PHRM 404

# Anti-oxidant and Analgesic Activity of *Phragmipedium Longifolium*

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**This thesis paper is dedicated**

**To my beloved Parents**

**and respected Teachers**

# CERTIFICATE

This research paper is submitted to the department of Pharmacy, East West University in conformity with the requirements for the degree of Bachelor of Pharmacy (B.Pharm) was carried out by Md. Ibtida-Bin-Shahid (2008-1-70-071) under my guidance and supervision and that no part of the thesis has been submitted for any other degree. I further certify that all the resources of the information in this research paper are duly acknowledged.

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# CERTIFICATE

This is to certify that, the research work on "In *vivo* assay of Analgesic Activity, and in *vitro* assay of Antioxidant Effect of the Ethanolic Extract of *Phragmipedium Longifolium* submitted to the department of pharmacy, East West University, Aftab Nagar, Dhaka, in partial fulfillment of the requirement for the degree of bachelor of pharmacy (B.Pharm) was carried out by Md. Ibtida-Bin-Shahid, ID# 2008-1-70-071 under our guidance and supervision and that no part of the thesis has been submitted for any other degree. I further certify that all the resources of the information in this research paper are duly acknowledged.

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## Abstracts

*In vivo* Analgesic and *in vitro* Antioxidant effects of the ethanolic extract of *Phragmipedium Longifolium* were tested in this project.

In Anti-oxident activity test of ethanolic extract of *Phragmipedium Longifolium* by quantitative DPPH method shows very good anti-oxidant activity. Result showed significant analgesic activities compared to control and the $IC_{50}$ is 63.8µg/ml compare to control ascorbic acid ($IC_{50}$ 10.81µg/ml). I hope further research on this plant can give a very good anti-oxidant source.

In Analgesic activity test of ethanolic extract of *Phragmipedium Longifolium* by Acetic acid induced writhing investigated method in mice, positive control group (Diclofenc-Na) showed writhing of 7.2±1.923538, ($P<0.001$) and the experimental group 200 mg/kg body weight dose group showed writhing with average of 4±1.581139. Further study is necessary to find out the active ingredient responsible for analgesic activities.



Key word: *Phragmipedium Longifolium*, Anti-oxidant, Analgesic

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# Chapter 1

# Introduction

## Introduction

All leaving system is a beautiful co-ordination of the bio-chemical reactions of different bio-molecules and these bio-molecules are different receptors, enzymes and different substrates. But which is really important that no system is perfect. So this system may be attacked by other system or there may be internal system breakdown by any interruption of any system. To stop those problems we must target associate receptor or enzyme with a suitable compound which can alter the problem back to normal. As these targets are very small and most of the time we do not know the structure of targets so we need a lot of compounds to trial and error sieving for the lead compound. Plants give us this opportunity to find the perfect compound or most of the time a lead to the most perfect compound to interact and alter the problem in system. Plant is really very good source of drug from ancient time.

According to the WHO, "A medicinal plants is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi-synthesis." When a plant is designated as 'medicinal', it is implied that the said plant is used as a drug or therapeutic agent or an active ingredient of a medicinal preparation.

For finding new activity and diversified compound library, we need to search in different compounds present in different plants. Because each plants can give a number of compounds with different activity. But it's very hard to find a good lead in thousand of compounds, for that partition of the extract of these, plant make it easy to find a specific compounds. Our target was specifically a complete new source, *Phragmipedium Longifolium* is one of new plant because very little research done on it and there is no old pharmacological activity test.

Natural products, including medicinal plants, have been the primary source for obtaining new drugs with therapeutic potential throughout history. It is estimated that approximately half of the drugs in use are derived from natural products (A. G. Paker et al., 2007). This means that around 4 billion people rely on natural products as a source of their primary medicinal needs. It is proved that half of the world's best selling drugs and many potential drugs under development are derived from plants. Some time ago the World Health Organization (WHO) reported that about 80% of the population in developing countries used some kind of traditional medicine in primary health care, emphasizing the use of herbal products (WHO, 1993). According to Farnsworth (1985), up until 1985, of the 119 chemicals extracted from plants and used in medicine (Farnsworth et al., 1985), 74% were first discovered through popular knowledge. Koehn & Carter (2005) reported that around 25% of prescribed drugs worldwide were obtained directly or indirectly

from plants. Moreover, about 50% of drugs developed between 1981 and 2002 were obtained from natural products, semi-synthetic analogues or synthetic compounds based on natural products (Koehn, Carter, 2005).

In Bangladesh, ninety percent of the medicinal plants are wild sourced (Ghani, 1998). My plant Phragmipedium Longifolium is also a wild sourced plant too. Though its medicinal use is not established yet but we may hope to find some new drug or lead from it. Out of approximately 5,000 species of indigenous and naturalized phanerogamic and pteridophytic plants growing in the country, more than a thousand of them, including many food, vegetable, beverage, spice and ornamental plants (Mia, 1990). Water plants are taxonomically different as there is generally a lack of adequate herbarium material and a paucity of critical studies in the development of various organs due to the high degree of adaptability in form and structure in relation to aquatic environment (Khan et al., 1987). Growing in the forests, jungles, wastelands, and along roadsides, the types of medicinal plants in Bangladesh are varied. Out of them more than a thousand have been claimed to posses medicinal or poisonous properties, of which 546 have recently been enumerated with their medicinal properties and therapeutic uses (Yusuf et al., 1994). Indigenous medicinal plants containing active and medicinal principles like glycerides, alkaloids, steroids, tannins etc. grow abundantly in Bangladesh. These indigenous medicinal plants are extremely used in both raw and semi-processed

forms in the preparation of pharmaceutical, Homeopathic, Unani, and Ayurbedic medicines. Although our country is rich with this vast natural resource but due to lack of knowledge none processes these indigenous medicinal plants or its extracts locally. As a result every year Bangladesh imports a huge quantity of processed indigenous medicinal plants or its extracts from abroad at the cost of our foreign exchange to meet the country's demand. So, efforts have been made to systematic processing and screening of indigenous medicinal plants as pharmaceutical raw materials.

*Phragmipedium Longifolium* is a new plant for medicinal research, as because it's a beautiful ornamental orchid it is generally used to increase the beauty of the garden. In wild condition these plants grows upon or are attached to another plant or object merely for physical support (Epiphyte). Epiphytes are found mostly in the tropics and are also known as air plants because they have no attachment to the ground or other obvious nutrient source. They obtain water and minerals from rain, moisture and from debris on the supporting plants. Orchids, ferns, and members of the pineapple family are common tropical epiphytes. Lichens, mosses, liverworts, and algae are epiphytes of temperate regions.

*Phragmipedium* is a genus of the Orchid family (Orchidaceae) (Subfamily Cypripedioideae) and the only genus comprised in the tribe *Phragmipedieae* and *subtribe Phragmipediinae*. The name of the genus is derived from the Greek

phragma, which means "division", and pedium, which means "slipper" (referring to the pouch). It is abbreviated 'Phrag' in trade journals.

About 20 species of these lady's slipper orchids are known from SW Mexico, Central and tropical South America.

All members of the genus Phragmipedium are listed in Appendix I of the Convention on International Trade in Endangered Species (CITES) (Wikipedia, 2009).

# Chapter 2

# Plant Detail

## Detail Information about the Plant *Phragmipedium Longifolium*

### 2.1 Family Profile (Orchidaceae)

The Orchidaceae or orchid family is a diverse and widespread family of flowering plants with colorful and fragrant blooms. Along with the Asteraceae, it is one of the two largest families of flowering plants, with between 21,950 and 26,049 currently accepted species, found in 880 genera. Selecting which of the two families is larger remains elusive because of the difficulties associated with putting hard species numbers on such enormous groups. Regardless, the number of orchid species equals more than twice the number of bird species, and about four times the number of mammal species. It also encompasses about 6–11% of all seed plants. The largest genera are *Bulbophyllum* (2,000 species), *Epidendrum* (1,500 species), *Dendrobium* (1,400 species) and *Pleurothallis* (1,000 species).

The family also includes Vanilla (the genus of the vanilla plant), Orchis (type genus), and many commonly cultivated plants such as *Phalaenopsis* and *Cattleya*. Moreover, since the introduction of tropical species in the 19th century, horticulturists have produced more than 100,000 hybrids and cultivars (Wikipedia, 2012).

## 2.2 Etymology

The name comes from the Greek ὄρχις (órkhis), literally meaning "testicle", because of the shape of the root. Linnaeus categorized the family as Orchidaceae. Orchid was introduced in 1845 by John Lindley in School Botany, due to an incorrect attempt to extract the Latin stem (orchis) from Orchidaceae (Corominas, 1980).

The Greek myth of Orchis explains the origin of the plants. Orchis, the son of a nymph and a satyr, came upon a festival of Dionysios (Bacchus) in the forest. He drank too much, and attempted to rape a priestess of Dionysios. For his insult, he was torn apart by the Bacchanalians. His father prayed for him to be restored, but the gods instead changed him into a flower (Wikipedia, 2012).

## 2.3 Distribution

Orchidaceae are cosmopolitan, occurring in almost every habitat apart from glaciers. The world's richest concentration of orchid varieties is found in the tropics, mostly Asia, South America and Central America, but they are also found above the Arctic Circle, in southern Patagonia, and even two species of Nematoceras on Macquarie Island, close to Antarctica.

The following list gives a rough overview of their distribution:

Tropical Asia: 260 to 300 genera

Tropical America: 212 to 250 genera

Tropical Africa: 230 to 270 genera

Oceania: 50 to 70 genera

Europe and temperate Asia: 40 to 60 genera

North America: 20 to 26 genera

## 2.4 Characteristics

Orchids are easily distinguished from other plants, as they share some very evident apomorphies. Among these, bilateral symmetry (zygomorphism), many resupinate flowers, a nearly always highly modified petal (labellum), fused stamens and carpels, and extremely small seeds are noticeable.

### 2.4.1 Stem and Roots

All orchids are perennial herbs, lack any permanent woody structure, and can grow according to two patterns.

**Monopodial:** The stem grows from a single bud, leaves are added from the apex each year and the stem grows longer accordingly. The stem of orchids with a monopodial growth can reach several metres in length, as in Vanda and Vanilla.

**Sympodial:** The plant produces a series of adjacent shoots which grow to a certain size, bloom and then stop growing, to be then replaced. Sympodial orchids grow laterally rather than vertically, following the surface of their support. The growth continues by development of new leads, with their own leaves and roots, sprouting from or next to those of the previous year, as in *Cattleya*. While a new lead is developing, the rhizome may start its growth again from a so-called 'eye', an undeveloped bud, thereby branching.

**Figure 2.1**: Root of *Paphiopedilum longifolium*

**Figure 2.2:** *Anacamptis lactea* (showing the two tubers)

Terrestrial orchids may be rhizomatous or form corms or tubers. The root caps of terrestrials are smooth and white.

Some sympodial terrestrials, such as Orchis and Ophrys, have two subterranean tuberous roots. One is used as a food reserve for wintry periods, and provides for the development of the other one, from which visible growth develops.

In warm and humid climates, many terrestrial orchids do not need pseudo bulbs.

Epiphytic orchids have modified aerial roots that can sometimes be a few meters long. In the older parts of the roots, a modified spongy epidermis, called velamen, has the function to absorb humidity. It is made of dead cells and can have a silvery-grey, white or brown appearance. In some orchids, the velamen includes spongy and fibrous bodies near the passage cells, called tilosomes.

The cells of the root epidermis grow at a right angle to the axis of the root to allow them to get a firm grasp on their support. Nutrients mainly come from animal droppings and other organic detritus on their supporting surfaces.

The base of the stem of sympodial epiphytes, or in some species essentially the entire stem, may be thickened to form a pseudo bulb that contains nutrients and water for drier periods.

**Figure 2.3:** The pseudo bulb of *Prosthechea fragrans*

The pseudo bulb has a smooth surface with lengthwise grooves, and can have different shapes, often conical or oblong. Its size is very variable; in some small species of *Bulbophyllum*, it is no longer than two millimeters, while in the largest orchid in the world, *Grammatophyllum speciosum* (giant orchid), it can reach three meters. Some *Dendrobium* species have long, cane like pseudo bulbs with short, rounded leaves over the whole length; some other orchids have hidden or extremely small pseudo bulbs, completely included inside the leaves.

With ageing, the pseudo bulb sheds its leaves and becomes dormant. At this stage it is often called a back bulb. A pseudo bulb then takes over, exploiting the last reserves accumulated in the back bulb, which eventually dies off, too. A pseudo bulb typically lives for about five years.

### 2.4.2 Leaves

Like most monocots, orchids generally have simple leaves with parallel veins, although some Vanilloideae have a reticulate venation. Leaves may be ovate, lanceolate, or orbiculate, and very variable in size. Their characteristics are often diagnostic. They are normally alternate on the stem, often plicate, and have no stipules. Orchid leaves often have siliceous bodies called stegmata in the vascular bundle sheaths (not present in the Orchidoideae) and are fibrous.

The structure of the leaves corresponds to the specific habitat of the plant. Species that typically bask in sunlight, or grow on sites which can be occasionally very dry, have thick, leathery leaves and the laminae are covered by a waxy cuticle to retain their necessary water supply. Shade species, on the other hand, have long, thin leaves.

The leaves of most orchids are perennial, that is, they live for several years, while others, especially those with plicate leaves, shed them annually and develop new leaves together with new pseudo bulbs, as in *Catasetum*.

The leaves of some orchids are considered ornamental. The leaves of the *Macodes sanderiana*, a semiterrestrial or lithophyte, show a sparkling silver and gold veining on a light green background. The cordate leaves of *Psychopsiella limminghei* are light brownish-green with maroon-puce markings, created by flower pigments. The attractive mottle of the leaves of lady's slippers from tropical and subtropical Asia (*Paphiopedilum*), is caused by uneven distribution of chlorophyll. Also, *Phalaenopsis schilleriana* is a pastel pink orchid with leaves spotted dark green and light green. The jewel orchid (*Ludisia discolor*) is grown more for its colorful leaves than its white flowers.

**Figure 2.4:** Leaves of *Paphiopedilum longifolium*

Some orchids, as *Dendrophylax lindenii* (ghost orchid), *Aphyllorchis* and *Taeniophyllum* depend on their green roots for photosynthesis and lack normally developed leaves, as do all of the heterotrophic species.

Orchids of the genus *Corallorhiza* (coralroot orchids) lack leaves altogether and instead wrap their roots around the roots of mature trees and use specialized fungi to harvest sugars (Jenny, 2011).

### 2.4.3 Flowers

Orchidaceae are well known for the many structural variations in their flowers.

Some orchids have single flowers, but most have a racemose inflorescence, sometimes with a large number of flowers. The flowering stem can be basal, that is, produced from the base of the tuber, like in *Cymbidium*, apical, meaning it grows from the apex of the main stem, like in *Cattleya*, or *axillary*, from the leaf axil, as in *Vanda*.

As an apomorphy of the clade, orchid flowers are primitively zygomorphic (bilaterally symmetrical), although in some genera like *Mormodes*, *Ludisia* and *Macodes*, this kind of symmetry may be difficult to notice.

The orchid flower, like most flowers of monocots, has two whorls of sterile elements. The outer whorl has three sepals and the inner whorl has three petals. The sepals are usually very similar to the petals (and thus called *tepals*), but may be completely distinct.

The upper medial petal, called the *labellum* or lip, is always modified and enlarged. The inferior ovary or the pedicel usually rotates 180 degrees, so that the labellum, goes on the lower part of the flower, thus becoming suitable to form a platform for pollinators. This characteristic, called resupination, occurs primitively

in the family and is considered apomorphic (the torsion of the ovary is very evident from the picture). Some orchids have secondarily lost this resupination, e. g. *Zygopetalum* and *Epidendrum secundum*.

The normal form of the sepals can be found in *Cattleya*, where they form a triangle. In *Paphiopedilum* (Venus slippers), the lower two sepals are fused into a synsepal, while the lip has taken the form of a slipper. In *Masdevallia*, all the sepals are fused.

Orchid flowers with abnormal numbers of petals or lips are called peloric. Peloria is a genetic trait, but its expression is environmentally influenced and may appear random.

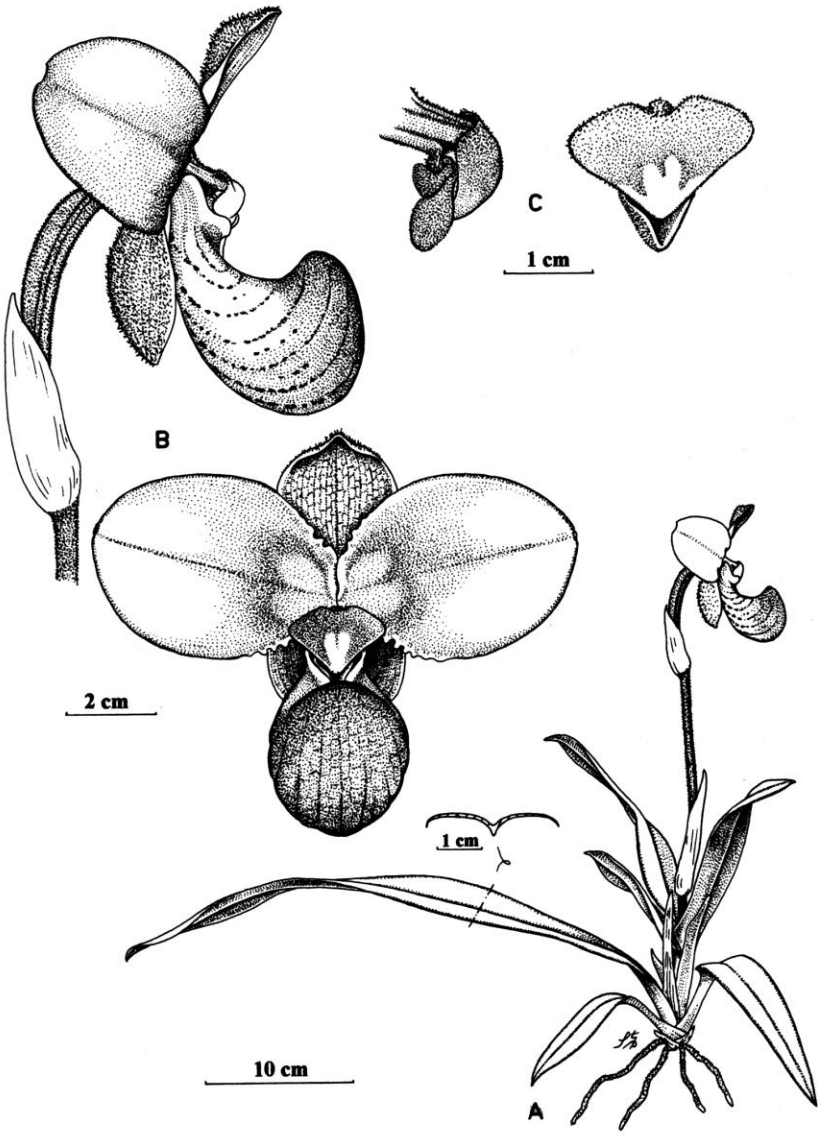



**Figure 2.5:** Illustration of *Paphiopedilum longifolium*, A. Whole plant, B. frontal and lateral view of flower, C. Staminode Frontal and lateral view

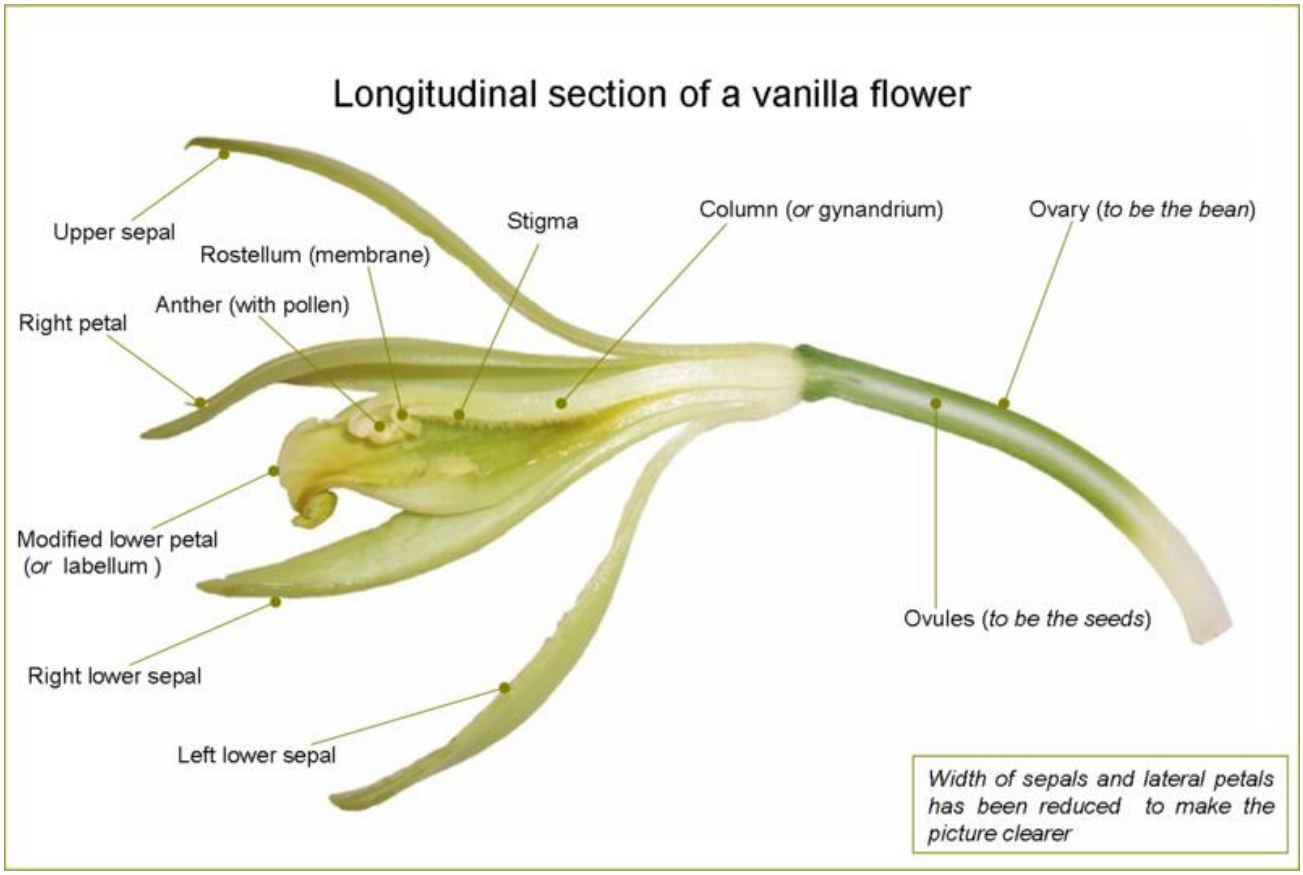



**Figure 2.6:** Longitudinal section of a flower of *Vanilla planifolia*

Orchid flowers primitively had three stamens, but this situation is now limited to the genus *Neuwiedia*. *Apostasia* and the *Cypripedioideae* have two stamens, the central one being sterile and reduced to a staminode. All of the other orchids, the clade called Monandria, retain only the central stamen, the others being reduced to staminodes . The filaments of the stamens are always adnate (fused) to the style to form cylindrical structure called the gynostemium or column. In the primitive Apostasioideae, this fusion is only partial; in the Vanilloideae, it is deeper; in Orchidoideae and Epidendroideae, it is total. The stigma is very asymmetrical, as

all of its lobes are bent towards the centre of the flower and lay on the bottom of the column.

Pollen is released as single grains, like in most other plants, in the Apostasioideae, Cypripedioideae and Vanilloideae. In the other subfamilies, that comprise the great majority of orchids, the anther, carries and two pollinia.

A pollinium is a waxy mass of pollen grains held together by the glue-like alkaloid viscin, containing both cellulosic strands and mucopolysaccharides. Each pollinium is connected to a filament which can take the form of a caudicle, as in *Dactylorhiza* or *Habenaria*, or a *stipe*, as in *Vanda*. Caudicles or stipes hold the pollinia to the viscidium, a sticky pad which sticks the pollinia to the body of pollinators.

At the upper edge of the stigma of single-anthered orchids, in front of the anther cap, there is the rostellum , a slender extension involved in the complex pollination mechanism.

As aforementioned, the ovary is always inferior (located behind the flower). It is three-carpelate and one or, more rarely, three-partitioned, with parietal placentation (axile in the Apostasioideae) (Wikipedia, 2012).

### 2.4.4 Fruits and seeds

The ovary typically develops into a capsule that is dehiscent by three or six longitudinal slits, while remaining closed at both ends. The ripening of a capsule can take two to 18 months.

The seeds are generally almost microscopic and very numerous, in some species over a million per capsule. After ripening, they blow off like dust particles or spores. They lack endosperm and must enter symbiotic relationships with various *mycorrhizal basidiomyceteous* fungi that provide them the necessary nutrients to germinate, so that all orchid species are mycoheterotrophic during germination and reliant upon fungi to complete their lifecycles.

As the chance for a seed to meet a fitting fungus is very small, only a minute fraction of all the seeds released grow into adult plants. In cultivation, germination typically takes weeks, while there is a report of one *paphiopedilum* that took fifteen years.

The main component for the sowing of orchids in artificial conditions is the agar agar. The substance is put together with some type of carbohydrate which provides qualitative organic feed.

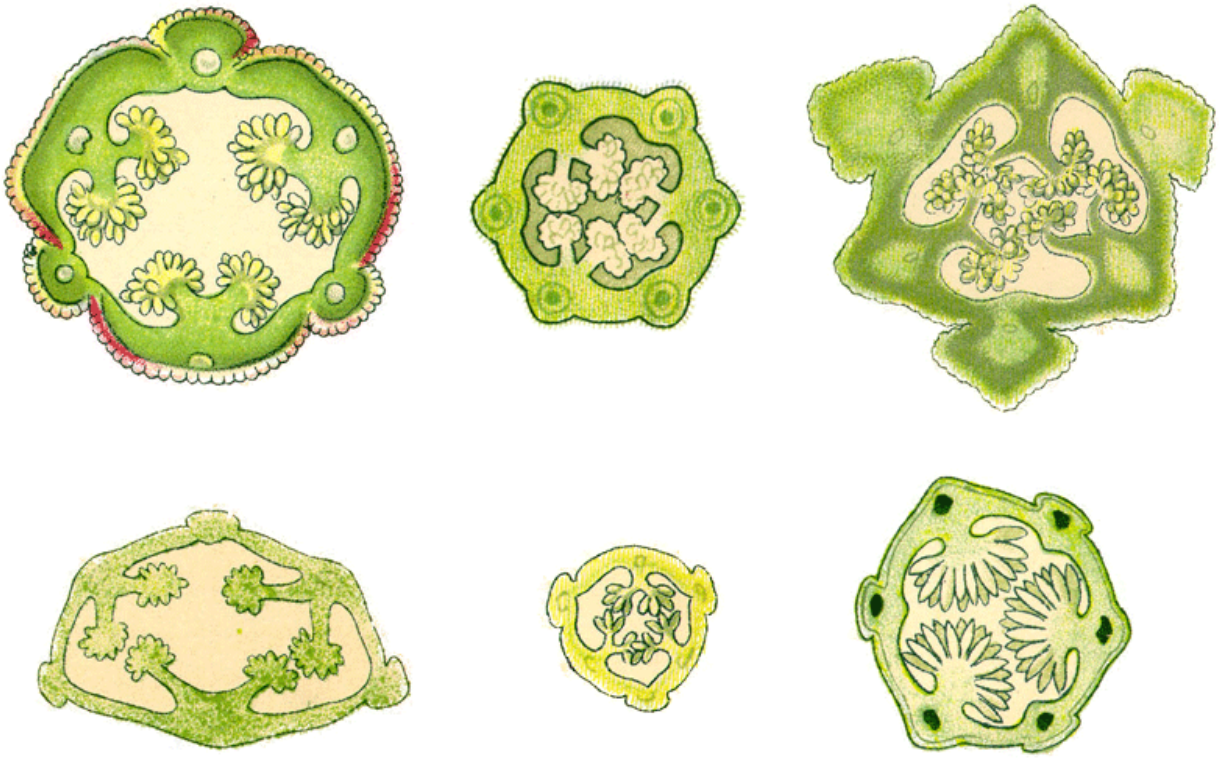


**Figure 2.7:** Cross-section of an orchid capsule, the longitudinal slits

## 2.5 Ecology

A majority of orchids are perennial epiphytes, which grow anchored to trees or shrubs in the tropics and subtropics. Species such as *Angraecum sororium* are lithophytes,(Whitman *et* al, 2011) growing on rocks or very rocky soil. Other orchids (including the majority of temperate Orchidaceae) are terrestrial and can be found in habitat areas such as grasslands or forest.

Some orchids, such as *Neottia* and *Corallorhiza*, lack chlorophyll, so are unable to photosynthesize. Instead, these species obtain energy and nutrients by parasitising soil fungi through the formation of orchid mycorrhizas. The fungi involved include those that form ectomycorrhizas with trees and other woody plants, parasites such as *Armillaria*, and *saprotrophs* (Leake, 2005). These orchids are known as myco-heterotrophs, but were formerly (incorrectly) described as saprophytes due to the belief that they gained their nutrition by breaking down organic matter. While only a few species are achlorophyllous holoparasites, all orchids are myco-heterotrophic during germination and seedling growth, and even photosynthetic adult plants may continue to obtain carbon from their *mycorrhizal* fungi.

## 2.6 Genus *Phragmipedium* Rolfe.

### 2.6.1 History

The first species of this group of South-American slipper orchids was named *Cypripedium vittatum* by Vellozo who described it in 1831. Almost all species discovered after that until 1854 were named Cypripedium. In 1846 Lindley named the species currently known as *Phragmipedium lindenii, Uropedium lindenii*. Reichenbach f. suggested in 1854, to put all tropical American slipper orchids in a new genus called *Selenipedium*. Later in 1886 Pfitzer united the phragmipediums and the paphiopedilums in one group called Paphiopedilum, where the American

species were first referred to as Paphiopedilum sect. Caudata and later in 1896 as Paphiopedilum sect. Phragmopedilum. Later on in 1896 Rolfe divided this group again in separate genera. All plants with a trilocular ovary were put together under the name of Phragmipedium. In 1896 Pfitzer agreed with this new division but changed the name into Phragmopedilum, which he thought was justified because he used this name earlier in 1886 when he called this group of plants Paphiopedilum sect. Phragmopedilum. It is true that the oldest name of Phragmipedium is Uropedium but it did not became a common name because Lindley only used it for a somewhat peculiar plant, the current Phragmipedium lindenii. With this species the lip is replaced by a third petal and therefore did not seem to fit in the group and was often called a monstrosity.

At the 12th international botanical congress in Leningrad (currently St. Petersburg) in 1975 the name Phragmipedium Rolfe was acknowledged to be the only valid name for this genus. It became a so called *Nomina conservanda*. The older name *Uropedium Lindl*. was rejected, and Phragmopedilum Pfitzer was officially recognized as a synonym.

Since the description of Phrag. sargentianum in 1893 it was quiet surrounding the genus, and interest faded until in 1979 Leslie A. Garay published his "The Genus Phragmipedium" in Orchid Digest. This renewed interested received an enormous boost when in 1981 Phrag. besseae with its spectacular colour was discovered. In

2002 the (slipper-)orchidworld was turned upside down again with the somewhat controversial situation surrounding the description of the spectacular Phrag. kovachii from Peru with its huge purple flowers.

## 2.6.2. Brief Detail of *Phragmipedium* Rolfe.

**Table 2.1:** Brief Detail of *Phragmipedium* Rolfe.

| | |
|---|---|
| **Synonyms:** | ***Phragmopedilum* Pfitzer; The genus *Uropedium* (Lindl.) is generally included in *Phragmipedium*.** |
| **Homonyms:** | *Uropedium Lindl.* in Orchid. Linden.: 28, 1846. |
| **Type species:** | *Phragmipedium* caudatum (Lindl.)Rolfe |
| **Etymology:** | Phragma=separation/demarcation, which points to the fact that the ovary is trilocular; pedium (or more correctly, pedilon)=slipper or sandal which of course points to the shape of the lip. |
| **Number of species :** | In 1903 Pfitzer described 11 species of Phragmipedium in 5 sections,<br>Leslie Garay mentions in his revision of the genus in 1979 in the Orchid Digest 22 species,<br>In the checklist of the genus in Orchid Digest (2003) Olaf Gruss lists 33 taxa; 20 at the species level; 8 at variety level and 2 at the level of form. He also mentions 2 natural hybrids. |
| **Chromosomes:** | Dressler (1990) mentions that the chromosomes are relatively large. |
| **Pollination:** | The flowers are so called trap flowers and, as far as known, none of the species offer any reward (pollen, honey) . |

| | |
|---|---|
| | According to Dressler (1990), both halictine bees and syrphid flies have been observed pollinating Phragmipedium. |

## 2.7 Plant Detail

### 2.7.1 Plant Taxonomy

| | |
|---|---|
| **Kingdom:** | Plantae |
| **(unranked):** | Angiosperms |
| **(unranked):** | Monocots |
| **Order:** | Asparagales |
| **Family:** | Orchidaceae |
| **Subfamily:** | Cypripedioideae |
| **Tribe:** | Phragmipedieae |
| **Subtribe:** | Phragmipediinae |
| **Genus:** | *Phragmipedium* |
| **Species:** | ***P. longifolium*** |

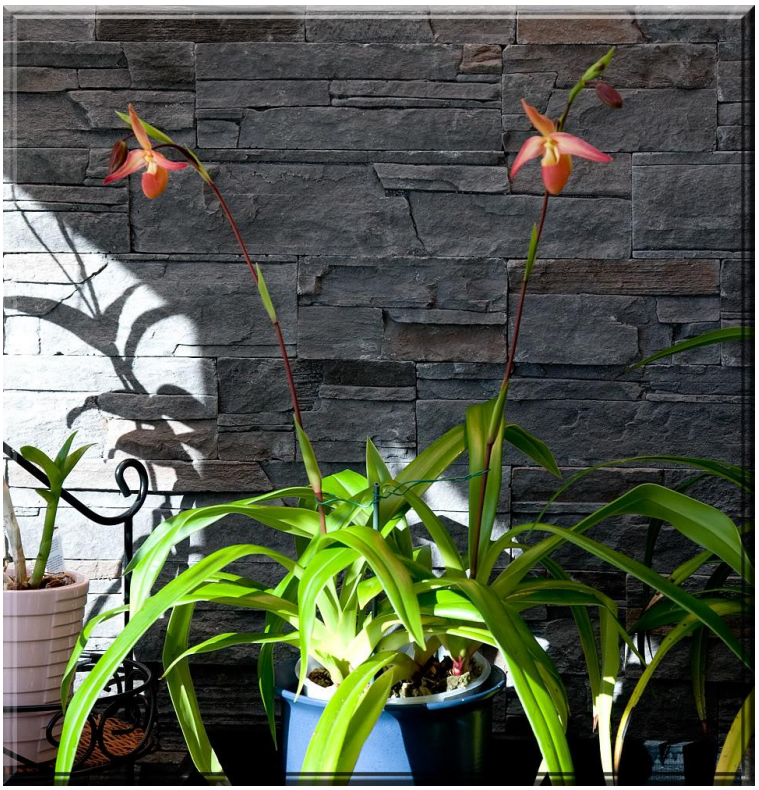


**Figure 2.8:** *P. longifolium* in full bloom

## 2.8 Other Species (name and country)

1. *Phragmipedium andreettae* P. J. Cribb & Pupulin (Ecuador)
2. *Phragmipedium besseae* Dodson & J.Kuhn (Ecuador to N. Peru).
3. *Phragmipedium besseae* var. besseae (E. Ecuador to N. Peru). Hemicr.
4. *Phragmipedium bessae* var. flavum (Braem) Gruss et Roeth 1999 (Peru) (now synonym of Phragmipedium besseae var. besseae)
5. *Phragmipedium besseae* var. dalessandroi (Dodson & O.Gruss) A.Moon & P.J.Cribb (S. Ecuador). Hemicr.
6. *Phragmipedium boissierianum* (Rchb.f.) Rolfe (S. Ecuador to Peru).
7. *Phragmipedium boissierianum* var. boissierianum (Peru). Hemicr.
8. *Phragmipedium boissierianum* var. czerwiakowianum (Rchb.f.) O.Gruss (S. Ecuador to Peru)
9. *Phragmipedium brasiliense* Quené & O.Gruss (Brazil)
10. *Phragmipedium caricinum* (Lindl. & Paxton) Rolfe (Bolivia).
11. *Phragmipedium caudatum* (Lindl.) Rolfe : Mandarin Orchid (Bolivia to Peru). (synonym : P. humboldtii subsp. humboldtii)
12. *Phragmipedium chapadense* Campacci & R.Takase (Brazil).
13. *Phragmipedium christiansenianum* O.Gruss & Roeth (Colombia) (now synonym of Phragmipedium longifolium (Warsz. & Rchb.f.) Rolfe)

14. *Phragmipedium exstaminodium* Castaño, Hágsater & E.Aguirre (Mexico - Chiapas to Guatemala).
15. *Phragmipedium fischeri* Braem & H.Mohr (Ecuador).
16. *Phragmipedium hirtzii* Dodson (N. Ecuador).
17. *Phragmipedium humboldtii* (Warsz. ex Rchb.f.) J.T.Atwood & Dressler (Costa Rica to Panama) (now synonym of : Phragmipedium popowii Braem, Ohlund & Quéné)
18. *Phragmipedium klotzschianum* (Rchb.f.) Rolfe (SE. Venezuela to Guyana and N. Brazil).
19. *Phragmipedium kovachii* J.T.Atwood, Dalström & Ric.Fernández (Peru – San Martin).
20. *Phragmipedium lindenii* (Lindl.) Dressler & N.H.Williams (Venezuela to Ecuador).
21. *Phragmipedium lindleyanum* (M.R.Schomb. ex Lindl.) Rolfe (N. South America to Brazil - Pernambuco).
22. *Phragmipedium longifolium* (Warsz. & Rchb.f.) Rolfe (Costa Rica to Ecuador, India).
23. *Phragmipedium pearcei* (Rchb.f.) Rauh & Senghas (Ecuador to N. Peru).
24. *Phragmipedium popowii* Braem, Ohlund & Quéné (Costa Rica to Panama)
25. *Phragmipedium reticulatum* (Rchb.f.) Schltr. (Ecuador to Peru).

26.*Phragmipedium richteri* Roeth & O.Gruss (Peru).

27.*Phragmipedium* × *roethianum* O.Gruss & Kalina (Ecuador) (P. hirtzii x P. longifolium)

28.*Phragmipedium schlimii* (Linden ex Rchb.f.) (Colombia).

29.*Phragmipedium tetzlaffianum* O.Gruss (Venezuela).

30.*Phragmipedium vittatum* (Vell.) Rolfe (WC. & SE. Brazil).

31.*Phragmipedium wallisii* (Rchb.f.) Garay (now synonym of : Phragmipedium warszewiczianum (Rchb.f.) Schltr.)

32.*Phragmipedium warszewiczianum* (Rchb.f.) Schltr. (Colombia to Ecuador) (Wikipedia, 2009)

# Chapter 3

# Evaluation of Tests

### 3.1 Evaluation of Antioxidant Activity

The main goal of antioxidant activity test is to find the oxidation reducing power of the plant extract.

Oxidation in living organisms is essential for the acquirement of energy in catabolism. However, oxygen-centered free radicals and other reactive oxygen species, which are continuously, produced in vivo result in cell death and tissue damage. Oxidative damage caused by free radicals may be related to aging, and diseases such as atherosclerosis, diabetes, cancer and cirrhosis (Halliwell and Gutteridge, 1999).

Free radicals are natural by-products of human metabolism. These are charged molecules which attack cells, breaking cellular membranes and reacting with the nucleic acids, proteins, and enzymes present in the cells. These attacks by free radicals, collectively known as oxidative stress, are capable of causing cells to lose their structure, function and eventually result in cell dysfunction. They are continuously produced by our body's use of oxygen, such as in respiration and some cell-mediated immune functions. Free radicals are also generated through environmental pollutants, cigarette smoke, automobile exhaust, radiation, air-pollution, pesticides, etc. (Li and Trush, 1994). Normally, there is a balance between the quantity of free radicals generated in the body and the antioxidant

defense systems which scavenge these free radicals preventing them from causing deleterious effects in the body (Nose, 2000). The antioxidant defense systems in the body can only protect the body when the quantity of free radicals is within the normal physiological level. But when this balance is shifted towards more free radicals, increasing their burden in the body either due to environmental conditions or infections, it leads to oxidative stress (Finkel and Holbrook, 2000).

When the production of reactive oxygen species (ROS) exceeds the antioxidant capacity of the system, oxidative stress occurs in cellular system, including the superoxide anion radical, the hydroxyl radical, hydrogen peroxide and the peroxyl are greatly reactive molecules, which consequently generate metabolic products that attack lipids in cel1 membrane or DNA (Halliwell and Gutteridge, 1999). Oxidative stress, involves a series of free radical chain reaction processes, is associated with several types of biological damage, DNA damage, diabetes, respiratory tract disorders, carcinogenesis and cellular degeneration related to aging (Anderson et al., 2000). Continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body beyond its capacity to control them and cause irreversible oxidative damage (Tseng et al., 1997). Improved antioxidant status helps to minimize the oxidative damage and thus can delay or decrease the risk for developing many chronic age related, free radical induced diseases (Karuna et al., 2009). The interest in natural antioxidants,

especially of plant origin, has greatly increased in recent years as the possibility of toxicity of synthetic antioxidants has been criticized (Jayaprakash and Rao, 2000).

Plants (fruits, vegetables, medicinal herbs, etc.) may contain a wide variety of free radical scavenging molecules, such as phenolic compounds (e.g. phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant activity (Zheng and Wang, 2001). Epidemiological studies have shown that many of these antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, or antiviral activities to a greater or lesser extent (Owen et al., 2000).

DPPH is reduced from a purple compound to a light yellow compound by electrons from oxidant compounds. Reaction of DPPH with hydroxyl groups involves a homolytic substitution of one of the phenyl rings of DPPH yielding 2-(4-hydroxyphenyl)-2-phenyl-1- picryl hydrazine as a major product whilst 2-(4nitrophenyl)-2phenyl-1-picrylhydrazine is also formed via a series of secondary processes. The concentration of DPPH at the end of a reaction will depend on the concentration and structure of the compound being scavenged (Naik et al., 2003).

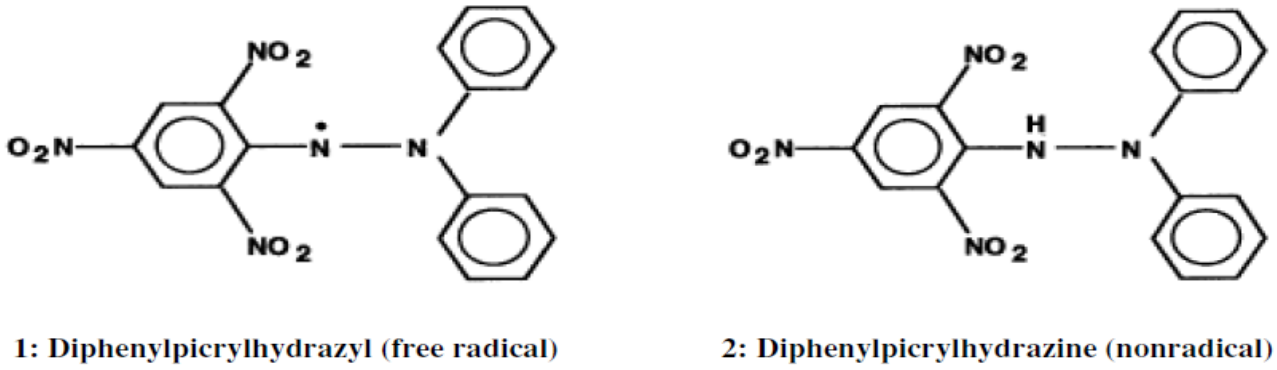



**Figure 3.1:** DPPH mechanism of Anti-oxidant test.

By absorbance test it is very easy to assay the %inhibition which shows the anti-oxidant property.

### 3.2 Evaluation of Analgesic Property

The goal of any analgesic property test is to find any analgesic particle in the plant crud extracts.

Pain is probably the most prevalent symptom in clinical practice, and characterization of pain is of major importance in the diagnosis and choice of treatment (Thumshirn *et al*., 1999). In the treatment of diseases associated with pain, the clinical effects typically guide the selection of the analgesics and titration of the dose. However, in practice, the different symptoms of the underlying diseases confound the characterization of pain. These confounders may include complaints relating to psychological, cognitive and social aspects of the illness, as well as systemic reactions such as fever and general malaise (Drewes *et al*., 2003).

Furthermore, treatment with analgesics often causes sedation and other side effects. This may bias the clinical evaluation, as the patients tend to interpret other effects of the medication– such as an effect on the anxiety and depression relating to the disease – as a relief of pain (Le Bars *et al*., 2001). Because of these confounding factors, *experimental pain models* are often advantageous in preclinical investigations of analgesics. With these models, the investigator can control the experimentally induced pain (including the nature, localization, intensity, frequency and duration of the stimulus), and provide quantitative measures of the psycho-physical, behavioral or the neurophysiological responses (Drewes *et al*., 2003). Experimental pain models have been used in *animal studies.* In these experiments, the neuronal nociceptive activity can be recorded or behaviour can be assessed (Sengupta & Gebhart 1994). However neuronal recordings or reactions do not reveal all aspects of pain, since pain is the net effect of complex multidimensional mechanisms that involve most parts of the central nervous system (Le Bars *et al*., 2001). Nociceptive reflexes or electrophysiological recordings from selected pathways in the animal nervous system are important in basic research and screening of analgesics. However, animal experiments typically suppress central pain mechanisms and associated complex reactions seen in man. Furthermore, the neurobiology of nociceptive systems differs between species, and

this limits the extrapolation of findings from animal studies to man even further (Le Bars *et al*., 2001).

Three types of test is possible

### 3.2.1 Analgesic Activity by Writhing Method:

In the present study analgesia was assessed according to the reported method. The back leg of the mice was writhing due to pain. Writhing method of mice counted after 10 minutes of acetic acid administration and after 5 minutes of drug (positive control) or extract administration, Count of writhing of each 5 minutes was taken up to 20 minutes.

### 3.2.2 Analgesic Activity by Tail Flick Test:

In this study analgesia was assessed according to the reported method. The terminal part of the tail (about 1cm) of the mice was placed on analgesiometer, at uniform distance from the nichrome wire. Temperature of heating element of the instrument was maintained at 52±0.5°C. Cut-off time 20 seconds was maintained.

### 3.2.3 Analgesic Activity by Tail Immersion Test:

In this study analgesia was assessed according to the reported method. 3-4 cm area of the tail was marked and immersed in the water bath thermo-statistically maintained at 51°C. The withdrawal time of the tail from hot water (in seconds) was noted as the reaction time or tail flick latency. The maximum cutoff time for immersion was 20 seconds to avoid the injury of the tissues of tail.

### 3.2.4 Mechanism of Pain Induction in Acetic Acid Induced Writhing Method

Intraperitonial administration of acetic acid causes localized inflammation in mice. Following inflammation, there is biogenesis of prostaglandins (cyclooxygenase pathway) & leukotrienes (lipooxigenase pathway). The released prostaglandin, mainly prostacyclin ($PGI_2$) & prostaglandin E have been reported responsible for pain sensation (Le Bars *et al*. 2001)

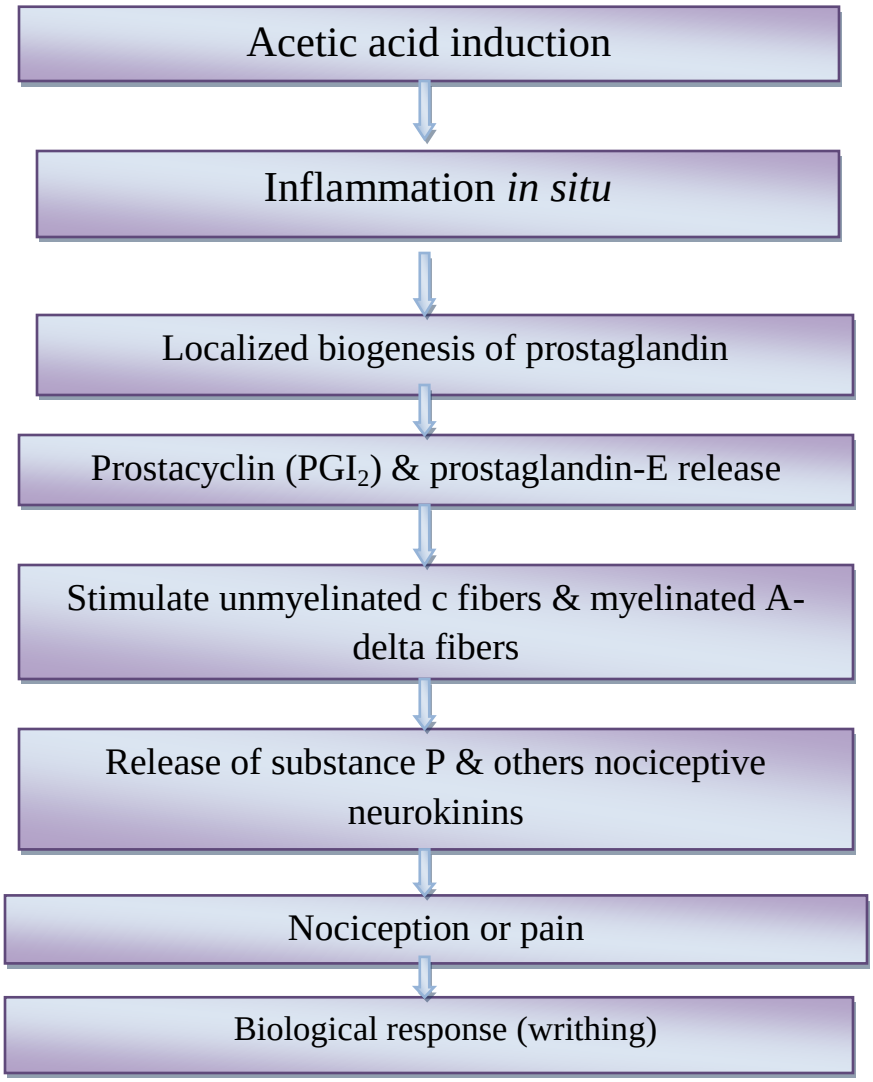



**Figure 3.2:** Schematic diagram of pain induction

# Chapter 4

# Materials and Methods

## 4.1 Extraction Process

### 4.1.1 Materials

**Table4.1:** List of chemicals used in Extraction Process.

| Name | Amount | Source | Justification |
|---|---|---|---|
| **Methanol (reagent grade)** | 500 ml | Merck, Germany | Extraction solvent |
| **Ethanol (rota)** | 100 ml | rota | Washing |
| **Acetone** | 20ml | Merck, Germany | Washing out extracts |

**Table4.1:** List of Apparatus used in Extraction Process.

| Name | Model | Justification |
|---|---|---|
| **Conical flask** | 500ml, 1000ml | BDH Laboratory Equipments |
| **Beaker** | 50ml, 100ml, 250ml | |
| **Funnel** | Medium | |
| **Test tube** | Medium | |
| **Vial** | 5ml | |
| **Glass rod** | | BD local lab sourse |
| **Filter paper** | Whiteman 2µm | |
| **Pipette** | 2ml,5ml,10ml,20ml | BDH Laboratory Equipments |
| **Automatic pipette puller** | | Bel-Art Products, USA |
| **Spatula** | | Local |
| **Weight machine** | M-220 | Denver Instruments |
| **Grinding machine** | | |
| **Rotary evaporator** | Rotary evaporator-HB4 basic IKA | IKA, India |

### 5.1.2 Collection

The plant collected from Baldha Garden a remote subdivision of the Botanical garden. In this garden there are several hundreds of plants nicely arranged and very nicely oriented with their scientific names. I have collect nearly two kg of the *Phragmipedium Longifolium.*

### 4.1.3 Process of powdering

After collection the whole amount of the collected plant was dried in sun for 10 days. Because of heavy cuticle a waxy layer on its leaves it was very difficult to dry. After 10 days it became one of the great nice brittle leaves which can easily grinder in a grinding machine. Before running in grinding machine I run two kg normal rice in the machine to be sure the machine is working perfectly and to wash out the contamination.

Leaves and roots of *Phragmipedium Longifolium* was cut into small pieces and then crushed and powdered by grinding machine which ensured a large surface area for extraction.

**Figure 4.1:** Grinding machine

### 4.1.4 Extraction

For extraction we use methanol. 140 gm of dried powdered *Phragmipedium Longifolium* was used in this extraction process. In a clean glass bottle I took 140 gm of *Phragmipedium Longifolium* powder with help of a funnel. Then 400ml of methanol (reagent grade) was added in the bottle. After that the bottle was tightly sealed. Vigorous shaking was done, which helps in extraction process. This bottle was saved in a cabinet for 7 days and everyday it shook well in every day. When the extraction process is going on I ensured that no air or light disturbance present in the time of extraction.

### 4.1.5 Filtration

After extraction we use whatman 2µm filter paper on a funnel and a conical flask, in the middle of the funnel tube to ensure that all insoluble particles are retain in filtrate.

### 4.1.6 Evaporation and extract collection

For evaporating the solvent and collect for reuse I have used rotary evaporator machine with a vacuum pump which helped to reduce the pressure of the inside of glass tube coil, as well as the whole system. Reduction of pressure causes quick evaporation. On the other part condenser recommenced the solvent so that I could reused it.

**Figure 4.2:** Rotary evaporator.

For this solvent almost 70% solvent get back into liquid form.

The extraction was collected from the evaporating flask and the solvent is collected from the receiving flask. Extract transferred into a 50 ml beaker and covered with aluminum foil.

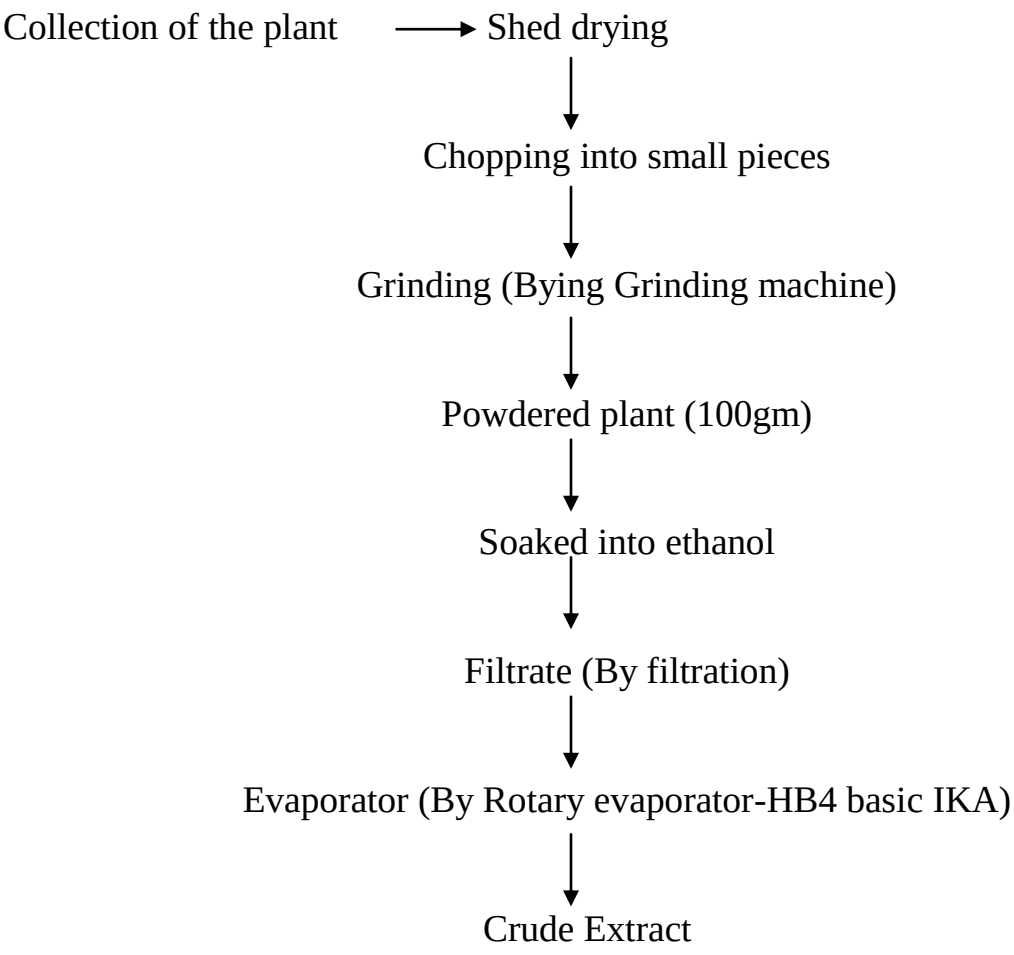



**Figure 4.3:** Schematic presentation of the crude preparation from the plant

## 4.2 Qualitative antioxidant study of *Phragmipedium Longifolium* Extract

### 4.2.1 Introduction

There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious effects of free radicals in the human body, and to prevent the deterioration of fats and other constituents of foodstuffs. In both cases, there is a preference for antioxidants from natural rather than from synthetic sources (Abdalla et al., 1999). There is therefore a parallel increase in the use of methods for estimating the efficiency of such substances as antioxidants (Schwarz, et al., 2001).

### 4.2.2 Materials

**Table4.3:** List of chemicals used in Extraction Process.

| Name | Justification |
|---|---|
| **DPPH** | Free radical source |
| **Ethanol** | solvent |
| **Methanol** | solvent |
| **Distilled water** | solvent |
| **Extracts** | Test sample |

**Table4.4:** List of Apparatus used in Extraction Process.

| Name | Justification |
|---|---|
| **Vial** | Vessel |
| **Pipette** | Measure liquids |
| **Pipette pumper** | To pump through pipette |
| **Vortex mixture** | To mix properly |
| **Ultra sonic Sonicator** | To crush and dissolve extract and other particle |
| **UV-visible spectroscopy** | To find absorbance |

### 4.2.2 Principle

The DPPH method measures electron-donating activity of other compounds in the mixture and hence provides an evaluation of antioxidant activity due to free radical scavenging. Any molecule that can donate an electron or hydrogen to a mixture will react with and bleach DPPH. DPPH is reduced from a purple compound to a light yellow compound by electrons from oxidant compounds. Reaction of DPPH with hydroxyl groups involves a homolytic substitution of one of the phenyl rings of DPPH yielding 2-(4-hydroxyphenyl)-2-phenyl-1- picryl hydrazine as a major product whilst 2-(4nitrophenyl)-2phenyl-1-picrylhydrazine is also formed via a series of secondary processes. The concentration of DPPH at the end of a reaction will depend on the concentration and structure of the compound being scavenged (Naik et al., 2003).

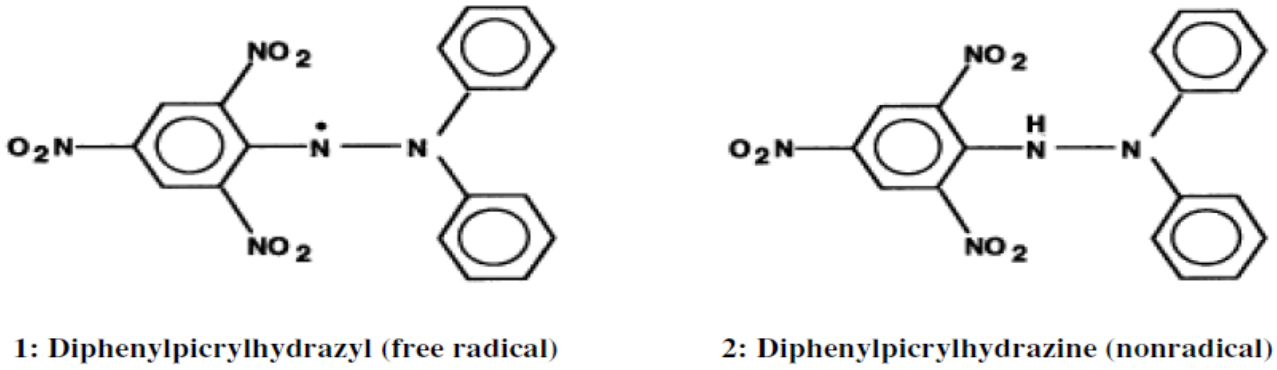



**Figure 4.3:** DPPH mechanism of Anti-oxidant test.

### 4.2.3 Method

Antioxidant activity was determined using quantitative analysis namely, the 2, 2,diphenyl-1-picrylhydrazyl (DPPH) assay and Trolox equivalent antioxidant capacity (TEAC) assay, respectively. In this paper quantitative result is given.

The molecule of 1,1-diphenyl-2-picrylhydrazyl (α,α-diphenyl-β-picrylhydrazyl; DPPH: **1**) is characterised as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalisation also gives rise to the deep violet colour, characterised by an absorption band in ethanol solution centred at about 520 nm. The range of highest absorbance wave length ($\lambda_{max}$) is 514-522. But our DPPH source shows highest absorbance at 517nm. So I measured all the absorbance at 517nm.

When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form, with the loss of this violet colour (although there would be expected to be a residual pale yellow colour from the picryl group still present). Representing the DPPH radical by Z• and the donor molecule by AH, the primary reaction is

$$Z\bullet + AH = ZH + A\bullet$$

Where ZH is the reduced form and A• is free radical produced in this first step. This latter radical will then undergo further reactions which control the overall stoichiometry, that is, the number of molecules of DPPH reduced (decolorised) by one molecule of the reductant.

The reaction is therefore intended to provide the link with the reactions taking place in an oxidising system, such as the autoxidation of a lipid or other unsaturated substance; the DPPH molecule Z• is thus intended to represent the free radicals formed in the system whose activity is to be suppressed by the substance AH.( Molyneux, 2004).

### 4.2.4 Procedure

In this method I have used 8 concentrations to prepare a standard line or curve for the evaluation of antioxidant activity. I used 5 ml vial for preparing the sample and controls. All absorbance is measured in 517nm.

## 4.3 Analgesic Effect of *Phragmipedium Longifolium* Extract

### 4.3.1 Mechanism of Pain Induction in Acetic Acid Induced Writhing Investigated Method

Intraperitonial administration of acetic acid causes localized inflammation in mice. Following inflammation, there is biogenesis of prostaglandins (cyclooxygenase pathway) & leukotrienes (lipooxigenase pathway). The released prostaglandin, mainly prostacyclin ($PGI_2$) & prostaglandin E have been reported responsible for pain sensation (Le Bars *et al*. 2001).

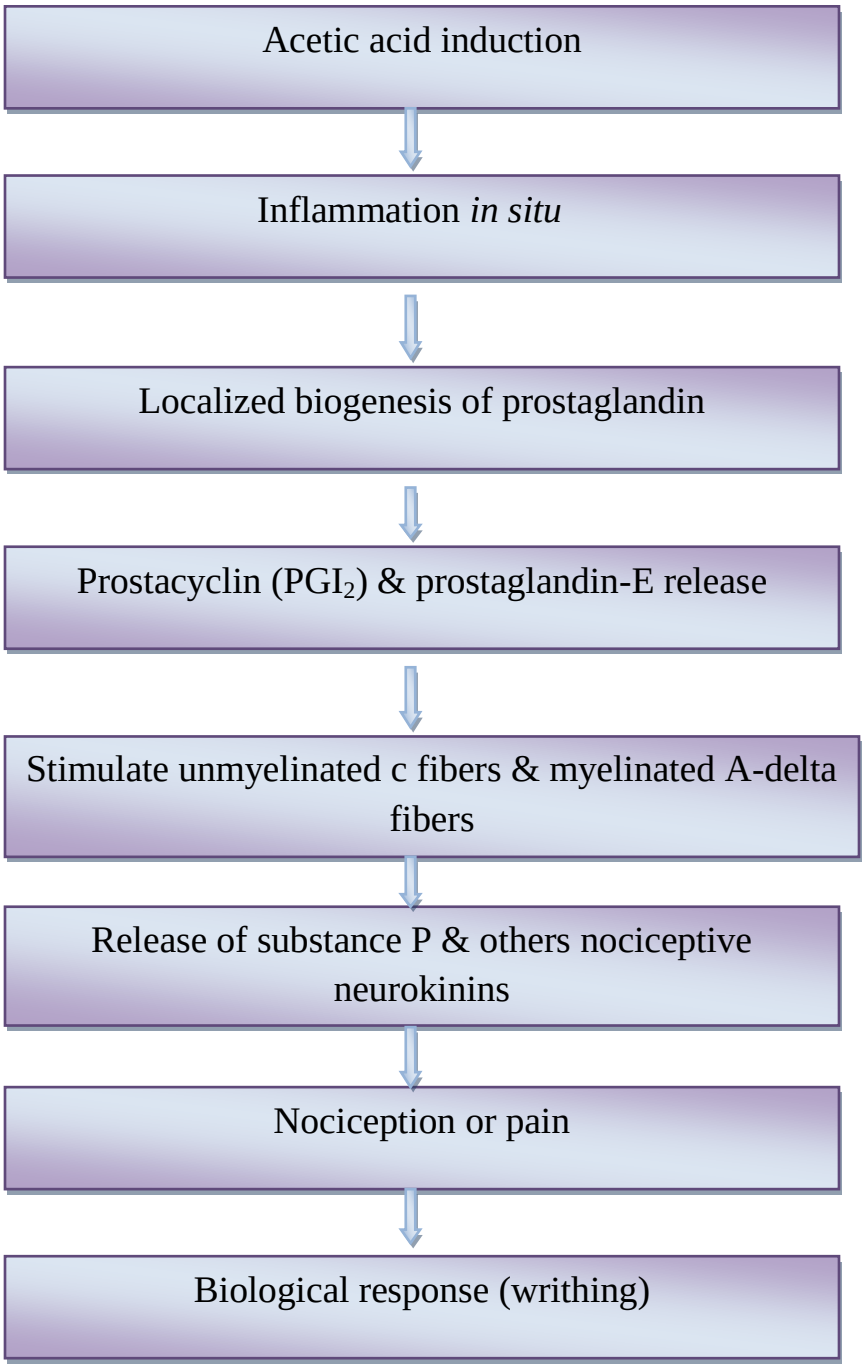



Figure 4.5: Schematic diagram of pain induction

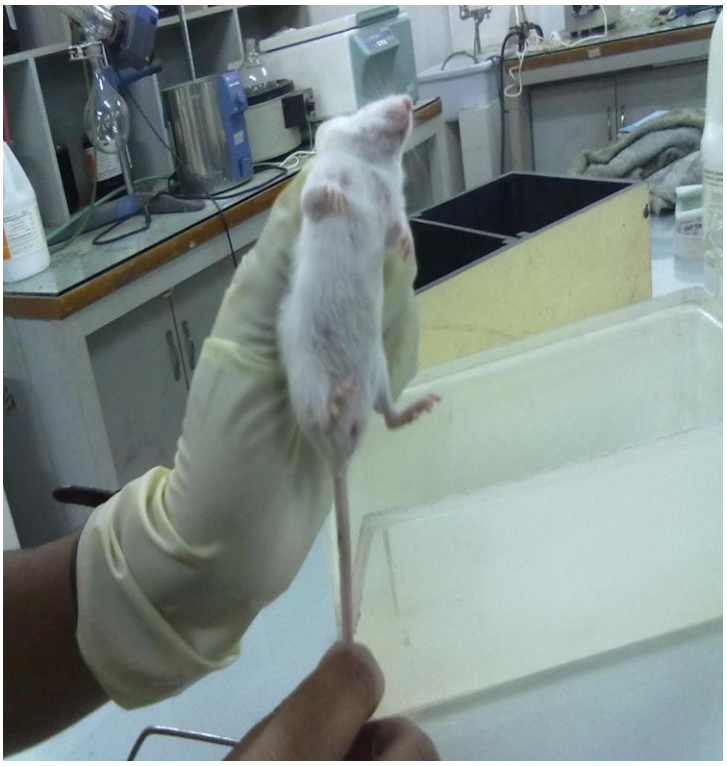


**Figure 4.4:** Swiss Albino mice

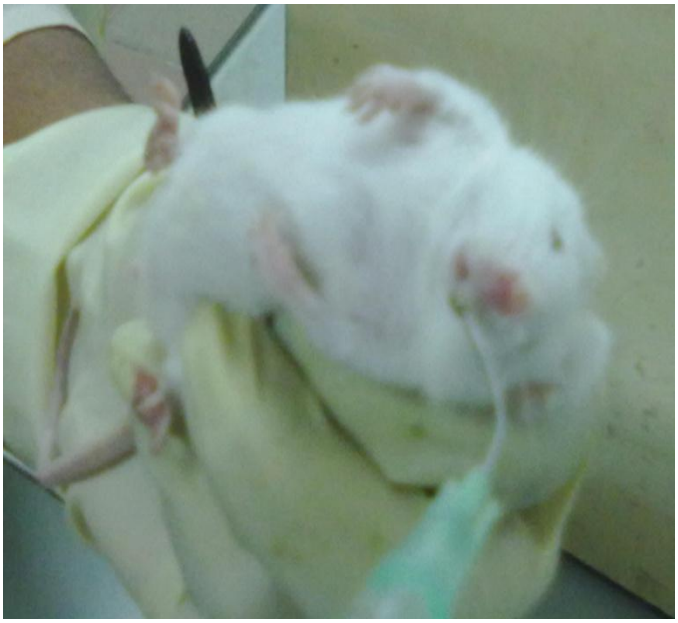


**Figure 4.5:** Oral administration of test sample

### 4.3.2Drugs and Chemicals

**Table 4.5:** Test sample used in the Evaluation Analgesic Activity of *Phragmipedium Longifolium* by Acetic Acid Induced writhing Method**.**

| Test samples | Group | Purpose | Dose(mg/kg) | Route of administration |
|---|---|---|---|---|
| **1% Tween-80 solution in saline** | 1 | Control Group | 0.1 ml/10gm of body weight | Oral |
| **diclofenac-Na** | 2 | Standard Group | 10 | Intraperitoneal |
| ***Phragmipedium Longifolium*** | 3 | Test sample | 400 | Oral |
| **Acetic acid (0.7%)** | **-** | **Writhing Inducer** | **0.1 ml/10gm of body weight** | **Intraperitoneal** |

Acetic acid was obtained from Merck, Germany. Tween-80 was obtained from BDH Chemicals, UK. Normal saline solution was purchased from Beximco Infusion Ltd., Bangladesh. Diclofenac was obtained from Square Pharmaceuticals Ltd., Bangladesh.

**Table 4.6:** Apparatus used for pharmacological investigation

| Apparatus used for pharmacological investigation | Source |
|---|---|
| **Sterile disposal syringe (1 ml, 100 divisions)** | CHPL, India |
| **Tuberculin syringe with ball shaped end** | Merck, Germany |
| **Electronic & digital balance** | Denver M-220 |
| **Glass vials** | JMI Bangladesh Co Ltd |

### 4.3.3 Animal

For the experiment male and female Swiss albino mice of 4 weeks of age, weighing between 25 gm± 3gm, were collected from the animal research branch of the international center for diarrheal disease & research, Bangladesh (ICDDRB). Animals were maintained under standard environmental conditions and had free access to feed and water which is ICDDRB formulated. The animals were acclimatized to laboratory condition for one week prior to experiments.

**Table 4.7:** Materials for pharmacological investigation

| **Test materials** | **Source** |
|---|---|
| **Crude extract of the selected plants, *cymbidium aloifolium*** | Dhaka |
| **Animals used** | **Source** |
| **Swiss Albino mice** | ICDDR,B |
| **wheat** | Mohakhali Bazar, Dhaka |
| **chick-pea** | Mohakhali Bazar,Dhaka |
| **Water** | East west university |
| **Materials used for mice housing** | **Source** |
| **Polyvinyl cages** | BIK industries, India |
| **Soft wood shavings for bedding** | Mohakhali Bazar, Dhaka |

### 4.3.4 Experimental Design

Ten experimental animals were taken randomly selected and divided into three groups denoted as experimental group, positive control group & negative control group. Each group mouse was weighed properly & dose of the test sample & control materials was adjusted accordingly.

### 4.3.5 Method of Identification of Animal

Each group consists of five animals. It was difficult to observe the biological response of five mice at a time receiving same treatment. It is quite necessary to identify individual animal of groups during treatment. The animals were individualized in the following way i.e. marked as M1=mice 1, M2=mice 2, M3=mice3, M4=mice 4 & M5=mice 5.

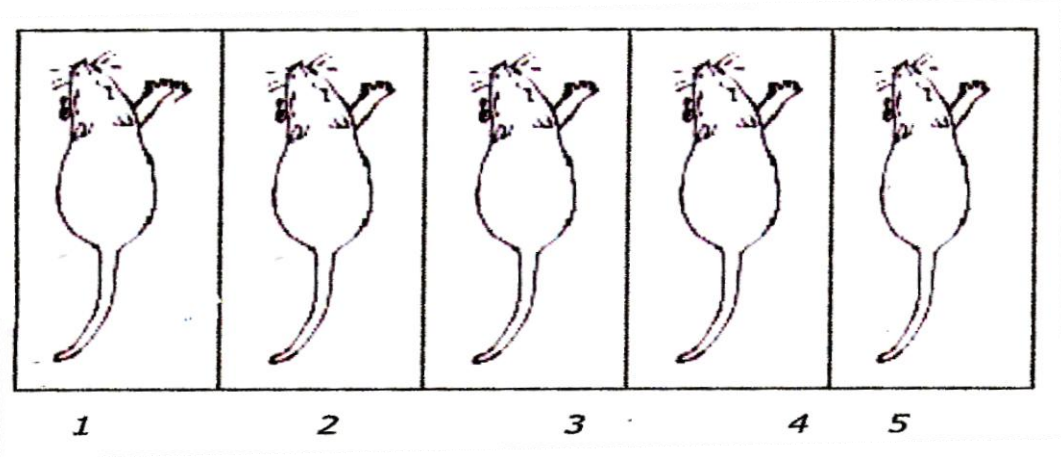



**Figure 4.6:** Identification of test animals for analgesic property screening

### 4.3.6 Preparation of Test Material

In order to administer the crude extract at dose 200 mg/kg body weight of mice. 150 mg of the extract was measured & was triturated unidirectional way by the addition of 5 ml of distilled water. After proper mixing, small amount of suspending agent Tween-80 was slowly added. The final volume of the suspension was made 5.01 ml. To stabilize the suspension it was stirred well. For the preparation of positive control group (10 mg/kg) 30 mg Diclofenac-Na is taken and a suspension of 3 ml is made. As average weight of mice was 25gm±3gm we need 5mg±.3mg for each mouse.

### 4.3.7 Procedure

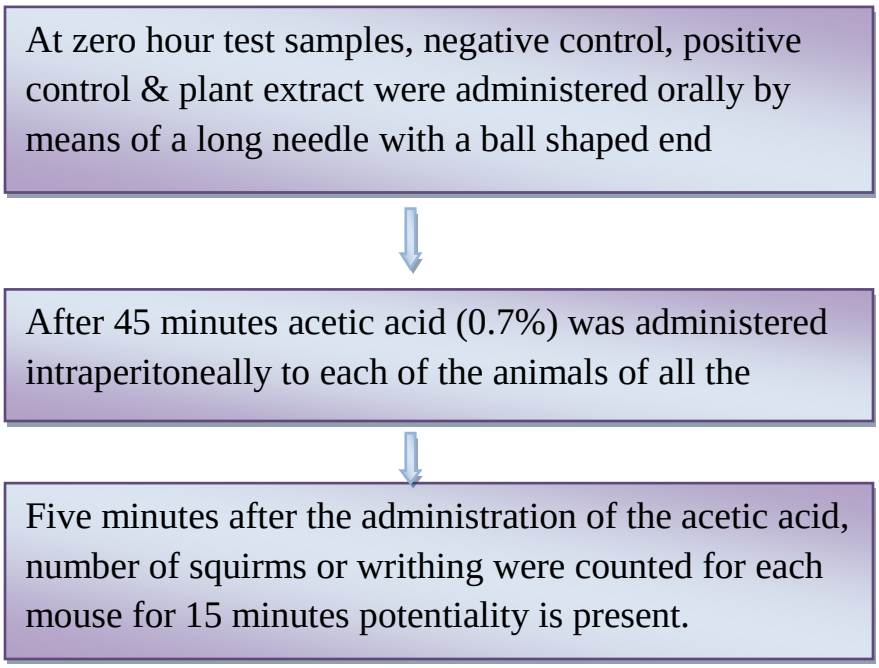



**Figure 4.7:** Schematic representation of procedure for screening of analgesic property on mice by acetic acid induced method.

### 4.3.8 Counting of Writhing Investigated

Each mouse of all groups were observed individually for counting the number of writhing they made in 20 minutes commencing just 5 minutes after the intraperitoneal administration of acetic acid solution. Full writhing was not always accomplished by the animal because sometimes the animals started to give writhing but they did not complete it. This incomplete writhing was considered as half writhing. Accordingly two half was writhing, I taken as one full writhing.

# Chapter 5

# Result and Discussion

## Anti-Oxidant Activity

### 6.1 Result and Discussion of Anti-Oxidant Activity of Ethanolic Extract of *Phragmipedium Longifolium* by DPPH Method.

Result of Anti-Oxidant Activity of Ethanolic Extract of *Phragmipedium Longifolium* by DPPH Method is given below:

**Table6.1:** DPPH scavenging activity of *Phragmipedium longifolium.*

| Concentration | Absorbance | % Inhibition | Blank | $IC_{50}$ | Ascorbic acid % inhibition |
|---|---|---|---|---|---|
| **1.5625** | 0.418 | 0.238663484 | 0.419 | 61.44 | - |
| **3.125** | 0.374 | 10.7398568 | | | - |
| **6.25** | 0.365 | 12.88782816 | | | - |
| **12.5** | 0.324 | 22.67303103 | | | 10 |
| **25** | 0.272 | 35.08353222 | | | 20 |
| **50** | 0.194 | 53.69928401 | | | 32 |
| **100** | 0.153 | 63.48448687 | | | 35 |
| **200** | 0.136 | 67.54176611 | | | 38 |
| **400** | 0.124 | 70.40572792 | | | 42 |

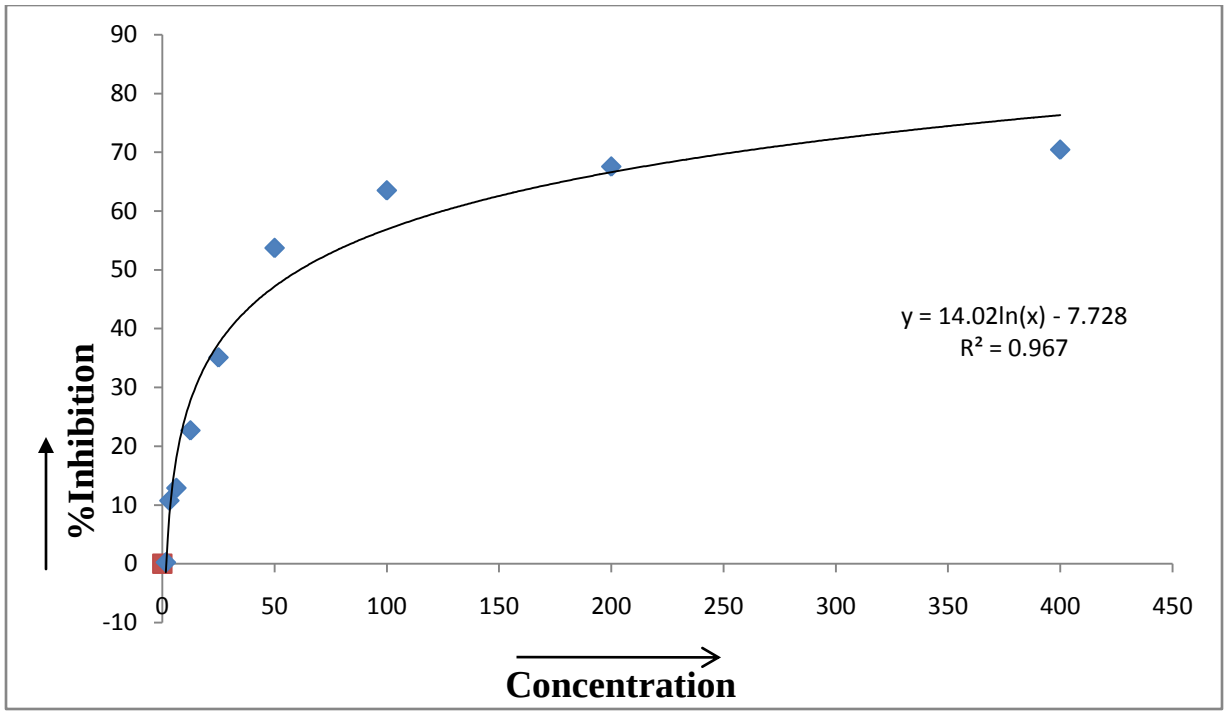



**Figure 6.1:** Diagram representing Anti-Oxidant activity ethanolic extract of *Phragmipedium Longifolium* by concentration vs. % Inhibition curve.

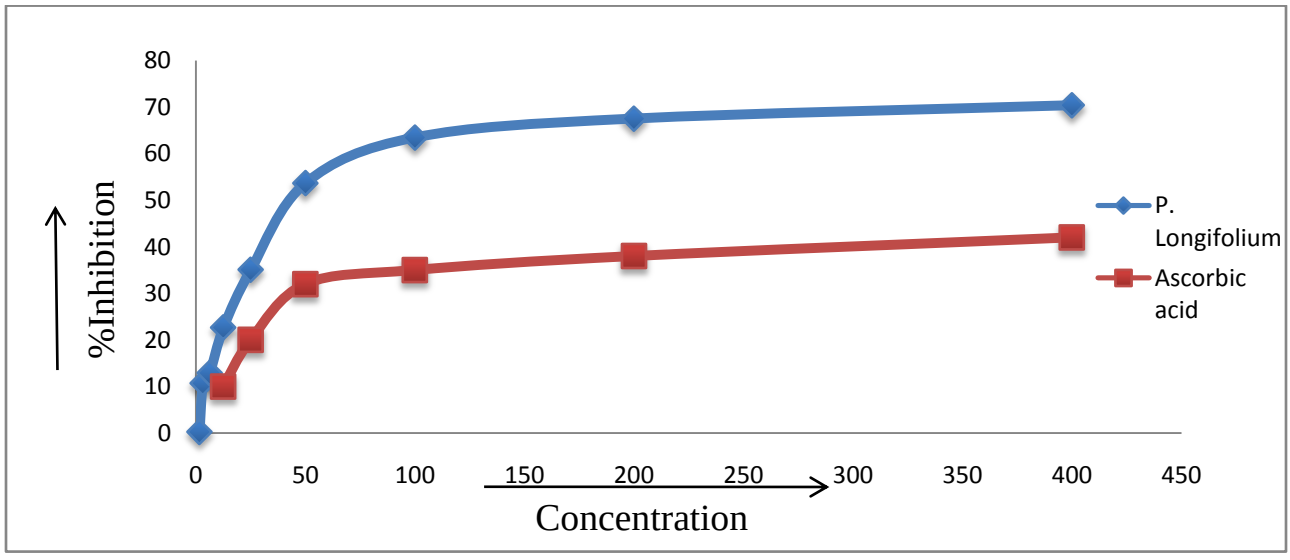



**Figure 6.2:** Diagram representing comparison between Anti-Oxidant activity of ethanolic extract of *Phragmipedium Longifolium* and positive control ascorbic acid by concentration vs. % Inhibition curve.

## Discussion

The half maximal inhibitory concentration ($IC_{50}$) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. It is the half maximal (50%) inhibitory concentration (IC) of a substance (50% IC, or $IC_{50}$). Sometimes, it is also converted to the $pIC_{50}$ scale (-log $IC_{50}$), in which higher values indicate exponentially greater potency. According to the FDA, $IC_{50}$ represents the concentration of a drug that is required for 50% inhibition *in vitro*.

In this study, the $IC_{50}$ is 61.44µg/ml. As anti-oxidant this is a very good anti-oxidant activity (best 1-50 µg/ml, good 50-150, not very good 150&above). The positive control ascorbic acid shows $IC_{50}$ 10.81 µg/ml.

Further study is needed to isolate the active compounds responsible for antioxidant activities. We may found a new good source of natural anti-oxidant from this plant.

## Analgesic Activity

### 6.2 Result and Discussion of Analgesic Activities of Ethanolic Extract of *Phragmipedium Longifolium* by Acetic Acid Induced Writhing Method in mice.

The result of ethanolic extract of *Phragmipedium Longifolium* by Acetic Acid Induced Writhing Method in mice is given below:

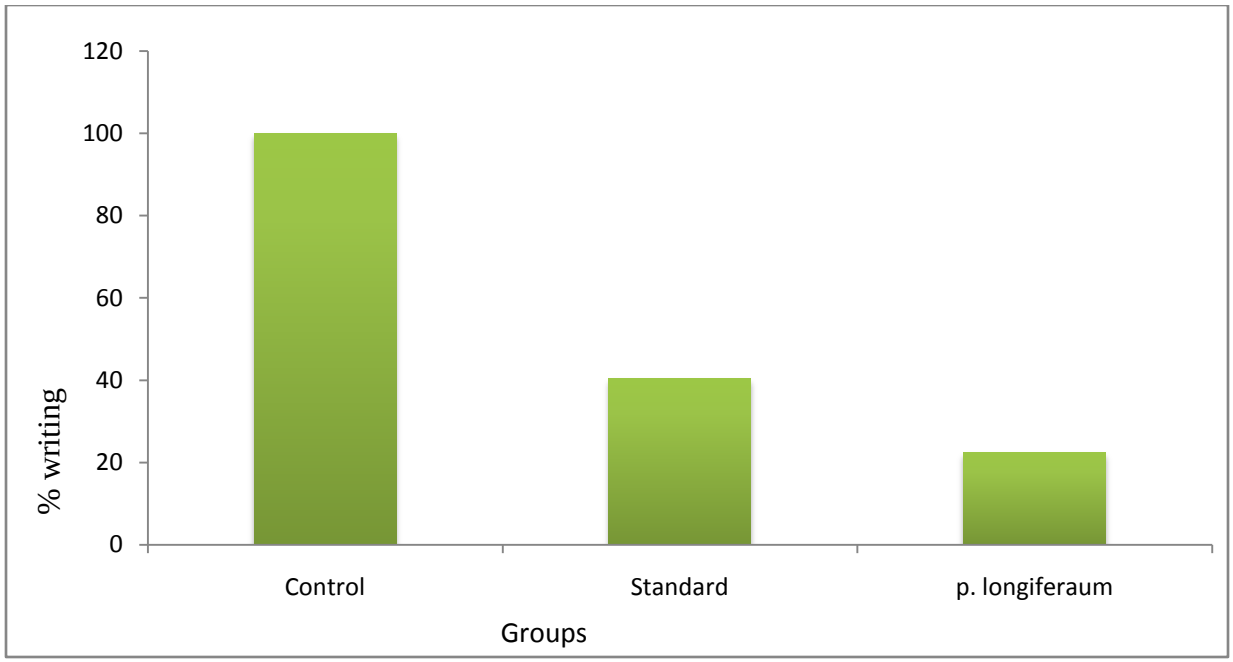
**Table 6.2:** Analgesic Activities of ethanolic extract of *Phragmipedium Longifolium* by Acetic Acid Induced Writhing Method in mice.

| | M1 | M2 | M3 | M4 | M5 | Mean | SD=standard deviation | % writhing | % inhibition |
|---|---|---|---|---|---|---|---|---|---|
| **Control** | 14 | 16 | 20 | 19 | 20 | 17.8 | 2.683282 | 100 | 0 |
| **Standard** | 10 | 7 | 6 | 5 | 8 | 7.2 | 1.923538 | 40.44944 | 59.55056 |
| ***p. longiferaum*** | 3 | 5 | 6 | 4 | 2 | 4 | 1.581139 | 22.47191 | 77.52809 |

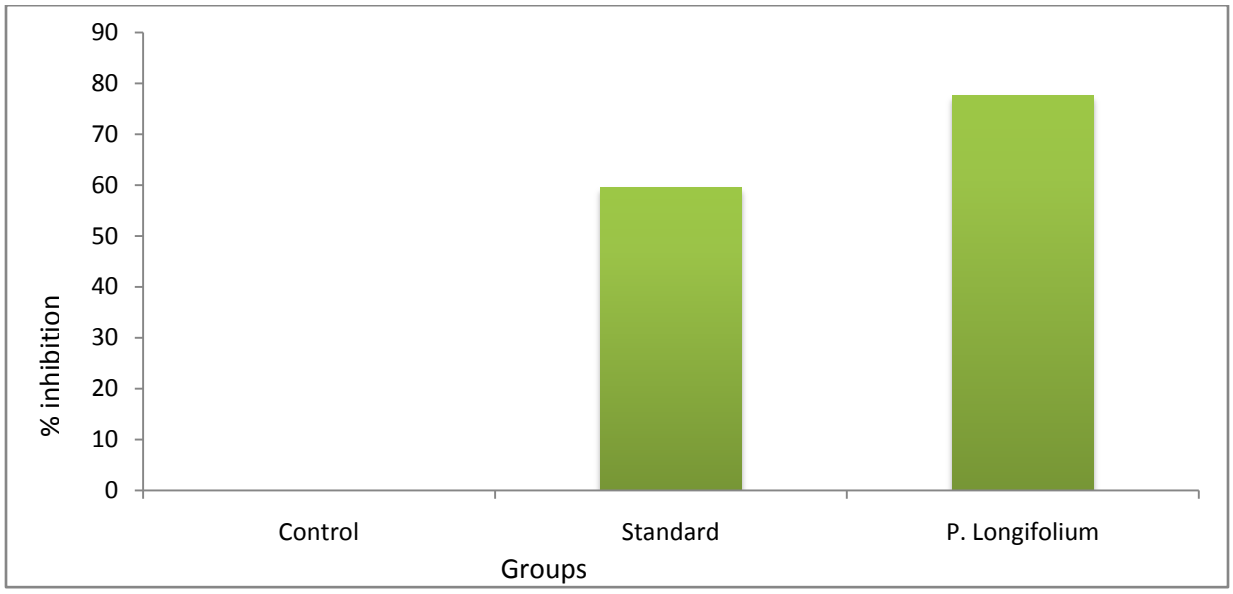
**Table6.3:** Summary and ANOVA test result of analgesic activity of *Phragmipedium Longiferaum*

| **SUMMARY** | | | | |
|---|---|---|---|---|
| ***Groups*** | *Count* | *Sum* | *Average* | *Variance* |
| **Control** | 5 | 89 | 17.8 | 7.2 |
| **Standard** | 5 | 36 | 7.2 | 3.7 |
| **p. longiferaum** | 5 | 20 | 4 | 2.5 |

| **ANOVA** | | | | | | |
|---|---|---|---|---|---|---|
| ***Source of Variation*** | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
| **Between Groups** | 521.7333 | 2 | 260.8667 | 58.40299 | 6.54E-07 | 3.885294 |
| **Within Groups** | 53.6 | 12 | 4.466667 | | | |
| **Total** | 575.3333 | 14 | | | | |




(A)




(B)

**Figure 6.2:** Diagram representing **(A)** % of writhing, **(B)** % of inhibition, the comparison of Analgesic Activities of Control, Diclofenc- Na and Ethanolic Extract of *Phragmipedium Longifolium* by Acetic Acid Induced Writhing Method in mice.

## Discussion

In acetic acid induced analgesic activities assay, all the three groups (Control, Diclofenc- Na, *Phragmipedium Longifolium* of 200 mg/kg body weight) were administered with 0.7% acetic acid. The writhing was counted accordingly and writhing of control group was assumed to be 100% .Control group showed writhing of 17.8±2.683282 Where as positive control group (Diclofenc-Na) showed writhing of 7.2±1.923538, which is highly significant$P<0.001$.The experimental group 200 mg/kg body weight dose group showed writhing with average of 4±1.581139.

On way ANOVA was conducted to compare between the mean of the three groups. The 'F' value from ANOVA was 58.40298507, which is statistically significant i.e. $P<0.001$. This indicates that, the groups are similar significantly different.

# Chapter 6

# Conclusion

The aim of this project paper is to measure phytochemical & pharmacological effect of ethanolic extracts of *Phragmipedium Longifolium*. In phytochemical experimental, Antioxidant assay by DPPH method shows very good anti-oxidant activity and the $IC_{50}$ is 63.8µg/ml compare to control ascorbic acid ($IC_{50}$ 10.81µg/ml). Further study is needed to isolate the active compounds responsible for antioxidant activities. Further study may show a new good source of natural anti-oxidant from this plant. In pharmacological investigation, *Phragmipedium Longifolium* plays a significant impact on mice. This study shows that ethanolic extract of *Phragmipedium Longifolium* show significance result in analgesic activity test, positive control group (Diclofenc-Na) showed writhing of 7.2±1.923538, ($P<0.001$) and the experimental group 200 mg/kg body weight dose group showed writhing with average of 4±1.581139. It indicates that plant contain chemicals which has analgesic activity much better than the Diclofenc-Na, so it would be necessary for further study.

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