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Bioseparation and Biochemical Activities of touch-menot Mimosa pudica l. (fabaceae) against Cryptolestes Pusillus and some harmful Microbes

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BIOSEPARATION AND BIOCHEMICAL ACTIVITIES OF TOUCH-ME-NOT *MIMOSA PUDICA* L. (FABACEAE) AGAINST *CRYPTOLESTES PUSILLUS* AND SOME HARMFUL MICROBES

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OF
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by

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Dedicated
To
My Beloved Daughters

DECLARATION

I do hereby declare that the entire work submitted as a thesis entitled **Bioseparation and Biochemical activities of Touch-me-not *Mimosa pudica* L. (Fabaceae) against *Cryptolestes pusillus* and some harmful microbes** in the Institute of Biological Sciences, University of Rajshahi, Bangladesh for the degree of **Doctor of Philosophy** is the result of my own investigation and was carried out under the supervision of Dr Md Wahedul Islam, Professor, Institute of Biological Sciences, University of Rajshahi. To the best of my knowledge, the thesis contains no material published previously except where due reference is made in the text.

May 2021

Ujjwal Kumar Mondol
Candidate

UNIVERSITY OF RAJSHAHI
BANGLADESH

CERTIFICATE

This is to certify that the thesis entitled **Bioseparation and Biochemical activities of Touch-me-not *Mimosa pudica* L. (Fabaceae) against *Cryptolestes pusillus* and some harmful microbes** submitted for the degree of **Doctor of Philosophy** is a bonafide research work of **Ujjwal Kumar Mondol** carried out at the Institute of Biological Sciences, University of Rajshahi, Bangladesh under my supervision. The thesis was an original one and has not been submitted anywhere for the diploma or a degree.

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The author

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ABSTRACT

The present study illustrates the antimicrobial potency against eight bacterial and four fungal strains and the determination of MIC of crude extracts as well as purified compounds of *Mimosa pudica* L (Fabaceae). The antioxidant activity was observed through 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. The phytoconstituents of crude extracts of leaf, stem and root were isolated through column chromatography, thin layer chromatography (TLC) and GC-MS (Gas Chromatography-Mass Spectrometry) analyses. Insecticidal and repellent activity was tested on *Cryptolestes pusillus.*

Three gram-positive bacterial strains *viz. Bacillus cereus, Staphylococcus aureus, Bacillus zhanjiangensis* and five gram-negative bacterial strains *viz. Klebsiella pneumoniae, Shigella boydii, Shigella sonnei, Escherichia coli* and *Salmonella typhi* were used for testing the antibacterial potency of petroleum ether, chloroform, ethyl acetate and methanol crude extracts of leaf, stem and root as well as isolated pure compounds of *M. pudica* and compared with standard antibiotic Ciprofloxacin (5µg/disc). The chloroform extract of root showed the highest potentiality among all the extracts of leaf, stem and root against all the bacterial strains where the zone of inhibition was 21.67±1.45mm against *S. sonnei* at 200µg/disc concentration. The susceptibility order of the extracts was: root > leaf > stem.

The antifungal activity was tested against four fungal strains *viz. Rhizopus oryzae, Fusarium proliferatum, Aspergillus niger* and *Candida albicans* with a standard Nystatin (50µg/disc). *R. oryzae* showed the highest (13mm in diam.) zone of inhibition against ethyl acetate extract at 200µg/disc concentration, and that of for *F. proliferatum* 12mm against ethyl acetate extract, for *A. niger*12mm against chloroform extract and for *C. albicans* 13mm against chloroform extract of root at the same concentration.

The minimum inhibitory concentrations (MICs) showed that the chloroform crude extract of leaf possessed a high potentiality to inhibit the growth of bacteria even at low concentration of 32µg/ml against *S. aureus*. For ethyl acetate crude extract, the lowest MIC value was 64µg/ml against *S. aureus, B. zhanjiangensis* and *E. coli.* In case of purified compounds 9-Octadecenamide and 13-Docosenamide, (Z)-, the zones of inhibition were 21mm and 22mm respectively against *E. coli* at 100µg/disc concentration.

The scavenging activity of different extracts of leaf, stem and root were examined by DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical with absorbance at 517nm and they showed moderate to higher antioxidant activities in comparison with ascorbic acid as standard. The ethyl acetate extracts showed the highest activity where the IC_{50} values were 65.152µg/ml, 76.036µg/ml and 65.000µg/ml, and the lowest activity was found in petroleum ether extracts where the IC_{50} values were 130.125µg/ml, 147.891µg/ml and 186.445µg/ml for leaf, stem and root respectively. The IC_{50} value of ascorbic acid was 18.012µg/ml.

GC-MS analyses were carried out to isolate the constituents of different extracts of leaf, stem and root. To detect the bioactive compounds from chloroform and petroleum ether extracts of leaf further subjected to chemical analysis. The extracts were fractioned using different polar and non-polar solvent systems followed by column chromatography and thin layer chromatography. The recorded purified compounds were 9-Octadecenamide from chloroform extract and 13-Docosenamide, (Z)- from petroleum ether extract of leaf using chemical and spectral analysis. 13-Docosenamide, (Z)- possessed good insecticidal property.

The effects of different doses of various extracts on the mortality of *Cryptolestes pusillus* were observed and the highest activity was found in chloroform extracts. The LD_{50} values were 2.891, 2.472 and 2.190mg/cm^2 for leaf; 3.144, 2.691 and 2.354mg/cm^2 for stem and 2.548, 2.218 and 2.024mg/cm^2 for root in 24h, 48h and 72h of exposures respectively. Among the extracts of leaf, stem and root, the chloroform extracts showed more potency in all the three exposures and the root extract showed the highest activity. The highest percentages in mortality for chloroform extracts in 24h, 48h and 72h of exposures were 73.3%, 70%, 80%; 80%, 76.7%,90% and 90%, 86.7%, 96.7% for leaf, stem and root respectively. The susceptibility order of the extracts were root > leaf > stem. The LD_{50} values were 5.069, 3.770 and 3.257mg/cm^2 for 9-Octadecenamide and 1.915, 1.761 and 1.634mg/cm^2 for 13-Docosenamide, (Z)- in 24h, 48h and 72h of exposures respectively. Obviously 13-Docosenamide, (Z)- was more potent for insecticidal activity and the mortality was dose and time dependent. In case of larvicidal assay no effectivity was observed in any of the extracts against the larvae.

The petroleum ether extract of root showed the highest repellent activity at 0.1% ($p<0.001$) level of significance. According to intensity the result could be arranged in a descending order: root (petroleum ether) > leaf (petroleum ether) > stem (ethyl acetate).

The detection of cytotoxic effects on *Artemia salina* showed that the rates of mortality increased with the raise of concentrations of the extracts. In case of leaf, the highest

potency was found in petroleum ether extract for both in 24h and 48h of exposures where the LC_{50} values were 2.632µg/ml and 1.464µg/ml respectively and according to the intensity of activity in 24h the result could be arranged in the following order: petroleum ether > ethyl acetate > methanol > chloroform. For stem, the highest activity was found in chloroform extract in both 24h and 48h of exposures where the LC_{50} values were 2.076µg/ml and 1.282µg/ml respectively and according to the intensity of activity in 24h the result could be arranged in the following order: chloroform > petroleum ether > ethyl acetate > methanol. And that of root, the highest activity was recorded also in petroleum ether extract for both in 24h and 48h of exposures where the LC_{50} values were 1.929µg/ml and 1.202µg/ml respectively and the intensity of activity in 24h could be arranged as: petroleum ether > chloroform > methanol > ethyl acetate. It was found that all the crude extracts were lethal and significantly effective to brine shrimp nauplii test which indicates the biological activeness of the extracts.

Chapter One

GENERAL INTRODUCTION

Since time immemorial, herbal medicines are using for the treatment of different ailments and almost all medicines were manufactured from plants in the ancient and medieval times. New drugs are being manufactured from the plants and animals with the advent of modern medicines. Still a huge portion of the population relies on herbal medicines to meet the medicinal needs because of the escalating prices of modern drugs (Fuller and Donald 1970). Herbalism is a practice of traditional or folk medicine which is based on the use of plants or plant extracts (Acharya and Srivastava 2008).

Recently the drugs are being prepared synthetically in the laboratory to meet greater necessity or to confirm purity and specificity. For example, the modern narcotic drugs like analgesics, anti-cardiovascular drugs like digitoxin, etc. Not only that but also a large number of plant metabolites are being used for the treatment of various diseases based on herbal and traditional medicines. Thus the primitive use of plants or herbs played a significant role in the development of modern medicines. The laboratory studies of many higher plants have showed insecticidal, antibacterial and antifungal properties (Okigbo and Ogbonnaya 2006 and Satish *et al.* 2007). Plant-produced drugs of the world are about 25% (Rates 2001), where 87% of human diseases including immunological disorders and bacterial infections are treated by natural products and related drugs (Newman 2007). In developing countries like Bangladesh, about 80% of the population relies on traditional medicine to treat serious diseases (FAO 2004).

The plant kingdom is a reservoir of biologically active molecules and only a small fraction of the medicinal plants have been assayed. About 50% of the drugs used in modern medicine are of plant origin. On the basis of ethnobotanical information, much current research is being performed for phytochemical investigation of higher plants. After isolation of the phytoconstituents they are screened for different types of biological activities. Cytotoxicity test through brine shrimp lethality bioassay is studied to reveal new anticancer compounds. The new hospital drug Taxol was discovered from the bark of *Taxus brevifolia* in this way. Conversely, crude plant extracts of particular activities can be assayed first and then phytochemically the active fractions can be analyzed (Harborne 1998).

Giasuddin (1998) described according to WHO (2000) that around 80% inhabitants of the world (5.86 billion) depend on traditional medicines; most of them use the plants or their active principles. WHO gave official recognition to these medicines in a resolution adopted in the 30th World Health Assembly 1977 which is quite encouraging. About one-third of the drugs used by the people are of plant origin and if the bacteria and fungi are included then it would be 60% of all the drugs (World of Science 1980).

Damage by insect pests

Stored-grains including wheat, maize, rice, flour, etc. are damaged by insect pests results in major economic loss, where subsistence grain production supports the livelihood of majority population (Udo 2005). The reduction in quality and quantity of the harvested crops is caused by insect-infestation of grains and in most cases pre-disposes the stored-grains to secondary attacks by various pathogens (Evans 1987). Generally, harvested grains such as wheat, rice, cereals, etc. are stored by small-scale farmers to provide food reserves as well as seed grains. Among the small-scale farmers grain losses are severe as they don't have access to modern storage techniques. In most cases they stored their grains in plastic drums, sacks, spread on the floor or hung on roofs (Odeyemi *et al*. 2006). In the developing countries grain losses have been reported as high as 80 percent (Pingali and Pandey 2000 and Tapondjou *et al*. 2002).

The insect pest causes damage to stored grains in two ways. Firstly, the direct feeding of grains, results in the reduction in weight, market cost, germination and nutritional value; and secondly, the infestation causes the degradation in quality of grains, down-grading the market value, odors, heat damage and moulds (Peairs 2010). Phillips and Throne (2010) denoted that the pests like *Cryptolestes* spp., *Rhizopertha dominica, Tribolium castaneum, Trogoderma granarium* and *Sitophilus oryzae* cause more than 20% post-harvest loss of stored grains in developing countries. Upadhaya and Ahmad (2011) denoted that the beetles (Coleoptera) are more diversified and extremely destructive stored-grains insects in comparison to moths. Both the grubs and adults beetles assault the stored-grains, but the larval stage of moth cause the damage. There are some particular insect pests that do not breed in stored-grains but their presence in stores is harmful; because they create excrement and nuisance, and generate noxious smell and debris.

Protection of stored-grains from insect attack

There are a large number of insect pests that attack the stored wheat, rice bran, maize, corn, wheat flour, beans, cocoa, cassava, groundnuts, cowpeas, shorghum, nere seed, etc. and cause a major amount of damage. *C. pusillus* is one of the most harmful and common insect pest of stored-grains. It is cosmopolitan in distribution and occurs throughout the world especially tropical and subtropical regions including Bangladesh. In worm and moist conditions it multiplies rapidly and causes huge damage, about 100% after six months of storage and the damage is caused both by the adult and larval stages of the pest. Storage conditions and climate, especially in tropics, are often very favorable for insect development. Control of these insects by chemical insecticides has then serious drawbacks (Sharaby 1988). The loss of food grains for insect infestation is a serious problem during storage. Around 2000 species of field and storage pests destroy about one-third production of the world's food annually that values more than US$ 100 billion. The loss occurs in developing Asian countries is approximately 43% of the potential production. Synthetic chemicals are used largely to control the insect pests in most parts of the world. However, the continuous and unsystematic use of the chemical pesticides give rise to genetic resistance of the pests, toxicity to other non-target organisms, pest resurgence, hazards from handling, increase the costs of application, pollution of the environmental, etc. (Ahmed *et al.* 1981, Pacheco *et al.* 1990, Rajendran and Narasimhan 1994, Jembere *et al*. 1995, Subramanyam and Hangstrum 1995, White and Leesch 1995, Okonkwo and Okoye 1996, Banwo and Adamu 2003 and Bughio and Wilkins 2004).

Post-harvest loss by insects

Rajendran and Sriranjini (2008) and Nenaah (2014) described that the insufficient and poor storage facilities cause tremendous loss annually in wheat storage. The insects not only intake the kernels, also accumulate the frass, exuvie, webbings and insect cadavers into the grains which deteriorate the quality of the grains. High degrees of insect detritus may also create the grains dangerous for human consumption. The changes in the storage condition may make heat and moist hotspots in the storage that helps the improvement of storage fungi. Safe storage of grains and food products is a major problem because of the damage by insect pests (Haq *et al.* 2005). Pimentel (2007) denoted that approximately 14% of the global potential food production is destroying by the insect pests. Raja *et al.* (2010) mentioned that the global post-harvest grain

damage due to insect infestation ranged from 10-40%. In the tropical countries, the loss may reach up to 30% and the loss of wheat in storage in USA costs over US$ 200 million annually (Weaver and Petroff 2004).

Insect damage, microbial deterioration and other factors cause 10-25 percent of the world's production as post-harvest loss annually (Matthews 1993). The post-harvest loss has great impact on both the micro and macro levels of economy and according to FAO and APO (2006) the post-harvest loss of grains in Bangladesh is 15% of the total production. Bala *et al.* (2010) described that in Bangladesh the post-harvest storage loss of wheat was 41.7%. The quality deterioration and post-harvest loss caused by storage pests are major problems for storage grains all over the world. *Cryptolestes pusillus* (Schon.) is a serious pest of stored legumes in most of the tropical countries. To protect the stored-grains from insect infestations, synthetic insecticides have been used successfully; but their massive and indiscriminate usage is a great problem (Sighamony *et al*. 1980).

General description about *Cryptolestes*

Cryptolestes is an important pest of oil seeds, dried processed foods of vegetables origin, cereals and cereal products. In the conditions of tropics, it is found on a wider range of commodities including cocoa, cassava, nuts and copra (David 2004). *Cryptolestes* spp. are incapable of damaging the whole kernel in good condition, but may be associated with primary invaders such as *Sitophilus* spp. and *R. dominica* (Singh *et al.* 2009).

C. pusillus multiplies and produces progeny on natural product or mixture of natural products at a high temperature of 29 ± 1^{0}C. Among the natural diets, the most favorable diet is wheat. The cracked food with better harborage and oviposition sites is the most favorable form of diet. Though it multiplies on all diets but wheat meal and cracked soybeans is unfavorable (LeCato 1974). The seeding adults generally die on diets which are not advantageous to multiply. *C. pusillus* produces relatively few progeny on whole grains, probably due to the small size of the adults and relative weakness of the mouthparts (Cotton 1963). It is reported that *C. pusillus* is unable to attack the undamaged grains (Payne 1946 and Davies 1949) and multiplies slowly on diets of whole corn, wheat and rice; apparently the germ of slightly damaged kernels served as the source of food.

C. pusillus lay their eggs on the grain kernels and in the cracks or furrows on the grain surface and hatch within five days at 30^0C and relative humidity of more than 65%. After hatching the larvae feed on the germ layer of the kernel, grain dust and broken kernels. Moulds grown in the endosperm when the grain moisture becomes high, renders the kernel more suitable as food for the larvae (Mason 2003). Among the four larval instars, the fourth instar larva secretes a small amount of silk which is used to fasten together a little bit of grains and other debris to make the cocoon for pupation. The silk originates from britles located on the anterior-laterial margins of the prothoracic segment of the larvae (Roberts and Rilett 1963). The larvae become contracted and inactive before pupation. They become translucent and white in color except the dark-brown compound eyes. The pupae are quite active and when disturbed they return the face to the downward position. Pupation takes about five days at 30^0C. On the fourth day of pupation, the color changes to light and gradually becomes darker until the beetles emergence (Mason 2003).

Currie (1967) reported that *C. pusillus* developed faster with the temperature above 25^0C. But that the growth is limited by low temperature and low relative humidity. It is most abundant in the wet tropical and warm temperate regions where it increases rapidly. The literature by Eden (1967) indicated that *C. pusillus* is the most economically important species in the southern United States. It is abundant and widely distributed species that infest a variety of stored commodities (Davies 1949 and Howe and Lefkovitch 1957).

Both the adults and larvae feed mainly on germs and endosperms of the grains, resulting in great damage to the grains (Peter and Ismaila 2015). *C. pusillus* is a worldwide cosmopolitan pest of stored-products particularly grains stored as grits; and behave as secondary pest following the infestation of primary stored-grain pests, such as *Sitophilus* spp. or *R. dominica* (Tuff and Telford 1964). However, *Cryptolestes* can attack apparently the sound grains when the grains become damaged by poor harvesting, storage and handling (David 2004). The adults as well as the larvae are cannibalistic and consume eggs, pupae and pre-pupae of other species co-habiting with them (Mason 2003).

Cryptolestes spp belongs to the beetles group in the family Cucujidae under order Coleoptera. There are six species under the genus *Cryptolestes* Ganglbauer namely *C.*

pusillus (Schon.), *C. ferrugineus* (Stephens), *C. pusilloides* (Steel & Howe), *C. turcicus* (Grouvelle), *C. capensis* (Waltl) and *C. ugandae* Steel & Howe recorded as stored products pests of the world. Generally husked rice, maize, wheat and wheat products, sorghum, barley and occasionally oil seeds are infested by them. The members of this genus have close external similarity often lead to confusion, but considerable differences exist in sclerites associated with male and female genitalia, habitat and in the range of geographical distribution. Among the known six pest species, the *C. pusilloides* (Steel & Howe), *C. turcicus* (Grouvelle), *C. capensis* (Waltl) and *C. ugandae* Steel & Howe do not occur in India. However, *C. ferrugineus* (Stephens) and *C. pusillus* (Schon.) are the most important pest species of the genus that are quite common in India; and serious pests of stored-grains and stored-products. It causes serious damage to unboiled rice, flour and suji and is more common. *C. pusillus* was considered to be a secondary pest prior to 1939, but it was recorded as a serious worldwide pest of rice during the Second World War (Sengupta *et al.* 1978).

Background information of *Mimosa pudica*

Carl Linnaeus first formally described *M. pudica* in *Species Plantarum* in 1753 (*Mimosa pudica.* Australian Plant Name Index (APNI), IBIS database). The term *Mimosa* is derived from Gk. *Mimos* = mimic, alluding to the fact that the leaves move in response to something and from L. *pudica* = bashful or shrinking to contact (Barneby 1991). It is also known as sleepy plant, sensitive plant, humble plant, chui-mui, action plant, ant plant (*Mimosa pudica.* Royal Horticultural Society 2018), shame plant or shy plant, dormilones, touch-me-not, etc. It is a creeping flowering plant and often grown for its curiosity value. When shaken or touched, the leaves fold inward and droop, and reopen a few minutes later (Klein 2016). This reflex may be an adaption to retain more water by shading itself and decrease water loss by heat evaporation or, is a defense mechanism to scare predators away from it.

M. pudica is a native species of North America, Central America and South America. But it is now also found in Bangladesh, India, Sri Lanka, Japan, Indonesia, Vietnam, Malaysia, Thailand, Philippines, Cambodia, Laos and Guam. It is an invasive species in many Pacific Islands and has been introduced to many other regions of the world (*Mimosa pudica.* Usambara Invesive Plants 2008). It is also regarded as an invasive plant in parts of Australia and is a declared weed in the Northern Territory (Declared Weeds. Natural Resources, Environment and The Arts 2008). In USA, it grows in

Florida, Virginia, Hawaii, Texus, Tennessee, Maryland, Georgia, North Carolina, Puerto Rico, Mississippi, Alabama and the Virgin Island (Distribution of *Mimosa pudica* in the United States of America). The largest center of *Mimosa* diversity is the central region of Brazil; and the biome 'Cerrado', in the central area of Brazil, is considered as important center of endemism of this genus (Flores-Cruz *et al.* 2004).

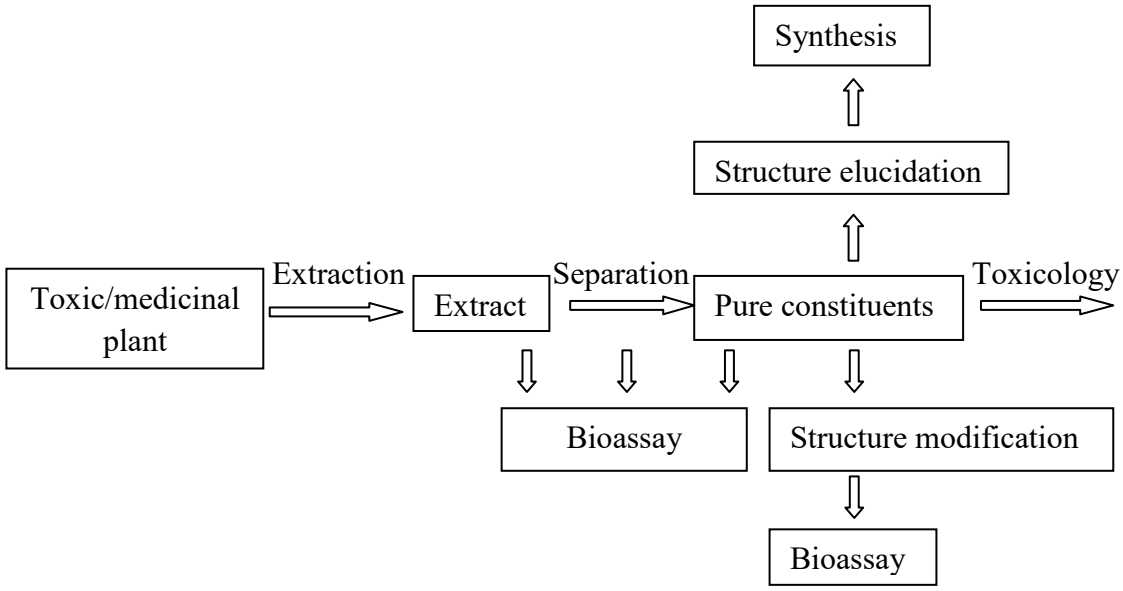


Fig 1. Basic pathway to synthesize bioactive constituents from plant (Hostettmann 1995)

Biology of *M. pudica*

The spread of the plant is performed by the seeds. The plant can complete its life cycle within 3 months. Propagation is performed by seeds and vegetative methods. The leaflets fold together and the rachises bend down at night. The flowers are insect and wind pollinated (*Mimosa pudica* L. US Forest Service 2008).

Habitat of *M. pudica*

M. pudica mainly grows on the hillside, jungle, glade and roadside of Asia. It occurs mostly in open marshy waste land areas; in sunny moist areas rather than heavily shaded areas (Nadkarani 1982 and Warrier *et al.* 1995). It has also been found in croplands, orchards, coconut plantations, scrub, pastures, in fences on river banks and areas disturbed by construction. It may grow singly or entangled thickets (Holm *et al.* 1977).

Table 1. Synonames or vernacular names of *M. pudica*

Language/Country	Synonames or Vernacular names	Meaning
Assamese	Adamalati, Lajubilata	-
Ayurveda	Lajjalu	Touch-me-not, shame plant, sleeping grass sensitive plant, humble plant
Bengali	Lojjaboti, Laajak, Lajjavathi	Shy virgin, bashful girl
Brazil/Portuguese	Dormideira	Roughly 'sleeper'
Central America	Dormilona	Sleepy head
Chinese	Memege (niue) Betguen sosa (guam) Limemeihr (phonpei) Mechiuain (palau) Ra Kau Pikikaa (cook islands) also- Hu he cao, Pa chou cao, Han xiu cao, Zhi xiu cao	Shyness grass
Danish	Almindelig mimose	-
Dutch	Kruidje-roer-me-niet	-
English	Sensitive plant, Touch-me-not, Humble plant, Shame plant, Bashful mimosa, Tickle-me plant	-
European	Nao-me-toque Sensitive Dormideira	Touch me not Sensitive Roughly 'sleeper'
Finnish	Tuntokasvi	-
French	Mimeuse commune, Mimosa pudique, Mimeuse pudique, Amourette herbe, Sensitive, Herbe sensible, Sensitive epineus	-
German	Gemeine mimose, Sinnpflanze	-
Haiti	Honte	-
Hindi	Laajvanti, Chhui-mui, Lajaalu	Unique property to drop, dies upon touch

Language/Country	Synonames or Vernacular names	Meaning
Hispaniola	Morivivi	-
Iloko	Bain-bain	-
Indonesia	Putri malu	Shy princess
Italian	Sensitiva	-
Kannada	Muttidare muni, Lajja, Nachika and Mudugu-davare	Angered by touch
Latin	Pudica	Shy shrinking
Liberia	Picker weed	-
Malayalam	Thottavadi, Tintarmani	Wilts by touch
Malaysia	Pakok semalu	Shy plant
Marathi	Lazalu	Shy
Mexico	Pinahuixtle, Quecupatli	-
Mayanmar (Burma)	Hti ka yoan	Crumbles when touched
Philippines	Makahiya	'Maka'- quite/tendency to be 'hiya'- shy/shyness
Punjabi	Lajan	-
Sanskrit	Namaskari, Namaskaar, Khadiraka, Lajjalu, Raktapaadi, Samangaa, Shamipatra, Lajja	-
Spanish	Mori-vivi, Dormidera, Sensitiva, Vergonzosa, Ten verguenza	'I died, I lived'
Sri Lanka (Sinhala)	Nidi-kumba	Sleeping plant
Swedish	Sensitiva	-
Tagalog	Makahiya	-
Tamil	Tottal sinungi, Tottalavaadi	Acts when touch
Telugu	Attapatti and Peddanidrakanni	-
Tonga	Mateloi	False death
Urdu	Chui-mui	-
West Indies	Mori vivi	'I died, I lived'
Siddha	Thottal Chinungi	-

Source: http://ijpsdr.com/pdf/vol5-issue2/1.pdf Churchward 1959, Union County College Biology Department 2008, Joseph *et al.* 2013.

Morphological attributes of *M. pudica*

The whole plant possesses thorny stem with branches, leaflets, flower heads, dry flowers and seed pods. It is a half-woody, diffusely spreading herb with branches that grow close to the ground. It is floppy, slender, branching and prickly with numerous bristly hairs. It grows up to 1.5m in length and 2.5cm in diameter, leaves bipinnate. The rachis (axis of compound leaf) is up to 5.5 cm in length; the pinnae (primary divisions of compound leaf) are sub-digitade into finger-like projections; the leaflets (the smallest segments of leaf) are 10 to 20 pairs, 0.3 to 0.4cm in breadth, 0.6 to 1.2cm in length, linear oblong or obliquely narrow. Peduncles prickly and cluster of (up to 5) flower heads is born in the leaf axil. The heads are 8 to 10mm in diameter. Floret petals red in upper part, filaments pink. Calyxes are very small about 0.2mm in length; corolla up to 2.3mm in length, ovate oblong, pink, lobes 4. In mid-spring to early autumn, flowers appear from leaf axils. Leaf axils are tufted with bristly hairs. Fruits are pods, up to 2.5cm in length, falcate (Saraswat and Pokharkar 2012). Seeds are compressed up to 0.3cm in length, 2.5mm in breadth, oval-elliptic, brown to gray, having a central ring on each surface and with seed coats that restrict germination. The seeds end dormancy mainly with high temperature (Chauhan and Johnson 2009). Roots are cylindrical, varying in length, thickness up to 2cm, tapering with secondary and tertiary branches, rough surface, brown, bark fibrous and fracture hard woody.

Uses of *M. pudica*

M. pudica is a traditional medicinal plant. Bioactivity studies have showed its hyperglycemic effect (Amalraj and Ignacimithu 2002) and anticonvulsant property (Bum *et al.* 2004). Moreover, phenolic and flavonoid compounds of this plant possess antidiabetic, anticancer and antiarthritic properties (McDonald *et al.* 2001). The leaf, stem, root, flowers, fruits and the whole plants are used in treatment of insomnia, neurasthenia, traumatic injury, pulmonary tuberculosis and others. Other bioactivities such as antibacterial, antioxidant, antihepatotoxic, etc. have also studied (Chowdhury *et al.* 2008, Samuel *et al.* 2008, Nazeema and Brindha 2009 and Adhikarimayum *et al.* 2010).

The phenolic compounds of methanolic extract of root exhibit good wound healing activities as well as cure skin diseases (Kokane *et al.* 2009). To reduce toothache, decoction of root is used with water and it is useful for wound healing, gynecological disorders, general weakness and impotence, bronchitis, etc. *Mimosa* root is used in

treatment of hemorrhoids, irritability, skin wounds, insomnia, menorrhagia and premenstrual syndrome in Western medicine (Nair *et al.* 2005). *M. pudica* leaves in infusion or decoction are used in sore throat, hypertension, glandular swelling and hoarseness; root is used as dysmenorrheal, diuretic and considered aphrodisiac and used for bladder gravel (Joseph *et al.* 2013).

Phylogenetic position of *M. pudica*

Kingdom : Plantae
Division : Magnoliophyta
Class : Magnoliopsida
Order : Fabales
Family : Fabaceae
Sub-family : Mimosoideae
Genus : *Mimosa*
Species : *M. pudica* L.

Phytoconstituents of *M. pudica*

M. pudica contains mimosine (Agharkar 1991 and Chauhan and Johnson 2009), a toxic alkaloid that have apoptotic and antiproliferative effects (Restivo *et al.* 2005). The leaf extract contains adrenalin like substance. The root contains flavonoids, alkaloids, tannins, phytosterol, glycoside, amino acids and fatty acids. The seeds contain mimosine. It also contains mucilage, composed of D-xylose and D-glucuronic acid 4-O-(3,5-dihydroxybenzoic acid)-β-D-glucoronide (Chatterjee and Pakrashi 2006). The plant contains up to 17% green yellow fatty oil. It also contains tubuline (Azmi *et al.* 2011). The leaf extracts possess the compounds as flavonoids, glycosides, alkaloids, terpenoids, phenols, coumarins, saponins, quinines, tannins, sterols and fatty acids (Gandhiraja *et al.* 2009, Rajendran and Sundararajan 2010 and Genest 2008).

Fresh tissues contain β-sitosterol and nor-epinephrine. The methanolic extract of leaves contain flavonoids, alkaloids, glycosides, terpenoids, phenols, quinines, tannins, saponins and coumarins (Mohan *et al.* 2015). Alkaloids are found in leaves (Khare 2004). The roots release SO_2, methyl sulfinic acid, pyruvic acid, ethane sulfinic acid, lactic acid, propane sulfinic acid, thioformaldehyde, 2-mercaptoaniline and S-propyl propane 1-thiosulfinate (Musah *et al.* 2015).

Chromatographic study showed that the plant contains glycosides, saponins, flavonoids and phenolic constituents (Vinothapooshan and Sundar 2010); tubuline, phytosterol, adrenaline, gums, green-yellow fatty oil, terpenoids, coumarins, quinines, c-glycosylflavone, phenolic ketone (Josewin *et al.* 1999).

The leaf contains phenolic ketones and β-sitosterol. The other compounds of the oil extract are 1-octanamine, methyl ester, 2-methylamino-N-phenylacetamide, N-methyl, 1-butanamine, 1,3-dioxolane-4-methanol, meglumine, 9,12-octadecadien-1-ol, 11,13-eicosadienoic acid, methyl ester and 2,5-dimethoxy-4(methylsulphonyl) amphetamines (Saraswat and Pokharkar 2012). The genus *Mimosa* contains an amino acid L-mimosine or leucenol (Thompson *et al.* 1969).

Importance of *M. pudica*

The paste of rhizome part of *M. pudica* (Nilagibon) was applied locally to treat toothache. The leaf powder was mixed in equal amount of Gandhan (*Cordia ghraf*) stem bark powder with a pinch of salt and boiled in a glass of water and gargled for relieving pain in gum and teeth. The decoction of root was used with water to gargle to reduce toothache (Joshi and Joshi 2006, Bora *et al.* 2012, Slave 2012 and Srivastava *et al.* 2012). Shu (1998) described that herbal drugs are using as medicines for treatment of diseases since ancient time. Paula *et al.* (2012) described that *M. pudica* was a common herb used for pain and inflammation.

Genest *et al.* (2008) evaluated that the major pharmacological activities of *M. pudica* were antimicrobial, antiviral, aphrodisiac, antihepatotoxic, antivenom, hyperglycemic, diuretic, wound healing, antioxidant effects, etc. Sharma (2006) denoted that the flavonoids possessed vasodilator, antileukemic, radical scavenger and antioxidant activity. Ghani (2003) reported that the plant *M. pudica* contained turgorins.

Aalok (1997) and Nayagam and Pushparaj (1999) mentioned that *M. pudica* roots in combination with other medicinal plants were used for gynecological disorders, diarrhea, amoebic dysentery, skin diseases and blood pressure disorders. Norton (1978) depicted that the flavonoids and glycosides produced by the plant was useful for antifertility activity.

Background of the study

Agricultural products are highly sensitive to insect pest and diseases. So the farmers apply the insecticides to restrain crop losses from pest assault. Use of pesticides in Bangladesh has increased dramatically during the last five decades. The government of our country has adopted chemical control measures to increase the production of crops in addition to prevent the pre and post-harvest losses of crops (Rahman *et al.* 1995, Matin 2003 and Aziz 2005). Most of our farmers apply pesticides without knowing the real necessities and effectiveness of them, and also they apply very excessive frequencies (Anonymous 2000). They use the pesticides unnecessarily and indiscriminately due to their ignorance and unconsciousness; and continuous exposure of pesticides poses great hazard to the environment. Some beneficial organisms also killed with the high degree of toxicity. Thus over use of pesticides creates serious problems not only on human health but also on wildlife and sensitive ecosystem (Stoate *et al.* 2001, Chitra *et al.* 2006, Berny 2007 and Power 2010). There are a great need and demand for the safety measures of the stored agricultural products and also for the production and introduction of the recent, less dangerous, less toxic and more secure insecticides (Fields 1999).

In order to develop new chemotherapeutic agents, plants are the important source and to achieve this goal *in vitro* antibacterial assay is the first step (Mojab *et al.* 2003). The development of antimicrobials into phytomedicines is of great importance in perspective of drug development (Iwu *et al.* 1999).

The farmers of Bangladesh lose a huge amount of their income from post-harvest losses of stored-grains. It is excessive and varies from 10-25%. According to GoB 2000, the losses can attain as excessive as 30%. *C. pusillus* causes a huge economic loss every year in our country. If we able to control the pest by bioactive compounds, it would minimize the economic loss in agriculture. So this research work was an attempt to control the pest, *C. pusillus* commonly known as flat grain beetle, biologically by using the bioactive components from *M. pudica* and also some microbial tests by using the crude extracts of leal, stem and root as well as purified compounds of *M. pudica* against some harmful microbes.

Objectives of the study

In both public and private sectors the Integrated Pest Management programs are being utilized. Moreover, eco-friendly, safe, more efficient and low-cost agents should be introduced. Thus it is necessary to find out new alternatives which are ecologically sound and without residual activity to non-target organisms. In this study *C. pusillus* is chosen as test insect as they are easy to culture, both the larvae and adult share the same food medium and cause serious damage and infestation to stored-grains. The present study aims to investigate the biochemical activities of different crude extracts of leaf, stem and root of *M. pudica*, followed by further fractionation by column chromatography, and analyze the fraction by Gas Chromatography-Mass Spectrometry coupled system (GC-MS). So we concentrate our mind:

- To evaluate the antimicrobial activities of the crude extracts as well as pure compounds of *Mimosa pudica*;
- to determine the antioxidant properties of the crude extracts through DPPH free radical scavenging assay;
- to isolate phytoconstituents of crude extracts of *M. pudica* as well as identification of bioactive pure compounds through chromatographic techniques and
- to evaluate the efficacy of *M. pudica* extracts for insecticidal potential through dose-mortality bioassay and repellent activity against *Cryptolestes pusillus,* and brine shrimp lethality bioassay.

Chapter Two

REVIEW OF LITERATURE

Antibacterial activity of *M. pudica*

Various studies have been conducted on the assessment of antimicrobial potential of *M. pudica.* The species showed mild to moderate antimicrobial potency against different bacterial and fungal strains.

Ahuchaogu *et al*. (2017) studied the ethanolic extracts for antibacterial activity of the wholesome parts of *M. pudica* using well diffusion method and the activity was observed against *E. coli, S. aureus, E. faecalis, P. aeruginosa* and *M. smegmatis.* Antibacterial activity was found against *E. coli, S. aureus* and *P. aeroginosa* at different concentrations and they concluded the presence of antimicrobial components which could be used for modern medicine. The potency of the plant extract of *M. pudica* was analysed to inhibit the growth of three microorganisms namely *B. subtilis, S. aureus* and *C. albicans* and found strong antibacterial activity against *B. subtilis* and *S. aureus* while a negative activity against *C. albicans* (Racadio 2016).

According to Amengialue *et al.* (2016), aqueous, methanol and ethanol leaf extracts of *M. pudica* were screened against some bacterial and fungal strains. The methanol extract showed better antimicrobial activity than ethanol extract; while the aqueous extract showed the least antimicrobial activity against the organisms. The study showed that the leaves of *M. pudica* could be a better antibacterial agent.

Chlorophyllin from leaf extract of *M. pudica* was assessed by Rajalakshmi and Banu (2016) for the antibacterial activity against human pathogenic bacteria (*K. pneumoniae, S. aureus, E. coli* and *P. aeruginosa*) and fungal (*C. albicans*) pathogens. The results showed that the natural chlorophyllin from the leaf extract of *M. pudica* had potential antibacterial activity against the tested microorganisms that might be useful for discovery of drugs against some diseases.

Ethanolic extracts of *M. pudica* was screened for antimicrobial potency against four bacterial strains *viz. Streptococcus pyogenes*, *S. aureus, P. aeruginosa* and *E. coli* and Ampicillin was used as standard. The results exhibited a strong antibacterial potential against all the bacterial strains (Sunil *et al.* 2016). Chinnathambi and Sathasivam

(2015) analysed the medicinal properties of leaves of *M. pudica* and antibacterial potency of active compounds against some human pathogenic bacteria and found the acetone and ethanol extracts most active. The aqueous extracts exhibited only limited inhibition against the tested organisms. Ethanol extracts of leaves of *M. pudica* were screened for phytochemical analysis and the leaves and roots samples were subjected to antimicrobial activity by Mohan *et al.* (2015) and revealed the presence of tannins, proteins and steroids which were biologically active compounds that aided in antimicrobial activity.

Thoa *et al.* (2015) evaluated the antibacterial potency of water and ethanol extracts of *M. pudica*, and found significant antibacterial activity against *B. subtilis, S. aureus, E. coli* and *S. typhi* by ethanol extract; while water extract inhibited the growth of *B. subtilis* and *S. aureus.*

Abhirami *et al*. (2014) studied the antimicrobial effectivity of petroleum ether, acetone, ethyl acetate and aqueous extracts of *M. pudica* against human pathogenic bacteria like *S. aureus, S. typhi, Lactobacillus, P. aeruginosa* and *E. coli* and plant pathogenic fungi like *Paecilomyces variotii, F. oxysporum* and *Pestalotia foedians* at different concentrations. In case of antibacterial test, the acetone extract exhibited the maximum inhibition against *S. aureus* while for antifungal test, the maximum inhibition was found for ethyl acetate extract against *F. oxysporum.*

The methanolic extract of leaves of *M. pudica* was tested for antibacterial activity against six bacterial strains *viz. B. subtilis*, *S. aureus, E. coli, S. typhi, K. pneumoniae* and *Proteus mirabilis* and the result showed a good antibacterial effect by Arokiyaraj *et al.* (2012).

Panda *et al.* (2012) carried out the phytochemical analysis, isolation of metabolites and antimicrobial activities of petroleum ether, ethyl acetate and methanol extracts of roots of *M. pudica*. Twelve bacteria and fungi were tested for antimicrobial potency in comparison to the control standard. Better antimicrobial activity was observed by the ethyl acetate root extract in comparison to petroleum ether and methanol extracts.

Tamilarasi and Ananthi (2012) screened the phytochemical constituents and antimicrobial effectivity of ethanol extracts of *M. pudica* leaves against *K. pneumoniae, P. aeruginosa, B. subtilis, Trycophyton rubrum* and *A. flavus* at different concentrations. The phytochemical analysis showed antimicrobial activity of the extract

due to the presence of active constituents as alkaloids and tannins. The susceptibility of microbial pathogens to different concentrations indicated the potential source of antimicrobial compounds of the plant.

Doss *et al.* (2011) isolated the flavonoid compounds of pharmacological interest from *M. pudica* and assayed against the bacterial strains *B. subtilis, S. aureus, S. typhi, E. coli* and *P. mirabilis.* Flavonoids showed antibacterial activity against the tested microorganisms. *M. pudica* leaves had long been recognized for antibacterial potency which inhibited bacterial pathogens.

The antibacterial activity of cold and hot methanol extracts of *M. pudica* was evaluated against seven pathogenic bacteria using agar well diffusion method by Jyothi and Rao (2011). The hot methanol extract showed large zone of inhibition than cold methanol extracts. *E. coli, B. subtilis, K. pneumoniae, E. faecalis* and *P. vulgaris* were highly susceptible to hot methanol extracts of *M. pudica*. The results provided promising information for the potential use of *M. pudica* methanol extract against bacterial infections.

Kaur *et al.* (2011) studied the antimicrobial activity of leaves of *M. pudica* extracted with absolute ethanol and distilled water. The extracts were tested against *Pyrogen, P. aeruginosa, E. coli, S. aureus* and *C. albicans* at different concentrations. The highest zone of inhibition was found against *S. aureus* and *E. coli* while *P. aeruginosa* showed good sensitivity.

The petroleum ether, chloroform and ethanol extracts of *M. pudica* were assessed for antibacterial activity against fifteen human pathogenic bacteria. The ethanol extract was more effective among the three types of extracts, and gram-negative bacteria were more sensitive to the plant extracts (Akter *et al*. 2010).

Rajendran and Sundararajan (2010) performed the phytochemical screening and antibacterial activity test of n-hexane, chloroform, ethyl acetate, methanol and methanolic fractions of leaves of *M. pudica* and the antibacterial efficacy was evaluated comparing with the standard Ciprofloxacin (5µg/disc). The results revealed that the plant *M. pudica* contained potential antibacterial compounds which might be used for the development of phytomedicine.

Antimicrobial activity of the methanol extract of *M. pudica* was examined against *A. fumigatus*, *C. divergens* and *K. pneumoniae* by Gandhiraja *et al.* (2009) and found antimicrobial activity against the microorganisms. The antimicrobial activity was found

for bioactive compounds like terpenoids, flavonoids, glycosides, alkaloids, quinines, phenols, tannins, saponins and coumarins. Chowdhury *et al.* (2008) screened the petroleum ether, chloroform and methanol crude extracts (500µg/disc) of aerial parts and roots of *M. pudica* against 13 bacterial and 3 fungal strains for antimicrobial activities. But none of the extracts showed significant inhibition of growth of the test microorganisms.

Ethyl alcohol extract of leaf of *M. pudica* was screened for the antibacterial activity determination by Kirby Bauer disc diffusion method against two test microorganisms *viz. S. aureus* and *E. coli* by Alpuerto and Daclan (2003). The extracts showed antibacterial potency against both the test organisms and the antibacterial potency of *M. pudica* extracts might be due to flavonoid compounds.

Antifungal activity of *M. pudica*

Khalimuthu *et al.* (2010) denoted the presence of phytochemical compounds of the plant part of *M. pudica* and highlighted the variations in the antifungal potency of the plants. The variation in the sensitivity could also be attributed to the differences in growth rate of isolated organism, inoculums size, nutritional requirement and temperature.

Stevia and Rebs (2008) described that the leaves of *M. pudica* contained alkaloid mimosine, root contained tannins, calcium oxalate crystals, ash and mimosine. It was susceptible to several herbicides including dicamba, glyphosate, picloram and triclopyr. Tsuchiya *et al.* (1996) denoted that the antifungal activity of *M. pudica* extract was probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. Muhammad *et al.* (2015) revealed that the results of antifungal test using the ethanol and aqueous extracts of *M. pudica* leaves showed antifungal effects against some isolated fungi. The potential sensitivity of the extracts was obtained against some fungal strains tested namely *Trichophyton verrucosum, Microsporum ferrugineum, T. schoenleinii, T. rubrum, T. concentricum, T. soudanense, M. canis,* and *M. gypseum*. The results showed that *T. verrucosum* and *T. soudanense* had the maximum zone of inhibition while *M. canis* showed the minimum zone of inhibition in ethanol and aqueous extracts of leaf.

The *M. pudica* whole plant was extracted in absolute ethanol, and the crude ethanolic extract and its isolated triterpenoid glycoside were observed for antifungal activity against *T. rubrum* and *A. flavus* by Chukwu *et al.* (2017). The crude extract possessed

antifungal activity against tested microorganisms indicated that the tested plant contained potential antimicrobial compounds.

Kakad *et al.* (2015) tested the antifungal potency of methanol extracts of leaves of twenty five plant species against *C. albicans, A. niger* and *Daedalea flavida*. Among the plants *M. pudica* was recorded antifungal activity to *C. albicans.* The antifungal activities of ethanol, methanol and aqueous extracts of leaf, stem and mixture of leaf and stem of *M. pudica* were investigated by using agar incorporation method by Dwivedy and Dubey (1983). The activity was observed against *A. niger, A. flavus, M. nanum, T. verrocusum* and *T. mentagrophytes.* The results showed that the extracts of *M. pudica* had no activity in water against *A. niger* in all three parts *i.e.* leaf, stem and mixture of leaf and stem. The stem extracts exhibied significant potency against the fungal strains. The activity was increased significantly with the increasing concentrations of extracts. At different concentrations the result revealed significant difference in percentage growth inhibition of leaf, stem and mixture of leaf and stem of the plant on all fungal isolates (Ibrahim *et al.* 2014).

Tamilarasi and Ananthi (2012) tested the ethanol extracts of leaf of *M. pudica* against the fungal strains *T. rubrum* and *A. flavus* at different concentrations through and the highest inhibition was found by *A. flavus.* They also described that the leaf methanol extract of *M. pudica* was active against microorganisms like *A. fumigates* and *C. divergens*. The activity might be due to the compounds like alkaloids, flavonoids, glycosides, terpenoids, tannins, saponins, quinines, coumarins and phenol.

Minimum Inhibitory Concentration

The minimum inhibitory concentrations (MIC) of different extracts were recorded against the test microorganisms at different concentrations (100, 50, 25, 12.5, 6.25 and 3.125mg/ml) with methanol and ethanol extracts of leaves of *M. pudica* and the results showed a significant MIC values against the test organisms (Amengialue *et al.* 2016).

Antioxidant activity of *M. pudica*

Phenolic compounds and some of their derivatives were very efficient to prevent autoxidation. But only a few phenolic compounds were permissible by law as food antioxidants. The activity and potential toxicity were the major concerns for acceptance of such antioxidants. The non-sugar part of saponins had a direct antioxidant activity. Triterpenes, a subclass of terpenes possessed antioxidant properties. Hence, the

presence of triterpene constituent in the plant extract was an indicator of antioxidant property (Racadio 2016).

Sahu *et al.* (2015) denoted that the defense mechanism of organisms against the attack of the free radicals was depended on antioxidants. Razali *et al.* (2008) described that the leaves of *M. pudica* possessed antioxidant property. The secondary metabolites in the plant extract interact with oxygen molecules to minimize the synthesis of nitric oxide.

Nagler *et al.* (2006) mentioned that DPPH was a stable radical and became unstable when received electrons from reducing agents. *M. pudica* acted as reducing agents, exhibiting antioxidant property and the antioxidant mechanism inhibited and scavenged free radicals to provide protection against infections and degenerative diseases. Couladis *et al.* (2003) reported that a large number of plants contain the antioxidants including vitamin C and E, carotenoids, flavonoids, tannins and that could be used to scavenge the excess free radicals from the human body. According to Gao *et al.* (1999), plants have potent antioxidant compounds such as flavonoids, isoflavonoids, flavones, coumarins, anthocyanins, lignans, catechins and isocatechins. Also vitamin C, vitamin E and betacarotene possess antioxidant activity.

Song *et al.* (2017) extracted the leaves of *M. pudica* with methanol to show the antioxidant activity of leaves through DPPH free radical scavenging activity. They suggested that the methanolic extract of the aerial part of the plant possessed good antioxidant potential compared to ascorbic acid.

Hexane crude extract of leaf of *M. pudica* was collected by vacuum distillation and the antioxidant potency was evaluated by DPPH (2,2-diphenyl-1-picrylhydrazil), nitric oxide hydroxyl and superoxide radicals by Almalki (2016) and revealed that the hexane extracts had significant scavenging activity on DPPH, nitric oxide, hydroxyl and superoxide radicals.

Patro *et al.* (2016) evaluated total phenolic and flavonoid contents from various extracts of *M. pudica* leaves and evaluated the antioxidant activity against 2-2-diphenyl-1-picrylhydrazyl (DPPH). The ethyl acetate extract of leaf in different doses were administered orally for seven consecutive days to albino rats and observed the oxidative stress markers. They observed the highest total phenolic and flavonoid contents by ethyl acetate extract than other extracts of plant leaves.

The antioxidant activity of aqueous and ethanolic thorn extracts of *M. pudica* was compared by Parmar *et al.* (2015) and found that the aqueous thorn extract had higher antioxidant activity than ethanolic thorn extract. The ethanol extract of leaf showed considerable antioxidant activity when scavenged with 2-2-diphenyl-1-picrylhydrazyl (DPPH) free radicals. He suggested that flavonoids might be responsible for the antioxidant properties. Das *et al.* (2014) evaluated the antioxidant and toxic properties of methanol extract of leaf of *M. pudica*. Antioxidant activity was determined through DPPH assay and the DPPH scavenging activity was compared with ascorbic acid. The methanolic crude extract showed a moderate antioxidant activity. The results showed that the DPPH reading was not very close to ascorbic acid. It was because the leaf extract of plant was in crude form.

Muthukumaran *et al.* (2011) also denoted that the methanolic crude extract of aerial part of *M. pudica* had moderate antioxidant potential.

Zhang *et al.* (2011) examined the antioxidant activity of the ethanol extracts of whole plant, leaves, stems, seeds and five flavonoid monomers of *M. pudica* by 2-2-diphenyl-1-picraylhydrazyl (DPPH) radical scavenging activity. They found the sequence of antioxidant activity of ethanol extracts as leaves > whole plant > seeds > stems and suggested that the plant could be a potential source of natural antioxidants.

Rajendran *et al.* (2010) screened the chloroform extract of *M. pudica* leaves for *in vitro* antioxidant activity against free radical scavenging by DPPH, nitric oxide, superoxide dismutage and reducing power ability. It was found that the chloroform extract of leaf had a strong antioxidant activity against free radical scavenging by DPPH, nitric oxide, superoxide dismutage and reducing power ability and the chloroform extract not only scavenged off the free radicals but also inhibited the generation of free radicals. Chowdhury *et al.* (2008) screened the methanol crude extract of the aerial part of *M. pudica* for antioxidant activity using 2-2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and found moderate antioxidant activity compared to ascorbic acid which suggested the presence of biologically active compounds in methanol extract.

Phytoconstituents of *M. pudica*

Umar *et al.* (2010) described that different classes of phytoconstituents extracted out by different solvents based on their polarity index. The flavonoids and phenolic compounds

would be successfully extracted out by the ethyl acetate as a semi-polar solvent, acetone as a polar solvent and methanol as a very polar solvent during the extraction and fractionation processes. As methanol was the most polar solvent, consequently methanol extract contained maximum flavonoids than other fractions.

Ramesh *et al.* (2014) screened for phytochemical compounds and GC-MS analysis of the leaves of *M. pudica* and revealed that the leaves of *M. pudica* contained various organic compounds *i.e.* fatty acids, amino acids, methyl and ethyl ester, flavonoids, phytol, vanillins, tyrosins, sugars, etc. which were responsible for biological activity.

Chandran *et al.* (2018) described that the *M. pudica* leaf extracts in hexane, chloroform, dichloromethane, ethyl acetate, acetone, methanol and aqueous possessed the phytochemicals like alkaloids, carbohydrate, coumarins, cardiac glycoside, saponins, glycosides, flavonoids, phenols, phytosterols, tannins and proteins. Methanol extract contained most of the phytochemicals. Ahuchaogu *et al.* (2017) showed that the whole plant of *M. pudica* consisted of 9.05% alkaloid, 8.32% flavonoid, 2.49% steroid, 8.15% saponin, 1.02% phenol, 0.083% tannin, o.122% cyanogenic glycoside and 1.913% anthocyanin. According to Amengialue *et al.* (2016), the results of the quantitative phytochemical screening of *M. pudica* showed the presence of all phytochemicals *i.e.* flavonoids, glycosides, alkaloids, steroids, saponins and tannins with some variations of extracting solvents used.

Kumarasamyraja *et al.* (2012) reported that *M. pudica* contained mimosine an alkaloid, oleic acid, linoleic acid, sitosterol and free amino acids. Aarthi and Murugan (2011) described that the hepatoprotective effect of *M. pudica* might be for the constituents like flavonoids, terpenoids, alkaloids, tannins, glycosides and saponins. According to Kaur *et al.* (2011), the secondary metabolite potential of *M. pudica* contained nitrogen-containing compounds, sitosterols, fatty compounds and rich source of flavonoids.

Gandhiraja *et al.* (2009) described that the ethanolic extracts of thorn of *M. pudica* contained steroids and the aqueous extracts of thorn contained the tannin. The preliminary phytochemical screening of leaf extract of *M. pudica* showed the presence of bioactive compounds such as alkaloids, flavonoids, terpenoids, glycosides, tannins, saponins, quinines, phenols, coumarins, fatty acids and mimosine. Rajendran *et al.* (2009) concluded that the methanol extract of *M. pudica* showed a hepatoprotective effect and the phytoconstituents like alkaloids, flavonoids and glycosides were responsible for the

significant hepatoprotective activity. Ghani (2003) reported that the leaves and stems of the plant contain mimosine, leaves also contain mucilage and roots contain tannins.

Vijayalakshmi and Udayakumar (2018) identified 23 bioactive compounds from ethanolic extract of leaf and 17 bioactive compounds from ethanolic extract of root of *M. pudica* through GC-MS analysis. Both the extracts of leaf and root possessed fatty acids, fatty alcohol, flavonoids, furans, terpenes and sugar derivatives. Ramesh *et al.* (2014) investigated the phytochemical analysis of ethyl acetate extract of leaves of *M. pudica* followed by further fractionation through column chromatography and analysis of the fraction by GC-MS coupled system and 52 compounds were identified. Among the constituents 1,3,5 cycloheptatrine-4-pentenal, 2-methyl, 1-butanol, 1-propene, 3-methyl acetate, vanillin tyrosine, cyclopentanol, acetic acid, phytol, etc. were present.

Vismayaviswan *et al.* (2019) identified 23 compounds from the essential oil of *M. pudica* through GC-MS and the major compounds were phthalic acid dioctyl ester, α-linolenic acid and cinnamaldehyde.

Saraswat and Pokharkar (2012) performed the gas chromatography electron ionization mass spectrometry (GC-MS) with oil extract of *M. pudica* and Lab solution software package was used for System Control and data evaluation. Esterification of oil extract was done with methanol and potassium hydroxide for GC-MS analysis. In the GC-MS chromatogram of *M. pudica* oil extract they found 35 peaks.

GC-MS was performed for the methanol extract of leaves of *M. pudica,* a medicinal plant, for identification of phytocompounds. They identified 19 compounds of which 10 had therapeutic values. Among the compounds phytol, glycerin, myo-inositol, trimethyl caffine, hexadecanoic acid, hexadecane-1ol, octadecadienoic acid, vitamin-E, etc. were present (Sridharan *et al.* 2011).

Insecticidal activity of *M. pudica*

Uyi *et al.* (2018) revealed that the low concentration of *M. diplotricha* caused significant mortality against the *Macrotermes* sp. The mortality caused by *M. diplotricha* root extract was high and both concentration and exposure time dependent. At the highest concentration (10% w/v) the root extract caused 100% mortality against *Macrotermes* sp. Addisu *et al.* (2014), Lawal *et al.* (2015) and Ileke and Olotuah (2012)

also reported the same findings *i.e.* increased the mortalities with increasing the concentrations of plant extracts, oils and powders against the pests. The reasons behind the insecticidal activities of *Mimosa* species could be attributed to the presence of sterols, tannins, flavonoids, alkaloids and terpenoids.

Aarthi *et al.* (2011) denoted that the leaf extract of *M. pudica* enhanced the larvicidal and pupicidal activity and hence it might be an effective alternative to conventional synthetic insecticides to control *Anopheles stephensi.* The smoke exposure affected the central nervous system and hence affected the neuroendocrine system to inhibit the hatchability of eggs and reduced the egg laying capacity.

Larvicidal activity of *M. pudica*

Astalakshmi *et al.* (2016) evaluated the larvicidal properties of aqueous extract of leaves of *M. pudica* against the larvae of *Aedes aegypti.* The percentage mortality was calculated against five different concentrations and it was noted that the aqueous extract of leaves of *M. pudica* possessed poor larvicidal activity and suggested the extract of *M. pudica* was not suitable for larvicidal actions on mosquitoes.

The results from preliminary screening, Kamaraj *et al.* (2010) prepared leaf and rhizome extracts of eight medicinal plants for testing larvicidal activity against the larvae of *C. gelidus* and *C. quinquefasciatus*. The dead larvae were counted after 24h of exposure. Among the eight plant species, the larvicidal activity of hexane, ethyl acetate and methanol extracts showed moderate toxic effect on the 4^{th} instar larvae; however, the highest percentage mortality was observed in ethyl acetate extract of *M. pudica* against the larvae of *C. gelidus* and *C. quinquefasciatus*.

Repellent activity of *M. pudica*

Uyi *et al.* (2018) investigated the repellent activity of aqueous root extract of *M. diplotricha* against *Macrotermes* sp. at different concentrations using filter paper impregnation technique and percentage repellency was observed for 30 minutes exposure period. The root extract of *M. diplotricha* significantly repelled *Macrotermes* sp. and this repellency was depended on the concentrations of the root extract used. The highest concentration of *M. diploticha* root extract (10% w/v) repelled 100% of the species.

The antivectorial activity of ethanolic extract of leaf of *M. pudica* was tested in the laboratory at different concentrations against different immature stages of *A. stephensi* by Aarthi *et al.* (2011) and considerable toxic effect against the vector species. The smoke toxicity effect of *M. pudica* leaf showed a good knock down effect when compared with commercially produced mosquito coils. The leaf extract of *M. pudica* possessed the smoke repellent activity.

Brine shrimp lethality bioassay

Methanol extract of leaves of *M. pudica* was tested by Das *et al.* (2014) for its cytotoxic properties through brine shrimp lethality bioassay. They showed lethality in a dose-reliant conduct. They suggested that the *M. pudica* leaf would be a significant source of anticancer compounds.

Chowdhury *et al.* (2008) screened the petroleum ether, chloroform and methanol crude extracts of aerial parts and roots of *M. pudica* for cytotoxicity study with brine shrimp lethality bioassay and they suggested that the petroleum ether and methanol crude extracts of roots of *M. pudica* posessed potent cytotoxicity whereas the chloroform extractives showed poor cytotoxic activity.

Genest *et al.* (2008) carried out the comperative general toxicity study of n-hexane, dichloromethane and methanol extracts of *M. pudica* and *M. rubicaulis*, two Banladeshi medicinal plants through brine shrimp lethality assay. They reported that none of the plants showed any significant toxicity to the brine shrimps.

Chapter Three

GENERAL METHODOLOGY

Collection of plant, identification and processing for extraction

The target plant touch-me-not, *M. pudica* was collected from the Botanical Garden of Rajshahi University Campus. It was then confirmed by the Taxonomist of the Department of Botany, University of Rajshahi. The leaf, stem and root of the herb were used as plant materials. So the whole plants with roots were collected on December 2017. The collected plants were taken in the laboratory and cleaned the soil attached with roots with a brush. The leaf, stem and root were separated with scissor. The separated parts of the plants were kept in wooden trays for drying in room temperature and the samples were kept contamination-free by any cost.

The fresh plant materials were processed through the following ways:

Leaf: Very young and greenish mature leaves avoiding old yellowish ones were collected from the stems and spread out to dry without heaping the material together. It was done in the laboratory in normal room temperature avoiding direct sunshine.

Stem: The stem were collected carefully and were chopped with the help of chopper and scissor as small as possible and spread out to dry in normal room temperature avoiding direct sunshine.

Root: The root, young primary and secondary, were separated from the stem and chopped with the help of chopper as small as possible. The chopped roots were dried in wooden trays in the laboratory in normal room temperature.

After drying, the leaf, stem and root were grinded separately in a grinding machine and the dusts were preserved separately in the air-tight bags. Every time 100g dust of each part of the plant *i.e*. leaf, stem and root were taken in envelopes, sealed and kept them in air-tight transparent polythine bags. The bags were marked with date and stored at 4^0C in refrigerator for extraction.

Chemical extraction of the plant materials

To yield the extracts of leaf, 500g dust of leaf was taken in five conical flasks of 500 ml (100g in each conical flask) and poured 400ml petroleum ether in each of the five flasks. All the flasks were covered by aluminum foil and shaken for 72 hours in an electric shaker. After shaking the extract was filtered by Double Rings Filter paper

No.102 and collected in a beaker. After filtering again the flasks were poured with 300ml petroleum ether, shaken for 48 hours and collected the extract in the same beaker. Repeated the work for third time, the beaker was kept at room temperature for evaporating the solvent with the help of aerator. After evaporation, the extract was collected in a glass vial and preserved in refrigerator at 4^0C for experiment. The same procedures were followed to yield the extracts of stem and root.

Fig 2. *M. pudica* in the field

Fig 3. *M. pudica* collected

Fig 4. Chopping of *M. pudica*

Fig 5. Grinding of *M. pudica* for dust

When the extracts of leaf, stem and root of petroleum ether were collected, the dusts in the conical flasks were dried in room temperature to evaporate the solvent. Again the dusts were taken in five conical flasks of 500 ml and used chloroform as solvent and the same procedures were followed as taken for the petroleum ether extracts of leaf, stem and root. The dried extracts were taken in glass vials and preserved at 4^0C. The same procedures were followed to yield the ethyl acetate and methanol crude extracts of leaf, stem and root of *M. pudica* and preserved at 4^0C for the future experiments.

Fig 6. Leaf of *M. pudica*

Fig 7. Stem of *M. pudica*

Fig 8. Root of *M. pudica*

Fig 9. Dust of leaf

Fig 10. Dust of stem

Fig 11. Dust of root

Crude extract bioassay

At first, suitable target organisms were chosen for the selection of bioassays to employ in research on plant constituents. The complexity of the bioassay had to be designed as a function of the facilities and resources available.

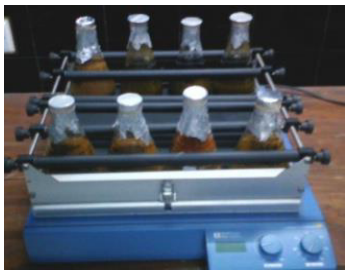


Fig 12. Shaking for extraction

Fig 13. Filtering of extraction

Fig 14. Evaporation of solvents

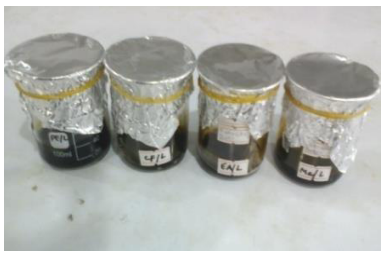


Fig 15. Extracts of leaf

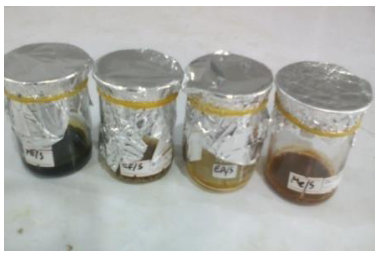


Fig 16. Extracts of stem

Fig 17. Extracts of root

Table 2. The list of bioassays taken for the investigation

Types of tests	Test agents
Antimicroibial assay:	
i. Antibacterial activity test	Eight pathogenic bacteria
ii. Antifungal activity test	Four pathogenic fungi
iii. Minimum Inhibitory Concentration	Six pathogenic bacteria
Antibacterial activity test of pure compounds	Eight pathogenic bacteria
Antioxidant activity test	DPPH free radical scavenging assay
Isolation of phytoconstituents from extracts	*Mimosa pudica* L.
Identification of pure compounds	$CHCl_3$ and petroleum ether extracts
Insecticidal assay	*Cryptolestes pusillus* (Schon.)
Insecticidal activity test of pure compounds	*C. pusillus*
Repellent activity test	*C. pusillus*
Brine shrimp lethality bioassay	Brine shrimp nauplii (*Artemia salina*)

The procedure of chemical extraction of plant materials was different for isolation of phytochemical constituents which was described in chapter six. Cold extraction procedure was followed adopted from Alam *et al.* (2002). Solvent-solvent partitioning of extracts was done using the protocol designed by Kupchan and modified by Wagenen *et al.* (1993).

Chapter Four

ANTIMICROBIAL ASSAY OF *MIMOSA PUDICA*

Introduction

Antibacterial activity

Plants are the rich source of bioactive molecules which help in the production of different types of drugs. A large number of drugs obtained from the natural sources are used as traditional system of medicines. Therefore, in the discovery of new drugs, it is logical to screen the traditional natural products. About 20% plants of the world have been submitted to the pharmaceuticals or designed for biological tests and new drugs have been introduced to the market. According to Cragg *et al.* (1999), between 1983 and 1994, the systematic screening of the plant extracts with antibacterial potential represents a continuous effort in order to find out new compounds that have the potential to act against multi-resistant bacteria. WHO described that to obtain a variety of drugs, the medicinal plants would be the best source (Santos *et al.* 1995). Ayurvedic system of medicines has a long history of therapeutic potentials. To prove the antimicrobial potentials of the medicinal plants a number of investigations have been conducted throughout the world in past decades (Nascimento *et al.* 1990 and Alonso-Paz *et al.* 1995).

Around 50,000 people killed by the infectious diseases per day which are the leading cause of premature deaths in the world. Today the resistance of drugs to human pathogenic bacteria has become a burning issue of the world (Piddock and Wise 1989, Singh *et al.* 1992, Mulligen *et al.* 1993, Davis 1994 and Robin *et al.* 1998). Both in the developed and developing countries the situation is going to the alarming because of the indiscriminate use of the antibiotics. The treatments of infectious diseases are being complicated with the drug-resistant bacterial and fungal pathogens (Rinaldi 1991 and Diamond 1993). Multiple drug resistance to human pathogenic organisms has become the issue to search for new antimicrobial substances from plants and other sources. The traditional medicinal plants produce compounds that have known therapeutic potential (Iyengar 1985, Chopra *et al.* 1992 and Harborne and Baxter 1995). The substances that are used to produce new antimicrobial drugs can either kill the pathogens or inhibit their growth and have least or no toxicity to the host cells. Now antimicrobial properties of the medicinal plants have been reported from all over the world

(Grosvenor *et al.* 1995, Ratnakar and Murthy 1995, Silva *et al.* 1996, David 1997, Saxena 1997, Nimri *et al.* 1999 and Saxena and Sharma 1999).

In the treatment of different human diseases a large number of medicinal plants from nature have been using in daily life for thousands of years (Nair *et al.* 2005). To alleviate illness and promote health of human beings herbal drugs can be used as they have natural necessary substances (Balakumar *et al.* 2011 and Rajan *et al.* 2011). To detect promising biological potentials of plants it is required to screen a number of medicinal plants, and it is reported that the potential source of better antibiotic properties are represented by the higher plants (Afolayan 2003). From the ancient, it is known that the plants have a useful degree of antimicrobial properties. Traditionally used plant and herb species have antimicrobial and antiviral potentials (Shelef 1983, Zaika 1988 and Beuchat and Golden 1989). Hence, the scientists are being optimistic with the future of phyto-antimicrobial agents (Das *et al.* 1999).

The demand of herbal medicine is increasing in the recent time as the antibiotics have harmful side effects and high cost to the poor as well as their non-availability to the people of remote areas. Also people are getting true relief from disease condition by using herbal medicines. Moreover, herbal drugs have eco-friendly attributes which are the causes of gaining interest of herbal drugs. The emergence of strains with reduced susceptibility to antibiotics and increasing prevalence of multi-drug resistant bacterial strains have raised the specter of untreatable bacterial infections and add the urgency of searching for new infection fighting strategies (Sieradzki *et al.* 1999). On the other hand, plant originated antimicrobial substances are free of side effects and have huge therapeutic potentials to heal a large number of infectious diseases (Iwu *et al.* 1999). In the ancient traditional system of medication, the medicinal plants had a significant role all over the world. The plants are the important raw materials for the production of drugs as they are main source of bioactive compounds (Goyal *et al.* 2007 and Uma *et al.* 2007). Recently, multi-drug resistant microorganisms have developed because of indiscriminate use of synthetic antimicrobial drugs for the treatment of infectious diseases (Joshi *et al.* 2009).

Antifungal activity

The pathogenic fungi are the main infectious agents of the plants that cause alterations during developmental stages and post-harvest. Fruits and vegetables are attacked by a wide variety of fungal genera that deteriorate the nutritional value, organoleptic characteristics and limited shelf life (Agrios 2004). In some cases, fungi are indirectly responsible for toxic or allergic disorders of the consumers as they produce mycotoxins or allergens. To control the phytopathogenic fungi, the synthetic fungicides are used; but the indiscriminate uses of these fungicides are strongly prohibited because of harmful effects on human health and environment (Harris *et al.* 2001). The demand of production is increasing with time but the use of agrochemicals is being regulated. Moreover, product resistant pathogens are emerging. So it is necessary to discover the novel active compounds for new control strategies.

A variety of compounds has been provided by the plant kingdom from the ancient time which have known therapeutic effects. Traditional medicinal plants are used by a huge portion of population in South America for the infectious diseases. In southern Brazil, the genus *Pterocaulon* known as 'quitoco' is commonly used as veterinary medicines popularly diagnosed as 'mycoses' (Demo and Oliva 2008). Different plant extracts of leaf, stem, root, seeds and flowers possess inhibitory potentials against the fungi that are demonstrated by several works in the laboratory (Davicino *et al.* 2007).

Plant metabolites and plant compounds are better alternatives in contrast to synthetic pesticides as they have minimal danger to the consumers and environmental impact (Verma and Dubey 1999). The natural products are safe for human as well as for ecosystem than chemical antifungal compounds and the general people are using them for a long time for the flavor and aroma as well as to increase the economic value of food (Shelef *et al.* 1980 and Shelef 1983). Traditional plant materials have been using in the treatment of infectious diseases caused by fungi, viruses, protozoa and bacteria (Yoshida *et al.* 2005 and Nejad and Deokule 2009). Hence, the study was designed to screen antifungal activity of the plant *M. pudica* with the aim to develop plant based formulations for disease management.

Materials and Methods

Antibacterial, antifungal, minimum inhibitory concentrations (MICs) of the extracts and effects of pure compounds on bacterial strains were observed in this investigation.

Antibacterial activity

Antibacterial activity of any plant or parts of a plant can be detected by observing the growth response of various microorganisms to the extracts of a plant or parts of a plant, which is placed in contact with them. Eight pathogenic bacterial strains were selected for this test, three were gram-positive and five were gram-negative. The strains were presented in table 3.

Table 3. List of pathogenic bacteria used in this investigation

Name of test organisms	Strain Number
Gram-stain positive	
Bacillus cereus	BMLRU1004
Staphylococcus aureus	BMLRU1002
Bacillus zhanjiangensis	JSM 099021(T)
Gram-stain negative	
Klebsiella pneumoniae	BMLRU1005
Shigella boydii	AL-17313
Shigella sonnei	BMLRU1015
Escherichia coli	BMLRU1001
Salmonella typhi	BMLRU1009

Collection and culture of test bacteria

Test organisms of pure culture were collected from the International Center for Diarrhoeal Disease Research, Bangladesh (ICDDR B), Mahakhali, Dhaka, Bangladesh and Department of Biochemistry and Molecular Biology, University of Rajshahi. The test bacteria were further cultured at Entomology and Insect Biotechnology Laboratory, Institute of Biological Sciences, University of Rajshahi, Bangladesh.

Culture media

A number of culture media are available. These are:

i) Nutrient Agar medium
ii) Nutrient Broth medium
iii) Mueller-Hinton medium
iv) Tryptic Soy broth (TSB) medium
v) Trypticase Soy Agar medium
vi) *Staphylococcus* defined medium
vii) Adams and Roe medium
viii) NTH agar or broth medium

The bacteria were cultured in Nutrient Broth media (HiMedia).

Standard Formula of Nutrient Broth medium (HiMedia)

Ingredients	**Gms/litre**
Peptic digest of animal tissue	5.00
Sodium chloride	5.00
Beef extract	1.50
Yeast extract	1.50
Final p^H 7.4±0.2 (at 25^0C)	

Preparation of the Nutrient Broth medium (HiMedia)

Nutrient Broth 13.0 grams was weighed and suspended in 1000 ml distilled water in a conical flask and heat the solution if necessary in a water bath to dissolve the medium completely. This solution was sterilized by autoclaving at 15 lbs pressure (121^0C) for 15 minutes.

Standard Formula of Nutrient Agar medium (HiMedia)

Ingredients	**Gms/litre**
Peptic digest of animal tissue	5.00
Beef extract	1.50
Yeast extract	1.50
Sodium chloride	5.00
Agar	15.00
Final p^H 7.4±0.2 (at 25^0C)	

Preparation of the Nutrient Agar medium (HiMedia)

Nutrient agar 28.0 grams was weighed and suspended in 1000 ml distilled water in a conical flask according to specification measurement and was heated in a water bath to dissolve the agar until a transparent solution was obtained. The solution was sterilized by autoclaving at 15 lbs/sq. inch pressure at 121^0C for 15 minutes.

Preparation of fresh culture of pathogenic organisms

The Nutrient Agar medium was prepared and dispersed in clean test tubes to prepare slants (5 ml in each test tube). By plugging with cotton the test tubes were sterilized in an autoclave at 121^0C and 15 lbs/sq. inch pressure for 15 minutes. Then the test tubes were kept in an inclined position for solidification and incubated at 37.5^0C to ensure sterilization. By maintaining an aseptic condition, test organisms were transferred to the agar slants from the supplied pure cultures with the help of inoculating loop. The loop was burnt after each transfer of microorganisms to avoid the contamination very carefully. The inoculated slants were incubated at 37.5^0C for 24 hours to assure the growth of test organisms and these fresh cultures were used in sensitivity tests.

Selection of test method

Three methods *i.e.* disc diffusion, dilution and bio-autographic method can be followed to evaluate the antibacterial activity. But the disc diffusion method (Bauer *et al.* 1966 and Reiner 1982) is widely acceptable for preliminary evaluation where different concentrations of the extracts were absorbed in sterile filter paper discs. In this study, disc diffusion method was followed to test the antibacterial efficiency.

To determine the most optimal concentration of extracts to be used in this study, sterile 5mm filter paper discs were treated with 200µg and 50µg of the petroleum ether, chloroform, ethyl acetate and methanol extracts while the only solvents were used as control. The bacteria were inoculated on full-strength nutrient agar (HiMedia Laboratories Pvt. Ltd.) by suspending loops in sterile de-ionized water. The bacterial suspension was then smeared on agar plates with a sterile glass-rod to ensure the entire surface of the agar had an even coating of the bacterial suspension. The test plates were divided into several areas and one filter paper disc was placed on each of the areas. The plates are then kept in an incubator (37.5^0C) for 18-24 hours to allow the growth of the organisms. If any of the test materials have antimicrobial activity, it would inhibit the

growth of microorganisms just giving a clear distinct zone called 'zone of inhibition'. Biological activity of the *M. pudica* components on bacterial growth was quantified in this way by measuring the diameter of zones of inhibition (in term of mm). The size of the inhibitory zone depends principally on the following factors:

- Intrinsic antimicrobial sensitivity of the test sample
- Growth rate of the test microorganisms
- Diffusion rate of the freshly seeded test organisms
- Concentration of the freshly seeded test organisms
- Amount of the test sample on disc
- Thickness of the test medium in the Petri dishes
- Composition of the culture medium
- Size of inoculums
- Time of incubation
- Temperature of incubation

Test materials used

i. Petroleum ether, chloroform, ethyl acetate and methanol extracts of leaf, stem and root of *M. pudica.*
ii. Ciprofloxacin (5µg/disc) as standard disc.

Apparatus and reagents to conduct antibacterial assay

- Crude extracts of petroleum ether, chloroform, ethyl acetate and methanol
- Standard disc (Ciprofloxacin 5µg/disc)
- Different solvents (petroleum ether, chloroform, ethyl acetate, methanol, alcohol, acetone, hexane, etc.)
- Hydrocloric acid
- Rectified spirit
- Nutrient Broth media (HiMedia)
- Nutrient Agar media (HiMedia)
- Filter paper discs (sterilized)
- Petri dishes (90mm diam.)
- Inoculating loop
- Sterile cotton
- Sterile forceps

- Test tubes
- Micropipette (10-100 µl, 20-200 µl, 1000 µl)
- Nose mask and hand gloves
- Spirit burner and match box
- Laminar air flow unit (Bio-craft & Scientific Industries, India)
- Incubator (Osk-9639A, Japan)
- Refrigerator (Artston, Italy)
- Autoclave (ALP Co. Ltd. KT-30L, Japan)

Sterilization procedure

The antibacterial screening was carried out in a laminar air flow unit and all types of precautions were highly maintained to avoid any type of contamination during the test. UV light was switched on for half an hour before working in the laminar hood to avoid any accidental contamination. Petri dishes and other glass-wares were sterilized in the autoclave at 121^0C temperature and a pressure of 15 lbs/sq. inch for 20 minutes. Micropipette, tips, culture media, cotton, forceps, blank discs, etc. were also sterilized.

Subculture of bacteria

Cleaned test tubes were autoclaved at 121^0C and 15 lbs/sq. inch pressure for 20 minutes. Approx. 10ml of autoclaved Nutrient Broth media (HiMedia) was poured into the test tubes. The bacterial suspensions of pure culture kept at 4^0C in a refrigerator were transferred into the test tubes containing broth media with the help of an inoculating loop in an aseptic condition. Every time the loop was burnt red and cool down again before transferring the suspensions. Then the test tubes were plugged with cotton pads and incubated at 37.5^0C in a refrigerator for 24 hours.

Preparation of the test samples

2mg extract was taken in a glass vial and 1ml of the respective solvent was added to make a solution of each of the petroleum ether, chloroform, ethyl acetate and methanol extracts of leaf, stem and root. The doses were prepared 200 µg/disc and 50 µg/disc separately along with a negative control of petroleum ether, chloroform, ethyl acetate and methanol extracts. Ciprofloxacin 5µg/disc was used as standard.

Preparation of test plates

Cleaned Petri dishes were sterilized in an autoclave at 121^0C and 15 lbs/sq. inch pressure for 20 minutes. The water vapor of the Petri dishes was removed with the help of sterilized cotton. Approximately 25ml of autoclaved Nutrient Agar media were poured into each of the Petri dishes so that it could give a uniform depth of approximately 4mm. These plates were kept in the laminar air flow unit for about 30 minutes to dry the nutrient agar media. Then the plates were smeared with bacterial suspensions with the help of a spreader. Thus the plates were ready to set the filter paper discs impregnated with specific extract.

Preparation of the discs treated with the test samples

Three types of discs were prepared for antibacterial screening. To prepare these discs containing petroleum ether, chloroform, ethyl acetate and methanol extracts, the following procedures were taken.

a) **Sample discs:** Sterilized filter paper discs (Double Rings Filter paper No. 102, 5mm in diameter) were taken in a blank Petri dish and sample solution of desired concentration was applied on these discs with micropipette in an aseptic condition. The discs were left for a few minutes for complete removal of solvent.
b) **Standard discs:** Standard discs are used to compare the antibacterial activities of the test materials. In this study the antibiotic Ciprofloxacin (5µg/disc) was used as standard disc for comparison of the treated extracts.
c) **Control/blank discs:** These were used as negative controls to ensure that the residual solvents on the filter papers were not active themselves. These were prepared by applying only solvent to the discs.

Application of doses on bacteria

Plates containing nutrient agar smeared with bacterial suspensions were dried in the laminar air flow unit. Prepared and dried crude extract filter paper discs of different doses along with a negative control with only solvent and a positive control or standard disc (Ciprofloxacin 5µg/disc) were placed gently on to the solidified agar plates containing bacterial suspensions with sterile fine forceps to ensure contact with the medium. Every time the forceps were made contamination free with the flame of a spirit lamp. The plates were then kept at 4^0C for 24 hours to provide sufficient time to

diffuse the antibiotics into the medium. Finally, the plates were incubated at 37.5^0C for 18-24 hours in an incubator.

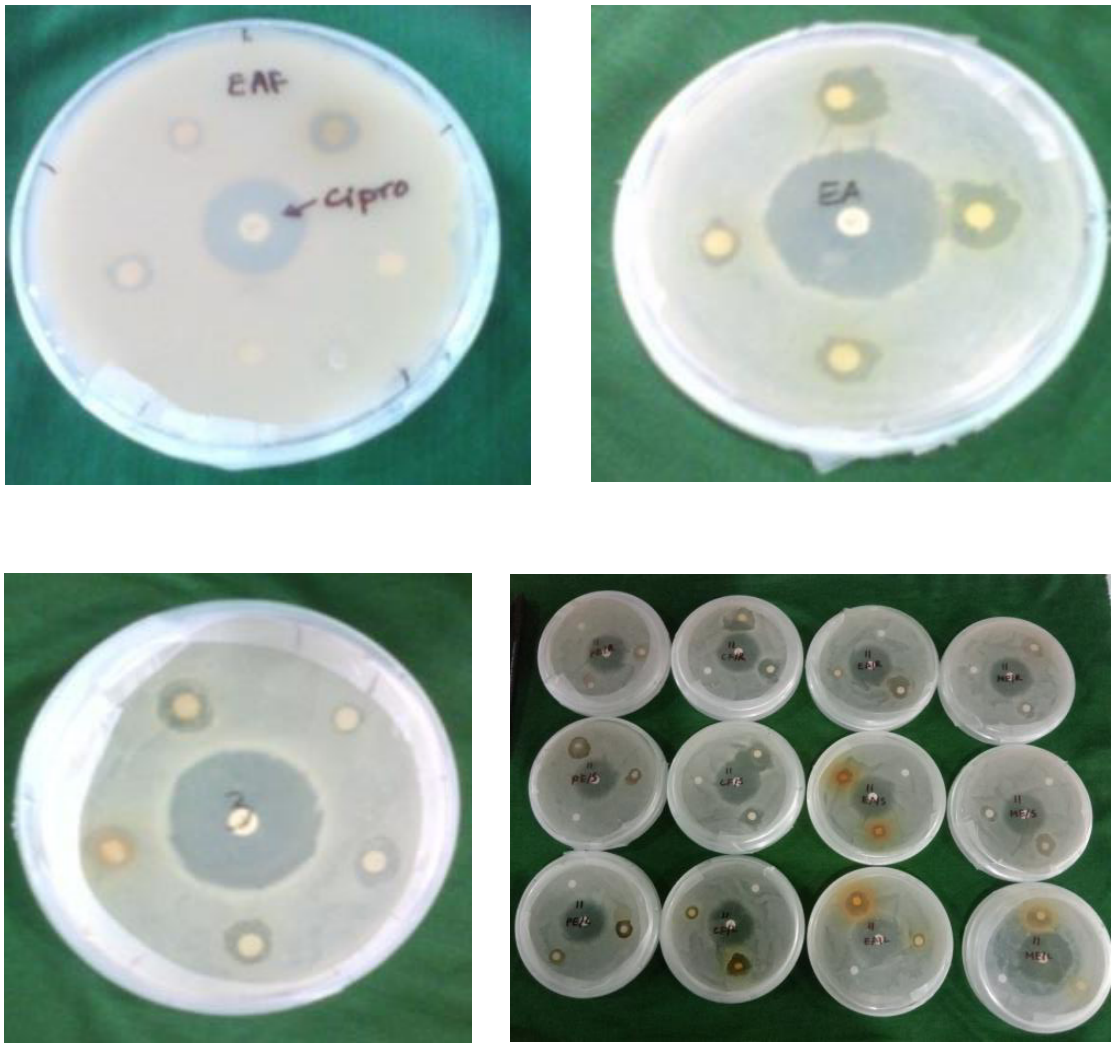


Fig 18. Antibacterial activity test of *M. pudica*

Measurement of zones of inhibition

The antibacterial activities of the plant extracts were determined by measuring the diameter of zones of inhibition in mm with a transparent scale.

Precaution

The discs were placed in such a way that they were not closer than 15mm to the edge of the plate and were placed apart enough to prevent overlapping of the zones of inhibition.

Antifungal activity

Plant derived compounds may offer potential leads for noble agents against systemic fungal diseases (Hufford and Clark 1988) in man and plants. Extracts from different parts of *M. pudica* were used for the detection of antifungal potentials and disc diffusion method was followed in this investigation.

Test agents for antifungal activity test

Four fungal strains were used in this experiment that was presented in table 4.

Table 4. List of pathogenic fungi used in this investigation

Serial No	Name of test organisms
1	*Rhizopus oryzae*
2	*Fusarium proliferatum*
3	*Aspergillus niger*
4	*Candida albicans*

Collection and culture of test fungi

The pure cultures of the strains were collected from the Pathology Department, Rajshahi Medical College, Rajshahi and Genetic Engineering and Molecular Biology Laboratory, Department of Genetic Engineering and Biotechnology, University of Rajshahi and were cultured in the Entomology and Insect Biotechnology Laboratory, Institute of Biological Sciences, University of Rajshahi, Bangladesh.

Culture media

Potato Dextrose Agar (PDA) media (HiMedia) were used in conducting the antifungal activity tests as well as for the maintenance of sub-cultures of the test organisms. The composition of medium is given below:

Standard Formula of Potato Dextrose Agar (PDA)

Ingredients	Gms/litre
Potatoes infusion from	200.00
Dextrose	20.00
Agar	15.00
Final p^H 5.6±0.2 (at 25^0C)	

Preparation of the Potato Dextrose Agar medium

Potato Dextrose Agar (PDA) 39.0 grams was weighed and suspended in 1000 ml distilled water in a conical flask and heated the solution in a water bath to dissolve the ingredients completely until a transparent solution was obtained. The p^H of the medium was adjusted to 5.6. The volume was adjusted by adding distilled water. This solution was sterilized by autoclaving at 15 lbs pressure and 121^0C temperature for 15 minutes.

Preparation of test plates

Following procedure was maintained to prepare the test plates:

i. Approximately 10ml of distilled water was poured in several clean test tubes and plugged with cotton pads.
ii. The test tubes, Petri dishes, glass rods, cotton pads and the medium were sterilized by autoclave and then transferred to the laminar air flow cabinet.
iii. About 20ml of the medium was poured carefully into the 90mm Petri dishes separately and were rotated clockwise and anti-clockwise to make homogenous thickness of the medium and allowed to cool down and solidify at about 30^0C.
iv. The test tubes containing distilled water were inoculated with fresh culture of the test fungi and were shaken gently to form a uniform suspension of the organism because of their high prevalence of sporulation process.
v. Separate pieces of cotton were immerged in the test tubes with individual glass rods and gently rubbed the medium. Then the pieces were discarded.
vi. Finally, the plates were stored overnight in a refrigerator at 4^0C.

Preparation of the discs containing the test samples

a) **Sample discs:** Sterilized filter paper discs (5mm in diam.) were transferred with the help of forceps in the plates. Crude extracts of petroleum ether, chloroform, ethyl acetate and methanol (200µg/disc and 50µg/disc) were applied on separate discs with micropipette in aseptic condition. The discs were left for some time for complete removal of the solvent.
b) **Standard discs:** These discs were used to compare the antifungal activities of test material. In this study, ready-made Nystatin 50µg/disc was used as standard disc for comparison with the efficacy of the test extracts.

c) **Control/blank disc:** These were used as negative controls to ensure that the residual solvent on the filter paper were not active themselves. These were prepared by applying only solvent to the discs and were used to examine the effect of the solvents used.

Application of doses on fungi

Plates containing Potato Dextrose Agar were swab with a piece of cotton inoculated with fresh culture of test fungi. The crude extract filter paper discs (200µg and 50µg) along with a positive control or standard disc (Nystatin 50µg) were placed gently on to the solidified agar plates with a sterile fine forceps to ensure contact with the medium. Every time the forceps were made contamination free with the flame of a spirit lamp. The plates were then kept in room temperature for a few days.

Fig. *Rhizopus oryzae*

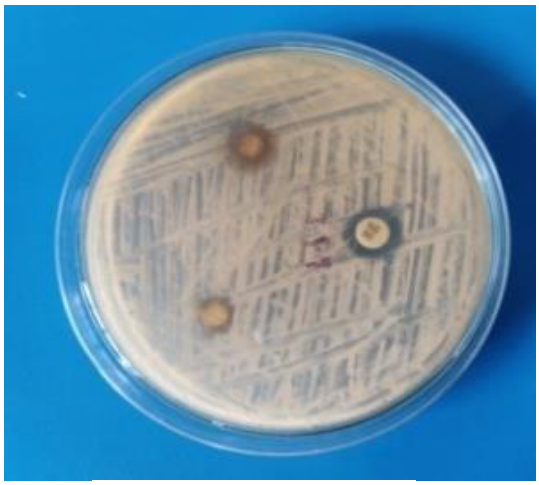


Fig. *Candida albicans*

Fig 19. Antifungal activity test of *M. pudica*

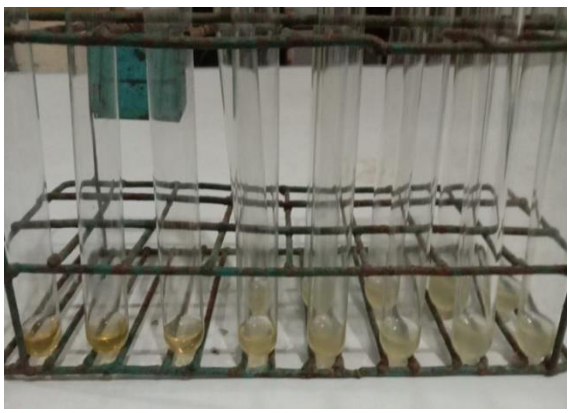


Fig 20. MIC test of *M. pudica* extract against pathogenic bacteria

Minimum Inhibitory Concentrations (MICs) for antibacterial agents

The MIC is the lowest concentration of an antimicrobial drug to inhibit the growth of the test organism. There are two methods of experiments to determine the MIC values are as follows:

i. Serial tube dilution technique or turbid metric assay (Reiner 1982)
ii. Paper disc plate technique or agar diffusion assay (Bauer *et al.* 1966)

Serial tube dilution test is the standard method of determining the levels of microbial resistance to antimicrobial agents. Serial dilution technique is followed for the test material in nutrient broth medium. The growth medium is inoculated with standardized number of organisms and inoculated for certain time. The highest dilution or the lowest concentration of the sample that prevent the appearance of growth or turbidity is considered to be the minimal or minimum inhibitory concentration (MIC) and at this level of concentration the test sample is bacteriostatic.

Here, 'Serial tube dilution technique' (Reiner 1982) was followed to determine the MIC values of chloroform and ethyl acetate extracts of leaf of *M. pudica* against the following pathogenic bacteria.

Gram-positive bacteria

i. *B. cereus*
ii. *S. aureus*
iii. *B. zhanjiangensis*

Gram-negative bacteria

i. *K. pneumoniae*
ii. *E. coli*
iii. *S. typhi*

Preparation of inoculums

Fresh cultures of test organisms were grown at 37.5^0C for two days on the Nutrient Agar medium. Bacterial suspensions were prepared in sterile Nutrient Broth medium in such a manner that the suspension contains 10^7CFU/ml which were used as inocula.

Preparation of the sample solution

The stock solution was prepared by taking 1.024 mg extract in 1ml of the respective solvent in a glass vial. When the extracts would not dissolve in the solvent well, a little amount of DMSO (Dimethylsulfoxide) was mixed with the extract.

Procedure of serial tube dilution technique

i. Thirteen autoclaved test tubes were taken, ten of which were marked as 1, 2, 3, 4, 5, 6, 7, 8, 9 & 10 and the rest three were assigned as Cm (medium), Cs (medium + sample) and Ci (medium + inoculums).
ii. One ml of sterile Nutrient Broth medium was added to each of the thirteen test tubes.
iii. One ml of the sample solution was added to the first test tube and mixed well.
iv. One ml content from the first test tube was transferred by the sterile pipette to the second test tube and mixed uniformly. Then one ml of this mixture was transferred to the third test tube. This process of serial dilution was continued up to the tenth test tube.
v. One drop (10µl) of properly diluted inoculums was added to each of the ten test tubes and mixed well.
vi. For the control test tube Cs, one ml of the sample solution was added and mixed well, while one ml of this mixed content was discarded. This was to check the clarity of the medium in presence of diluted solution of the compound.
vii. To the control test tube Ci, 10µl of the inoculums was added to observe the growth of the organism in the medium used.
viii. The control test tube Cm containing medium only was used to confirm the sterility of the medium.
ix. All the test tubes were incubated at 37.5^0C for 18-24 hours. The MIC was the lowest drug concentration at which there was no growth of the organisms.

The effects of pure compounds were tested against eight bacterial strains and the minimum inhibitory concentrations were observed against four pathogenic bacteria. Two pure compounds were identified from chloroform and petroleum ether extracts of leaf. The isolation technique of pure compounds was described in chapter six.

Results

Antibacterial activity

Antibacterial activity of extracts was examined by following the agar disc diffusion method and activity was determined by measuring the diameter of zones of inhibition (in mm). The crude extracts of leaf, stem and root of different solvents of *M. pudica* were tested against three gram-positive bacterial strains *viz. B. cereus, S. aureus* and *B. zhanjiangensis* and five gram-negative bacterial strains *viz. K. pneumoniae, S. boydii, S. sonnei, E. coli* and *S. typhi.* All the extracts possessed antibacterial activity with different potency against a variety of microorganisms pathogenic to human beings. In antibacterial screening, the crude extracts were used at concentrations of 200 µg/disc and 50 µg/disc and the pure compounds were used at concentrations of 100 µg/disc and 50 µg/disc along with a negative control against human pathogenic bacterial strains and the sensitivities were compared with the standard antibiotics Ciprofloxacin (5 µg/disc) and considering the size of clear-zones in millimeter, the efficacy of extracts against the test microbes were recorded. The results showed effectivity of the extracts against both the gram-positive and gram-negative bacteria that have been presented in tables 5-17.

Table 5. Antibacterial activity of petroleum ether extract of leaf of *M. pudica*

Sl no	Name of organisms	Diameter of zone of inhibition in mm			
		200 µg/disc	50 µg/disc	Negative control	Ciprofloxacin (5 µg/disc)
1	*Bacillus cereus*	11.67±0.33	7.67±0.33	-	36.00±0.58
2	*Staphylococcus aureus*	13.00±0.58	8.67±0.33	-	32.67±0.33
3	*Bacillus zhanjiangensis*	13.33±0.33	9.00±0.58	-	33.33±1.20
4	*Klebsiella pneumoniae*	13.00±0.58	8.33±0.33	-	38.00±0.58
5	*Shigella boydii*	12.67±0.33	9.00±0.58	-	33.67±0.33
6	*Shigella sonnei*	12.00±0.58	8.00±0.58	-	31.33±0.33
7	*Escherichia coli*	14.33±0.33	10.33±0.33	-	34.00±0.58
8	*Salmonella typhi*	13.00±0.58	9.00±0.58	-	37.00±0.58

The result of antibacterial activity of petroleum ether extract of leaf of *M. pudica* showed in table 5. At 200 µg/disc concentration the highest antibacterial activity (diameter of zone of inhibition 14.33±0.33 mm) was found against gram-negative bacterial strain *E. coli*; while against the gram-positive bacterial strain *B.*

zhanjiangensis, the zone of inhibition was recorded 13.33±0.33 mm. The lowest activity (diameter of zone of inhibition 11.67±0.33 mm) was recorded against the gram-positive bacterial strain *B. cereus.* At 50 µg/disc concentration, the highest activity was found 10.33±0.33 mm against *E. coli* and the lowest was 7.67±0.33 mm against *B. cereus*.

Table 6. Antibacterial activity of chloroform extract of leaf of *M. pudica*

Sl no	Name of organisms	Diameter of zone of inhibition in mm			
		200 µg/disc	50 µg/disc	Negative control	Ciprofloxacin (5 µg/disc)
1	*Bacillus cereus*	15.00±0.58	9.67±0.33	-	32.00±0.58
2	*Staphylococcus aureus*	17.67±0.67	11.33±0.33	-	32.33±0.33
3	*Bacillus zhanjiangensis*	15.00±0.58	9.00±0.58	-	37.67±0.88
4	*Klebsiella pneumoniae*	14.00±0.58	8.67±0.33	-	38.00±0.58
5	*Shigella boydii*	14.67±0.88	9.33±0.67	-	35.00±0.58
6	*Shigella sonnei*	16.00±0.58	10.00±0.58	-	30.67±0.33
7	*Escherichia coli*	19.67±0.88	11.67±0.33	-	35.00±0.58
8	*Salmonella typhi*	15.00±0.58	9.33±0.33	-	36.00±0.58

Table 6 showed the antibacterial activity of chloroform extract of leaf of *M. pudica.* At 200 µg/disc concentration also the highest antibacterial activity was found against gram-negative bacterial strain *E. coli* where the diameter of zone of inhibition was 19.67±0.88 mm; whilst against gram-positive bacterial strain *S. aureus,* the zone of inhibition was recorded 17.67±0.67 mm. The lowest activity (diameter of zone of inhibition 14.00±0.58 mm) was recorded against the gram-negative bacterial strain *K. pneumoniae.* At 50 µg/disc concentration, the highest activity was found 11.67±0.33 mm against *E. coli* and the lowest was 8.67±0.33 mm against *K. pneumoniae*.

Table 7. Antibacterial activity of ethyl acetate extract of leaf of *M. pudica*

Sl no	Name of organisms	Diameter of zone of inhibition in mm			
		200 µg/disc	50 µg/disc	Negative control	Ciprofloxacin (5 µg/disc)
1	*Bacillus cereus*	15.00±0.58	9.33±0.33	-	30.00±0.58
2	*Staphylococcus aureus*	16.00±1.15	10.67±0.33	-	29.33±0.67
3	*Bacillus zhanjiangensis*	15.00±0.58	9.00±0.58	-	37.00±0.58
4	*Klebsiella pneumoniae*	13.33±0.33	8.33±0.33	-	34.67±0.88
5	*Shigella boydii*	15.00±0.58	10.00±0.58	-	32.00±0.58
6	*Shigella sonnei*	14.00±0.58	9.33±0.33	-	29.67±1.20
7	*Escherichia coli*	15.67±0.88	10.67±0.33	-	32.00±0.58
8	*Salmonella typhi*	14.00±0.58	9.33±0.88	-	33.67±0.33

In table 7 the ethyl acetate extract of leaf of *M. pudica* showed that at 200 µg/disc concentration the highest antibacterial activity was found against gram-positive bacterial strain *S. aureus* where the diameter of zone of inhibition was 16.00±1.15 mm and against gram-negative bacterial strain *E. coli* where the zone of inhibition was 15.67±0.88 mm. The lowest activity (zone of inhibition 13.33±0.33 mm) was recorded against the gram-negative bacterial strain *K. pneumoniae.* At 50 µg/disc concentration, the highest activity was found 10.67±0.33 mm against *S. aureus* and *E. coli* and the lowest was 8.33±0.33 mm against *K. pneumoniae*.

Table 8. Antibacterial activity of methanol extract of leaf of *M. pudica*

Sl no	Name of organisms	Diameter of zone of inhibition in mm			
		200 µg/disc	50 µg/disc	Negative control	Ciprofloxacin (5 µg/disc)
1	*Bacillus cereus*	14.00±0.58	9.33±0.33	-	28.00±0.58
2	*Staphylococcus aureus*	16.33±0.33	11.33±0.33	-	29.00±0.58
3	*Bacillus zhanjiangensis*	15.00±0.58	10.00±0.58	-	31.67±0.33
4	*Klebsiella pneumoniae*	13.67±0.88	9.67±0.33	-	33.00±0.58
5	*Shigella boydii*	13.00±0.58	8.33±0.33	-	32.67±0.88
6	*Shigella sonnei*	14.00±0.58	9.67±0.88	-	28.33±0.33
7	*Escherichia coli*	15.00±1.15	9.33±0.88	-	31.67±0.33
8	*Salmonella typhi*	13.00±0.58	8.00±0.58	-	32.00±0.58

Table 8 showed the antibacterial activity of methanol extract of leaf of *M. pudica.* At 200 µg/disc concentration the highest value was found against gram-positive bacterial strain *S. aureus* where the diameter of zone of inhibition was 16.33±0.33 mm; while against gram-negative bacterial strain *E. coli* the diameter of zone of inhibition was 15.00±1.15 mm. The lowest activity was recorded against the gram-negative bacterial strain *S. boydii* and *S. typhi* where the diameter of zone of inhibition was 13.00±0.58 mm. At 50 µg/disc concentration, the highest activity was found against *S. aureus* (11.33±0.33 mm) and the lowest activity was found against *S. typhi* (8.00±0.58 mm).

Table 9. Antibacterial activity of petroleum ether extract of stem of *M. pudica*

Sl no	Name of organisms	Diameter of zone of inhibition in mm			
		200 µg/disc	50 µg/disc	Negative control	Ciprofloxacin (5 µg/disc)
1	*Bacillus cereus*	13.00±0.58	7.67±0.33	-	35.00±0.58
2	*Staphylococcus aureus*	15.00±0.58	9.00±0.58	-	31.67±0.88
3	*Bacillus zhanjiangensis*	14.33±0.33	8.33±0.33	-	33.00±0.58
4	*Klebsiella pneumoniae*	13.00±0.58	8.00±0.58	-	37.33±0.88
5	*Shigella boydii*	13.00±0.58	8.00±0.58	-	32.67±0.33
6	*Shigella sonnei*	13.33±0.88	8.33±0.33	-	30.00±0.58
7	*Escherichia coli*	14.00±0.58	8.67±0.33	-	32.33±0.33
8	*Salmonella typhi*	12.00±0.58	8.00±0.58	-	36.67±0.88

As shown in table 9, the petroleum ether extract of stem of *M. pudica* at 200 µg/disc concentration, the highest activity was found against gram-positive bacterial strain *S. aureus* where the diameter of zone of inhibition was 15.00±0.58 mm and against gram-negative bacterial strain *E. coli* where the zone of inhibition was 14.00±0.58 mm. The lowest activity was recorded against gram-negative bacterial strain *S. typhi* where the diameter of zone of inhibition was 12.00±0.58 mm at the same concentration. At 50 µg/disc concentration, the highest activity was found against *S. aureus* (9.00±0.58 mm) and the lowest activity was found against *B. cereus* (7.67±0.33 mm).

Table 10. Antibacterial activity of chloroform extract of stem of *M. pudica*

Sl no	Name of organisms	Diameter of zone of inhibition in mm			
		200 µg/disc	50 µg/disc	Negative control	Ciprofloxacin (5 µg/disc)
1	*Bacillus cereus*	14.00±0.58	8.00±0.58	-	30.00±0.58
2	*Staphylococcus aureus*	16.67±0.33	10.33±0.33	-	30.33±0.33
3	*Bacillus zhanjiangensis*	15.00±0.58	9.00±0.58	-	35.33±1.45
4	*Klebsiella pneumoniae*	14.33±0.88	8.00±0.58	-	35.00±0.58
5	*Shigella boydii*	14.00±0.58	8.33±0.88	-	32.67±0.33
6	*Shigella sonnei*	14.00±0.58	8.67±0.33	-	30.00±0.58
7	*Escherichia coli*	15.67±0.88	10.00±0.58	-	31.67±0.33
8	*Salmonella typhi*	14.00±0.58	9.00±0.58	-	33.00±0.58

From the results shown in table 10, we found that the highest antibacterial activity of chloroform extract of stem against *S. aureus* was 16.67±0.33 mm for gram-positive bacterial strain and against *E. coli* was 15.67±0.88 mm for gram-negative bacterial strain at 200 µg/disc concentrations. The lowest activity (diameter of zone of inhibition 14.00±0.58 mm) was recorded against *B. cereus, S. boydii, S. sonnei* and *S. typhi* at same concentration. At 50 µg/disc concentration, the highest activity was found 10.33±0.33 mm against *S. aureus* and the lowest was 8.00±0.58 mm against *B. cereus* and *K. pneumoniae*.

Table 11. Antibacterial activity of ethyl acetate extract of stem of *M. pudica*

Sl no	Name of organisms	Diameter of zone of inhibition in mm			
		200 µg/disc	50 µg/disc	Negative control	Ciprofloxacin (5 µg/disc)
1	*Bacillus cereus*	14.00±0.58	8.00±0.58	-	31.33±0.33
2	*Staphylococcus aureus*	16.00±0.58	10.00±0.58	-	30.67±0.33
3	*Bacillus zhanjiangensis*	14.67±0.88	9.67±0.33	-	35.33±1.20
4	*Klebsiella pneumoniae*	13.67±0.88	8.67±0.67	-	36.00±0.58
5	*Shigella boydii*	13.33±0.33	9.00±0.58	-	32.33±0.33
6	*Shigella sonnei*	13.00±0.58	8.33±0.88	-	30.00±0.58
7	*Escherichia coli*	16.67±0.88	10.67±0.33	-	33.67±0.88
8	*Salmonella typhi*	15.00±0.58	10.00±1.00	-	32.00±0.58

In table 11 showed the result of the ethyl acetate extract of stem. At 200 µg/disc concentration, the highest antibacterial activity was found against gram-negative bacterial strain *E. coli* where the diameter of zone of inhibition was 16.67±0.88 mm and against gram-positive bacterial strain *S. aureus* the zone of inhibition was 16.00±0.58 mm. The lowest activity was recorded against gram-negative bacterial strain *S. sonnei* where the diameter of zone of inhibition was 13.00±0.58 mm. At 50 µg/disc concentration, the highest activity was found against *E. coli* (10.67±0.33 mm) and the lowest was against *B. cereus* (8.00±0.58 mm).

Table 12. Antibacterial activity of methanol extract of stem of *M. pudica*

Sl no	Name of organisms	Diameter of zone of inhibition in mm			
		200 µg/disc	50 µg/disc	Negative control	Ciprofloxacin (5 µg/disc)
1	*Bacillus cereus*	13.00±0.58	8.67±0.33	-	29.00±0.58
2	*Staphylococcus aureus*	16.33±0.33	10.67±0.33	-	29.67±0.33
3	*Bacillus zhanjiangensis*	14.00±0.58	9.33±0.88	-	32.00±0.58
4	*Klebsiella pneumoniae*	13.00±0.58	8.00±0.58	-	34.00±0.58
5	*Shigella boydii*	13.33±0.88	8.67±0.67	-	33.67±0.88
6	*Shigella sonnei*	13.00±0.58	8.00±0.58	-	28.00±0.58
7	*Escherichia coli*	16.00±0.58	10.00±0.58	-	32.00±0.58
8	*Salmonella typhi*	12.67±0.33	9.67±0.67	-	31.33±0.67

In table 12, methanol extract of stem showed that at 200 µg/disc concentration, the highest value was found against gram-positive bacterial strain *S. aureus* where the diameter of zone of inhibition was 16.33±0.33 mm; while against gram-negative bacterial strain *E. coli* it was 16.00±0.58 mm. The lowest activity was recorded against gram-negative bacterial strain *S. typhi* where the diameter of zone of inhibition was 12.67±0.33 mm. At 50 µg/disc concentration, the highest activity was found against *S. aureus* (10.67±0.33 mm) and the lowest activity was found against *K. pneumoniae* and *S. sonnei* (8.00±0.58 mm).

Table 13. Antibacterial activity of petroleum ether extract of root of *M. pudica*

Sl no	Name of organisms	Diameter of zone of inhibition in mm			
		200 µg/disc	50 µg/disc	Negative control	Ciprofloxacin (5 µg/disc)
1	*Bacillus cereus*	14.00±0.58	9.00±0.58	-	34.67±0.88
2	*Staphylococcus aureus*	14.33±0.33	9.00±0.58	-	35.00±1.15
3	*Bacillus zhanjiangensis*	13.00±0.58	7.67±0.33	-	33.67±0.33
4	*Klebsiella pneumoniae*	12.00±0.58	7.33±0.33	-	38.33±0.88
5	*Shigella boydii*	14.67±0.88	9.67±0.88	-	32.67±0.67
6	*Shigella sonnei*	15.00±0.58	10.00±0.58	-	31.67±0.33
7	*Escherichia coli*	15.33±0.88	10.67±0.88	-	34.00±0.58
8	*Salmonella typhi*	13.00±0.58	8.00±0.58	-	36.67±0.88

In case of petroleum ether extract of root (table 13), the highest activity was found against gram-negative bacterial strain *E. coli* where the diameter of zone of inhibition was 15.33±0.88 mm and against gram-positive bacterial strain *S. aureus* the zone of inhibition was 14.33±0.33 mm. The lowest activity was recorded against gram-negative bacterial strain *K. pneumoniae* where the diameter of zone of inhibition was 12.00±0.58 mm. At 50 µg/disc concentration, the highest activity was found against *E. coli* (10.67±0.88 mm) and the lowest activity was found against *K. pneumoniae* (7.33±0.33 mm).

Table 14. Antibacterial activity of chloroform extract of root of *M. pudica*

Sl no	Name of organisms	Diameter of zone of inhibition in mm			
		200 µg/disc	50 µg/disc	Negative control	Ciprofloxacin (5 µg/disc)
1	*Bacillus cereus*	17.33±0.33	11.00±0.58	-	30.67±0.33
2	*Staphylococcus aureus*	20.00±0.58	12.33±0.33	-	31.00±0.58
3	*Bacillus zhanjiangensis*	16.00±0.58	10.00±0.58	-	36.00±1.15
4	*Klebsiella pneumoniae*	19.67±0.88	12.00±0.58	-	35.00±0.58
5	*Shigella boydii*	16.67±0.88	10.67±0.88	-	34.00±0.58
6	*Shigella sonnei*	21.67±1.45	12.67±0.88	-	31.33±0.88
7	*Escherichia coli*	19.00±0.58	11.00±0.58	-	34.00±0.58
8	*Salmonella typhi*	15.00±0.58	9.00±0.58	-	32.33±0.88

From the results shown in table 14, it was found that the highest antibacterial activity of chloroform extract of root against *S. sonnei* was 21.67±1.45 mm for gram-negative bacterial strain and against *S. aureus* was 20.00±0.58 mm for gram-positive bacterial strain at 200 µg/disc concentrations. The lowest activity was recorded against *S. typhi* was 15.00±0.58 mm for gram-negative bacterial strain. At 50 µg/disc concentration, the highest activity was found against *S. sonnei* (12.67±0.88 mm) and the lowest was found against *S. typhi* (9.00±0.58 mm).

Table 15. Antibacterial activity of ethyl acetate extract of root of *M. pudica*

Sl no	Name of organisms	Diameter of zone of inhibition in mm			
		200 µg/disc	50 µg/disc	Negative control	Ciprofloxacin (5 µg/disc)
1	*Bacillus cereus*	16.00±0.58	9.67±0.67	-	31.67±0.33
2	*Staphylococcus aureus*	17.00±0.58	10.00±0.58	-	32.00±0.58
3	*Bacillus zhanjiangensis*	15.67±1.20	9.67±0.67	-	35.33±0.88
4	*Klebsiella pneumoniae*	18.33±0.88	11.00±0.58	-	38.00±0.58
5	*Shigella boydii*	16.00±0.58	9.67±0.33	-	34.33±0.33
6	*Shigella sonnei*	18.00±0.58	11.00±0.58	-	30.67±0.67
7	*Escherichia coli*	16.33±0.88	10.00±1.00	-	35.00±0.58
8	*Salmonella typhi*	14.00±0.58	9.33±0.88	-	36.33±0.67

The results of ethyl acetate extract of root presented in table 15. At 200 µg/disc concentration, the highest antibacterial activity was found against gram-negative bacterial strain *K. pneumoniae* where the diameter of zone of inhibition was 18.33±0.88 mm and against gram-positive bacterial strain *S. aureus* the zone of inhibition was 17.00±0.58 mm. The lowest activity was recorded against gram-negative bacterial strain *S. typhi* where the zone of inhibition was 14.00±0.58 mm. At 50 µg/disc concentration, the highest activity was found against *K. pneumoniae* and *S. sonnei* (11.00±0.58 mm) and the lowest was against *S. typhi* (9.33±0.88 mm).

Table 16. Antibacterial activity of methanol extract of root of *M. pudica*

Sl no	Name of organisms	Diameter of zone of inhibition in mm			
		200 µg/disc	50 µg/disc	Negative control	Ciprofloxacin (5 µg/disc)
1	*Bacillus cereus*	13.00±0.58	7.67±0.33	-	31.00±0.58
2	*Staphylococcus aureus*	16.33±0.88	10.00±0.58	-	33.33±1.45
3	*Bacillus zhanjiangensis*	13.00±0.58	8.00±0.58	-	34.00±0.58
4	*Klebsiella pneumoniae*	16.00±1.15	9.33±0.88	-	35.00±0.58
5	*Shigella boydii*	14.33±0.88	8.67±0.67	-	35.67±0.33
6	*Shigella sonnei*	16.00±0.58	10.00±0.58	-	29.67±0.33
7	*Escherichia coli*	17.33±0.88	11.00±0.58	-	34.00±0.58
8	*Salmonella typhi*	14.00±0.58	9.33±0.33	-	32.67±0.88

In table 16, methanol extract of root showed that at 200 µg/disc concentration the highest value was found against gram-negative bacterial strain *E. coli* where the diameter of zone of inhibition was 17.33±0.88 mm; while against gram-positive bacterial strain *S. aureus* the zone of inhibition was 16.33±0.88 mm. The lowest activity was recorded against gram-positive bacterial strain *B. cereus* and *B. zhanjiangensis* where the diameter of zone of inhibition was 13.00±0.58 mm. At 50 µg/disc concentration, the highest activity was found against *E. coli* (11.00±0.58 mm) and the lowest activity was found against *B. cereus* (7.67±0.33 mm).

Table 17. Antibacterial activity of petroleum ether, chloroform, ethyl acetate and methanol extracts of leaf, stem and root of *M. pudica* at 200 µg/disc concentrations

Test material	Extracts	Diameter of zone of inhibition in mm at 200 µg/ml concentrations							
		Gram-positive bacterial strains				Gram-negative bacterial strains			
		B. cereus	*S. aureus*	*B. zhanjian gensis*	*K. pneumo niae*	*S. boydii*	*S. sonnei*	*E. coli*	*S. typhi*
Leaf	Petroleum ether	11.67±0.33	13.00±0.58	13.33±0.33	13.00±0.58	12.67±0.33	12.00±0.58	14.33±0.33	13.00±0.58
	Chloroform	15.00±0.58	17.67±0.67	15.00±0.58	14.00±0.58	14.67±0.88	16.00±0.58	19.67±0.88	15.00±0.58
	Ethyl acetate	15.00±0.58	16.00±1.15	15.00±0.58	13.33±0.33	15.00±0.58	14.00±0.58	15.67±0.88	14.00±0.58
	Methanol	14.00±0.58	16.33±0.33	15.00±0.58	13.67±0.88	13.00±0.58	14.00±0.58	15.00±1.15	13.00±0.58
Stem	Petroleum ether	13.00±0.58	15.00±0.58	14.33±0.33	13.00±0.58	13.00±0.58	13.33±0.88	14.00±0.58	12.00±0.58
	Chloroform	14.00±0.58	16.67±0.33	15.00±0.58	14.33±0.88	14.00±0.58	14.00±0.58	15.67±0.88	14.00±0.58
	Ethyl acetate	14.00±0.58	16.00±0.58	14.67±0.88	13.67±0.88	13.33±0.33	13.00±0.58	16.67±0.88	15.00±0.58
	Methanol	13.00±0.58	16.33±0.33	14.00±0.58	13.00±0.58	13.33±0.88	13.00±0.58	16.00±0.58	12.67±0.33
Root	Petroleum ether	14.00±0.58	14.33±0.33	13.00±0.58	12.00±0.58	14.67±0.88	15.00±0.58	15.33±0.88	13.00±0.58
	Chloroform	17.33±0.33	20.00±0.58	16.00±0.58	19.67±0.88	16.67±0.88	21.67±1.45	19.00±0.58	15.00±0.58
	Ethyl acetate	16.00±0.58	17.00±0.58	15.67±1.20	18.33±0.88	16.00±0.58	18.00±0.58	16.33±0.88	14.00±0.58
	Methanol	13.00±0.58	16.33±0.88	13.00±0.58	16.00±1.15	14.33±0.88	16.00±0.58	17.33±0.88	14.00±0.58

Table 17 showed that, among the petroleum ether, chloroform, ethyl acetate and methanol extracts of leaf the highest value of zone of inhibition was 19.67±0.88 mm found in chloroform extract against *E. coli* and the lowest was 11.67±0.33 mm in petroleum ether extract against *B. cereus* for both the gram-positive and gram-negative bacterial strains. In case of stem, the highest value was 16.67±0.33 mm and 16.67±0.88 mm found in chloroform and ethyl acetate extracts against *S. aureus* and *E. coli* respectively and the lowest was 12.00±0.58 mm in petroleum ether extract against *S. typhi*. And for the root, the chloroform extract showed the highest activity where the zone of inhibition was 21.67±1.45 mm against *S. sonnei* and the lowest was 12.00±0.58 mm in petroleum ether extract against *K. pneumoniae*. Against both the gram-positive and gram-negative bacterial strains, chloroform extract showed the highest activity among all the extracts. In comparison, the root extracts showed more potent antibacterial activity than the leaf and stem extracts as it showed the highest zone of inhibition (21.67±1.45 mm in diameter) at 200 µg/disc concentration. Hence, the antibacterial activity order of the test plant *M. pudica* was: root > leaf > stem.

Antifungal activity

The petroleum ether, chloroform, ethyl actate and methanol extracts of leaf, stem and root of *M. pudica* were tested against four species of fungal stains *viz. R. oryzae, F. proliferatum, A. niger* and *C. albicans,* and the results were presented in the tables 18, 19, 20 and 21. The zone of inhibition was prominent for Nystatin (50µg/disc).

Table 18. Antifungal activity of different extracts of *M. pudica* and the standard Nystatin against *R. oryzae*

Solvents	Diameter of zone of inhibition in mm								
	Leaf			Stem			Root		
	200µg/ disc	50µg/ disc	Nystatin (50µg/ disc)	200µg/ disc	50µg/ disc	Nystatin (50µg/ disc)	200µg/ disc	50µg/ disc	Nystatin (50µg/ disc)
Petroleum ether	7	5	12	6	-	13	8	5	12
Chloroform	8	6	11	7	5	12	10	7	13
Ethyl acetate	8	6	12	8	6	10	**13**	8	11
Methanol	7	5	13	7	-	12	12	6	10

The zone of inhibition for *R. oryzae* was tabulated in table 18. The highest zone of inhibition was found 13mm for ethyl acetate extract of root followed by 12mm and 10mm for methanol and chloroform extracts of root respectively, and 8mm for petroleum ether extract of root at 200µg/disc concentration. For chloroform extract of leaf and ethyl acetate extracts of leaf and stem also the zone of inhibition was 8mm at same concentration. On the other hand, at 50µg/disc concentration, the highest zone of inhibition was found 8mm for ethyl acetate extract of root; whereas petroleum ether and methanol extracts of stem showed no activity.

Table 19. Antifungal activity of different extracts of *M. pudica* and the standard Nystatin against *F. proliferatum*

Solvents	Diameter of zone of inhibition in mm								
	Leaf			Stem			Root		
	200µg/ disc	50µg/ disc	Nystatin (50µg/ disc)	200µg/ disc	50µg/ disc	Nystatin (50µg/ disc)	200µg/ disc	50µg/ disc	Nystatin (50µg/ disc)
Petroleum ether	6	-	11	7	-	12	8	6	10
Chloroform	7	5	10	6	-	11	11	7	12
Ethyl acetate	8	6	12	7	-	13	**12**	7	11
Methanol	8	6	11	7	-	11	10	6	10

In table 19, the zone of inhibition for *F. proliferatum* was tabulated. The highest zone of inhibition was found 12mm in diameter for ethyl acetate extract of root followed by 11mm, 10mm and 8mm for chloroform, methanol and petroleum ether extracts of root respectively. Also the ethyl acetate and methanol extracts of leaf showed the zone of inhibition 8mm at same concentration. On the other hand, at 50µg/disc concentration, the highest zone of inhibition was found 7mm in diameter for chloroform and ethyl acetate extract of root; whereas petroleum ether extract of leaf and all the extracts of stem showed no effectivity.

Table 20. Antifungal activity of different extracts of *M. pudica* and the standard Nystatin against *A. niger*

Solvents	Diameter of zone of inhibition in mm								
	Leaf			Stem			Root		
	200µg/ disc	50µg/ disc	Nystatin (50µg/ disc)	200µg/ disc	50µg/ disc	Nystatin (50µg/ disc)	200µg/ disc	50µg/ disc	Nystatin (50µg/ disc)
Petroleum ether	6	5	10	6	-	12	9	5	11
Chloroform	7	6	11	7	5	11	**12**	8	13
Ethyl acetate	8	6	12	7	5	12	11	7	12
Methanol	7	5	11	6	-	10	10	6	10

Table 20 showed the effectivity of different extracts against *A. niger.* The highest zone of inhibition was found 12mm in diameter for chloroform extract of root followed by 11mm, 10mm and 9mm for ethyl acetate, methanol and petroleum ether extracts of root respectively at 200µg/disc concentration. On the other hand, at 50µg/disc concentration, the highest zone of inhibition was found 8mm in diameter for chloroform extract of root; whereas petroleum ether and methanol extracts of stem showed no activity.

Table 21. Antifungal activity of different extracts of *M. pudica* and the standard Nystatin against *C. albicans*

Solvents	Diameter of zone of inhibition in mm								
	Leaf			Stem			Root		
	200µg/ disc	50µg/ disc	Nystatin (50µg/ disc)	200µg/ disc	50µg/ disc	Nystatin (50µg/ disc)	200µg/ disc	50µg/ disc	Nystatin (50µg/ disc)
Petroleum ether	6	-	11	6	-	13	10	6	10
Chloroform	7	6	10	6	5	12	**13**	9	12
Ethyl acetate	8	6	12	7	5	10	12	7	11
Methanol	7	5	11	6	-	11	11	6	11

The table 21 showed the zone of inhibition for *C. albicans*. The highest zone of inhibition was found 13mm in diameter for chloroform extract of root followed by 12mm, 11mm and 10mm for ethyl acetate, methanol and petroleum ether extracts of root respectively at 200µg/disc concentration. On the other hand, at 50µg/disc concentration, the highest zone of inhibition was found 9mm in diameter for chloroform extract of root; whereas petroleum ether extract of leaf and petroleum ether and methanol extracts of stem showed no effectivity.

Determination of Minimum Inhibitory Concentrations (MICs)

Serial tube dilution technique was followed using Nutrient Broth media (Himedia, Mumbai, India) to determine the MIC values of chloroform and ethyl acetate extracts of leaf against three gram-positive *viz. B. cereus, S. aureus, B. zhanjiangensis* and three gram-negative bacterial strains *K. pneumoniae, E. coli* and *S. typhi*.

Table 22. Minimum Inhibitory Concentrations (MICs) of chloroform extract of leaf of *M. pudica* against six pathogenic bacteria

Test tube no	Nutrient Broth Medium added (ml)	Diluted soln. of extract (µg/ml)	Inoculums added (µg/ml)	Gram-positive bacteria			Gram-negative bacteria		
				B. cereus	*S. aureus*	*B. zhanjiangensis*	*K. pneumoniae*	*E. coli*	*S. typhi*
1	1	512	10	–	–	–	–	–	–
2	1	256	10	–	–	–	–	–	–
3	1	128	10	–	–	–	–	–	–
4	1	64	10	+	–	–	+	–	+
5	1	32	10	+	–	+	+	+	+
6	1	16	10	+	+	+	+	+	+
7	1	8	10	+	+	+	+	+	+
8	1	4	10	+	+	+	+	+	+
9	1	2	10	+	+	+	+	+	+
10	1	1	10	+	+	+	+	+	+
Cs	1	512	–	–	–	–	–	–	–
Ci	1	–	10	+	+	+	+	+	+
Cm	1	–	–	–	–	–	–	–	–
Results of MIC (µg/ml)				128	**32**	64	128	64	128

Notes: '+ve' = indicates Growth, '-ve' = indicates no Growth, Cs = Medium + Sample, Ci = Medium + Inoculums, Cm = Medium

The results in table 22 indicated that the MICs of chloroform extract of leaf of *M. pudica* against gram-positive bacterial strains *B. cereus, S. aureus* and *B. zhanjiangensis* were 128, 32 and 64 µg/ml and that of gram-negative bacterial strains *K. pneumoniae, E. coli* and *S. typhi* were 128, 64 and 128 µg/ml respectively. Obviously the MIC value of chloroform extract of leaf of *M. pudica* was more potent against *S. aureus* than other strains.

Table 23. Minimum Inhibitory Concentrations (MICs) of ethyl acetate extract of leaf of *M. pudica* against six pathogenic bacteria

Test tube no	Nutrient Broth Medium added (ml)	Diluted soln. of extract (µg/ml)	Inoculums added (µg/ml)	Gram-positive bacteria			Gram-negative bacteria		
				B. cereus	*S. aureus*	*B. zhanjiangensis*	*K. pneumoniae*	*E. coli*	*S. typhi*
1	1	512	10	−	−	−	−	−	−
2	1	256	10	−	−	−	−	−	−
3	1	128	10	−	−	−	+	−	−
4	1	64	10	+	−	−	+	−	+
5	1	32	10	+	+	+	+	+	+
6	1	16	10	+	+	+	+	+	+
7	1	8	10	+	+	+	+	+	+
8	1	4	10	+	+	+	+	+	+
9	1	2	10	+	+	+	+	+	+
10	1	1	10	+	+	+	+	+	+
Cs	1	512	−	−	−	−	−	−	−
Ci	1	−	10	+	+	+	+	+	+
Cm	1	−	−	−	−	−	−	−	−
Results of MIC (µg/ml)				128	64	64	256	64	128

Notes: '+ve' = indicates Growth, '-ve' = indicates no Growth, Cs = Medium + Sample, Ci = Medium + Inoculums, Cm = Medium

In table 23, the MIC values of ethyl acetate extract of leaf of *M. pudica* against gram-positive bacterial strains were 128 µg/ml for *B. cereus* and 64 µg/ml for both *S. aureus* and *B. zhanjiangensis* and that of gram-negative bacterial strains the values were 256, 64 and 128 µg/ml for *K. pneumoniae, E. coli* and *S. typhi* respectively.

Antibacterial activity of pure compounds

The antibacterial activity of 9-Octadecenamide and 13-Docosenamide, (Z)- were tested against eight bacterial strains using concentrations of 100µg/disc and 50µg/disc along with a negative control and the sensitivities were compared with standard antibiotic Ciprofloxacin (5µg/disc).

Table 24. Antibacterial activity of 9-Octadecenamide of *M. pudica* and Ciprofloxacin

Sl no	Name of the organisms	Diameter of zone of inhibition in mm			
		100 µg/disc	50 µg/disc	Negative control	Ciprofloxacin (5 µg/disc)
1	*Bacillus cereus*	18	11	-	29
2	*Staphylococcus aureus*	19	12	-	32
3	*Bacillus zhanjiangensis*	17	10	-	31
4	*Klebsiella pneumoniae*	18	11	-	30
5	*Shigella boydii*	16	10	-	33
6	*Shigella sonnei*	19	12	-	34
7	*Escherichia coli*	21	10	-	32
8	*Salmonella typhi*	15	9	-	30

The results of antibacterial effect of 9-Octadecenamide of *M. pudica* was presented in table 24. At 100 µg/disc concentration, the highest activity was found against gram-negative bacterial strain *E. coli* where the diameter of zone of inhibition was 21 mm and against gram-positive bacterial strain *S. aureus* where the diameter of zone of inhibition was 19 mm. The lowest activity was recorded against gram-negative bacterial strain *S. typhi* where the zone of inhibition was 15 mm and against gram-positive bacterial strain *B. zhanjiangensis* where the zone of inhibition was 17 mm. At 50 µg/disc concentration, the highest activity was found against *S. aureus* and *S. sonnei* (12 mm) and the lowest was against *S. typhi* (9 mm).

Table 25. Antibacterial activity of 13-Docosenamide, (Z)- of *M. pudica* and Ciprofloxacin

Sl no	Name of the organisms	Diameter of zone of inhibition in mm			
		100 µg/disc	50 µg/disc	Negative control	Ciprofloxacin (5 µg/disc)
1	*Bacillus cereus*	19	12	-	31
2	*Staphylococcus aureus*	21	13	-	33
3	*Bacillus zhanjiangensis*	17	11	-	35
4	*Klebsiella pneumoniae*	19	12	-	34
5	*Shigella boydii*	16	10	-	32
6	*Shigella sonnei*	20	13	-	33
7	*Escherichia coli*	22	11	-	32
8	*Salmonella typhi*	16	10	-	30

From the results shown in table 25, we found that the highest antibacterial activity of 13-Docosenamide, (Z)- of *M. pudica* against *E. coli* was 22 mm for gram-negative bacterial strain and against *S. aureus* was 21 mm for gram-positive bacterial strain at 100 µg/disc concentrations. The lowest activity was recorded against *S. boydii* and *S. typhi* were 16 mm for gram-negative bacterial strains and against *B. zhanjiangensis* was 17 mm for gram-positive bacterial strain. At 50 µg/disc concentration, the highest activity was found against *S. aureus* and *S. sonnei* (13 mm) and the lowest was found against *S. boydii* and *S. typhi* (10 mm).

Minimum Inhibitory Concentrations of pure compounds against test bacteria

Two gram-positive *viz. B. sereus, S. aureus* and two gram-negative *viz. E. coli* and *S. typhi* bacterial strains were selected to determine the MIC values of purified compounds following serial tube dilution technique by using nutrient broth medium.

Table 26. Minimum Inhibitory Concentrations (MICs) of 9-Octadecenamide of *M. pudica* against four pathogenic bacteria

Test tube no	Nutrient Broth Medium added (ml)	Diluted soln. of extract (µg/ml)	Inoculums added (µg/ml)	*B. cereus*	*S. aureus*	*E. coli*	*S. typhi*
1	1	512	10	−	−	−	−
2	1	256	10	−	−	−	−
3	1	128	10	−	−	−	−
4	1	64	10	+	−	−	+
5	1	32	10	+	+	+	+
6	1	16	10	+	+	+	+
7	1	8	10	+	+	+	+
8	1	4	10	+	+	+	+
9	1	2	10	+	+	+	+
10	1	1	10	+	+	+	+
Cs	1	512	−	−	−	−	−
Ci	1	−	10	+	+	+	+
Cm	1	−	−	−	−	−	−
Results of MIC (µg/ml)				128	64	64	128

Notes: '+ve' = indicates Growth, '-ve' = indicates no Growth, Cs = Medium + Sample, Ci = Medium + Inoculums, Cm = Medium

In table 26, the MIC values of 9-Octadecenamide of *M. pudica* against gram-positive bacterial strains were 128 µg/ml for *B. cereus* and 64 µg/ml for *S. aureus* and that of gram-negative bacterial strains the values were 64 µg/ml and 128 µg/ml for *E. coli* and *S. typhi* respectively. Evidently, the MIC value of 9-Octadecenamide of *M. pudica* was more effective against *S. aureus* and *E. coli* than *B. cereus* and *S. typhi.*

Table 27. Minimum Inhibitory Concentrations (MICs) of 13-Docosenamide, (Z)- of *M. pudica* against four pathogenic bacteria

Test tube no	Nutrient Broth Medium added (ml)	Diluted soln. of extract (µg/ml)	Inoculums added (µg/ml)	*B. cereus*	*S. aureus*	*E. coli*	*S. typhi*
1	1	512	10	−	−	−	−
2	1	256	10	−	−	−	−
3	1	128	10	−	−	−	−
4	1	64	10	−	−	−	+
5	1	32	10	+	−	+	+
6	1	16	10	+	+	+	+
7	1	8	10	+	+	+	+
8	1	4	10	+	+	+	+
9	1	2	10	+	+	+	+
10	1	1	10	+	+	+	+
Cs	1	512	−	−	−	−	−
Ci	1	−	10	+	+	+	+
Cm	1	−	−	−	−	−	−
Results of MIC (µg/ml)				64	**32**	64	128

Notes: '+ve' = indicates Growth, '-ve' = indicates no Growth, Cs = Medium + Sample, Ci = Medium + Inoculums, Cm = Medium

Table 27 showed that the minimum inhibitory concentrations of 13-Docosenamide, (Z)- of *M. pudica* against gram-positive bacterial strains *B. cereus* and *S. aureus* were 64 µg/ml and 32 µg/ml and that of gram-negative bacterial strains *E. coli* and *S. typhi* were 64 µg/ml and 128 µg/ml respectively. Obviously, the MIC value of 13-Docosenamide, (Z)- of *M. pudica* was more potent against *S. aureus* than other strains.

Discussion

Antibacterial activity

In this study twelve crude extracts were used to evaluate the antibacterial activity against eight harmful bacterial strains. Each extract showed more or less equal effectivity except a few cases. If the zones of inhibition were considered then it was found that the root extracts of *M. pudica* showed more potentiality than leaf and stem against both gram-positive and gram-negative bacterial strains, and among the extracts of root, the chloroform extract showed the highest activity.

Othman (2003) described that the gram-positive microorganisms were typically more susceptible to antimicrobial agents than gram-negative bacteria. The antibacterial activity of *M. pudica* extract was screened by Gandhiraja *et al.* (2009) against some selected microorganisms and found good sensitivity against *K. pneumoniae* at 1µg/200µl concentration. In the present study, moderate to high antibacterial activity against all the tested microorganisms for petroleum ether, chloroform, ethyl acetate and methanol crude extracts of leaf, stem and root were found.

Three types of extracts of *M. pudica* (petroleum ether, chloroform and ethanol) tested by Akter *et al.* (2010) and reported that ethanol extract possessed the maximum antibacterial activity. The gram-negative bacterial strains were more sensitive than gram-positive bacterial strains. Khan *et al.* (2009) also reported that the ethanol extract of *Achyranthus aspera* was much effective in gram-negative bacterial strains than gram-positive strains. Several types of glycosides, alkaloids, proteins and steroids were reported to have antibacterial activity (Barnabas and Nagarajan 1988). In case of ethanol extract of *M. pudica,* the highest and lowest zones of inhibition were 13.40 mm and 9.35 mm against *P. aeruginosa* and *B. cereus* respectively. Balakrishnan *et al.* (2006) showed a close agreement with this result. On the other hand, the corresponding highest zone of inhibition for petroleum ether and chloroform extracts was 15.30 mm and 12.40 mm for *B. subtilis*. Present study showed the same findings *i.e.* the gram-negative bacterial strains were more sensitive than gram-positive bacterial strains except for ethyl acetate (16.00±1.15 mm) and methanol (16.33±0.33 mm) extracts of leaf, and petroleum ether (15.00±0.58 mm), chloroform (16.67±0.33 mm) and methanol (16.33±0.33 mm) extracts of stem against *S. aureus*.

Panda *et al.* (2012) showed the ethyl acetate root extract of *M. pudica* exhibited better inhibition of bacteria and fungi in comparison to petroleum ether and methanol extract. In a concentration of 250 mg/ml the ethyl acetate extract showed a zone of inhibition 23.1±1.68 mm against the gram-negative bacteria *P. vulgaris,* which was even more than that of the standard drug Chloramphenicol where the zone of inhibition was 15.7±1.06 mm in a concentration of 200 µg/ml. And at a same concentration of 250 mg/ml, the zone of inhibitions were 1.9±0.24 mm and 7.6±0.94 mm against *P. vulgaris* for petroleum ether and methanol extracts of root respectively. In the present study the petroleum ether, ethyl acetate and methanol extracts of root showed moderate to high antibacterial activity where the ethyl acetate extract exhibited the highest zone of inhibition (18.33±0.88 mm against *K. pneumoniae*) followed by methanol extract (13.33±0.88 mm against *E. coli*) and petroleum ether (15.33±0.88 mm against *E. coli*) at 200 µg/ml concentration.

Tamilarasi and Ananthi (2012) indicated that the ethanol extracts of leaves of *M. pudica* exhibited antibacterial activity against *B. subtilis, P. aeruginosa* and *K. pneumoniae* at four different concentrations of 25, 50, 75 and 100 µl/disc. The potential sensitivity was obtained against all the tested microorganisms and the highest zone of inhibition at 100 µl/disc was recorded against *B. subtilis* (20 mm). Ahuchaogu *et al.* (2017) tested the antibacterial activity of ethanol extract of *M. pudica* at three different concentrations of 25, 50 and 100 mg/disc. The result exhibited good antibacterial activity against *E. coli, P. aeruginosa* and *S. aureus.* The inhibitory effect was stronger on *P. aeruginosa* (18.0 mm) than *E. coli* (14.0 mm) and *S. aureus* (9.8 mm) at 100 mg/ml concentration. Gandhiraja *et al.* (2009) showed that the *M. pudica* leaf extract exhibited a good sensitivity where the zone of inhibition was 20 mm at 200 µl/disc against *K. pneumoniae*. Thoa *et al.* (2015) described that the ethanol extracts of leaf and stem of *M. pudica* had stronger inhibition to gram-positive bacteria compared to gram-negative bacteria. The zone of inhibition showed by ethanol extract against *E. coli, S. aureus, B. cereus* and *S. typhi* were 11 mm, 19 mm, 17 mm and 16 mm respectively. Sunil *et al.* (2016) assessed that at low concentrations the ethanolic extract of *M. pudica* did not show any antibacterial activity. But exhibited a significant antibacterial activity against *E. coli, P. aeruginosa, S. pyogenes* and *S. aureus* at the test dose of 50, 100 and 250 µg/ml. The highest zone of inhibition was found against *E. coli* (17 mm), followed by *P. aeruginosa* and *S. aureus* (16 mm) and *S. pyogenes* (15 mm) at a concentration of 250 µg/ml. Kaur *et al.* (2011) revealed the antibacterial activity of the ethanolic extract of leaf of *M. pudica* against three potentially pathogenic

microorganisms and found the zone of inhibitions 23.96 mm, 23.3 mm and 21.43 mm for *S. aureus, E. coli* and *P. aeruginosa* respectively at 30 mg/ml concentration, where the zone of inhibition of the standard Amphicilline was found 25.5 mm at 2.0 mg/ml concentration. In the present study it was found that all the petroleum ether, chloroform, ethyl acetate and methanol extracts of leaf of *M. pudica* showed moderate antibacterial activity and the highest activity was shown by chloroform extract against *E. coli* (19.67±0.88 mm) at 200 µg/ml concentrations and a stronger inhibition to gram-positive bacteria compared to gram-negative bacteria was observed in methanol extracts of leaf and stem against *S. aureus* (16.33±0.33 mm).

M. pudica leaf extract was reported to have strong antibacterial activity against *S. aureus* (76.67%), *B. subtilis* (73.89%) and *P. aeruginosa* (82.3%) (Doss *et al.* 2011). Arokiyaraj *et al.* (2012) described that the methanolic extract of leaves of *M. pudica* effectively inhibited the tested bacterial strains where the inhibition was found to be higher than that of the standard Streptomycin (30 µg/disc). The methanolic extract showed significant antibacterial effect against *B. subtilis* (16 mm), *S. aureus* (15 mm), *S. typhi* (14.5 mm), *E. coli* (12 mm), *P. mirabilis* (11 mm) and *K. pneumoniae* (20 mm). The highest zone of inhibition was exhibited by *K. pneumoniae* (20 mm) at 5 µg/disc concentration. In the present study the methanolic extract of leaf also showed significant antibacterial effectivity between 13.00±0.58 to 16.33±0.33 mm zone of inhibition at 200 µg/disc concentration. Rajendran and Sundararajan (2010) denoted that the methanolic fraction (methanol: ethyl acetate = 75:25) produced a greater antibacterial activity than that of the standard Ciprofloxacin (5 µg/disc) against tested organisms. The present study showed a moderate antibacterial activity against tested organisms by methanolic extract of leaf where the highest zone of inhibition was 16.33±0.33 mm against *S. aureus.*

According to Amengialue *et al.* (2016), at 100 mg/ml concentration, the highest antimicrobial activity of aqueous leaf extract of *M. pudica* was obtained against *E. coli* (6.3±1.5) followed by *S. aureus* (3.3±0.9), while the least was recorded against *K. pneumoniae* (2.3±0.3)*;* for ethanol extract, the highest activity was recorded against *S. aureus* (15.0±0.6) followed by *E. coli* (14.0±0.6), while the least was recorded against *P. aeruginosa* (2.3±0.3); and for methanol extract, the highest activity was recorded against *B. subtilis* (15.7±1.9) followed by *K. pneumoniae* (15.3±0.7), while the least was recorded against *E. coli* (14.3±0.9). In case of 50 mg/ml concentration, aqueous leaf extract showed antimicrobial activity against *E. coli* (3.0±0.6) only; for ethanol extract, the highest antimicrobial activity was found against *S. pyogenes* (11.3±0.9)

followed by *E. coli* (10.7±0.9), while the least was recorded against *B. subtilis* (3.7±0.7); and for methanol extract, the highest activity was found against both for *B. subtilis* and *K. pneumoniae* (12.7±0.9) and the least was recorded against *S. aureus* (7.3±0.9). In the present investigation the similar results were found for methanolic leaf extract of *M. pudica* and the highest zone of inhibition was found against *S. aureus* (16.33±0.33 mm) and the lowest was recorded against *S. boydii* and *S. typhi* (13.0±0.58 mm) at 200 µg/ml concentration. In case of 50 mg/ml concentration, the highest zone of inhibition was found against *S. aureus* (11.33±0.33 mm) and the lowest was recorded against *S. typhi* (8.00±0.58 mm).

Sharma and Sharma (2010) investigated the inhibitory activity of aqueous extracts of *M. pudica* by agar disc well diffusion method against *E. coli, B. subtilis, S. aureus, M. luteus* and *K. pneumoniae* and showed a good antimicrobial activity (28 mm) in *E. coli.* Abhirami *et al.* (2014) described that the antibacterial activity of acetone leaf extract of *M. pudica* showed the highest zone of inhibition against *S. aureus;* while aqueous leaf extract showed the highest zone of inhibition against *E. coli* and petroleum ether extract showed the highest zone of inhibition against *S. typhi.* The present study showed moderate antibacterial activity for petroleum ether extract of leaf of *M. pudica* where the highest zone of inhibition was found against *E. coli* (14.33±0.33 mm).

Srivastava *et al.* (2012) mentioned that the leaf extract possessed a strong antibacterial activity against *S. aureus* and *B. cereus*. Successive extracts of the whole plant of *M. pudica* was reported to have antibacterial activity. Muthukumaran *et al.* (2011) revealed that the leaf extract inhibited the bacterial growth at different concentrations. For *E. coli* the zone of inhibition was 15±3 mm at 1 mg/ml, 7±3 mm at 0.5 mg/ml, 4±3 mm at 0.1 mg/ml and for *Pseudomonas* 13±2 mm at 1 mg/ml, 6±2 mm at 0.5 mg/ml, 3±2 mm at 0.1 mg/ml concentration. In the present study also the leaf extracts of *M. pudica* of different solvents showed moderate to strong antibacterial activity for tested isolates. Among the petroleum, chloroform, ethyl acetate and methanol extracts of leaf the highest zone of inhibition was found against *E. coli* which was 19.67±0.88 mm at 200 µg/disc concentration.

Racadio (2016) denoted that the *M. pudica* extract had a strong antibacterial activity against *S. aureus* (21.8 mm) and *B. subtilis* (23.5 mm). The strong antimicrobial activity was due to flavonoids. The whole plant extract possessed good antimicrobial activity between 7-18 mm (inhibition zone) against *S. albus, S. aureus, E. coli, S. paratyphi A, S. paratyphi B, S. typhi, S. flexneri, P. aeruginosa* and *K. pneumoniae* (Pawaskar and Kale 2006). The present study showed the highest activity for chloroform extract of root against *S. sonnei* (21.67±1.45 mm).

Antifungal activity

The antifungal activity test was conducted on four fungal species and more or less effectivity was observed for different solvent extracts of leaf, stem and root of *M. pudica.* The root extracts showed the higher activity than leaf and stem at 200 µg/disc and 50 µg/disc concentration.

Abhirami *et al.* (2014) showed that for antifungal study the ethyl acetate extract of leaf showed the maximum zone of inhibition against *F. oxysporum,* acetone extract showed the highest zone of inhibition against *P. variotii* and petroleum ether showed the highest zone of inhibition against *Pestalotia foedians, F. oxysporum* and *P. variotii.* In the present study, the petroleum ether and ethyl acetate extracts of leaf of *M. pudica* showed poor antifungal activity for the fungal isolates. In the concentration of 250 mg/ml, the ethyl acetate extract of root of *M. pudica* showed the zone of inhibition 31.5±2.96 mm against *A. flavus*; which was well comparable with the zone of inhibition of the standard drug Ketokonazole (33.8±2.06 mm) in a concentration of 200 µg/ml by Panda *et al.* (2012). The present study showed more or less similar results with this study where the root extract exhibited higher potency than that of leaf and stem, and the ethyl acetate and chloroform extracts of root showed the highest zones of inhibition (13 mm) against *R. oryzae* and *C. albicans* respectively at 200 µg/disc concentrations.

Tamilarasi and Ananthi (2012) indicated that the ethanol extracts of leaves of *M. pudica* exhibited antifungal activity against fungal strains of *A. flavus* and *T. rubrum* at four concentrations of 25, 50, 75 and 100 µl/disc. The potentiality was obtained against both the microorganisms and the highest zone of inhibition at 100 µl/disc was recorded against *A. flavus* (21 mm). Muhammad *et al.* (2015) revealed that the ethanol extracts of leaves of *M. pudica* showed antifungal effect against some of the isolated fungi from the razor bumps. Potential sensitivity was found against *T. verrucosum, T. schoeileinii, T. soudanense, M. ferrugineum, M. canis* and *M. gyseum* using four different concentrations *i.e.* 150, 200, 250 and 300 mg/ml. The highest zone of inhibition was found against *T. verrucosum* and *T. soudanense* which was 5 mm for both the isolates at 300 mg/ml concentration. Gandhiraja *et al.* (2009) showed that the *A. fumigatus* exhibited a good sensitivity where the zone of inhibition was 12 mm at 200 µl/disc against *M. pudica* leaf extract. But in the present study, the antifungal experiment showed a poor sensitivity against all the extracts of leaf and stem of *M. pudica.* But the root extracts showed a good sensitivity against all fungal isolates at 200 µg/disc concentration.

Ibrahim *et al.* (2014) revealed that at 10 to 40 mg/ml concentrations the percentages of growth inhibition of ethanolic extract of leaf of *M. pudica* ranged from 57.96-86.85% against *T. verrucosum,* 30.0-70.37% against *T. mentagrophyte,* 39.81-69.26% against *M. nanum* and 23.52-65.74% against *A. flavus.* When the concentration of ethanolic extract of stem was increased, the percentages of growth inhibition was also increased significantly ($p<0.05$). At 10 mg/ml concentration the percentages of growth inhibitions were 59.44% against *T. verrucosum,* 29.44% against *T. mentagrophyte,* 48.52% against *M. nanum* and 11.1% against *A. flavus;* while at 40 mg/ml concentration of the extract, the growth inhibition was increased significantly ($p<0.05$) to 83.89% against *T. verrucosum,* 70.0% against *T. mentagrophyte,* 72.59% against *M. nanum* and 35.53% against *A. flavus.* At 40 mg/ml concentration the activities were higher than that of positive control (Greseofulvin, 10 mg/ml) against all the fungal isolates except *A. flavus.* The study also revealed that the ethanolic extract of the mixture of leaf and stem of *M. pudica* showed significant increase ($p<0.05$) in percentage of growth inhibition with the increase of concentration of extracts. The percentages of growth inhibition of methanol extract of leaf, stem and mixture of leaf and stem showed significant differences ($p<0.05$) against the fungal pathogens. At 40 mg/ml concentration the methanol extract of leaf of *M. pudica* showed a strong growth inhibition activity against *T. verrucosum* (90%), *T. mentagrophytes* (71.85%), *M. nanum* (73.70%) and *A. flavus* (73.33%). The activity of methanolic extract of leaf was significantly ($p<0.05$) higher than that of Greseofulvin (40 mg/ml). At 40 mg/ml concentration the methanol extract of stem of *M. pudica* showed very high activity in growth inhibition against the fungal isolates. At 40 mg/ml concentration the methanol extract of stem showed higher activity than Greseofulvin (10 mg/ml) against *T. verrucosum, T. mentagrophyte, M. nanum* and *A. flavus.* At 40 mg/ml concentration the mixture of leaf and stem of *M. pudica* extract showed the highest activity of 87.04% against *T. verrucosum.* In the present study, the methanolic extract of leaf and stem showed antifungal activity where the zones of inhibition were 6 mm to 8 mm at 200 µg/disc concentration for the fungal isolates.

At 40 mg/ml concentration the range of percentage inhibition of *M. pudica* methanol extracts against *A. flavus* was 62.59-73.33% which was comparable to the reported 70% inhibition showed by *Tamarix dioica* against *A. flavus* as reported by Khan *et al.* (2004). Among the tested fungal isolates, only the *C. albicans* showed significant susceptibility to ethanol and methanol extracts of *M. pudica* leaf with the highest zones of inhibition of 11.2±2.5 and 12.3±1.2 at 100 mg/ml concentration. The findings of the

research work had clearly denoted that the *M. pudica* extract was a better antibacterial agent than antifungal agent (Amengialue *et al.* 2016). In the present study, the root extract showed the highest activity against the fungal isolates at 200µg/disc concentration among the methanolic extracts of leaf, stem and root.

Muthukumaran *et al.* (2011) described that the leaf extract of *M. pudica* inhibited the fungal growth at different concentrations. For *C. albicans* the zone of inhibition was 12±2 mm at 1 mg/ml, 6±2 mm at 0.5 mg/ml and 3±1 mm at 0.1 mg/ml concentration. More or less similar results were obtained in the present study. Kakad *et al.* (2015) recorded that the methanol extracts of *M. pudica* leaf showed antifungal activity to *C. albicans* (3 mm) and *A. niger* (1 mm); while no antifungal activity was found against *Daedalea flavida.* In the present investigation, the methanolic extract of leaf showed poor activity at 200 µg/disc concentration where the zones of inhibition were 7 mm to 8 mm for the fungal isolates.

Minimum Inhibitory Concentrations (MICs)

MIC test was performed with chloroform and ethyl acetate crude extracts of leaf where the range of MIC value was 32-128 µg/ml for chloroform extract and 64-256 µg/ml for ethyl acetate extract. In case of pure compounds, the range of MIC value was 64-128 µg/ml for 9-Octadecenamide and 32-128 µg/ml for 13-Docosenamide, (Z)-. The MIC results indicated remarkable antibacterial potency of the test plant. Very limited works have conducted to identify the MIC values of different solvent extracts of *M. pudica* against harmful bacterial strains.

In the present study, the MIC of the chloroform extract of leaf of *M. pudica* was 32 to 128 µg/ml against different bacterial strains that were similar to the study of Rajendran and Sundararajan (2010). They showed that the MICs of n-hexane, chloroform, ethyl acetate, methanol and methanolic fractions of leaves of *M. pudica* was found 133.33 µg/ml; but one methanolic fraction was found 33.33 µg/ml against the tested bacterial organisms which was comparable with the standard antibiotic Ciprofloxacin. They also showed that the ethyl acetate extract was not effective at lower concentration but effective only at higher concentration of 133.33 µg/ml. But the chloroform and methanolic fractions were effective at a lower concentration of 33.33 µg/ml. The present study differed from their study with ethyl acetate extract; where lower concentrations of ethyl acetate extract were also effective.

Akter *et al.* (2010) described that the minimum inhibitory concentrations (MICs) of ethanol extract of *M. pudica* were 312.5 µg/ml and 615 µg/ml against *P. aeruginosa* and *B. cereus* respectively. Balakrishnan *et al.* (2006) showed a close agreement with this result. On the other hand, the corresponding MIC for petroleum ether and chloroform extracts was 156.25 µg/ml for *B. subtilis*. In the present study, the MIC for *B. cereus* was 128 µg/ml for both chloroform and ethyl acetate extracts.

Amengialue *et al.* (2016) conducted the MIC of different extracts of *M. pudica* where six concentrations were taken (100, 50, 25, 12.5, 6.25 and 3.125 mg/ml). In case of aqueous extract, the minimum inhibitory concentrations were 100 mg/ml, 50 mg/ml and 100 mg/ml for *K. pneumoniae, E. coli* and *S. areus* respectively. The ethanol extract showed a potential inhibitory effect. *S. pyogenes* and *E. coli* both had the minimum inhibitory effect at 12.5 mg/ml concentration; *K. pneumoniae, B. subtilis* and *S. aureus* had the minimum inhibitory concentration 25 mg/ml; while *P. aeruginosa* had 100 mg/ml. And for the methanol extract, the minimum inhibitory concentration was 12.5 mg/ml for *E. coli, B. subtilis* and *S. pyogenes;* 25 mg/ml for *K. pneumoniae* and *S. aureus* and the least 100 mg/ml for *P. aeruginosa.* In the present study, the result showed a close agreement where the MIC of chloroform extract of leaf of *M. pudica* was 32 µg/ml against *S. aureus.* Panda *et al.* (2012) showed that the ethyl acetate extract of root of *M. pudica* exhibited the lowest MIC which was 3.12 mg/ml against gram-positive bacteria *S. epidermidis* and gram-negative bacteria *E. coli.* In the present discussion, the lowest MIC value of ethyl acetate extract of leaf was 64 µg/ml against *S. aureus, B. zhangiangensis* and *E. coli.*

The present investigation was conducted with twelve extracts from three parts of the test plant *M. pudica.* Four different solvents *i.e.* petroleum ether, chloroform, ethyl actate and methanol were used for the extraction. Due to lack of available related papers limited comparison was presented in the discussion.

Chapter Five

ANTIOXIDANT ACTIVITY OF *MIMOSA PUDICA*

Introduction

Plants contain bioactive constituents that are used as traditional medicines as well as modern medicine and these natural compounds are used for phytotherapy and pharmaceutical drugs (Sahu *et al.* 2015). Antioxidants react through molecular oxygen quenching or free radical, being capable of either inhibiting or delaying oxidation process that occur under the influence of reactive oxygen species or molecular oxygen. A large number of factors are responsible for inducing oxidative stress and enhancing production of free radicals such as xenobiotics, radiation or exposure to heavy metals (Shinde *et al.* 2016). The medicinal plants that possess the antioxidant activities may be explained by the presence of phenolic compounds containing the hydroxyl group which confers the hydrogen donating capacity (Narayanaswamy and Balakrishnan 2011).

Normally metabolism produces free radicals in body. However, the environmental factors as the energy from the sun, cigarette smoking, pollution, etc. may destroy molecules in the body through oxidation. It may cause artery hardening, premature aging, edema, arthritis and even cancer susceptibility (www.youtube.com/watch? V=ZwU8xy5VnQK). Today, attention has been given to the therapeutic potential of antioxidants for controlling degenerative diseases associated with oxidative damage. The plant extracts with phytochemicals have prominent antioxidant potentials (Tripathi *et al.* 1996, Sreejayan and Rao 1997, Vani *et al.* 1997 and Larson 1988).

Antioxidants derived from natural sources have less adverse effects and very much promising for its better efficacy. The damage of cells are prevented by the antioxidants significantly by scavenging the free radicals and reactive oxygen species developed in different diseases as hepatic failure, diabetes mellitus, renal failure, atherosclerosis, inflammation, cancer, etc. (Bulkley 1983, Halliwell and Gutteridge 1993, Niki 1995 and Frei 1999).

Herbal plants, vegetables and fruits possess the antioxidants such as Vitamin C, Vitamin E, flavonoids, polyphenol, phenolics, tannins and proanthocyanidins. So the consumption of antioxidant rich diet may prevent the oxidative stress induced degenerative diseases (Habib and Ibrahim 2011, Hendra *et al.* 2011 and Gulcin 2012). The present study serves as a basis for further research to isolate the bioactive compounds for discovery of new herbal drugs.

Materials and Methods

DPPH (2,2-diphenyl-1-picrylhydrazyl) was used to evaluate the free radical scavenging activity of petroleum ether, chloroform, ethyl acetate and methanol extracts of leaf, stem and root of *M. pudica* .

DPPH free radical scavenging principle

The DPPH radical has been widely used to evaluate the free radical scavenging capacity of antioxidants (Choi *et al.* 2000). DPPH free radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors. DPPH can make stable free radicals in aqueous or methanol solution.

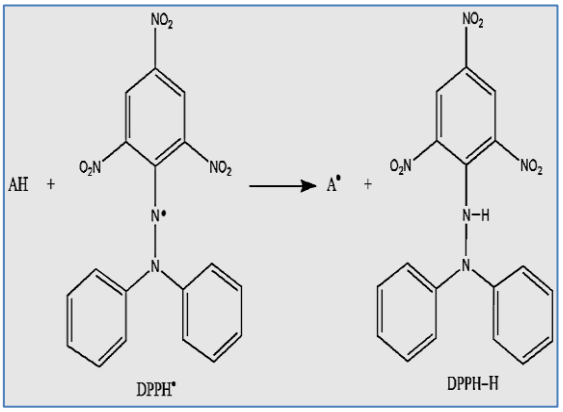


Fig 21. Principle of antioxidant molecule

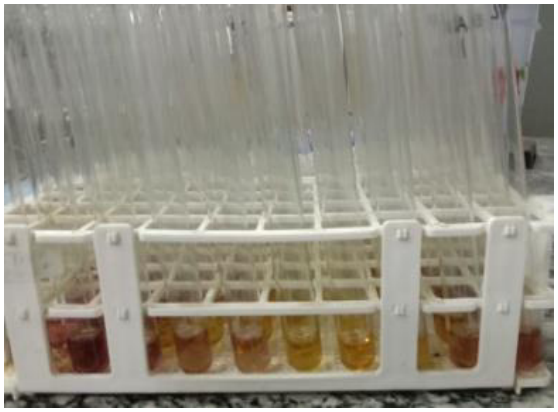


Fig 22. Antioxidant activity of *M. pudica*

By following this method, it is possible to determine the antiradical power of an antioxidant by measuring the decrease in the absorbance of DPPH at 517 nm. Resulting from a color change from purple to yellow, the absorbance decreased when the DPPH was scavenged by an antioxidant through donation of hydrogen to form a stable DPPH molecule. In the radical form, this molecule had an absorbance at 517 nm which disappeared after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule.

Test materials:

- DPPH (Aldrich, India)
- Methanol (Merck, Germany)
- Ascorbic acid
- Micropipette (10-100µl)
- Pipette (1-10ml)

- Digital Weigh machine
- UV Spectrophotometer (Shimadzu, USA)
- Test tubes (25ml)
- Conical flask
- Centrifuge machine
- Distilled H_2O

Experimental Procedure

DPPH radical scavenging activity of the extracts was measured by the method developed by Manzorro *et al*. (1998). Stock solution was made by mixing 1mg extract in 1ml methanol. 3.94 mg DPPH was added in 100 ml methanol and vortex well. Extracts and standard of each concentration (12.5, 25, 50, 100, 200 µg/ml) were mixed with 1.5 ml of DPPH solution (0.1 mM). The reaction was carried out at room temperature in a dark place for 30 minutes and the absorbance was measured at 517 nm. IC_{50} values (concentration of samples required to scavenge 50% of free radicals) were calculated from the regression equation, developed by plotting concentration of samples versus percentage inhibition of free radicals. Ascorbic acid was used as positive control.

Preparation for antioxidant activity test

At first, all equipments were washed and sterilized carefully. Different concentrations *i.e.* 12.5, 25, 50, 100, 200 µg/ml of the extracts and a standard were taken in test tubes. 1.5 ml of methanol solution of DPPH was added into each of the test tubes. The test tubes were then incubated at room temperature for 30 minutes in dark place to complete the reaction. Then the absorbances of the solutions were measured at 517 nm using a spectrophotometer against blank. Methanol was used as a blank. Control sample was prepared containing the same amount of methanol and DPPH without plant extracts and it was incubated under the same conditions as rest of the sample solution.

Reading and analysis of data for antioxidant activity test

The percentage (%) inhibition activity of DPPH was calculated from the following equation:

$$\% I = \{(A_0 - A_1)/A_0\} \text{ X } 100$$

Where, A_0 is absorbance of the control and A_1 is the absorbance of the extract/standard. The percent inhibitions were plotted against concentration and IC_{50} was calculated from graph.

Results

DPPH radical scavenging activity

The free radical scavenging capacity of different solvent extracts of leaf, stem and root of *M. pudica* were estimated using the stable DPPH (2-2-Diphenyl-1-picrylhydrazyl) radical with absorbance at 517 nm. Ethyl acetate crude extracts showed well antioxidant activity in comparison to ascorbic acid as standard.

Different solvent extracts of leaf, stem and root of *M. pudica* showed moderate to high antioxidant properties in comparison to ascorbic acid as standard. Absorbance at 517 nm was gradually decreased with higher concentrations of the samples in respect to control. It was quantitatively measured from the change in absorbance and percent of scavenging activity was calculated. The activity was increased by increment of the concentration of the extracts. All the extracts of leaf, stem and root of *M. pudica* possessed significant DPPH free radical scavenging activity.

Table 28. DPPH free radical scavenging activity of different extracts of leaf of *M. pudica* and Ascorbic acid as standard at different concentrations

Concentrations (µg/ml)	Scavenging (%)				
	Petroleum ether	Chloroform	Ethyl acetate	Methanol	Ascorbic acid
12.5	7.45	9.67	41.29	26.69	45.41
25.0	16.92	22.66	50.45	38.97	72.47
50.0	32.23	36.96	63.75	53.17	88.96
100.0	48.74	67.88	69.39	66.97	94.25
200.0	66.67	86.40	74.62	76.03	96.11

DPPH free radical scavenging activity of different extracts of leaf and ascorbic acid as standard were shown in table 28. Among the extracts, the highest scavenging activity was found in chloroform extract at 200 µg/ml concentration that was slightly lower than that of standard ascorbic acid, and petroleum ether, ethyl acetate and methanol extracts showed moderate scavenging activity.

Table 29. DPPH free radical scavenging activity of different extracts of stem of *M. pudica* and Ascorbic acid as standard at different concentrations

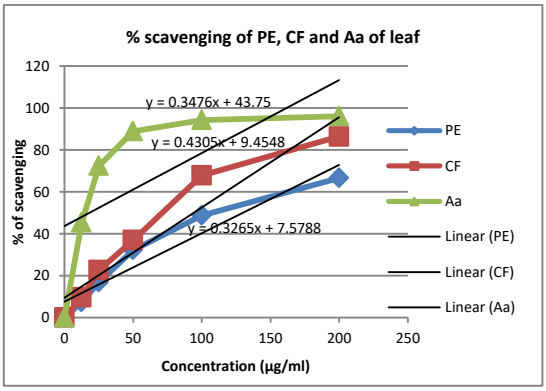
Concentrations (µg/ml)	Scavenging (%)				
	Petroleum ether	Chloroform	Ethyl acetate	Methanol	Ascorbic acid
12.5	3.02	16.82	36.15	10.07	45.41
25.0	9.06	27.09	47.73	16.31	72.47
50.0	21.95	37.76	56.29	33.53	88.96
100.0	43.00	63.24	67.27	50.55	94.25
200.0	62.24	72.61	73.72	62.24	96.11

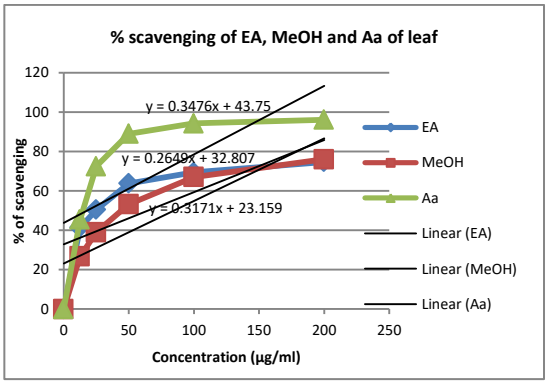
Table 29 showed the DPPH free radical scavenging activity of different extracts of stem and ascorbic acid as standard. Among the extracts, the highest scavenging activity was found in ethyl acetate extract at all concentrations and all the extracts showed moderate scavenging activity in comparison with ascorbic acid.

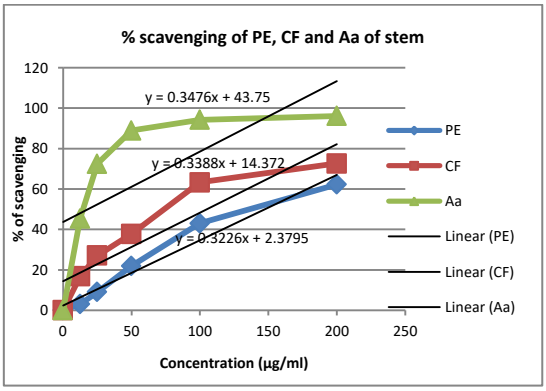
Table 30. DPPH free radical scavenging activity of different extracts of root of *M. pudica* and Ascorbic acid as standard at different concentrations

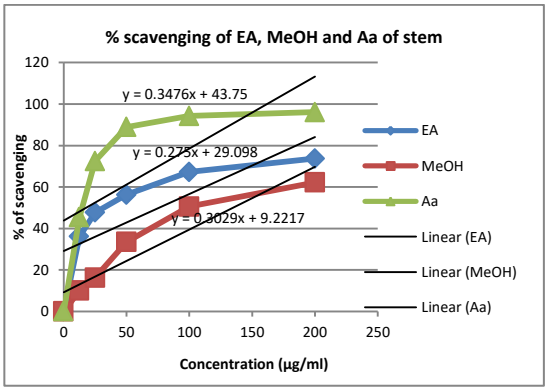
Concentrations (µg/ml)	Scavenging (%)				
	Petroleum ether	Chloroform	Ethyl acetate	Methanol	Ascorbic acid
12.5	10.57	9.06	27.09	35.95	45.41
25.0	15.11	17.02	48.74	43.50	72.47
50.0	24.17	26.38	65.56	50.76	88.96
100.0	34.84	38.17	74.22	64.35	94.25
200.0	49.35	57.40	83.79	78.95	96.11

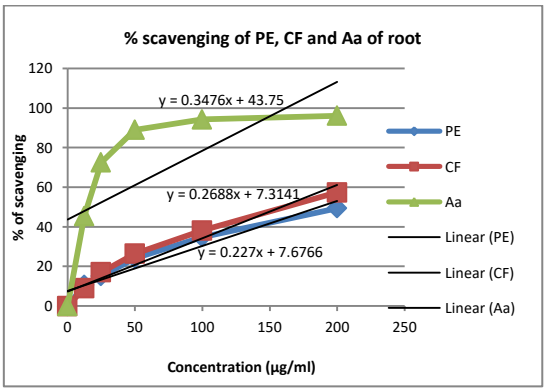
In table 30 the DPPH free radical scavenging activity of different extracts of root and ascorbic acid as standard were shown. Among the extracts, the highest scavenging activity was found in ethyl acetate extract at all concentrations which was slightly lower than that of standard ascorbic acid. On the other hand, petroleum ether and chloroform extracts showed less scavenging activity and methanol extract showed moderate scavenging activity.











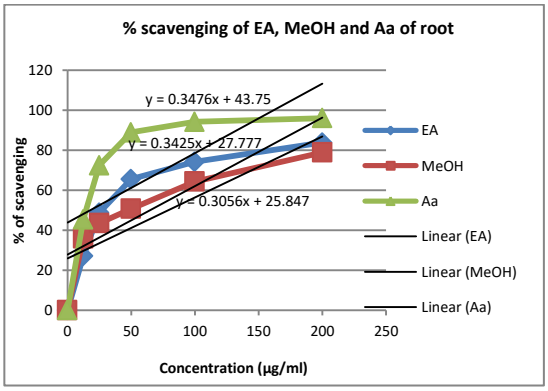


Fig 23. Regression lines between concentration and % of scavenging of different solvent extracts of *M. pudica* and the standard for DPPH free radical scavenging activity.

PE- Petroleum ether
CF- Chloroform
EA- Ethyl acetate
MeOH- Methanol
Aa- Ascorbic acid as Standard

Table 31. IC_{50} values of of different extracts of leaf, stem and root of *M. pudica* and Ascorbic acid as standard

Extracts	IC_{50} values (µg/ml)				
	Petroleum ether	Chloroform	Ethyl acetate	Methanol	Ascorbic acid
Leaf	130.129	94.293	65.152	84.700	
Stem	147.891	105.414	76.036	135.030	18.012
Root	186.449	159.276	65.000	79.213	

From table 31, the ethyl acetate extracts showed the highest activity where the IC_{50} values were 65.152, 76.036 and 65.000 µg/ml and the lowest activity was found in petroleum ether extracts where the IC_{50} values were 130.129, 147.891 and 186.449 µg/ml for leaf, stem and root respectively. The IC_{50} value of the standard ascorbic acid was found 18.012 µg/ml.

Discussion

In the present study the antioxidant activity of different solvent extracts of *M. pudica* was evaluated at different concentrations. Among the extracts, the highest activity or the lowest IC_{50} value was observed in ethyl acetate extracts and the lowest activity or the highest IC_{50} value was observed in petroleum ether extracts of leaf, stem and root. But in respect of scavenging activity, the highest percentage was found in chloroform extract of leaf (86.40%) at 200 µg/ml concentration.

The chloroform extract obtained from *M. pudica* have significant antioxidant activity and the antioxidant potential might be due to the phytoconstituents like alkaloids, glycosides, flavonoids, steroids and phenolic compounds. The reducing power of *M. pudica* leaf extracts was very potent and the power increased with quantity of sample (Rajendran *et al.* 2010). The chloroform extract not only scavenged off free radicals but also inhibited the generation of free radicals. The present study showed more or less similar results.

DPPH free radical scavenging assay was carried out by Tunna *et al.* (2015) to determine the free radical scavenging potential of the extracts of aerial parts of *M. pudica* against DPPH free radical. The initial ethanol extract (mother extract) and subsequent subfractions (hexane, ethyl acetate, acetone and methanol) were evaluated for their free radical scavenging activity. In their study they found that the methanol extract (initial or mother extract) possessed the lowest IC_{50} value (7.18±0.0005) for DPPH free radical scavenging assay; and the lower the IC_{50} value the stronger the free radical scavenger. The hexane fraction showed a higher IC_{50} value for DPPH assay but

a weaker antioxidant activity of 92.302±0.0077 which might be attributed to the fraction for the presence of dodecarboranes in the GC results which might be the cause for radical scavenging activity. This fact could also be attributed to hexane fraction supposedly contains mostly fatty acids, lower hydrocarbons and wax of the plant and some small phenolic acids might had been extracted. The fatty acids were reported to show the free radical scavenging activity in some studies (Hayouni *et al.* 2007). In the present investigation, the lowest IC_{50} values were observed in ethyl acetate extracts of leaf, stem and root and the IC_{50} values of DPPH radical scavenging activity could be arranged as ethyl acetate > methanol > chloroform > petroleum ether for leaf and root, and ethyl acetate > chloroform > methanol > petroleum ether for stem.

The methanol crude extract of aerial part of *M. pudica* showed moderate antioxidant activity compared to ascorbic acid as standard where the IC_{50} values were 296.62 µg/ml and 131.29 µg/ml for methanol extract of aerial part and ascorbic acid respectively against DPPH free radical scavenging assay (Chowdhury *et al.* 2008). The present study also showed moderate antioxidant activity for methanol crude extracts of leaf and stem *i.e.* the aerial parts of the plant in comparison to ascorbic acid where the IC_{50} values were 84.700 µg/ml, 135.030 µg/ml and 18.012 µg/ml for methanol extracts of leaf, stem and ascorbic acid respectively.

Das *et al.* (2014) depicted that the IC_{50} values of methanolic leaf extract of *M. pudica* through DPPH free radical scavenging assay was 126.71 µg/ml and that of for ascorbic acid was 20.13 µg/ml. They mentioned that the DPPH reading was very close to ascorbic acid as the leaf extract of the plant was in crude form. These findings were nearly close to the present result where the IC_{50} values of DPPH free radical scavenging activity of methanolic leaf extract and ascorbic acid were 84.700 µg/ml and 18.012 µg/ml respectively. But Arokiyaraj *et al.* (2012) showed somewhat different result; where they mentioned that the methanolic extract of leaf of *M. pudica* had a significant free radical scavenging activity generated by DPPH where the IC_{50} was 9.0 mg/ml.

Almalki (2016) denoted that the hexane extract of leaves of *M. pudica* showed a significant scavenging effect on DPPH free radical (IC_{50} 20.83 mM) and vitamin C (22.6 mM). The leaves of *M. pudica* were good antioxidant agent and it was predicted that the hexane extract contained the phytochemicals which could be exhibiting the scavenging activity for maintaining the redox level. But in the present study the petroleum ether extract of leaf showed a moderate DPPH scavenging effect where the IC_{50} was 130.129 µg/ml and that of ascorbic acid was 18.012 µg/ml.

Chapter Six

PHYTOCHEMICAL CONSTITUENTS OF *MIMOSA PUDICA*

Introduction

Plants have been using as food as well as medicine with pharmacologically active compounds. The medicinal properties of the plants are being investigated at present throughout the world due to their potent antioxidant activity, therapeutic efficiency, economic importance and no side effects. Medicinal plants are using as raw materials for manufacturing drugs and also for synthesizing phytochemicals which are beneficial for health and are not synthesized in human body (Martinez *et al.* 2008). Alkaloids, flavonoids, tannins and phenolic compounds are the most important bioactive constituents of plants (Doss 2009). Awareness is growing with phytochemicals to correlate with their biological activities (Summer *et al.* 2003, Fernie *et al.* 2004 and Robertson 2005).

To discover the therapeutic agents from plants, knowledge of chemical constituents of plants is necessary and such information has a great value in disclosing new sources of phytocompounds to synthesis complex chemical substances and also to discover the significance of folkloric remedies (Milne 1993). Gas chromatography coupled with mass spectrometry is used for direct analysis of compounds in traditional medicinal plants.

M. pudica contains phenolic compounds, nitrogen-containing compounds, rich source of flavonoids, sitosterols and fatty compounds (Kaur *et al.* 2011). It is famous for anticancer alkaloid mimosine (Renz and Mimosine 1936) and includes 5-deoxyflavonols. A large number of phytoconstituents were reported from root, stem, leaf, seed whole aerial parts of the plant (Choudhary and Chakrabarti 1980, Chatterjee and Pakrashi 2006, Yuan *et al.* 2006 and Jha 2007).

Phytochemistry is mainly devoted to the study of physiologically or biologically active constituents obtained from plants. It is an interdisciplinary science that encompasses a broad range of studies. To identify the phytoconstituents, several new techniques have been introduced during the past decades, leading to acceleration and simplification of different separation problems (Hostettmann *et al.* 1991 & 1998 and Marston and Hostettmann 1991). Though, there is no universal technique to solve the isolation problems, the best results are often obtained by a combination of two or more of these

techniques. All methods have advantages and limitations. Among the important preparative separation techniques employed in the isolation and purification of plant constituents, thin layer chromatography and open column chromatography have been used simultaneously. Thin layer chromatography is used to select the slurry or the solvent system for the successful run of the open column chromatography.

Technique such as Gas chromatography coupled with mass spectrometry is used for direct analysis of phytoconstituents of medicinal plants. Recently, emphasis have been given to GC-MS technique for the analysis of phytocompounds of medicinal plants as the technique has proved to be a valuable method for analysis of fatty acids, alkaloids, lipids, terpenoids, steroids, volatile essential oils, non-polar components, etc. using a few grams of plant extracts (Jie and Choi 1991, Betz 1997 and Sermakkani and Thangapandian 2012).

The availability and choice of chromatographic techniques are essential for the successful investigation of biologically active plant constituents to separate the pure substances from the desired plants. As the preparative separation techniques may be tedious, the aim is to have the maximum yield with minimum effort to reduce the time and cost of the separation procedure.

Materials and Methods

Collection of sample plants

M. pudica was collected from the Botanical Garden of Rajshahi University Campus on November 2019. In the laboratory the soil or sand particles of the plants were cleaned and the three parts *i.e.* leaf, stem and root were separated of the plants. The petioles were separated from the rachis and the stem and root were chopped into small pieces. Then the leaf, stem and root were dried in room temperature keeping them into wooden trays. Drying of the plant parts was carried out under shed to prevent the changes of the constituents in it due to drying. After drying they were grinded into dust with the pulverizer.

Extraction and Isolation procedure

The dusts of the plant parts were used for extraction through the following scheme. The method employed mainly for the isolation of chemical constituents based on fractioned by solvents of varying polarity. After extraction with different solvents the fractions

were subjected to separation by different techniques as thin layer chromatography, open column chromatography, gas chromatography-mass spectrometry, etc. to yield the pure compounds (Harbone 1984, Mann *et al.* 1994 and Houghton and Raman 1998).

Fig 24. Separation of leaf from stem of *M. pudica*

Fig 25. Grinding for dust

Fig 26. Dust of leaf of *M. pudica*

Fig 27. Extracts of different solvents of *M. pudica*

Cold extraction

The extraction procedure was adopted from Alam *et al.* (2002). The collected whole plant materials had a weight of about 5.5kg. After drying and grinding of the leaf, the total weight of the powdered material (leaf) was 650g. That amount was taken in an amber colored extraction bottle and soaked in 100% methanol (2.0L x 3 times). The bottle was kept first time for 7 days with occasional shaking and stirring and also submitted to ultrasonic agitation for an hour daily in a sonicator (Power Sonic 510).

The organic phase or the supernatant was then filtered separately through cotton followed by Double Rings Filter paper No. 102 and collected in a beaker. Second time the bottle was kept for 5 days and third time for 3 days with occasional shaking and stirring and also ultrasonic agitation daily in a sonicator and the filtered mixture was collected in the same beaker. Then the extraction was kept open in room temperature, aeration by an aerator for evaporation of the solvent to afford crude extract (32g).

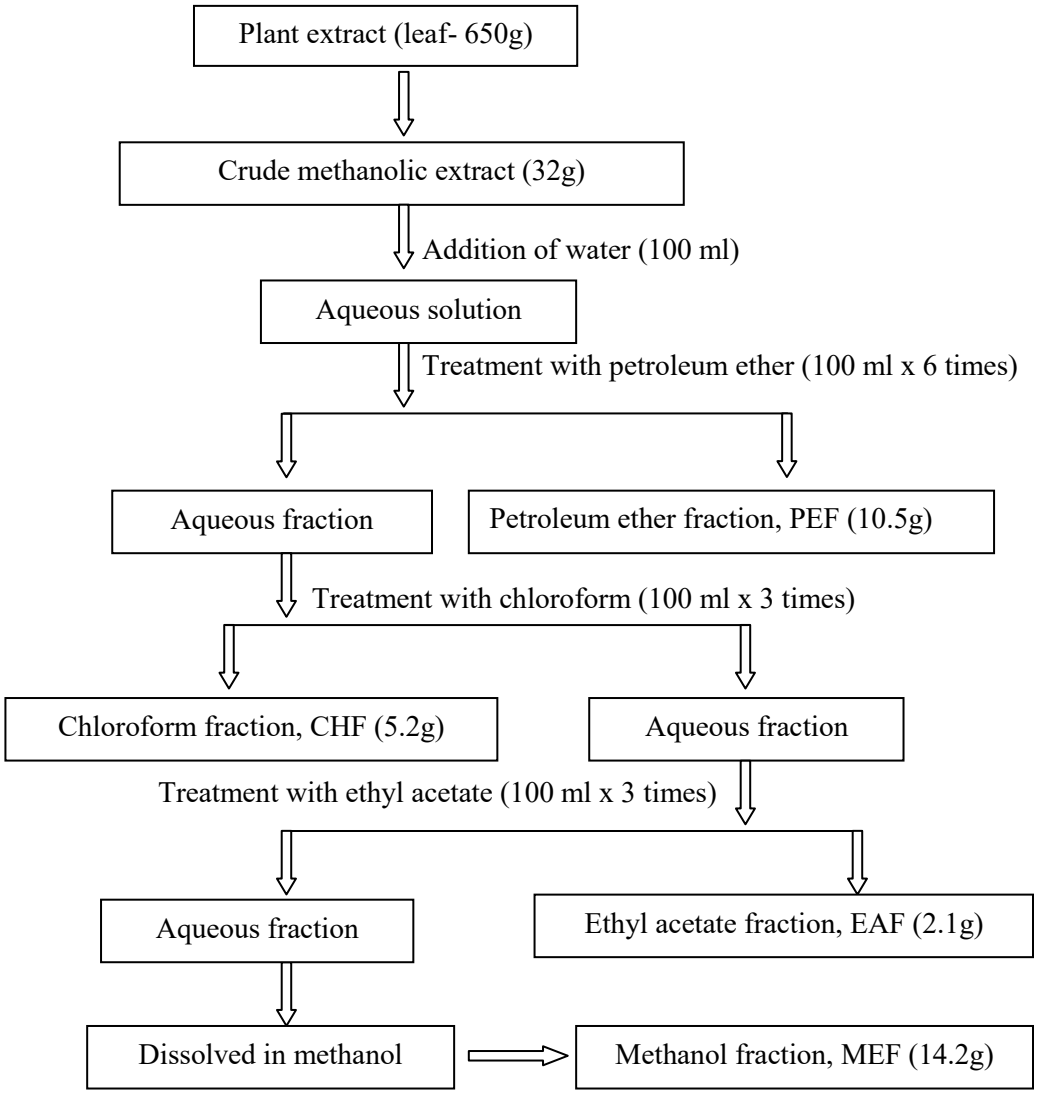


Fig 28. Schematic representation of solvent-solvent partitioning of crude methanolic extract of *M. pudica*

Solvent-solvent partitioning of crude extracts

Solvent-solvent partitioning of extracts was done using the protocol designed by Kupchan and modified by Wagenen *et al.* (1993).

Extraction with petroleum ether

The crude methanolic extract was made slurry with water (100 ml) and taken in a separating funnel of 500 ml. Then 100 ml petroleum ether was added with that aqueous methanolic solution and shaken well. The mixture was kept untouched for some time until the layers were separated. The upper organic layer was then collected and the process was repeated five times. The collected combined petroleum ether extract was filtered and allowed the solvent to evaporate off in room temperature. The dried extract afforded a bluish-black colored oily mass (10.5g).

Extraction with chloroform

After extraction of petroleum ether fraction, the aqueous fraction was treated with chloroform (100 ml) and shaken well. When the layers were separated the lower organic layer was collected in a beaker. The process was repeated twice. The combined chloroform extract was filtered and evaporated off the solvent in normal room temperature. The dried, concentrated extract obtained a coffee-colored mass (5.2g).

Fig 29. Filtering for extracts of *M. pudica*

Fig 30. Solvent-solvent partitioning of extracts

Extraction with ethyl acetate

After extraction of chloroform fraction, the aqueous fraction was again extracted with ethyl acetate (100 ml x 3 times) and shaken well. The combined ethyl acetate extract was collected, filtered and evaporated off the solvent. The dried extract obtained a coffee-colored mass (2.1g).

Extraction with methanol

Finally the left aqueous fraction was dissolved in methanol and the combined methanol extract was evaporated off, filtered and dried to obtain a blackish mass (14.2g).

The same procedures were followed to yield the extracts of stem and root of *M. pudica.* All the output extracts were removed to glass vials and preserved in refrigerator at 4^0C with proper labeling for future experiments.

Identification of bioactive constituents by GC-MS

The GC-MS analysis of the plant extract was made in a Shimadzu QP 2020 (Japan) instrument. About 1µL of the methanol extract was injected into the GC-MS using a micro syringe and the scanning was done for 55 minutes.

Before analyzing the compounds using Gas Chromatography electron ionization and Mass Spectroscopy (GC-MS), the temperature of the oven, the flow rate of the gas used and the electron gun, etc. were programmed initially. The injection port temperature was set at 220^0C and the initial oven temperature was set at 80^0C for two minutes and then gradually increased to 280^0C (8 min hold) at a flow rate of 6^0C/min. Helium gas was used as a carrier as well as an eluent and the flow rate of helium was set to 1ml per minute. Separation was carried out using a HP-5MS capillary column (30 meter, 0.25mm ID, 0.25µm df) and spectra were obtained over m/z 45-350. The electron gun of mass detector liberated electrons having energy of about 70eV. The split sampling technique was used to inject the sample in a ratio of 50.0.

GC-MS analysis is a common confirmation test. It is best used to make an effective chemical analysis. This analysis provides a representative spectral output of all the compounds that get separated from the sample. The injection of the sample to the injected port of the GC device is the first step of GC-MS method. The GC-MS instrument vaporizes the sample and then separates and analyzes of the various components. Each component ideally produces a specific spectral peak that may be

recorded on a paper chart electronically. The time elapsed between elution and injection is called retention time. Differentiation among the compounds is identified using the retention time. The peak is measured from the base to the tip of the peak.

Retention indices (RI) of the compounds are determined by comparing the retention time of a series and identification of each component is confirmed by comparison of its RI with data in the literature. Interpretation of mass spectrum is carried out by using the database of National Institute of Standards and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown components is compared with the spectrum of known components which is stored in the NIST library. The name, molecular weight, chemical structure and molecular formula of the components of the test materials are ascertained.

Fig 31. Gas Chromatograph Mass Spectrometer

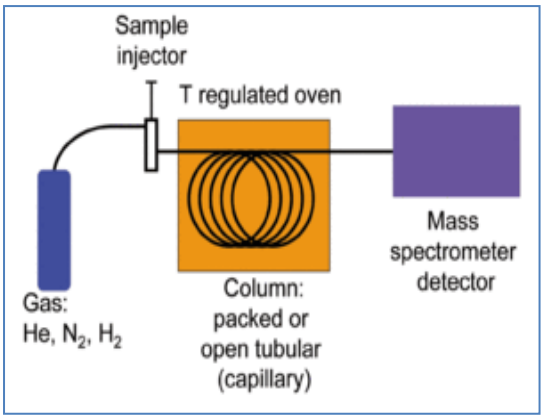


Fig 32. GC-MS schematic

Isolation and purification of compounds

The pure compounds are isolated from the fractioned chloroform and petroleum ether extracts of leaf of *M. pudica* applying different chromatographic methods and other techniques that are described below.

Chromatography

The most commonly used and convenient method for the separation, isolation and purification of the compounds from the extracts is the chromatography. The method involves the principle of selective distribution (partition and adsorption) of components of a mixture between two phases *i.e.* a stationary phase and a mobile phase. The compounds are separated with this technique on the basis of differences in affinity for stationary phase and a mobile phase.

Isolation of pure compounds is done mainly by open column chromatography (OCC); while thin layer chromatography (TLC) is used as a supporting tool. The selection of the test extract for the isolation is done depending on their biological activities.

The different chromatographic techniques that are followed for the identification of pure compounds in this study are described below:

A. Thin Layer Chromatography (TLC)
B. Column Chromatography (CC)

A. Thin Layer Chromatography (TLC)

Thin layer chromatography is a simple, inexpensive and quick procedure that gives the chemist a quick answer as to how many components are in a mixture. TLC is also used to support the identity of a compound in a mixture, when the R_f value of a compound is compared with the R_f of a known compound. TLC plate is a sheet of metal, plastic or glass which is coated with a thin layer of a solid adsorbent usually silica or alumina. A small amount of the mixture is spotted near the bottom of the plate which is to be analyzed. The plate is then placed longitudinally in a shallow pool of a solvent in a developing chamber so that only the very bottom of the plate is in liquid. The liquid or the eluent is the mobile phase and it slowly rises up the TLC plate by capillary action. As the solvent moves part the spot that was applied, equilibrium is established for each constituents of the mixture between the molecules of that constituent which are adsorbed on the solid and the molecules which are in solution. In principle the constituents will differ in solubility and in the strength of their adsorption to the adsorbent and some components will be carried farther up the plate than others.

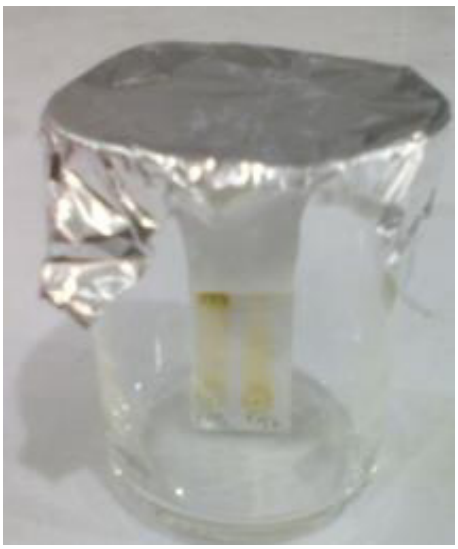
TLC plates for the separation of compounds

Thin layer chromatographic plates were used to select the solvent system for the run of an open column separation technique. Aluminum backed pre-coated preparative thin layer chromatographic (TLC) plates (20 x 20cm) with silica gel 60 GF_{254} with 0.5mm thickness and active in the usual manner (Merck, Germany) were used in this regard. The sample was applied on the active plates with the help of a gradient micropipette as a narrow band at 1cm above the lower edge of the plate to make sure that the sample was not washed away when the plates were placed inside the TLC chamber with the solvent system. The plates were then developed in the usual manner. A concentration of

10mg/ml of the sample in the solvent of extraction offered 100µg/spot when 10µl for each of the samples spotted. The chromatograms then developed within a conventional chamber (Camag) using the following solvent systems:

Preparation of TLC Tank

To develop the chromatoplates, cylindrical glass chamber or TLC tank with airtight lid is used. The selected solvent system (10 ml) is poured into the tank and a smooth sheet of filter paper is laid and allowed to soak in the solvent. This filter paper helps to facilitate the process of saturation. The chamber is then made airtight and kept for few minutes to saturate the internal atmosphere with solvent vapor.



TLC chamber

Plate under UV light

After iodine vapor

Chromatograms of compounds

Column run of the extracts

Fig 33. TLC chamber, developed chromatogram and column run of the extracts

Sample applying

According to Harborne (1980), a small amount of dried sample is dissolved in a suitable solvent to get a solution of approx. 1%. The solution (1-5µg/µl) is applied as a small spot or band on the activated silica plate with a capillary tube (spotter) just 2 cm above the lower edge of the plate. The spot is dried by blowing the air before developing the plates. A straight line is drawn 2 cm below the upper edge of the activated plate that marks the upper limit of the solvent flow.

Development of the plate

Spotted plate is placed in the tank in such a way as to keep the applied spot above the surface of the solvent system and the cap is placed again. The plate is left for development and when the solvent front reaches up to a certain limit, the plate is taken out and air dried (Harborne 1976 and Touchstone and Dobbins 1978). The properly developed plates are viewed under UV light of various wave lengths, kept in iodine chamber and treated with suitable spray (*viz.* Godin and after Godin reagents) in order to detect the different classes of compounds.

B. Column Chromatography (CC)

Column chromatography is the most commonly used separation technique based on the principle of distribution (partition/adsorption) of compounds between a stationary phase and a mobile phase. The stationary phase is a macromolecule and the mobile phase is the selected solvent(s).

A normal chromatographic column is packed with silica gel 60 F_{254} (Kiesel gel 60), the most commonly used stationary phase. Silica gel is suspended in a suitable solvent or solvent mixture. A finely cleaned glass column, typically 25-100 cm in length and 2.5-7.5 cm in diameter (although, many longer and wider columns are used), is fitted with a stopcock in order to control the flow rate of the mobile phase. The packing material is supported inside the column by means of a piece of glass wool and fitted with a strand. The suspended silica is then poured into the column of appropriate height and diameter. When a desired height of adsorbent bed is obtained, few hundred milliliters of solvent/solvent mixture is allowed to run for proper packing of the column. Care must be taken so that the solvent sufficiently cover the adsorption bed to avoid cracking. After packing the column the sample can be applied in two ways:

i. Dry column
ii. Wet column

i. Dry column

Dry column preparation becomes necessary when the extract is sticky or gummy in nature. The extract is ground finely with an optimum amount of silica gel and mixed well using mortar and pestle in order to obtain a non-stick free flowing mass. The amount thus obtained is poured on the packed column carefully in such a way that upper layer of the bed is not disturbed.

ii. Wet column

In this case the sample is applied to the packed column as a concentrated solution in a suitable solvent.

After application of the sample by either of the above ways different solvents/ solvent systems are passed through the column with progressively increased polarity. The flow is maintained at a constant rate (2 ml per minute) in order to better separation. Elutes are collected in a volume either in test tubes or in conical flasks.

Detection of Compounds

Different methods are applied for the detection of compounds resolved on the development chromatogram.

i. Visual detection

The developed chromatogram is examined visually to detect the presence of color compounds.

ii. UV light

The developed and dried plates are observed under UV light (254 and 366 nm) to locate UV absorbing/quenching compounds.

iii. Iodine vapor

The developed chromatogram is placed in a closed jar containing crystals of iodine and kept for few minutes. The compounds, which appeared as brown spots, are marked. Unsaturated compounds absorbed iodine. Bound iodine is removed from the plate by air blowing.

iv. TLC spray reagents (Stahl 1969)

Different types of spray reagents are used in the chemical study of plates depending upon the nature of the compounds expected to be present in the fractions or in the crude extracts. According to Godin (1954) the spraying reagent is as follows:

Godin-A	: 1% ethanolic solution of vanillin (1g vanillin + 125.32 ml ethanol)
Godin-B	: 3% aqueous solution of perchloric acid (3 ml perchloric acid + 97 ml Distilled H_2O)
Godin	: Equal mixture of Godin-A and Godin-B
After Godin	: 10% ethanolic solution of H_2SO_4
Treatment	: The chromatogram was sprayed with Godin and heating by hair dryer at 60-80^{0}C. Further was sprayed with after Godin and then heating by hair dryer at 60-80^{0}C until the spots attain maximum intensity.

Determination of R_f value (Retention Factor)

The retention factor or R_f is defined as the distance traveled by the compound divided by the distance travelled by the solvent (described in CU Boulder Organic Chemistry Undergraduate Courses). The R_f values were calculated by the following formula:

$$R_f = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent}}$$

The R_f value for a compound is a constant from one experiment to the next only if the chromatographic conditions below are also constant:

- Solvent system
- Amount of material spotted
- Adsorbent
- Thickness of adsorbent
- Temperature

Revelation of compounds on TLC by reagent spray

The Godin reagent (Godin 1954) is the mixture of the equal volume of 1% ethanolic solution of vanillin and 3% aqueous solution of perchloric acid. After spraying the reagent on the dried TLC 10% ethanolic solution of H_2SO_4 is also applied in the same way before drying the plate at 60-80^{0}C to reveal the compound spots in different colors.

The properly developed plates were dried and viewed visually after Godin reagent spray. The developed chromatogram was examined carefully to find different bands on the basis of the differences in colors and concentrations of substances in each of the bands. Thus several compounds were detected. The R_f values of the separated compounds were calculated on a developed chromatogram using the pre-established solvent system.

Characterization of isolated compounds

Physical properties as well as spectral data are fully studied to characterize the isolated compounds.

Physical Characterization

Physical properties of the isolated compounds such as physical form, color, odor, R_f value, solubility, melting point, etc. are observed carefully. The solubility studies of the compounds are performed by adding different solvents (2 ml) to 1 mg of the dried compounds in the glass vials and shaking well to allow thorough mixing. The change in the solid form represented the extent of solubility.

Spectroscopic Characterization

GC-MS (Gas Chromatography and Mass Spectroscopy) technique was used for spectroscopic characterization of the compounds.

GC-MS is a technique which is extensively employed for chemical profiling of plant extracts and its components, essential oils, fatty acids, bioactives, etc. The use of this technique in detection of phenolic acid, flavonoids and polyphenolic compounds is still a new prospect as they have high molecular weight and high vaporizing points which make their detection much difficult. However, in order to reduce the polarity and to make the compounds more prone to fragmentation, the derivetisation is done to overcome this cumbersome problem.

GC is a type of chromatography where as the mobile phase is a carrier gas, usually an inert gas such as helium or an un-reactive gas such as nitrogen and the stationary phase is a microscopic layer of liquid or polymer on an inert solid support coated with a non-volatile liquid. By the stream of helium gas the sample is swept through the column. The components in a sample are separated from each other as some of the components take longer to pass through the column than others. MS is a detector for the GC.

As the sample exits from the end of the GC column, it is fragmented by ionization and the fragments are stored by mass to form a fragmentation pattern. Like the retention time, the fragmentation pattern for a given component of a sample is unique and therefore is an identifying characteristic of that component. It is so specific that it is often referred to as a molecular fingerprint. GC-MS is an analytical method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample. GC can separate volatile and semi-volatile compounds with great resolution, but it cannot identify them. MS can provide detailed structural information on most compounds such that they can be exactly identified, but it cannot readily separate them. It is used to analyze complex organic and biochemical mixtures (Skoog *et al.* 2007).

Results

Gas Chromatography-Mass Spectrometry analysis of the samples

GC-MS analysis was carried out for eight crude extracts in different solvents of leaf, stem and root of the sample plant *M. pudica.* Those were petroleum ether extract of leaf (PE/L), chloroform extract of leaf (CF/L), ethyl acetate extract of leaf (EA/L), chloroform extract of stem (CF/S), ethyl acetate extract of stem (EA/S), petroleum ether extract of root (PE/R), chloroform extract of root (CF/R) and ethyl acetate extract of root (EA/R).

Petroleum ether extract of leaf

Petroleum ether extract of leaf was analyzed by GC-MS method and was identified sixteen compounds. The major compounds were benzene,1-ethyl-3-methyl- (14.830%), mesitylene (13.267%), vitamin E (13.117%), nonadecane (7.248%), tetratetracontane (6.486%), benzene,1-ethyl-2-methyl- (6.305%) and benzene,1,2,4-trimethyl (5.649%).

Chloroform extract of leaf

The GC-MS analysis of chloroform extract of leaf showed eleven compounds of which the major compounds were fumaric acid, ethyl 2-methylallyl ester (16.959%), 1-(2-[3-(2-acetyloxiran-2-yl)-1,1-dimethyl)propyl]cycloprop-2-enyl)ethanone (15.829%), 1H-

pyrrole-2,5-dione, 3-ethyl-4-methyl- (14.966%), 2(4H)-benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)- (12.960%) and 6-hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one (7.907%).

Ethyl acetate extract of leaf

Three compounds were identified from ethyl acetate extract of leaf through GC-MS analysis, and those were glycerin (74.711%), 9-octadecenamide (15.297%) and hexadecenoic acid, methyl ester (9.989%).

Chloroform extract of stem

GC-MS analysis of chloroform extract of stem showed 26 compounds. Among them tigloidine (80.902%) was the major compound.

Ethyl acetate extract of stem

Three compounds were identified by the GC-MS analysis of ethyl acetate extract of stem. Those were hexadecanoic acid, methyl ester (69.947%), 9-octadecenamide (15.620%) and 9-octadecenoic acid (Z)-, methyl ester (14.433%).

Petroleum ether extract of root

Thirty seven compounds were identified through GC-MS method for petroleum ether extract of root. The major compounds were identified as hexadecanoic acid, methyl ester (30.255%), methyl stearate (10.175%), 9,12-octadecadienoic acid, methyl ester (9.674%), 9-octadecenoic acid, methyl ester (E)- (7.416%), squalene (6.661%) and dl-alpha-tocopherol (6.302%).

Chloroform extract of root

The GC-MS analysis of chloroform extract of root showed forty compounds; among them tigloidine (56.694%) was the major compound.

Ethyl acetate extract of root

GC-MS analysis was carried out for ethyl acetate extract of root and identified four compounds. Among them the major compound was hexadecanoic acid, methyl ester (46.343%). The other compounds were diisooctyl phthalate (24.408%), 13-docosenamide, (Z)- (21.384%) and 9-octadecenoic acid, (Z)-, methyl ester (7.864%).

Sample Information

Analyzed : 6/7/2020 10:37:01 AM

Sample Name : Petroleum ether extract of leaf (PE/L)

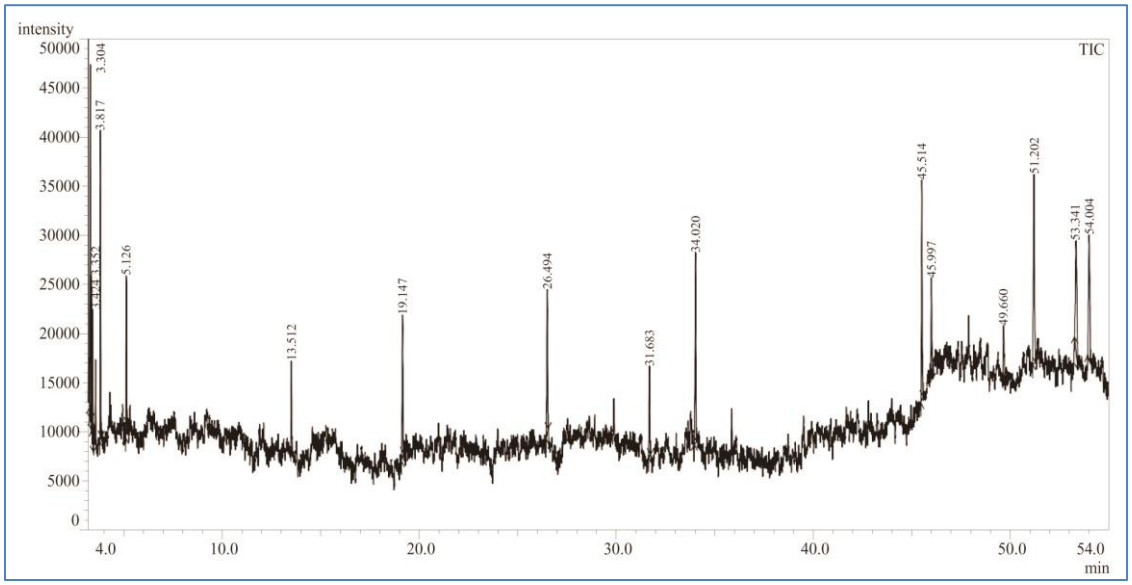


Fig 34. Mass spectrum graph of petroleum ether extract of leaf

Table 32. Quantitative result of petroleum ether extract of leaf

ID #	Name	R.Time	Area	Height	Conc.	Conc.Unit
1	Benzene, 1-ethyl-3-methyl-	3.305	22980	12893	14.830	%
2	Benzene, 1-ethyl-2-methyl-	3.353	9770	5601	6.305	%
3	Benzene, 1,2,4-trimethyl-	3.423	8754	5202	5.649	%
4	Mesitylene	3.817	20558	11526	13.267	%
5	Aniline, N-methyl-	5.127	7733	3746	4.990	%
6	Hexadecane	13.513	5307	2303	3.425	%
7	Nonadecane	19.150	11231	3694	7.248	%
8	Tetratetracontane	26.494	10051	3531	6.486	%
9	10-Methylnonadecane	31.676	5625	2272	3.630	%
10	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	34.022	5159	1755	3.329	%
11	13-Docosenamide, (Z)-	45.514	7337	2945	4.735	%
12	Squalene	45.999	5709	2310	3.684	%
13	.gamma.-Tocopherol	49.664	4394	1291	2.836	%
14	Vitamin E	51.200	20325	5154	13.117	%
15	.beta.-Sitosterol	53.346	3498	531	2.257	%
16	Stigmasterol	54.006	6524	1154	4.210	%

Sample Information

Analyzed : 6/7/2020 11:38:33 AM

Sample Name : Chloroform extract of leaf (CF/L)

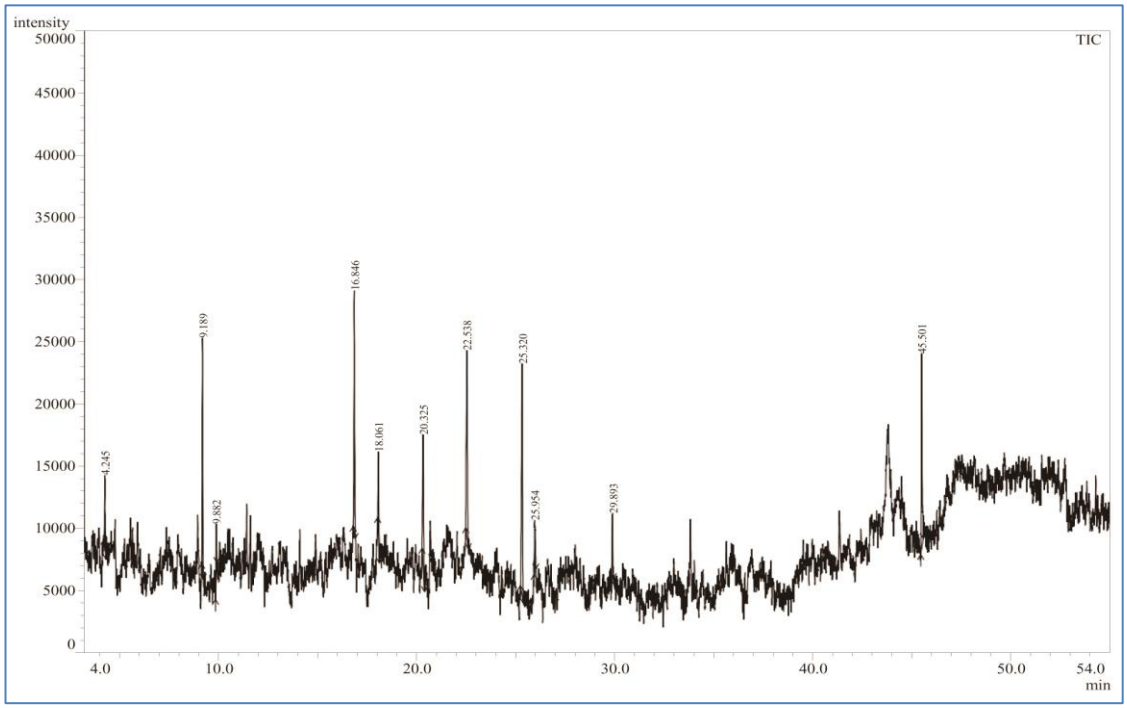


Fig 35. Mass spectrum graph of chloroform extract of leaf

Table 33. Quantitative result of chloroform extract of leaf

ID#	Name	R.Time	Area	Height	Conc.	Conc.Unit
1	Allyl(methoxy)dimethylsilane	4.247	3091	1551	3.808	%
2	1H-Pyrrole-2,5-dione, 3-ethyl-4-methyl-	9.190	12149	4314	14.966	%
3	5-exo-Vinyl-5-endo-norbornenol	9.878	4502	1338	5.546	%
4	2(4H)-Benzofuranone,5,6,7,7\a-tetrahydro-4,	16.846	10521	3514	12.960	%
5	Fumaric acid, ethyl 2-methylallyl ester	18.058	13767	3745	16.959	%
6	3-Hydroxy-.alpha.-ionene	20.324	4519	1085	5.567	%
7	1-(2-[3-(2-Acetyloxiran-2-yl)-1,1-dimethylpr	22.523	12850	3174	15.829	%
8	6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahy	d 25.320	6419	1630	7.907	%
9	Phenethyl alcohol, 2,5-dihydroxy-.alpha.-me	25.968	4654	1018	5.733	%
10	Hexadecanoic acid, methyl ester	29.885	3862	1407	4.757	%
11	9-Octadecenamide	45.502	4844	1819	5.967	%

Sample Information

Analyzed : 6/7/2020 12:43:41 PM

Sample Name : Ethyl acetate extract of leaf (EA/L)

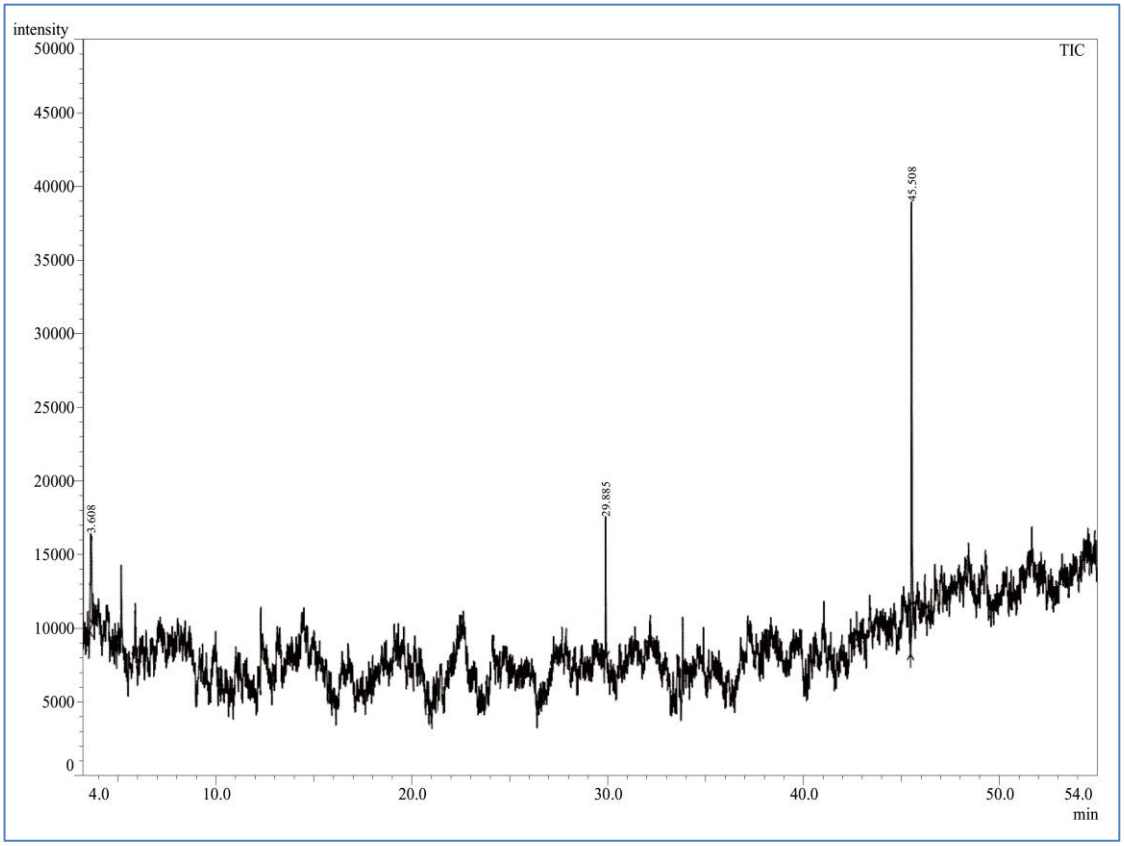


Fig 36. Mass spectrum graph of ethyl acetate extract of leaf

Table 34. Quantitative result of ethyl acetate extract of leaf

ID#	Name	R.Time	Area	Height	Conc.	Conc.Unit
1	Glycerin	3.615	39005	5321	74.714	%
2	Hexadecanoic acid, methyl ester	29.884	5215	2016	9.989	%
3	9-Octadecenamide	45.507	7986	3031	15.297	%

Sample Information

Analyzed : 6/14/2020 1:31:50 PM

Sample Name : Chloroform extract of stem (CF/S)

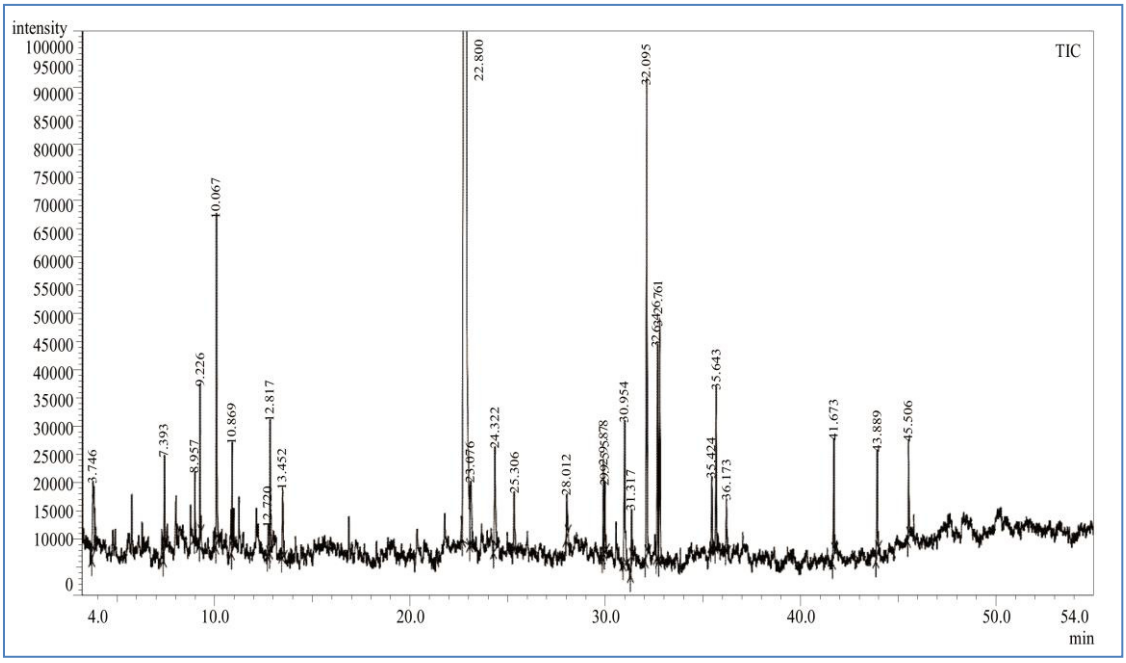


Fig 37. Mass spectrum graph of chloroform extract of stem

Table 35. Quantitative result of chloroform extract of stem

ID#	Name	R.Time	Area	Height	Conc.	Conc. Unit
1	Hexanoic acid	3.745	13626	2949	1.028	%
2	Dehydromevalonic lactone	7.392	16578	7079	1.250	%
3	L-Proline, 1-acetyl-	8.958	9913	4073	0.748	%
4	Isoneral	9.224	7509	2997	0.566	%
5	Bicyclo[3.1.1]hept-3-en-2-ol,4,6,6-trimethyl	10.066	16277	6753	1.228	%
6	2,7-Octadiene-1,6-diol, 2,6-dimethyl-	10.869	4745	2024	0.358	%
7	cis-Verbenol	12.721	1353	345	0.102	%
8	2-Cyclohexen-1-ol, 1-methyl-4-(1-methyleth	12.818	5098	1883	0.384	%
9	Vanillin	13.451	10852	2395	0.818	%
10	Tigloidine	22.800	1072712	132995	80.902	%
11	4-[3,4-Dimethoxycyclohexyl]-n-butanol	23.097	4705	1220	0.355	%
12	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyp	24.324	6863	1522	0.518	%
13	6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahy	d 25.309	3663	951	0.276	%
14	Acetic acid,10,11-dihydroxy-3,7,11-trimethy	28.011	2626	673	0.198	%
15	Bicyclo[4.2.0]octa-1,3,5-triene, 7-(3-butenyl	29.948	7441	2277	0.561	%
16	(5-Nitrohex-1-enyl)benzene	30.954	15037	3869	1.134	%
17	Acetohydrazide, 2-(2-isopropyl-5-methylphe	31.315	2722	763	0.205	%
18	Bicyclo[4.2.0]octa-1,3,5-triene, 7-(3-butenyl	32.095	43668	14490	3.293	%
19	1,5-Diphenyl-1,5-hexadiene	32.646	19515	6491	1.472	%
20	Benzeneethanol, .beta.-ethenyl-	32.763	18086	5682	1.364	%
21	3-Buten-2-ol,4-(2,6,6-trimethyl-2-cyclohexe	35.422	4904	1605	0.370	%
22	[1R-(1.alpha.,7a.beta.)]-[1-((Z)-2-Methyl-1-o	35.644	7749	2722	0.584	%
23	Heliosupine	36.171	2937	815	0.222	%
24	1,2-Benzenedicarboxylic acid, diisooctyleste	41.671	13935	5420	1.051	%
25	7-Hydroxy-3-(4-methoxyphenyl)chromen-2-	43.889	7533	2316	0.568	%
26	9-Octadecenamide	45.506	5892	2114	0.444	%

Sample Information

Analyzed : 6/14/2020 12:29:33 PM

Sample Name : Ethyl acetate extract of stem (EA/S)

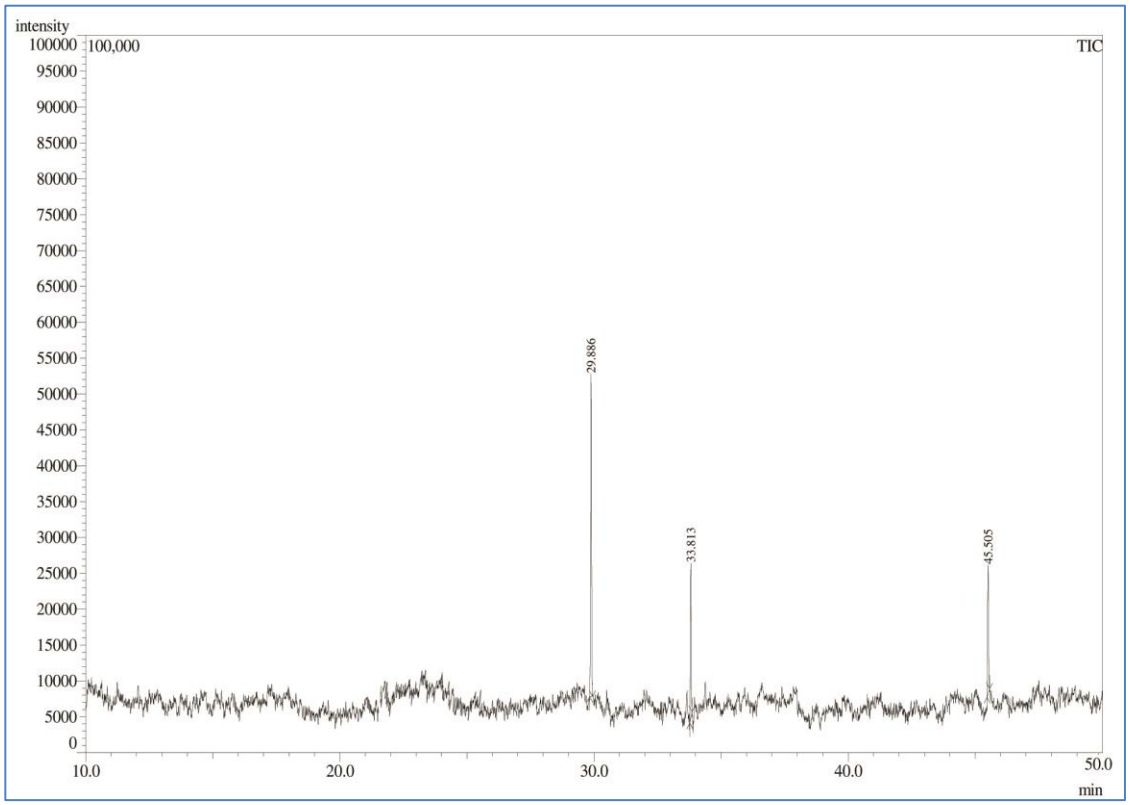


Fig 38. Mass spectrum graph of ethyl acetate extract of stem

Table 36. Quantitative result of ethyl acetate extract of stem

ID#	Name	R.Time	Area	Height	Conc.	Conc. Unit
1	Hexadecanoic acid, methyl ester	29.886	27760	10385	69.947	%
2	9-Octadecenoic acid (Z)-, methyl ester	33.813	5728	2153	14.433	%
3	9-Octadecenamide	45.505	6199	1991	15.620	%

Sample Information

Analyzed : 6/7/2020 2:46:36 PM
Sample Name : Petroleum ether extract of root (PE/R)

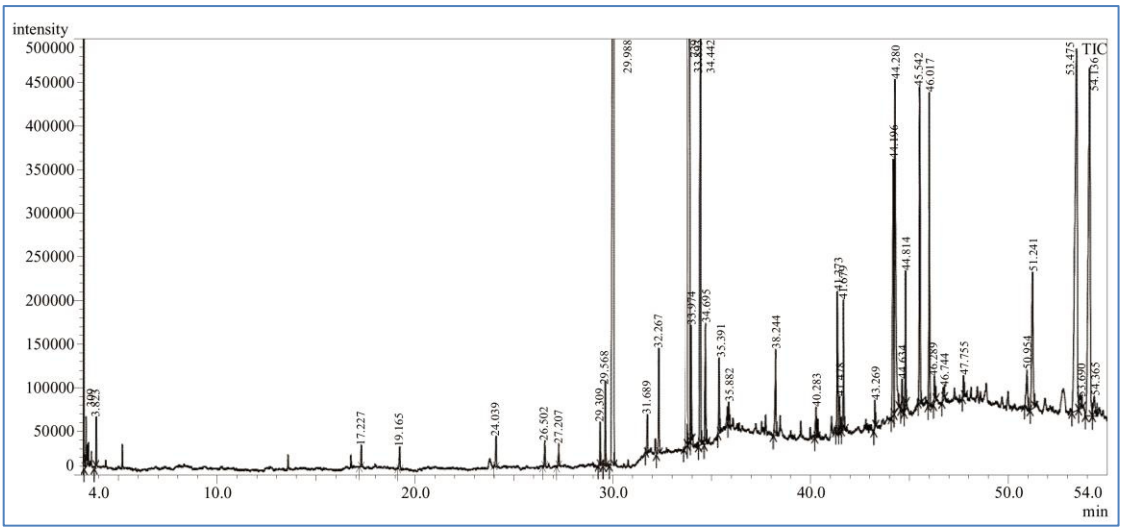


Fig 39. Mass spectrum graph of petroleum ether extract of root

Table 37. Quantitative result of petroleum ether extract of root

ID#	Name	R.Time	Area	Height	Conc.	Conc.Unit
1	Benzene, 1-ethyl-3-methyl-	3.310	33620	21985	0.806	%
2	Benzene, 1,2,3-trimethyl-	3.822	34510	20962	0.827	%
3	1H-2-Benzopyran-1-one,3,4-dihydro-8-hydr	17.227	9699	3375	0.232	%
4	Hexadecane	19.164	25892	7590	0.621	%
5	Methyl tetradecanoate	24.039	31917	9481	0.765	%
6	Heneicosane	26.505	25774	8305	0.618	%
7	Pentadecanoic acid, methyl ester	27.207	18016	6356	0.432	%
8	9-Hexadecenoic acid, methyl ester, (Z)-	29.308	15539	5110	0.372	%
9	(Z)-Methyl hexadec-11-enoate	29.569	35305	12256	0.846	%
10	Hexadecanoic acid, methyl ester	29.988	1262312	243898	30.255	%
11	Nonadecane	31.691	25752	8956	0.617	%
12	Methyl stearate	32.267	73960	27385	1.773	%
13	9,12-Octadecadienoic acid, methyl ester	33.739	403618	93762	9.674	%
14	9-Octadecenoic acid, methyl ester, (E)-	33.894	309419	73036	7.416	%
15	11-Octadecenoic acid, methyl ester	33.974	33853	13553	0.811	%
16	Methyl stearate	34.443	424509	136767	10.175	%
17	cis-Trismethoxyresveratrol	34.694	40926	15087	0.981	%
18	1-Naphthalenepropanol,.alpha.-ethenyldecah	35.391	14221	4844	0.341	%
19	Heneicosane	35.883	22420	7397	0.537	%
20	Methyl 18-methylnonadecanoate	38.245	49048	19290	1.176	%
21	Retinol, acetate	40.284	6824	2204	0.164	%
22	Hexadecanoicacid, 2-hydroxy-1-(hydroxym	41.374	32587	10947	0.781	%
23	2-Pentenoic acid, 5-(decahydro-5,5,8a-trimet	41.480	6127	1635	0.147	%
24	Triacontanoic acid, methyl ester	41.678	55914	20671	1.340	%
25	Methyl 18-methylnonadecanoate	43.270	13155	5262	0.315	%
26	9,12-Octadecadienoic acid (Z,Z)-,2-hydroxy	44.196	96943	23536	2.324	%
27	9-Octadecenoic acid, 1,2,3-propanetriyl ester	44.280	143298	28094	3.435	%
28	Octadecanoicacid, 2,3-dihydroxypropyl este	44.636	8613	2525	0.206	%
29	Methyl 18-methylnonadecanoate	44.815	67597	26679	1.620	%
30	13-Docosenamide, (Z)-	45.542	182483	56778	4.374	%
31	Squalene	46.018	277898	98369	6.661	%
32	Triacontanoic acid, methyl ester	46.289	9639	3722	0.231	%
33	Triacontanoic acid, methyl ester	47.754	9545	3724	0.229	%
34	Cholesterol	50.954	6668	1343	0.160	%
35	dl-.alpha.-Tocopherol	51.241	262928	54334	6.302	%
36	3Beta-hydroxy-5-cholen-24-oic acid	53.473	95904	11530	2.299	%
37	5,6-Dihydrostigmasterol, acetate	54.364	5844	1138	0.140	%

Sample Information

Analyzed : 6/10/2020 10:28:58 AM
Sample Name : Chloroform extract of root (CF/R)

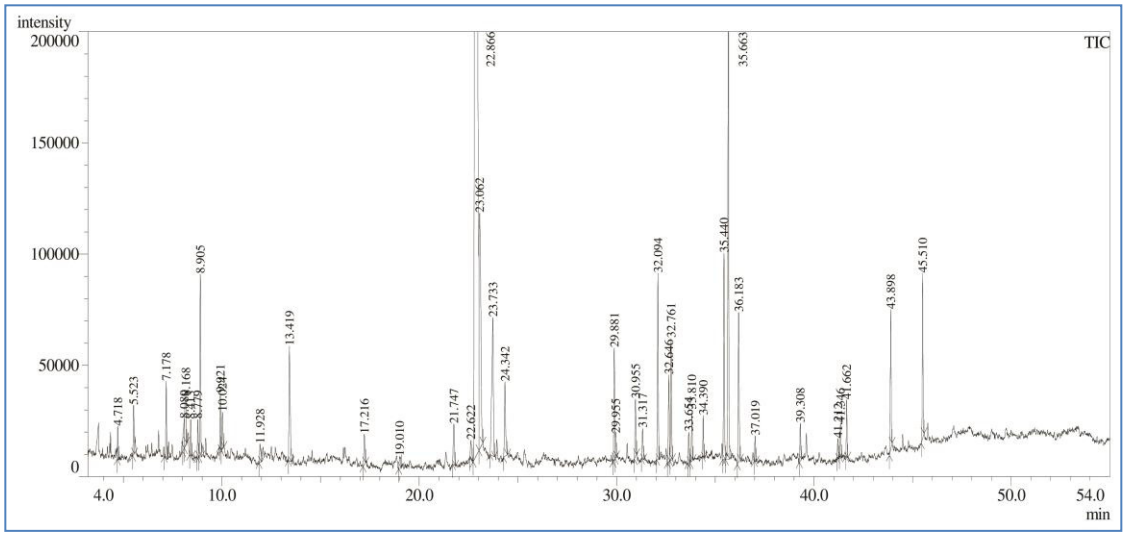


Fig 40. Mass spectrum graph of chloroform extract of root

Table 38. Quantitative result of chloroform extract of root

ID#	Name	R.Time	Area	Height	Conc.	Conc.Unit
1	l-Pantoyl lactone	4.719	13234	6567	0.619	%
2	5H-1-Pyrindine	5.518	13537	6265	0.633	%
3	1-Penten-3-ol, 3-methyl-	7.177	39489	15564	1.847	%
4	Silane, methoxytriphenyl-	8.078	116	58	0.005	%
5	5-Methoxypyrrolidin-2-one	8.167	13211	8440	0.618	%
6	5-Thiazoleethanol, 4-methyl-	8.412	10203	4218	0.477	%
7	1,2-Cyclopropanedicarboxylic acid, cis-	8.784	7494	2931	0.351	%
8	L-Proline, 1-acetyl-	8.905	66133	26554	3.094	%
9	2H-Pyran-2-one, 5,6-dihydro-6-propyl-	9.919	24849	10065	1.162	%
10	2,7-Octadiene-1,6-diol, 2,6-dimethyl-	10.031	14423	2920	0.675	%
11	2-Undecene, 4,5-dimethyl-, [R*,R*-(E)]-	11.931	6305	1448	0.295	%
12	Vanillin	13.417	29606	10097	1.385	%
13	1H-2-Benzopyran-1-one,3,4-dihydro-8-hydr	17.222	5656	1729	0.265	%
14	2-Isopropyl-5-methylcyclohexylethylphosph	19.028	3178	823	0.149	%
15	4-[3,4-Dimethoxycyclohexyl]-n-butanol	21.752	15068	3646	0.705	%
16	2,6,8-Trimethylbicyclo[4.2.0]oct-2-ene-1,8-d	22.620	5579	1285	0.261	%
17	Tigloidine	22.866	1211985	122531	56.694	%
18	Tigloidine	23.058	189164	31102	8.849	%
19	Benzaldehyde, 3-ethoxy-	23.731	83279	14894	3.896	%
20	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyp	24.341	12801	2878	0.599	%
21	Hexadecanoic acid, methyl ester	29.883	25290	8877	1.183	%
22	Bicyclo[4.2.0]octa-1,3,5-triene, 7-(3-butenyl	29.950	6523	2136	0.305	%
23	(5-Nitrohex-1-enyl)benzene	30.954	16240	4386	0.760	%
24	Acetohydrazide, 2-(2-isopropyl-5-methylphe	31.316	3490	1112	0.163	%
25	4H-1,2,4-Triazole-3-thiol,4-(2,3-dihydro-1H	32.093	45005	14960	2.105	%
26	1,5-Diphenyl-1,5-hexadiene	32.647	21821	7032	1.021	%
27	Benzeneethanol, .beta.-ethenyl-	32.763	24973	7544	1.168	%
28	9,12-Octadecadienoic acid, methyl ester	33.652	3216	1200	0.150	%
29	11-Octadecenoic acid, methyl ester	33.810	6100	2126	0.285	%
30	Methyl stearate	34.389	10375	4043	0.485	%
31	Menthyl salicylate	35.440	38396	13740	1.796	%
32	[1R-(1.alpha.,7a.beta.)]-[1-((Z)-2-Methyl-1-o	35.663	60270	22795	2.819	%
33	Heliosupine	36.191	20806	7503	0.973	%
34	Neo-triangularine	37.018	2575	811	0.120	%
35	Citronellylpalmitoleate	39.308	5776	1876	0.270	%
36	trans-.beta.-Terpinyl benzoate	41.212	2502	844	0.117	%
37	3-Buten-2-ol,4-(2,6,6-trimethyl-2-cyclohexe	41.349	5038	1680	0.236	%
38	Di-n-octyl phthalate	41.662	18217	7045	0.852	%
39	7-Hydroxy-3-(4-methoxyphenyl)chromen-2-	43.896	28523	8289	1.334	%
40	13-Docosenamide, (Z)-	45.511	27324	9959	1.278	%

Sample Information

Analyzed : 6/29/2020 10:26:58 AM

Sample Name : Ethyl acetate extract of root (EA/R)

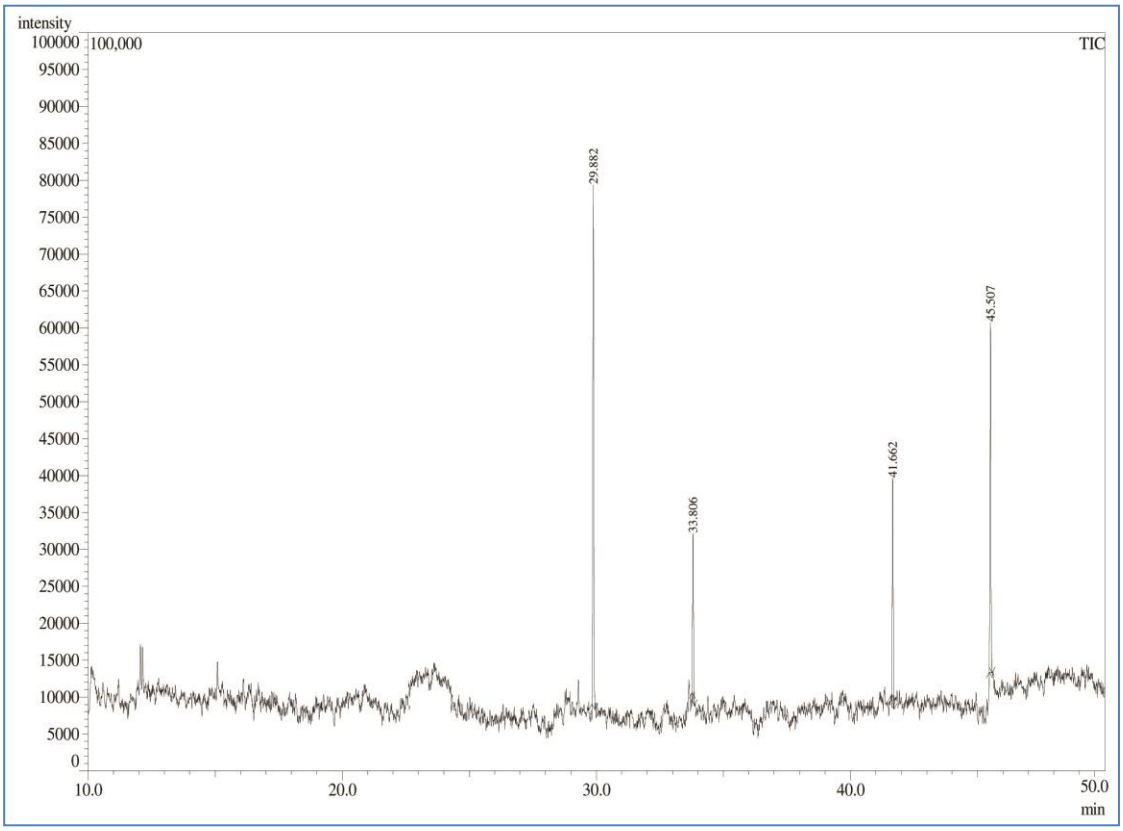


Fig 41. Mass spectrum graph of ethyl acetate extract of root

Table 39. Quantitative result of ethyl acetate extract of root

ID#	Name	R.Time	Area	Height	Conc.	Conc.Unit
1	Hexadecanoic acid, methyl ester	29.881	36937	13817	46.343	%
2	9-Octadecenoic acid (Z)-, methyl ester	33.807	6268	2373	7.864	%
3	Diisooctyl phthalate	41.660	19454	7958	24.408	%
4	13-Docosenamide, (Z)-	45.506	17044	6454	21.384	%

Identification of pure compounds

Open column chromatography is very popular and extensively used in the solid phase category. It can include non-exchange resins polymeric columns, gel-filtration and chromatography over silica gel or chemically modified silica gel. Open column chromatography has a high load capacity but the separation time is long and the resolution is respectively low.

In the stationary phase of open column chromatography silica gel Si60 (60-120 mesh) (Merck) and glass column of size 32x2.5cm was used. Cotton pads washed with acetone, chloroform and methanol were used at the base of the gel column. A similar cotton cloud was used at the top of the column (after application of the sample and the solvent) to protect destruction of the sample layer and the elution rate was 2ml/min.

To fractionate the selected extracts with a view to isolate biologically active compounds they were subjected to biological assay. In case of antibacterial or insecticidal test, the chloroform extract of root showed more potentiality and chloroform extract of leaf showed moderate activity. However, the amount of chloroform extract of root was very poor. And for brine shrimp lethality test, the petroleum ether showed the highest activity among the petroleum ether, chloroform, ethyl acetate and methanol extracts of leaf. Considering the bioactive potentials, the chloroform and petroleum ether extracts of leaf of *M. pudica* were the target extracts for activity guided fractionations.

Investigation of chloroform extract of leaf by column chromatography

The TLC analysis of chloroform soluble leaf extract showed three prominent spots of compounds when developed with chloroform : methanol = 5:1. The chloroform leaf extract (3.0g) was mixed well with an optimum amount of silica gel (column grade) in a mortar with pestle to get a free flowing powder and was subjected to a column of silica gel (column height 32cm and diameter 2.5cm).

The elution was successively done with 100% n-hexane and increasing proportion of chloroform and finally methanol as shown in the table 40. Elutes were collected in a number of test tubes marked from 1 to 78. The fractions obtained were spotted successively on the TLC plates, allowed to develop by chloroform : methanol = 5:1 as mobile phase and were viewed usually under UV, in iodine chamber and with spraying Godin and after Godin reagent. The test tubes containing similar compounds were combined together.

Table 40. Solvent system used in column chromatography of chloroform extract of leaf

Fraction No	Solvent system	Proportion	Volume eluted (ml)	Toxicity test
1	n-hexane	100%	100	
2	n-hexane : chloroform	80 : 20	100	
3	n-hexane : chloroform	60 : 40	100	
4	n-hexane : chloroform	40 : 60	100	
5	n-hexane : chloroform	20 : 80	100	
6	chloroform	100%	100	
7	chloroform : methanol	98 : 2	100	
8*	**chloroform : methanol**	**95 : 5**	**100**	
9*	**chloroform : methanol**	**90 : 10**	**100**	
10*	**chloroform : methanol**	**85 : 15**	**100**	++
11*	**chloroform : methanol**	**80 : 20**	**100**	
12*	**chloroform : methanol**	**75 : 25**	**100**	
13	chloroform : methanol	70 : 30	100	
14	chloroform : methanol	60 : 40	100	
15	chloroform : methanol	50 : 50	100	
16	chloroform : methanol	40 : 60	100	
17	chloroform : methanol	30 : 70	100	
18	chloroform : methanol	20 : 80	100	
19	chloroform : methanol	10 : 90	100	
20	methanol	100%	100	

*9-Octadecenamide isolated from this fraction, ++ = moderate toxicity response

Analysis of fraction 8-12

The fractions 8, 9, 10, 11 and 12 showed similar spots on TLC using solvent system chloroform : methanol = 4:1 and were combined. The solvent was evaporated off to a certain volume and allowed to stand overnights. Few crystals were appeared. Recrystallization from chloroform and methanol afforded compound-1 (50mg). Each of the fractions was subjected to toxicity test against *C. pusillus* for insecticidal effect and moderate activity was observed. The fractions showed positive test under UV, vanillin-sulfuric acid reagent and Godin and after Godin reagent. The combined fractions were analyzed by GC-MS method and isolated 9-Octadecenamide.

Investigation of petroleum ether extract of leaf by column chromatography

The TLC analysis of petroleum ether soluble leaf extract showed six prominent spots of compounds when developed with n-hexane : chloroform = 1:9. The petroleum ether leaf extract (3.0g) was mixed well with an optimum amount of silica gel (column grade) in a mortar with pestle to get a free flowing powder and was subjected to a column of silica gel (column height 32cm and diameter 2.5cm).

Table 41. Solvent system used in column chromatography of petroleum ether extract of leaf

Fraction No	Solvent system	Proportion	Volume eluted (ml)	Toxicity test
1	n-hexane	100%	100	
2	n-hexane : chloroform	90 : 10	100	
3	n-hexane : chloroform	80 : 20	100	
4	n-hexane : chloroform	70 : 30	100	
5	n-hexane : chloroform	60 : 40	100	
6	n-hexane : chloroform	50 : 50	100	
7	n-hexane : chloroform	40 : 60	100	
8*	**n-hexane : chloroform**	**30 : 70**	**100**	
9*	**n-hexane : chloroform**	**20 : 80**	**100**	
10*	**n-hexane : chloroform**	**10 : 90**	**100**	+++
11*	**chloroform**	**100%**	**100**	
12	chloroform : ethyl acetate	90 : 10	100	
13	chloroform : ethyl acetate	80 : 20	100	
14	chloroform : ethyl acetate	70 : 30	100	
15	chloroform : ethyl acetate	60 : 40	100	
16	chloroform : ethyl acetate	50 : 50	100	
17	chloroform : ethyl acetate	40 : 60	100	
18	chloroform : ethyl acetate	30 : 70	100	
19	chloroform : ethyl acetate	20 : 80	100	
20	chloroform : ethyl acetate	10 : 90	100	
21	ethyl acetate	100%	100	
22	ethyl acetate : methanol	95 : 5	100	
23	ethyl acetate : methanol	90 : 10	100	
24	ethyl acetate : methanol	80 : 20	100	
25	ethyl acetate : methanol	60 : 40	100	
26	ethyl acetate : methanol	40 : 60	100	
27	ethyl acetate : methanol	20 : 80	100	
28	methanol	100%	100	

*13-Docosenamide, (Z)- isolated from this fraction, +++ = strong toxicity response

The elution was successively done with 100% n-hexane and increasing proportion of chloroform, ethyl acetate and finally with methanol as shown in the table 41. Elutes were collected in a number of test tubes marked from 1 to 122. The fractions obtained were spotted successively on the TLC plates, allowed to develop by n-hexane : chloroform = 1:9 as mobile phase and were viewed usually under UV, in iodine chamber and with spraying Godin and after Godin reagent. The test tubes containing similar compounds were combined together.

Analysis of fraction 8-11

The fractions 8, 9, 10 and 11 showed similar spots on TLC using solvent system n-hexane : chloroform = 1:12 and were combined. The solvent was evaporated off to a certain volume and allowed to stand overnights. Few crystals were appeared. Recrystallization from n-hexane and chloroform afforded compound-2 (55mg). Each of the fractions was subjected to toxicity test against *C. pusillus* for insecticidal effect and strong effectivity was observed. The fractions showed positive test under UV, vanillin-sulfuric acid reagent and Godin and after Godin reagent. The combined fractions were analyzed by GC-MS method and isolated the 13-Docosenamide, (Z)-.

R_f value of isolated compounds

Two pure compounds 9-Octadecenamide and 13-Docosenamide, (Z)- were isolated from partitioning of chloroform and petroleum ether extracts of crude methanolic extract of leaf of *M. pudica.* Commercially available pre-coated silica gel (Kiesel gel 60 GF_{254}) plates were used for this purpose. Different developing solvent systems with R_f values were presented in the following table 42.

Table 42. Developing solvent system and R_f value of the compounds

Compound	Extract	Solvent system	R_f value
9-Octadecenamide	Chloroform	Chloroform : Methanol (5:1)	0.51
13-Docosenamide, (Z)-	Petroleum ether	n-Hexane : Chloroform (1:12)	0.55

Observation of the isolated compounds

To characterize both the isolated compounds physical as well as spectral data were studied. The physical properties such as physical form, color, melting point, R_f value, solubility, etc. were observed. Among the spectroscopic methods GC-MS technique was carried out for structure elucidation.

Properties of 9-Octadecenamide

Molecular formula	: $C_{18}H_{35}NO$
Molecular weight	: 281.5 g/mol
Physical form	: Pellets large crystals
Color	: Pinkish/Brick
Melting point	: 70^0C (158^0F)
Boiling point	: >200^0C (392^0F)
R_f value	: 0.51
Solubility	: Insoluble in H_2O
Synonym	: Oleamide, 9-Octadecenamide, (9Z)-, cis-9,10-Octadecenamide, octadec-9-enamide.

Properties of 13-Docosenamide, (Z)-

Molecular formula	: $C_{22}H_{43}NO$
Molecular weight	: 337.6 g/mol
Physical form	: A crystalline solid
Color	: Colorless
Melting point	: 77.5^0C
R_f value	: 0.55
Solubility	: Soluble in isopropanol, slightly soluble in alcohol, acetone.
Synonym	: 13Z-Docosenemide, Armoslip E, cis-13-Docosenamide, Erucamide, (Z)-Docos-13-enamide.

Fig 42. 9-Octadecenamide

Fig 43. 13-Docosenamide, (Z)-

Spectral characteristics of 9-Octadecenamide

Analyzed : 6/16/2020 12:53:29 PM
Sample Name : Chloroform extract of leaf

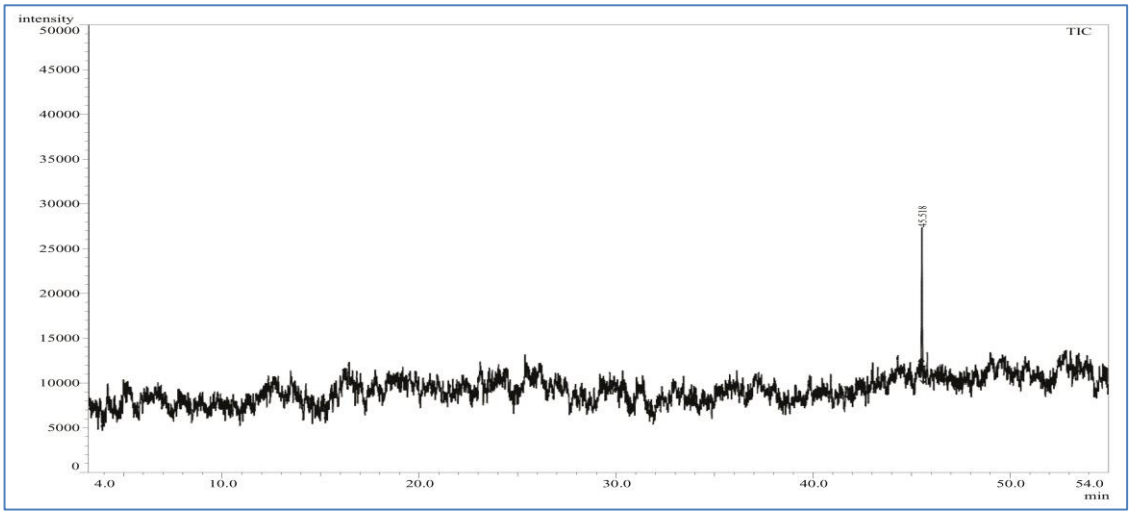
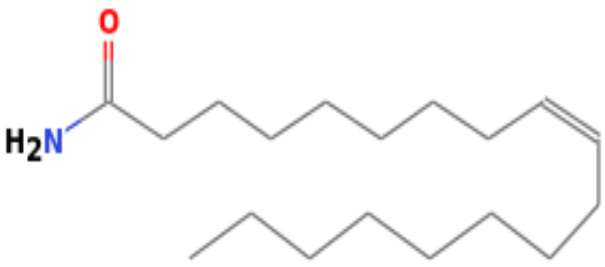


Fig 44. Mass spectrum graph of 9-Octadecenamide

Table 43. Quantitative result of 9-Octadecenamide

ID#	Name	R.Time	Area	Height	Conc.	Conc.Unit
1	9-Octadecenamide	45.516	5004	1668	100.00	%



9-Octadecenamide

Spectral characteristics of 13-Docosenamide, (Z)-

Analyzed : 6/16/2020 12:53:29 PM

Sample Name : Petroleum ether extract of leaf

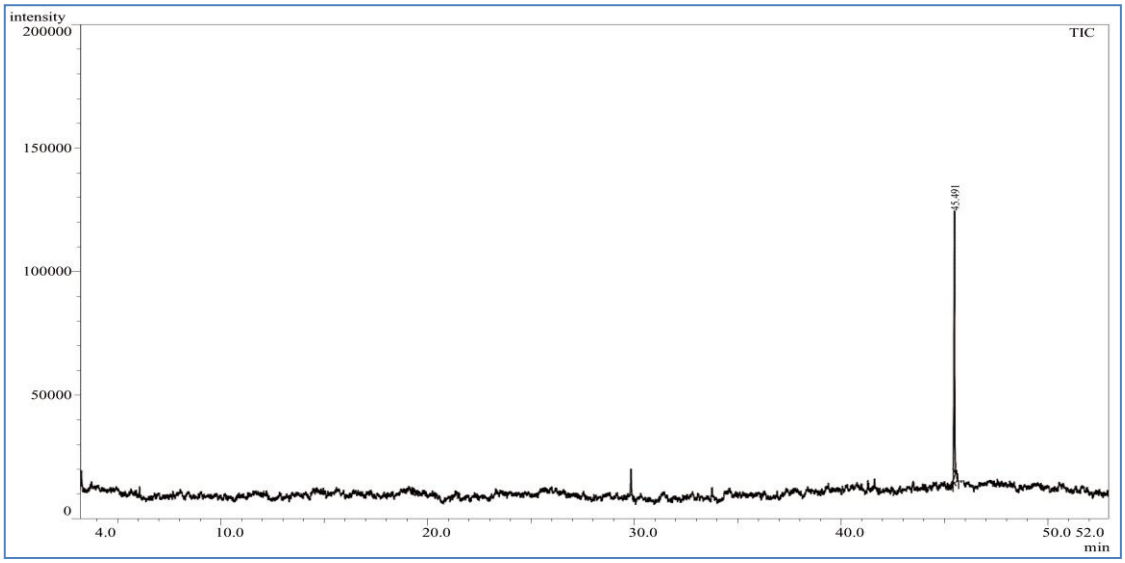
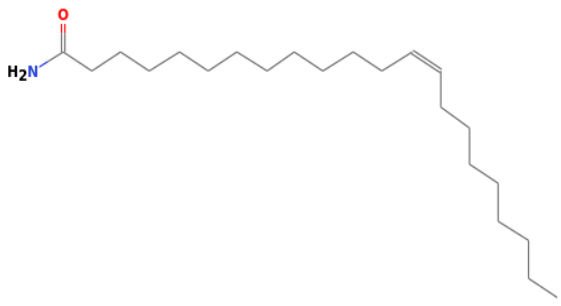


Fig 45. Mass spectrum graph of 13-Docosenamide, (Z)-

Table 44. Quantitative result of 13-Docosenamide, (Z)-

ID#	Name	R.Time	Area	Height	Conc.	Conc.Unit
1	13-Docosenamide, (Z)-	45.491	36002	13696	100.00	%



13-Docosenamide, (Z)-

Discussion

The medicinal plant *M. pudica* is used in herbal medicine all over the world in the treatment of various diseases. The researchers have been making efforts to isolate and identify the biologically active principles and other major phytoconstituents of the plant. In the present study, a large number of phytocompounds were isolated through GC-MS method.

Moncao *et al.* (2014) observed the chemical composition of ethanol extract from leaves of *M. caesalpiniifolia* and the chemical derivatization was followed by GC-MS analysis which showed a total of thirty two compounds. The compounds of high relative abundance were phytol (11.66%), lactic acid-2 (9.16%), α-tocopherol (7.34%) and β-sitosterol (6.80%). Araujo (2010) identified he compound β-sitosterol in hexane fraction of leaves, fruits, branches and barks of the same species. Galic acid was identified from the aerial parts of another species of the same genus, *M. hamata* by Hussain *et al.* (1979). As well as lupeol was isolated from the leaves of *M. artemisiana* (Nascimento *et al.* 2012), from the aerial parts of *M. hostiles* (Ohsaki *et al.* 2006) and flowers of *M. caesalpiniifolia* (Araujo 2010). In the present investigation, more or less similar compounds were isolated through GC-MS analysis. For example, β-sitosterol (2.26%), stigmasterol (4.21%), γ-tocopherol (2.84%), mesitylene (13.27%), etc.

Tunna *et al.* (2015) analysed the sample by gas chromatography time of flight mass spectra (GC-Q-TOF-MS) for identification of the chemical compounds from the aerial part of *M. pudica* and matched to the NIST library. The result showed that the methanol extract and its fractions of hexane, ethyl acetate and methanol contained 46, 34, 22 and 33 different compounds respectively based on the mass spectra peaks. Phenlos like benzenethanol, diphenols, alkaloids like benzeneamide, quinine and quinolinone, iminocarbonitrile, etc. were identified. The acetone fraction showed the presence of compounds like tyrosol, glycine, dodecaboranes, benzothiazole, carbamic acid, benzyloxypropanoic acid, butanoic acid, quinine and tyrophanamide. As methanol fraction was the last fraction, most of the components had already been successfully fractioned with ethyl acetate and acetone fractions. Although, methanol fraction showed pretty much similar compounds like its counter fractions and also identified some compounds like quinolinone, fluoro-anisidine and a high molecular wt. compound $C_{27}H_{38}O_4S_2$. Lastly, the initial methanolic extract (mother extract) was

analyzed and found maximum number of compounds. Among them some were larger and important molecules as phenylbutanol, napthalein, carboxylic acid, dioxolane, iminocarbonitrile, β-D-glucopyranosiduronic acid and eicosatriynoic acid together with some compounds found in the fractions. It also showed some unidentified compounds that could be the fact that the initial methanol extract underwent continuous heat under reflux during the fractionation procedure which might be responsible for degradation of some key compounds. The undetected compounds could be new or the ones that were not derivetised enough to identify through GC-Q-TOF-MS analysis. Glysine was found in all the samples as a major compound. Harborne (1984), revealed that a non-polar solvent, hexane, brought out the fatty acid and wax components from the initial methanol extract that were basically long chain carbohydrates. Oboh *et al.* (2012), showed that the hexane fraction contained methyl sulfide, dioxolane, carotene and acids like aspartic, malonic and mannopyranosyl dodecaborane. From acetone fraction, dodecaborane, tyrosol, benzothiazole, dioxime, p-quinone etc. were identified. Ethyl acetate was a semi-polar solvent mainly used for the extraction of polyphenolic compounds due to its particular affinity mostly to phenolic compounds. In the present study, the result showed that the petroleum ether extract of leaf contained 16 compounds based on the mass spectra peaks and benzene, 1-ethyl-3-methyl- (14.83%), aniline, N-methyl- (4.99%), hexadecane (3.43%), nonadecane (7.25%), 13-docosenamide, (Z)- (4.74%), squalene (3.68%), vitamine E (13.12%), etc. were identified. The ethyl acetate extract of leaf contained glycerin (74.71%), hexadecanoic acid, methyl ester (9.99%) and 9-octadecenamide (15.30%) and ethyl acetate extract of stem contained hexadecanoic acid, methyl ester (69.95%), 9-octadecenoic acid (Z)-, methyl ester (14.43%) and 9-octadecenamide (15.62%).

Through GC-MS analysis of methanol extract of *M. pudica,* Sridharan *et al.* (2011) reported the compounds as glycerine (alcohol), myo-inositol (aromatic compound), trimethyl caffine (alkaloid), hexadecen-1-ol (terpene alcohol), hexadecanoic acid (palmictic acid), phytol (diterpene), octadecadienoic acid (linoleic acid), vitamin E and squalene (tri-terpene). The highest peak area (46.61%) was obtained by myo-inositol and the lowest peak area (0.90%) was obtained by glycerine. Saraswat and Pokharkar (2012) reported the compounds of N-dl-alanylglycine, dl-alanyl-dl-valine, d-alanin, dl-alanin ethyl ester, 1-alanine ethyl amide, 9,12-octadecadienoic acid (Z,Z), methyl ester, 9,12-octadecadienoic acid, methyl ester, 11,13-eicosadienoic acid, methyl ester and

meglumine by GC-MS analysis of oil extract of *M. pudica.* Vismayaviswan *et al.* (2019) found that the major compounds were phthalic acid dioctyl ester (27.76%), α-linolenic acid (20.34%) and cinnamaldehyde (16.24%) by GC-MS analysis from the essential oil of *M. pudica*. Ramesh *et al.* (2014) analyzed the methanolic extract leaf of *M. pudica* by gas chromatography coupled to mass-selective detector and 52 compounds were separated from peak value. 3,7,11,15-tetramethyl-2-hexadecen-1-ol possessed the highest peak area (18.80%) and the main compounds obtained from the sample were 1,3,5-cycloheptatriene-4-pentenal, 2-methyl, 1-propene, 1-(2-propenyloxy)-E, p-xylene, 2-cyclopentene-1, 4-dione, 3-hydroxybutanamade, w-phenylmethoxy, tetrahydro-4H-pyran-4-ol, carbamic acid, phenyl ester 2(3H)-furanone, benzenacetaldehyde, heptanal, 1-butanol, 3-methyl-acetate, 4H-pyran-4-one, 2,3-dihydroxymethyl, hydroxyethyl-hydroxymethyl benzene, benzene, coumaranone, 3-oxo-4-phenyl butyranitrile nonanoic acid, 5H-1-pyrindine, 2-methoxy-4-vinylphenol naphthalene, decanoic acid, 3-isopropoxybenzaldehyde, octose, vanillin tyrosine, benzofuranone, octadiene, 3-hydroxy-7,8-dihydro-a-ionol, trimethyl-tetraclopenta, 3-buten 3-O-methyl-d-glucose, acetic acid, buten, cyclohexane, cyclopentanol, phytol, etc.

Vijayalakshmi and Udayakumar (2018) carried out the GC-MS analysis of ethanolic extracts of leaf and root of *M. pudica* and the chromatogram showed the presence of various compounds with corresponding peaks at different retention time. The % peak area, retention time, molecular weight, molecular formula, nature and biological activities of leaf and root compounds were presented. The biological activities were predicted based on Dr Duke's Phytochemical and Ethnobotanical Databases created by Dr Jim Duke's of Agricultural Research Service/USDA. Among the compounds, the percentage of peak area of n-hexadecanoic acid was predominant for ethanolic leaf extract and Z,E-2-methyl-3,13-octadecadien-1-ol was predominant for ethanolic root extract. Both the ethanolic extracts of leaf and root possessed the compounds of 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl ($C_6H_8O_4$); phenol, 2,4-bis(1,1-dimethylethyl) ($C_{14}H_{22}O$); dodecanoic acid ($C_{12}H_{24}O_2$); 3-O-methyl-d-glucose ($C_7H_{14}O_6$); 3,7,11,15-tetramethyl-2-hexadecen-1-ol ($C_{20}H_{40}O$); n-hexadecanoic acid ($C_{16}H_{32}O_2$) and octadecanoic acid ($C_{18}H_{36}O_2$). The ethanolic leaf extract possessed the compounds of D-arabinitol ($C_5H_{12}O_5$); 1-methyl-2,4,5-trioxoimidazolidine ($C_4H_4N_2O_3$); piperidine, 3-phenyl- ($C_{11}H_{15}N$); 3-hexadecene, (Z)- ($C_{16}H_{32}$); sucrose ($C_{12}H_{22}O_{11}$); 5-dodecanol ($C_{12}H_{26}O$); myo-inositol, 4-C-methyl- ($C_7H_{14}O_6$); 2-

pentadecanone-6,10,14-trimethyl ($C_{18}H_{36}O$); methyl-α-d-mannofuranoside ($C_7H_{14}O_6$); phytol ($C_{20}H_{40}O$); 9,17-octadecadienal, (Z)- ($C_{18}H_{32}O$); E-11-hexadecenal ($C_{16}H_{30}O$); hexadecanoic acid, 2-hydroxy-1,3-propanediyl ester ($C_{35}H_{68}O_5$); 7,11-hexadecadienal ($C_{16}H_{28}O$); 16-heptadecenal ($C_{17}H_{32}O$) and 2,6,10,14,18,22-tetracosahexane, 2,6,10,15,19,23-hexamethyl-,(all-E)- ($C_{30}H_{50}$). The ethanolic root extract showed the presence of compounds such as ethane, 1,1-diethoxy- ($C_6H_{14}O_2$); furan,2,5-dimethyl- (C_6H_8O); 2-pyrazoline,1,3,4-trimethyl- ($C_6H_{12}N_2$); 2,5-furandicarboxaldehyde ($C_6H_4O_3$); 2-furandicarboxaldehyde,5-(hydroxylmethyl)- ($C_6H_6O_3$); cyclohexane-carboxylic acid, 4-methoxyphenyl ester ($C_{14}H_{18}O_3$); pentadecanal- ($C_{15}H_{30}O$); heptadecanoic acid ($C_{17}H_{34}O_2$); Z,E-2-methyl-3,13-octadecadien-1-ol ($C_{19}H_{36}O$) and 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester ($C_{16}H_{22}O_4$).

Chapter Seven

INSECTICIDAL ASSAY OF *MIMOSA PUDICA*

Introduction

Insecticidal activity

The continuous use of the conventional insecticides has necessitated the search for possible alternatives. Among the small-scale farmers the use of plants for the protection of grains against insect-infestation is an age-long practice (Hassanali *et al*. 1990 and Poswal and Akpa 1991). A huge volume of insecticides are used annually; but botanical insecticides comprise a small portion of the total volume of insecticides. But they are important in insect pest management. Plant derived insecticides pose less risk to non-target organisms as they are short-lived in the environment. They are accepted by organic certification programs as well as certain consumer groups as they are naturally occurring (Isman 2000 and Weinzierl 2000).

Natural products of the local plants with insecticidal activity represent a low-cost sustainable alternative in protecting agricultural products. Moreover, botanical insecticides are suitable alternative to control mites and insect pests worldwide and pose little threat to human health or the environment compared to synthetic insecticides. It is a common traditional method in rural areas of the world to apply plant extracts with insecticidal or repellent properties to stored-grains to control the insect pests (Isman 2006, Regnaut-Roger *et al*. 2012 and Kedia *et al*. 2013). Plant-derived constituents are used as insecticides as well as antifeedants (potential seed protectant) (Pavela 2007). Biologically active compounds of the plants are expected to produce chemical defense against their enemies (Oly *et al*. 2011).

The synthetic insecticides have produced a fascinating result against pests which have been over shadowed by recent debates on hazards to human health and effects on non-target organisms (Adedire *et al.* 2011). However, their frequent usages sometimes result in the development of insecticidal resistance to the target species. The challenge of finding sustainable alternatives to these synthetic insecticides has led to the bio-prospecting of plants with repellent and toxicological properties (Osipitan *et al.* 2012). As plant-originated natural products are effective, they are reported to be useful and

desirable tools in pest management. Plant extracts and plant powders are used in several experiments for agriculture and household pest management; but little is known about the use of extracts or powders from invasive alien plants to control the noxious species (Uyi and Osarieme 2016, Chaubey 2017 and Uyi and Obi 2017).

The most effective pest control technology is often considered to be the use of pesticides. But continuous and huge use of some pesticides have created serious problems such as, direct toxicity to predators, pollinators, parasites, fish and man (Munakata 1977 and Pimental 1981), pesticidal resistance (Georghiou and Taylor 1977, Schmutterer 1981 and Waiss *et al.*1981) and susceptibility to insect pests of crop plants (Pimental *et al.* 1980). Botanical insecticides have the advantages over synthetic insecticides. Recently, plant products or plant derived compounds are used as the promising alternatives to synthetic insecticides in controlling the insect pests of stored products (Rajapakse *et al*. 1998 & 2002 and Rajapakse and Ratnasekera 2009).

Today, almost no crop is free from attack by insects at least to some degree in the world (Berenbaum 1995). Botanical insecticides are believed to possess certain attributes that have higher advantage over conventional insecticides. These include non-phytotoxicity, less persistence to the environment, selectivity towards the target pests and low mammalian toxicity (Rosenthal 1986 and Isman 2006). Thus plant derived insecticides are safer than synthetic products. No serious attempts were taken to observe the insecticidal efficacy of *M. pudica.* The present study reveals that the extracts of *M. pudica* possess some pesticidal potential which can be effective in biological control of insect pests.

Repellent activity

Plant products are used traditionally by human communities throughout the world against different species of insects and vectors. Plants are the rich source of phytochemicals that can act as repellents, larvicides, ovipositional attractants, insect growth regulators and have deterrent activities (Babu and Murugan 1998). In order to protect human beings from bite of arthropods repellents provide a mechanism. To design of an arthropod repellent the desired quality should be low toxicity to humans, non-irritating and long-lasting repellent activity (Murugan *et al.* 2003). The present study was designed to investigate the repellent activity of *M. pudica.*

Brine shrimp lethality bioassay

Artemia salina is an aquatic crustacean, a species of brine shrimp which is closely related to the cladocerans and *Triops* than the true shrimps. It belongs to the lineage which does not appear to have changed much in 100 million years. It is found in ponds, saline lakes and temporary waters in the Mediterranean region of southern Europe, Anatolia and North Africa. In pollution research it has an extensive use as a test organism and in some cases it becomes an acceptable alternative for testing the toxicity of mammals (Lewan *et al.* 1992).

Artemia species are able to avoid combating with predators by their ability to live in very high salinity up to 25%. Their eggs are known as cysts and their ability to produce dormant eggs has led to extensive use of *Artemia* in aquaculture. The cyst can be stored for a long time and can be hatched if necessary to supply a convenient form of live feed for larval fish and crustaceans (Daintith M 1996).

The toxicity of extracts, fractions or chemical constituents of plants is often evaluated against brine shrimp nauplii because of its simple, rapid and low-cost properties. The substances tested having lethal dose 50% of the specimens (LD_{50}) of less than 1000 $mg.ml^{-1}$, are considered active (toxic) (Nunes 2008). The bioassay provides an advantage in the evaluation of bioactivity of natural products in traditional medical practices, confirming their therapeutic potentials as well as evaluating their cytotoxic profiles that represent a support for bioguided obtention of plant-derived chemicals and toxicological studies in animal models (Arcanjo *et al.* 2012).

For the preliminary assessment of toxicity, the brine shrimp lethality assay is considered as a useful bench-top method. It is used to detect the plant extract toxicity, cytotoxicity, pesticides, fungal toxins, heavy metals and testing of dental materials. The lethality is evaluated by exposing the nauplii to the test sample in a saline solution (Harwing and Scott 1971, McLaughlin *et al.* 1991, Martinez *et al.* 1998, Barahona and Sanchez-Fortun 1999 and Pelka *et al.* 2000). To isolate the bioactive components from plant extracts this assay is a very useful tool (Sam 1993). In traditional medicines, some crude plant extracts possess an extensive diversity of molecules that have indefinite biological effects (Konan *et al.* 2007). The scientific data regarding the medicinal

potential of the plants are not provided with credible information. Therefore, the scientists are devoted to determine the toxicity of medicinal plants through brine shrimp lethality test (Sasidharan *et al.* 2009).

To biomonitor the isolation of insecticidal (Oberlies *et al.* 1998), antifeedant (Labbe *et al.* 1993), antimalarial (Perez *et al.* 1997) and cytotoxic (Siqueira *et al.* 1998) compounds from the plant extracts this assay has been used successfully. Brine shrimp lethality test is an inexpensive test that requires a small amount of test materials (2-20 mg or less). It is a simple, rapid and easily mastered test as no aseptic techniques are required. It is a bioassay capable of detecting a broad spectrum of bioactivity in crude extracts. This technique provides a front-line screen that can be backed up by more expensive and more specific bioassays when the active compounds are isolated. The test is a predictive of cytotoxicity and pesticidal activity (Ghisalberti 1993). Therefore, the brine shrimp lethality bioassay was conducted to observe the cytotoxicity of the medicinal plant *M. pudica.*

Materials and Methods

Insecticidal activity

Selection of test organisms

C. pusillus was selected as the test insect to carry on tests for insecticidal and larvicidal activities and also for repellent potentials of the extracts of *M. pudica* as it is an easy cultivable and noble laboratory animal. Life history of the insect made it popular choice as test insect for biological studies. Brine shrimp nauplii (*A. salina*) were selected for the cytotoxicity test, since it is being used as a model test agent in such experiments.

Collection of *C. pusillus*

Adult beetles of *C. pusillus* were collected from the stock culture of Entomology and Insect Biotechnology Laboratory, Institute of Biological Sciences, University of Rajshahi, Bangladesh and rared as mass-cultures and sub-cultures to use in the experiments. They were rared in the control temperature room (30 ± 0.5^0C), Institute of Biological Sciences, University of Rajshahi, Bangladesh.

Culture of *C. pusillus*

Mass-cultures and sub-cultures were maintained in the plastic containers and Petri dishes (15 cm) with the food medium. The Petri dishes were kept at 30±0.5^{0}C in an incubator without controlling the light and humidity. Each container and Petri dish contained 200g and 100g of food respectively. Each container and Petri dish was introduced about 200 and 100 adults respectively. The cultures were checked regularly and the larvae were separated to increase properly. The containers and Petri dishes were covered with muslin cloth tightly to avoid possible escape of the beetles.

Preparation of food medium for maintenance of insect culture

The cracked wheat with flour was used as food medium for the insects. The food was sterilized at 60^{0}C for 24 hours in an oven. The food was mixed with powdered dry yeast in a ratio of 19:1 (Park 1962 and Khalequzzaman *et al.* 1994) and was used throughout the experiment period. Both the food and the dry yeast were sterilized at 60^{0}C for six hours in an oven. The food was used two weeks after sterilization to allow its moisture content to equilibrate with the environment (Khan and Selman 1981a).

Maintenance of stock culture

About 100 adults of *C. pusillus* were released in container containing food medium for oviposition and after two days the adults were transferred to another container with fresh food for further oviposition. Thus a series of stock culture was maintained in the laboratory for constant supply of insects to conduct the experiments.

Phylogenetic position of *C. pusillus*

Kingdom: Animalia
Phylum: Arthropoda
Sub-Phylum: Hexapoda
Class: Insecta
Sub-Class: Pterygota
Order: Coleoptera
Family: Cucujidae
Genus: *Cryptolestes*
Species: *C. pusillus*

C. pusillus (Schon.) (Coleoptera: Cucujidae) is a small and most common insect pest of stored-grains. It is an important stored-products pest of the world. It is established in Australia but is less common than *C. ferrugineus* (Schon.). It is cosmopolitan in distribution and occurs all over the world, mainly the tropical and subtropical regions including Bangladesh. It is most prevalent in warmer climates and probably occurs in crop-growing regions throughout the world. Though, it is common in wet tropical and warmer temperate regions, unable to survive in cooler temperate regions (Halstead 1993 and CABI Crop Protection Compendium 2008). It scavenges by nature and infests grains of poor conditions. Mainly, it follows up the attack of *T. castaneum* (Herbst) and *S. Oryzae* (L.). Particularly, their larvae are fond of germ of wheat (Sengupta *et al.* 1978). In Canada, it has been recorded in grain elevators and flour mills feeding on damaged grains, preferably wheat (Bousquet 1990). Though, above 20^0C are required for flight but it is not known how far *C. pusillus* can disperse by flying. It spreads internationally with movement of grains and other stored commodities.

Adults are small (1.5 to 2.0 mm), reddish brown, elongated, flattened, parallel-sided, densely and finely pubescent and with long bead-like antennae. Newly-hatched larvae are translucent white, becoming less translucent, pale and white to yellow with age, with a darkened head capsule and dark urogomphi. Body is elongated and depressed vertically with well developed legs. They have four larval instars and a quiescent pre-pupal stage. The length of first-instar larva is about 1 mm and the later instars are similar to the first-instar larva. The length of mature fourth instar larva is 3-4 mm. The pupal stage finishes within a silken cocoon. Newly-formed pupa is white, becomes darker with age. Cocoon is slightly longer than the mature larva. (Sengupta *et al.* 1978 and CABI Crop Protection Compendium 2008).

Newly-emerged adults are light-brown that rapidly becomes a reddish-brown. Tarsi are five-segmented except male's hind tarsi that are four-segmented. As the basal segment is small, it may be only possible to count 4 segments on each tarsus, or 3 on the male hind tarsus. Female antennae are about half the length of body, while about two-third of the length of body in male. Elytra have 5 parallel ridges and adults cannot climb up glass.

Biology of *C. pusillus*

Females lay eggs in crevices or loosely amongst the food. At 17^0C it may take 20 days to hatch, but at 37^0C only 3 days. In an optimum condition of 25^0C and 75% relative humidity the females begin laying eggs within 4 days after emergence and continue for

more than 34 weeks. Though, the oviposition rate declines towards the end, it may increase with higher temperatures and humidities. Also, if the larval density or the female proportion of the population increases, the oviposition rate decreases (White and Bell 1993). The larvae may be free-living, though, generally they live within a grain kernel for protection. At 37^0C, they take 11 days to complete development and the higher the humidity the more larvae complete development. Usually, within the food materials, the mature larvae spin cocoons in which they pupate. The pupal stage lasts for 12 days at 17^0C and 3 days at 37^0C. The humidity has a considerable effect on the rate of pupal development. A female lays about 200-300 eggs during her lifetime (Ashby 1961 and CABI Crop Protection Compendium 2008).

Developmental stages of *C. pusillus* depend on varied combinations of high temperature and humidity. Larval development is quicker at 90% than at 70% relative humidity, and the optimum temperature is about 37^0C. The developmental period is less than that of other species of *Cryptolestes* under optimal conditions and mean duration of the larval stage is as little as $8^1/_2$ days at 33^0C and 90% relative humidity. The average oviposition rate may be 7 eggs per female per day under optimal conditions which is about 17% of the weight of adult. *C. pusillus* completes life cycle within 30 to 34 days at 30^0C temperatures, but the development period increases at lower temperature and relative humidity (Ashby 1961).

Pilot experiment for application of *M. pudica*

Surface-film method was used to carry on the pilot experiment with chloroform extract of leaf along with a control experiment where the treatment was made only with solvent. The experiment was carried out to obtain doses where the mortality rate was in between 10-95%. Different doses were used with five replications and in each replication 3 to 5 day old 20 adult insects were used in the experiment. Then the actual doses were calculated from the amount of extract present in 1 ml of solution.

Preparation of doses for insecticidal activity test

Washed and properly autoclaved Petri dishes were marked with permanent marker for different doses of the extracts. Insects were collected before setting up the experiments. Doses were prepared according to the results of pilot experiment and poured down into the Petri dishes of known surface area with the help of micropipette. As the solvents

were volatile, they evaporated out immediately simply with the atmospheric temperature. Thus the ingredients went to make film on the surfaces of the Petri dishes. The insects that were released within this captivity might have contact with the substances distributed evenly on the floor.

All extracts were diluted with the selected solvents in which they were extracted and the amount extracts in every dose were recorded. The dose per square centimeter was calculated by dividing the amount of extract present in 1 ml of mixture with the area of the Petri dish. The test was carried out by residual film method (Busvine 1971). 254.34 mg extracts was mixed in 2ml of respective solvents to make the stock solutions. From these stock solutions other successive doses were prepared by serial dilution method. Thus four doses were prepared as 2.0, 1.0, 0.5 and 0.25 mg/cm^2. Then the Petri dishes were kept in an incubator at 30±0.5^{0}C.

Application of doses on insects

To conduct surface film activity test 90 mm Petri dishes were taken for all the doses and their replications. One ml of each dose was poured into the lower part of Petri dishes and allowed them to dry out. Ten insects were released in each of the Petri dishes. Control experiments by applying the only solvents into the Petri dishes were also set at the same time.

Observation of mortality of the insects

The plates were kept at a secured place in room temperature. The whole experiment was observed from time to time and the mortality of the insects was recorded after every 24, 48 and 72 hours of exposures. A simple microscope was used to check each and every beetle by tracing natural movement of its organs. In some cases hot needle was taken closer to the bodies (without movement) to confirm death.

Reading and statistical analysis of data for insecticidal activity

The mortality recorded was corrected by the Abbott's (1925) formula in the following manner:

$$P_r = \{(P_o - P_c) / (100-P_c)\} \times 100$$

Where, P_r = Corrected mortality (%)

P_o = Observed mortality (%)

P_c = Control mortality (%), sometimes called natural mortality (%).

The bioassay test showing more than 10% control mortality were discarded and repeated. However, when the control mortality ranged from 5-10%, the corrected mortality was calculated using Abbott's formula. The LD_{50} with 95% confidence limits of different extracts were determined using log probit analysis test. The analysis was performed using IBM SPSS statistical package for WINDOWS (version 22, SPSS Inc., Chicago). Regression lines were drawn by using MS-Excel. The results with $p<0.05$ were considered to be statistically significant. The dose-mortality relationship was expressed as median lethal dose (LD_{50}).

Larvicidal activity

Preparation of doses for larvicidal activity test

Toxic effect of the extracts of *M. pudica* against the larvae of *C. pusillus* was observed and the mortality was recorded. In this case, 800 mg of extracts were taken in 2 ml of the respective solvents and used as stock solutions. One gram of whole wheat flour was taken in each Petri dish for each dose and thus treated with different doses (400, 200, 100 and 50 mg/g of flour) by serial dilution technique. The flour was mixed well with the extracts so that the solvent might dry out. Then the larvae were released in each Petri dish as well as a control with only solvent mixed with flour.

Application of doses on larvae

To conduct the test 90 mm Petri dishes were taken for all the doses and their replications. Ten larvae were released in each Petri dish as well as a control with only solvent mixed with flour. Any type of change in the developmental stages was observed from time to time including mortality.

Reading and analysis of data for larvicidal activity

The Petri dishes containing the larvae along with their treated food were kept in an incubator at 30 ± 0.5^0C. The mortality of the larvae was counted after every 24, 48 and 72 hours and recorded. The LD_{50} with 95% fiducial limits of upper confidence limit and lower confidence limit and chi-square values were calculated for extracts using log probit analysis test. The analysis was done by IBM SPSS statistical package for WINDOWS (version 22, SPSS Inc., Chicago). The results with $p<0.05$ were considered to be statistically significant. The dose-mortality relationship was expressed as median lethal dose (LD_{50}).

Repellent activity

Preparation of doses for repellent activity test

Stock solutions (20 mg/2ml) were prepared for each of the petroleum ether, chloroform, ethyl acetate and methanol extracts of leaf, stem and root. From this stock solution five doses (10.0, 5.0, 2.5, 1.25 and 0.625 mg/ml) were prepared by serial dilution technique at the proportion 1:2 from the stock solution and were used in the repellency treatments of the insects. One control was taken using only the respective solvent. Half-disc filter papers (Double Rings Filter paper No. 102) were prepared and applied the doses onto half-disc filter papers with the help of a micropipette and allowed to dry out in the air. Each treated half-disc then attached lengthwise, edge-to-edge to a control half disc with a scotch tape and placed in a Petri dish (9 cm diameter).

Application of doses to test repellent activity of *C. pusillus*

The repellency test was adopted from the method of McDonald *et al.* (1970) with some modifications by Talukder and Howse (1993). Half-disc filter papers (Double Rings Filter paper No.102, 9 cm diameter) were prepared by cutting filter paper circles in half. 20 mg of each sample extract was taken in a vial and 2 ml of the respective solvent was added to the extract. If the extract was insoluble in the respective solvent, few drops of pure DMSO were added to dissolve the extract. So the concentration of the dose became 10 mg/ml. From this dose, 1 ml was dropped to the half-disc filter paper. Then another 1 ml solvent was added to the rest 1 ml solution to give the concentration 5 mg/ml. From this dose, 1 ml was dropped to another half-disc filter paper. In the same way, 2.5 mg/ml, 1.25 mg/ml and 0.625 mg/ml doses were prepared with three replications for each dose. The half-disc filter papers with doses were left for sometimes in the air in normal room temperature to dry up the solvent. After drying the two half-discs, one with dose and the other blank, were joined by scotch tape and placed in the Petri dishes (9 cm diam.). Three half-disc filter papers were soaked with only solvent as control (C_1, C_2 and C_3). All the Petri dishes were marked and 10 adult individuals were released in the middle of each filter paper circle and Petri dishes were covered immediately and observed.

Reading and statistical analysis for repellent activity test

Insects were counted on the untreated half filter paper discs after one hour and then at hourly intervals up to five hours. No significant difference was detected between the

repellent activity of only solvent impregnated and untreated filter papers in tests designed to check for any possible influence of the solvents. Three replicates were taken and the averages of the counts were converted to 'percentage repulsion (PR)' using the formula:

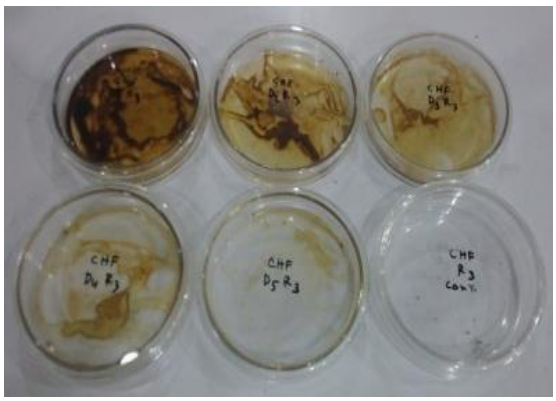
$$PR = (N_c - 5) \times 20$$

Where, N_c is the number of insects on the untreated half of the disc. Positive values expressed repellency and negative values for attractant activity.

The percentage repulsion (PR) data was again developed by arcsine transformation for calculation of analysis of variance (ANOVA). The significance of difference among the groups was assessed using one way analysis of variance. The results with $p<0.05$ were considered to be statistically significant.

Fig 46. Mass-culture of *C. pusillus*

Fig 47. *Cryptolestes pusillus* (Flat Grain Beetle)



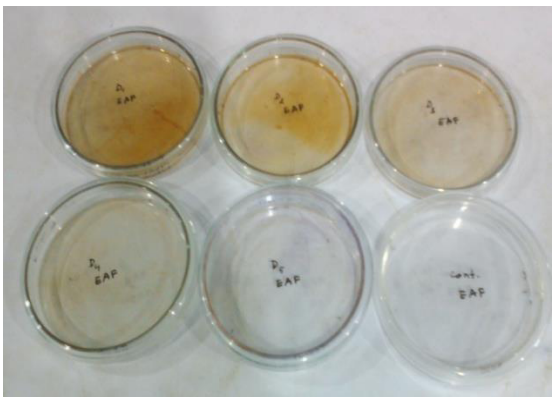


Fig 48. Insecticidal test of *M. pudica* extract

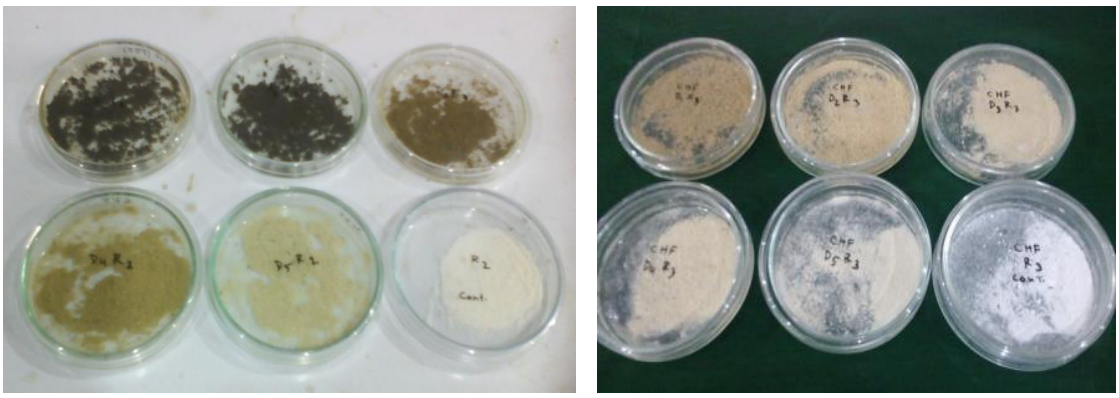


Fig 49. Larvicidal test of *M. pudica* extract

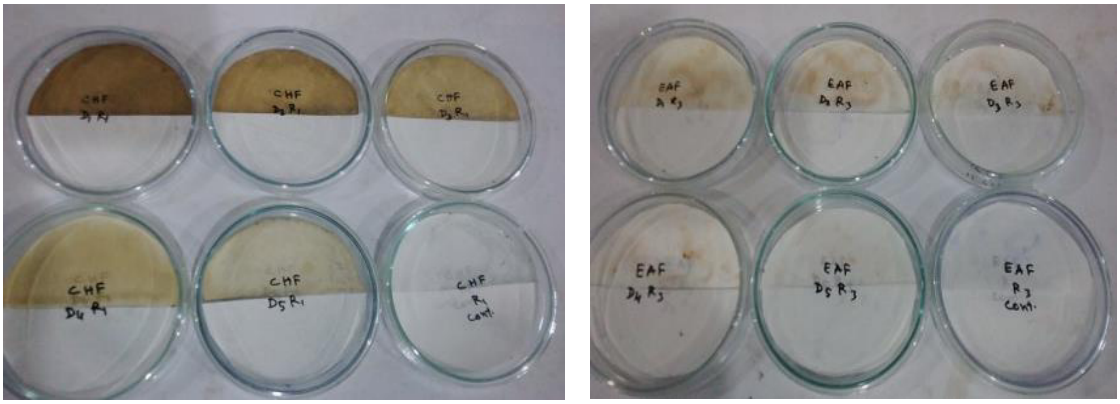


Fig 50. Repellent activity test of *C. pusillus*

Brine shrimp lethality bioassay

Collection and culture of brine shrimp nauplii (*A. salina*)

A. salina is a species within the Family Artemiidae. Its hatching rate is very high and very nice to grow. Because of its easy hatching; and easy to use in the experiment brine shrimp nauplii were used to conduct the cytotoxicity test. The brine shrimp cysts were collected from the aquarium shop of Kataban Market, Dhaka. For their easy hatching the requirements were as follows:

- Salt water: 35g NaCl was added to 1 litre of filtered pond water;
- Temperature: Room temperature (29-30^{0}C);
- Light: The beaker is placed near a window with sunlight;
- Aeration: Oxygen was supplied in the water with the help of a dropper in every hour (but a small aquarium pump with a little air-stone is better);
- Special attention: Brine shrimp eggs are sometimes very buoyant. Swirling of the water was done to knock down the eggs.

The cysts absorbed water and hatched after 24-48 hours depending on the environment. Freshly hatched *A. salina* called nauplii and have a size of just 0.25mm (0.01inch). They molt like any other crawfish. They molt about 17 times to reach up to the adult stage. Freshly hatched nauplii were used in this experiment.

Test materials:

- *A. salina* Leach (brine shrimp eggs or cysts)
- Sea salt (non-ionized NaCl)
- Beaker to hatch the shrimp
- Aerator
- Digital Weigh machine
- Lamp to attract the nauplii
- Dropper
- Micropipette (10-200µl and 1ml adjustable)
- Test tubes
- Petri dishes
- Magnifying glass
- Test tube strands

As the brine shrimp nauplii grown in salt water contains approx. 3.5% of NaCl; so 3.5% NaCl solution was prepared by dissolving 35g sodium chloride in 1 litre of filtered pond water. The p^H of the brine water thus prepared was maintained between 8 and 9 using $NaHCO_3$.

One litre of brine water was taken in a beaker and 1g of brine shrimp eggs was added; by shaking well it was kept near the window in room temperature. Sufficient oxygen supply and light were maintained. Within 24-48 hours nauplii (larvae) were formed and were collected for the experiment.

Preparation of doses for cytotoxicity test

In a glass vial 4mg extract was taken and added 100µl of pure Dimethylsulfoxide (DMSO) to make it hydrophilic. Then 10ml brine water was added to the mixture. Thus the concentration became 400µg/ml which was used as stock solution for the specific extract. Thus 12 stock solutions were prepared for the twelve extracts. From these concentrations other successive doses were prepared separately for each of the extracts through serial dilution method. Thus a series of concentrations were made from the

stock solutions *i.e.* 200, 100, 50 and 25µg/ml for each of the extracts separately. At the same time control was taken for each extract and three replications were taken.

Application of doses on *A. salina*

Different extracts of leaf, stem and root of *M. pudica* were applied against the brine shrimp nauplii. To get the nauplii, the brine shrimp eggs were hatched in simulated sea water. Ten nauplii were counted by visual inspection and were taken in every test tube containing 5ml of brine water. Then the samples of different concentrations were added to the pre-marked test tubes with the help of a micropipette. The test tubes were observed for 30 minutes, 24 hours and 48 hours and the dead nauplii were recorded.

Reading and analysis of data for cytotoxicity test

The strands with test tubes containing the nauplii along with the treated brine water were kept near the window in the laboratory. The recorded mortality was analyzed according to Finney (1947) and Busvine (1971).

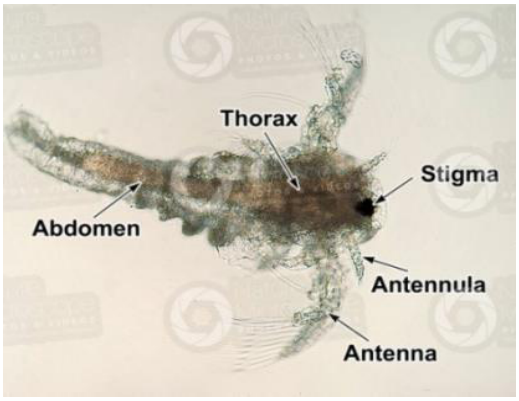


Fig 51. Brine shrimp (*Artemia salina*)

Fig 52. Hatching of brine shrimp eggs

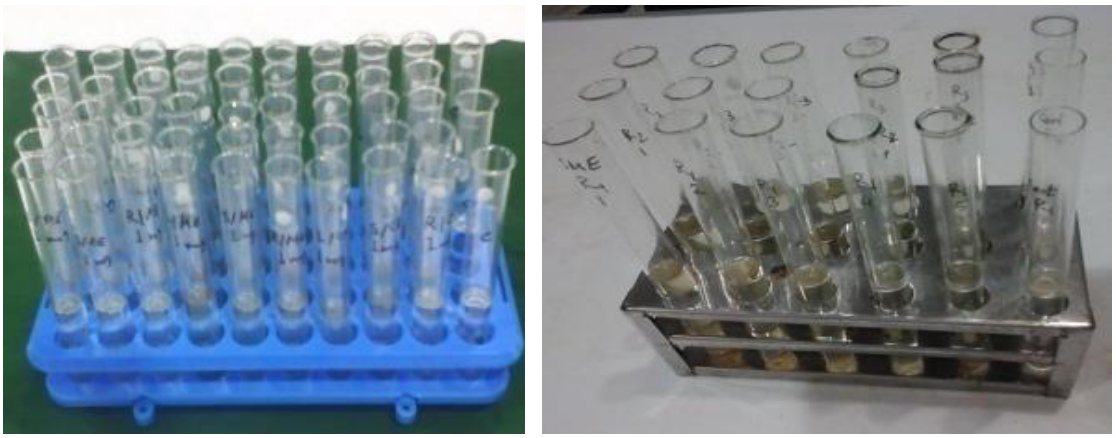


Fig 53. Brine shrimp lethality test of *A. salina*

Results

Insecticidal activity

The test was carried out by residual film method. Data was subjected to probit analysis. The extracts, duration of application, LD_{50} value, 95% confidence limits, regression equation and chi-square value with degrees of freedom were presented in tables 45-47. However, the degree of toxicity was found to be different. Chi-square values with 3 degrees of freedom indicate no heterogeneity for the dose-mortality tests done.

Table 45. LD_{50}, 95% confidence limits and regression equations of different extracts of leaf of *M. pudica* against adult *C. pusillus*

Test extracts	Time exposed (hours)	LD_{50} value (mg/cm^2)	95% confidence limits		Regression equation	χ^2 value (df)
			Lower limit	Upper limit		
Petroleum ether	24	5.560	4.296	10.250	y= -1.37+1.64x	2.300 (3)
	48	4.535	3.674	6.711	y= -1.13+1.59x	3.298 (3)
	72	3.870	3.206	5.134	y= -0.92+1.53x	4.493 (3)
Chloroform	24	2.891	2.442	3.409	y= -0.75+1.91x	5.549 (3)
	48	2.472	2.074	2.866	y= -0.48+1.87x	7.475 (3)
	72	2.190	1.854	2.503	y= -0.55+2.57x	6.779 (3)
Ethyl acetate	24	4.314	3.540	6.067	y= -1.15+1.72x	3.166 (3)
	48	3.577	2.997	4.516	y= -0.93+1.69x	4.356 (3)
	72	2.936	2.470	3.489	y= -0.67+1.71x	6.026 (3)
Methanol	24	3.456	2.898	4.313	y= -0.86+1.65x	4.892(3)
	48	2.980	2.510	3.549	y= -0.72+1.75x	5.782 (3)
	72	2.506	2.106	2.907	y= -0.52+1.91x	7.226 (3)

The effect of different doses of petroleum ether, chloroform, ethyl acetate and methanol extracts of leaf of *M. pudica* on the mortality of *C. pusillus* was shown in table 45. The highest activity was found in chloroform extract where the LD_{50} values were 2.891 mg/cm^2, 2.472 mg/cm^2 and 2.190 mg/cm^2 in 24, 48 and 72 hours of exposures respectively. The LD_{50} values of 72 hours of exposure showed significant along with 95% confidence than 48 and 24 hours of application.

Table 46. LD_{50}, 95% confidence limits and regression equations of different extracts of stem of *M. pudica* against adult *C. pusillus*

Test extracts	Time exposed (hours)	LD_{50} value (mg/cm^2)	95% confidence limits		Regression equation	χ^2 value (df)
			Lower limit	Upper limit		
Petroleum ether	24	3.809	3.196	4.891	y= -1.13+1.93x	3.299 (3)
	48	3.025	2.560	3.591	y= -0.85+1.96x	4.822 (3)
	72	2.648	2.226	3.091	y= -0.58+1.84x	6.790 (3)
Chloroform	24	3.144	2.651	3.691	y= -1.02+2.22x	3.826 (3)
	48	2.691	2.274	3.134	y= -0.71+2.04x	5.745 (3)
	72	2.354	1.987	2.706	y= -0.59+2.3x	7.021 (3)
Ethyl acetate	24	5.085	4.013	8.459	y= -1.23+1.57x	2.873 (3)
	48	4.435	3.573	6.614	y= -0.94+1.3x	4.322 (3)
	72	3.576	2.946	4.668	y= -0.58+1.08x	6.790 (3)
Methanol	24	4.210	3.477	5.784	y= -1.17+1.79x	3.014 (3)
	48	3.554	2.996	4.430	y= -1.04+1.91x	3.826 (3)
	72	2.980	2.510	3.549	y= -0.72+1.75x	5.782 (3)

The effect of similar doses of different extracts of stem of *M. pudica* on the mortality of *C. pusillus* was shown in table 46. Stem extracts were somewhat less toxic than the leaf except the petroleum ether extract. The highest activity was also found in chloroform extract where the LD_{50} values were 3.144 mg/cm^2, 2.691 mg/cm^2 and 2.354 mg/cm^2 in 24, 48 and 72 hours of exposures respectively. Also the LD_{50} values of 72 hours of exposure showed significant along with 95% confidence than 48 and 24 hours of application.

Table 47. LD_{50}, 95% confidence limits and regression equations of different extracts of root of *M. pudica* against adult *C. pusillus*

Test extracts	Time exposed (hours)	LD_{50} value (mg/cm^2)	95% confidence limits		Regression equation	χ^2 value (df)
			Lower limit	Upper limit		
Petroleum ether	24	3.119	2.638	3.732	y= -0.87+1.92x	4.873 (3)
	48	2.722	2.289	3.193	y= -0.6+1.79x	7.009 (3)
	72	2.241	1.895	2.566	y= -0.57+2.5x	7.256 (3)
Chloroform	24	2.548	2.151	2.948	y= -0.65+2.12x	6.142 (3)
	48	2.218	1.879	2.534	y= -0.6+2.61x	6.782 (3)
	72	2.024	1.730	2.292	y= -0.74+3.49x	6.574 (3)
Ethyl acetate	24	3.805	3.154	5.011	y= -0.88+1.48x	4.632 (3)
	48	3.081	2.579	3.729	y= -0.62+1.48x	6.470 (3)
	72	2.611	2.192	3.046	y= -0.54+1.8x	7.039 (3)
Methanol	24	4.649	3.705	7.274	y= -0.96+1.27x	4.508 (3)
	48	3.765	3.103	5.000	y= -0.7+1.26x	5.638 (3)
	72	3.184	2.662	3.897	y= -0.64+1.44x	6.569 (3)

The effect of contact poisoning of root extracts showed the highest activity among the leaf, stem and root of *M. pudica* against *C. pusillus* to all the three exposures of application except methanol extract which was shown in table 47. Among the root extracts, the highest activity was also found in chloroform extract where the LD_{50} values were 2.548 mg/cm^2, 2.218 mg/cm^2 and 2.024 mg/cm^2 in 24, 48 and 72 hours of exposures respectively. The recorded LD_{50} values of 72 hours of exposure observed higher mortality along with 95% confidence than 48 and 24 hours of application.

Table 48. LD_{50} values of different solvent extracts of leaf, stem and root of *M. pudica* in 24h, 48h and 72h of exposure

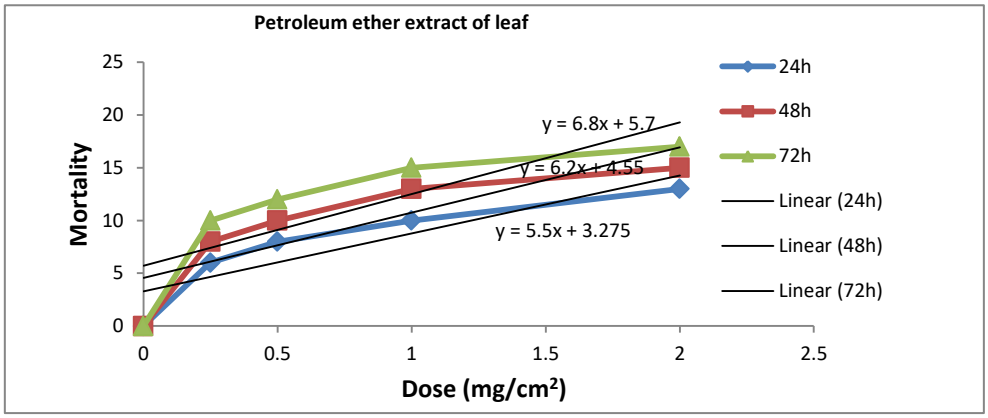
Test extracts	24 hour			48 hour			72 hour		
	LD_{50} value (mg/cm^2)			LD_{50} value (mg/cm^2)			LD_{50} value (mg/cm^2)		
	Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root
Petroleum ether	5.560	3.809	3.119	4.535	3.025	2.722	3.870	2.648	2.241
Chloroform	2.891	3.144	2.548	2.472	2.691	2.218	2.190	2.354	2.024
Ethyl acetate	4.314	5.085	3.805	3.577	4.435	3.081	2.936	3.576	2.611
Methanol	3.456	4.210	4.649	2.980	3.554	3.765	2.506	2.980	3.184

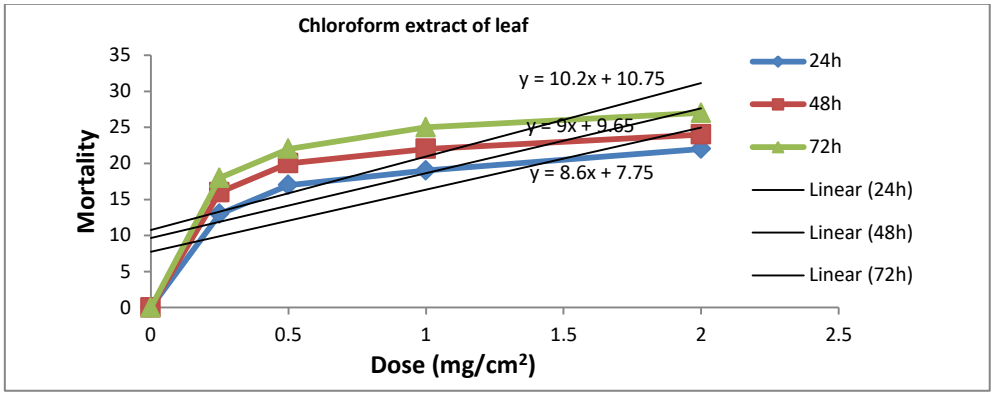
Among the extracts of leaf, stem and root, the chloroform extracts of root showed the highest activity. In 24 hours, the lowest LD_{50} value was 2.548 mg/cm^2 for chloroform extract of root and the highest was 5.560 mg/cm^2 for petroleum ether extract of leaf. In case of 48 hours, the lowest LD_{50} value was 2.218 mg/cm^2 for chloroform extract of root and the highest was 4.535 mg/cm^2 also for petroleum ether extract of leaf. And that of 72 hours, the lowest LD_{50} value was 2.024 mg/cm^2 also for the chloroform extract of root and the highest was 3.870 mg/cm^2 for petroleum ether extract of leaf (table 48). The exposure wise dose mortality order was 72h > 48h > 24h and the susceptibility order of the extracts was root > leaf > stem.

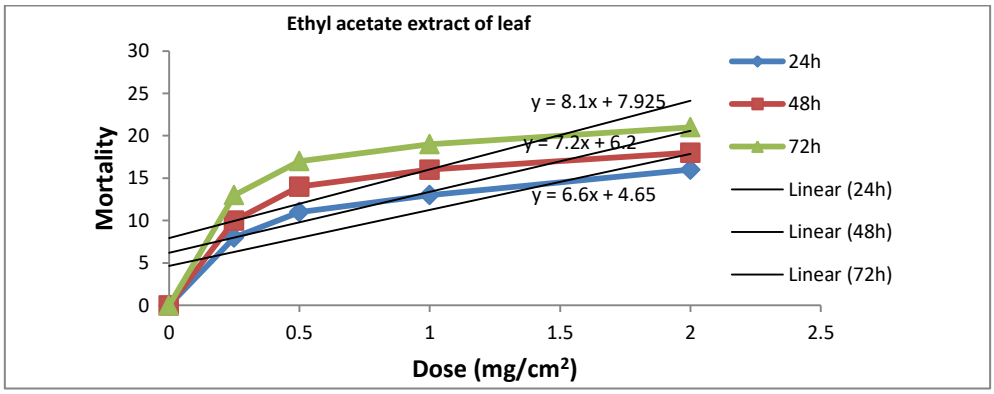
Table 49. % Mortality of *C. pusillus* for different extracts of *M. pudica*

Exposure (h)	Dose (mg/cm^2)	Kill (%)											
		Petroleum ether			Chloroform			Ethyl acetate			Mehtanol		
		Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root
24	0.25	20.0	30.0	40.0	43.3	36.7	50.0	26.7	23.3	33.3	36.7	26.7	30.0
	0.50	26.7	40.0	50.0	56.7	50.0	63.3	36.7	30.0	43.3	46.7	36.7	33.3
	1.00	33.3	50.0	60.0	63.3	63.3	73.3	43.3	36.7	50.0	53.3	46.7	40.0
	2.00	43.3	60.0	70.0	73.3	70.0	80.0	53.3	46.7	56.7	63.3	53.3	50.0
48	0.25	26.7	40.0	50.0	53.3	46.7	60.0	33.3	30.0	43.3	43.3	33.3	36.7
	0.50	33.3	53.3	56.7	66.7	60.0	70.0	46.7	36.7	53.3	53.3	43.3	43.3
	1.00	43.3	63.3	66.7	73.3	70.0	83.3	53.3	43.3	60.0	63.3	53.3	50.0
	2.00	50.0	70.0	76.7	80.0	76.7	90.0	60.0	50.0	66.7	70.0	63.3	56.7
72	0.25	33.3	50.0	60.0	60.0	56.7	66.7	43.3	40.0	50.0	53.3	43.3	43.3
	0.50	40.0	60.0	70.0	73.3	66.7	76.7	56.7	46.7	63.3	63.3	53.3	50.0
	1.00	50.0	70.0	80.0	83.3	76.7	90.0	63.3	53.3	70.0	73.3	63.3	56.7
	2.00	56.7	76.7	90.0	90.0	86.7	96.7	70.0	56.7	76.7	80.0	70.0	66.7

Among the different solvent extracts, the highest percentages of mortality were found in chloroform extracts. The percentages of mortality for chloroform extracts of leaf, stem and root were 73.3%, 70.0%, 80.0%; 80.0%, 76.7%, 90.0% and 90.0%, 86.7%, 96.7% in 24, 48 and 72 hours of exposures respectively (table 49).







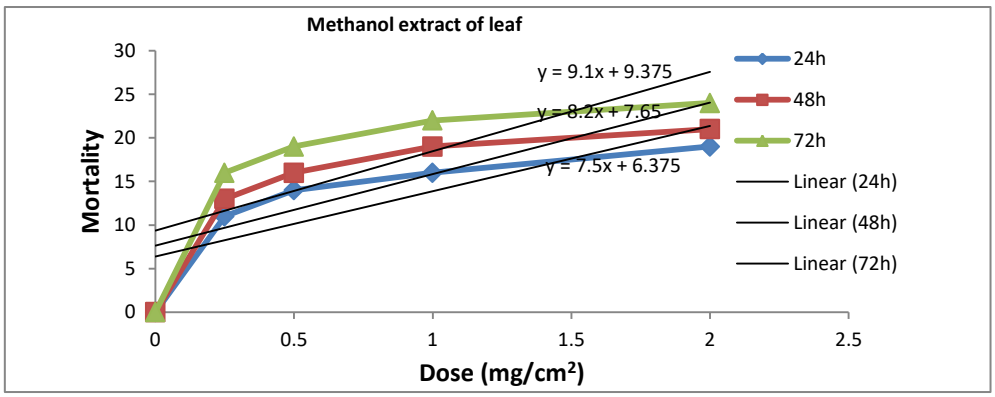


Fig 54. Regression lines between dose and mortality of *C. pusillus* of different solvent extracts of leaf of *M. pudica* after 24h, 48h and 72h of exposures

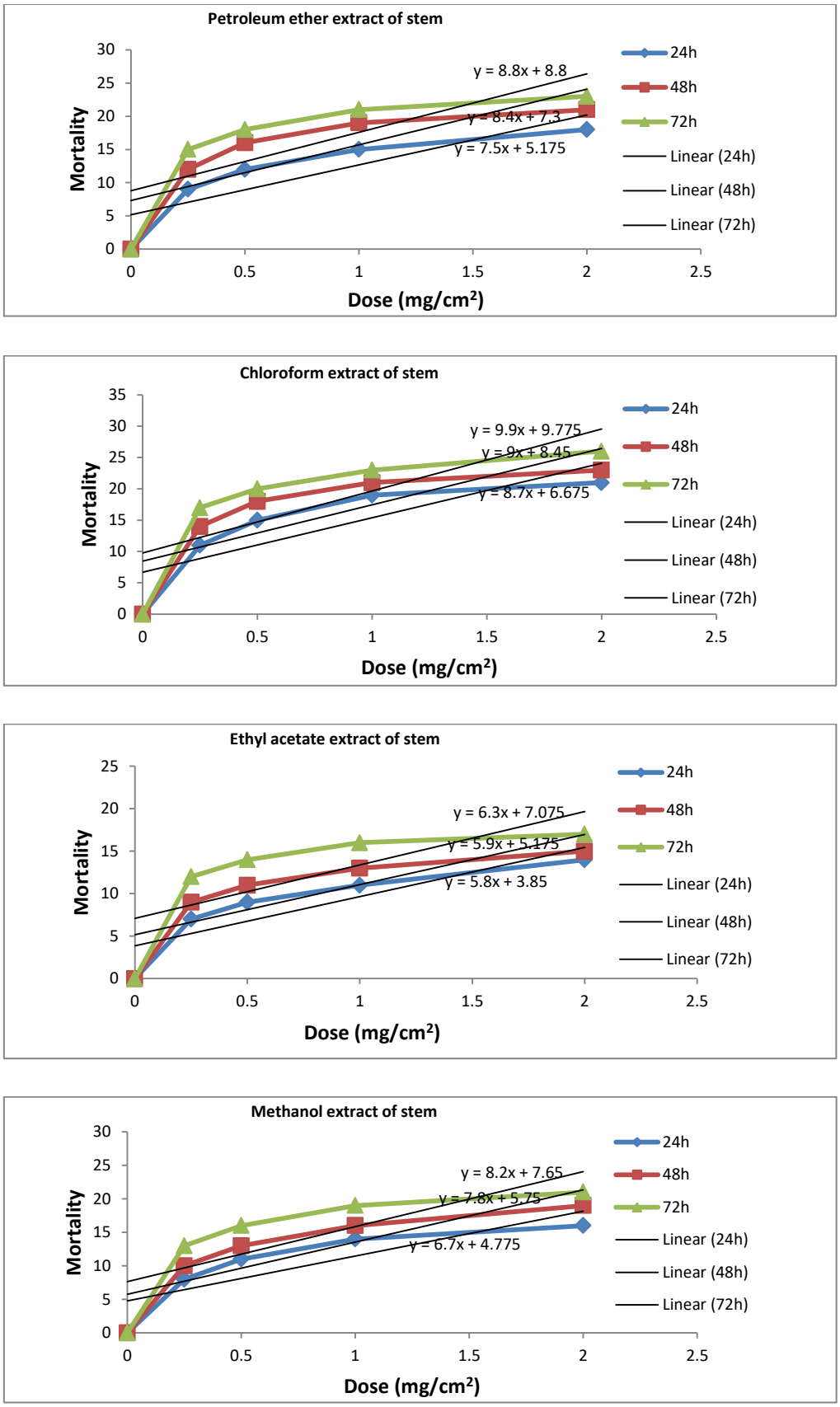


Fig 55. Regression lines between dose and mortality of *C. pusillus* of different solvent extracts of stem of *M. pudica* after 24h, 48h and 72h of exposures

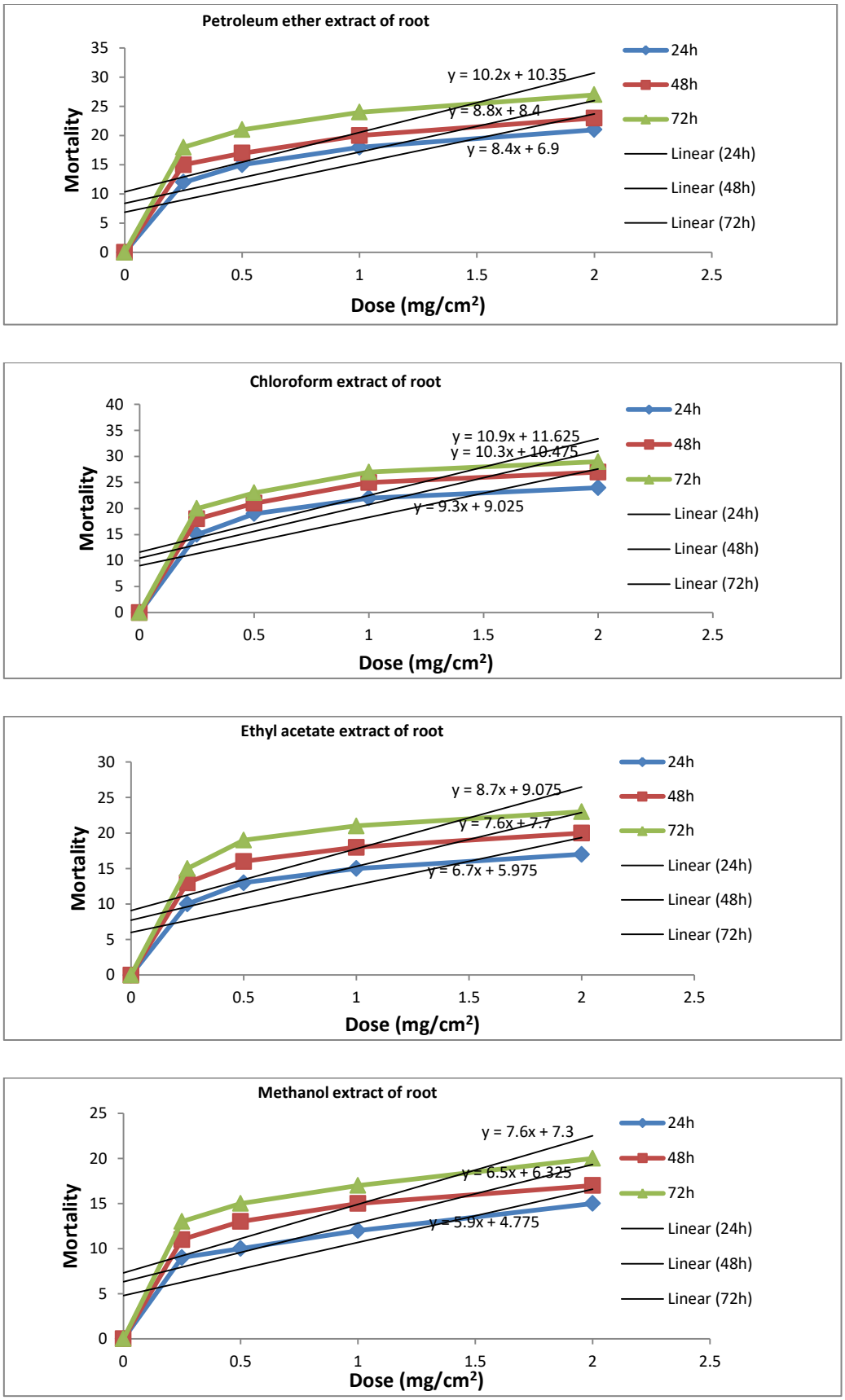


Fig 56. Regression lines between dose and mortality of *C. pusillus* of different solvent extracts of root of *M. pudica* after 24h, 48h and 72h of exposures

Larvicidal activity

The larvicidal activity test was carried out for petroleum ether, chloroform, ethyl acetate and methanol extracts of leaf, stem and root of *M. pudica.* Very poor effectivity was observed for the extracts and sometimes no effectivity was found. In some cases, after three-four days of experiment the larvae formed cocoons within the treatments.

Insecticidal test of pure compounds

The dose-mortality test was carried out by residual film method and the data were subjected to probit analysis.

Table 50. LD_{50}, 95% confidence limits and regression equations of pure compounds 9-Octadecenamide and 13-Docosenamide, (Z)- against adult *C. pusillus*

Compound	Time exposed (hours)	LD_{50} value (mg/cm^2)	95% confidence limits		Regression equation	χ^2 value (df)
			Lower limit	Upper limit		
9-Octadecenamide	24	5.069	4.043	8.157	y= -1.41+1.85x	2.079 (3)
	48	3.770	3.192	4.739	y= -1.31+2.25x	2.468 (3)
	72	3.257	2.772	3.903	y= -1.09+2.22x	4.079 (3)
13-Docosenamide, (Z)-	24	1.915	1.651	2.154	y= -0.47+3.2x	6.545 (3)
	48	1.761	1.521	1.978	y= -0.17+3.24x	7.706 (3)
	72	1.634	0.002	2.637	y= 0.91+1.25x	10.922 (3)

The effect of 9-Octadecenamide of chloroform extract of leaf and 13-Docosenamide, (Z)- of petroleum ether extract of leaf of *M. pudica* on the mortality of adult *C. pusillus* was shown in the table 50. In case of 9-Octadecenamide the LD_{50} values were 5.069 mg/cm^2, 3.770 mg/cm^2 and 3.257 mg/cm^2 and that of for 13-Docosenamide, (Z)- were 1.915 mg/cm^2, 1.761 mg/cm^2 and 1.634 mg/cm^2 in 24h, 48h and 72h of exposures respectively. Obviously 13-Docosenamide, (Z)- was more potent for insecticidal activity.

Table 51. % Mortality of *C. pusillus* for 9-Octadecenamide and 13-Docosenamide,(Z)- from *M. pudica*

Exposure (hour)	Dose (mg/cm^2)	Kill (%)			
		9-Octadecenamide		13-Docosenamide, (Z)-	
		No of death	%	No of death	%
24	0.25	6	20.0	21	70.0
	0.50	9	30.0	25	83.3
	1.00	11	36.7	28	93.3
	2.00	14	46.7	30	100.0
48	0.25	8	26.7	24	80.0
	0.50	12	40.0	27	90.0
	1.00	16	53.3	29	96.7
	2.00	18	60.0	30	100.0
72	0.25	11	36.7	27	90.0
	0.50	13	43.3	28	93.3
	1.00	18	60.0	30	100.0
	2.00	21	70.0	30	100.0

The table 51 showed the percent mortality of adult *C. pusillus* for 9-Octadecenamide of chloroform extract of leaf and 13-Docosenamide, (Z)- of petroleum ether extract of leaf of *M. pudica.* The percent mortality was higher for 13-Docosenamide, (Z)- than that of 9-Octadecenamide and the mortality was dose and time dependant *i.e* the mortality increased with increasing dose and time.

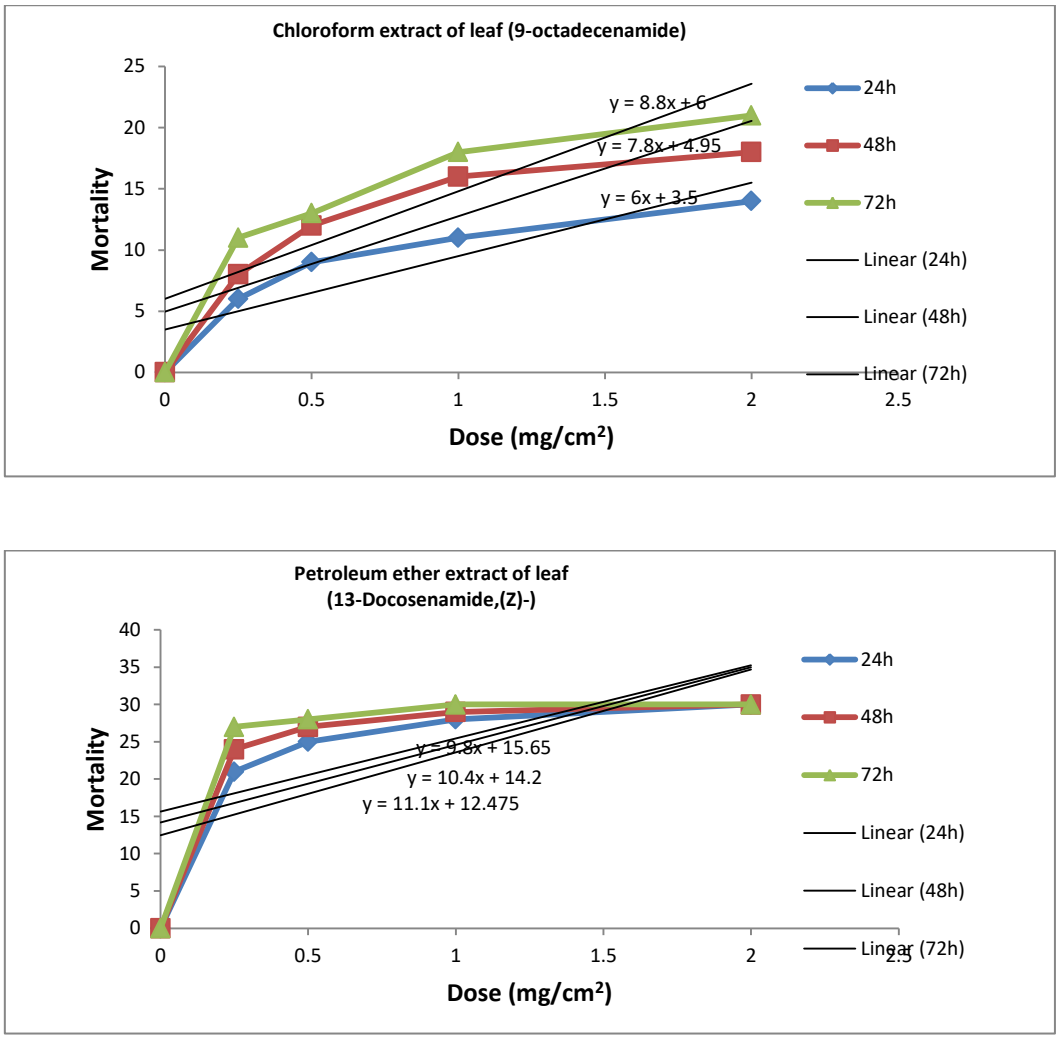


Fig 57. Regression lines between dose and mortality of *C. pusillus* for 9-octadecenamide of chloroform extract of leaf and 13-Docosenamide, (Z)- of petroleum ether extract of leaf of *M. pudica* after 24h, 48h and 72h of exposures

Repellent activity

The repellent activity test was performed with doses of 314.56, 157.28, 78.64, 39.32 and 19.66 µg/cm^2 on half-disc filter papers. The data was recorded with one hour interval for up to five consecutive hours of exposure and the percentage repulsion data was then subjected to ANOVA after transforming into arcsine percentage values.

Among the extracts tested the petroleum ether extract of root offered the highest repellent activity at 0.1% ($P<0.001$) level of significance. According to the intensity of repellent activity the result could be arranged in a descending order: Root (petroleum ether) > Leaf (petroleum ether) > Stem (ethyl acetate).

The following results were found during repellent activity test of *C. pusillus* with extracts of different parts of *M. pudica* using different solvents.

Table 52. ANOVA results for repellent activity of leaf extracts of *M. pudica* against adult *C. pusillus*

Test material	Extracts	Source of Variation	SS	df	MS	F	P-value	F crit
Leaf	Petroleum ether	Dose	1685.274	4	421.3184	49.74491	7.91E-09	3.006917
		Time	578.5058	4	144.6265	17.07599	1.26E-05	3.006917
		Error	135.5133	16	8.469579			
		Total	2399.293	24				
	Chloroform	Dose	1821.602	4	455.4006	13.11165	6.36E-05	3.006917
		Time	332.332	4	83.083	2.392082	0.093914	3.006917
		Error	555.7202	16	34.73251			
		Total	2709.655	24				
	Ethyl acetate	Dose	1651.455	4	412.8639	19.99361	4.57E-06	3.006917
		Time	643.0277	4	160.7569	7.784918	0.001107	3.006917
		Error	330.3966	16	20.64979			
		Total	2624.88	24				
	Methanol	Dose	1280.406	4	320.1015	46.44371	1.31E-08	3.006917
		Time	451.473	4	112.8683	16.37612	1.64E-05	3.006917
		Error	110.2759	16	6.892247			
		Total	1842.155	24				

The tables showed that the F-values were 49.74491, 13.11165, 19.99361 and 46.44371 for the analysis between doses and 17.07599, 2.392082, 7.784918 and 16.37612 for the analysis between time interval for petroleum ether, chloroform, ethyl acetate and methanol extracts of leaf respectively (table 52).

Table 53. ANOVA results for repellent activity of stem extracts of *M. pudica* against adult *C. pusillus*

Test material	Extracts	Source of Variation	SS	df	MS	F	P-value	F crit
Stem	Petroleum ether	Dose	1472.515	4	368.1288	13.4846	5.38E-05	3.006917
		Time	1052.202	4	263.0505	9.635567	0.000365	3.006917
		Error	436.7991	16	27.29995			
		Total	2961.516	24				
	Chloroform	Dose	1800.978	4	450.2444	9.968747	0.000303	3.006917
		Time	685.2142	4	171.3035	3.792788	0.023581	3.006917
		Error	722.6496	16	45.1656			
		Total	3208.841	24				
	Ethyl acetate	Dose	1855.095	4	463.7739	36.56298	7.34E-08	3.006917
		Time	925.2233	4	231.3058	18.23568	8.29E-06	3.006917
		Error	202.9479	16	12.68425			
		Total	2983.267	24				
	Methanol	Dose	1675.989	4	418.9973	20.1373	4.37E-06	3.006917
		Time	1093.728	4	273.4319	13.14133	6.27E-05	3.006917
		Error	332.9124	16	20.80702			
		Total	3102.629	24				

In case of stem, the F-values were 13.4846, 9.968747, 36.56298 and 20.1373 for the analysis between doses and 9.635567, 3.792788, 18.23568 and 13.14133 for the analysis between time interval for petroleum ether, chloroform, ethyl acetate and methanol extracts respectively (table 53).

Table 54. ANOVA results for repellent activity of root extracts of *M. pudica* against adult *C. pusillus*

Test material	Extracts	Source of Variation	SS	df	MS	F	P-value	F crit
Root	Petroleum ether	Dose	1547.642	4	386.9106	189.6542	2.93E-13	3.006917
		Time	681.239	4	170.3098	83.48169	1.65E-10	3.006917
		Error	32.64136	16	2.040085			
		Total	2261.523	24				
	Chloroform	Dose	1126.007	4	281.5017	66.00051	9.71E-10	3.006917
		Time	530.9797	4	132.7449	31.1232	2.29E-07	3.006917
		Error	68.2423	16	4.265144			
		Total	1725.229	24				
	Ethyl acetate	Dose	1278.837	4	319.7093	102.7792	3.37E-11	3.006917
		Time	581.417	4	145.3543	46.72803	1.25E-08	3.006917
		Error	49.7703	16	3.110644			
		Total	1910.025	24				
	Methanol	Dose	1000.317	4	250.0793	127.6997	6.34E-12	3.006917
		Time	526.9535	4	131.7384	67.27046	8.42E-10	3.006917
		Error	31.33342	16	1.958339			
		Total	1558.604	24				

For the root, the F-values were 189.6542, 66.00051, 102.7792 and 127.6997 for the analysis between doses and 83.48169, 31.1232, 46.72803 and 67.27046 for the analysis between time interval for petroleum ether, chloroform, ethyl acetate and methanol extracts respectively (table 54).

Brine shrimp lethality bioassay

The petroleum ether, chloroform, ethyl acetate and methanol extracts of leaf, stem and root of *M. pudica* were subjected to apply against brine shrimp nauplii (*A. salina*) for the detection of their biological activity. Several doses were selected by a pilot experiment and a final experiment was set up with three replications and control doses. The rates of mortality increased with the increase of concentrations of crude extracts. The LC_{50} values along with 95% confidence limits, regression equations and chi-squared value of the crude extracts of different solvents of leaf, stem and root for 30 minutes, 24 hours and 48 hours of exposure were determined using probit analysis (McLaughlin 1990). The results were shown in tables 55-58.

Table 55: LC_{50}, 95% confidence limits and regression equations of *M. pudica* leaf extracts against *A. salina*

Test extracts	Time exposed	LC_{50} value (µg/ml)	95% confidence limits		Regression equation	χ^2 value (df)
			Lower limit	Upper limit		
Petroleum ether	30 min	9.808	-	-	-	0.208 (3)
	24h	2.632	2.210	3.102	y= -1.28+3.11x	2.249 (3)
	48h	1.464	1.016	1.830	y= -0.48+2.83x	2.553 (3)
Chloroform	30 min	-	-	-	-	-
	24h	4.184	3.377	6.024	y= -1.5+2.42x	0.259 (3)
	48h	1.985	1.575	2.374	y= -0.86+2.99x	4.150 (3)
Ethyl acetate	30 min	-	-	-	-	-
	24h	3.505	2.923	4.440	y= -1.51+2.77x	0.640 (3)
	48h	1.891	1.513	2.242	y= -0.87+3.2x	1.335 (3)
Methanol	30 min	-	-	-	-	-
	24h	4.096	3.269	6.021	y= -1.35+2.2x	0.849 (3)
	48h	2.244	1.809	2.684	y= -0.97+2.82x	1.270 (3)

From table 55 it was found that the LC_{50} values of leaf were 2.632 and 1.464 µg/ml for petroleum ether, 4.184 and 1.985 µg/ml for chloroform, 3.505 and 1.891 µg/ml for ethyl acetate and 4.096 and 2.244 µg/ml for methanol extract in 24h and 48h respectively. The highest activity was found in petroleum ether extract for both in 24h and 48h exposure and the lowest activity was recorded in chloroform extract in 24h and methanol extract in 48h exposure. According to the intensity of activity in 24h the result of the extracts of leaf of *M. pudica* against the brine shrimp nauplii could be arranged in the following order: petroleum ether > ethyl acetate > methanol > chloroform.

Table 56: LC_{50}, 95% confidence limits and regression equations of *M. pudica* stem extracts against *A. salina*

Test extracts	Time exposed	LC_{50} value (µg/ml)	95% confidence limits		Regression equation	χ^2 value (df)
			Lower limit	Upper limit		
Petroleum ether	30 min	-	-	-	-	-
	24h	2.475	2.091	2.872	y= -1.35+3.45x	3.468 (3)
	48h	1.457	1.052	1.794	y= -0.49+2.98x	1.987 (3)
Chloroform	30 min	-	-	-	-	-
	24h	2.076	1.659	2.466	y= -1.04+3.12x	3.823 (3)
	48h	1.282	0.916	1.583	y= -0.33+3.08x	0.027 (3)
Ethyl acetate	30 min	-	-	-	-	-
	24h	2.758	2.230	3.413	y= -1.1+2.5x	0.025 (3)
	48h	1.414	0.992	1.761	y= -0.4+2.69x	0.268 (3)
Methanol	30 min	-	-	-	-	-
	24h	3.178	2.540	4.192	y= -1.17+2.31x	1.080 (3)
	48h	1.520	0.968	1.959	y= -0.39+2.13x	0.215 (3)

Table 56 showed that, the LC_{50} values of stem were 2.475 and 1.457 µg/ml for petroleum ether, 2.076 and 1.282 µg/ml for chloroform, 2.758 and 1.414 µg/ml for ethyl acetate and 3.178 and 1.520 µg/ml for methanol extract in 24h and 48h respectively. The highest activity was found in chloroform extract and the lowest activity was recorded in methanol extract for both in 24h and 48h exposure. According to the intensity of activity in 24h the result of the extracts of stem of *M. pudica* against the brine shrimp nauplii could be arranged in the following order: chloroform > petroleum ether > ethyl acetate > methanol.

Table 57: LC_{50}, 95% confidence limits and regression equations of *M. pudica* root extracts against *A. salina*

Test extracts	Time exposed	LC_{50} value (µg/ml)	95% confidence limits		Regression equation	χ^2 value (df)
			Lower limit	Upper limit		
Petroleum ether	30 min	-	-	-	-	-
	24h	1.929	1.603	2.230	y= -1.25+4.11x	3.910 (3)
	48h	1.202	0.915	1.443	y= -0.27+3.64x	0.670 (3)
Chloroform	30 min	-	-	-	-	-
	24h	1.972	0.702	2.958	y= -1.18+3.68x	7.069 (3)
	48h	1.463	1.148	1.737	y= -0.54+3.28x	4.340 (3)
Ethyl acetate	30 min	-	-	-	-	-
	24h	2.723	2.274	3.238	y= -1.31+3.03x	0.092 (3)
	48h	1.800	1.459	2.111	y= -0.95+3.7x	1.597 (3)
Methanol	30 min	-	-	-	-	-
	24h	2.712	2.201	3.325	y= -1.12+2.57x	0.574 (3)
	48h	2.043	1.656	2.410	y= -0.98+3.18x	0.333 (3)

Table 57 showed that the LC_{50} values of root were 1.929 and 1.202 µg/ml for petroleum ether, 1.972 and 1.463 µg/ml for chloroform, 2.723 and 1.800 µg/ml for ethyl acetate and 2.712 and 2.043 µg/ml for methanol extract in 24h and 48h respectively. The highest activity was found in petroleum ether extract for both in 24h and 48h exposure and the lowest activity was recorded in ethyl acetate extract in 24h and methanol extract in 48h exposure. According to the intensity of activity in 24h the result of the extracts of root of *M. pudica* against the brine shrimp nauplii could be arranged in the following order: petroleum ether > chloroform > methanol > ethyl acetate.

Table 58: LC_{50} values of different solvents of leaf, stem and root extracts of *M. pudica* against *A. salina* in 24h and 48h

Test extracts	24 hour			48 hour		
	Leaf LC_{50} value (µg/ml)	Stem LC_{50} value (µg/ml)	Root LC_{50} value (µg/ml)	Leaf LC_{50} value (µg/ml)	Stem LC_{50} value (µg/ml)	Root LC_{50} value (µg/ml)
Petroleum ether	2.632	2.475	1.929	1.464	1.457	1.202
Chloroform	4.184	2.076	1.972	1.985	1.282	1.463
Ethyl acetate	3.505	2.758	2.723	1.891	1.414	1.800
Methanol	4.096	3.178	2.712	2.244	1.520	2.043

Table 58 showed the LC_{50} values of the extracts of leaf, stem and root of *M. pudica* against brine shrimp nauplii in 24 and 48 hours. Among all the extracts the highest activity was found in petroleum ether extract of root, where the LC_{50} values were 1.929 µg/ml and 1.202 µg/ml in 24 and 48 hours respectively. On the other hand the lowest activity was found in chloroform and methanol extracts of leaf, where the LC_{50} values were 4.184 µg/ml and 2.244 µg/ml in 24 and 48 hours respectively.

The study showed that the crude extracts of the plant *M. pudica* were significantly effective and lethal to the brine shrimp nauplii (*A.* salina) indicating that the extracts were biologically active.

Discussion

Insecticidal activity

From the result it was found that the LD_{50} values were time dependent *i.e.* if the exposure time increased, the activity of the extracts also increased. Though the chloroform extracts showed the highest potentiality, but all the extracts of leaf, stem and root showed more or less similar potentiality.

Waliullah *et al.* (2014) tested the insecticidal activity of root, leaf and stem of *Clerodendrum viscosum* vent. (Verbenaceae) against *S. oryzae* (Coleoptera: Curculionidae). The ethyl alcohol extracts of root and stem showed the highest percent mortality (44-84%) and the leaf extract showed moderate percent mortality (32-72%) at 72h of exposure. Whereas, at 24h of exposure the highest percent mortality was found for root extract (40-80%) and the stem and leaf showed 36-76% and 28-60% respectively. They also revealed the insecticidal activity of ethyl acetate and chloroform fractions of root, leaf and stem of *C. viscosum.* The ethyl acetate fraction of root and leaf showed the highest percent mortality (40-80%) and the stem showed 48-76% percent mortality at 72h of exposure; whereas, at 24h of exposure the percent mortality of root, leaf and stem of *C. viscosum* was found 28-68%, 28-56% and 32-60% respectively. In case of chloroform fraction, the highest percent mortality was shown by root extract (24-56%) and that of the stem and leaf were 20-52% and 20-44% respectively at 72h of exposure. Whereas, at 24h of exposure, the chloroform fraction of root, stem and leaf showed 16-48%, 16-40% and 12-28% mortality respectively. Among the three kinds of fractions the root extracts showed most potency. Though the ethyl alcohol extracts of root and stem showed the equal percentage mortality (44-84%) at 72h of exposure, the LC_{50} of root extract (0.39 mg/ml) was more effective than that of stem extract (0.43 mg/ml). In the present study, the chloroform extracts showed the highest percent mortality among the petroleum ether, chloroform, ethyl acetate and methanol extracts of *M. pudica* and the root extract showed the highest percent mortality among the extracts of root, leaf and stem. In 72h of exposure the percent mortality for chloroform extracts were 66.7-96.7%, 60-90% and 56.7-86.7% and that of ethyl acetate extracts were 50-76.7%, 43.3-70% and 40-56.7% for root, leaf and stem extracts respectively.

According to Waliullah *et al.* (2014) the susceptibility of contact toxicity in intensity of solvents were found as ethyl alcohol > ethyl acetate > chloroform and the dose

mortality order of *C. viscosum* extracts were root > stem > leaf against *S. oryzae.* Yankanchi and Gadache (2010) reported the similar findings with this study. In the present study, the susceptibility of contact toxicity in intensity of solvents were found as chloroform > petroleum ether > methanol > ethyl acetate and the dose mortality order of *M. pudica* extracts were root > leaf > stem against *C. pusillus.*

The root extract of *M. diplotricha* showed a high mortality for *Macrotermes* species and the mortality were observed to be concentration and exposure time dependent. The highest concentration [10% (w/v)] of root extract of *M. diplotricha* accounted for 100% mortality against *Macrotermes* species after a 36 hour exposure period. Following a 36 hour exposure period, the median lethal concentration (LC_{50}) of *M. diplotricha* against the termites was 4.12% (w/v) (Uyi *et al.* 2018). In our study we found the related results *i.e.* root extracts were most effective where the highest percent mortality (96.7%) was observed in72 hour ofexposure.

The leaf and stem powders of *M. diplotricha* plant exhibited some degrees of mortality against *S. zeamais* Motschulsky which was also both concentration and exposure time dependent. At the highest concentration (3.5g), the leaf and stem powders of *M. diplotricha* accounted for only 28% mortality of *S. zeamais* after a five hour exposure period (Uyi and Samugana 2018). In present study, the chloroform extracts showed the highest percent mortality among the petroleum ether, chloroform, ethyl acetate and methanol extracts of leaf, stem and root; and the root extracts were the most potential in all three exposure periods (24, 48 and 72 hours).

Larvicidal activity

Astalakshmi *et al.* (2016) depicted that the *M. pudica* leaf aqueous extract was not suitable for larvicidal properties against *Aedes aegypti.* In the present experiment, more or less similar results were observed against the larvicidal effectivity of *C. pusillus* (Schon.) for different crude extracts of leaf, stem and root of *M. pudica.* Due to lack of available data on larvicidal activity of *C. pusillus* the comparison on this event was limited.

Repellent activity

The present research showed that the repellent activity differed in the extracts of leaf, stem and root of *M. pudica* where the root extracts revealed a strong repellent activity for both dose and time effect at $p<0.01$ level of significance against *C. pusillus.* In fact, the reports on the repellent effects of extracts of *M. pudica* against the insect pest *C. pusillus* were not available.

Uyi and Samugana (2018) revealed that the leaf and stem powders of *M. diplotricha* exhibited some degrees of repellent activity against *S. zeamais* Motschulsky (Coleoptera: Curculionidae), although, the repellent activity was a function of both concentration and exposure time. After a 3 hour exposure period, the 3.5g of the leaf (53%) and stem (58%) powders exhibited the highest repellent activity against *S. zeamais*. Uyi *et al.* (2018) denoted that the highest concentration [10% (w/v)] of the root extract of *M. diplotricha* significantly repelled 100% of *Macrotermes* species following a 30 min. exposure period. In the present investigation, the root extracts possessed the higher potentiality (highest 90% repellency for petroleum ether extract of root, appendix table 68) than leaf or stem extracts.

Waliullah *et al.* (2014) carried out the repellent activity using the ethyl alcohol fraction of root, leaf and stem of *C. viscosum.* The result was found to be 100% repellency (class V category repellency) for the higher doses. The degree of repellency was significantly ($p<0.05$) different for root, stem and leaf. The strong repellent activity was observed in root extract of the plant. This result was supported with the observation of Husain and Rahman (2006) and Husain and Hasan (2008) who performed both the toxicity and repellent activity test on larvae and adults of *T. confusum* and a correlation was observed between the insecticidal and repellent activity. The present study also showed a close agreement with repellent activity for the extracts of root of *M. pudica* ($p<0.01$).

Brine shrimp lethality bioassay

Brine shrimp lethality test is performed to investigate the presence of bioactive compounds of a test material. When the effectivity is observed on the brine shrimp nauplii, it indicates the lethality and pharmacological activities of the plant extract (Persoone *et al.* 1980 and Mayer *et al*. 1982). Therefore, in the present study, the result of mortality of brine shrimp nauplii by the crude extracts of *M. pudica* indicated that the plant extracts contained bioactive compounds that possessed antimicrobial as well as insecticidal properties.

Chowdhury *et al.* 2008 showed that the petroleum ether and methanol crude extracts of root of *M. pudica* showed potential cytotoxic activities where LC_{50} was 0.05 µg/ml and 0.035 µg/ml respectively; whereas petroleum ether, chloroform and methanol extracts of aerial parts and chloroform extract of root showed poor cytotoxicity (LC_{50} were 23.44 µg/ml, 20.89 µg/ml, 80.0 µg/ml and 78.87 µg/ml respectively) by brine shrimp lethality bioassay. In the present study, all three parts *i.e.* root, stem and leaf of *M. pudica* showed moderate potential cytotoxic activity and the petroleum ether extract of

root showed the highest potentiality where LC_{50} were 1.929 µg/ml in 24h and 1.202 µg/ml in 48h of exposure. In case of 24h, the root extracts showed the higher potentiality, but in 48h, the stem extracts showed the higher potentiality except petroleum ether extract of root. In case of root and leaf, the highest activity was observed by petroleum ether extracts. But for the stem, the chloroform extract showed the highest activity where the LC_{50} values were 2.076 µg/ml and 1.282 µg/ml in 24h and 48h of exposures respectively.

Waliullah *et al.* (2015) depicted that the brine shrimp nauplii mortality were increased with the increase of concentrations of the crude extracts of *C. infortunatum* (Verbenaceae). The LC_{50} of root, leaf and stem of the tested plant were 20.85, 24.02 and 31.38 mg/L for ethanol; 30.70, 32.91 and 42.56 mg/L for chloroform respectively and 14.68 mg/L for standard drug Tetracycline. The antibiotic Tetracycline showed a good correlation with the LC_{50} of crude extracts, and the root extract of ethanol fraction was very effective. They also described that the plant extracts were biologically active and the cytotoxic evidence was emerged from the result of brine shrimp bioassay. In the present study, the LC_{50} of chloroform extracts of root, leaf and stem of *M. pudica* were 1.972, 4.184 and 2.076 µg/ml in 24h of exposures and 1.463, 1.985 and 1.282 µg/ml in 48h of exposures respectively.

Moncao *et al.* (2014) described that the ethanol extract of leaves showed low cytotoxicity against brine shrimp (*A. salina*) at the concentrations analyzed and Vieira (2005) showed that the ethanol extract of leaf from *M. caesalpiniifolia* Benth. showed no toxicity against *A. salina* where the LC_{50} was 1765 mg/L. In another study, Das *et al.* (2014) showed that the leaf extract possessed a significant lethality after 24h of exposure in brine shrimp lethality test. The LC_{50} was 282.35 µg/ml for methanolic extract of leaf. The plant showed lethality in a dose-reliant conduct and the leaf showed a significant source of anticancer compounds. They concluded that the methanolic leaf extract of *M. pudica* possessed moderate cytotoxic property. The present results showed somewhat different findings where the methanolic extract of leaf of *M. pudica* showed potential activity and the LC_{50} was 4.096 µg/ml in 24h of exposure.

SUMMARY AND CONCLUSION

Summary

In antibacterial test, among the petroleum ether, chloroform, ethyl acetate and methanol extracts of leaf of *M. pudica,* the highest value of zone of inhibition was found in chloroform extract (19.67±0.88 mm) against *E. coli* and the lowest was in petroleum ether extract (11.67±0.33 mm) against *B. cereus* for both the gram-positive and gram-negative bacterial strains. In case of stem, the highest value was found in both chloroform and ethyl acetate extracts (16.67±0.33 and 16.67±0.88 mm) against *S. aureus* and *E. coli* respectively and the lowest was in petroleum ether extract (12.00±0.58 mm) against *S. typhi*. And for the root, the chloroform extract showed the highest activity where the zone of inhibition was 21.67±1.45 mm against *S. sonnei* and the petroleum ether extract showed the lowest activity (12.00±0.58 mm) against *K. pneumoniae*. Among all the extracts of leaf, stem and root, chloroform extract showed the highest activity against both gram-positive and gram-negative bacterial strains. In comparison, the root extracts showed more potentiality in antibacterial activity as it showed the highest zone of inhibition (21.67±1.45 mm in diameter), followed by leaf (19.67±0.88 mm) and stem (16.67±0.33 mm) at 200 µg/disc concentration. Therefore, the antibacterial activity order of the plant parts was: root > leaf > stem.

The petroleum ether, chloroform, ethyl acetate and methanol extracts of leaf, stem and root were tested against four species of fungal strains *viz. R. oryzae, F. proliferatum, A. niger* and *C. albicans*. The activity was very low for the extracts of leaf and stem. But the root extracts showed moderate activities. In case of *R. oryzae* and *F. proliferatum* the highest zones of inhibition were found in ethyl acetate extracts of root that were 13mm and 12mm in diameter respectively at 200µg/disc concentration. But for *A. niger* and *C. albicans* the highest zones of inhibition were found in chloroform extract of root which were 12mm and 13mm in diameter respectively at the same concentration.

The Minimum Inhibitory Concentration (MIC) tests of bacteria were carried out with chloroform and ethyl acetate extracts of leaf against *B. cereus, S. aureus, B. zhanjiangensis, K. pneumoniae, E. coli* and *S. typhi*. The highest activity was found against *S. aureus* (32µg/ml) for chloroform extract and that of for ethyl acetate extract was 64µg/ml against *S. aureus, B. zhanjiangensis* and *E. coli*.

The pure compounds 9-Octadecenamide and 13-Docosenamide, (Z)- were tested against the bacterial strains and found considerable effects. In case of 9-Octadecenamide, the highest antibacterial activity was found against *E. coli* for gram-negative bacterial strain where the diameter of zone of inhibition was 21mm at 100µg/disc concentration and that of for gram-positive bacterial strain the zone of inhibition was 19mm against *S. aureus*. In case of 13-Docosenamide, (Z)-, also the highest antibacterial activity was found against *E. coli* (zone of inhibition was 22mm) for gram-negative bacterial strain and against *S. aureus* (21mm) for gram-positive bacterial strain at 100µg/disc concentration.

The MIC value of 9-Octadecenamide among the four bacterial strains was more effective against *S. aureus* and *E. coli* (64µg/ml) than *B. cereus* and *S. typhi* (128µg/ml). Whereas, the MIC value of 13-Docosenamide, (Z)- was more potent against *S. aureus* (32µg/ml) than other strains.

In antioxidant activity test, the ethyl acetate extracts showed the highest scavenging activity among the extracts of different solvents, where the IC_{50} values were 65.152µg/ml, 76.036µg/ml and 65.000µg/ml and the lowest scavenging activity was found in petroleum ether extracts, where the IC_{50} values were 130.129µg/ml, 147.891µg/ml and 186.449µg/ml for leaf, stem and root respectively. The IC_{50} value of the standard ascorbic acid was found 18.012µg/ml.

GC-MS analysis was carried out for petroleum ether, chloroform and ethyl acetate extracts of leaf; chloroform and ethyl acetate extracts of stem; and petroleum ether, chloroform and ethyl acetate extracts of root. Sixteen compounds were identified from petroleum ether extract of leaf. The major compounds were benzene,1-ethyl-3-methyl- (14.830%), mesitylene (13.267%), vitamin E (13.117%), nonadecane (7.248%), tetratetracontane (6.486%), benzene,1-ethyl-2-methyl- (6.305%) and benzene,1,2,4-trimethyl (5.649%). Chloroform extract of leaf showed eleven compounds where the major compounds were fumaric acid, ethyl 2-methylallyl ester (16.959%), 1-(2-[3-(2-acetyloxiran-2-yl)-1,1-dimethyl)propyl]cycloprop-2-enyl)ethanone (15.829%), 1H-pyrrole-2,5-dione, 3-ethyl-4-methyl- (14.966%), 2(4H)-benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)- (12.960%) and 6-hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one (7.907%). Three compounds were identified from ethyl acetate extract of leaf and those were glycerin (74.714%), 9-octadecenamide

(15.297%) and hexadecenoic acid, methyl ester (9.989%). Chloroform extract of stem showed 26 compounds. Among them tigloidine (80.902%) was the major compound. Three compounds were identified from ethyl acetate extract of stem; hexadecanoic acid, methyl ester (69.947%), 9-octadecenamide (15.620%) and 9-octadecenoic acid (Z)-, methyl ester (14.433%). Thirty seven compounds were identified from petroleum ether extract of root. The major compounds were identified as hexadecanoic acid, methyl ester (30.255%), methyl stearate (10.175%), 9,12-octadecadienoic acid, methyl ester (9.674%), 9-octadecenoic acid, methyl ester (E)- (7.416%), squalene (6.661%) and dl-alpha-tocopherol (6.302%). Chloroform extract of root showed forty compounds and tigloidine (56.694%) was the major compound. Through GC-MS analysis four compounds were identified from ethyl acetate extract of root and the major compounds were hexadecanoic acid, methyl ester (46.343%), diisooctyl phthalate (24.408%) and 13-docosenamide, (Z)- (21.384%).

Two pure compounds 9-Octadecenamide and 13-Docosenamide, (Z)- were isolated from partitioning of chloroform and petroleum ether extracts of crude methanolic extract of leaf of *M. pudica.*

In insecticidal activity test, the highest activity was shown by the chloroform extracts of root where the LD_{50} values were 2.548µg/cm^2, 2.218µg/cm^2 and 2.024µg/cm^2 in 24h, 48h and 72h of exposures respectively and the lowest activity was shown by the petroleum ether extracts of leaf where the LD_{50} values were 5.560µg/cm^2, 4.535µg/cm^2 and 3.870µg/cm^2 in 24h, 48h and 72h of exposures respectively. The exposure wise dose mortality was 72h > 48h > 24h and the order of dose-mortality effect of *M. pudica* extracts was: root > leaf > stem.

The LD_{50} values of 9-Octadecenamide were 5.069mg/cm^2, 3.770mg/cm^2 and 3.257mg/cm^2 and that of for 13-Docosenamide, (Z)- were 1.915mg/cm^2, 1.761mg/cm^2 and 1.634mg/cm^2 in 24h, 48h and 72h of exposures respectively. Obviously 13-Docosenamide, (Z)- was more potent for insecticidal activity. The percent mortality was higher for 13-Docosenamide, (Z)- than that of 9-Octadecenamide and the mortality was dose and time dependent *i.e* the mortality increased with the increasing dose and time.

The extracts of *M. pudica* showed very poor effectivity or ineffective to the larvae of *C. pusillus* in the larvicidal activity test.

The highest repellent activity was observed in petroleum ether extract of root ($p<0.001$). According to the intensity of repellent activity the result could be arranged in a descending order: root (petroleum ether) > leaf (petroleum ether) > stem (ethyl acetate).

Brine shrimp lethality test was performed to access the biological activity of the extracts of leaf, stem and root of *M. pudica* and found that the rates of mortality increased with the raise of concentrations of crude extracts. In case of leaf, the petroleum ether extract showed the highest activity (LC_{50} 2.632µg/ml) in 24h exposure and the intensity of activity could be arranged in the following order: petroleum ether > ethyl acetate > methanol > chloroform. In case of stem, the highest activity (LC_{50} 2.076µg/ml) was found in chloroform extract in 24h exposure and the intensity of activity could be arranged in the following order: chloroform > petroleum ether > ethyl acetate > methanol. And that of the root, the highest activity (LC_{50} 1.929µg/ml) was also observed in petroleum ether extract in 24h exposure and the intensity of activity could be arranged as: petroleum ether > chloroform > methanol > ethyl acetate. All of the crude extracts were found to be lethal and significantly effective to brine shrimp nauplii (*A. salina)* indicating that the extracts were biologically active.

Conclusion

The findings of antimicrobial screening indicate that the plant *M. pudica* possesses promising antibacterial and antifungal properties against harmful pathogens. Therefore, this plant may be an effective natural source for development of new drugs and also eco-friendly biopesticides. It contains potential antimicrobial components which can be used for the development of therapy for infectious diseases. Various phytochemical constituents are responsible for the antimicrobial potency of the plant and this study indicates that the sample plant may be a potential source of antimicrobial agents. The isolation and purification of phytoconstituents as well as bioactive compounds can reveal the potency of *M. pudica* which can inhibit the pathogenic microbes and thus control the microbial diseases.

Stable, biologically active components are represented by the traditional plants which may be new sources of antimicrobials and may establish a scientific base to use the plants in modern medicine. These ethnomedical plant sources should be evaluated scientifically and the botanical preparations can be disseminated for further investigation for discovery of new drugs.

In case of fungal strains, the leaf and stem extracts showed very poor activity. But the root extracts showed moderate activity which was more or less equal to the standard Nystatin (50 µg/disc). The susceptibility to different concentrations indicates that the plant possesses the potentiality for antimicrobial compounds. So, further approaches should be taken for the determination of bioactive principles and their mode of action.

The MIC of chloroform extract of leaf ranges from 32-128µg/ml and that of ethyl acetate extract of leaf ranges from 64-256µg/ml which indicates that the extracts possess relatively good antibacterial compounds. Between the two pure compounds, 13-docosenamide, (Z)- was more potential in antibacterial assay. For the MIC tests, the compounds showed good effectiveness to inhibit bacterial growth.

Among the extracts, the ethyl acetate extracts possessed significant antioxidant potentiality that encouraged enough to pursue the isolation and characterization of active compounds of leaf, stem and root of *M. pudica* which were responsible for the significant antioxidant activity. The presence of antioxidant principles of the plant extracts might be due to the presence of flavonoids, tannins and phenolic compounds (Larson 1988). The plant can be used for various ailments as ethnomedicinal agents which can be evidenced by the free radical scavenging mechanisms. Both the external and internal sources of body generate the free radicals that can damage proteins, lipids, carbohydrates and nucleic acids and may attribute to cancer (Thirumalai *et al.* 2011). Antioxidants play an important role by inhibiting free radicals and thus save human beings from diseases. Phytotherapy based on active principles and natural products from plants have received attention for their pharmacological properties. The present study indicates that *M. pudica* is enough active in scavenging DPPH radicals and further study is required to detect the active compounds responsible for this activity.

Veeramuthu *et al.* (2006) denoted that a very few screening programs have been initiated on crude plant materials and the medicinal value are observed in the bioactive phytocomponents of the plants. Phytochemical constituents of different parts of *M. pudica* were detected through GC-MS method. But it was quite unknown that which compounds were active for antimicrobial, insecticidal, antioxidant or other form of activities. So, mass spectrum investigation is necessary to identify the bioactive compounds responsible for different biological activities. Sometimes a crude extract may not show remarkable activity to any biological test, which does not mean the

inactivity of the extract. Rather it may contain the active compounds in the minimum. If it is possible to identify the active compound then it would show a remarkable activity to the biological test.

Insecticidal activity test of *M. pudica* showed that the plant contained bioactive compounds. The dose-mortality effect was different for different extracts of leaf, stem and root. Also it was exposure time dependent; as the exposure time increased, the mortality also increased. The chloroform extracts of leaf, stem and root showed the highest activity than the other solvent extracts which indicated that the chloroform extracts possessed one or more bioactive compounds that were required further investigation to isolate.

The pure compound 13-Docosenamide, (Z)- was more effective on insecticidal test, was found in all the extracts of root.

More or less repellent activity was observed in both dose and time effects. But the dose effects showed higher efficiency and the root extracts showed the highest activity. An intensive investigation of the phytoconstituents is required to observe the repellent activity of the extracts.

Brine shrimp lethality bioassay indicates that the plant possesses potent bioactive materials which may be useful as pesticidal, antiproliferative and other bioactive agents (Mayer *et al.* 1982). Insecticidal, antimicrobial, cytotoxicity and other pharmacological activities of plant extracts were evaluated by brine shrimp lethality bioassay method. Different solvent extracts of *M. pudica* were found to be lethal to different degrees. In 24h of exposure the root extracts showed higher potentiality than stem and leaf. But in 48h of exposure the stem extracts showed the higher activity except the petroleum ether extract of root.

The present result shows that extracts as well as pure compounds have good antimicrobial and insecticidal effects against *C. pusillus.* They could be good bioinsecticide alternatives for chemical control, while preserving human health and the environment. These new molecules are biodegradable and less likely to cause the resistance of the target species. Therefore, the present investigation is designed to evaluate the antimicrobial and insecticidal potency of different parts of *M. pudica* in different solvents with the ultimate goal of developing new drugs.

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APPENDICES

Insecticidal activity test

Table1. Dose-mortality effect of petroleum ether extract of leaf of *M. pudica* against *C. pusillus* adults after 24 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	6	4.004	1.996	.133
	0.50	.477	30	8	7.544	.456	.251
	1.00	.602	30	10	10.810	-.810	.360
	2.00	.699	30	13	13.623	-.623	.454
LD_{50} is 5.560 mg/cm^2				Log LD_{50} is 0.745			
95% conf. limits are 4.296 to 10.250				X^2 = 2.300 with 3 df			

Table 2. Dose-mortality effect of petroleum ether extract of leaf of *M. pudica* against *C. pusillus* adults after 48 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	8	5.303	2.697	.177
	0.50	.477	30	10	9.593	.407	.320
	1.00	.602	30	13	13.302	-.302	.443
	2.00	.699	30	15	16.320	-1.320	.544
LD_{50} is 4.535 mg/cm^2				Log LD_{50} is 0.657			
95% conf. limits are 3.674 to 6.711				X^2 = 3.298 with 3 df			

Table 3. Dose-mortality effect of petroleum ether extract of leaf of *M. pudica* against *C. pusillus* adults after 72 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	10	6.555	3.445	.218
	0.50	.477	30	12	11.464	.536	.382
	1.00	.602	30	15	15.465	-.465	.516
	2.00	.699	30	17	18.556	-1.556	.619
LD_{50} is 3.870 mg/cm^2				Log LD_{50} is 0.588			
95% conf. limits are 3.206 to 5.134				X^2 = 4.493 with 3 df			

Table 4. Dose-mortality effect of chloroform extract of leaf of *M. pudica* against *C. pusillus* adults after 24 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	13	9.027	3.973	.301
	0.50	.477	30	17	15.626	1.374	.521
	1.00	.602	30	19	20.314	-1.314	.677
	2.00	.699	30	22	23.431	-1.431	.781
LD_{50} is 2.891 mg/cm^2				Log LD_{50} is 0.461			
95% conf. limits are 2.442 to 3.409				X^2 = 5.549 with 3 df			

Table 5. Dose-mortality effect of chloroform extract of leaf of *M. pudica* against *C. pusillus* adults after 48 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	16	11.169	4.831	.372
	0.50	.477	30	20	18.512	1.488	.617
	1.00	.602	30	22	23.112	-1.112	.770
	2.00	.699	30	24	25.820	-1.820	.861
LD_{50} is 2.472 mg/cm^2				Log LD_{50} is 0.393			
95% conf. limits are 2.074 to 2.866				X^2 = 7.475 with 3 df			

Table 6. Dose-mortality effect of chloroform extract of leaf of *M. pudica* against *C. pusillus* adults after 72 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	18	12.998	5.002	.433
	0.50	.477	30	22	21.586	.414	.720
	1.00	.602	30	25	26.016	-1.016	.867
	2.00	.699	30	27	28.094	-1.094	.936
LD_{50} is 2.190 mg/cm^2				Log LD_{50} is 0.341			
95% conf. limits are 1.854 to 2.503				X^2 = 6.779 with 3 df			

Table 7. Dose-mortality effect of ethyl acetate extract of leaf of *M. pudica* against *C. pusillus* adults after 24 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	8	5.480	2.520	.183
	0.50	.477	30	11	10.033	.967	.334
	1.00	.602	30	13	13.937	-.937	.465
	2.00	.699	30	16	17.071	-1.071	.569
LD_{50} is 4.314 mg/cm^2				Log LD_{50} is 0.635			
95% conf. limits are 3.540 to 6.067				X^2 = 3.166 with 3 df			

Table 8. Dose-mortality effect of ethyl acetate extract of leaf of *M. pudica* against *C. pusillus* adults after 48 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	10	7.019	2.981	.234
	0.50	.477	30	14	12.393	1.607	.413
	1.00	.602	30	16	16.665	-.665	.555
	2.00	.699	30	18	19.862	-1.862	.662
LD_{50} is 3.577 mg/cm^2				Log LD_{50} is 0.554			
95% conf. limits are 2.997 to 4.516				X^2 = 4.356 with 3 df			

Table 9. Dose-mortality effect of ethyl acetate extract of leaf of *M. pudica* against *C. pusillus* adults after 72 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	13	9.013	3.987	.300
	0.50	.477	30	17	15.350	1.650	.512
	1.00	.602	30	19	19.896	-.896	.663
	2.00	.699	30	21	22.975	-1.975	.766
LD_{50} is 2.936 mg/cm^2				Log LD_{50} is 0.468			
95% conf. limits are 2.470 to 3.489				X^2 = 6.026 with 3 df			

Table 10. Dose-mortality effect of methanol extract of leaf of *M. pudica* against *C. pusillus* adults after 24 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	11	7.389	3.611	.246
	0.50	.477	30	14	12.887	1.113	.430
	1.00	.602	30	16	17.184	-1.184	.573
	2.00	.699	30	19	20.354	-1.354	.678
LD_{50} is 3.456 mg/cm^2				Log LD_{50} is 0.539			
95% conf. limits are 2.898 to 4.313				X^2 = 4.892 with 3 df			

Table 11. Dose-mortality effect of methanol extract of leaf of *M. pudica* against *C. pusillus* adults after 48 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	13	8.810	4.190	.294
	0.50	.477	30	16	15.107	.893	.504
	1.00	.602	30	19	19.667	-.667	.656
	2.00	.699	30	21	22.779	-1.779	.759
LD_{50} is 2.980 mg/cm^2				Log LD_{50} is 0.474			
95% conf. limits are 2.510 to 3.549				X^2 = 5.782 with 3 df			

Table 12. Dose-mortality effect of methanol extract of leaf of *M. pudica* against *C. pusillus* adults after 72 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	16	10.948	5.052	.365
	0.50	.477	30	19	18.258	.742	.609
	1.00	.602	30	22	22.893	-.893	.763
	2.00	.699	30	24	25.650	-1.650	.855
LD_{50} is 2.506 mg/cm^2				Log LD_{50} is 0.399			
95% conf. limits are 2.106 to 2.907				X^2 = 7.226 with 3 df			

Table 13. Dose-mortality effect of petroleum ether extract of stem of *M. pudica* against *C. pusillus* adults after 24 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	9	6.178	2.822	.206
	0.50	.477	30	12	11.416	.584	.381
	1.00	.602	30	15	15.747	-.747	.525
	2.00	.699	30	18	19.069	-1.069	.636
LD_{50} is 3.809 mg/cm^2				Log LD_{50} is 0.581			
95% conf. limits are 3.196 to 4.891				X^2 = 3.299 with 3 df			

Table 14. Dose-mortality effect of petroleum ether extract of stem of *M. pudica* against *C. pusillus* adults after 48 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	12	8.436	3.564	.281
	0.50	.477	30	16	14.859	1.141	.495
	1.00	.602	30	19	19.561	-.561	.652
	2.00	.699	30	21	22.771	-1.771	.759
LD_{50} is 3.025 mg/cm^2				Log LD_{50} is 0.481			
95% conf. limits are 2.560 to 3.591				X^2 = 4.822 with 3 df			

Table 15. Dose-mortality effect of petroleum ether extract of stem of *M. pudica* against *C. pusillus* adults after 72 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	15	10.211	4.789	.340
	0.50	.477	30	18	17.178	.822	.573
	1.00	.602	30	21	21.819	-.819	.727
	2.00	.699	30	23	24.729	-1.729	.824
LD_{50} is 2.648 mg/cm^2				Log LD_{50} is 0.423			
95% conf. limits are 2.226 to 3.091				X^2 = 6.790 with 3 df			

Table 16. Dose-mortality effect of chloroform extract of stem of *M. pudica* against *C. pusillus* adults after 24 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	11	7.848	3.152	.262
	0.50	.477	30	15	14.355	.645	.478
	1.00	.602	30	19	19.226	-.226	.641
	2.00	.699	30	21	22.575	-1.575	.753
LD_{50} is 3.144 mg/cm^2				Log LD_{50} is 0.493			
95% conf. limits are 2.651 to 3.691				X^2 = 3.826 with 3 df			

Table 17. Dose-mortality effect of chloroform extract of stem of *M. pudica* against *C. pusillus* adults after 48 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	14	9.836	4.164	.328
	0.50	.477	30	18	16.942	1.058	.565
	1.00	.602	30	21	21.721	-.721	.724
	2.00	.699	30	23	24.714	-1.714	.824
LD_{50} is 2.691 mg/cm^2				Log LD_{50} is 0.430			
95% conf. limits are 2.274 to 3.134				X^2 = 5.745 with 3 df			

Table 18. Dose-mortality effect of chloroform extract of stem of *M. pudica* against *C. pusillus* adults after 72 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	17	11.761	5.239	.392
	0.50	.477	30	20	19.745	.255	.658
	1.00	.602	30	23	24.406	-1.406	.814
	2.00	.699	30	26	26.917	-.917	.897
LD_{50} is 2.354 mg/cm^2				Log LD_{50} is 0.372			
95% conf. limits are 1.987 to 2.706				X^2 = 7.021 with 3 df			

Table 19. Dose-mortality effect of ethyl acetate extract of stem of *M. pudica* against *C. pusillus* adults after 24 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	7	4.614	2.386	.154
	0.50	.477	30	9	8.460	.540	.282
	1.00	.602	30	11	11.895	-.895	.397
	2.00	.699	30	14	14.780	-.780	.493
LD_{50} is 5.085 mg/cm^2				Log LD_{50} is 0.706			
95% conf. limits are 4.013 to 8.459				X^2 = 2.873 with 3 df			

Table 20. Dose-mortality effect of ethyl acetate extract of stem of *M. pudica* against *C. pusillus* adults after 48 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	9	5.849	3.151	.195
	0.50	.477	30	11	10.095	.905	.337
	1.00	.602	30	13	13.669	-.669	.456
	2.00	.699	30	15	16.545	-1.545	.552
LD_{50} is 4.435 mg/cm^2				Log LD_{50} is 0.647			
95% conf. limits are 3.573 to 6.614				X^2 = 4.322 with 3 df			

Table 21. Dose-mortality effect of ethyl acetate extract of stem of *M. pudica* against *C. pusillus* adults after 72 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	12	7.726	4.274	.258
	0.50	.477	30	14	12.660	1.340	.422
	1.00	.602	30	16	16.498	-.498	.550
	2.00	.699	30	17	19.391	-2.391	.646
LD_{50} is 3.576 mg/cm^2				Log LD_{50} is 0.553			
95% conf. limits are 2.946 to 4.668				X^2 = 6.790 with 3 df			

Table 22. Dose-mortality effect of methanol extract of stem of *M. pudica* against *C. pusillus* adults after 24 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	8	5.554	2.446	.185
	0.50	.477	30	11	10.250	.750	.342
	1.00	.602	30	14	14.262	-.262	.475
	2.00	.699	30	16	17.458	-1.458	.582
LD_{50} is 4.210 mg/cm^2				Log LD_{50} is 0.624			
95% conf. limits are 3.477 to 5.784				X^2 = 3.014 with 3 df			

Table 23. Dose-mortality effect of methanol extract of stem of *M. pudica* against *C. pusillus* adults after 48 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	10	6.822	3.178	.227
	0.50	.477	30	13	12.385	.615	.413
	1.00	.602	30	16	16.834	-.834	.561
	2.00	.699	30	19	20.144	-1.144	.671
LD_{50} is 3.554 mg/cm^2				Log LD_{50} is 0.551			
95% conf. limits are 2.996 to 4.430				X^2 = 3.826 with 3 df			

Table 24. Dose-mortality effect of methanol extract of stem of *M. pudica* against *C. pusillus* adults after 72 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	13	8.810	4.190	.294
	0.50	.477	30	16	15.107	.893	.504
	1.00	.602	30	19	19.667	-.667	.656
	2.00	.699	30	21	22.779	-1.779	.759
LD_{50} is 2.980 mg/cm^2				Log LD_{50} is 0.474			
95% conf. limits are 2.510 to 3.549				X^2 = 5.782 with 3 df			

Table 25. Dose-mortality effect of petroleum ether extract of root of *M. pudica* against *C. pusillus* adults after 24 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	12	8.141	3.859	.271
	0.50	.477	30	15	14.364	.636	.479
	1.00	.602	30	18	19.003	-1.003	.633
	2.00	.699	30	21	22.232	-1.232	.741
LD_{50} is 3.119 mg/cm^2				Log LD_{50} is 0.494			
95% conf. limits are 2.638 to 3.732				X^2 = 4.873 with 3 df			

Table 26. Dose-mortality effect of petroleum ether extract of root of *M. pudica* against *C. pusillus* adults after 48 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	15	9.890	5.110	.330
	0.50	.477	30	17	16.658	.342	.555
	1.00	.602	30	20	21.268	-1.268	.709
	2.00	.699	30	23	24.230	-1.230	.808
LD_{50} is 2.722 mg/cm^2				Log LD_{50} is 0.435			
95% conf. limits are 2.289 to 3.193				X^2 = 7.009 with 3 df			

Table 27. Dose-mortality effect of petroleum ether extract of root of *M. pudica* against *C. pusillus* adults after 72 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	18	12.588	5.412	.420
	0.50	.477	30	21	20.958	.042	.699
	1.00	.602	30	24	25.480	-1.480	.849
	2.00	.699	30	27	27.716	-.716	.924
LD_{50} is 2.241 mg/cm^2				Log LD_{50} is 0.350			
95% conf. limits are 1.895 to 2.566				X^2 = 7.256 with 3 df			

Table 28. Dose-mortality effect of chloroform extract of root of *M. pudica* against *C. pusillus* adults after 24 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	15	10.569	4.431	.352
	0.50	.477	30	19	18.028	.972	.601
	1.00	.602	30	22	22.799	-.799	.760
	2.00	.699	30	24	25.633	-1.633	.854
LD_{50} is 2.548 mg/cm^2				Log LD_{50} is 0.406			
95% conf. limits are 2.151 to 2.948				X^2 = 6.142 with 3 df			

Table 29. Dose-mortality effect of chloroform extract of root of *M. pudica* against *C. pusillus* adults after 48 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	18	12.741	5.259	.425
	0.50	.477	30	21	21.307	-.307	.710
	1.00	.602	30	25	25.810	-.810	.860
	2.00	.699	30	27	27.962	-.962	.932
LD_{50} is 2.218 mg/cm^2				Log LD_{50} is 0.346			
95% conf. limits are 1.879 to 2.534				X^2 = 6.782 with 3 df			

Table 30. Dose-mortality effect of chloroform extract of root of *M. pudica* against *C. pusillus* adults after 72 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	20	14.690	5.310	.490
	0.50	.477	30	23	24.128	-1.128	.804
	1.00	.602	30	27	27.931	-.931	.931
	2.00	.699	30	29	29.266	-.266	.976
LD_{50} is 2.024 mg/cm^2				Log LD_{50} is 0.306			
95% conf. limits are 1.730 to 2.292				X^2 = 6.574 with 3 df			

Table 31. Dose-mortality effect of ethyl acetate extract of root of *M. pudica* against *C. pusillus* adults after 24 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	10	6.744	3.256	.225
	0.50	.477	30	13	11.699	1.301	.390
	1.00	.602	30	15	15.703	-.703	.523
	2.00	.699	30	17	18.778	-1.778	.626
LD_{50} is 3.805 mg/cm^2				Log LD_{50} is 0.580			
95% conf. limits are 3.154 to 5.011				X^2 = 4.632 with 3 df			

Table 32. Dose-mortality effect of ethyl acetate extract of root of *M. pudica* against *C. pusillus* adults after 48 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	13	8.698	4.302	.290
	0.50	.477	30	16	14.592	1.408	.486
	1.00	.602	30	18	18.930	-.930	.631
	2.00	.699	30	20	21.975	-1.975	.732
LD_{50} is 3.081 mg/cm^2				Log LD_{50} is 0.489			
95% conf. limits are 2.579 to 3.729				X^2 = 6.470 with 3 df			

Table 33. Dose-mortality effect of ethyl acetate extract of root of *M. pudica* against *C. pusillus* adults after 72 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	15	10.426	4.574	.348
	0.50	.477	30	19	17.427	1.573	.581
	1.00	.602	30	21	22.042	-1.042	.735
	2.00	.699	30	23	24.909	-1.909	.830
LD_{50} is 2.611 mg/cm^2				Log LD_{50} is 0.417			
95% conf. limits are 2.192 to 3.046				X^2 = 7.039 with 3 df			

Table 34. Dose-mortality effect of methanol extract of root of *M. pudica* against *C. pusillus* adults after 24 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	9	5.587	3.413	.186
	0.50	.477	30	10	9.649	.351	.322
	1.00	.602	30	12	13.105	-1.105	.437
	2.00	.699	30	15	15.920	-.920	.531
LD_{50} is 4.649 mg/cm^2				Log LD_{50} is 0.667			
95% conf. limits are 3.705 to 7.274				X^2 = 4.508 with 3 df			

Table 35. Dose-mortality effect of methanol extract of root of *M. pudica* against *C. pusillus* adults after 48 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	11	7.100	3.900	.237
	0.50	.477	30	13	11.953	1.047	.398
	1.00	.602	30	15	15.822	-.822	.527
	2.00	.699	30	17	18.785	-1.785	.626
LD_{50} is 3.765 mg/cm^2				Log LD_{50} is 0.576			
95% conf. limits are 3.103 to 5.000				X^2 = 5.638 with 3 df			

Table 36. Dose-mortality effect of methanol extract of root of *M. pudica* against *C. pusillus* adults after 72 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	13	8.405	4.595	.280
	0.50	.477	30	15	14.109	.891	.470
	1.00	.602	30	17	18.374	-1.374	.612
	2.00	.699	30	20	21.421	-1.421	.714
LD_{50} is 3.184 mg/cm^2				Log LD_{50} is 0.503			
95% conf. limits are 2.662 to 3.897				X^2 = 6.569 with 3 df			

Table 37. Dose-mortality effect of 9-Octadecenamide from chloroform extract of leaf of *M. pudica* against *C. pusillus* adults after 24 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	6	4.237	1.763	.141
	0.50	.477	30	9	8.166	.834	.272
	1.00	.602	30	11	11.765	-.765	.392
	2.00	.699	30	14	14.811	-.811	.494
LD_{50} is 5.069 mg/cm^2				Log LD_{50} is 0.705			
95% conf. limits are 4.043 to 8.157				X^2 = 2.079 with 3 df			

Table 38. Dose-mortality effect of 9-Octadecenamide from chloroform extract of leaf of *M. pudica* against *C. pusillus* adults after 48 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	8	5.863	2.137	.195
	0.50	.477	30	12	11.357	.643	.379
	1.00	.602	30	16	15.958	.042	.532
	2.00	.699	30	18	19.464	-1.464	.649
LD_{50} is 3.770 mg/cm^2				Log LD_{50} is 0.576			
95% conf. limits are 3.192 to 4.739				X^2 = 2.468 with 3 df			

Table 39. Dose-mortality effect of 9-Octadecenamide from chloroform extract of leaf of *M. pudica* against *C. pusillus* adults after 72 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	11	7.357	3.643	.245
	0.50	.477	30	13	13.613	-.613	.454
	1.00	.602	30	18	18.430	-.430	.614
	2.00	.699	30	21	21.835	-.835	.728
LD_{50} is 3.257 mg/cm^2				Log LD_{50} is 0.513			
95% conf. limits are 2.772 to 3.903				X^2 = 4.079 with 3 df			

Table 40. Dose-mortality effect of 13-Docosenamide, (Z)- from chloroform extract of leaf of *M. pudica* against *C. pusillus* adults after 24 hours of exposure

	Dose (mg/cm²)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	21	16.323	4.677	.544
	0.50	.477	30	25	26.234	-1.234	.874
	1.00	.602	30	28	29.106	-1.106	.970
	2.00	.699	30	30	29.788	.212	.993
LD_{50} is 1.915 mg/cm^2				Log LD_{50} is 0.282			
95% conf. limits are 1.651 to 2.154				X^2 = 6.545 with 3 df			

Table 41. Dose-mortality effect of 13-Docosenamide, (Z)- from chloroform extract of leaf of *M. pudica* against *C. pusillus* adults after 48 hours of exposure

	Dose (mg/cm^2)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	24	19.312	4.688	.644
	0.50	.477	30	27	28.159	-1.159	.939
	1.00	.602	30	29	29.738	-.738	.991
	2.00	.699	30	30	29.963	.037	.999
LD_{50} is 1.761 mg/cm^2				Log LD_{50} is 0.246			
95% conf. limits are 1.521 to 1.978				X^2 = 7.706 with 3 df			

Table 42. Dose-mortality effect of 13-Docosenamide, (Z)- from chloroform extract of leaf of *M. pudica* against *C. pusillus* adults after 72 hours of exposure

	Dose (mg/cm^2)	Log Dose	Observed	Observed Responses	Expected Responses	Residual	Probability
PROBIT	0.25	.301	30	27	23.148	3.852	.772
	0.50	.477	30	28	29.620	-1.620	.987
	1.00	.602	30	30	29.985	.015	1.000
	2.00	.699	30	30	29.999	.001	1.000
LD_{50} is 1.634 mg/cm^2				Log LD_{50} is 0.213			
95% conf. limits are 0.002 to 2.637				X^2 = 10.922 with 3 df			

Brine shrimp lethality bioassay

Table 43. Dose-mortality effect of petroleum ether extract of leaf of *M. pudica* against *A. salina* after 30 minutes of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	0	.000	.000	.000
	50	.477	30	0	.012	-.012	.000
	100	.602	30	0	.166	-.166	.006
	200	.699	30	1	.845	.155	.028
Log LD_{50} is 0.992				X^2 = 0.208 with 3 df			
LD_{50} is 9.808 µg/ml				95% conf. limits are-			

Table 44. Dose-mortality effect of petroleum ether extract of leaf of *M. pudica* against *A. salina* after 24 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	8	10.542	-2.542	.351
	50	.477	30	16	17.167	-1.167	.572
	100	.602	30	23	21.592	1.408	.720
	200	.699	30	25	24.416	.584	.814
Log LD_{50} is 0.420				X^2 = 2.249 with 3 df			
LD_{50} is 2.632 µg/ml				95% conf. limits are 2.210 to 3.102			

Table 45. Dose-mortality effect of petroleum ether extract of leaf of *M. pudica* against *A. salina* after 48 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	19	19.061	-.061	.635
	50	.477	30	21	23.610	-2.610	.787
	100	.602	30	26	26.030	-.030	.868
	200	.699	30	29	27.407	1.593	.914
Log LD_{50} is 0.166				X^2 = 2.553 with 3 df			
LD_{50} is 1.464 µg/ml				95% conf. limits are 1.016 to 1.830			

Table 46. Dose-mortality effect of chloroform extract of leaf of *M. pudica* against *A. salina* after 24 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	7	6.533	.467	.218
	50	.477	30	10	10.879	-.879	.363
	100	.602	30	14	14.432	-.432	.481
	200	.699	30	18	17.240	.760	.575
Log LD_{50} is 0.622				X^2 = 0.259 with 3 df			
LD_{50} is 4.184 µg/ml				95% conf. limits are 3.377 to 6.024			

Table 47. Dose-mortality effect of chloroform extract of leaf of *M. pudica* against *A. salina* after 48 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	11	15.113	-4.113	.504
	50	.477	30	21	20.930	.070	.698
	100	.602	30	24	24.304	-.304	.810
	200	.699	30	28	26.298	1.702	.877
Log LD_{50} is 0.298				X^2 = 4.150 with 3 df			
LD_{50} is 1.985 µg/ml				95% conf. limits are 1.575 to 2.374			

Table 48. Dose-mortality effect of ethyl acetate extract of leaf of *M. pudica* against *A. salina* after 24 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	8	7.459	.541	.249
	50	.477	30	11	12.760	-1.760	.425
	100	.602	30	18	16.904	1.096	.563
	200	.699	30	20	19.989	.011	.666
Log LD_{50} is 0.545				X^2 = 0.640 with 3 df			
LD_{50} is 3.505 µg/ml				95% conf. limits are 2.923 to 4.440			

Table 49. Dose-mortality effect of ethyl acetate extract of leaf of *M. pudica* against *A. salina* after 48 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	14	15.909	-1.909	.530
	50	.477	30	21	22.050	-1.050	.735
	100	.602	30	26	25.382	.618	.846
	200	.699	30	28	27.215	.785	.907
Log LD_{50} is 0.227				X^2 = 1.335 with 3 df			
LD_{50} is 1.891 µg/ml				95% conf. limits are 1.513 to 2.242			

Table 50. Dose-mortality effect of methanol extract of leaf of *M. pudica* against *A. salina* after 24 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	7	7.200	-.200	.240
	50	.477	30	10	11.385	-1.385	.380
	100	.602	30	14	14.721	-.721	.491
	200	.699	30	19	17.338	1.662	.578
Log LD_{50} is 0.612				X^2 = 0.849 with 3 df			
LD_{50} is 4.096 µg/ml				95% conf. limits are 3.269 to 6.021			

Table 51. Dose-mortality effect of methanol extract of leaf of *M. pudica* against *A. salina* after 48 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	11	13.312	-2.312	.444
	50	.477	30	19	19.185	-.185	.639
	100	.602	30	23	22.843	.157	.761
	200	.699	30	26	25.133	.867	.838
Log LD_{50} is 0.351				X^2 = 1.270 with 3 df			
LD_{50} is 2.244 µg/ml				95% conf. limits are 1.809 to 2.684			

Table 52. Dose-mortality effect of petroleum ether extract of stem of *M. pudica* against *A. salina* after 24 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	8	11.142	-3.142	.371
	50	.477	30	22	18.496	3.504	.617
	100	.602	30	23	23.106	-.106	.770
	200	.699	30	25	25.819	-.819	.861
Log LD_{50} is 0.394				X^2 = 3.468 with 3 df			
LD_{50} is 2.475 µg/ml				95% conf. limits are 2.091 to 2.872			

Table 53. Dose-mortality effect of petroleum ether extract of stem of *M. pudica* against *A. salina* after 48 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	17	19.501	-2.501	.650
	50	.477	30	25	24.304	.696	.810
	100	.602	30	26	26.709	-.709	.890
	200	.699	30	29	27.994	1.006	.933
Log LD_{50} is 0.163				X^2 = 1.987 with 3 df			
LD_{50} is 1.457 µg/ml				95% conf. limits are 1.052 to 1.794			

Table 54. Dose-mortality effect of chloroform extract of stem of *M. pudica* against *A. salina* after 24 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	18	14.415	3.585	.481
	50	.477	30	22	20.554	1.446	.685
	100	.602	30	23	24.143	-1.143	.805
	200	.699	30	25	26.254	-1.254	.875
Log LD_{50} is 0.317				X^2 = 3.823 with 3 df			
LD_{50} is 2.076 µg/ml				95% conf. limits are 1.659 to 2.466			

Table 55. Dose-mortality effect of chloroform extract of stem of *M. pudica* against *A. salina* after 48 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	22	21.706	.294	.724
	50	.477	30	26	26.149	-.149	.872
	100	.602	30	28	28.065	-.065	.935
	200	.699	30	29	28.958	.042	.965
Log LD_{50} is 0.108				X^2 = 0.027 with 3 df			
LD_{50} is 1.282 µg/ml				95% conf. limits are 0.916 to 1.583			

Table 56. Dose-mortality effect of ethyl acetate extract of stem of *M. pudica* against *A. salina* after 24 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	11	10.906	.094	.364
	50	.477	30	16	16.091	-.091	.536
	100	.602	30	20	19.704	.296	.657
	200	.699	30	22	22.227	-.227	.741
Log LD_{50} is 0.441				X^2 = 0.025 with 3 df			
LD_{50} is 2.758 µg/ml				95% conf. limits are 2.230 to 3.413			

Table 57. Dose-mortality effect of ethyl acetate extract of stem of *M. pudica* against *A. salina* after 48 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	20	19.743	.257	.658
	50	.477	30	25	24.346	.654	.812
	100	.602	30	26	26.672	-.672	.889
	200	.699	30	28	27.931	.069	.931
Log LD_{50} is 0.150				X^2 = 0.268 with 3 df			
LD_{50} is 1.414 µg/ml				95% conf. limits are 0.992 to 1.761			

Table 58. Dose-mortality effect of methanol extract of stem of *M. pudica* against *A. salina* after 24 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	11	9.823	1.177	.327
	50	.477	30	16	14.336	1.664	.478
	100	.602	30	17	17.638	-.638	.588
	200	.699	30	19	20.076	-1.076	.669
Log LD_{50} is 0.502				X^2 = 1.080 with 3 df			
LD_{50} is 3.178 µg/ml				95% conf. limits are 2.540 to 4.192			

Table 59. Dose-mortality effect of methanol extract of stem of *M. pudica* against *A. salina* after 48 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	19	18.010	.990	.600
	50	.477	30	22	22.071	-.071	.736
	100	.602	30	24	24.453	-.453	.815
	200	.699	30	26	25.954	.046	.865
Log LD_{50} is 0.182				X^2 = 0.215 with 3 df			
LD_{50} is 1.520 µg/ml				95% conf. limits are 0.968 to 1.959			

Table 60. Dose-mortality effect of petroleum ether extract of root of *M. pudica* against *A. salina* after 24 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	20	15.744	4.256	.525
	50	.477	30	23	23.314	-.314	.777
	100	.602	30	26	26.882	-.882	.896
	200	.699	30	28	28.500	-.500	.950
Log LD_{50} is 0.285				X^2 = 3.910 with 3 df			
LD_{50} is 1.929 µg/ml				95% conf. limits are 1.603 to 2.230			

Table 61. Dose-mortality effect of petroleum ether extract of root of *M. pudica* against *A. salina* after 48 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	25	24.239	.761	.808
	50	.477	30	28	28.233	-.233	.941
	100	.602	30	29	29.403	-.403	.980
	200	.699	30	30	29.778	.222	.993
Log LD_{50} is 0.080				X^2 = 0.670 with 3 df			
LD_{50} is 1.202 µg/ml				95% conf. limits are 0.915 to 1.443			

Table 62. Dose-mortality effect of chloroform extract of root of *M. pudica* against *A. salina* after 24 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	21	15.255	5.745	.508
	50	.477	30	22	22.084	-.084	.736
	100	.602	30	24	25.693	-1.693	.856
	200	.699	30	27	27.578	-.578	.919
Log LD_{50} is 0.295				X^2 = 7.069 with 3 df			
LD_{50} is 1.972 µg/ml				95% conf. limits are 0.702 to 2.958			

Table 63. Dose-mortality effect of chloroform extract of root of *M. pudica* against *A. salina* after 48 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	24	20.733	3.267	.691
	50	.477	30	25	26.225	-1.225	.874
	100	.602	30	27	28.375	-1.375	.946
	200	.699	30	30	29.253	.747	.975
Log LD_{50} is 0.165				X^2 = 4.340 with 3 df			
LD_{50} is 1.463 µg/ml				95% conf. limits are 1.148 to 1.737			

Table 64. Dose-mortality effect of ethyl acetate extract of root of *M. pudica* against *A. salina* after 24 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	10	10.241	-.241	.341
	50	.477	30	16	16.535	-.535	.551
	100	.602	30	21	20.847	.153	.695
	200	.699	30	24	23.692	.308	.790
Log LD_{50} is 0.435				X^2 = 0.092 with 3 df			
LD_{50} is 2.723 µg/ml				95% conf. limits are 2.274 to 3.238			

Table 65. Dose-mortality effect of ethyl acetate extract of root of *M. pudica* against *A. salina* after 48 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	19	16.933	2.067	.564
	50	.477	30	22	23.526	-1.526	.784
	100	.602	30	26	26.716	-.716	.891
	200	.699	30	29	28.264	.736	.942
Log LD_{50} is 0.255				X^2 = 1.697 with 3 df			
LD_{50} is 1.800 µg/ml				95% conf. limits are 1.459 to 2.111			

Table 66. Dose-mortality effect of methanol extract of root of *M. pudica* against *A. salina* after 24 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	10	10.992	-.992	.366
	50	.477	30	18	16.351	1.649	.545
	100	.602	30	20	20.053	-.053	.668
	200	.699	30	22	22.607	-.607	.754
Log LD_{50} is 0.433				X^2 = 0.574 with 3 df			
LD_{50} is 2.712 µg/ml				95% conf. limits are 2.201 to 3.325			

Table 67. Dose-mortality effect of methanol extract of root of *M. pudica* against *A. salina* after 48 hours of exposure

	Dose (µg/ml)	Log Dose	Observed	No of Death	Expected Responses	Residual	Probability
PROBIT	25	.301	30	14	14.646	-.646	.488
	50	.477	30	22	21.067	.933	.702
	100	.602	30	24	24.702	-.702	.823
	200	.699	30	27	26.758	.242	.892
Log LD_{50} is 0.310				X^2 = 0.333 with 3 df			
LD_{50} is 2.043 µg/ml				95% conf. limits are 1.656 to 2.410			

Table 68. % repellent activity of extracts

Petroleum ether extract of leaf

Dose	1h	2h	3h	4h	5h
10	66.67	70.00	70.00	73.33	73.33
5	60.00	63.33	63.33	66.67	66.67
2.5	56.67	60.00	60.00	63.33	63.33
1.25	53.33	56.67	56.67	60.00	63.33
0.625	50.00	53.33	53.33	56.67	60.00

Chloroform extract of leaf

Dose	1h	2h	3h	4h	5h
10	60.00	63.33	60.00	66.67	66.67
5	56.67	60.00	56.67	60.00	63.33
2.5	53.33	53.33	56.67	56.67	60.00
1.25	50.00	50.00	53.33	53.33	56.67
0.625	46.67	46.67	50.00	50.00	53.33

Ethyl acetate extract of leaf

Dose	1h	2h	3h	4h	5h
10	60.00	63.33	63.33	66.67	70.00
5	56.67	60.00	60.00	63.33	63.33
2.5	53.33	56.67	56.67	60.00	60.00
1.25	50.00	50.00	53.33	56.67	56.67
0.625	46.67	50.00	53.33	53.33	56.67

Methanol extract of leaf

Dose	1h	2h	3h	4h	5h
10	63.33	63.33	66.67	66.67	70.00
5	60.00	63.33	63.33	66.67	66.67
2.5	56.67	60.00	60.00	63.33	63.33
1.25	53.33	56.67	56.67	60.00	60.00
0.625	50.00	53.33	53.33	56.67	56.67

Petroleum ether extract of stem

Dose	1h	2h	3h	4h	5h
10	63.33	66.67	66.67	70.00	73.33
5	60.00	60.00	63.33	66.67	70.00
2.5	53.33	56.67	60.00	66.67	66.67
1.25	50.00	56.67	56.67	60.00	63.33
0.625	46.67	50.00	53.33	56.67	60.00

Chloroform extract of stem

Dose	1h	2h	3h	4h	5h
10	60.00	63.33	63.33	66.67	70.00
5	56.67	56.67	60.00	63.33	66.67
2.5	53.33	53.33	56.67	56.67	63.33
1.25	50.00	50.00	53.33	53.33	60.00
0.625	43.33	46.67	50.00	50.00	56.67

Ethyl acetate extract of stem

Dose	1h	2h	3h	4h	5h
10	70.00	70.00	73.33	73.33	76.67
5	63.33	66.67	70.00	70.00	73.33
2.5	56.67	60.00	66.67	70.00	70.00
1.25	53.33	56.67	60.00	63.33	66.67
0.625	50.00	53.33	56.67	60.00	60.00

Methanol extract of stem

Dose	1h	2h	3h	4h	5h
10	63.33	66.67	66.67	70.00	73.33
5	56.67	60.00	63.33	66.67	70.00
2.5	53.33	56.67	56.67	60.00	66.67
1.25	50.00	53.33	53.33	56.67	63.33
0.625	46.67	50.00	53.33	56.67	60.00

Petroleum ether extract of root

Dose	1h	2h	3h	4h	5h
10	80.00	83.33	86.67	86.67	90.00
5	76.67	80.00	83.33	83.33	86.67
2.5	70.00	73.33	76.67	80.00	83.33
1.25	63.33	70.00	73.33	76.67	80.00
0.625	60.00	63.33	66.67	70.00	73.33

Chloroform extract of root

Dose	1h	2h	3h	4h	5h
10	66.67	66.67	73.33	73.33	76.67
5	63.33	66.67	66.67	70.00	73.33
2.5	60.00	63.33	63.33	66.67	66.67
1.25	56.67	56.67	60.00	63.33	66.67
0.625	53.33	53.33	56.67	60.00	63.33

Ethyl acetate extract of root

Dose	1h	2h	3h	4h	5h
10	66.67	70.00	73.33	76.67	76.67
5	63.33	66.67	66.67	70.00	73.33
2.5	60.00	63.33	63.33	66.67	70.00
1.25	56.67	56.67	60.00	63.33	66.67
0.625	53.33	53.33	56.67	60.00	63.33

Methanol extract of root

Dose	1h	2h	3h	4h	5h
10	73.33	76.67	80.00	80.00	83.33
5	70.00	73.33	76.67	76.67	80.00
2.5	66.67	70.00	73.33	76.67	76.67
1.25	63.33	66.67	70.00	73.33	73.33
0.625	56.67	60.00	63.33	66.67	70.00

1. Acceptance letter from the **Journal of Life and Earth Science** of the entitled paper

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Bangladesh

Memo No. JLES – **02/2019** Date : 27.09.2020

Ujjwal Kumar Mondol
Institute of Biological Sciences
Rajshahi University
Rajshahi – 6205, Bangladesh.

Dear U. K. Mondol,

Your paper entitled **"ANTIBACTERIAL POTENCY OF CRUDE EXTRATS OF LEAVES OF *MIMOSA PUDICA* L. (FAMILY : MIMOSACEAE)"** under the authorship of **Ujjwal Kumar Mondol and Md. Wahedul Islam** has been accepted for publication in the ensuing **Volume -14, 2019** of the Journal of Life & Earth Science.

Thank you very much indeed.

Nazrul Islam
27.09.2020
(Professor Dr. M. Nazrul Islam)
Chief Editor

2. Acceptance letter from the **International Journal of Current Microbiology and Applied sciences (IJCMAS)** of the entitled paper

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ACCEPTANCE LETTER

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Date: 06-09-2020

To

Ujjwal Kumar Mondol and W Islam*

Institute of Biological Sciences, University of Rajshahi, Bangladesh

Sub: Acceptance of Manuscript - IJCMAS- 202009364– **reg.**

Dear Author(s),

It is pleasure to inform you that your manuscript No. 202009364- IJCMAS entitled **"Insecticidal and Repellent activities of *Mimosa pudica* L. (Fabaceae) against *Cryptolestes pusillus* (Schon) (Coleoptera:Cucujidae)"** has been accepted for publication in International Journal of Current Microbiology and Applied Sciences and published in the Vol 9 (09) to be released in September-2020.

Thank you

With Regards

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