kept exposed to 50 µg/mL of samples for 24-72 hrs at 37°C in a humidified atmosphere of 5% CO_2.Scratch wound closure was analyzed under the inverted microscope (Magnus INVI, Noida) equipped with a digital CCD camera, by acquiring digital images at different time 0^{th} (T0), 2^{nd} (T1), 3^{rd} (T2) and 4^{th} (T3) days (static imaging) and found that the extract promoted 7.69%, 42.31% and 80.77% during its 24^{th}, 48^{th} and 76^{th} hour of observation respectively. Through this study, we showed that the Water hyacinth (*E. crassipes*) have anti-bacterial, anti-oxidant and anti-inflammatory and wound healing Property. Also, using Water hyacinth in medicinal treatment for wound healing, we found a more effective way to exploit the Abundant resource of Water hyacinth and to minimise the pollution caused by it.

APPENDIX

NUTRIENT BROTH:

Composition		gm/1000ml
Beef extract	-	1
Yeast extract	-	2
Peptone	-	5
Sodium chloride	-	5

REFERENCES

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